Purification and Partial Characterization of a Murine Melanoma-associated Antigen*

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A murine B16 melanoma-associated antigen was detected and partially purified from spent culture medium and intracellular material. The antigen was also purified and partially characterized from the detergent extract of cells cultured in the presence of [3H]glucosamine alone or together with [14C]leucine. During purification, the melanoma-associated antigen activity was monitored by a double antibody antigen binding assay (Bystryn, J.-C., Shenken, I., Baur, S., and Uhr, J. W. (1974) J. Nat. Cancer Inst. 52, 1263). A radiochemically pure preparation of this glycoprotein antigen was obtained by gel filtration on Sepharose CL-4B and Sepharose CL-6B, ion exchange chromatography on DEAE-Sepharose, and chromatography on concanavalin A-Sepharose. Gel filtration on a calibrated Sepharose CL-6B column in the presence of detergent gave an apparent molecular weight of 375,000. Prolonged treatment with sodium dodecyl sulfate and 2-mercaptoethanol followed by gel filtration or acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated a subunit molecular weight in the range of 44,000 to 52,000. The tritium label in the antigen was distributed mainly between sialic acid and N-acetylgalcosamine, with a small amount in N-acetylgalactosamine. Pronase digestion of antigen followed by fractionation on a Bio-Gel P-10 column yielded several glycopeptides. The results of the action of exo- and endoglycosidases on these glycopeptides and their behavior on lectin affinity columns suggested the presence of both complex (N-acetyllactosaminyl type) and simple (oligomannosyl type) oligosaccharides linked via N-acetylglucosamine to asparagine. The glycopeptide fractions isolated after proteolytic digestion were devoid of antigenic activity. Significant antigen activity was detected in the nuclei isolated from the labeled cells.

It is widely believed that tumors possess unique tumor-associated antigens that are absent in normal adult tissues and which play an important but as yet not properly understood role in tumor growth. The isolation, purification, and characterization of these antigens is of paramount importance if their influence on tumor growth is to be elucidated.

The B16 murine melanoma has been extensively investigated (1-15), and it has been proposed as a model for human malignant melanoma (16, 17). Previous publications from our laboratories have reported on the nature of the glycosaminoglycans and glycopeptides isolated from B16 melanoma cells, culture media, and tumors (5-7, 18, 19). In addition, the presence of melanoma-associated antigens on the cells and their release into culture medium by viable cells was reported (8, 10). We have also shown that the partially purified antigens retained their biological activity since it was possible to immunize syngeneic mice with these antigens and inhibit the subsequent growth of otherwise lethal numbers of melanoma cells (11).

This study describes further purification and partial characterization of a radiochemically and immunologically pure melanoma-associated antigen.

EXPERIMENTAL PROCEDURES

Materials

The B16 mouse melanoma cell line described previously was used in these studies (7). Sepharose CL-4B and CL-6B and DEAE-Sepharose CL-6B were from Pharmacia. Bio-Gel P-2 and Bio-Gel P-10, both 200 to 400 mesh, and protein standards for gel electrophoresis were obtained from Bio-Rad. Pronase CB and Vibrio cholerae neuraminidase were from Calbiochem; leech hyaluronidase from Biotics, Arlington, Mass.; endoglycosidase D and H (endo-β-N-acetylgalcosaminidases), concanavalin A-agarose (glycosylx A), and protein standards for gel filtration were from Miles. Neuraminidase, endo-α- and β-N-acetylgalcosaminidase, concanavalin A-galactosidase, and protein standards for gel filtration were from Miles. Neuraminidase, endo-α- and β-N-acetylgalcosaminidase, β-hexosaminidase, and β-galactosidase from Diplococcus pneumoniae were isolated as described (20, 21). Concanavalin A, wheat germ agglutinin, Rávicus communis agglutinin 120 and 60 were isolated and conjugated to Sepharose 4B essentially as described (7). Concanavalin A-polyacrylamide was prepared as described by Lotan et al. (22).

Tritiated glucosamine ([6-3H]glucosamine hydrochloride, 10 to 30 Ci/mmol) and Na235SO4 (carrier-free; 10 to 1000 mCi/mmol) were obtained from New England Nuclear. L-[U-14C]Leucine (330 mCi/mmol) was from Amersham. [14C]Acetylovalbumin glycopeptides were isolated as previously reported (21, 23). Isotopically labeled and unlabeled glycoprotein, α-acid glycoprotein, fetuin, and ovomucoid were prepared by published procedures (24).

Methods

Cell Cultures—The conditions of cell culture and of labeling of the complex saccharides produced by the cells have been described (7). For simultaneous labeling of saccharides and proteins, the cells were grown for 48 h in the presence of [3H]glucosamine at 10 μCi/ml and [14C]leucine at 1.5 μCi/ml of media. The cultured cells were harvested at confluence by treatment with 0.02% EGTA* in NaCUP. The cells were pelleted by centrifugation at 2000 X g.

The abbreviations used are: EGTA, ethylene glycol bis(β-aminomethyl ether)N,N,N',N'-tetraacetic acid; MA.A, melanoma-associated antigen; MAA-M, melanoma-associated antigen isolated from culture medium; MAA-C, melanoma-associated antigen isolated from cells; NP-40, Nonidet P-40 from Shell Chemical Corp., N. Y.; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; Con A, concanavalin

* This investigation was supported by Research Grants CA 17886 and CA 13844 from the National Cancer Institute, National Institutes of Health, and the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 16 U.S.C. Section 1734 solely to indicate this fact.

5145
Mouse Melanoma Antigen

(200 x g, 10 min) and washed thrice with serum-free media by resuspension and centrifugation.

Assay for MAA—MAA were identified and quantitated by a double antibody antigen binding assay. The assay is based on the coprecipitation by anti-lg of immune complexes formed between excess antimalanoma antibodies and radiolabeled MAA (9). Briefly, triplicate 0.1-ml aliquots of material to be tested for MAA activity were diluted 1/5 in NaCl/P, and incubated with 0.1 ml of antimalanoma or normal rabbit serum diluted 1/50 in NaCl/P. The assay was performed in the presence of 0.1 ml of fetal calf serum to reduce nonspecific binding of proteins. After 30 min of incubation at 37°C, antigen-antibody complexes were precipitated by the addition of goat anti-rabbit IgG. After an additional 30 min of incubation, 0.1 ml of normal rabbit serum diluted 1/5 in NaCl/P, was added to all tubes to provide carrier immunoglobulin, and the incubation continued for 30 min at 37°C and then for 1 h at 4°C. The precipitates were collected by centrifugation and washed three times with 3 ml of NaCl/P, dissolved in 0.5 ml of 0.5 M NaOH, mixed with 10 ml of Beckman scintillation fluid and radioactivity was determined. Specific binding is defined as the radioactivity (counts/min) bound by antimalanoma serum less that bound by normal serum. The results are expressed as the percentage of added radioactivity bound specifically. In some cases the ratio of radioactivity bound by antimalanoma serum to that bound by normal serum, the binding index, was also calculated. Prior experiments have shown (9) that under these conditions there is a linear relationship between the amount of radioactivity bound specifically and the amount of labeled MAA present, indicating that the assay provides a quantitative measure of MAA concentration.

Column Chromatography—Sepharose CL-6B and CL-4B columns were equilibrated and eluted with Tris-HCl buffer, pH 8.0, with or without 0.1% Triton X-100. In preparative experiments 0.1 ml of normal rabbit serum diluted 1/5 in NaCl/P, was added to all tubes to provide carrier immunoglobulin, and the incubation continued for 30 min at 37°C and then for 1 h at 4°C. The precipitates were collected by centrifugation and washed three times with 3 ml of NaCl/P, dissolved in 0.5 ml of 0.5 M NaOH, mixed with 10 ml of Beckman scintillation fluid and radioactivity was determined. Specific binding is defined as the radioactivity (counts/min) bound by antimalanoma serum less that bound by normal serum. The results are expressed as the percentage of added radioactivity bound specifically. In some cases the ratio of radioactivity bound by antimalanoma serum to that bound by normal serum, the binding index, was also calculated. Prior experiments have shown (9) that under these conditions there is a linear relationship between the amount of radioactivity bound specifically and the amount of labeled MAA present, indicating that the assay provides a quantitative measure of MAA concentration.

Isolation of Melanoma-associated Antigen from Culture Medium—The medium (200 ml) was dialyzed at 4°C against 0.2 M NaCl for 2 days, followed by distilled water for 5 days, and then lyophilized. The powder was dissolved in NaCl/P, (200 ml), an equal volume of saturated (NH₄)₂SO₄ added and the pH adjusted to 8 with 1 M Tris base. After allowing the suspension to stand for 4 h at 4°C, the precipitate was collected by centrifugation, dissolved in 20 ml of 50 mM Tris- HCl, pH 8.0, and the resulting solution made 52% saturated by addition of solid (NH₄)₂SO₄. After standing overnight at 4°C the precipitate was recovered by centrifugation, washed with 52% saturated (NH₄)₂SO₄ by resuspension and centrifugation, and dissolved in 10 ml of 50 mM Tris-HCl buffer, pH 8.0. The percentage of radioactivity in the (NH₄)₂SO₄ precipitate specifically bound by antimalanoma serum varied from 21 to 74% in different preparations; the binding index was between 1.5 and 3.0.

Purification of MAA—The fractionation of the (NH₄)₂SO₄ precipitate of the culture medium on columns of Sepharose CL-4B is illustrated in Fig. 1. The material from each peak was tested for MAA activity with highest activity present in the void peak (Peak I). When the fractionation was repeated using buffer containing 0.1% Triton X-100, only negligible radioactivity eluted in the position of peak I (void
aggregates. In agreement with this, when the glycoprotein found in peak I (Fig. 2b). Material with high MAA activity eluted in areas designated peaks I, II, and III in Fig. 2b. Chromatography of peak I material on a Sepharose CL-6B column using 3 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 resulted in further purification of MAA-M. Of the radioactivity in the main peak from the Sepharose CL-6B column 63% was specifically bound by antimelanoma serum and the binding index of this fraction was 38.2. The results of gel filtration in the presence of Triton X-100 suggest that the antigens found in peak I (Fig. 1) in the absence of detergent are aggregates. In agreement with this, when the glycoprotein from peak I (Fig. 1) was rechromatographed on Sepharose CL-4B using 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100, the radioactive antigenic material was essentially all included in the column, whereas if the rechromatography was carried out in buffer without Triton, then a portion of the material eluted at the void volume and the rest as a continuous trail (no sharp peaks).

Detection of MAA in the Intracellular Material—The EGTA supernatant obtained during harvesting of cultured labeled cells was exhaustively dialyzed at 4°C against 0.1 M NaCl for 2 days and against water for 5 days. The dialyzed solution was lyophilized, dissolved in Triton buffer, and tested for MAA activity. The percentage of radioactivity specifically bound by antimelanoma serum was 3.6%. Chromatography of the above material on a Sepharose CL-4B column gave an elution profile identical with that subsequently obtained with material in the NP-40 extract of cells (cf. Fig. 2b). The percentages of radioactivity specifically bound by antimelanoma serum were 9.0, 9.5, and 0.6% and the binding indices were 2.2, 2.3, and 1.3 for the material eluting in positions corresponding to peaks I, II, and III, respectively, in Fig. 2b. The melanoma antigens in the EGTA supernatant were not further investigated.

Isolation of MAA from Cells—The cell pellet was suspended in 3 M KCl (1.4 to 2.0 × 10^7 cells/ml) and stirred at 4°C for 24 h. The resulting suspension was centrifuged at 64,000 × g for 60 min at 4°C, and the supernatant (KCl supernatant) saved. The pellet was then extracted with 0.5% Nonidet P-40 in 50 mM Tris-HCl, pH 8.0, at 4°C for 24 h and centrifuged as above. The KCl and NP-40 extracts were separately dialyzed against 6 liters of 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 at 4°C, the buffer was changed at 24 h and the dialysis stopped at 48 h. The dialyzed material was assayed for MAA activity. In a typical experiment, the percentage of radioactivity specifically bound by antimelanoma serum in the NP-40 extract was 2.8% compared to 0.7% for the KCl extract; the binding indices were 1.6 and 1.2, respectively.

Purification of MAA C—Since Triton X-100 prevented the aggregation of MAA, all further chromatographic procedures were carried out using buffers containing 0.1% Triton X-100. The protease inhibitor, phenylmethylsulfonyl fluoride (0.1 mM), was also included in the buffer.

The KCl and NP-40 cell extracts were fractionated on Sepharose CL-4B columns, and individual fractions tested for MAA activity (Fig. 2 and Table I). Since the peak I material obtained from the NP-40 extract had the highest antigenic activity, it was further purified by sequential chromatography on columns of Sepharose CL-6B and DEAE-Sepharose CL-6B. The percentage of radioactivity in the purified material...
The percentage of label specifically bound by antimelanoma after gel filtration on Sepharose 6B but before chromatography increased severalfold (Table I). This suggests that the combination of the chromatography on Sepharose 6B and DEAE-Sepharose may have removed some labeled non-antigenic contaminants and perhaps unlabeled contaminants capable of binding to the antibody. The material after gel filtration on Sepharose 6B but before chromatography on DEAE-Sepharose was not assayed for MAA activity.

The percentage of label specifically bound by antimelanoma serum and the binding index of other purified preparations of labeled non-antigenic contaminants and perhaps unlabeled contaminants capable of binding to the antibody. The material after gel filtration on Sepharose 6B but before chromatography on DEAE-Sepharose was not assayed for MAA activity.

which was specifically bound by the antimelanoma serum, as well as the binding index, increased severalfold (Table I). This suggests that the combination of the chromatography on Sepharose 6B and DEAE-Sepharose may have removed some labeled non-antigenic contaminants and perhaps unlabeled contaminants capable of binding to the antibody. The material after gel filtration on Sepharose 6B but before chromatography on DEAE-Sepharose was not assayed for MAA activity.

The percentage of label specifically bound by antimelanoma serum and the binding index of other purified preparations of MAA-C varied between 70 to 85 and 21 to 54, respectively.

Nature of Labeled Contaminants in MAA and Attempts at Further Purification—Chromatography of MAA-C on a calibrated column of DEAE-Sepharose CL-6B gave a single peak eluting at a concentration of 0.50 M LiCl. The peak elution positions of standards on this column were as follows: glycoporin, 0.505 M LiCl; hyaluronic acid, 0.55 M LiCl; heparan sulfate, 0.78 M LiCl; chondroitin sulfate, 1.05 M LiCl; and heparin, 1.25 M LiCl.

Since the antigen eluted close to the position of hyaluronic acid and had a high glucosamine content, the preparation was examined for the presence of hyaluronic acid. Treatment of the antigen fraction with leech hyaluronidase (phosphate/citrate buffer, pH 5.6, 37°C, 24 h) followed by examination of the digest on controlled pore glass (6) and Sepharose CL-4B columns indicated that less than 5% of the radioactive material was degraded. The low molecular weight material produced eluted close to the position of glucuronic acid on the Sepharose 4B column and was not further characterized, but is presumably hyaluronic acid oligosaccharides. In an attempt to remove the glycosaminoglycans in the antigen preparations, MAA-C (Sepharose 4B Peak II in Fig. 2b) was treated with cetylpyridinium chloride in the presence of carrier anionic polysaccharides (6). Greater than 90% of the labeled glycoprotein material remained in the cetylpyridinium chloride supernatant but showed only 1.5% MAA activity, suggesting that the cetylpyridininum chloride treatment caused denaturation of the antigen.

On treatment of MAA-C with 8% trichloroacetic acid at 4°C, 82% of the radioactivity was precipitable. The precipitate showed only 6% MAA activity compared to about 60% in the starting material, suggesting inactivation of the antigen.

Chromatography of MAA-C on Immobilized Lectins—Chromatography on various concanavalin A-Sepharose or concanavalin A-acrylamide columns as described under "Experimental Procedures", results in recoveries varying from 22 to 32%. The reason for these poor recoveries is not clear. However, chromatography on a concanavalin A-acrylamide column at 37°C, instead of 25°C, and elution with a 5% solution of methyl-a-mannoside improved the recovery to about 80%. About 72% of the applied material passed through the column and was antigenically active.

Chromatography of MAA-C on WGA-Sepharose 4B and Ricinus communis agglutinin 120-Sepharose 4B columns resulted in 92 and 82%, respectively, of the radioactivity binding. However, the material eluted from these columns did not show any increase in MAA activity. Asialo-MAA, prepared by treatment of the purified material with neuraminidase, was poorly bound by the WGA-Sepharose (25%) but still interacted well with RCA-120 or RCA-60; 85% and 82% bound.

Estimation of the Molecular Size of MAA-C—Gel filtration of the purified MAA on a calibrated Sepharose CL-6B column using buffer with 0.1 or 1.0% Triton X-100 gave a single homogeneous peak (not illustrated). A semilogarithmic plot of molecular weights versus $K_v$ of glycoproteins with carbohydrate contents greater than 20% is linear (Fig. 3). Standards with lower carbohydrate contents (<10%) fall on a different straight line. Even though our results indicate that MAA-C is a sialoglycoprotein, we have no information on the percentage of carbohydrate in this molecule. If it is assumed that MAA-C has a carbohydrate content comparable to the glycoprotein standards falling on the linear plot, then MAA-C would have an apparent molecular weight of 375,000, whereas, if the carbohydrate content is low, a larger size is indicated.

![Fig. 3. Molecular weight of MAA-C](image-url)

Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radioactivity specifically bound by antimelanoma IgG</th>
<th>Binding index</th>
<th>Tritium activity specifically bound by WGA-Sepharose 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% NP-40 extract</td>
<td>2.8 1.7 1.6 1.5</td>
<td>ND 53</td>
<td>ND 92</td>
</tr>
<tr>
<td>After fractionation on Sepharose CL-4B</td>
<td>2.8 1.7 1.6 1.5</td>
<td>ND 53</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak I</td>
<td>0.5 1.4 1.1 1.2</td>
<td>ND ND</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak II</td>
<td>27.4 16.2 19.4 17.7</td>
<td>4.6 90</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak III</td>
<td>6.8 4.0 6.7 5.8</td>
<td>9.0 58</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak IV</td>
<td>0.6 0.4 1.6 1.4</td>
<td>49.0 12</td>
<td>ND 92</td>
</tr>
<tr>
<td>3 M KCl extract</td>
<td>6.7 2.7 1.2 1.5</td>
<td>ND ND</td>
<td>ND 92</td>
</tr>
<tr>
<td>After fractionation on Sepharose CL-4B</td>
<td>6.7 2.7 1.2 1.5</td>
<td>ND ND</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak I</td>
<td>2.9 1.2 2.6 2.2</td>
<td>ND ND</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak II</td>
<td>2.1 0.6 2.0 1.5</td>
<td>ND ND</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak III</td>
<td>0.5 0.1 1.5 1.1</td>
<td>ND ND</td>
<td>ND 92</td>
</tr>
<tr>
<td>NP-40 Sepharose CL-4B</td>
<td>76.8 68.7 70.7 99.6</td>
<td>14.0 92</td>
<td>ND 92</td>
</tr>
<tr>
<td>Peak II after purification on Sepharose CL-6B and DEAE-Sepharose</td>
<td>76.8 68.7 70.7 99.6</td>
<td>14.0 92</td>
<td>ND 92</td>
</tr>
</tbody>
</table>

* ND, not determined.
Since gel filtration of MAA-M in aqueous buffer resulted in elution as an aggregate, we examined the possibility of further dissociating MAA-C. When the sample was treated with 1% sodium dodecyl sulfate and 10 mM 2-mercaptoethanol at 25°C for 30 min and rechromatographed on the Sepharose CL-6B column using buffer containing 1% SDS and 10 mM 2-mercaptoethanol, there was no significant alteration in its elution position or its $K_w$. However, prolonged incubation (64 h or more) with 1% SDS at 50°C followed by a 4-h incubation with 10% 2-mercaptoethanol as described by Kawasaki and Ashwell (31) resulted in dissociation of MAA into smaller subunits (Fig. 4). The apparent subunit molecular weight was 44,000 based on a calibration curve obtained by running the reference glycoproteins in buffer containing 1% SDS and 2-mercaptoethanol. It is known that glycoproteins behave anomalously in SDS systems (32) and this may be the reason for the four points of the standards not lying on a straight line (Fig. 4). In this system, glycoporphin gave two peaks, a major one apparently corresponding to the dimer with $K_w$ 0.34, and a minor peak which is probably the monomer (33). A value of 23,500 was obtained for the apparent molecular weight of asialo-MAA, indicating that MAA has about 47% sialic acid. However, since it is known that sialylglycoproteins behave anomalously in the presence of SDS (32) and on gel filtration (34) other independent confirmations of the subunit molecular weight and sialic acid content are needed.

Electrophoresis of MAA-C using 6.5% or 5% acrylamide gels resulted in the sample failing to enter the gel; on 4% polyacrylamide gel the major portion of MAA-C entered the gel and had a mobility comparable to that of thyroglobulin. The samples, however, trailed instead of giving a sharp band. Polycrylamide gel electrophoresis of MAA in 5% gels in the presence of sodium dodecyl sulfate is illustrated in Fig. 5. A radioactive band was obtained corresponding to a molecular weight of 52,000 or 97,000 based on glycoprotein or pure protein standards, respectively, provided the sample was subjected to prolonged incubation with SDS and 2-mercaptoethanol. Shorter treatment with SDS (for example, heating for 10 min at 100°C) gave a band of radioactivity diffused over 6 cm of the gel (Fig. 5a).

**Sialic Acid and Hexosamine Analysis of MAA-C—**The proportion of sialic acid in the carbohydrate moiety of MAA-C was estimated by assessing the percentage of incorporated radioactivity released by mild acid hydrolysis (0.1 M H$_2$SO$_4$, 80°C, 1 h) or neuraminidase digestion followed by separation of the cleaved product by exclusion or ion exchange chromatography. The values ranged from 23 to 33%, probably reflecting some methodological variation. The identity of the monosaccharide released by neuraminidase treatment was confirmed as N-acetylmuramic acid by chromatography on Dowex 1 (formate) followed by paper chromatography utilizing Solvent A.

The ratio of radioactivity in glucosamine to that in galactosamine was found to be 14.0, whereas NP-40-extracted Sepharose CL-4B peak II material had a ratio of 4.6 (Table I) suggesting the removal of galactosamine-rich contaminants.

**Isolation and Characterization of Glycopeptides from MAA-C—**The MAA-C was treated with pronase, the digest frozen and thawed three times, centrifuged at 1000 $\times$ g for 10 min and the glycopeptides in the supernatant fractionated by gel filtration on a Bio-Gel P-10 column (Fig. 6). The distribution of radioactivity in the major glycopeptide fractions is given in Table II.

![Fig. 4. Gel filtration of MAA-C. Gel filtration of MAA-C (•) after prolonged treatment with SDS and 2-mercaptoethanol (see text for conditions) and of [14C]glucose (○) on a Sepharose CL-6B column (1.5 × 36 cm) using 50 mM Tris-HCl buffer, pH 8.0, containing 1% SDS and 10 mM 2-mercaptoethanol. The inset illustrates the calibration curve obtained with glycoprotein standards chromatographed under identical conditions. The peak elution position of asialo-MAA-C on the same column is also indicated.](image-url)
These glycopeptides as well as glycopeptides produced by the treatment of antigen with trypsin did not have appreciable antigenic activity. In agreement with this, the Class I and II glycopeptides isolated from culture medium and cells (7) were also devoid of antigenic activity. 

**Sialic Acid and Hexosamine Analysis of the Glycopeptides—**This distribution of tritium activity in sialic acid and hexosamines in the various glycopeptides is shown in Table II.

The chromatographic elution profiles of the asialoglycopeptides II and III on the Bio-Gel P-10 column are illustrated in Fig. 6.

**Treatment of Glycopeptides with Glycosidases—**The asialoglycopeptide III on treatment with *D. pneumoniae* β-galactosidase and β-N-acetylhexosaminidase released a labeled compound which was isolated by chromatography on a Bio-Gel P-2 column and desalted by passage through Dowex 50 (H⁺) and Dowex AG-1 (formate) columns. On a paper chromatogram the major product had a mobility identical with that of GlcNAc. A minor product had a mobility similar to that of lactose and is probably the product of the endoglycosidase D activity present in trace amounts in the β-galactosidase used. The major product was further characterized as [3H]GlcNAc by acid hydrolysis (3 M HCl at 100°C for 8 h) followed by detection of [3H]GlcNH₂ on a paper chromatogram developed in Solvent System B. In control experiments treatment of asialoglycopeptide III with the hexosaminidase failed to release any labeled products.

The treatment of glycopeptide VI and standard [3H]N-acetylovalbumin glycopeptides with endoglycosidase H followed.

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**Table II**

<table>
<thead>
<tr>
<th>Glycopeptide fraction</th>
<th>Distribution of radioactivity</th>
<th>Tritium activity in Specific activity bound to</th>
<th>Radioactivity specifically bound to</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sialic acid</td>
<td>GlcNH₂</td>
</tr>
<tr>
<td>I</td>
<td>4.0</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>II</td>
<td>19.0</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>36.0</td>
<td>28</td>
<td>66</td>
</tr>
<tr>
<td>IV</td>
<td>16.0</td>
<td>ND</td>
<td>98</td>
</tr>
<tr>
<td>V</td>
<td>5.0</td>
<td>ND</td>
<td>98</td>
</tr>
<tr>
<td>VI</td>
<td>14.0</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>VII</td>
<td>6.0</td>
<td>ND</td>
<td>94</td>
</tr>
</tbody>
</table>

*ND, not determined.*
by gel filtration on a Bio-Gel P-4 column gave the results shown in Fig. 7. The treatment of asialoagalacto-glu-
cosaminoglycopeptide III (see above) with endoglycosidase H gave several peaks, but only a minor peak eluted in the position of GlcNAc → Asn (Fig. 7c). Treatment of glycopeptide VI with endoglycosidase D did not result in the release of significant label.

**Affinity Chromatography of the Glycopeptides on Lectin-Sepharose Columns**—Glycopeptides VI and VII bound to a Con A-Sepharose 4B column and were specifically eluted with a 2% solution of methyl-α-mannoside. None of the glycopeptides showed affinity to a WGA-Sepharose 4B column, in contrast to the parent glycoprotein which was bound by this column (Tables I and II).

Asialoglycopeptide III bound to both *Ricinus communis* agglutinin 60- and 120-Sepharose 4B columns and was specifically eluted with 0.1 M lactose. The asialoglycopeptide III was subjected to hydrazinolysis (35) followed by chromatography on a Bio-Gel P-4 column; results are shown in Fig. 8.

Treatment of glycopeptide II with alkaline borohydride (1 M NaBH₄, 0.1 M NaOH, 37°C for 72 h) followed by neutralization and gel filtration on a Bio-Gel P-6 column indicated the presence of some oligosaccharides linked O-glycosidically to the peptide (Fig. 9). This is in agreement with the higher proportion of galactosamine in this glycopeptide fraction. Similar treatment of MAA-C resulted in the release of insignificant quantities of oligosaccharides (Fig. 9).

**Subcellular Location of MAA**—To obtain information on the localization of MAA in the cells, plasma membranes and nuclei were isolated from [³H]glucosamine-labeled cells. The isolation procedure involved differential and sucrose gradient centrifugation which has been shown to yield plasma membranes and nuclei free of significant contamination by other organelles. The membrane and nuclear pellets were then sequentially extracted with 3 M KCl and NP-40 detergent and dialyzed as described under "Isolation of MAA from Cells." The extracts were tested for MAA activity and the percentage of radioactivity specifically bound by the melanoma antisera were as follows: plasma membrane KCl extract, 3.9; plasma membrane NP-40 extract, 0.0; nuclei KCl extract, 5.8; and nuclei NP-40 extract, 6.3.

**Discussion**

Earlier studies indicated that some mouse melanoma-associated antigens are glycoproteins which were detectable both on the cells and in the culture medium (8, 10). We have now partially purified one of these antigen(s) and studied its biochemical properties.

Initial studies were conducted on the [³H]glucosamine- and/or [¹⁴C]leucine-labeled macromolecules released following gel filtration on Sepharose CL-4B and CL-6B. Column chromatography in the presence of detergents resulted in a marked shift of the antigenic material to lower molecular weight regions, suggesting that the MAA aggregated in the absence of detergents.

Examination of the material isolated from cells showed that the KCl-solubilized components were of lower molecular size and also had lower antigenic activity than the material subsequently extracted with NP-40 (Fig. 2 and Table I). It is possible that the material solubilized by KCl may have undergone partial proteolysis, since it has been demonstrated by Mann (36) that the release of histocompatibility antigens from lymphoid cells by 3 M KCl requires the presence of cell sap proteases. The 3 M KCl-extracted material was not further investigated. Subsequent treatment of the cell pellet after KCl extraction with 0.5% NP-40 solubilized over 90% of the labeled macromolecules in the pellet. The NP-40 extract was chosen for detailed studies of MAA because it had high quantities of the antigenic material and was also expected to have less fetal calf serum contaminants. Even though unlikely, we cannot completely exclude the possibility that the membrane-bound proteases in the cell pellet may have been active during the extraction with 0.5% NP-40 at 4°C and subsequent operations. Mann (36) found that only 3 to 6% of histocompatibility antigen was solubilized when the lymphoid cell membranes, free of cell sap, were extracted with 3 M KCl at 4°C for 24 h. Further, the treatment of MAA with Pronase or trypsin (see "Results") or with papain (8) completely abolishes its anti-genic activity. Whereas MAA exhibited a macromolecular structure with a tendency to precipitate in the absence of detergents, the glycopeptides derived from MAA were easily

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**Figure 8. Chromatography of glycopeptides after hydrazinolysis.** Chromatography of asialoglycopeptide III (●-●) and [³H]NeuAc α-1-acid glycoprotein (□-□) on a Bio-Gel P6 column (0.9 × 105 cm) after hydrazinolysis. Fractions of 1.0 ml were collected and analyzed for radioactivity. Peak elution positions of references are indicated. Prior to treatment with hydrazine asialoglycopeptide III and α-1-acid glycoprotein gave single peaks with peak positions at Fractions 45 and 29 (void), respectively.

**Figure 9. Gel filtration of MAA-C and glycopeptide II after borohydride treatment.** Gel filtration of MAA-C (●-●) and glycopeptide II (□-□) after treatment with alkaline borohydride on a Bio-Gel P6 column (0.9 × 105 cm). Fractions of 1.0 ml were collected and analyzed for radioactivity.
soluble in water and had molecular weights ranging from 4,000 to 12,000 (7). These data indicate that the MAA-C purified and characterized in this study has not been extensively degraded by proteolysis. The requirement of a detergent for solubilization from cells and aggregation behavior in the absence of detergents suggests that this macromolecule may be membrane-associated. However, MAA-C may not be the native form of the antigen present in the cell organelles (nuclei, membrane), and further studies are needed to resolve this problem.

Sequential chromatography of NP-40-extracted material having the highest MAA activity (Peak II, Fig. 2b) on Sepharose CL-4B, DEAE-Sepharose, and concanavalin A-Sepharose 4B columns gave apparently homogenous peaks of labeled material with increased specific antigenic properties.

Attempts to further purify the antigen were unsuccessful due to loss of antigenic activity. It was apparent from these results that both high pH (about 8.0) and detergent (0.1% Triton X-100) are essential to protect the antigenic properties of the isolated glycoprotein. Conditions of low pH, as encountered during chromatography using acetate buffer of pH 5.0, trichloroacetic acid precipitation, preparative isoelectric focusing, or desialylation with acid all resulted in the loss of antigenic activity. Recognition by antibody was also significantly reduced in the absence of detergent probably due to precipitation of the antigen.

The MAA-C preparations appeared radiochemically homogeneous when chromatographed on Sepharose CL-4B, Sepharose CL-4B, and DEAE-Sepharose (not illustrated). After prolonged treatment (72 h at 50°C) with SDS followed by gel electrophoresis in the presence of SDS a single but broad radioactive band was obtained. The band covers about 2 cm of the gel; however, 79 and 53% of the radioactivities fall within 1.0 and 0.6 cm of the gel, respectively. This diffuse band of 2 cm may be due to incomplete dissociation or partial reassociation of subunits. Electrophoresis of samples treated with SDS for shorter periods (10 min at 100°C (Fig. 5a) or 24 h at 50°C (not illustrated)) gave diffuse radioactive areas over 6 and 5 cm of the gel, respectively. An additional contribution to the gel behavior may be introduced by microheterogeneity in the carbohydrate moiety of the glycoprotein. The bulk (75 to 85%) of the labeled material in the purified MAA-C was specifically precipitated by an antisemur that reacted selectively with melanoma cells. These data suggest that our preparations consist of either a single glycoprotein exhibiting microheterogeneity or a group of antigenically related glycoproteins not separated by the above techniques. The presence of trace amounts of unlabeled material cannot be excluded even though other components were not detectable in column fractions (90) or gels (Coomasie blue staining). Since the characterization of MAA is based on the behavior of the labeled material, trace contaminants (if present) should not alter the major findings.

The antisemur used for monitoring the antigen had been raised to freshly excised B16 melanoma cells, a procedure which obviates the possibility of generating antibodies to neoantigens or other artifacts of cultured cells. On the basis of the specificity studies conducted to date with this antisemur (11, 25), the possibilities that the antigens it identifies are histocompatibility antigens, tissue-specific antigens of melanocytes, or one of the major type C viral proteins can be excluded. However, as in all studies of tumor antigens, the possibility that the purified antigen is simply one which is expressed to a greater degree on melanoma as opposed to unrelated cells cannot be completely discounted.

The molecular weight of MAA based on its mobility on a Sepharose CL-6B column in buffer containing 0.1 or 1.0% Triton X-100 was estimated to be about 375,000. Evidence for a strong association of monomer subunits even in the presence of 1.0% Triton X-100 was obtained when the sample was subjected to prolonged treatment with sodium dodecyl sulfate. Provided the MAA has a carbohydrate composition comparable to the glycoprotein standards used, values of 375,000 and 44,000 to 52,000 may be assumed for the native and subunit molecular weight, respectively. B16 mouse melanoma antigens having molecular weight in the range of 20,000 to 25,000 have been reported by Poskitt et al. (14). A more accurate determination of the molecular weight will be possible only after isolation of sufficient quantities of MAA for sedimentation equilibrium study and for quantitation of the carbohydrate content.

Some problems were experienced in determining the percentage of radioactivity present as sialic acid in MAA probably because of the insolubilization of the asialo-MAA in H2SO4 and/or the effect of the low pH (5.0) of the pyridine acetate buffer used to elute the column. Based on several experiments, we conclude that approximately 30% of the 3H radioactivity in the MAA isolated from cells is in sialic acid and that virtually all of it is released as N-acetylneuraminic acid by V. cholerae neuraminidase. The balance of the radioactivity was distributed between glucosamine (64%) and galactosamine (6%).

The glycopeptides obtained by fractionation of a Pronase digest were investigated to obtain some information on the structure of the parent glycoprotein. Seven fractions were isolated, three of which contained significant levels of radioactivity in sialic acid; the 3H-label in other glycopeptides was mainly in GlcNH2. Glycopeptides VI and VII interacted with concanavalin A-Sepharose and were cleaved by endoglycosidase H to release [3H]GlcNAc → Asn (Fig. 7). This indicated the presence of an oligomannosyl core saccharide attached to the peptide backbone.

The results of the chromatography of the asialoglycopeptides II and III on a Bio-Gel P-10 column (Fig. 6) suggest that the differences between these two and the other glycopeptides were due to reasons other than different degrees of sialylation. This was further apparent in the high proportion of GalNH2 (GaNH2/GlCNH2 = 0.2) in glycopeptide II compared to III. The release of oligosaccharides on treatment of glycopeptide II with alkaline borohydride was consistent with the high GalNHa content of this glycopeptide. However, the failure of the sialglycopeptide II to interact with WGA-Sepharose 4B indicates the absence of clustered sialooligosaccharides (O-glycosidically linked to peptide via serine and threonine) such as those present in the Class I glycopeptides isolated from B16 mouse melanoma cells (7, 24).

The release of GlcNAc on treatment of asialoglycopeptide III with β-galactosidase and β-N-acetylgalactosaminidase but not with β-N-acetylgalactosaminidase alone indicates the sequence Gal → GlcNAc → in this glycopeptide. The presence of this sequence was also suggested by the interaction of the asialo-glycopeptide III and of the asialo-MAA itself with Ricinus communis agglutinin.

The release of oligosaccharide from the asialoglycopeptide III on hydrazinolysis and the susceptibility to endoglycosidase H of glycopeptide III which has been pretreated with neuraminidase, β-galactosidase, and β-N-acetylgalactosaminidase indicated the presence of GlcNAc → Asn in this glycopeptide, as well.

In summary, we have succeeded in isolating an antigen associated with B16 mouse melanoma cells and purifying it to

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3 V. P. Bhavanandan, J. G. Kemper, and J.-C. Bystryn, unpublished results.
apparent homogeneity. The isolated antigen is a glycoprotein with a heterogenous population of oligosaccharides, which consist mainly of those terminating with the sequence NeuAc → Gal → GlcNAc→; some are terminated with mannosyl residues. These oligosaccharides, because of their susceptibility to endoglycosidase H and to hydrazinolysis, are presumably linked via N,N'-diacet tylcithitobiose to asparagine residues. These oligosaccharides, because of their susceptibility to endoglycosidase H and to hydrazinolysis, are presumably linked via N,N'-diacetylchitobiose to asparagine residues of the peptide chain. The presence of a few GaINAc-containing О-glycosidically linked oligosaccharides is indicated. The antigen was also partially purified from culture medium and some activity was associated with nuclei.

The information obtained in this study on the characteristics of MAA would enable us to isolate milligram quantities of this antigen from solid tumors (19) or from serum-free spent culture medium (18). Future studies will be directed toward this antigen.

Acknowledgments—We wish to thank Ms. Anne Katlic, Ms. Cory Ann Tedholm, and Mr. Michael Atchison for technical assistance; Mr. John Banks for help with cell culture; Mrs. Joy Sizgorich for typing the manuscript and Dr. E. A. Davidson for his interest in this work, support, and encouragement.

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