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Immunology

AU cell-surface antigen of human malignant melanoma: Solubilization and partial characterization

(cancer immunology/autologous typing/class 1 tumor antigen/glycoprotein)

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ABSTRACT **AU antigen is defined by reactions of sera from patient AU with cell-surface antigens of cultured autologous melanoma cells (SK-MEL-28). Past studies established that no available cell type other than AU melanoma expressed AU antigen. By use of antibody inhibition tests for antigen detection, limited papain digestion of AU melanoma cells was found to result in the solubilization of AU antigen along with β_2-microglobulin (β_2m) and HLA allogeneic and xenogeneic specificities. Comparable papain treatment of other melanoma and nonmelanoma cell lines solubilized β_2m and HLA, but did not result in the release of antigen with AU reactivity. Maximum yield of AU antigen from AU melanoma cells was obtained after very short (5-15 min) digestion times in contrast to the more prolonged proteolysis required for maximum HLA and β_2m release. AU antigen was not immunoprecipitated by rabbit antiserum against β_2m or HLA under conditions leading to partial or complete removal of β_2m and HLA. At least a proportion of the molecules with AU determinants appear to be glycoproteins, as indicated by specific affinity for *Lens culinaris* hemagglutinin (LcH). After affinity chromatography on LcH-agarose, the specific activity of AU antigen was increased 50-fold. As determined by gel filtration chromatography, AU antigen has a molecular weight in the range of 20,000-50,000.**

In a serological study of cultured melanoma cells from a series of 35 patients, one patient (AU) was found to have high-titered antibody to surface antigens of autologous melanoma cells (1). Absorption analysis of AU sera indicated that the antigen being detected was restricted to AU melanoma cells and could not be found on autologous fibroblasts or peripheral blood cells or on any of the allogeneic melanoma cell lines or other cell types tested. Four additional examples of such restricted antigens have been defined by comparable autologous typing of cultured human cancer cells—the BD and BI antigens of melanoma (2, 3) and the AC and BC antigens of astrocytoma (4). These individually distinct or unique tumor antigens, of which AU is the prototype, have been termed class 1 antigens to distinguish them from various classes of shared tumor antigens (4).

As an approach to the characterization of AU antigen, we attempted to immunoprecipitate AU antigen from extracts of radiolabeled AU melanoma cells by AU serum, but these initial efforts proved unsuccessful. Because of this, we turned to the use of antibody inhibition tests as a way to follow the solubilization and characterization of AU antigen.

MATERIALS AND METHODS

Tissue Culture. Cell lines and tissue culture procedures have been described (1, 2). Two T-cell lines (HSB-CEM and JURKET) and a B-cell line (35M) were obtained from John Hansen (Sloan-Kettering Institute).

Antisera. The following typing reagents were used. (*i*) Sera from patient AU that detect AU cell-surface antigen on SK-MEL-28, the cultured line of autologous melanoma. The AU antigen was originally detected by mixed hemadsorption assays (1); the same specificity can be detected by protein A hemadsorption assays (see below). These two techniques are now used interchangeably to detect AU. (*ii*) Rabbit antiserum against human β_2-microglobulin (β_2m) (DAKO-immunoglobulin), purchased from Accurate Chemical and Scientific Corp. (Hicksville, NY). (*iii*) Rabbit antiserum against human HLA (no. 6095-3), provided by N. Tanagaki (Roswell Park Memorial Institute, Buffalo, NY). (*iv*) Human HLA typing sera, provided by M. Fotino (New York Blood Center) and C. Whitsett, J. Lee, and B. Dupont (Sloan-Kettering Institute).

Serological Procedures. The mixed hemadsorption assay (MHA) and the protein A hemadsorption assay (PA-HA) were carried out as described (1, 4).

Antibody Inhibition Tests. For quantitation of AU, β_2m, and HLA activity in papain digests, we used antibody inhibition tests. Antisera were tested at a concentration falling two doubling dilutions below their end point for target cells (see Fig. 1). Serial dilutions of the papain digests were prepared and each dilution was mixed with an equal volume (25 μl) of appropriately diluted antiserum. The mixtures were allowed to stand for 20 min at 23°C and 20 min at 4°C. Starting with the highest dilution of antigen, the mixtures were pipetted into the wells of microtest plates containing washed target cells. After incubation with target cells for 45 min at room temperature, unbound antibody was washed away and MHA or PA-HA indicator cells were added for a further 45-min incubation at room temperature. The plates were then washed and read.

Inhibitory units were calculated from the dilution of papain digest that inhibited antibody reactivity by one-half. Thus, if the unabsorbed serum resulted in 60% positive cells and the digest reduced that to 30% at a dilution of $^1/_{16}$, then the $^1/_{16}$ antigen dilution contained 1 inhibitory unit and the starting material had 16 units of inhibitory activity in its initial volume. In most experiments the initial volume of undiluted digest was 25 μl; therefore, in the example cited there would be 16 units/25 μl × 1000 μl/ml = 640 units/ml.

Papain Solubilization. Papain was purchased from Worthington. Immediately prior to use, it was suspended in NaCl/Tris buffer (0.15 M NaCl/0.01 M Tris·HCl, pH 7.2/0.05% NaN_3) and activated by 10 mM cysteine (ICN) and 1 mM

Abbreviations: MHA, mixed hemadsorption assay; PA-HA, protein A hemadsorption assay; LcH, *Lens culinaris* lectin; β_2m, β_2-microglobulin.

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EDTA. In initial studies, monolayer cells were harvested by scraping with a rubber policeman. The cells were washed three times in phosphate-buffered saline and resuspended in activated papain solution (4–10 units/ml) to a concentration of 5–10 × 10^7 cells per ml and the cell suspension was agitated in a shaking water bath at 37°C for 30 min. If DNA clots appeared, DNase (Worthington), was added to the digest. At the end of the incubation, the cells were sedimented at 1000 × *g* for 10 min at 4°C and the supernatant was mixed with iodoacetamide (ICN) to a final concentration of 13 mM. The supernatant was then filtered through 0.45- and 0.22-μm membrane filters (Nalge Co., Rochester, NY) to remove particulate material and dialyzed overnight at 4°C against Earle's balanced salt solution. Precipitates that formed during dialysis were removed by centrifugation at 4000 × *g* for 10 min. The clarified supernatants were concentrated by ultrafiltration on PM10 retention filters (Amicon, Lexington, MA) to the point that each milliliter of the concentrated papain digest represented solubilized material from 2–4 × 10^8 cells. The samples were then centrifuged at 100,000 × *g* for 1 hr and tested for inhibitory activity as described above.

In later experiments (see *Results*), papain digestion was carried out with monolayer cells *in situ*. Roller bottles with confluent monolayers were decanted to remove culture fluid and washed with NaCl/Tris buffer. Activated papain was added in aliquots of 15 ml/490 cm^2 roller bottle (equivalent to 6–10 units/5–10 × 10^7 cells per bottle), and the roller bottles were returned to the roller apparatus at 37°C for the appropriate time. During incubation with papain, the cells generally became detached within 3–5 min and could be recovered for counting. After incubation, the cells and papain digest were poured into chilled tubes in ice, the bottles were washed with 15 ml of NaCl/Tris buffer, and the wash and cell suspensions were combined. The cells were removed by centrifugation at 1000 × *g* for 20 min at 4°C, and iodoacetamide (final concentration 13 mM) and 20 units of Aprotinin per ml (Trasylol, FBN Pharmaceuticals, New York) were added to the supernatant. The digests were then filtered, centrifuged, and concentrated as described above. Direct papain treatment of monolayer cells rather than of mechanically detached cells in suspension resulted in extracts with higher specific activity of AU, β_2m, and HLA.

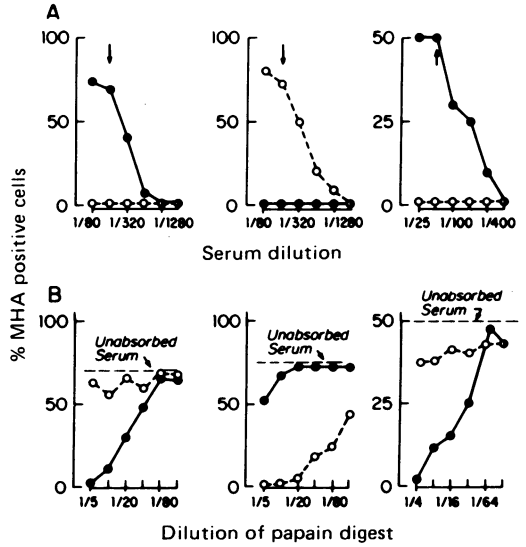

FIG. 1. (*A*) Titrations of HLA-A11 (*Left*) and HLA-A2 (*Center*) alloantisera and AU serum (*Right*). Target cells: ●, SK-MEL-28 melanoma cells; ○, SDE-1 epithelial cells. Serological assay: MHA. Arrows indicate the serum dilution selected for antibody inhibition tests. (*B*) Antibody inhibition tests of papain digests of SK-MEL-28 (●) and SDE-1 (○). Tests for HLA-A11 (*Left*), HLA-A2 (*Center*), and AU (*Right*) antigens.

Protein Assays. Protein content was determined by measuring the absorbance at 280 nm when the A_{260}/A_{280} ratio was less than 1.0. More accurate values were obtained by the Coomassie blue procedure (5) using bovine serum albumin as a protein standard.

Immunoprecipitation Tests. Papain digests (300–400 μl) were mixed with 100 μl of (*i*) undiluted rabbit anti-β_2m serum, (*ii*) undiluted rabbit anti-HLA serum, or (*iii*) phosphate-buffered saline and allowed to stand at 4°C for 3–4 hr. One milliliter of a 10% suspension (in phosphate-buffered saline) of washed, heat-killed, and fixed *Staphylococcus aureus* (Cowan Strain-I; IgG Sorb, New England Enzyme Center, Boston, MA) were added to each mixture. After 1 hr at room temperature with occasional mixing, the Staph A cells were removed by centrifugation at 4000 × *g* for 20 min at 4°C. The supernatant was concentrated to the original digest volume and tested for residual β_2m or HLA antibody by the PA-HA assay with SK-MEL-28 as target cells. If residual antibody was present, a second batch of Staph A was added and the procedure was repeated. The digests were then tested for AU, β_2m, and HLA activity by antibody inhibition tests.

RESULTS

Solubilization of AU and HLA Antigens by Papain Treatment of Cultured Cells. Suspensions of SK-MEL-28 cells (AU melanoma cell line) and SDE-1 cells (an unrelated epithelial cell line of human origin) were exposed to papain under conditions that result in HLA release from other cell types. The papain digests were assayed by antibody inhibition tests for AU antigenic activity and for two HLA-A alloantigens that distinguish SK-MEL-28 and SDE-1 (Fig. 1). In conformity with their surface phenotypes, papain digests of SK-MEL-28 contained HLA-A11 and AU activity but lacked HLA-A2 activity, whereas papain digests of SDE-1 contained HLA-A2 activity but lacked HLA-A11 and AU activity. Comparable papain digests were prepared from cultured fibroblasts of patient AU. Typing of AU fibroblasts indicated that these cells express HLA-A11 but not AU antigen. In antibody inhibition tests, HLA-A11 activity was detected in papain digests of AU fibroblasts, whereas AU activity was not.

Tests for AU Antigen in Papain Digests of Allogeneic Melanoma Cell Lines and Other Human Cell Lines. Papain

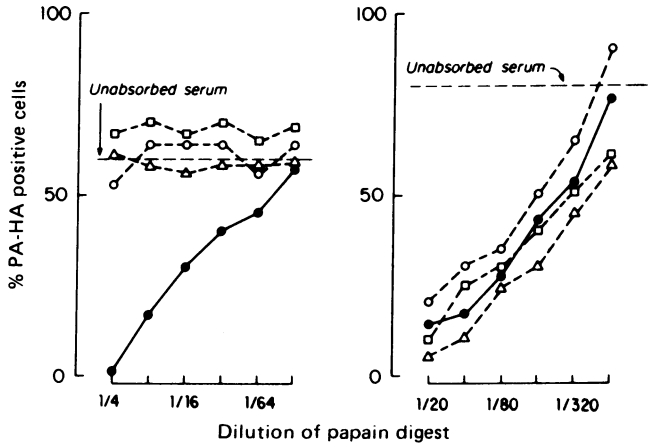

FIG. 2. Antibody inhibition tests of papain digests of SK-MEL-41 (□), SK-OV-3 (△), T-24 (○), and SK-MEL-28 (●). Tests for AU (*Left*) and β_2m (*Right*) activity. Serological assay: PA-HA. Target cells: SK-MEL-28 melanoma cells.

Table 1. Summary of antibody inhibition tests for AU antigen in papain digests of cultured human cells

Inhibition	No inhibition		
	Cultured normal cells	Cultured nonmelanoma cells	Cultured melanoma cells
SK-MEL-28 (AU)	AU fibroblasts	SDE-1	SK-MEL-5
	HFLF-1 (fetal lung fibroblasts)	HSB-CEM (T-cell line)	SK-MEL-13
		JURKET (T-cell line)	SK-MEL-26
		35M (B-cell line)	SK-MEL-27
		T-24 (bladder carcinoma)	SK-MEL-29
			SK-MEL-37
		SK-OV-3 (ovarian carcinoma)	SK-MEL-41
			SK-MEL-50
			Me Wo

digests were prepared from 16 human cell lines under standard conditions and tested for AU activity. As a way to monitor papain solubilization, each extract was also tested for β_2m or HLA activity. The results of one such inhibition test is illustrated in Fig. 2, and the results of all tests are summarized in Table 1. AU activity was demonstrable only in extracts of SK-MEL-28. Extracts of other lines, including nine allogeneic melanoma cells, lacked AU antigen. Thus, these antibody inhibition tests with papain extracts and past absorption tests with viable cells (1) lead to the same conclusion: AU antigen expression is restricted to autologous melanoma cells.

Rate of AU, β_2m, and HLA Solubilization by Papain. Monolayers of SK-MEL-28 were exposed to papain for various time intervals and the resulting digests were tested for AU, β_2m, and HLA activity. As indicated by representative tests in Table 2, the maximum yield of AU was obtained after brief exposure to papain. Longer digestion resulted in a lower yield of AU. In contrast, the amount of HLA and β_2m increased as a function of the time of digestion (up to 90 min). In light of these results, 5-min papain digests were used in subsequent studies.

Immunoprecipitation with Rabbit Antisera against β_2m and HLA. To determine whether molecules with AU activity carry β_2m or HLA determinants, we immunoprecipitated papain digests of SK-MEL-28 with antisera against β_2m or HLA. As described in *Materials and Methods*, *S. aureus* was used to remove antigen–antibody complexes and the supernatants were tested for AU antigen and for residual β_2m and HLA. As shown in Fig. 3*A*, β_2m activity in the papain digest was reduced by β_2m antiserum, whereas AU activity was unaffected. Similarly, HLA antiserum reduced HLA activity but did not affect AU activity (Fig. 3*B*).

Lectin-Binding Characteristics of Papain-Solubilized Antigens. Fractionation of an SK-MEL-28 papain digest by affinity chromatography on a *Lens culinaris* (LcH)-agarose column is shown in Fig. 4. Fractions were combined to give two pools of material corresponding to unbound (fractions 1–10) and eluted (fractions 19–30) samples, and the AU, β_2m, and HLA activity of each sample was determined by antibody inhibition tests. In terms of total antigenic activity, unbound and mannoside-eluted fractions contain similar amounts of AU, β_2m, and HLA. However, the specific activity (inhibitory units/mg of protein) of AU, HLA, and β_2m was 30–50 times greater in the mannoside-eluted fraction (Fig. 4).

Gel Filtration of Papain-Solubilized Antigens. A papain digest of SK-MEL-28 was fractionated on a Sephadex G-150 column; the elution profile is shown in Fig. 5*A*. The fractions were combined to form five pools (A–E) and each pool was tested for AU, β_2m, and HLA activity (Fig. 5*B*). The major peak of AU activity was eluted immediately after the major protein

Table 2. Rate of AU, β_2m, and HLA solubilization by papain

Exp.	Antigen	Time of digestion, min*				
		5	15	30	60	90
1	AU	640	160	160	160	
2	AU		256	80	80	40
3	AU	320	160	40	40	20
	β_2m	1600	6400	6,400	12,800	>25,600
	HLA-A11	80	160	160	640	640
4	AU	320		80		
	β_2m	2560		10,240		
	HLA (xenogeneic determinants)	640		1,280		

For details concerning concentration of papain extracts and calculations of inhibitory units, see *Materials and Methods*.
*Values are inhibitory units/ml.

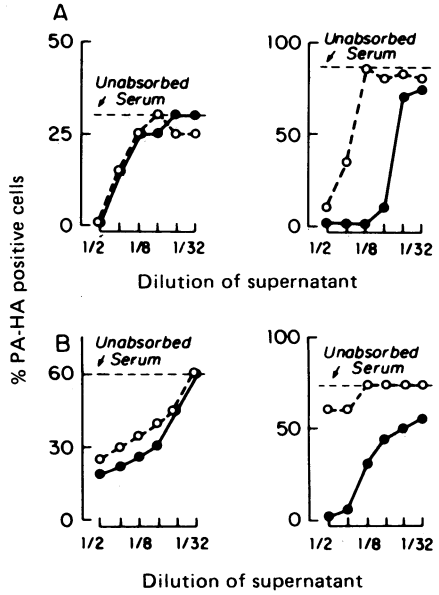

FIG. 3. (*A*) Immunoprecipitation of SK-MEL-28 papain digest by rabbit anti-β_2m serum. Residual AU and β_2m activity in the digest was assayed by antibody inhibition tests with AU serum (*Left*) and anti-β_2m serum (*Right*). ○, Anti-β_2m serum + *S. aureus*; ●, *S. aureus*. (*B*) Immunoprecipitation of SK-MEL-28 papain digest by rabbit anti-HLA serum. Residual AU and HLA activity in the digest was assayed by antibody inhibition tests with AU serum (*Left*) and anti-HLA serum (*Right*). ○, Anti-HLA serum + *S. aureus*; ●, *S. aureus*. Target cells: SK-MEL-28 melanoma. Assay system: PA-HA.

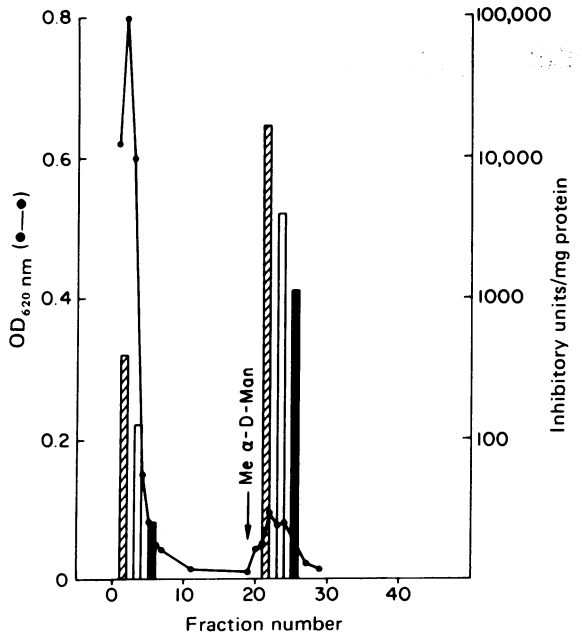

FIG. 4. LcH-agarose affinity chromatography of a papain digest of SK-MEL-28 cells. The sample (containing solubilized material from 5×10^8 cells) was applied to a column (10×1.1 cm) containing 8 ml of gel (1.2 mg of lectin per ml) at 4°C and unbound material was eluted with NaCl/Tris buffer. Bound glycoproteins were then removed by elution with 0.5 M methyl α-D-mannopyranoside in NaCl/Tris buffer. The protein content of the fractions was monitored by the Coomassie blue method ($OD_{620\ nm}$). Unbound fractions (1–10) and mannoside-eluted fractions (19–30) were separately pooled, dialyzed against NaCl/Tris buffer, concentrated by ultrafiltration (Amicon, PM10 membrane), and assayed for AU (■), β_2m (▨), and HLA (□) activity by antibody inhibition tests. Inhibitory units per mg of protein in the pooled fractions are indicated by the bars located in the region of the respective protein peaks.

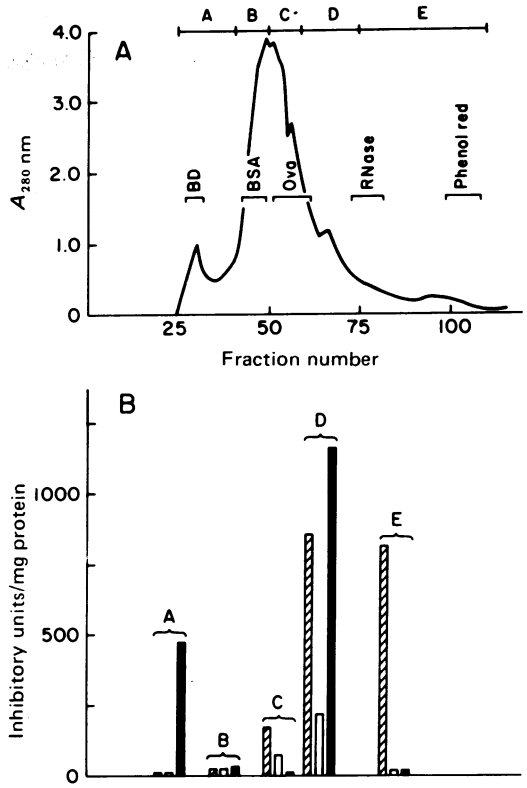

FIG. 5. (*A*) Sephadex G-150 gel filtration chromatography of a papain digest of SK-MEL-28 cells. The gel column (0.9×45 cm) was equilibrated with NaCl/Tris buffer and the sample (concentrated 10-fold to contain approximately 5×10^8 cell equivalents in 100 μl) was applied. The column was eluted with NaCl/Tris buffer at 4°C and the protein in the eluate was monitored at 280 nm. The fractions were combined into five pools, labeled A–E as shown. Molecular weight markers used to calibrate the column: blue dextran (BD), 1×10^6; bovine serum albumin (BSA), 68,000; ovalbumin (Ova), 45,000; ribonuclease (RNase), 14,500; phenol red, 354. (*B*) Antibody inhibition tests of pooled fractions from Sephadex G-150 column. Pools A–E were concentrated by ultrafiltration (Amicon, PM10 membrane) to the original volume (100 μl) and assayed for AU (■), β_2m (▨), and HLA (□) inhibitory units/mg of protein.

peak in a molecular weight range of 20,000–50,000; HLA inhibitory activity also eluted in this region. Two peaks of β_2m activity were found: one in the D pool (presumably associated with HLA) and one in the E pool (presumably in the free form). Approximately one-third of the AU activity eluted in the void volume of the column.

DISCUSSION

Tumor antigens such as AU, whose expression is restricted to a single tumor, have now been identified by serological typing with autologous sera in four human tumors (1–4). These antigens, which have been referred to as class 1 antigens to distinguish them from other classes of tumor antigens, resemble the individually distinct or unique transplantation antigens that characterize chemically induced sarcomas and other tumor types of rodents (see ref. 6). Considerable interest is presently focused on the serological identification of class 1 antigens of experimental tumors as a way to facilitate analysis of these antigens. Thus far, two antigens with class 1 characteristics have been serologically defined in chemically induced sarcomas of the mouse (6, 7), and biochemical and genetic analysis of these systems is under way (8, 9). Comparable studies with class 1 antigens of human cancer are now also possible, and the present report records our initial efforts to solubilize and characterize AU, a defined class 1 antigen of melanoma.

AU antigen is rapidly solubilized by papain, and this feature distinguishes AU from β_2m and HLA, which require more prolonged papain treatment for maximum release. Co-immunoprecipitation experiments provide another way to distinguish AU from β_2m and HLA, and the results indicate that AU determinants are not carried by molecules with either β_2m or HLA heavy chain determinants. Expression of Ia-like antigens on the surface of melanoma cells has recently been reported (10). These antigens, which are presumed to be products of the *HLA-D* locus, had previously been thought to be restricted to B cells, macrophages, and other cells of hematopoietic derivation (11, 12). The level of Ia antigen on different melanoma cell lines varies greatly, with some lines exhibiting strong Ia antigen expression, others intermediate levels, and the remainder having little, if any, demonstrable Ia antigen. Because the AU melanoma cell line belongs to the last category, it seems unlikely that AU is related to the Ia system. Nevertheless, this possibility is being explored.

At least a proportion of molecules with AU determinants appear to be glycoproteins, as indicated by affinity for *Lens culinaris* lectin and elution from an LcH-agarose column by methyl α-D-mannoside. However, approximately half of the AU activity in the papain digest is not retained by LcH-agarose; this could be due to heterogeneity in the structure of the carbohydrate moieties (13) or to AU molecules lacking carbohydrate chains. With regard to the molecular weight determination of AU antigen, the estimate of 20,000–50,000 cannot be considered more than provisional, especially in light of the apparent sensitivity of the antigen to progressive degradation by papain. For this reason, more definitive measurement of the

size of the native AU antigen awaits solubilization by other methods.

Lens culinaris lectin chromatography provides a useful purification step for AU, with the bound fraction showing a 50-fold increase in specific activity over the initial extract. This procedure also concentrates HLA and β_2m, but these components can easily be removed by affinity chromatography with anti-β_2m and anti-HLA heavy chain sera. An important next step will be to produce heteroantibody, in the form of either conventional immune sera or hybridoma products, against partially purified AU antigen. Heterologous reagents of this sort should permit the development of simpler and more sensitive assays for AU and be of sufficient titer to precipitate AU from radiolabeled extracts (something that has not been possible with human AU sera) for a more decisive characterization of the AU molecule. A key question about AU is whether it represents a unique determinant or set of determinants on a molecule common to all melanomas or a unique molecule present only on a single melanoma. With heteroantibody to the range of determinants on the AU molecule, it should be possible to answer this and other critical questions about class 1 tumor antigens.

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