Melanoma Antigens That Produce Cell-Mediated Immune Responses in Melanoma Patients: Joint U.S.–U.S.S.R. Study

Abstract. *Melanoma extracts used in the U.S.S.R. for immunochemotherapy of melanoma patients are found to contain a glycolipoprotein that is very similar to the melanoma tumor-associated antigen used in the United States for skin tests.*

Since 1956, under the guidance of Professor L. A. Zilber, cancer immunotherapy studies have been in progress at the Hertsen Oncology Institute, U.S.S.R. A current method of immunochemotherapy of melanoma patients includes the use of polysaccharide complexes extracted from melanoma tissue (*1*).

In the United States, 113 cancer patients have now been skin tested for delayed hypersensitivity reactions with purified proteins (antigens) separated from the surface membranes of malignant melanoma cells (*2 4*). One of these proteins, a tumor-associated antigen (TAA), is highly specific for melanoma, producing positive skin reactions in (43 of 49) 88 percent of early stage skin melanoma and ocular melanoma (where the tumor is confined to one site) patients as compared to (1 of 34) only 3 percent positivity in patients with other types of cancer. In patients with advanced stages of melanoma (7 of 19) 37 percent were skin positive. Among the 34 control patients were 5 patients with ocular lesions simulating melanoma, 9 breast cancer patients, and 20 patients with cancers other than melanoma and without ocular disease. The melanoma-specific antigen, prepared from primary melanoma tumor cells, is a glycolipoprotein. A search was made for the antigen in five types of metastatic tissues of melanoma patients and skin tests were conducted in 11 melanoma patients (6 early stage and 5 late stage); some positive delayed hypersensitivity skin test reactions were seen to the antigen from liver (5 of 6, early stage; 2 of 5, late stage), lung (3 of 11), and adrenal (4 of 11) metastases, whereas negative skin tests were seen for antigens from skin and colon metastatic tissues (*4*).

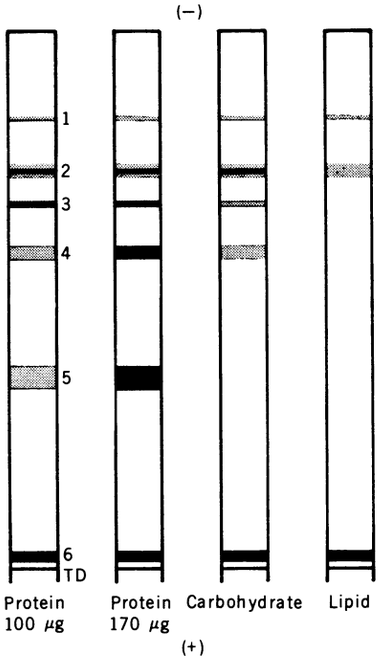

Fig. 1. PAGE drawings of U.S.S.R. polysaccharide complexes extracted from melanoma tissue. A good concentration of material positive for periodic acid Schiff (PAS) stain for carbohydrate is shown. In addition, protein (stained by CBB) and lipid (stained by ORO) components are present. Region 1 contains a glycolipoprotein which is a TAA. This is also the TAA identified in PAGE separations of U.S. primary melanoma soluble membrane antigens. Tracking dye (*TD*) was bromphenol blue. Delayed hypersensitive reactions at 48 hours to skin tests were specific for the glycolipoprotein of region 1.

In the U.S. studies of the separation, identification, and analysis of the melanoma antigens (*2, 4*), we have used a special method (*5*) of polyacrylamide gel electrophoresis (PAGE), free of sodium dodecyl sulfate (SDS), in which the separations were performed with discrete, carefully assessed melanoma cells, membrane extraction, Sephadex G-200 separation of the soluble membrane components, and subsequent PAGE in which the materials are separated on stacked gels of 3.5 percent, 4.75 percent, 7 percent, and 10 percent. The bands were stained with Coomassie brilliant blue (CBB) for protein, with periodic acid–Schiff reagent (PAS) for carbohydrate and with oil red O (ORO) for lipid. By conducting a similar study on the antigen from the U.S.S.R. we could determine whether or not the material which produced a cell-mediated immune response in the patients undergoing immunotherapy in the U.S.S.R. might contain similar components.

As shown in Fig. 1, the Soviet extracts of melanoma tissue did contain highly concentrated amounts of polysaccharide. In addition, certain protein and lipid components were also present. A component of very low molecular weight settled near the tracking dye and stained black rather than blue for protein, and gold rather than pink for carbohydrate. This material was probably a mixture of minor blood components or melanin components and did not interfere with the separation. Of even greater importance was the presence of glycolipoprotein band (PAGE region 1) as illustrated in Fig. 1 and shown in a photograph of the gel separations in Fig. 2.

An analysis of the separated, primary melanoma, soluble membrane antigens (*4*) of cancer cells from American patients indicated that the cathodic components separated by PAGE consisted of three major band regions. Although present in different quantities, the same bands, 1, 2, and 3, are present in the U.S.S.R. and U.S. materials. The PAGE region 1 band contained glycolipoprotein, and it was this material that produced specific delayed hypersensitivity skin reactions in melanoma patients. The same glycolipoprotein is present in the U.S.S.R. antigen, as shown in Figs. 1 and 2.

This glycolipoprotein (TAA), isolated by PAGE (*4*), was injected into New Zealand white rabbits at a dose of 100 μg with Freund's complete adjuvant, followed by a 100-μg booster injection 1 month later. Two weeks after the last injection the rab-

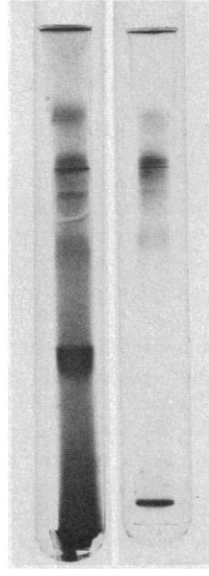

Fig. 2. Photograph of the PAGE separations of the U.S.S.R. antigens; drawings of the separations are shown in Fig. 1 in order to more clearly show bands present. On the left is the protein stained with CBB and on the right the carbohydrate components stained with PAS.

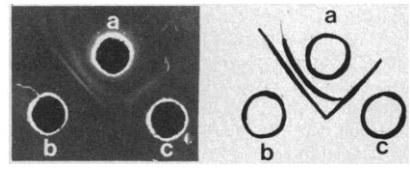

Fig. 3. Precipitin patterns observed in double diffusion agarose plate tests with melanoma TAA antiserum (a) (for specificity, see text) for testing U.S.S.R. melanoma carbohydrate extract (b) and U.S. melanoma TAA (c).

bits were bled. Precipitin patterns were observed in double diffusion agarose plate tests with the rabbit melanoma TAA antiserum for testing against melanoma TAA, neuroblastoma antigens, colon cancer antigens, fetal skin antigens, other less specific melanoma-associated antigens (*4*), and purified albumin. A specific precipitin band formed with the melanoma TAA, but not with the other antigens tested. A nonintersecting, different band formed with purified albumin. The U.S.S.R. melanoma carbohydrate extract was then compared with melanoma TAA with the antiserum for analysis of common precipitin patterns. As shown in Fig. 3, the melanoma TAA present in the U.S.S.R. extract (70 μg) was serologically identical to the U.S. melanoma TAA (10 μg). A slightly skewed pattern developed since a stronger concentration of the U.S.S.R. antigens was used. The additional band formed was similar to that for albumin. When antiserums were absorbed against purified albumin this band disappeared.

In conclusion, a very similar major specific TAA was isolated from melanoma tissue in both countries. This glycolipoprotein produces a specific cell-mediated immune response in melanoma patients of both places. This assures us that a continued comparison of ongoing studies of the immune responses of melanoma patients carried out in both countries for diagnostic, monitoring, or therapeutic purposes will be possible.

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Thermal Transitions in Human Plasma Low Density Lipoproteins

Abstract. *Thermal analysis of human plasma low density lipoproteins reveals a broad reversible transition encompassing body temperature. The calorimetric and x-ray scattering data identify this transition as a cooperative, liquid-crystalline to liquid phase change involving the cholesterol esters in the lipoprotein. This behavior requires the presence of a region rich in cholesterol ester within the lipoprotein.*

Although low density lipoprotein (LDL) is the major cholesterol-carrying lipoprotein in human serum and has been implicated in the etiology of atherosclerosis, its structure and the intermolecular forces required to maintain its individually insoluble lipid and protein moieties as a soluble particle have not been well defined. Essential to such an understanding is a knowledge of the lipid-lipid and lipid-protein interactions within the particle. The interactions between different lipid classes, such as phospholipids, free and esterified cholesterol, and triglycerides have been studied by, for example, differential scanning calorimetry, polarizing light microscopy, x-ray diffraction, and various spectroscopic methods (*1–3*).

Thermal transitions, reflecting either polymorphic or liquid crystalline transitions, have been observed with phospholipids, cholesterol esters, and triglycerides, and in addition, calorimetric methods have revealed phase transitions associated with the lipid and protein components of cell membranes (*4*). In this report, we describe a reversible transition due to a cooperative order-disorder phase change involving the cholesterol esters within intact LDL, and an irreversible transition associated with lipoprotein denaturation.

We isolated LDL from plasma of normal human donors by preparative salt density ultracentrifugation (*5*), and concentrated it to about 200 mg of LDL per milliliter (*6*). The protein concentration was determined by the method of Lowry *et al.* (*7*), and the total LDL concentration was estimated from the protein content and by dry weight determination. Lipid composition was analyzed by quantitative thin-layer chromatography (*8*) of the chloroform-methanol-extracted lipids (*9*). Individual lipid classes were isolated by preparative thin-layer chromatography.

Six preparations of LDL were studied with a Perkin-Elmer DSC-2 differential scanning calorimeter at a full-scale sensitivity of 0.1 to 0.2 mcal/second with heating and cooling rates of 10°C per minute. Differential scanning calorimetry curves of a typical LDL preparation are shown in Fig. 1. Concentrated native LDL shows a reversible transition between 20° and 45°C. The peak of the endothermic transition occurs, depending on the sample (*10*), between 29° and 38°C, and the enthalpy (ΔH) of this transition is 0.4 ± 0.1 cal per gram of LDL lipid (mean ± standard deviation). The transition is unchanged on repeated heating between 0° and 45° (Fig. 1a), and it is not significantly affected by cooling the LDL samples to −60°C and reheating (Fig. 1, b and d). On cooling, little supercooling, if any, is observed (Fig. 1c). Finally, the transition is unaffected over a 30-fold change in LDL concentration (13 to 400 mg/ml) or by NaBr concentrations up to 2*M* NaBr.

An irreversible transition (ΔH = 0.9 ± 0.2 cal per gram of LDL protein), seen only on initial heating, occurs between 70° and 90°C (Fig. 1d) and corresponds to irreversible changes in the optical properties of LDL observed by polarizing light microscopy (*11*). We have defined these irreversible changes as "lipoprotein denaturation." Following denaturation and cooling to −60°C, a transition is present at 5° to 45°C (Fig. 1e) with an enthalpy at least five times greater than that of the initial reversible transition (Fig. 1, a to d).

X-ray scattering and diffraction from LDL and its cholesterol ester extracts (Fig. 2) were recorded at temperatures above (45°C) and below (10°C) the low temper-

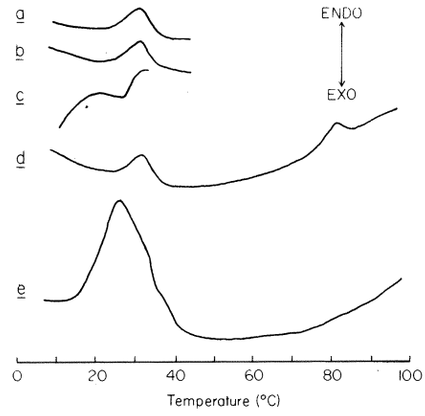

Fig. 1. Differential scanning calorimetry curves of a single LDL sample. Approximately 10 μl of concentrated solution containing 2.05 mg of LDL was placed in a sealed sample pan and an equivalent amount of the solvent 0.19*M* NaCl in the reference pan. Samples were studied between 0° and 45°C and between 0° and 100°C, with and without prior cooling to −60°C. Samples cooled to −60°C were heated at 10°C per minute and held at 1°C until the ice-liquid water transition was complete, and then the heating run was continued. (a) Initial heating curve from 0° to 45°C; (b) heating curve 0° to 45°C after cooling to −60°C; (c) cooling curve from 45° to 0°C; (d) heating curve from 0° to 100°C after cooling to −60°C; (e) heating curve from 0° to 100°C after heating to 100°C and cooling to −60°C.