Brief Definitive Report

HUMAN MELANOMA ANTIGEN AH IS AN AUTOANTIGENIC GANGLIOSIDE RELATED TO G_{D2}*

By TADASHI WATANABE, CLIFFORD S. PUKEL, HIDEO TAKEYAMA, KENNETH O. LLOYD, HIROSHI SHIKU, LUCY T. C. LI, LUIZ R. TRAVASSOS, HERBERT F. OETTGEN, AND LLOYD J. OLD

From the Memorial Sloan-Kettering Cancer Center, New York 10021

AH antigen was detected during our initial analysis of the humoral immune response of melanoma patients to autologous melanoma cells (1). Serum from patient AH was found to react with a cell surface antigen expressed by cultured autologous melanoma cells, but not by autologous skin fibroblasts or peripheral blood lymphocytes. Absorption tests indicated that AH antigen was expressed by some allogeneic melanoma cell lines, but not by any other normal or malignant cell types examined (1). An antigen with related properties was found during a comparable study of the autologous reactivity of sera from patients with astrocytoma (2). This antigen, AJ, was expressed by all astrocytoma cell lines and a high proportion of melanoma cell lines, but was not detected on cultured fibroblasts or epithelial cancers. Comparison of the AJ and AH phenotypes of a series of melanoma cell lines showed only two phenotypes, AJ^+/AH^+ or AJ^-/AH^-, suggesting a serological relatedness of AH and AJ antigens. Of the 75 patients with melanoma that have been analyzed for autologous reactivity, <5% have had demonstrable AH antibody in their sera (3). Overt cancer is not a prerequisite for the presence of AH antibody, because sera from 6 of 106 normal individuals were also found to have AH reactivity (4). In the present report, we summarize the results of typing an extensive panel of human cell types for AH antigen and provide evidence that the AH determinant is related to G_{D2}[1] ganglioside.

Materials and Methods

Cells and Tissues. The derivation and maintenance of melanoma and other cell lines are described in refs. 1, 2, 4–6. Noncultured cells from autopsy specimens were prepared by finely mincing the specimen in RPMI 1640 medium supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml); the resulting cell suspension was washed repeatedly in the same medium before use.

Reference Gangliosides. The source of purified gangliosides are given in ref. 7. G_{D2}, isolated from adult brain (8, 9), was generously provided by Dr. R. K. Yu, Yale University School of Medicine, New Haven, CT. G_{D1b} and G_{T1b} were purchased from Seromed Co., Munich, Federal Republic of Germany. G_{D3} and G_{M4} were isolated from SK-MEL-28 cells and chicken brain, respectively, in our laboratory.

Isolation of Gangliosides from SK-MEL-13 Melanoma Cell Line. Packed SK-MEL-13 cells (10 ml) were extracted sequentially with 30 vol of chloroform/methanol (C/M) (2:1, 1:1, and 1:2). The sample was filtered, evaporated, and resuspended in 50 ml of C/M (2:1), and glycolipids were precipitated at −20°C with 10 vol of acetone. Acidic glycolipids were isolated from this

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[1] G_{D3}, NANA α2→8 NANA α2→3 Gal β1→4 Glc-Cer; G_{D2}, GalNac β1→4 Gal [3←2 αNANA 8←2 αNANA] β1→4 Glc-Cer.

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fraction by Florisil and DEAE-Sephadex chromatography (see ref. 7). Individual gangliosides were isolated by silica gel chromatography. The sample (1 ml) was applied to a column (60 × 1 cm) of Iatrobeads (Iatron Chemical Co., Tokyo, Japan) equilibrated in *n*-propanol: NH_4OH:H_2O, (60:9.5:11.5) (solvent 1). Gangliosides were eluted with the same solvent, and 400-μl fractions were collected after discarding the first 10 ml of the eluate. Gangliosides were detected by applying aliquots of the fractions to an activated silica gel plate (Rediplates; Fisher Scientific Co., Pittsburgh, PA), and the plates were developed in solvent 1 and visualized with resorcinol-HCl.

Serological Assays. The immune adherence (IA) assay was performed as described (1, 2, 4, 5). Qualitative and quantitative absorption tests were done according to procedures developed in our past work (1, 2, 4, 5, 6). Glycolipid inhibition tests were performed as described in (7). Diluted AH serum was mixed with 50 μg of the acidic glycolipid fraction; the mixture was incubated for 30 min at room temperature and 30 min on ice and assayed for residual AH antibody. Purified gangliosides and SK-MEL-13 ganglioside fractions were tested in the same manner.

Results

Reactivity of AH Serum with Autologous and Allogeneic Melanoma Cell Lines. Fig. 1 illustrates the reaction of AH serum with AH melanoma cells (SK-MEL-13) and three other melanoma cell lines. Tests with these and other cell lines showed that cells reacting with AH serum in direct tests absorbed the reactivity of AH serum for autologous SK-MEL-13 cells, whereas direct test-negative cells did not absorb autologous AH reactivity. One of the AH^+ cell lines, SK-MEL-31, showed higher reactivity with AH sera than did autologous SK-MEL-13 melanoma cells (Fig. 1). Although this could have been caused by contaminating antibodies in AH sera, this proved not to be the case; preabsorption of AH sera with skin fibroblasts from the same donor as SK-MEL-31 did not lower reactivity for SK-MEL-31, and quantitative absorption tests indicated that SK-MEL-31 had >10 times more AH antigen than AH melanoma. Because of this higher sensitivity to AH antibody, SK-MEL-31, rather than AH melanoma, was used as the target cell in subsequent absorption and inhibition tests.

AH Phenotype of Normal and Malignant Human Cells. Table I summarizes the results of absorption tests with a panel of human cell types. AH expression is restricted to cells of neuroectodermal origin: melanoma, astrocytoma, neuroblastoma, fetal, and adult brain. The presence of AH on chondrosarcomas is an unexpected exception to this pattern. In a series of 44 melanoma cell lines, 30 typed AH^+ and 14 typed AH^-.

Biochemical Characterization of AH Antigen. The fact that AH antigenicity was not destroyed by trypsin, Pronase, formaldehyde (0.33%), or heating (100°C for 5 min) suggested that AH antigen might be a glycolipid. Consequently, neutral and acidic glycolipids were isolated from AH^+ SK-MEL-13 cells and tested for AH inhibitory activity. Acidic glycolipids were inhibitory, whereas neutral glycolipids had no detectable effect. The acidic glycolipid fraction from a series of AH^+ and AH^- cells and tissues was subsequently tested (Table I). Fractions from AH^+ sources were inhibitory, whereas fractions from AH^- sources were not inhibitory. Partial purification of AH antibody-reactive ganglioside from SK-MEL-13 cells was carried out by Florisil, DEAE-Sephadex, and Iatrobead chromatography. Fig. 2A shows the pattern of gangliosides eluted from the Iatrobead column. G_{M3} (fractions 86–98) and G_{D3} (fractions 99–122) are the major gangliosides of SK-MEL-13, as they were found to be in other melanomas (7). Appreciable amounts of G_{M2} (fractions 99–107) and a number of minor components were also observed. Testing representative ganglioside fractions for AH reactivity showed that only one series of fractions (140–150) was active in inhibition tests. This corresponded to a minor component that co-migrated

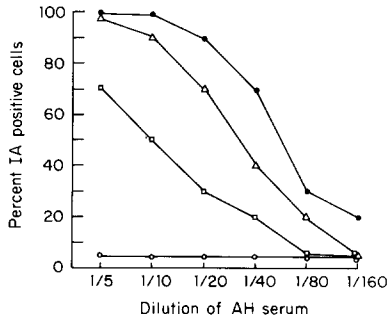

FIG. 1. Serological reactivity of AH serum with four human melanoma cell lines: SK-MEL-13 (autologous AH melanoma) (□); SK-MEL-31 (●); SK-MEL-29 (△); SK-MEL-28 (○).

TABLE I

Serological Definition of the AH Antigenic System: Summary of Absorption Tests with Cells and Tissues and Inhibition Tests with Acidic Glycolipids

Qualitative absorption tests with cells and tissues		
AH^+ phenotype	AH^- phenotype	
Melanoma	Melanoma	Other cancer cells
SK-MEL-13, 23, 26, 27, 29, 31, 36, 42, 44, 52, 57, 68, 73, 75, 79, 80, 86, 88, 89, 91, 94, 95, 97, 100, 104, 106, 110, 112, 113, MeWo	SK-MEL-19, 21, 25, 28, 30, 40, 61, 72, 78, 80, 83, 87, 93, 108	Hep-2 (larynx), SK-SC-1 (squamous cell carcinoma), MOLT-4 (T cell leukemia), Raji (B cell lymphoma)
Astrocytoma	Renal cancer	Fetal cells
SK-MG-AJ, AN, AS, BE, BQ, BT, BU, U251-MG, U373-MG	SK-RC-1, 2, 6, 7, 9, 11	Skin fibroblasts
Retinoblastoma	Bladder cancer	WI-38
WERI, Y-79	T-24, J-82	Retina (non-cultured)
Neuroblastoma	Colon cancer	Liver (non-cultured)
SK-NMC, SK-N-SH	HT-29, SW-837, SW-1083, SW-1222	Kidney (non-cultured)
Meningioma	Cervical and ovarian cancer	Normal adult cells
SK-MEN-1	HeLa, ME-180, SK-OV-3, RAOC	Skin fibroblasts
Chondrosarcoma	Breast cancer	(AX, AS, AT, BD)
SK-CH-H, SK-CH-M	AlAb, BT-20, CAMA, MCF-7, MDA-MBA-361	Liver (non-cultured)
Neural tissue	Lung cancer	Kidney (non-cultured)
Fetal brain	SK-LC-LL	EBV*-transformed B cells
Adult brain	Human erythrocytes (RBC)	AH, AV, BD, BT, CO, CZ, DE, DP, DY
	A, B, AB, O	Xenogeneic cells
		VERO (monkey kidney), CMS-4 (chemically induced mouse sarcoma), guinea pig kidney, spleen, liver; sheep RBC, mouse RBC, rat RBC

Inhibition tests with acidic glycolipids from cells and tissues		
Melanoma	Melanoma	Renal Cancer
SK-MEL-13, 29, 31, 113	SK-MEL-21, 28	SK-RC-6, 7
Astrocytoma	Colon Cancer	Cervical Cancer
SK-MG-AJ, AS	SW-1083	ME-180
Neural Tissue	Other Cells and Tissues	Xenogeneic Cells
Fetal brain	Raji (B cell lymphoma); A, B, AB, RBC; fetal liver, spleen; adult liver, kidney, spleen	Sheep RBC, Horse RBC
Adult brain		

* Epstein-Barr virus.

with G_{D2} in thin layer chromatograms (Fig. 2B). Because of the low yield of this ganglioside, a complete structural analysis was not possible.

Inhibition Tests with Purified Gangliosides. G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D2}, G_{D1a}, G_{D1b}, and G_{T1b} were tested for AH inhibitory capacity (Fig. 3). Only G_{D2} was found to be inhibitory. Further inhibition tests showed that 1 μg of G_{D2} or fraction 140–150 from SK-MEL-13 was sufficient for a 50% reduction in AH serum reactivity.

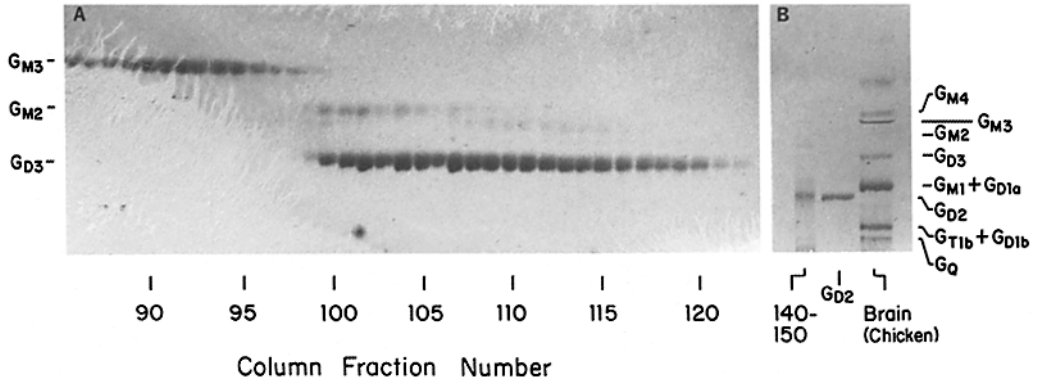

FIG. 2. (A) Fractionation of gangliosides from SK-MEL-13 cells by Iatrobead chromatography. Column fractions were assayed by thin-layer chromatography, and gangliosides were detected by resorcinol-HCl. Fractions 1–73 did not contain detectable gangliosides; fractions 74–85 contained G_{M3}; fractions 86–122 are shown; fractions 123–150 contained minor ganglioside bands that were too weak to photograph. (B) Thin-layer chromatography of AH-reactive fractions 140–150 (pooled) from Iatrobead chromatography of SK-MEL-13 gangliosides. Comparison with reference G_{D2} and standard gangliosides from chicken brain shows that the AH-reactive fraction co-migrates with G_{D2}.

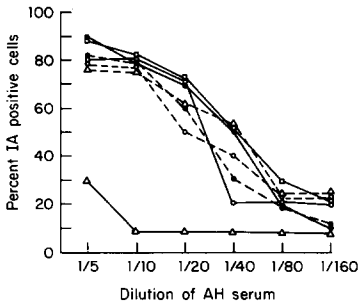

FIG. 3. Inhibition of AH serum reactivity by purified gangliosides: unabsorbed serum (●——●); G_{M1} (●- - -●); G_{D2} (△——△); G_{M3} (△- - -△); G_{D1a} (○——○); G_{M2} (○- - -○); G_{D3} (□——□). Three other gangliosides (G_{M4}, G_{D1b}, and G_{T1b}) were also tested and found to be noninhibitory.

Discussion

Three classes of antigens have been distinguished in our analysis of the specificity of antibodies found in patients with melanoma (3). Antigens referred to as class 1 antigens show an absolute restriction to autologous melanoma cells, not being detected on any other malignant or normal cell type. Class 2 antigens, such as the AH antigen, are expressed by allogeneic as well as autologous melanoma and can be found on a limited range of normal cell types and other malignancies. Class 3 antigens are widely distributed on normal and malignant cells.

From the pattern of cellular distribution, AH antigen has the characteristics of a differentiation antigen of cells of neuroectodermal origin. This also seems to be the case for G_{D2}, which was originally identified as a minor ganglioside in neural tissue (9, 10). G_{D2} represents 8% of the total ganglioside content in adult grey matter (9) and 2% in human spinal cord (11). The fact that AH antigen was detected initially by an autologous antibody indicates that this cell surface component can be autoantigenic. AH antibody belongs to the IgM class and is preferentially reactive at 4°C; in this respect it resembles other autoantibodies reactive with glycoconjugates, e.g., I, i (12),

Pr (13, 14), Gd (15), lacto-*N*-neotetraose (16), and P blood group (17). The stimulus for the production of AH antibody is unclear, particularly because AH antibody has also been detected in normal individuals (4) and astrocytoma patients (2).

Irie (18) and Gupta et al. (19) detected a cell surface antigen designated OFA or OFA-1 that has features in common with AH. However, OFA-1 has been reported to be absent from adult brain and present on fibroblasts, the converse of AH distribution. To clarify the relation between OFA-1 and AH, we have recently exchanged reagents with Dr. R. Irie. The two antigens appear to be related serologically, because OFA typing antibody reacted with AH^+ cells but not with AH^- cells in a panel of 42 cell types. This finding was surprising, as OFA-1 has been reported to be C/M insoluble (19) and relatively unstable at 4°C (20).

We recently reported (7) that another ganglioside, G_{D3}, is a prominent glycolipid in melanoma, in contrast to other cell types. As G_{D2} is derived from the addition of GalNAc to G_{D3}, it will be interesting to determine whether there is an inverse relationship between G_{D3} and G_{D2} levels in melanomas and whether there is a difference in the specific transferase catalyzing G_{D2} biosynthesis in AH^+ and AH^- melanomas. An additional point has to do with the protective effect of antibody directed against AH/G_{D2} in patients with AH^+ melanoma, a possibility that is raised by the unexpected favorable course of the initial melanoma patient with AH antibody. AH ganglioside appears to be weakly immunogenic, as indicated by the low frequency of antibody in melanoma patients and normal individuals. Moreover, vaccination of melanoma patients with AH^+ melanoma cells did not elicit AH antibody, despite the inclusion of adjuvants such as BCG and *C. parvum* in the vaccine (21). The weak immunogenicity of AH may be the result of several factors: (*a*) its presence in brain and other neuroectodermally derived tissues (leading to variable degrees of tolerance), (*b*) the low levels of AH ganglioside in melanoma cells, and (*c*) the recognized difficulty of raising antibody to gangliosides (22). Use of G_{D2} or the oligosaccharide derived from G_{D2} coupled to an immunogenic carrier provides another approach to increasing the immune response to AH antigen in melanoma patients.

Summary

AH antigen, initially defined by an antibody present in a melanoma patient, is a cell surface antigen found on ~65% of melanoma cell lines. Absorption analysis indicates that AH is a differentiation antigen marking normal and malignant cells of neuroectodermal origin. The AH determinant has been found to be related to G_{D2} ganglioside.

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References

1. Shiku, H., T. Takahashi, H. F. Oettgen, and L. J. Old. 1976. Cell surface antigens of human malignant melanoma. II. Serological typing with immune adherence assays and definition of two new surface antigens. *J. Exp. Med.* **144**:873.
2. Pfreundschuh, M., H. Shiku, T. Takahashi, R. Ueda, J. Ransohoff, H. F. Oettgen, and L. J. Old. 1978. Serological analysis of cell surface antigens of malignant human brain tumors. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5122.
3. Old, L. J. 1981. Cancer immunology: the search for specificity. G. H. A. Clowes Memorial Lecture. *Cancer Res.* **41**:361.

4. Houghton, A. N., M. C. Taormina, H. Ikeda, T. Watanabe, H. F. Oettgen, and L. J. Old. 1980. Serological survey of normal humans for natural antibody to cell surface antigens of melanoma. *Proc. Natl. Acad. Sci. U. S. A.* **77:**4260.
5. Ueda, R., H. Shiku, M. Pfreundschuh, T. Takahashi, L. T. C. Li, W. F. Whitmore, H. F. Oettgen, and L. J. Old. 1979. Cell surface antigens of human renal cancers defined by autologous typing. *J. Exp. Med.* **150:**564.
6. Carey, T. E., T. Takahashi, L. A. Resnick, H. F. Oettgen, and L. J. Old. 1976. Cell surface antigens of human malignant melanoma. I. Mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **73:**3278.
7. Pukel, C. S., K. O. Lloyd, L. R. Travassos, W. G. Dippold, H. F. Oettgen, and L. J. Old. 1982. G_{D3}, a prominent ganglioside of human melanoma. Detection and characterization by mouse monoclonal antibody. *J. Exp. Med.* **155:**1133.
8. Ando, S., and R. K. Yu. 1977. Isolation and characterization of a novel trisialoganglioside, G_{T1a}, from human brain. *J. Biol. Chem.* **252:**6247.
9. Ando, S., N-C. Chang, and R. K. Yu. 1978. High-performance thin-layer chromatography and densitometric determination of brain ganglioside composition of several species. *Anal. Biochem.* **89:**437.
10. Klenk, V. E., and M. Nasi. 1968. Über eine komponente des gemisches der Gehirnganglioside, die durch neuraminidaseeinwirkung in das Tay-Sachs-Gangliosidübergeht. *Hoppe-Seylers' Z. Physiol. Chem.* **349:**288.
11. Yu, R. K., K. Ueno, G. H. Glaser, and W. W. Tourtellotte. 1982. Lipid and protein alterations of spinal cord and cord myelin of multiple sclerosis. *J. Neurochem.* **39:**464.
12. Feizi, T., and E. A. Kabat. 1972. Immunochemical studies on blood groups. LIV. Classification of anti-I and anti-i sera into groups based on reactivity patterns with various antigens related to the blood group A, B, H, Le^a, Le^b, and precursor substances. *J. Exp. Med.* **135:**1247.
13. Roelcke, D., W. Ebert, and H. P. Geisen. 1976. Anti-P_{r3}: serological and immunochemical identification of a new anti-P_r subspecificity. *Vox Sang.* **30:**122.
14. Tsai, C-M., D. A. Zopf, R. K. Yu, R. Wistar, Jr., and V. Ginsburg. 1977. A Waldenström macroglobulin that is both a cold agglutinin and a cryoglobulin because it binds *N*-acetylneuraminosyl residues. *Proc. Natl. Acad. Sci. U. S. A.* **74:**4591.
15. Roelke, D., W. Reisen, H. P. Geisen, and W. Ebert. 1977. Serological identification of the new cold agglutinin specificity anti-Gd. *Vox Sang.* **33:**304.
16. Tsai, C-M., D. A. Zopf, R. Wistar, Jr., and V. Ginsburg. 1976. A human cold agglutinin which binds lacto-N-neotetraose. *J. Immunol.* **117:**717.
17. Schwarting, G. A., S. K. Kundu, and D. M. Marcus. 1979. Reaction of antibodies that cause paroxysmal cold hemoglobulin (PCH) with globoside and Forssman glycospingolipids. *Blood.* **53:**186.
18. Irie, R. F. 1980. Oncofetal antigen (OFA-I): a human tumor-associated fetal antigen immunogenic in man. Serologic analysis of human cancer antigens. *In* Serologic Analysis of Human Cancer Antigens. S. A. Rosenberg, editor. Academic Press, Inc., New York. 493–513.
19. Gupta, R. K., R. F. Irie, D. O. Chee, D. H. Kern, and D. L. Morton. 1979. Demonstration of two distinct antigens in spent tissue culture medium of a human malignant melanoma cell line. *J. Natl. Cancer Inst.* **63:**347.
20. Rees, W. V., R. F. Irie, and D. L. Morton. 1981. Oncofetal antigen-I: distribution in human tumors. *J. Natl. Cancer Inst.* **67:**557.
21. Livingston, P. O., T. Takeyama, A. N. Houghton, A. P. Albino, C. M. Pinsky, H. F. Oettgen, and L. J. Old. 1981. Serological response to vaccination with cultured allogeneic melanoma cells. *Proc. Am. Assoc. Cancer Res.* 283.
22. Kundu, S. K., D. M. Marcus, and R. W. Veh. 1980. Preparation and properties of antibodies to G_{D3} and G_{M1} gangliosides. *J. Neurochem.* **34:**184.