DR (Ia-LIKE) ANTIGENS ON HUMAN MELANOMA CELLS

Serological Detection and Immunochemical Characterization*

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The DR antigens are a group of human histocompatibility antigens which have attracted great interest because of their role in cell-cell interactions (1). These antigens have a two-chain structure consisting of noncovalently associated glycoproteins of 34,000 and 29,000 mol wt (2–5) and exhibit considerable amino acid sequence homology at the NH_2-terminal end with murine Ia E/C subregion antigens (6). Like their murine analogs, DR antigens have a restricted tissue distribution compared with the HLA-A, B, and C antigens because they are mainly expressed by cells associated with immune functions (7).

Some adenocarcinoma cells of mice have been reported to produce Ia antigens as indicated by their ability to elicit anti-Ia alloantibodies (8). If so, human tumor cells of nonlymphoid origin, in contrast to their nonmalignant counterparts, may express DR antigens. Because these antigens are involved in immune phenomena and have been reported to control cell proliferation (9), their detection on malignant cells may relate to the disordered proliferation of tumor cells and aid our understanding of their mechanism(s) of escape from immune destruction.

Cultured human melanoma cells were studied for the presence of DR antigens to test the hypothesis that malignant cells of nonlymphoid origin can express DR antigens. The melanoma system was selected because melanocytes have been reported to lack DR antigens (10, 11), and the cellular and humoral immune reactions to melanoma-associated antigens have been suggested to affect the clinical course of this disease (12). Results to be presented indicate that (*a*) some melanoma cell lines can express DR antigens; (*b*) the DR antigens on cultured melanoma cells are immunologically functional as they can bind DR xeno- and alloantibodies and can elicit DR antibodies in a xenogeneic combination; and (*c*) DR antigens on cultured melanoma cells have a two-chain structure like those on B-lymphoid cells, however, the DR β-chain derived from melanoma cells appears to have a slightly lower molecular weight than the DR β-chain derived from the Victor B-lymphoid cell line.

Materials and Methods

Cultured Human Cells. The lymphoblastoid cell lines Victor and WI-L2, used in this study, have been described previously in terms of sources, cell surface antigens, and receptors (13, 14).

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The following 11 cultured human melanoma cell lines were used in this study: 1) Colo 38, Colo 53, Colo 55, M14, M16, M21, M51, BW-5, Allen, Danison, and Vega. Background on the origin of the Colo cell series and the M series (15) and the BW-5 cell line (16) is available in the literature. The cell lines Allen, Danison, and Vega, which were established by primary explant from surgical specimens obtained from three melanoma patients, were a gift from Dr. Y. H. Pilch, Department of Surgery, University of California, San Diego, Calif.

Allo- and Xenoantisera to Cell Surface Markers. DR alloantisera were obtained from parous women whose sera lacked cytotoxic activity to the peripheral lymphocytes of at least 80 unrelated donors (17). The specificity of these DR alloantisera was determined by testing with a panel of DR-typed B-lymphoid cells (14). The DR alloantibody eluate (78-E8) was obtained by acid elution (18) from 1×10^9 Daudi cells incubated with 50 ml of an HLA alloantiserum. This eluate was cytotoxic to cultured B-lymphoid cells and enriched preparations of B-peripheral lymphocytes but not to T lymphocytes from 10 unrelated donors (prepared as previously published) (19). Furthermore, 78-E8 did not react with the cultured T-lymphoid cell lines Molt 4 and 1301 in an indirect rosette microassay.[1] In indirect immune precipitation, 78-E8 reacted with antigenic structures of 34,000 and 29,000 mol wt which are characteristic of the α- and β-chains of DR antigens (2–5).

A specific xenoantiserum to DR antigens (rabbit 8612) was produced as previously described.[2] Briefly, an anti-Daudi cell xenoantiserum which was made specific for B-lymphoid cells by absorption with 1301 and Molt 4 T-cell lines was reacted with *Staphylococcus aureus,* Cowan I strain (SACI)[3] (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) The anti-DR-loaded SACI preparation was then mixed with an NP-40 extract of B-lymphoid cells (e.g. RPMI 4098) to bind DR antigens. These SACI-antibody-DR antigen complexes were injected into rabbits. The resulting antisera were shown by serological and immunochemical tests to react specifically with the same antigenic structures detected by DR alloantisera.[2]

Xenoantiserum 3634 which contained antibodies to the heavy chain of HLA-A and B antigens and to DR antigens (20) was obtained from a rabbit immunized with histocompatibility antigens solubilized from cultured WI-L2 lymphoid cells and partially purified by ultracentrifugal flotation in KBr (21). This xenoantiserum has been used successfully to purify DR antigens for amino acid sequence analysis (6).

Anti-human β_2-microglobulin (β_2-μ) xenoantiserum was from a cow immunized with purified urinary β_2-μ. Anti-Molt 4 antiserum was from a rabbit (6521) immunized with Molt 4 cells.

Rabbits 7428 and 7617 were primed and boosted with intramuscular injections of $3–5 \times 10^6$ BW-5 and M16 melanoma cells, respectively. The cells had been washed in phosphate-buffered saline and mixed with 300 μls of Mylantha (Stuart Pharmaceuticals, Wilmington, Del.) for each injection.

Serological Tests. The indirect rosette microassay was performed as described.[1] Briefly, 20 μl of a cell suspension (5×10^6 cells/ml) was mixed with 20 μl of an appropriate dilution of antisera in microtiter plates. This mixture was incubated at 4°C for 60 min and then washed six times with minimum essential medium. 20 μls of a 2% suspension of sheep erythrocytes coupled with purified antibody to either rabbit or human gamma globulin was added. The cells were pelleted, resuspended, and stained with toluidine blue (0.1% in saline), after which the percentage of rosettes was determined by counting 200 cells. The cytotoxic test with melanoma cells (22) and cultured human lymphoid cells (23) and the lysostrip test (24) (resistance to complement-dependent lysis after redistribution of antigens with corresponding antibodies) were performed as described.

Indirect Immunoprecipitation and Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE). Indirect immunoprecipitation was performed following the method of Cullen and Schwartz (25) with minor modifications described in detail elsewhere (26). Briefly, lentil-lectin-

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[1] Indiveri, F., B. S. Wilson, M. A. Pellegrino, and S. Ferrone. 1978. Detection of human histocompatibility antigens with an indirect rosette microassay. *J. Immunol.* In press.

[2] Wilson, B. S., F. Indiveri, M. A. Pellegrino, and S. Ferrone. 1978. Production and characterization of DR xenoantisera: use for detection of serum DR antigens. Manuscript submitted for publication.

[3] *Abbreviations used in this paper:* SACI, *Staphylococcus aureus* Cowan I strain; β_2-μ, Beta 2-microglobulin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

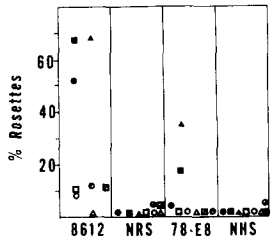

FIG. 1. Detection of DR antigens on cultured human melanoma cells by an indirect rosette microassay. Eight melanoma cell lines, BW-5 (●), M14 (■), M16 (▲), and others (Colo 53, Colo 55, M21, M51, and Allen) were treated with either anti-DR xenoantiserum 8612 (1:40 dilution), normal rabbit serum (NRS) (1:40 dilution), anti-DR alloantibody eluate 78-E8 (1:10 dilution) or normal human serum (NHS) (1:10 dilution) and were then rosetted with either anti-rabbit Ig or anti-human Ig-coated sheep erythrocytes.

purified glycoproteins from ^{3}H-leucine-labeled cells (25) were reacted with antibody-loaded SACI. The labeled glycoproteins were eluted from the SACI by boiling with 2% 2-mercaptoethanol and 2% SDS and then electrophoresed on SDS-PAGE as described by Laemmli (27). The gels were cut into 1-mm slices and analyzed by liquid scintillation counting.

Results

Serological Detection of DR Antigens on Cultured Human Melanoma Cells by Indirect Rosetting. Of the 11 melanoma cell lines available in our laboratory, 3 (BW-5, M14, M16) grow as monolayers and the remaining 8 grow as suspension cultures. When the 11 melanoma cell lines to be tested for DR antigens were treated with a specific xenoantiserum (8612) to common structures of DR antigens in an indirect rosette microassay, only the three cell lines BW-5, M14, and M16 consistently formed rosettes with the DR xenoantiserum (Fig. 1). In addition, the melanoma lines BW-5 and M16 reacted in the indirect rosette microassay with the antibody eluate 78-E8, an alloantibody preparation which is highly specific for DR antigens. The cell line Colo 38 varied in its reactivity with the DR xenoantiserum 8612 and the eluate 78-E8 in separate experiments, the percentage of rosettes ranging from 0 to 30% (data not shown); the remaining seven cell lines, only five of which are shown in Fig. 1, did not react with the DR xeno- and alloantibodies. Because the monolayer cell lines were harvested with trypsin, it was not known whether trypsin treatment had increased the expression of DR antigens on these cell lines. However, monolayer cell lines harvested wtih 0.2 M EDTA were as reactive as trypsin-treated cells to DR antibodies. Furthermore, treatment of suspension melanoma cell lines with trypsin or EDTA did not increase their reactivities with the DR antibodies (data not shown).

The three melanoma cell lines BW-5, M14, and M16 were compared with cultured B-lymphoid cell lines for reactivity with the DR xenoantiserum 8612 in the indirect rosette microassay. The titer of the xenoantiserum was about 1/160 for the melanoma cells and about 1/8,000 for a representative lymphoid cell line, RPMI 4098 (Fig. 2). These data suggest that expression of DR antigens is much lower on melanoma cells than on B-lymphoid cells; a low level of expression of DR antigens on melanoma cells would also explain why we were unable to obtain consistent results when the melanoma cell lines BW-5, M14, and M16 were reacted with the xenoantisera 8612 and rabbit complement in the microcytotoxic test (data not shown).

Detection of DR-Specific Xenoantibodies in Anti-Melanoma Antisera. The melanoma lines BW-5 and M16, which react with DR allo- and xenoantisera, were injected into

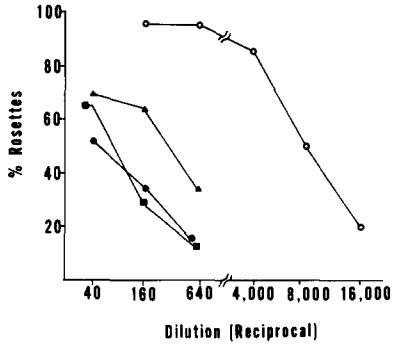

FIG. 2. Titration of the anti-DR xenoantiserum 8612 with a B-lymphoid cell line RPMI 4098 (○) and with cultured melanoma cells BW-5 (●), M14 (■), and M16 (▲) in an indirect rosette microassay.

TABLE I

Susceptibility of WI-L2 Cells to Complement-Dependent Lysis after Redistribution (Stripping) of Cellular Antigens with Xenoantisera to Melanoma Cells

Stripping* antiserum	Susceptibility to lysis		
	Anti-HLA-A2	Anti-DRw4	Anti-Molt 4(6521)
Rabbit anti-BW-5	−	−	+
Rabbit anti-M16	−	−	+
Bovine anti-β_2-μ	−	+	+

* WI-L2 cells were treated with antiserum for 30 min at 25°C and then washed and incubated with goat anti-rabbit Ig or anti-bovine Ig antiserum for 1 h at 37°C to induce redistribution of cellular antigens.

rabbits to determine whether the DR antigens derived from melanoma cells are immunogenic. The resulting antisera were then analyzed for reactivity with DR antigens in lysostrip and indirect immunoprecipitation experiments because both tests detect DR xenoantibodies even though heterogeneous populations of antibodies to other cell surface markers are present. In this way, xenoantisera to melanoma cells could be analyzed for their content of DR antibodies without prior absorption.

In the lysostrip experiments, WI-L2 lymphoid cells (HLA-A1, A2, B5, B17, DRw, 4, 7) were first treated with xenoantisera to melanoma cells BW-5 (7428), M16 (7617), and anti-β_2-μ. After redistribution of antigens in cell membrane (capping), the cells were tested with anti-HLA-A2, anti-DRw4 alloantisera and with anti-Molt 4 xenoantiserum (6521) in the cytotoxic text. As a result, cells pretreated with 7428 and 7617 xenoantisera resisted complement-dependent lysis mediated by anti-HLA-A2 and DRw4 alloantisera but were still susceptible to lysis mediated by 6521 xenoantiserum (Table I). This resistance of WI-L2 cells to lysis by alloantisera after stripping surface antigens with anti-melanoma antisera was specific, because WI-L2 cells treated with xenoantiserum to β_2-μ remained susceptible to lysis by anti-DR alloantiserum and 6521 antiserum (Table I).

To characterize the molecules reactive with anti-melanoma antisera, we used the indirect immunoprecipitation technique and reacted 7428 and 7617 xenoantisera with ^{3}H-leucine-labeled glycoproteins purified from the Victor B-lymphoid cell line. The SDS-PAGE analysis of these immunoprecipitates showed that the melanoma xenoantiserum 7428 reacted with antigenic structures having mol wt of 90,000, 45,000, 34,000

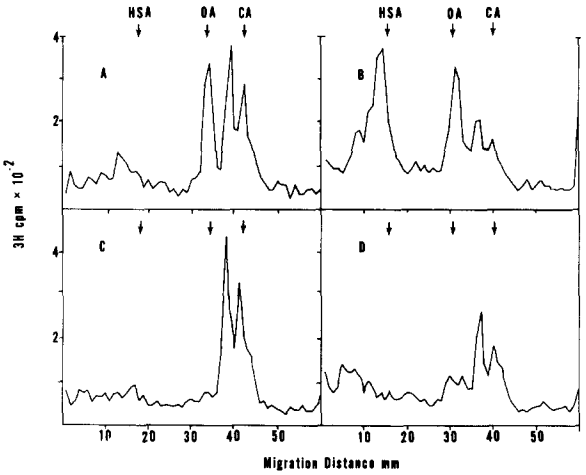

FIG. 3. Immunoprecipitation and SDS-PAGE analysis of anti-melanoma (7428) and anti-B-lymphoid (3634) cell xenoantisera reactive with B-cell (Victor) glycoproteins. Xenoantiserum 3634 unadsorbed (panel A) and adsorbed with 1301 cells (panel C), 7428 antiserum unadsorbed (panel B), and adsorbed with 1301 cells (panel D) were first bound to SACI and then reacted with ^{3}H-leucine-labeled Victor glycoprotein. Immunoprecipitated glycoproteins were electrophoresed under reducing conditions on 10% SDS-PAGE. Human serum albumin (HSA, 65,000 mol wt), ovalbumin (Ov, 44,000 mol wt) and carbonic anhydrase (CA, 29,000 mol wt) were run on companion gels as standards.

and 29,000 (Fig. 3). Similar results were also obtained using 7617 (data not shown). The latter three components also immunoprecipitated by the xenoantiserum 3634 (produced against high density lipoprotein-associated histocompatibility antigens) corresponded to the allotypic heavy chain of HLA-A, B alloantigens and the α-chain and β-chain of the DR antigen molecule, respectively. Absorption of 7428 xenoantiserum with the lymphoid cells 1301, which do not express DR antigens, removed antibodies to the 90,000 and 45,000 mol wt components but not to the 34,000 and 29,000 mol wt components. Similar results, which are shown for comparative purposes, were also obtained with the xenoantiserum 3634. Thus, xenoantisera to the melanoma cells BW-5 and M16 contained antibodies to 34,000- and 29,000-mol wt structures which are characteristic of the α- and β-chains of DR-antigen molecules.

Sequential immunodepletions were then performed to establish whether the 34,000 and 29,000 mol wt B-lymphoid cell components detected by anti-melanoma antisera are in fact DR antigens. For this purpose, antibody-loaded SACI were used to remove various components from a labeled glycoprotein preparation of Victor cells. ^{3}H-leucine-labeled glycoproteins were divided into two aliquots; one aliquot was treated with 7428-loaded SACI and the other with normal rabbit serum-loaded SACI. After three successive depletions, the remaining glycoproteins were reacted with the xenoantiserum 3634, specific for HLA-A, B, and DR antigens. Antigen-antibody complexes in the mixture were precipitated with SACI, and labeled antigens eluted from the precipitates were analyzed by SDS-PAGE. As the results shown in Fig. 4 indicate, 7428 (anti-BW-5) antibodies specifically depleted the Victor glycoprotein preparation of almost all the DR antigens reactive with the DR xenoantiserum 3634. These results indicate that anti-melanoma antisera react with the DR antigens

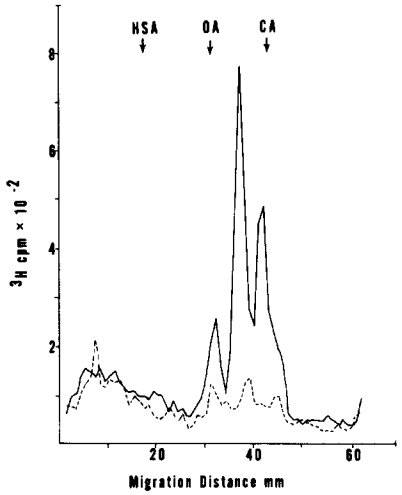

FIG. 4. Reactivity of DR xenoantisera with B-cell glycoproteins immunodepleted with anti-BW-5 (7428) antiserum. ^{3}H-leucine-labeled glycoprotein from Victor cells was immunodepleted three times with SACI loaded with either 7428 antiserum (- - -) or normal rabbit serum (——). The remaining glycoproteins were precipitated by xenoantiserum 3634 bound to SACI and analyzed by SDS-PAGE (under reducing conditions on 10% gels).

synthesized by B-lymphoid cells. In contrast, neither SACI-absorbed normal rabbit serum nor antiserum to Molt 4 cells (6521) (28) reduced the amount of DR antigens precipitated by the antiserum 3634 (Fig. 4).

Immunochemical Characterization of Melanoma DR Antigens. The melanoma cell lines BW-5 and Colo 38 were both used in these experiments because one cell line grows as a monolayer and the other as a suspension culture. The labeled glycoproteins purified from these cells were reacted with the xenoantiserum 8612 and with the DR alloantibody eluate 78-E8. The antigen-antibody complexes were precipitated with SACI, eluted, and the labeled immunoprecipitates were then electrophoresed on SDS-PAGE gels. Immunoprecipitation of Colo 38 glycoproteins with 8612 detected two components; one component was ≅34,000 mol wt and the other was ≅27,000 mol wt. This electrophoretic pattern is shown and compared to that of Victor DR antigens in Fig. 5. The specificity of this immunoprecipitation reaction was indicated by the absence of any reactivity when Colo 38 glycoprotein was reacted with normal rabbit serum (Fig. 6). The fact that the two peaks detected by 8612 antiserum were the α- and β-chain of the DR molecule from melanoma cells is substantiated by an experiment in which identical 34,000 and 27,000 mol wt components were immunoprecipitated from Colo 38 glycoproteins with the highly specific anti-DR alloantibody eluate 78-E8 (Fig. 7). These results were repeated and confirmed when the melanoma cell line BW-5 was substituted for Colo 38 (data not shown).

Xenoantiserum 3634, which contain antibodies to DR antigens and to the HLA heavy chain was used to compare the expression of DR antigens to that of HLA antigens on both melanoma cells and B-lymphoid cells. With 3634, the amount of radioactivity precipitated from a Colo 38 glycoprotein preparation by DR antibodies was much lower than that precipitated by HLA heavy chain antibodies (Fig. 6), whereas, the opposite result was obtained with Victor B-cell glycoprotein (Fig. 4).

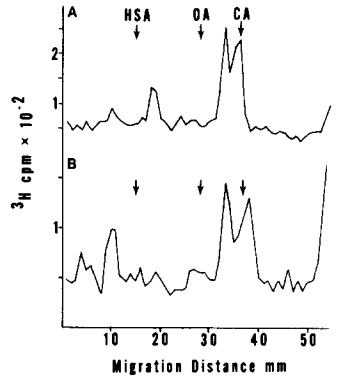

FIG. 5. Detection of melanoma DR antigens with a DR xenoantiserum. SACI loaded with anti-DR xenoantiserum 8612 were reacted with ^{3}H-leucine-labeled glycoprotein from Victor cells (A) and Colo 38 cells (B). The immune complexes were eluted and electrophoresed on SDS-PAGE as described in Fig. 4.

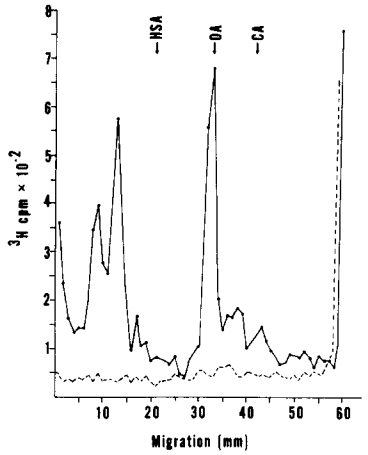

FIG. 6. Immunoprecipitation and SDS-PAGE analysis of melanoma glycoproteins reactive with DR xenoantiserum 3634 and normal rabbit serum. DR xenoantiserum 3634 (●——●) and normal rabbit serum (- - -) bound to SACI were reacted with ^{3}H-leucine-labeled glycoprotein from Colo 38 cells. The immune complexes were eluted and run on SDS-PAGE as described in Fig. 3.

Discussion

Several lines of evidence indicate that cultured human melanoma cells can express DR antigens: (*a*) cultured melanoma cells reacted with specific DR allo- and xenoantibodies in an indirect rosette assay; (*b*) melanoma cells synthesize antigenic molecules with molecular structures similar to the DR antigens synthesized by B-lymphoid cells; (*c*) immunization of rabbits with melanoma cell lines serologically positive for DR antigens (BW-5 and M16) resulted in the production of antibodies showing serological specificity for DR antigens. Immunochemical studies with these anti-melanoma antisera indicated that they reacted with the same molecules of 34,000 and 29,000 mol wt detected by DR xenoantiserum 3634; in previous studies these molecules, reactive with 3634, were found to exhibit considerable amino acid sequence homology at the amino terminal end with DR antigens purified by a conventional biochemical

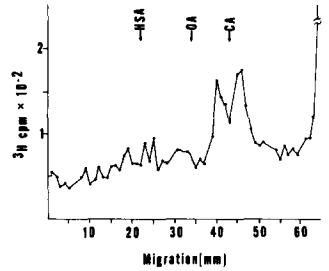

FIG. 7. Immunochemical detection of melanoma DR antigen with an anti-DR alloantibody eluate. Anti-DR alloantibody eluate 78-E8, bound to SACI, was reacted with ^{3}H-leucine-labeled glycoproteins from Colo 38. The immune complexes were eluted and run on SDS-PAGE as described in Fig. 3.

approach (29) and with the murine Ia E/C subregion antigens (6). These data combined overrule the possibility that the reaction of melanoma cells with DR antisera was actually a result of contaminating anti-viral antibodies or cross-reactive antigens. These two alternatives were previously offered to account for the unexpected reactions of histocompatibility alloantisera with some human and murine tumor cells (30, 31).

Human melanoma cells seem to express a smaller amount of DR antigens as compared with B-lymphoid cells because: (*a*) the endpoint titer of the DR xenoantiserum (8612) with melanoma cell lines was many fold lower than the titer with a typical B-lymphoid cell line (RPMI 4098); (*b*) approximately six times more labeled melanoma glycoprotein than B-lymphoid glycoprotein was required to detect even minimal amounts of DR molecules with anti-DR xenoantiserum by indirect immunoprecipitation and SDS-PAGE; (*c*) xenoantiserum 3634 which reacts very strongly with DR antigens but only weakly with HLA-A, B antigens from B-lymphoid cells, reacted better with HLA-A and B than with DR antigens from melanoma cells. Consequently, either the DR antigens from melanoma cells are synthesized in relatively small amounts or the DR xenoantisera used in this study cross-reacted weakly with these melanoma-derived DR antigens because these antisera (8612, 3634) were elicited against DR antigens from B-lymphoid cells. We favor the former alternative, because the DR antibodies present in xenoantisera to melanoma cells also reacted more strongly with DR antigens of B-lymphoid cells than with DR antigens from melanoma cells.

The fact that some melanoma cell lines are DR positive and others are DR negative may reflect the development of melanoma cells from different precursors. Alternatively, if one accepts the hypothesis that DR antigens are differentiation antigens and are expressed at various stages of maturation, then the variable expression of DR antigens by different melanoma cell lines may reflect the stage of differentiation at which the melanocyte becomes a tumor cell. Furthermore, the variable expression of DR antigens by different melanoma cell lines may be quantitative, not qualitative, in the sense that DR-negative melanoma cells express DR antigens but at a density too low to be detected by our serologic techniques. In fact, some of the serologically DR-negative melanoma cells (e.g. Colo 38 and M51) were able to elicit anti-DR antibodies after injection into rabbits (B. S. Wilson and L. E. Walker, unpublished observations). These data are similar to findings with some murine mammary adenocarcinoma cells

which can elicit production of Ia alloantibodies even though they lack serologically detectable Ia antigens (8). Thus, in view of the strong immunogenicity of DR antigens, other research may reveal that these antigens exist on all melanoma cells and possibly on other tumor cells of nonlymphoid origin.

SDS-PAGE analysis of the melanoma DR antigen indicates that its α-chain is $\cong$34,000 mol wt and its β-chain is $\cong$27,000 mol wt or slightly less than the β-chain of the Victor B-lymphoid cell line (29,000 mol wt). This lower molecular weight of the DR β-chain derived from melanoma cells may result either from proteolytic cleavage or from differences in carbohydrate content between β-chains derived from lymphoid and melanoma cells, respectively. This notion is based on the observation that carbohydrate content affects migration rates of glycoproteins on SDS-PAGE (32). Besides clarifying these structural differences, further studies will be directed at determining whether DR antigens on melanoma and B-lymphoid cells differ functionally, e.g. in mixed lymphocyte reactions.

The observation that melanoma cells can produce DR antigens whereas their normal counterparts (melanocytes) do not (10, 11), raises some important questions about how these new antigens develop and their relationship, if any, to the malignant state of the tumor cell. The expression of DR antigens by melanoma cells might result from (*a*) derepression or activation of gene(s) coding for DR antigens, (*b*) repression of genes coding for substances masking DR antigens, or (*c*) changes in the rate of synthesis and/or shedding of DR antigens. Because this study involved cultured melanoma cells, we are not yet certain whether in vivo melanoma cells express DR antigens. If so, the expression of DR antigens may relate to the immunogenicity of melanoma-associated antigens. For example, in experiments with guinea pigs, the presence of Ia antigens was necessary for the immunogenicity of leukemia-associated antigens (33). Studies are currently in progress to test this hypothesis further.

Besides these theoretical considerations, the expression of DR antigens by melanoma cells requires consideration when melanoma cells are typed for HLA, -A, and -B antigens, because the vast majority of HLA-, A-, and B-typing sera are contaminated with antibodies to DR antigens. These contaminant antibodies may cause unexpected reactions as was previously found for the HLA-A and B typing of B-lymphoid cells (7). Finally, the detection of DR antigens on melanoma cells may improve the classification of melanoma as a disease and bear on the clinical association between the course of disease and/or response to therapy.

Summary

11 cultured human melanoma cell lines were tested for the expression of DR antigens by using specific allo- and xenoantisera in an indirect rosette microassay. Four of these melanoma cell lines expressed DR antigens, but in lower amounts than expressed on cultured human B-lymphoid cells. Rabbits injected with the DR-positive melanoma cells produced antibodies that were serologically and immunochemically reactive with B-cell-derived DR antigens. Immunochemical studies indicate that melanoma cell-derived DR antigens have a two-chain structure with 34,000 and 27,000 mol wt components. The melanoma cell-derived DR β-chain at 27,000 mol wt is slightly smaller than that of the Victor cell DR β-chain whose mol wt is 29,000.

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