Brief Definitive Report

ANTIBODIES TO NORMAL HUMAN MELANOCYTES IN VITILIGO

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Vitiligo is a dermatological disorder characterized by destruction of melanocytes and loss of pigmentation. It affects approximately 500,000 persons in the United States (1). The cause of vitiligo is not known. It has been suggested that immune mechanisms may be involved since vitiligo is frequently associated with disorders of the immune system and the presence of autoantibodies to various organs (1, 2). In addition, vitiligo is more common in persons and animals with malignant melanoma who are believed to have immune reactions to pigment cells.

To date, however, no consistent abnormalities in specific immune responses to melanocytes have been found in vitiligo. Earlier reports of antibodies to melanocytes in vitiligo (3–5) have either not been confirmed by other investigators using the same technique (6) or have been found to result from other diseases concurrently afflicting the patients (7).

Recently, human melanocytes have been established in tissue culture, permitting more sensitive assays of antibody to melanocytes (8). In the present studies we have used specific immunoprecipitation of detergent-soluble, radioiodinated macromolecules of normal human melanocytes to search for antibodies to melanocytes. We have found that the majority of individuals with active vitiligo have antibodies to melanocyte-associated antigens. These antibodies are not found in patients with nonpigmentary skin diseases.

Materials and Methods

Sera. Sera were collected from 120 patients. Their diagnosis is shown in Table I. 61 patients had vitiligo; of these, 14 had common vitiligo without evidence of other autoimmune disorders, 42 had vitiligo associated with various immune disorders, and 5 had in addition chronic mucocutaneous candidiasis (MCC). All patients with vitiligo had active disease as evidenced by continued appearance of new lesions or extension of old lesions. 59 patients without vitiligo served as controls; of these, 32 had nonpigmentary dermatosis, 24 had malignant melanoma, and 3 had chronic MCC without vitiligo. Sera were aliquoted into 2-ml fractions and stored at −70°C until used.

Cells. Neonatal human melanocytes from three individuals were established and maintained in culture as recently described by one of us (8). The cultures were used in the second or third passage. >95% of the cells were melanocytes. Control cells included human melanoma cells HM31 and HM49, established in our laboratory, and M21 and M3M, kindly provided by Dr. J. Fogh of the Sloan-Kettering Institute, Rye, NY. The

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M21 cells were pigment producing. Normal human keratinocytes and fibroblasts were established in culture as previously published (9) and used in the first to third passages. All melanoma cells were grown in plastic culture dishes in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.02 mg/ml each of gentamycin, spectinomycin, and penicillin. Cells were used when forming confluent cultures.

Lactoperoxidase-catalyzed radioiodination. Surface macromolecules on melanocytes and control cells were radioiodinated by the lactoperoxidase method (10). Briefly, cells were collected gently with a rubber policeman, washed three times with 15 ml of ice-cold Hank's balanced salt solution (HBSS) without calcium and magnesium, and the pellet resuspended in 1 ml of the HBSS. 2–2.5 × 10^6 cells were radioiodinated with 1 mCi of ^{125}I-sodium (New England Nuclear, Boston, MA) in the presence of 0.5 mg of lactoperoxidase and 0.025 ml of 0.03% hydrogen peroxide in phosphate-buffered saline (PBS). Additional 0.025 ml aliquots of 0.03% hydrogen peroxide were added at 3 min intervals. After 10 min the cells were washed four times with 50 ml of HBSS. Cell viability following radioiodination was 90–95% by trypan blue exclusion. Labeled macromolecules were solubilized by lysing the cells in 1.0 ml of 0.5% Nonidet P-40 (NP-40) (Shell Chemical Corp., Houston, TX) for 1 h at 4°C, a procedure that solubilized >95% of the radioactivity associated with labeled macromolecules. Insoluble material was removed by centrifugation at 12,000 *g* for 20 min. Radioactivity associated with acid-insoluble radiolabeled macromolecules was measured by precipitation with 10% trichloroacetic acid (TCA).

Immunoprecipitation Assays. Antibodies to melanocytes and control cells were measured by specific immunoprecipitation with protein A-Sepharose. 0.1 ml of test serum, diluted 1:10 in PBS, pH 7.4, and 0.1 ml of radioiodinated cell lysate, diluted 1:2.5 in PBS and containing 8–11 × 10^4 TCA-insoluble cpm, were added to 0.2 ml of 0.5% NP-40 with 0.25% bovine serum albumin and incubated at 4°C. After 12 h, 0.02 ml of a 1:1 suspension of protein A-Sepharose (Pharmacia Inc., Piscataway, NJ) in PBS was added to all tubes and agitated continuously at 4°C. After 2 h, samples were centrifuged at 2,000 *g* for 3 min in a microfuge, the pellets washed four times with 0.2 ml of PBS with 0.5% NP-40, and their radioactivity determined. All assays were performed in duplicate, and the average value used. Variation between replicate samples was ±1.0%. Specific binding was calculated by subtracting from the average cpm bound by test serum, the cpm bound by an equal volume of a normal human serum. The same normal serum was used as control in all studies. Sera were considered positive for antibodies when 2.5% or more of the acid-insoluble radioactivity was bound specifically.

Polyacrylamide Gel Electrophoresis (PAGE). Immunoprecipitates generated with vitiligo and normal sera were examined by sodium dodecyl sulfate (SDS)-8% PAGE under reducing conditions and radioautography as previously described (11). Immunoprecipitates were solubilized by heating for 3 min at 100°C in buffer containing a final concentration of 10% glycerol, 1% SDS, 2% 2-mercaptoethanol, 0.1 M Tris-HCl, pH 8.0, and 0.01% bromophenol blue, and run in buffer containing 0.1% SDS and 1.0% 2-mercaptoethanol.

Immunofluorescence Assays. Replicate aliquots of melanocyte or control cells were plated in microtiter plates (Lab-Tek Div., Miles Laboratories, Inc., Naperville, IL) at a density of 5 × 10^3 cells/well in 0.5 ml of culture media, and incubated at 37°C for 24 h. The cells were washed three times with 0.7 ml of HBSS per well, incubated with 0.5 ml of test serum diluted 1:10 in PBS for 30 min at 37°C, washed three times with 0.7 ml of PBS, and reincubated for 30 min with fluorescein-conjugated goat anti-human IgG (Hyland Diagnostics, Deerfield, IL) diluted 1:40 in PBS. The cells were washed again three times with PBS and examined immediately with a Nikon binocular microscope (Nikon Inc., Garden City, NY) equipped for immunofluorescence.

Results

Antibodies to Melanocytes. Human sera from patients with vitiligo (61 patients), melanoma (24), nonpigmentary diseases (32), and MCC (3) were tested by specific immunoprecipitation for antibodies to melanocytes. The results are presented in Fig. 1 and summarized in Table I. 50 (82%) of 61 patients with vitiligo had

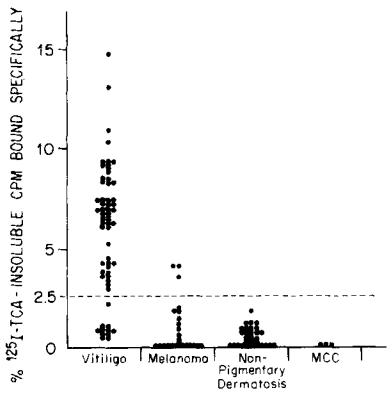

FIGURE 1. Incidence of melanocyte antibodies in sera of patients with vitiligo, melanoma nonpigmentary dermatoses, and MCC without vitiligo. 8–11 × 10^4 input TCA-insoluble cpm per test.

TABLE I

Antigen-binding Antibodies to Melanocytes in Vitiligo

Diagnosis	Number of patients	Percent with antibodies* to surface antigens
Vitiligo	61	82
Common vitiligo	14	100
Vitiligo and autoimmune diseases	42	74
Thyroiditis	18	89
Thyroiditis and diabetes	8	61
Hypoparathyroidism and alopecia areata	9	66
Pernicious anemia	2	50
Vitiligo and MCC	5	100
Melanoma	24	12
Nonpigmentary diseases	32	0
MCC without vitiligo	3	0

* Sera binding specifically at least 2.5% of cpm associated with acid-insoluble melanocyte macromolecules.

antibodies to melanocytes. This included 14 of 14 patients with common vitiligo, 5 of 5 patients with vitiligo associated with chronic MCC, and 31 (74%) of 42 patients with vitiligo associated with other immune diseases. By contrast, no antibodies to melanocytes were found in 32 patients with nonpigmentary skin diseases or in 3 patients with chronic MCC without vitiligo. 3 (12%) of 24 patients with melanoma had antibodies to melanocytes, but their binding activity was much lower than that of vitiligo patients (Fig. 1). There was a linear relationship between the proportion of radioactivity bound specifically and the volume of melanocyte antibody-positive serum used in the assay, suggesting that the assay provided a quantitative estimate of melanocyte antibody level. Five of the antibody-positive sera and five of the negative sera were tested against the same line of melanocytes on three different occasions. The results were similar, indicating that the assay was reproducible.

Eight of the melanocyte antibody-positive vitiligo sera and four normal sera were tested for reactivity to melanocytes derived from three different individuals. All vitiligo, but none of the normal, sera were positive against all three lines, indicating that the antibodies were directed to antigen(s) common to melanocytes.

The SDS-PAGE profile of radioiodinated surface macromolecules immuno-

precipitated by three vitiligo (lanes 1, 2, 3) and two normal human sera (lanes 4, 5) is shown in Fig. 2. The vitiligo sera immunoprecipitated a labeled protein of ~75,000 mol wt, which was not immunoprecipitated by the normal sera.

Reactions to Control Cells. To study the specificity of antibodies to melanocytes, all sera were tested for binding activity to radioiodinated surface macromolecules prepared and solubilized from control cells in a manner identical to that used to prepare material from melanocytes. The results obtained with vitiligo sera are illustrated in Fig. 3. No significant binding was detected to normal allogeneic keratinocytes or fibroblasts. Subsequently, the vitiligo sera were found to be unreactive against an additional three lines of allogeneic fibroblasts. Several vitiligo sera reacted weakly to three of four melanoma lines. The three melanoma sera previously shown to react weakly to melanocytes were unreactive to melanomas or normal cells. Sera of patients with nonpigmentary dermatosis or chronic MCC without vitiligo did not react to any of the cells studied.

Antibodies to Melanocytes by Immunofluorescence. To confirm that the immunoprecipitation assay was measuring antibodies directed to antigens on melanocytes, five randomly selected antibody-positive vitiligo sera and five negative control sera were tested for antibodies to melanocytes by indirect immunofluorescence using unfixed cells as substrate. All vitiligo sera reacted strongly to melanocytes, giving a granular pattern of fluorescence. When appropriately focussed, the staining could be seen to involve the entire surface of the cells. There was a correlation between the intensity of the staining and the binding activity of the sera as found by specific immunoprecipitation. By contrast, none of the control sera reacted to melanocytes. Neither vitiligo or normal sera reacted to melanoma cells used as controls. Identical results were obtained in two similar experiments.

Melanocyte Antibodies in Chronic MCC. Because prior studies have shown that

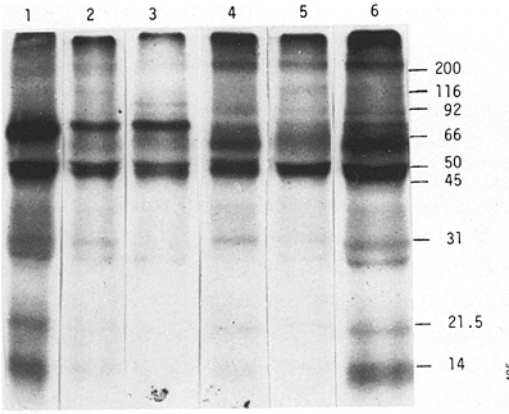

FIGURE 2

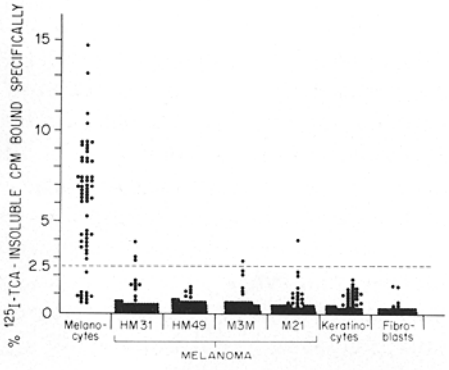

FIGURE 3

FIGURE 2. SDS-PAGE profile of detergent-soluble, radioiodinated macromolecules of normal human melanocytes immunoprecipitated by sera from three patients with vitiligo (lanes 1–3) and two normal individuals (lanes 4 and 5). A protein with a mol wt of ~75,000 is immunoprecipitated by all three vitiligo sera but not by the normal sera. Lane 6 represents the pattern of detergent-soluble radioiodinated proteins in the melanocyte lysate.

FIGURE 3. Reaction of 61 vitiligo sera with radioiodinated surface macromolecules solubilized from melanocytes, pigmented and nonpigmented melanoma cells, and normal human keratinocytes and fibroblasts.

complement-fixing antibodies to melanocytes are associated with chronic MCC (7), the relation of melanocyte antibodies detected by specific immunoprecipitation to chronic MCC was examined. The 120 sera tested included 8 from patients with chronic MCC. Of these, five had vitiligo and three did not. Melanocyte antibodies detected by specific immunoprecipitation were present in all of the patients with both MCC and vitiligo but in none of the MCC patients who did not have vitiligo.

Discussion

The most important finding of this study is that the majority of patients with vitiligo have circulating antibodies to antigens of normal human melanocytes that can be detected by specific immunoprecipitation and indirect immunofluorescence. These antibodies do not react to several unrelated normal human cells and are not found in patients with nonpigmentary diseases.

Although the cause of vitiligo is not presently known, it is believed that immune mechanisms may be involved in its pathogenesis. However, repeated attempts to demonstrate abnormalities in humoral and/or cellular immunity in vitiligo have failed. Recent reports that a few patients with vitiligo have complement-fixing antibodies to cytoplasmic antigens of melanocytes (4, 5) have been superceded by a subsequent study of a much larger number of vitiligo patients that found this to be a result of chronic MCC, which was concurrently afflicting the antibody-positive patients (7).

The recent development of methods to grow human melanocytes in culture free of contaminating cells permits the study of humoral immunity to melanocytes with more sensitive assays. We have taken advantage of this opportunity by studying melanocyte antibodies with a sensitive and quantitative antigen-binding assay based on the specific immunoprecipitation of lactoperoxidase-radioiodinated melanocyte macromolecules that have been solubilized with detergent. Using this approach, we found that the majority of patients with active vitiligo have antibodies to melanocytes. Antibodies were found in 82% of 61 patients with vitiligo. The antibodies were as common in patients with common vitiligo (100% of 14 patients) as in the patients with vitiligo associated with other autoimmune diseases (74% of 42) or chronic MCC (100% of 5). These antibodies were not detected in 32 patients with nonpigmentary skin disorders or in 3 patients with chronic MCC without vitiligo. They were found infrequently (12%) and in low titers in patients with malignant melanoma. The antibodies could also be detected by indirect immunofluorescence using unfixed cells as substrate.

The antigen(s) appear to be melanocyte associated, since antibodies to it do not react to normal human fibroblasts or keratinocytes. The antigen(s) is common to melanocytes, since positive sera reacted equally well to melanocytes obtained from several different individuals. Preliminary SDS-PAGE analysis of immunoprecipitates generated with vitiligo sera suggests that the antigen(s) has an approximate molecular weight of 75,000. Antibodies in a few patients with vitiligo reacted to some malignant melanoma cells and, conversely, some patients with melanoma reacted weakly to melanocytes, suggesting that one or more of the antigen(s) may be expressed, though in smaller amounts, on malignant melanoma cells. The nature of the antigen defined by vitiligo sera remains to be determined; it could be an unusual alloantigen.

The presence of antibodies of melanocytes in the vast majority of patients with active common vitiligo provides direct evidence that alterations in specific immunity to pigment cells occurs in this disease. The finding suggests, but does not prove, that vitiligo is mediated by antibodies to melanocyte-associated antigen(s).

Summary

Most patients with active vitiligo (82% of 61) have antibodies to antigens of normal human melanocytes that can be detected by specific immunoprecipitation of radioiodinated, detergent-soluble, melanocyte macromolecules. Such antibodies were present in only 12% of patients with melanoma and in none of 35 patients with nonpigmentary skin diseases. The antibodies were directed to a common antigen(s) on melanocytes that was not present on normal fibroblasts or keratinocytes. These observations suggest that vitiligo is an autoimmune disease mediated by antibodies to melanocyte-associated antigen(s).

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