Vitiligo Antibodies Are Not Directed to Tyrosinase

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Background: Patients with vitiligo have a markedly increased incidence of antibodies to melanocytes, referred to as vitiligo antibodies. Antibodies to tyrosinase have been reported in some patients with vitiligo, suggesting that vitiligo antibodies may be directed to this enzyme. However, there is considerable controversy as to the frequency with which these antibodies occur, and, hence, about their relevance to the pathogenesis of vitiligo. The frequency with which anti-tyrosinase antibodies occur in vitiligo is critical to evaluate their potential role in the pathogenesis of this disease.

Objective: To examine the prevalence of antibodies to tyrosinase in a large group of patients with vitiligo.

Design: We examined the incidence of antibodies to enzymatically and immunologically active tyrosinase in patients with and without vitiligo.

Setting: Outpatient clinic in referral center.

Patients: The study was conducted on serum samples obtained from 54 patients with active (n = 40) and inactive (n = 14) uncomplicated vitiligo and from 52 age- and sex-matched individuals without vitiligo.

Main Outcome Measure: Presence in the serum of antibodies to enzymatically and/or immunologically active tyrosinase.

Results: By immunoblotting, 20 patients (50%) with active vitiligo, 9 of those (64.3%) with inactive vitiligo, and 29 control individuals (55.8%) had antibodies to an antigen that comigrated with tyrosinase. However, by immunoprecipitation DOPA stain and by sandwich enzyme-linked immunosorbent assay, none of the vitiligo or control individuals had antibodies to tyrosinase, even though both assays easily detected control antityrosinase antibodies.

Conclusion: These results indicate that while antibodies to an antigen(s) that comigrates with tyrosinase are common in patients with or without vitiligo, vitiligo antibodies are not directed to tyrosinase.

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VITILIGO, a disease characterized by the selective destruction of melanocytes, is suspected to result from an autoimmune reaction to these cells.1,2 The most convincing evidence in support of this hypothesis is that most patients and animals with vitiligo have antibodies to melanocyte that can be detected by a variety of techniques, including indirect immunofluorescence, immunoprecipitation, immunoblotting, and complement-dependent cytolysis.3-8 Such antibodies are rare in healthy individuals. The melanocyte antibodies that occur in many patients with vitiligo but are rare in healthy persons are called vitiligo antibodies. Vitiligo antibodies are believed to be involved in the pathogenesis of the disease because they can kill pigment cells in vitro,9,9 their presence correlates with the activity and extent of depigmentation,5,7 and their appearance can precede the development of vitiligo in animals.10

Recently, it has been suggested that one of the antigens defined by vitiligo antibodies is tyrosinase, based on the presence of antibodies to tyrosinase in patients with vitiligo.11-13 However, there is considerable controversy on the incidence of these antibodies and, hence, about their relevance to the pathogenesis of vitiligo. Antityrosinase antibodies were initially reported in almost two thirds of patients with vitiligo.11 However, 2 subsequent studies found these antibodies in an ever-declining proportion of patients. Baharav et al12 reported these in 40% or fewer of vitiligo patients, and most recently Kemp et al13 reported these in 11% of patients. As the frequency with which

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PATIENTS, MATERIALS, AND METHODS

PATIENTS AND CONTROLS

The study was conducted on serum samples of 54 patients with vitiligo (Table 1). Forty patients had active disease as evidenced by appearance of new lesions and/or enlargement of old lesions in the 3 months preceding collection of serum samples, and 14 had inactive disease. None of the patients had segmental vitiligo or other autoimmune or endocrine diseases. The serum samples of 52 age-matched, randomly selected individuals without vitiligo served as controls. These included patients with bullous diseases (pemphigus or bullous pemphigoid [n = 22]), alopecia areata (n = 11), systemic lupus erythematous (n = 2), and healthy individuals (n = 17).

MELANOCYTES

Normal human neonatal melanocytes obtained from 5 individuals (4 white and 1 black) were established in tissue culture by the method of Eisinger and Marko14 as modified by Tobin et al.15 The melanocytes were maintained in melanocyte medium, a mixture of Eagle minimal essential medium (Mediatech, Washington, DC) and serum-free keratinocyte culture medium (Keratinocyte-SFM; Life Technologies, Grand Island, NY) supplemented with 10-3-mol/L phorbol myristate acetate (Sigma, St Louis, Mo), 10-5-mol/L cholera toxin (List Biological Laboratories, Inc, Campbell, Calif), 10% fetal bovine serum (Life Technologies), 2-mmol/L levoglutamide (L-glutamine [L-2-aminoglutaramine acid]) (Life Technologies), 0.01-mmol/L nonessential amino acids (Life Technologies), 100-mg/mL penicillin, and 100-mg/mL streptomycin. The serum-free keratinocyte culture medium was supplemented with 2.5 mg of human epidermal growth factor and 25 mg of bovine pituitary extract. Contaminating fibroblasts were removed by treating the cultures with Geneticin (Life Technologies) for 1 cycle of 48 hours. Contaminating epidermal keratinocytes were removed by differential trypsinization. After 2 to 3 passages, the remaining cells were more than 99% melanocytes, as determined by immunostaining with antimalanocyte antibodies.

Following 6 passages, the melanocytes were harvested, washed twice in Hanks balanced salt solution (Bio-Whitaker, Walkersville, Mass), resuspended in 1.0 mL of 0.25-mol/L sucrose buffer (pH 7.2) containing 2 mmol/L of phenylmethylsulfonyl fluoride (Sigma), 1-mg/mL leupeptin, chymostatin, antipain, and pepstatin (Sigma). A soluble extract was prepared by homogenizing the cells in a glass tissue homogenizer on ice for 20 minutes, centrifuged at 200g for 10 minutes, and the supernatant (postnuclear supernatant) was collected and stored at −80°C until used. For use, equal amounts of protein extract from the 5 different lines of melanocytes were pooled.

Control extracts were prepared similarly from 6 other allogenic cell lines, ie, pigmented (SK-Mel 23) and nonpigmented (SK-Mel 28) melanoma cells, breast carcinoma (MDA-MB-231), colon cancer (Co-1), fibroblasts, and keratinocytes.

IMMUNOBLOTTING

Melanocyte extracts, prepared as above, were mixed with Laemmli sample buffer16 under nondenaturing condition without 2-mercaptoethanol or boiling. Sodium dodecyl sulfate–polyacrylamide gel (8%) electrophoresis (SDS–PAGE) was performed by the Laemmli method using 400 μg of protein per gel. Separated proteins were electrophoretically transferred to polyvinylidene fluoride micro porous membrane (PVDF membrane) (Millipore Corp, Bedford, Mass), blocked with 5% milk/phosphate-buffered saline (PBS) (pH 7.4) for 2 hours at room temperature, cut into strips, and incubated with patients’ or control serum diluted 1:100 in 5% milk/PBS at 4°C overnight. The strips were washed, incubated with biotin-conjugated anti-human IgG (Organon Teknika, Durham, NC), diluted 1:100 with 5% milk/PBS for 2 hours at room temperature, then incubated with avidin-conjugated peroxidase (Organon Teknika) diluted 1:100 with 5% milk/PBS for 1 hour at room temperature. Between each treatment, the strips were washed for 10 minutes with 4 changes of PBS containing 0.05% polyoxyethylene-sorbitan monolaureate

RESULTS

The presence of tyrosinase in blots of soluble, mechanical extracts of human melanocytes was examined by the DOPA reaction and by immunostaining with polyclonal antityrosinase antibody. As illustrated in Figure 1, tyrosinase was detected by both procedures (lane 1, Figure 1, A and B) in the extract, and migrated with a molecular weight of approximately 62 kDa. This molecular weight is within the range previously reported for tyrosinase, which can vary from 53 to 80 kDa depending on its degree of glycosylation, which in turn is affected by the cell line from which it is derived and the methods used to purify it and to measure its size. As expected, enzymatically and immunologically active tyrosinase was only detected in pigmented cells (normal melanocytes and pigmented melanoma cells) but not in a variety of nonpigmented cells, ie, nonpigmented melanoma cells, breast cancer and colon cancer cells, fibroblasts, and keratinocytes (Figure 1).

The melanocyte extract was then used as an antigen source to assay by immunoblotting the serum samples of 54 patients with vitiligo and 52 control individuals for antibodies to antigens that comigrate with tyrosinase. The results are illustrated in Figure 2 and summarized in Table 2. Antibodies that reacted to an antigen(s) of 62 kDa that comigrated with enzymatically (Figure 2, lane A) and immunologically (Figure 2, lane B) active tyrosinase were present in 20 (50%) of the 40 patients with active vitiligo, in 9 (64.3%) of 14 patients with inactive vitiligo, and in 29 (55.8%) of the 52 control individuals. These differences are not significant (P = .45).

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ASSAY OF TYROSINASE

Tyrosinase in melanocyte extracts and in immunoblots was assayed for enzymatic activity by L-3,4-dihydroxyphenylalanine (levodopa [L-DOPA]) stain and for immunological activity by immunostaining with polyclonal rabbit antimalanosine tyrosinase antibody (SL61290; provided by Bryan Fuller, PhD, University of Oklahoma, Oklahoma City), or with murine monoclonal antihuman tyrosinase (Chemicon, Temecula, Calif). Both antibodies react with non-denatured tyrosinase. For DOPA stain, melanocyte proteins were separated on SDS-PAGE, blotted onto PVDF membranes, blocked with 5% milk/PBS for 2 hours at room temperature, and incubated with polyclonal antityrosinase antibody diluted in 5% milk/PBS overnight at 4°C. The membranes were washed, incubated with biotin-conjugated anti–rabbit IgG (Organon Teknika), diluted with 5% milk/PBS for 2 hours at room temperature, and then with avidin-conjugated peroxidase for 1 hour at room temperature. Between each treatment, membranes were washed for 10 minutes with 4 changes of PBS containing 0.05% Tween 20. The reaction was visualized with 4-chloro-1-naphthol. Tyrosinase in the gels was visualized by DOPA stain and by immunostaining with specific polyclonal antibody to tyrosinase. Serum samples that contained antibodies to antigen(s) that comigrated with tyrosinase were then tested for antibodies that reacted specifically to tyrosinase by immunoprecipitation DOPA stain and by a double-sandwich ELISA assay. Both assays measure reactivity to non-denatured molecules.

For immunoprecipitation DOPA stain, 10 µL of patients’ serum was incubated with 100 µg of melanocyte extract overnight at 4°C. Fifty microliters of protein A Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) (diluted 1:1 with PBS, pH 7.4) was added and incubated at room temperature for 2 hours. The beads were washed 3 times with PBS, 30 µL of Laemmli sample buffer was added, and the samples were loaded onto 8% SDS-PAGE and electrophoresed. Tyrosinase in the gels was visualized by DOPA stain as described above. The polyclonal antityrosinase antibody (SL61290) was used as a positive control.

For double-sandwich ELISA assay, monoclonal mouse antihuman tyrosinase antibody15 was precoated onto a 96-well microtiter plate (Nagle Nunc International, Roskilde, Denmark) at 4°C overnight and blocked with 0.3% Tween 20/PBS for 2 hours at 37°C. Wells were then incubated with 50 µL of 2-mg/mL melanocyte extract at 4°C overnight, washed 4 times with 0.05% Tween-20/PBS, and then with 50 µL of patient serum diluted 1:100 in 0.3% Tween-20/PBS for 3 hours at 37°C. Human antibodies reacting with bound tyrosinase were visualized with anti–human IgG conjugated with horseradish peroxidase (Sigma), followed by tetramethylberizidine substrate (Kirkegaard & Perry, Gaithersburg, Md), and the absorption at 450 nm was recorded. Polyclonal rabbit antihuman tyrosinase and monoclonal mouse antihuman tyrosinase antibody were used as positive controls in all studies. Fibroblast extract was used as the negative control in this assay.

STATISTICAL ANALYSIS

We used the χ² test to analyze the significance of differences in the incidence of antibodies to different antigens between patients with and without vitiligo. A t test was used to analyze the results of double-sandwich ELISA assay.
Vitiligo is associated with antibodies to melanocytes that are present in the circulation of a high proportion of patients with the disease but are uncommon in healthy individuals. These vitiligo antibodies have been demonstrated by several investigators using different techniques, which include indirect immunofluorescence, immunoprecipitation, whole-cell ELISA, complement-mediated cytolysis, and antibody-dependent cellular cytotoxicity. They react in part to antigens of 90, 75, and 40 to 45 kD that are expressed on the external cell surface of melanocytes and have been denominated VIT90, VIT75, and VIT40, respectively.

In this study, we examined whether vitiligo antibodies are directed to tyrosinase, a possibility suggested by several prior studies. As this enzyme is intracellular, we used several assay procedures that can detect antibodies to internal antigens. We found that 50% of patients with active vitiligo, 64.3% of those with inactive vitiligo, and 55.8% of control individuals had antibodies to a 62-kD antigen in soluble extracts of human melanocytes that comigrated with enzymatically and immunologically active tyrosinase. These antibodies were not vitiligo antibodies for the reason that they occurred with equal frequency in patients with or without vitiligo. They probably are “natural” antibodies, as they are present in healthy individuals. The 62-kD antigen to which these antibodies were directed was a pigmented cell–associated antigen that was expressed on melanocytes and pigmented melanoma cells but not by nonpigmented melanoma or other control cells. This antigen was not tyrosinase based on the failure of antibodies to the unknown antigen to react to tyrosinase using 2 different assay procedures, immunoprecipitation DOPA stain and double-sandwich ELISA. Antibodies to tyrosinase were not detected by either procedure in patients with vitiligo. The inability to detect tyrosinase antibodies was not due to their being directed to conformational epitopes that might have been lost during the assay procedure, as both assays detect antibodies to native, nondenatured, enzymatically, and immunologically active tyrosinase. No

Table 1. Characteristics of Patients*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Patients</th>
<th>Male</th>
<th>Female</th>
<th>Average Age, y (Range)</th>
<th>Mean % Body Area Depigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitiligo</td>
<td>54</td>
<td>28</td>
<td>26</td>
<td>38.4 (17-56)</td>
<td>15</td>
</tr>
<tr>
<td>Active</td>
<td>40</td>
<td>22</td>
<td>18</td>
<td>39 (18-56)</td>
<td>15</td>
</tr>
<tr>
<td>Inactive</td>
<td>14</td>
<td>6</td>
<td>8</td>
<td>38 (17-54)</td>
<td>14.8</td>
</tr>
<tr>
<td>Controls</td>
<td>52</td>
<td>25</td>
<td>27</td>
<td>37.6 (17-59)</td>
<td>0</td>
</tr>
<tr>
<td>Healthy individual</td>
<td></td>
<td>17</td>
<td>8</td>
<td>33 (25-40)</td>
<td>0</td>
</tr>
<tr>
<td>Alopecia area</td>
<td></td>
<td>11</td>
<td>6</td>
<td>34.6 (19-45)</td>
<td>0</td>
</tr>
<tr>
<td>SLE</td>
<td></td>
<td>2</td>
<td>0</td>
<td>32 (30-34)</td>
<td>0</td>
</tr>
<tr>
<td>Bullous disease</td>
<td></td>
<td>22</td>
<td>11</td>
<td>39.7 (20-60)</td>
<td>0</td>
</tr>
</tbody>
</table>

* SLE indicates systemic lupus erythematosus.

Table 2. Incidence of Antibodies to Antigens That Comigrate With Tyrosinase in Patients With and Without Vitiligo*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Patients</th>
<th>No. (%) With Antibodies to Antigen(s) That Comigrate With Tyrosinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitiligo</td>
<td>54</td>
<td>29 (53.7)</td>
</tr>
<tr>
<td>Active</td>
<td>40</td>
<td>20 (50.0)</td>
</tr>
<tr>
<td>Inactive</td>
<td>14</td>
<td>9 (64.3)</td>
</tr>
<tr>
<td>Control</td>
<td>52</td>
<td>29 (55.8)</td>
</tr>
<tr>
<td>Healthy individual</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Alopecia area</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>SLE</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Bullous disease</td>
<td></td>
<td>22</td>
</tr>
</tbody>
</table>

* SLE indicates systemic lupus erythematosus.
antibodies to VIT 90, VIT 75, or VIT 40 were detected in the current study, which reflects that these antigens are on the external cell surface and are detected by lactoperoxidase radioiodination, a procedure specifically designed not to react with internal cytoplasmic antigens. While we have not formally excluded the possibility that failure to detect tyrosinase antibodies in vitiligo was due to low sensitivity of the assays, we believe this is unlikely as both assays easily detected control antityrosinase antibodies.

These results differ from 3 prior reports that antibodies to tyrosinase are associated with vitiligo. However, the differences are more apparent than real as close examination of the earlier studies indicates that the associations described are not very strong and in any case are not associated with uncomplicated vitiligo. The strongest correlation was described by Song et al, who reported that 16 (61%) of 26 vitiligo patients but none of 31 healthy individuals had antibodies to recombinant human tyrosinase expressed in Escherichia coli. However, no analysis was performed to confirm that these antibodies were directed to tyrosinase, as opposed to unrelated contaminants in transfected E coli, nor were functional studies to confirm that the targeted molecule was indeed tyrosinase based on enzymatic activity conducted. Furthermore, these antibodies were detected only in patients who also had associated endocrine disorders. No antibody was detected in patients with uncomplicated vitiligo, such as those in our study. This may be of importance, since it has previously been reported that antibodies that react to melanoctyes can be present independently of vitiligo in patients with certain associated endocrine disorders. Subsequently, Baharav et al reported that antibodies to mushroom tyrosinase were present in a group of 18 patients with vitiligo but not in healthy individuals. However, the antibodies were present only in a subgroup of 7 patients with diffuse vitiligo, and the actual percentage of these patients who had antibodies was not provided. There were no tyrosinase antibodies in 11 patients with localized vitiligo. Thus at best, the antibodies were present in no more than 39% of patients with the disease. As the antibodies were directed to mushroom tyrosinase, it is uncertain whether they were actually directed to human tyrosinase or to an unrelated epitope on mushroom tyrosinase, as mushroom tyrosinase has less than 50% similarity with the human molecule. More recently, Kemp et al reported that antibodies to radiolabeled human tyrosinase were present in only 5 (10.9%) of 46 patients with vitiligo. The level of antibodies in 4 (80%) of the 5 patients was barely above that of normal values, suggesting that the true incidence of antityrosinase antibodies was less than 10%.

In summary, all 4 studies that have examined the presence of antibodies to tyrosinase in patients with uncomplicated vitiligo have found these to be absent or to be present in only a small proportion of such patients. Because of their low incidence, tyrosinase antibodies are not vitiligo antibodies and are unlikely to play a primary role in the pathogenesis of this disease. A more likely

<table>
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<th>No. of Patients</th>
<th>No. (%) With Tyrosinase Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitiligo</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Active</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Inactive</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Healthy individual</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Alopecia areata</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>SLE</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bullous disease</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Antityrosinase antibody†</td>
<td>1</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>

*SLE indicates systemic lupus erythematosus. The patients’ serum samples were selected based on having antibodies that react to melanoctye antigen(s) that comigrate with tyrosinase (see Table 2).†Rabbit antimouse tyrosinase polyclonal antibody.
explanation is that they are an epiphenomenon similar to the small increase in incidence of many other types of autoantibodies that occur in patients with vitiligo.2

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REFERENCES


Vitiligo Antibodies

Our patients often demand an explanation for otherwise mysterious skin ailments, even if there is no effective treatment. Based on a significant amount of data, it appears that vitiligo is, at least in part, an autoimmune phenomenon. In this article, Xie and colleagues have tried to give us more information to provide for our patients by trying to pinpoint a specific antibody that may produce vitiligo. If this antibody proved to be pathogenic, serum samples from those with an undiagnosed disorder of pigmentation could be tested for its presence. Unfortunately, the investigators did not uncover the diagnostic antibody for vitiligo, but their work may set the stage for further evaluation of other possible pathogenic antibodies in this complex skin disease.

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Tucson, Ariz

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