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 antityrosinase 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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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 erythematosus (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 Marko as modified by Tobin et al. 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-ng/mL phorbol myristate acetate (Sigma, St Louis, Mo), 10^{-5} \text{mol/L} \text{cholera} \text{toxin} \text{(List Biological Laboratories, Inc., Campbell, Calif)}, 10\% \text{fetal} \text{calf serum} \text{(Life Technologies), 2-mmol/L levoglutamidet (L-glutamine [L-2-aminoglutaramine acid])} \text{(Life Technologies),} \text{0.01-mmol/L nonessential amino acids} \text{(Life Technologies),} \text{100-mg/ml penicillin, and 100-mg/ml streptomycin}. The serum-free keratinocyte culture medium was supplemented with 2.3 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 Hank’s balanced salt solution (Bio-whittaker, 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 allogeneic 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 buffer under nondenaturing condition without 2-mercaptoethanol or boiling. Sodium dodecyl sulfate–polycrylamide gel (8%) electrophoresis (SDS-PAGE) was performed by the Laemmli method using 400 ng 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 3% milk/PBS for 2 hours at room temperature, then incubated with avidin-conjugated peroxidase (Organon Teknika) diluted 1:100 with 3% 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 kd. This molecular weight is within the range previously reported for tyrosinase, which can vary from 53 to 80 kd 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 kd 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).
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 antimouse tyrosinase antibody (SL61290; provided by Bryan Fuller, PhD, University of Oklahoma, Oklahoma City), or with murine monoclonal antityrosinase (Chemicon, Temecula, Calif). Both antibodies react with non-natured tyrosinase. For DOPA stain, melanocyte proteins were separated on SDS-PAGE, blotted onto PVDF membranes, and incubated with 0.15% L-DOPA (Sigma) in PBS (pH 7.4) for 20 to 30 minutes at 37°C until a brown band appeared. For immunostaining, proteins in melanocyte extracts or immunoprecipitate were similarly separated and blotted onto PVDF membranes, blocked with 3% milk/PBS for 2 hours at room temperature, and incubated with polyclonal antityrosinase antibody diluted in 3% 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 was also assayed by double-sandwich enzyme-linked immunosorbent assay (ELISA) as described below.

**ASSAY OF ANTIBODIES TO TYROSINASE**

Serum samples were initially screened by immunoblotting for antibodies directed to antigens that comigrate with tyrosinase. This was conducted by a standard procedure as described above, using as an antigen source extracts of melanocyte and 1:100 dilution of patients’ serum. In all cases, the extracts were demonstrated to contain enzymatically and immunologically active tyrosinase as described above.

To examine whether the 62-kd antigen comigrating with tyrosinase bound by these antibodies was tyrosinase, an immunoprecipitation DOPA assay was used. Melanocyte extract containing immunoreactive tyrosinase (Figure 1) was incubated individually with the serum samples of each of the 29 patients with vitiligo who had antibodies to this antigen and with serum of each of the 29 control individuals who also had these antibodies. As a positive control, the extract was also incubated with antityrosinase polyclonal antibody. Bound antigens were precipitated with protein A Sepharose, the precipitates were analyzed by SDS-PAGE, and the gels incubated with DOPA to test for the presence of tyrosinase.

The results are illustrated in Figure 3 and summarized in Table 3. Nondenatured tyrosinase in the melanocyte extract could be immunoprecipitated by control antityrosinase polyclonal antibody and visualized by DOPA in the gel, as illustrated by Figure 3, lane 6. However, none of the 29 vitiligo and 29 control serum samples with antibodies to 62-kd antigen(s) could immunoprecipitate tyrosinase.

A double-sandwich ELISA assay was then used to further test all vitiligo and control serum samples for antibodies to tyrosinase. In this assay, nondenatured tyrosinase in melanocyte extract was bound to microtiter plates precoated with monoclonal mouse antihuman tyrosinase, and antibodies in the test serum that reacted to the bound tyrosinase were detected with peroxidase-labeled anti-human IgG. The results are shown in Figure 4. Controls of polyclonal and monoclonal tyrosinase antibodies reacted strongly to tyrosinase (mean optical density [OD], 1.082 ± 0.035). By contrast, there was no difference in binding activity between vitiligo and the control serum samples (mean OD, 0.558 ± 0.022 and 0.577 ± 0.019, respectively) or between patients with and without vitiligo or with active vs inactive vitiligo. Nor were any individual serum samples positive for tyrosinase antibodies.
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 extract 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, nonnaturated, enzymatically, and immunologically active tyrosinase. No

**Table 1. Characteristics of Patients**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Patients</th>
<th>Sex</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>38.4 (17-56)</td>
<td>15</td>
</tr>
<tr>
<td>Active</td>
<td>40</td>
<td>22</td>
<td>39 (18-56)</td>
<td>15</td>
</tr>
<tr>
<td>Inactive</td>
<td>14</td>
<td>6</td>
<td>38 (17-54)</td>
<td>14.8</td>
</tr>
<tr>
<td>Healthy individual</td>
<td>52</td>
<td>25</td>
<td>37.6 (17-59)</td>
<td>0</td>
</tr>
<tr>
<td>Alopecia areata</td>
<td>11</td>
<td>6</td>
<td>34.6 (19-45)</td>
<td>0</td>
</tr>
<tr>
<td>SLE</td>
<td>2</td>
<td>0</td>
<td>32 (30-34)</td>
<td>0</td>
</tr>
<tr>
<td>Bullous disease</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>
<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>17</td>
<td>6 (35.5)</td>
</tr>
<tr>
<td>Alopecia areata</td>
<td>11</td>
<td>5 (45.5)</td>
</tr>
<tr>
<td>SLE</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Bullous disease</td>
<td>22</td>
<td>16 (72.7)</td>
</tr>
</tbody>
</table>

*SLE indicates systemic lupus erythematosus.*

**Figure 1. Identification of tyrosinase in extracts of melanocytes.** Soluble mechanical extracts of pigmented and nonpigmented cells were tested for presence of immunologically active tyrosinase by immunoblotting using polyclonal antityrosinase (A) and for enzymatically active tyrosinase by DOPA stain (B). Lane 1, normal human melanocyte; lane 2, SK-Mel 28 (nonpigmented melanoma cell); lane 3, SK-Mel 23 (pigmented melanoma cell); lane 4, MDA-MB-231 (breast carcinoma); lane 5, Co-1 (colon cancer); lane 6, fibroblast; and lane 7, keratinocyte.

**Figure 2. Immunoblotting assay of vitiligo and control serum for antibodies to human melanocytes.** Six representative vitiligo (lanes 1-6) and 6 normal (lanes 7-12) serum samples were tested for antibodies to antigens in soluble mechanical extracts of normal human melanocytes by immunoblotting. Note that several vitiligo and normal serum samples have antibodies to a 62-kd antigen that comigrates enzymatically and immunologically with active tyrosinase in the same blots that were detected by DOPA stain (lane A) and reaction with antityrosinase antibody (lane B).
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 radiiodination, 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,11 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 melanocytes can be present independently of vitiligo in patients with certain associated endocrine disorders.23 Subsequently, Baharav et al12 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 al13 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
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


Clinical Implications

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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