Detection of Tyrosinase Autoantibodies in Patients With Vitiligo Using ^{35}S-Labeled Recombinant Human Tyrosinase in a Radioimmunoassay

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Tyrosinase antibodies recently have been reported to occur frequently in patients with vitiligo. We describe the detection of tyrosinase antibodies in vitiligo patients using *in vitro* ^{35}S-labeled human tyrosinase in a radioimmunoassay. Of 46 vitiligo sera examined in the assay, five (10.9%) were found to be positive for tyrosinase antibodies. In contrast, 20 control sera and sera from 10 patients with Hashimoto's thyroiditis were negative. Four of the sera positive in the radioimmunoassay were also positive in an ELISA using mushroom tyrosinase as antigen. Absorption studies indicated that pre-incubation with mushroom tyrosinase absorbed out the immunoreactivity of the positive sera in the radioimmunoassay, suggesting cross-reactivity, but this absorption was never complete, indicating that there are tyrosinase antibodies in human sera that do not react with the mushroom protein. There was no obvious association between the presence of tyrosinase antibodies and the age of the patients (range: 22–62 y), their duration of disease (range: 5–20 y), or the type of vitiligo (one segmental, one symmetrical/periorificial, three symmetrical), although the three patients with the highest antibody levels also had an associated autoimmune disorder (one with Graves' disease; two with autoimmune hypothyroidism). The results confirm that tyrosinase autoantibodies are present in the sera of vitiligo patients but at a low frequency. The technique described is sensitive and quantitative and allows the detection of conformational epitopes. It will be useful in longitudinal studies to determine the relation between the clinical features of vitiligo and tyrosinase antibody levels. *Key word: autoimmunity.*

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Vitiligo is a common skin disorder, the precise etiology of which remains obscure. Some studies have shown that anti-melanocyte autoantibodies are often present in the sera of vitiligo patients (Cui *et al*, 1992) and that there is a correlation between the presence and level of these antibodies and the extent (Naughton *et al*, 1986) and activity (Harning *et al*, 1991) of vitiligo. These observations and the fact that these antibodies can kill human melanocytes *in vitro* (Norris *et al*, 1988) suggest they may be involved in the pathogenesis of vitiligo, although it is also possible that antibody production may merely reflect a secondary immunologic response to melanocytes damaged by other mechanisms.

Recent work has tried to identify the melanocyte autoantigens against which vitiligo antibodies react. Some of the antigens are unique to pigmented cells, whereas others are also expressed on nonpigmented cells, and all appear to be located on the cell surface (Cui *et al*, 1992). Tyrosinase, a key enzyme involved in melanin synthesis, has been implicated as an autoantigen in two studies (Song *et al*, 1994; Baharav *et al*, 1996), but others have dismissed any role.[1] The current study aimed to analyze vitiligo sera for the presence of tyrosinase antibodies using a novel radioimmunoassay (RIA) with ^{35}S-labeled recombinant human tyrosinase. This type of assay recently has been widely used, for example, to detect antibodies against glutamic acid decarboxylase, steroid 21-hydroxylase, and candidal antigens in insulin-dependent diabetes mellitus, Addison's disease, and autoimmune polyglandular syndrome type 1, respectively (Falorni *et al*, 1994, 1995; Peterson *et al*, 1996).

MATERIALS AND METHODS

Patients Sera from 46 sequential patients with vitiligo (14 men, 32 women; mean age 48 y, range 23–79 y) collected in dermatology and endocrinology clinics between January 1990 and September 1996 were used. Patients were characterized with respect to the presence of associated autoimmune diseases: 20 had no other disease and no family history of autoimmune disease; nine had no other disease but had a family history of autoimmune disease; 17 had an autoimmune disorder. Autoimmune diseases were: Graves' disease, 4; autoimmune hypothyroidism, 9; alopecia, 2; Addison's disease with hypothyroidism and type 1 diabetes mellitus, 1; and pernicious anemia with hypothyroidism, 1.

Sera from 20 healthy laboratory personnel (eight men, 12 women; age range 25–43 y, mean 33 y) were used as controls. As a further set of controls, 10 sera from patients (two men, eight women; age range 34–73 y, mean 48 y) with Hashimoto's thyroiditis but without vitiligo were tested.

All sera were kept frozen at −20°C. The study was approved by the

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Abbreviations: TRP, tyrosinase-related protein; TyrAb index, tyrosinase antibody index.

[1] Xie Z, Bystryn JC: Anti-tyrosinase antibodies in vitiligo. *J Invest Dermatol* 106:938, 1996 (abstr).

Ethics Committee of the Northern General Hospital, Sheffield, and all subjects gave informed consent.

Rabbit Antisera Rabbit polyclonal antisera αPEP7 (Tsukamoto *et al*, 1992), generated against a synthetic peptide that corresponds to the carboxyl terminus of mouse tyrosinase, and αPEP8 (Tsukamoto *et al*, 1992), generated against a synthetic peptide that corresponds to the carboxyl terminus of mouse tyrosinase-related protein 2 (TRP2), were a gift of Prof. Vincent Hearing (National Institutes of Health, Bethesda, MD).

Cloning of Human Tyrosinase cDNA into pBluescript A full-length human tyrosinase cDNA fragment in pRHOHT2 (Takeda *et al*, 1989) was a gift of Prof. Shigeki Shibahara (Tohoku University School of Medicine, Sendai, Japan). Initially, the cDNA was subcloned into pBluescript SK (Stratagene, La Jolla, CA) to allow *in vitro* transcription-translation of tyrosinase under control of the T7 promoter.

Plasmid pRHOHT2 (1 μg) was cleaved with enzymes *Xba*I and *Sal*I (Promega, Southampton, U.K.) in a 50-μl reaction volume with buffer containing 6 mM Tris HCl (pH 7.9), 6 mM $MgCl_2$, and 150 mM NaCl at 37°C for 90 min. The fragments generated were resolved on a 1% low-melting-point agarose gel (Sambrook *et al*, 1989), and the 2-kb DNA band containing tyrosinase cDNA was excised and purified using a Wizard DNA clean-up system (Promega). *Xba*I-*Sal*I–restricted pBluescript SK (2.96 kb) was prepared in the same way. Ligations were conducted overnight at 16°C in a 15-μl reaction mixture with 1 U of T4 DNA ligase (Promega), 50 ng of restricted pBluescript SK, 100 ng of tyrosinase cDNA, and buffer containing 66 mM Tris HCl (pH 7.6), 6 mM $MgCl_2$, 1 mM dithiothreitol, and 66 μM ATP. A 2-μl aliquot of the ligation mixture was used to transform 100 μl of competent *Escherichia coli* XL1Blue (Sambrook *et al*, 1989). Transformed cells were selected on LB agar (Sambrook *et al*, 1989) containing 50 μg ampicillin per ml. Plasmid was prepared from 10 transformants using a Wizard miniprep DNA purification system (Promega). After restriction with *Xba*I and *Sal*I, each plasmid was analyzed by agarose gel electrophoresis (Sambrook *et al*, 1989) to identify those carrying a DNA insert. One appropriate recombinant plasmid was designated pBSTYR. The construct was verified by dideoxy sequencing using a Sequenase version 2.0 kit (Amersham, Aylesbury, U.K.) with [α-^{35}S]dATP (Amersham) and T7 primer (Promega). Large-scale preparation of pBSTYR was done using the Wizard midiprep DNA purification system.

***In Vitro* Coupled Transcription-Translation of Human Tyrosinase** Full-length human tyrosinase was produced *in vitro* from the cDNA in pBSTYR using a TnT T7-coupled reticulocyte lysate system (Promega). Plasmid DNA (2 μg) was incubated for 120 min at 30°C in 50 μl reaction mixture containing 25 μl rabbit reticulocyte lysate, 1 μl T7 TnT RNA polymerase, 1 μl amino acids minus methionine, 40 U RNasin (Promega), 2 μl TnT reaction buffer, and 4 μl translation-grade [^{35}S]methionine (1000 Ci/mmol, 10 mCi per ml; Amersham). The reaction was stored at −20°C until needed.

To determine the percentage incorporation of [^{35}S]methionine, we added 2 μl of the reaction mixture to 98 μl of 1 M NaOH/2% H_2O_2 and incubated it at 37°C for 10 min. After this, 900 μl of ice-cold 25% trichloroacetic acid (TCA)/2% casamino acids (Difco, Detroit, MI) was added, and the reaction was incubated on ice for 30 min. To collect the precipitated translation products, 250 μl of the TCA reaction mix was vacuum filtered onto a Whatman GF/A glass fiber filter prewetted with cold 5% TCA. The filter was rinsed three times with 1 ml of ice-cold 5% TCA and once with 1 ml of acetone and then allowed to dry at room temperature before counting in an LKB 1217 Rackbeta liquid scintillation counter. To determine total counts per min (cpm) present in the reaction, a 5-μl aliquot of the TCA reaction mix was spotted directly onto a filter. This was dried for 10 min before counting. The percent incorporation of [^{35}S]methionine was determined as (cpm of washed filter/cpm of unwashed filter × 50) × 100.

Electrophoretic Analysis and Autoradiography Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Sambrook *et al*, 1989) of *in vitro* translated products was performed in a 10% polyacrylamide resolving gel containing 325 mM Tris HCl (pH 8.8) and 0.1% SDS and a 4% polyacrylamide stacking gel containing 125 mM Tris HCl (pH 6.8) and 0.1% SDS. The gel running buffer contained 25 mM Tris HCl (pH 8.3), 192 mM glycine, and 0.1% SDS.

A 5-μl aliquot of the *in vitro* translation reaction mixture was added to 20 μl of SDS sample buffer containing 63 mM Tris HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue and heated to 100°C for 2 min before loading a 10-μl sample. To visualize protein markers, the gel was stained with 0.05% Coomassie blue in 10% glacial acetic acid/25% isopropanol and destained with 10% glacial acetic acid/25% isopropanol, each for 30 min at room temperature. The gel was then soaked in Amplify scintillant (Amersham) for 30 min at room temperature before drying at 80°C for 2 h on 3 MM filter paper under vacuum. Autoradiography was carried out at −70°C using x-ray film (Genetic Research Instrumentation Ltd, Essex, U.K.). Protein molecular weight standards (Sigma, Dorset, U.K.) consisted of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (20.1 kDa).

RIA for Tyrosinase Antibodies For each assay, an aliquot of the *in vitro* translation reaction mixture (equivalent to 15,000–20,000 cpm of TCA precipitable material) was suspended in 50 μl immunoprecipitation buffer containing 20 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% Triton X100, and 10 mg aprotinin per ml. Serum was then added to a final dilution of 1:10 unless stated otherwise. After incubation overnight with shaking at 4°C, 50 μl of protein G Sepharose 4 Fast Flow slurry (Pharmacia Biotech, Uppsala, Sweden), prepared according to the manufacturer, was added and incubated for 1 h at 4°C. The protein G Sepharose–antibody complexes were then collected by centrifugation and washed six times for 15 min in immunoprecipitation buffer at 4°C. Immunoprecipitated radioactivity was evaluated in a liquid scintillation analyzer. Three aliquots of each sample were counted and the mean cpm calculated. The SD of the mean was always within 12%.

For analysis by SDS-PAGE, the protein G Sepharose–antibody complexes were resuspended in 100 μl SDS sample buffer, boiled, and centrifuged, and the supernatant was recovered for electrophoresis.

For absorption studies, each positive patient serum at a final dilution of 1:10 was incubated with either 1 μg, 5 μg, 10 μg, 20 μg, or 50 μg of mushroom tyrosinase (Sigma) in 50 μl of immunoprecipitation buffer for 2 h at 4°C. Bovine serum albumin (Sigma) was used as a control in the same amounts. After pre-incubation, ^{35}S-tyrosinase was added as above, and the RIA was performed as described.

Enzyme-Linked Immunosorbent Assay (ELISA) for Tyrosinase Antibodies This was performed essentially as described elsewhere (Baharav *et al*, 1996). Corning polystyrene 96-well microtiter plates (Bibby Sterilin Ltd, Mid Glamorgan, U.K.) were coated with 100 μl of mushroom tyrosinase at a concentration of 50 μg per ml in buffer containing 1.5 mM Na_2CO_3, 3.5 mM $NaHCO_3$, and 3.0 mM NaN_3 (pH 9.2) and incubated overnight at 4°C. The plates were then washed six times with phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4), and Tween-20 at 0.05% (PBS/Tween) and then blocked with 0.5% gelatin in PBS at room temperature for 1 h. After washing six times with PBS/Tween, 100 μl of each serum, diluted 1:50 in PBS/Tween, was added to a well and the plates were incubated at 37°C for 2 h. The plates were washed six times with PBS/Tween and then 100 μl of anti-human IgG (γ-chain specific) alkaline phosphatase conjugate (Sigma), diluted 1:1000 in PBS/Tween, was added for 2 h at room temperature. After washing six times in PBS/Tween, 100 μl of Sigma 104 phosphatase substrate (Sigma) was applied to each well at a concentration of 1 mg per ml in buffer containing 11.9 mM Na_2CO_3, 16 mM $NaHCO_3$, and 2.1 mM $MgCl_2$ (pH 9.3). A LabSystems Integrated EIA Management System (Life Sciences International, Hampshire, U.K.) was used to read absorption at 405 nm. In each assay, sera were tested in duplicate and the average value was taken.

Expression of Tyrosinase Antibody Levels To control for interassay variation, we expressed tyrosinase antibody levels as a relative index (TyrAb index). A TyrAb index for each serum tested in the RIA was calculated as cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by the 20 healthy controls. Each serum was tested in at least three experiments, and the mean TyrAb index was calculated from these. The SD of the mean was always within 12%. The upper level of normal for the assay was calculated using the mean TyrAb index + 3 SD of a population of 20 healthy individuals.

For the ELISA, the TyrAb index for each serum sample tested was calculated as absorbance at 405 nm for the tested serum/mean absorbance at 405 nm for 20 healthy controls. Each serum was tested in at least three experiments, and the mean TyrAb index was calculated from these. The SD of the mean was always within 10%. The upper level of normal for the assay was calculated using the mean TyrAb index + 3 SD of a population of 20 healthy controls.

RESULTS

***In Vitro* Translation of Recombinant Human Tyrosinase and Immunoprecipitation of ^{35}S-Tyrosinase** *In vitro* translation of pBSTYR resulted in an incorporation of [^{35}S]methionine of 9.3 ± 1.4% (mean ± SD) in four separate experiments. The quality of the *in vitro* translated tyrosinase was evaluated by SDS-PAGE **(Fig 1)**, showing a product with an estimated molecular weight of 57 kDa, which agrees well with the molecular weight of 56.2 kDa

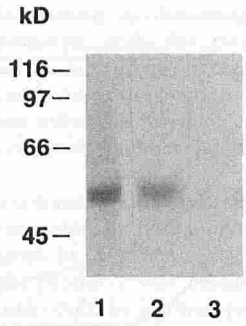

Figure 1. SDS-PAGE of *in vitro* translated ^{35}S-tyrosinase and of immunoprecipitated ^{35}S-tyrosinase. ^{35}S-tyrosinase was produced *in vitro* in a TnT T7-coupled reticulocyte lysate system as described in *Materials and Methods*. Five microliters of the reaction mixture were added to 20 μl of SDS sample buffer, and 10 μl of this mix were then analyzed by SDS-PAGE. The immunoreactivity of ^{35}S-tyrosinase was tested using two rabbit polyclonal sera: tyrosinase-specific αPEP7 and TRP2-specific αPEP8. These sera were incubated at a dilution of 1:100 with an aliquot of the *in vitro* translation reaction mixture before incubation with protein G Sepharose as described in *Materials and Methods*. The protein G Sepharose–antibody complexes were resuspended in 100 μl of SDS sample buffer and boiled, and the supernatant was recovered for analysis by SDS-PAGE. *Lane 1*, *in vitro* translated ^{35}S-tyrosinase; *lane 2*, ^{35}S-tyrosinase immunoprecipitated with tyrosinase-specific αPEP7 antiserum; *lane 3*, ^{35}S-tyrosinase immunoprecipitated with TRP2-specific αPEP8 antiserum.

predicted from the amino acid sequence of the protein (Takeda *et al*, 1989).

The immunoreactivity of the *in vitro* translated recombinant human tyrosinase was tested using rabbit antisera at a 1:100 dilution. ^{35}S-tyrosinase was immunoprecipitated by the tyrosinase-specific αPEP7 antiserum but not by the TRP2-specific αPEP8 antiserum **(Fig 1)**. The average percentage of ^{35}S-tyrosinase immunoprecipitated in four assays by the αPEP7 antiserum was 65 ± 10% (mean ± SD) at a dilution of 1:100. To test the sensitivity of the RIA, we immunoprecipitated ^{35}S-tyrosinase with serial dilutions of the αPEP7 antiserum. From this, the end point dilution of the antiserum was 1:18,000.

RIA of Vitiligo and of Healthy Control Sera Sera from 46 vitiligo patients and 20 healthy controls, at a dilution of 1:10, were tested for their ability to immunoprecipitate ^{35}S-tyrosinase. For each serum, a TyrAb index was assigned as the mean TyrAB index of at least three experiments with an SD of less than 12%. The upper level of normal for the RIA (mean TyrAb index + 3 SD of 20 healthy controls) was estimated as a TyrAb index of 1.27 **(Fig 2)**.

None of the healthy individuals was positive for tyrosinase antibodies. Among 46 vitiligo patients, five (10.9%) had a TyrAb index above 1.27 **(Fig 2)** and were considered positive for tyrosinase antibodies. Sera from 10 patients with Hashimoto's thyroiditis had a mean TyrAb index of 0.96 ± 0.10 (mean ± SD), and all 10 were negative for antibodies to tyrosinase.

SDS-PAGE was used to check that the radioactivity immunoprecipitated by each of the positive sera was due to ^{35}S-tyrosinase. **Figure 3** indicates that the five positive sera immunoprecipitated a band of the correct size when compared with that precipitated by tyrosinase-specific αPEP7 antiserum.

The three most strongly reacting sera were analyzed at different dilutions in the RIA along with a group of six healthy controls. A TyrAb index for each dilution was calculated and plotted as a function of serum dilution **(Fig 4)**. For these three sera, saturated binding was observed at dilutions up to 1:100.

ELISA of Vitiligo and of Healthy Sera Using Mushroom Tyrosinase Sera from 46 vitiligo patients and 20 healthy individuals were tested in the ELISA with mushroom tyrosinase. For each sample, a TyrAb index was calculated as the mean TyrAb index of at least three experiments with an SD of less than 10%.

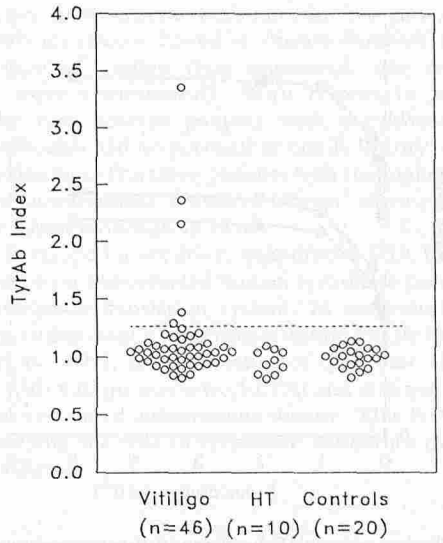

Figure 2. TyrAb index of vitiligo patients, Hashimoto's thyroiditis (HT) patients, and healthy controls in the RIA. Sera were incubated at a dilution of 1:10 with an aliquot of *in vitro* translation reaction mix before incubation with protein G Sepharose as described in *Materials and Methods*. The cpm immunoprecipitated by each serum were determined, and a TyrAb index was calculated as cpm immunoprecipitated by serum sample/mean cpm immunoprecipitated by 20 healthy controls. The TyrAb index shown for each serum is the mean TyrAb index of at least three experiments, and the SD of the mean was always within 12%. The *dotted line* shows the upper level of normal of 1.27 (mean TyrAb index of 20 healthy controls + 3 SD) for the RIA. The baseline data giving a TyrAb index of 1 are 2340 cpm.

The upper level of normal for the ELISA (mean TyrAb index + 3 SD of 20 healthy controls) was estimated as a TyrAb index of 1.96 **(Fig 5)**.

All the controls were negative. Of the 46 vitiligo patients, four (8.7%) had an antibody index greater than 1.96 and were considered positive for tyrosinase antibodies. These four were also positive by the RIA, and the ranking of their reactivity was the same in both assays. Patients with Hashimoto's thyroiditis had a mean TyrAb index of 0.74 ± 0.22 (mean ± SD), and all 10 sera were negative.

Absorption Studies To determine whether the immunoreactivity of sera in the ELISA and in the RIA was identical, we performed absorption studies as described in *Materials and Methods* using the

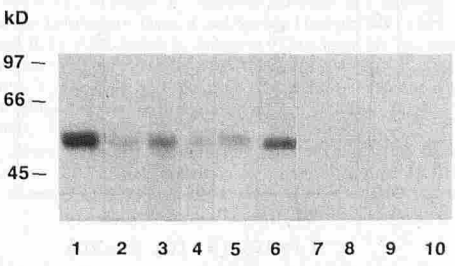

Figure 3. SDS-PAGE of ^{35}S-tyrosinase immunoprecipitated with either vitiligo or healthy serum. Sera were incubated at a dilution of 1:10, except for αPEP7, which was used at 1:100, with an aliquot of the *in vitro* translation reaction mixture before incubation with protein G Sepharose as described in *Materials and Methods*. The protein G Sepharose–antibody complexes were resuspended in 100 μl of SDS sample buffer and boiled, and the supernatant was recovered for analysis by SDS-PAGE. *Lane 1*, ^{35}S-tyrosinase immunoprecipitated with αPEP7 antiserum; *lanes 2–6*, ^{35}S-tyrosinase immunoprecipitated with positive vitiligo sera; *lanes 7–8*, ^{35}S-tyrosinase immunoprecipitated with negative vitiligo sera; *lanes 9–10*, ^{35}S-tyrosinase immunoprecipitated with healthy control sera.

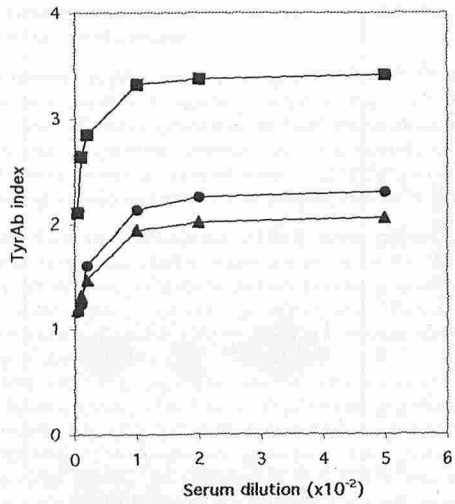

Figure 4. Dilution curves of the three highest tyrosinase antibody-positive sera. A dilution series in PBS was made of each of the three most positive sera and six healthy controls. Five microliters of each dilution were added to 50 μl of immunoprecipitation buffer containing an aliquot of *in vitro* translation reaction mixture, and the RIA was performed as in *Materials and Methods*. The TyrAb index for each serum at each dilution was calculated as cpm immunoprecipitated by serum sample/mean cpm immunoprecipitated by six healthy controls. The TyrAb index of each positive serum is plotted as a function of the serum dilution. Positive serum 1 (■); positive serum 2 (●); positive serum 3 (▲).

three most positive sera. **Figure 6** shows the percentage cpm immunoprecipitated by each serum in the RIA after pre-incubation with varying amounts of either mushroom tyrosinase or bovine serum albumin. The results indicate that the mushroom tyrosinase was able to absorb out the immunoreactivity of these sera in the

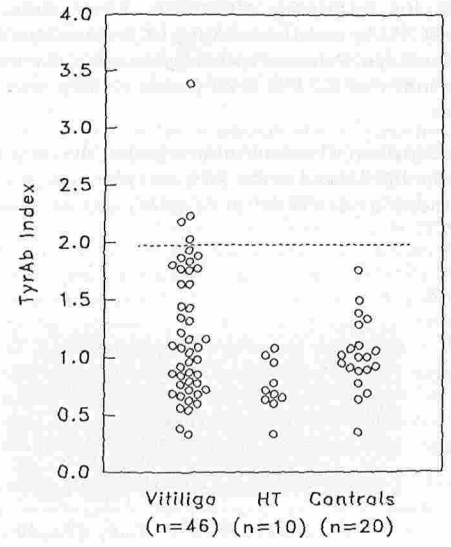

Figure 5. TyrAb index of vitiligo patients, Hashimoto's thyroiditis (HT) patients, and healthy controls in the ELISA. Sera were tested for the presence of tyrosinase antibodies in an ELISA using mushroom tyrosinase as in *Materials and Methods*. A TyrAb index for each serum tested was calculated as absorbance at 405 nm for the serum sample/mean absorbance at 405 nm for 20 healthy controls. The TyrAb index shown is the mean TyrAb index of at least three experiments for each serum sample, and the SD of the mean was always within 10%. The *dotted line* shows the upper level of normal as 1.96 (mean TyrAb index of 20 healthy controls + 3 SD) for the ELISA. The baseline data giving a TyrAb index of 1 are an absorbance at 405 nm of 0.398.

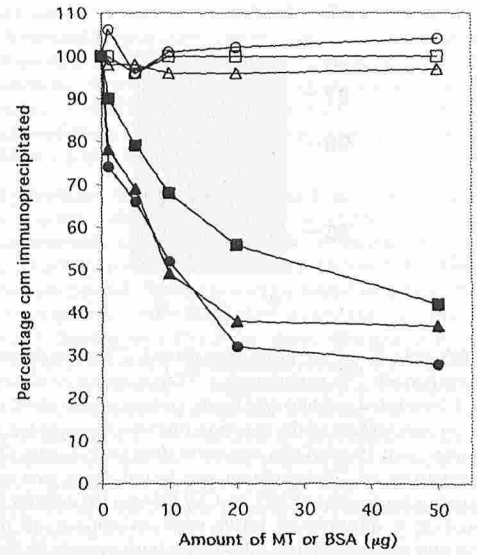

Figure 6. Absorption of tyrosinase antibodies from positive sera with mushroom tyrosinase. Each serum, at a final dilution of 1:10, was pre-incubated with either 1 μg, 5 μg, 10 μg, 20 μg, or 50 μg of mushroom tyrosinase. Bovine serum albumin was used as a control in the same amounts. After pre-incubation, ^{35}S-tyrosinase was added and the RIA was carried out as described in *Materials and Methods*. The cpm immunoprecipitated are expressed as a percentage of the cpm immunoprecipitated by each serum without pre-incubation and are plotted as a function of the amount of mushroom tyrosinase (MT) or bovine serum albumin (BSA) pre-incubated with the sera. Positive serum 1 + MT (■); positive serum 2 + MT (●); positive serum 3 + MT (▲); positive serum 1 + BSA (□); positive serum 2 + BSA (○); positive serum 3 + BSA (△).

RIA. This was never complete, however; there was 58–72% loss of reactivity with the highest amount of mushroom tyrosinase used. Bovine serum albumin at similar concentrations had no effect on tyrosinase binding in the RIA.

It was possible that proteolytic activity in the mushroom tyrosinase extract was degrading the labeled protein during incubation and thereby causing a decrease in cpm in the RIA. To test this, we incubated ^{35}S-tyrosinase overnight with either 20 μg or 50 μg of mushroom tyrosinase and then analyzed the samples by SDS-PAGE and autoradiography. Degradation of ^{35}S-tyrosinase was not detected by this analysis.

DISCUSSION

Tyrosinase antibodies have been detected in the sera of patients with vitiligo in two previous studies (Song *et al*, 1994; Baharav *et al*, 1996), implicating the enzyme tyrosinase as an autoantigen. The first study used bacterially synthesized human tyrosinase and immunoblotting (Song *et al*, 1994), which is a relatively insensitive technique and detects linear epitopes that are unlikely to be as important as conformational epitopes in the pathogenesis of autoimmune disease. The second study used mushroom tyrosinase in an ELISA (Baharav *et al*, 1996). Although this method is sensitive and allows the detection of conformational epitopes, the use of mushroom tyrosinase is unlikely to allow the detection of all epitopes present on the human protein. In contrast to these two studies, others have reported the absence of tyrosinase antibodies in all vitiligo sera examined by immunoblotting (n = 40), immunoprecipitation (n = 20), and ELISA (n = 13) using human melanocyte extract as a source of tyrosinase.[2]

Recently, RIAs have been developed to detect specific antibodies in the sera of patients with autoimmune disease (Falorni *et al*, 1994, 1995; Peterson *et al*, 1996). These involve *in vitro* translation

[2]Xie Z, Bystryn JC: Anti-tyrosinase antibodies in vitiligo. *J Invest Dermatol* 106:938, 1996 (abstr).

and concomitant radiolabeling of the antigen of interest. The method combines sensitivity with the possibility of detecting conformational epitopes. It also allows quantitative measurement of the level of antibodies and avoids the need to express and purify the protein of interest from either bacterial or mammalian cells. For these reasons, we have developed an RIA for the detection of tyrosinase antibodies.

Using coupled *in vitro* transcription-translation, we were able to produce ^{35}S-labeled recombinant human tyrosinase. This was of high quality as evaluated by SDS-PAGE, and contamination by lower-molecular-weight products was minimal. The protein was also immunoreactive and could be immunoprecipitated by specific anti-tyrosinase antiserum.

The technique was used to examine vitiligo and control sera for tyrosinase antibodies. Healthy control sera and sera from patients with Hashimoto's thyroiditis did not contain tyrosinase antibodies. Among 46 vitiligo sera tested, five (10.9%) contained tyrosinase antibodies. This was a much lower frequency than found in the study of Song *et al* (1994), in which 61% of vitiligo patients had tyrosinase antibodies. There are several possible explanations for this discrepancy. The use of immunoblotting in the previous report is a major difference from our study, and no quantitative data were provided to validate the definition of positivity. The differences may also reflect variations in the groups of patients studied, such as race and geographic location. In addition, all of the patients in the study of Song *et al* (1994) had a personal or family history of autoimmune disease, but even considering the 26 similar patients in the present study, only 3 (11.5%) had tyrosinase antibodies.

The state of the disease may also affect the frequency of positive sera. In patients with active vitiligo, melanocytes are still being destroyed, and sera are therefore more likely to have antibodies to melanocyte antigens (Harning *et al*, 1991), although formation of immune complexes could theoretically reduce detectability. When the disease is inactive, tyrosinase antibodies may decrease to undetectable levels. It is difficult, however, to assess the activity of vitiligo at a single clinic visit, and there are no reliable serologic markers for activity. These findings suggest that it may be worth a prospective study to explore the value of tyrosinase antibody levels in this capacity. Finally, it is possible that some epitopes are not present on the tyrosinase used in this assay, as it may not be properly glycosylated in the *in vitro* transcription-translation system. This could account for the low frequency of tyrosinase antibody-positive sera. The tyrosinase used in the study of Song *et al* (1994), however, was produced in bacteria and would therefore not be glycosylated or processed, as it would be in the melanocyte; yet, still a high frequency of tyrosinase antibody-positive sera was found.

Four of the patients positive in the RIA were also positive for tyrosinase antibodies in the ELISA using mushroom tyrosinase. This may have been due to antibodies reacting with a conformational epitope, as the amino acid homology between mushroom and human tyrosinase is low: the region of highest homology between the two proteins occurred in a 111–amino acid overlap, where 39.6% similarity and 23.4% identity were found. Incomplete absorption of antibodies by mushroom tyrosinase **(Fig 6)** indicated that there may be tyrosinase antibodies in human sera that do not react with the mushroom protein, and this could account for the negative result in one patient in the ELISA.

The presence of tyrosinase antibodies in five patients was not associated with age (range: 22–62 y), disease duration (range: 5–20 y), or the type of vitiligo (one segmental; one symmetrical/periorificial; three symmetrical). With respect to autoimmune disorders, the two positive patients with the lowest levels of tyrosinase antibodies had no personal or family history of associated autoimmune diseases. The three patients with the highest levels had an associated autoimmune disorder: Graves' disease in one and autoimmune hypothyroidism in two.

We have developed a sensitive, quantitative RIA for tyrosinase antibodies based on radiolabeled human tyrosinase produced in an *in vitro* transcription-translation system. In contrast to a recent report testing vitiligo sera for tyrosinase antibodies by immunoblotting (Song *et al*, 1994), the prevalence of tyrosinase antibodies in our patients with vitiligo was low (10.9%) and was not confined to those with an associated autoimmune disease. This RIA should be useful in clarifying the role of tyrosinase antibodies in the pathogenesis of vitiligo.

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