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 relationship between the clinical features of vitiligo and tyrosinase antibody levels. **Key word:** autoimmunity. J Invest Dermatol 109:69–73, 1997

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 possibly 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. 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 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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the n soaked acid/25% isopropanol, each for 10% glycerol, 2% resolving gel containing 325 mM Tris HCl (pH 8.8) and 0.1% SDS. The gel was incubated on ice for 10 min.

Products, 250 J.LI of the TCA reaction mix was vacuum filtered onto a Whatman GF/A glass fiber filter prerinsed 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 was passed directly onto a filter. This was dried for 10 min before counting. The percent incorporation of [35S]methionine was determined as (cpm of washed filter/cpm of unwashed filter × 100).
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 aPep7 antisera but not by the TRP2-specific aPep8 antisera (Fig 1). The average percentage of $^{35}$S-tyrosinase immunoprecipitated in four assays by the aPep7 antisera 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 aPep7 antisera. From this, the end point dilution of the antisera 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 aPep7 antisera.

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%.
three most positive sera. Figure 6 shows the percentage cpm immunoprecipitated by each serum in the RIA after pre-incubation with varying amounts of 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, 35S-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 35S-tyrosinase overnight with either 20 µg or 50 µg of mushroom tyrosinase and then analyzed the samples by SDS-PAGE and autoradiography. Degradation of 35S-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

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 \textit{in vitro} transcription-translation, we were able to produce \textsuperscript{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. 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 \textit{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; three generalized). 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 \textit{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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