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Immunoprecipitation of melanogenic enzyme autoantigens with vitiligo sera: evidence for cross-reactive autoantibodies to tyrosinase and tyrosinase-related protein-2 (TRP-2)

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SUMMARY

In the present study we describe the detection of TRP-2 antibodies in vitiligo patients using *in vitro* ^{35}S-labelled human TRP-2 in a radioimmunoassay. Of 53 vitiligo sera examined in the assay, three (5·9%) were found to be positive for TRP-2 antibodies. In contrast, 20 control sera, sera from 10 patients with Hashimoto's thyroiditis and sera from 10 patients with Graves' disease were all negative. All three patients positive for TRP-2 antibodies (mean age 54 years, age range 50–63 years) had had vitiligo of the symmetrical type for more than 1 year and all of them also had an associated autoimmune disorder: Graves' disease in one and autoimmune hypothyroidism in two. In addition, antibodies to the melanogenic enzyme tyrosinase were present in their serum. To examine any immunological cross-reactivity between TRP-2 and tyrosinase, the three vitiligo sera positive for TRP-2 antibodies were preabsorbed with COS-7 cell extract containing either expressed TRP-2 or tyrosinase, and subsequently used in the radioimmunoassay. These absorption studies indicated that preincubation with both proteins inhibited the immunoreactivity of the positive sera in the immunoassay using *in vitro* translated ^{35}S-TRP-2. This antibody cross-reactivity suggests the humoral response to the two melanogenic enzymes in these patients may not be entirely independent.

Keywords vitiligo autoantigen tyrosinase tyrosinase-related protein-2 autoimmunity

INTRODUCTION

Vitiligo is a common skin disorder characterized by areas of depigmentation resulting from loss of melanocytes in the epidermis. It is often associated with autoimmune diseases such as Hashimoto's thyroiditis (HT), diabetes mellitus and Addison's disease [1]. Although the precise aetiology remains obscure, some studies have shown that anti-melanocyte autoantibodies are often present in the sera of vitiligo patients [2] and that there is a correlation between the presence and level of these antibodies and the activity [3] and extent of vitiligo [4]. These observations, and the fact that these antibodies can kill human melanocytes *in vitro* [5], suggest they may be involved in disease pathogenesis, although it is also possible that antibody production may merely reflect a secondary immunological response to melanocytes damaged by other mechanisms.

Recent work has tried to identify the autoantigens against which vitiligo antibodies react. It is clear from immunoprecipitation studies that several autoantigens are recognized by vitiligo sera: some of these antigens are unique to pigment cells, while others are also expressed on non-pigmented cells [2]. Tyrosinase, a key enzyme involved in melanin synthesis, has been implicated as an autoantigen in a number of studies [6–8]. TRP-2, a protein related to tyrosinase and also referred to as DOPAchrome tautomerase, catalyses the conversion of DOPA(3,4-dihydroxyphenylalanine)chrome to 5,6-dihydroxyindole-2-carboxylic acid as part of the pathway of melanin production [9]. The cDNA encoding TRP-2 has been cloned and the deduced amino acid sequence of the protein shows 40% identical amino acid residues to that of human tyrosinase [10,11].

The present work aimed to analyse vitiligo sera for the presence of antibodies to TRP-2 using a radioimmunoassay (RIA) with ^{35}S-labelled recombinant human TRP-2. This type of assay is sensitive, quantitative, allows the detection of conformational epitopes and has recently been widely used to identify autoantigens [8,12–14]. Since TRP-2 and tyrosinase share substantial amino acid homology and our previous study had detected

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tyrosinase antibodies in some vitiligo patients [8], we also wanted to examine any sera positive for TRP-2 antibodies for cross-reactivity with tyrosinase using absorption experiments.

MATERIALS AND METHODS

Patients

Sera from 53 sequential vitiligo patients (16 men, 37 women; mean age 49 years; age range 18–79 years) collected in dermatology and endocrinology clinics between January 1990 and September 1996 were used in this study. Patients were characterized with respect to the presence of associated autoimmune diseases: 22 had no other disease and no family history of autoimmune disease; 12 had no other disease but had a family history of autoimmune disease; 19 had an autoimmune disorder. These were: Graves' disease (GD), 4; autoimmune hypothyroidism, 9; alopecia areata, 2; Addison's disease with hypothyroidism and type 1 diabetes mellitus, 1; pernicious anaemia with hypothyroidism, 1; diabetes mellitus, 2.

Sera from 20 healthy laboratory personnel (nine men, 11 women; age range 23–47 years; mean age 31 years) were used as controls. As a further two sets of controls, 10 sera from patients (one man, nine women; age range 30–74 years; mean age 51 years) with HT and 10 sera from patients (three men, six women; age range 27–66 years; mean age 42 years) with GD were tested.

All sera were kept frozen at −20°C. The study was approved by the Ethics Committee of the Northern General Hospital, Sheffield, and all subjects gave informed consent.

Rabbit antisera

Rabbit polyclonal antisera αPEP7 [15], generated against a synthetic peptide which corresponds to the carboxyl terminus of mouse tyrosinase, and αPEP8 [15], generated against a synthetic peptide which corresponds to the carboxyl terminus of mouse TRP-2, were a gift of Professor Vincent Hearing (National Institutes of Health, Bethesda, MD).

Cloning of human tyrosinase cDNA into pcDNA3

A full-length human tyrosinase cDNA fragment in pBSTYR [8] was cloned into pcDNA3 (Invitrogen, Abingdon, UK) for expression of tyrosinase in COS-7 cells. Briefly, 1 μg of pBSTYR was cleaved with enzymes *Kpn*I and *Xba*I (Promega, Southampton, UK). The fragments generated were resolved on a 1% low melting point agarose gel [16] and the 2·0-kb DNA band containing tyrosinase cDNA was excised and purified using a Wizard DNA clean-up system (Promega). *Kpn*I-*Xba*I restricted pcDNA3 (5·4 kb) was prepared in the same way. Ligations were conducted overnight at 16°C with T4 DNA ligase (Promega), 50 ng of restricted pcDNA3 and 100 ng of tyrosinase cDNA in ligase buffer (Promega). A 2-μl aliquot of the ligation mixture was used to transform 100 μl of competent *Escherichia coli* XL1Blue [16]. Transformed cells were selected on LB agar [16] containing 100 μg/ml ampicillin. Plasmid was prepared from 10 transformants using a Wizard miniprep DNA purification system (Promega). After restriction with *Kpn*I and *Xba*I, each plasmid was analysed by agarose gel electrophoresis [16] in order to identify those carrying a DNA insert. One appropriate recombinant plasmid was designated pcDNA3TYR. The construct was verified by dideoxy sequencing using a Sequenase version 2·0 kit (Amersham, Aylesbury, UK) with α-^{35}S-dATP (Amersham) and T7 primer (Promega). Large scale preparation of pcDNA3TYR was carried out using a Qiagen plasmid maxi kit (Qiagen Ltd, Dorking, UK).

In vitro *coupled transcription-translation of human TRP-2 and tyrosinase*

Full-length human TRP-2 cDNA in pCMVDTS [11] was a gift of Professor Shigeki Shibahara (Tohoku University School of Medicine, Sendai, Japan). The TRP-2 cDNA was in the correct orientation for expression of TRP-2 from the T7 promoter in the vector. Large scale preparation of pCMVDTS from *E. coli* XL1 Blue was carried out with a Qiagen plasmid maxi kit. The plasmid was then used in a TnT T7 coupled reticulocyte lysate system (Promega), to produce and label TRP-2 with ^{35}S-methionine *in vitro*. Briefly, pCMVDTS (2 μg) was incubated for 120 min at 30°C in a 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/ml; Amersham). The reaction was stored at −20°C until needed. The percentage incorporation of ^{35}S-methionine was determined by TCA precipitation according to the manufacturer's protocol. ^{35}S-tyrosinase was produced by the same method, but using pBSTYR [8].

SDS–PAGE and autoradiography

SDS–PAGE of *in vitro* translated products was performed in a 10% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel, as described elsewhere [8].

A 5-μl aliquot of the *in vitro* translation reaction mixture was added to 20 μl of SDS sample buffer [16] 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 onto 3 MM chromatography paper (Whatman International Ltd, Maidstone, UK) under vacuum. Autoradiography was carried out at −70°C using x-ray film (Genetic Research Instrumentation Ltd, Dunmow, Essex, UK). Protein molecular weight standards (Sigma, Poole, UK) consisted of myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD) and soybean trypsin inhibitor (20·1 kD).

RIA for TRP-2 antibodies

For each assay, an aliquot of the *in vitro* translation reaction mixture (equivalent to 12 000 ct/min of TCA precipitable material) was suspended in 50 μl immunoprecipitation buffer containing 20 mM Tris–HCl pH 8·0, 150 mM NaCl, 1% Triton X-100 and 10 mg/ml aprotinin. 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, were 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 analyser. Three aliquots of each sample were counted and the mean ct/min calculated. The s.d. of the mean was always within 12%. For analysis by SDS–PAGE and autoradiography, the protein G Sepharose–antibody complexes were resuspended in 100 μl SDS sample buffer, boiled, centrifuged and the supernatant recovered for electrophoresis. For dilution

experiments, each positive serum and six healthy control sera were used in the RIA at final dilutions of 1:23, 1:58, 1:116, 1:232, 1:580, 1:1160 and 1:2320.

Expression of TRP-2 antibody levels

TRP-2 antibody levels were expressed as a relative index (TRP-2Ab index). A TRP-2Ab index for each serum tested in the RIA was calculated as: ct/min immunoprecipitated by tested serum/mean ct/min immunoprecipitated by 20 healthy controls. Each serum was tested in three experiments and the mean TRP-2Ab index was calculated from these. The s.d. of the mean was always within 15%. The upper level of normal for the assay was calculated using the mean TRP-2Ab index + 3 s.d. of a population of 20 healthy individuals. For dilution experiments, a TRP-2Ab index was calculated for each serum tested at each dilution as: ct/min immunoprecipitated by tested serum at each dilution/mean ct/min immunoprecipitated by six healthy controls at each dilution. For each serum at each dilution, a mean TRP-2Ab index was calculated from three experiments, and this value plotted against 1/serum dilution. The s.d. of the mean was < 15%.

Transfection of COS-7 cells

Both TRP-2 and tyrosinase were transiently expressed in COS-7 cells (European Collection of Animal Cell Cultures, Salisbury, UK) for use in absorption experiments. COS-7 cells were grown in 100-mm dishes (Corning, New York, NY) in RPMI 1640 (Life Technologies Ltd, Paisley, UK) containing 2 mM L-glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin and 10% fetal calf serum (FCS). When 50% confluent, cells were treated with 10 μg of either pCMVDTS or pcDNA3TYR and Transfectam reagent (Promega) according to the manufacturer's protocol. The cells were incubated for 48 h at 37°C after transfection and then washed once in PBS containing 137 mM NaCl, 2·7 mM KCl, 10·1 mM Na_2HPO_4 and 1·8 mM KH_2PO_4 pH 7·4. One millilitre of buffer containing 40 mM Tris–HCl pH 7·5, 1 mM EDTA and 150 mM NaCl was added to each plate and left for 5 min at room temperature. Cells were then scraped from the surface of the plate and transferred to a microcentrifuge tube. After spinning at 12 000 ***g*** for 1 min at 4°C, the supernatant was removed and the cells resuspended in 150 μl of 0·25 M Tris–HCl pH 8·0 containing 10 μg/ml aprotinin (Bayer, Newbury, UK), 100 μM Nα-tosyl-phenylalanyl chloromethyl ketone (Novobiochem, Nottingham, UK), 100 μM Nα-tosyl-lysyl chloromethyl ketone (Sigma) and 10 μM pepstatin A (Novobiochem). Cell extracts were made by freezing the cell suspension in ethanol/dry-ice followed by rapid thawing at 37°C and unlysed cells were removed by centrifugation. The total protein content of the extracts was determined by the method of Bradford [17] and the extracts were diluted where appropriate to contain equivalent amounts of total protein.

Expression of TRP-2 and tyrosinase in COS-7 cells

Expression of TRP-2 in COS-7 cells from pCMVDTS was assessed using absorption experiments. Antiserum αPEP8 was first incubated at 4°C for 16 h with serial dilutions of COS-7 cell extract containing expressed TRP-2 and with serial dilutions of COS-7 cell extract alone. After preincubation, *in vitro* translated ^{35}S-TRP-2 was added and the RIA carried out as previously described. Expression of tyrosinase in COS-7 cells from pcDNA3-TYR was also assessed using absorption experiments: αPEP7 antiserum was preabsorbed with serial dilutions of COS-7 cell extract containing expressed tyrosinase and with serial dilutions of COS-7 cell extract alone before use in the RIA with ^{35}S-tyrosinase as the antigen. The ct/min immunoprecipitated in the RIA were expressed as a percentage of the ct/min immunoprecipitated by each serum without preincubation, and were plotted as a function of the cell extract dilution preincubated with the serum.

Absorption experiments with vitiligo patient sera

In absorption experiments, αPEP8 antiserum, positive patient sera and six healthy control sera were first incubated at 4°C for 16 h with COS-7 cell extract containing either expressed TRP-2 or tyrosinase and with COS-7 cell extract as control. The extracts used contained equivalent amounts of total protein. After preincubation, *in vitro* translated ^{35}S-TRP-2 was added and the RIA carried out as previously described. For each serum, a TRP-2Ab index was calculated as: ct/min immunoprecipitated by tested serum/mean ct/min immunoprecipitated by six healthy controls. A mean TRP-2Ab index was calculated for each sample tested from three experiments, the s.d. of the mean being < 15%.

RESULTS

In vitro *translation of recombinant human TRP-2 and immunoprecipitation of ^{35}S-TRP-2*

In vitro transcription-translation of pCMVDTS resulted in an incorporation of ^{35}S-methionine into TRP-2 of 6·8 ± 1·5% (mean ± s.d.) in four separate experiments. The quality of the *in vitro* translated ^{35}S-TRP-2 was evaluated by SDS–PAGE and autoradiography, which revealed a protein product with an estimated mol. wt of 57 kD (Fig. 1). This agrees well with the molecular weight of 59 kD predicted from the amino acid sequence of the protein [11].

The immunoreactivity of the *in vitro* translated ^{35}S-TRP-2 was tested using rabbit antisera at a 1:10 dilution: ^{35}S-TRP-2 was immunoprecipitated by the TRP2-specific αPEP8 antiserum but not by the tyrosinase-specific αPEP7 antiserum (Fig. 1). The average percentage of ^{35}S-TRP-2 immunoprecipitated in four assays by the αPEP8 antiserum was 58 ± 7·5% (mean ± s.d.) at a dilution of 1:10. To test the sensitivity of the RIA, ^{35}S-TRP-2 was immunoprecipitated with serial dilutions of the αPEP8 antiserum. From this, the end-point dilution of the antiserum was > 1:5800.

RIA of patient and control sera

Sera from 53 vitiligo patients, 20 healthy controls, 10 HT patients and 10 patients with GD were tested, at a dilution of 1:10, for their ability to immunoprecipitate ^{35}S-TRP-2. For each serum a TRP-2Ab index was assigned, this being the mean TRP-2AB index of three experiments with a s.d. of < 15%. The upper level of normal for the RIA (mean TRP-2Ab index + 3 s.d. of 20 healthy controls) was estimated as a TRP-2Ab index of 1·33 (Fig. 2).

None of the healthy individuals was positive for TRP-2 antibodies. From 53 vitiligo patients, three (5·9%) had a TRP-2Ab index > 1·33 (Fig. 2) and were considered positive for TRP-2 antibodies. Sera from 10 patients with HT and 10 patients with GD had a mean TRP-2Ab index of 0·94 ± 0·21 (mean ± s.d.) and 0·84 ± 0·31, respectively. All 20 sera were negative for antibodies to TRP-2.

SDS–PAGE and autoradiography were used to check that the radioactivity immunoprecipitated by each of the positive sera was due to ^{35}S-TRP-2. All positive sera immunoprecipitated a band of

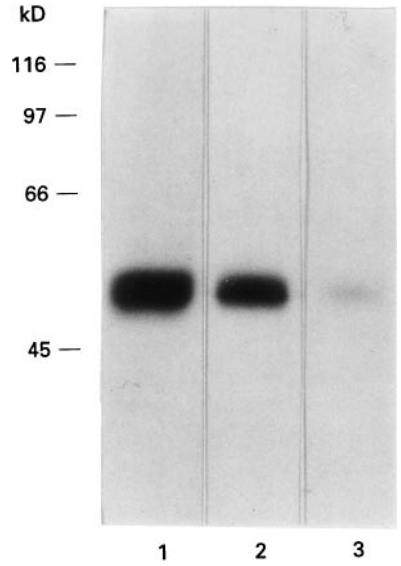

Fig. 1. SDS–PAGE and autoradiography of *in vitro* translated and immunoprecipitated ^{35}S-TRP-2. *In vitro* translated ^{35}S-TRP-2 (lane 1) and immunoprecipitation of ^{35}S-TRP-2 with TRP-2-specific αPEP8 antiserum (lane 2) and with tyrosinase-specific αPEP7 antiserum (lane 3).

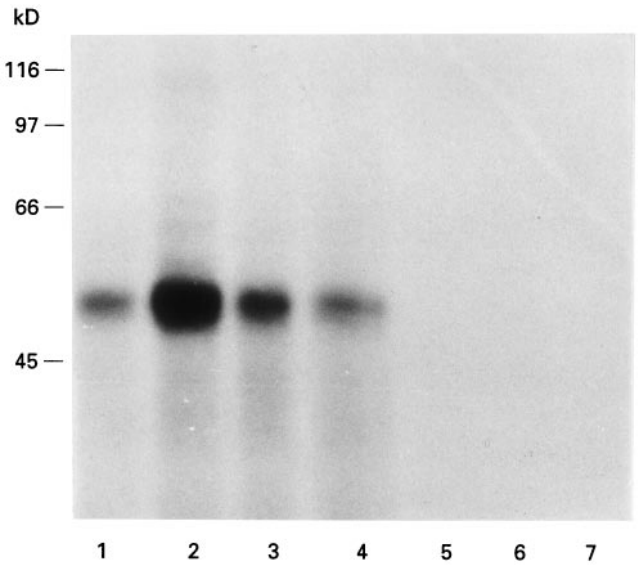

Fig. 3. SDS–PAGE and autoradiography of ^{35}S-TRP-2 immunoprecipitated with either vitiligo or healthy sera**.** ^{35}S-TRP2 immunoprecipitated with αPEP8 antiserum (lane 2), with positive vitiligo sera (lanes 1,3,4), with negative vitiligo sera (lanes 5–6) and with a healthy control serum (lane 7).

the correct size when compared with that precipitated by TRP2-specific αPEP8 antiserum (Fig. 3).

The three positive sera were analysed at different dilutions in the RIA along with a group of six healthy controls. A TRP-2Ab index for each serum sample at each dilution was plotted as a function of 1/serum dilution (Fig. 4). Each TRP-2Ab index was the mean TRP-2Ab index of three experiments, with a s.d. of the mean being within 15%. For all three sera, saturated binding was observed at dilutions up to 1:100.

Expression of TRP-2 and tyrosinase in COS-7 cells

Antiserum αPEP8 was preabsorbed with serial dilutions of COS-7 cell extract containing expressed TRP-2 and with serial dilutions of control COS-7 cell extract. The subsequent immunoprecipitation using *in vitro* translated ^{35}S-TRP-2 showed that anti-TRP-2 reactivity was decreased after preabsorption with COS-7 cell extract which contained expressed TRP-2 (Fig. 5). Likewise, αPEP7 reactivity in the RIA with *in vitro* translated ^{35}S-tyrosinase was diminished after preincubation with COS-7 cell extract containing expressed tyrosinase (Fig. 5). However, absorption was never

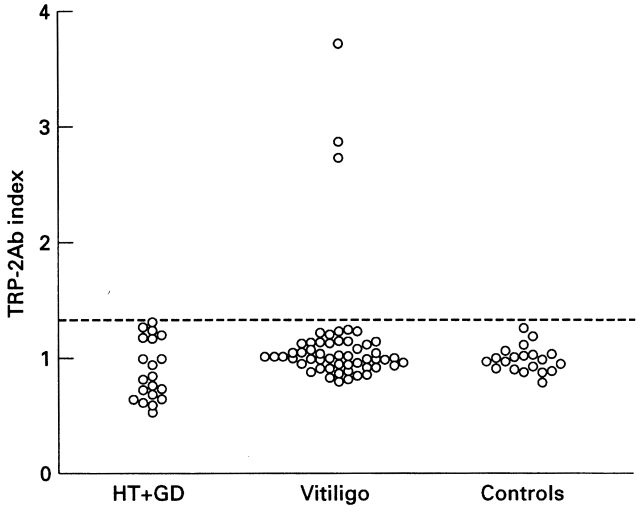

Fig. 2. TRP-2 antibody (TRP-2Ab) index of vitiligo patient sera ($n = 53$), Hashimoto's thyroiditis (HT) patient sera ($n = 10$), Graves' disease (GD) patient sera ($n = 10$) and healthy control sera ($n = 20$) in the radioimmunoassay (RIA)**.** The TRP-2Ab index shown for each serum is the mean TRP-2Ab index of three experiments. The s.d. of the mean was always within 15%. The dotted line shows the upper level of normal of 1·33 (mean TRP-2Ab index + 3 s.d. of 20 healthy controls) for the RIA.

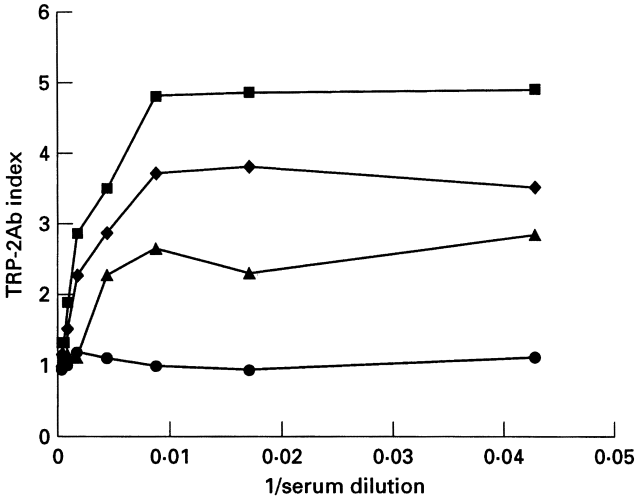

Fig. 4. Dilution curves of TRP-2 antibody-positive sera and one healthy control serum**.** The TRP-2Ab index of each positive serum and one healthy control serum at each dilution is plotted as a function of 1/serum dilution. Each TRP-2Ab index is the mean TRP-2Ab index of three experiments with a s.d. of within 15%. ■, Positive serum 1; ◆, positive serum 2; ▲, positive serum 3; ●, healthy control serum.

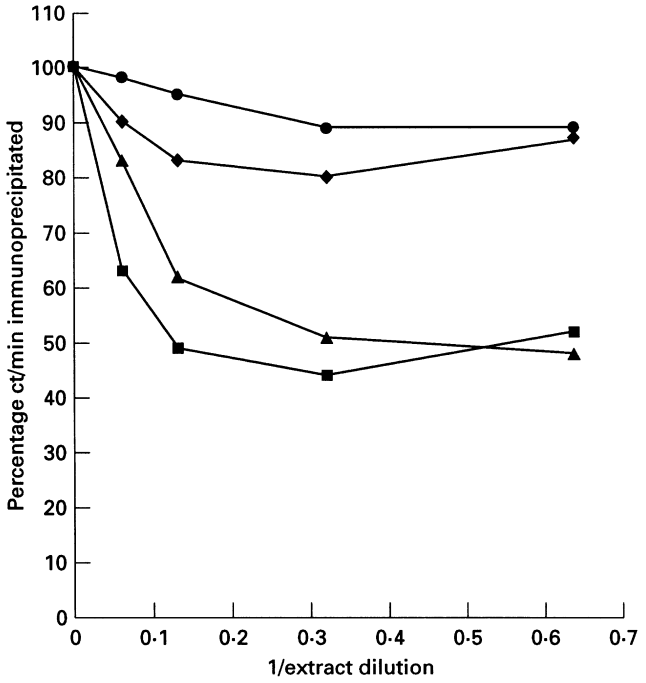

Fig. 5. Absorption experiments with αPEP8 and αPEP7 antisera to detect expressed TRP-2 and tyrosinase, respectively, in COS-7 cells. The percentage ct/min immunoprecipitated in the radioimmunoassay (RIA) is shown for: ^{35}S-TRP-2 with αPEP8 preincubated with serial dilutions of COS-7 cell extract containing expressed TRP-2 (■); ^{35}S-TRP-2 with αPEP8 preincubated with serial dilutions of COS-7 cell extract (◆); ^{35}S-tyrosinase with αPEP7 preincubated with serial dilutions of COS-7 cell extract containing expressed tyrosinase (▲); and ^{35}S-tyrosinase with αPEP7 preincubated with serial dilutions of COS-7 cell extract (●).

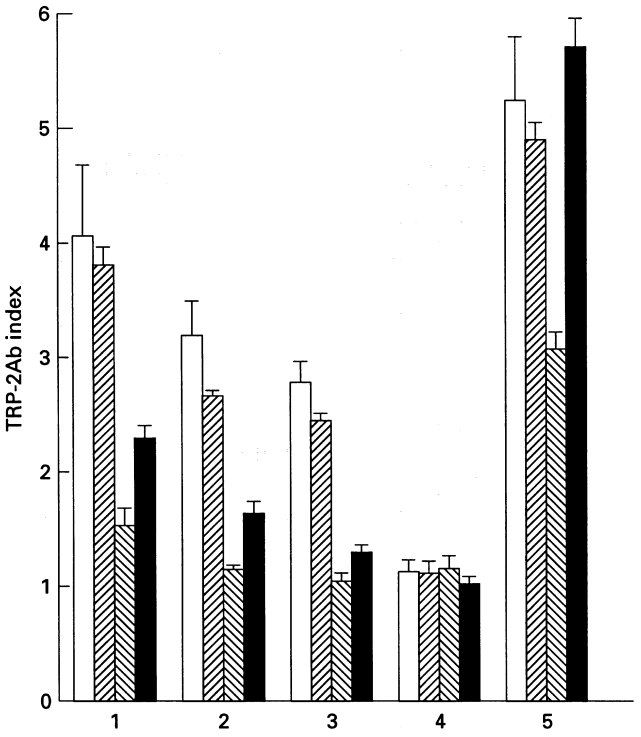

Fig. 6. Absorption experiments with vitiligo patient sera positive for TRP-2 antibodies (1–3), healthy control serum (4) and αPEP8 antiserum (5). The mean TRP-2Ab index and s.d. of each tested serum from three separate experiments are shown for immunoprecipitation of ^{35}S-TRP-2 with sera (□), with sera preincubated with COS-7 cell extract (▨), with sera preincubated with COS-7 cell extract containing expressed TRP-2 (▧), and with sera preincubated with COS-7 cell extract containing expressed tyrosinase (■).

complete with either serum, which may be due to the high antibody titres of both αPEP8 and αPEP7. The reactivity of both sera was unaffected by preincubation with COS-7 cell extract alone (Fig. 5). One representative experiment is shown, which was confirmed by two further experiments.

Absorption experiments with vitiligo patient sera

Positive vitiligo sera reacting with ^{35}S-TRP-2 were preabsorbed with COS-7 cell extracts containing expressed TRP-2 or tyrosinase, and with control COS-7 cell extract. The subsequent immunoprecipitation using *in vitro* translated ^{35}S-TRP-2 showed that the anti-TRP-2 reactivity of the positive vitiligo sera was greatly decreased when preabsorbed with COS-7 cell extract which contained either expressed TRP-2 or tyrosinase (Fig. 6), indicating antibody cross-reactivity to the two proteins. In contrast, the binding ability of serum samples absorbed with control extract was not affected (Fig. 6). Antiserum αPEP8 could be preabsorbed with COS-7 cell extracts containing expressed TRP-2, but not with extracts containing tyrosinase, indicating the specificity of the antiserum (Fig. 6).

DISCUSSION

Recently, RIAs have been developed to detect specific antibodies in sera of patients with autoimmune disease [8,12–14]. These involve *in vitro* translation and concomitant radiolabelling of the antigen of interest. The method combines sensitivity with the possibility of detecting conformational epitopes which may be of more importance in the pathogenesis of autoimmune disorders than reactivities detected by immunoblotting. It also allows a 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. We describe here the use of an immunoprecipitation assay to detect TRP-2 antibodies in sera from patients with vitiligo.

Using coupled *in vitro* transcription-translation we were able to produce ^{35}S-labelled recombinant human TRP-2. 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-TRP-2 antiserum.

The technique was used to examine vitiligo and control sera for TRP-2 antibodies. Healthy control sera and sera from patients with HT or GD did not contain TRP-2 antibodies. From 53 vitiligo sera tested, three (5·9%) contained TRP-2 antibodies. All three positive patients (mean age 54 years; age range 50–63 years) had had vitiligo of the symmetrical type for more than 1 year and all of them also had an associated autoimmune disorder: GD in one and autoimmune hypothyroidism in two. In addition, all three sera contained tyrosinase antibodies, as determined in our previous study using *in vitro* translated ^{35}S-tyrosinase in a RIA [8].

Since all three patient sera contained both TRP-2 and

tyrosinase antibodies, and considering the amino acid homology of the two enzymes, we examined the possibility of antibody cross-reactivity using absorption experiments. Pre-incubation with both TRP-2 and tyrosinase inhibited the immunoreactivity of the positive sera in the RIA using *in vitro* translated ^{35}S-TRP-2. This indicates antibody cross-reactivity and suggests that the humoral response to the two melanogenic proteins in these patients may not be entirely independent, although it is not possible to say which (if either) of the enzymes is the primary target of the antibody response.

The antibody cross-reactivity to TRP-2 and tyrosinase is not surprising when one considers the overall amino acid homology of the two enzymes: 40% identical residues and 25 conservative changes [10,11] are found when the two amino acid sequences are aligned using the multiple sequence alignment program ALIEN. Fifteen cysteine residues are aligned throughout the two proteins and a high degree of homology is found in the putative copper-binding domains: 53% identical amino acids and 22% conservative changes in a 32 amino acid sequence (residues 189–220 in TRP-2 and 180–211 in tyrosinase) and 46% identical amino acids and 32% conservative changes in a 28 amino acid sequence (residues 369–396 in TRP-2 and 363–390 in tyrosinase). Work is in progress to define the epitope common to the two enzymes.

The low frequency of antibodies to TRP-2 in vitiligo sera as found in this study suggests the protein is not a major autoantigen in patients with this disease. However, it is possible that some epitopes are not present on the ^{35}S-TRP-2 used in this immunoprecipitation assay, as it is not glycosylated in the *in vitro* transcription-translation system used and this may affect the reactivity of vitiligo sera to the protein. In addition, antibodies to linear epitopes present on TRP-2 may not necessarily be detected by this method, although they may be present in vitiligo patients and could possibly be detected by immunoblotting.

Although antibodies to melanocyte proteins are found in some vitiligo patients, we do not yet know if the humoral autoimmune response to these antigens is important in the initial onset of this disease or if it is provoked by destruction of the melanocyte and the subsequent release of autoantigen proteins. However, identification of both tyrosinase and TRP-2 as autoantigens should lead to studies of T cell responses to these proteins, which may elucidate whether a shared T cell epitope also exists. This may be important in disease pathogenesis even in patients who do not produce tyrosinase or TRP-2 antibodies.

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