

Identification of Autoantibody to Melanocytes and Characterization of Different Autoantigens in Western Indian Vitiligo Patients

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Abstract

Vitiligo is a skin disorder that is characterized by selective melanocyte destruction and simultaneous appearance of de-pigmented macules. Vitiligo is caused by an autoimmune response that is usually mediated by the cytotoxic T cells against the melanocytes. Auto antibodies against pigment cells might result from a genetic predisposition to immune dysregulation at the T cell level. Vitiligo auto antibodies could also result from an immune response to melanocyte antigens. But an immune response to damaged pigment cells might further aggravate melanocyte loss. *In vitro* studies have shown that vitiligo auto antibodies are able to destroy melanocytes by complement mediated damage and ADCC. And *in vivo* following the passive immunization of nude mice grafted with human skin. Hence the present study was conducted in our laboratory on 80 Vitiligo patients and 30 Healthy Normals for their anti-melanocyte antibody against melanocyte antigens using Electrochemical detection to understand the real cause of melanocyte destruction. This is the first study conducted in India.

Introduction

Vitiligo is a skin disorder that is characterized by selective melanocyte destruction and simultaneous appearance of de-pigmented macules that over the time enlarge, coalesce and form patches [1]. It has been suggested that vitiligo is caused by an autoimmune response that is usually mediated by the cytotoxic T cells against the melanocytes [2-4]. The destruction or the lyses of melanocytes give a direct proof of an autoimmune phenomenon [5].

Auto antibodies against pigment cells might result from a genetic predisposition to immune dysregulation at the T cell level. Vitiligo auto antibodies could also result from an immune response to melanocyte antigens. But an immune response to damaged pigment cells might further aggravate melanocyte loss [6]. *In vitro* studies have shown that vitiligo auto antibodies are able to destroy melanocytes by complement mediated damage and Antibody Dependent Cellular Cytotoxicity (ADCC) and *in vivo* following the passive immunization of nude mice grafted with human skin [7-9].

Hence the present study was conducted in our laboratory to understand the real cause of melanocyte destruction. This is the first study conducted in India.

Materials and Methods

The Study involved Human Vitiligo patients and Healthy Normal individuals.

Patient group

Clinically confirmed cases (N=80) of Human Vitiligo were included in this study. These patients were selected in our OPD of skin department. These patients were clinically examined by a well known dermatologist and the patient's clinical findings and family history were noted in the proforma. Informed consent was taken from the patients before the collection of blood for the autoantibody study.

Ethical approval was taken from the Scientific Advisory Committee of the Sir.H.N.Hospital and Research Centre and the project was sanctioned by the Management of the Medical Research Society.

These patients were broadly categorized into 2 groups such as Group I and Group II depending on the involvement of the disease.

Group I (N=10) with Localized lesions further divided according to the stage of the disease, as

- i) Active disease (N=9) ii) Inactive disease (N=1).

Group II (N=70)

- i) Active (N=49) and ii) Inactive (N=21).

A Control group of Healthy Normal individuals (N=30) which were age and sex matched were taken for comparison in this study. The individuals were free of any infections and previous illness. Anti-Melanocyte Antibody in the serum of vitiligo patients and controls were detected using SDS-PAGE and Western Blotting using the Enhanced chemiluminescence (ECL) method.

Melanocyte culture

Cultured Human Melanocytes taken from Neonatal foreskin after 4 passages having a confluency of 80% were received from Co-centre on request. Melanocytes were centrifuged and supernatant was discarded.

Extraction of melanocyte protein

The pellet was washed with PBS and again centrifuged. To this is added 1ml of NP-40 (containing 5M NaCl, 1M Tris-HCL) and the cells were homogenized using a homogenizer. The lysate was centrifuged

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and the supernatant was removed in a different tube and processed for protein estimation. Protein concentration was estimated by Bradford assay using Coomassie plus Protein Assay [10].

SDS-PAGE electrophoresis

SDS-PAGE is performed by the following method. Briefly 7.5% of resolving gel is prepared containing (29% w/v acrylamide and 1% bisacrylamide in a 1.5 M Tris HCL buffer pH 8.8, 10% SDS, and 10% APS with 10ul of TEMED added just before making the volume with milli-Q water.) The gel is poured through the glass plates upto the desired level. The resolving gel was covered with water saturated butanol to keep the gel surface straight so that there is a flat and sharp interface. After polymerization of resolving gel for 15-20 minutes a 4% stacking gel is added that contained (29% w/v acrylamide and 1% bisacrylamide in a 1.5 M Tris HCL buffer pH 8.8, 10% SDS, and 10% APS with 10ul of TEMED added just before making the volume with milli-Q water). The comb was fully inserted depending on the number of wells. The stacking gel is allowed to polymerize completely.

The gel was assembled in the electrophoresis apparatus and the electrophoresis buffer was added till all the electrodes were properly submerged. The protein was mixed with Laemmli buffer and loaded in the wells. Each well contained 40ug proteins. A prestained protein marker (Sigma-USA) was also added to determine the molecular weight of the proteins of our interest. The Electrophoresis was carried out at 200V and 150mAmp for 1 hour. Proteins were electrophorised till the dye front reached to the end of the gel. The gels were then removed and processed for staining for proper transfer of proteins and later the proteins were transferred for further electro-chemical detection.

Assay of melanocyte antibodies by western blotting

This was performed according to the method of Towbin et al. [11]. Briefly (0.45µm) nitrocellulose paper was cut into 6x10 cm size. Filter papers were also cut in the same size about 7x11cm. Electrophorized gel, nitrocellulose membrane, filter papers and pressure pads with the gel placed in the transfer cassette inbetween the two filter papers and transfer pads and then were kept for half an hour in a tank containing transfer buffer (Toubins transfer buffer-Tris HCL-0.63%, glycine 1.38% and Methanol about 50% of the reconstituted volume of buffer with milli-Q water and a pH of 8.5. The transfer module or cassette kept in the tank and western blot is run at 100V and 75mA for 2 hours. The temperature of the buffer is kept low.

Immunoassay by electrochemical detection

After the 2 hr run the membrane is removed and separated from the gel carefully. 4ml of the blocking buffer was added onto the blot to cover the whole blot and incubated at RT for 2 hours with constant shaking to block the non-specific sites. 1:100 diluted serum (Patients or Healthy controls) was then added after removing the excess blocking buffer on to the individual strips cut in a single lane. The blot is then incubated overnight at 4°C. Diluted serum is then removed and the blot is then washed 3 times for 5 mins with wash buffer. A 1:1000 diluted Clean Blot IP detection Reagent which is the secondary antibody is then added to the blot and incubated for 1 hr at RT. The Secondary antibody is then removed and the blot was then washed 3 times for 5 mins with wash buffer.

ECL working solution is then prepared by combining equal portions of Pierce ECL detection Reagent 1(Peroxide solution) and ECL detection 2 (Luminol enhancer solution). A 0.125 ml of this

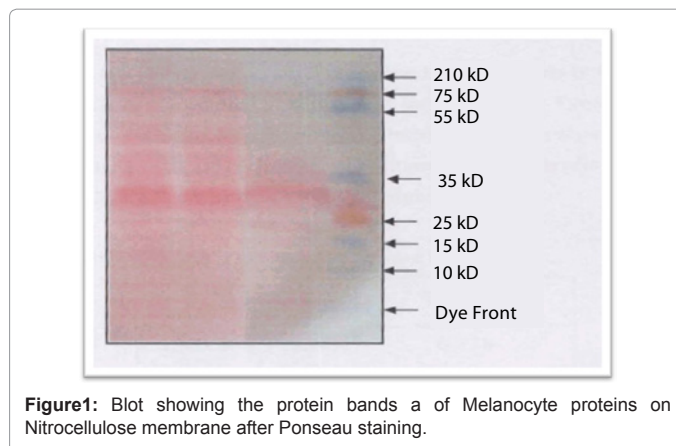


Figure 1: Blot showing the protein bands a of Melanocyte proteins on Nitrocellulose membrane after Ponceau staining.

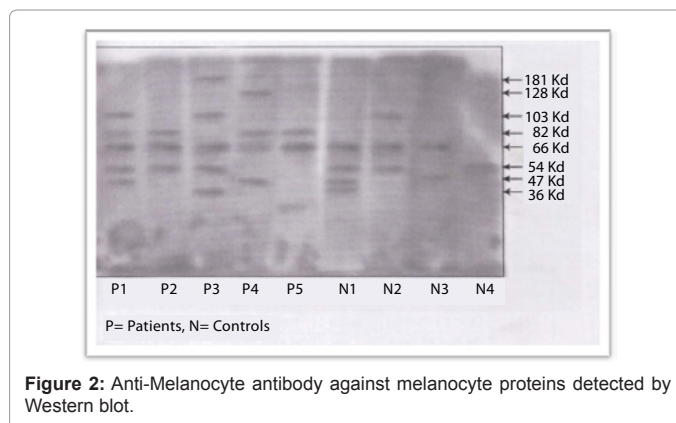


Figure 2: Anti-Melanocyte antibody against melanocyte proteins detected by Western blot.

solution mixture is then added to the blot and then incubated for 5 minutes. The ECL working solution was removed and exposed to the X-ray film for 1 minute. The exposed X-ray film was dipped in the developer for appropriate time till the bands were clearly visualized in Infra-Red light and then immediately washed in water, fixed for a few seconds and then allowed to dry for further observations.

Statistical analysis

Statistical analysis was performed using SPSS version 16 and student 't' test is used for analysis.

Results

Table 1 gives the general characteristics of the study group. The ratio of male to female being 1. This study included Group I Vitiligo patients (N=10) showing localized patches and further divided into 2 groups as Active Disease N=9 and Static disease N=1. Similarly Group II showed generalized vitiligo (N=70) and divided into 2 groups as Active disease N=49 and Static stage of the disease as N=21.

Vitiligo is divided in to 2 types-Stable or static and Active. Stable disease is described as, when neither spread of existing lesions nor development of new lesions have appeared in the previous lesions during 6 months. The results were compared to a control group (N=30) of healthy individuals.

We observed that most of the patients showed active stage of disease showing increased development in the disease.

Table 2 shows the Melanocyte antibody as detected by Western

blot. Anti-Melanocyte antibody was detected in the circulating sera and its reactivity was checked against the extracted proteins on Western blot. This table compares the reactivity shown by Group I and Group II patients towards proteins such as 66.10kD, 53.75kD, and 46.52 kD using Fisher's Exact test. Significant reactivity was seen against these protein bands when compared with controls. However Group I patients showed non-significant reactivity towards these bands.

Besides the Normal bands seen in Controls. Table 3 gives the reactivity of Group I and Group II patients towards 128kD protein wherein 2/10 and 6/70 patients showed reactivity. Similarly 4/70 of Group II patients showed reactivity towards 292 kD protein. Table 4 shows the different Normal bands and the specific bands.

Table 5 represents the number of patients showing reactivity towards the different melanocyte antigens by western blot analysis. Out of 30 healthy controls 24 reacted towards the 66kDa protein which is normal albumin band present in the melanocyte extract. Similarly 12 healthy controls also reacted towards the 82.50 antigen. Similarly 11/49-103.742kDa, 21/49 -82.50 and 49/49 -66.097 kDa showed positive reactivity from Group II patients thus proving that these are the normal protein bands also present in the controls.

Sr.No	Group	No. of Subjects(N)	Stage		Sex male/ female	Age Range Yrs.
			Active	Static		
1	Group I	10	9	1	4:6	5-52
2	Group II	70	49	21	30:40	5-72
3	Control(Healthy individuals)	30	-	-	13:17	21-45

Table 1: General Characteristics of Vitiligo patients and Controls.

Bands kDa	Significance of serum antibodies showing Reactivity to Melanocyte Protein Bands (pValue)	
	Group I(N=10)	Group II(N=70)
292	-	0.313
181.38	1.00	1.00
128.06	0.058	0.174
103.74	0.696	0.44
82.50	-	0.822
66.10	1.00	0.0027***
53.75	1.00	0.00037***
46.52	0.556	0.05*
35.65	1.00	0.087

p<0.01, **p<0.05, ***p<0.001

Table 2: Comparison of serum Anti-Melanocyte antibody reactivity of group I and Group II patients against melanocyte protein bands compared with controls by Western Blot and analysed by Fisher's Exact test.

Bands	Group I	GroupII
128	2/10(20 %)	6/70(8.5 %)
292	0/10(0 %)	4/70(5.7 %)

Table 3: Percentage reactivity of patients towards specific bands.

Normal Bands -kDa	Specific Bands -kDa	
181.38	128.06	292
103.74	Group I-2/10(20%),	Group I-0/10 (0%),
82.50	GroupII-6/70(8.5%).	Group II 4/70(5.7%).
66.10		
53.75		
46.52		
35.65		

Table 4: Represents the Normal Bands and Specific Bands.

Figure 1 shows the different bands with the marker bands after ponceau staining, while figure 2 shows the blot with the reactivity of the Patients and Normal serum towards the different bands.

In the current study we found anti-melanocyte antibodies against multiple protein bands as compared to previous studies where bands were found against 2 or 4 melanocyte proteins. Group I patient did not show any significant difference in the number of bands when compared to Controls. But Group II patients showed significant difference at 66.10kDa, 53.75kDa and 46.52kDa bands when compared to controls. Also other bands such as 128 kDa were seen in 20% of Group I patients and 8.5% of Group II patients. 5.7% of Group II patients showed reactivity against 292kDa protein though they were not significant but they were just found in Vitiligo patients.

Discussion

Norris et al. [7] showed that the presence of anti-melanocyte antibody is detected by the lysing the cultured human melanocytes by both complement activation and antibody-dependent cellular cytotoxicity (ADCC).He found significant antibodies by both the methods in the vitiligo patients when compared to controls.

Cui et al. [14] analysed the response of sera of vitiligo patients and controls by immunoprecipitation and SDS-PAGE analysis using ¹²⁵I labeled cell antigens on pigment and control cells. 78% of vitiligo and 14% of controls showed the presence of antibodies. These were directed to one or more of the antigens of Molecular weight 35, 40-45, 90 or 150kDa. These antibodies are directed to several cell surface antigens some of which are expressed on pigment cells. He also studied VIT 90, VIT 75 and VIT 40 antigens and found that 83% of vitiligo patients had antibodies while only 7% of controls showed reactivity against these antigens.

Park et al. [15] performed indirect immunofluorescence and live cell ELISA as well as immunoblotting for detection of antibodies. 44% of vitiligo patients showed the presence of antibodies which were directed to molecular weight 65kDa melanocyte antigen.

But Hann et al. [16] showed that 93% of Vitligo patients and 62% of controls had the presence of antibodies and these reacted to 83kDa protein. In addition the patients with vitiligo had antibody responses to antigens with molecular weight of 45,65,and 110kDa.The heterogeneity of the antibody responses was further confirmed by the presence of antibodies to at least 3 distinct antigens in one third of vitiligo patients but absent in the healthy controls. There was no difference in antibody responses between patients with generalized and segmental vitiligo suggesting that pathogenesis of the disease is similar in both cases.

From our studies we observed that patients of Group I did not show any significant difference in the number of bands but Group II patients showed significant difference in the reactivity towards 66.10, 53.75 and 46.52 kDa bands when compared with controls. Besides this 20% of Group I and 8.57% of Group II patients reacted to 292kD which were not significant.

Thus this study indicates that antibodies are present in the patient's serum and have different functional ability to selectively kill the melanocytes. These are more common in the active disease and are directed against the pigment cell antigens.

Antibodies to melanocytes are present in circulation in significant proportion of patients with vitiligo. In our present study we observed

	Bands	292 kDa	181.381 kDa	128.058 kDa	103.742 kDa	82.50 kDa	66.097 kDa	53.754 kDa	46.518 kDa	35.650 kDa
Group II	Active (N = 49)	0	0	4	11	21	49	33	21	14
	Static (N = 21)	4	4	2	4	4	20	21	3	1
Group I	Active (N = 1)	0	0	0	0	0	1	1	0	0
	Static (N = 9)	0	0	2	2	0	5	2	0	0
Controls	(N=30)	0	1	0	9	12	24	8	4	2

Table 5: Number of positive bands representing the number of patients positive towards the anti-melanocyte antibody.

that patients of Group I did not show any significant difference in the number of bands when compared to Controls. But Group II patients showed significant difference in the reactivity towards 66.10, 53.75 and 46.52 bands when compared with controls. Besides this 20% of Group I and 8.57% of Group II patients reacted to 292kD which were not significant.

It has been proved that the presence of auto antibodies in patients serum directed against the pigment cells have a direct correlation between the level of these auto antibodies and the disease activity [12].

Studies conducted by Naughton et al. [13]. According to Naughton et al. (1983) 82% of 14/14 common vitiligo, 5/5 vitiligo associated with chronic MCC, 31/42 vitiligo associated with other immune diseases showed the presence of antibodies to melanocyte. None of the controls showed any antibodies. In our study, we detected antibodies against certain melanocyte antigens in controls and specific bands such as 128kDa and 292kDa in Group I and Group II patients. None of Group I patients showed reactivity towards 292 kDa protein bands. Antibodies to pigment cell antigens were present in 75% of the patients but 14% of the controls showed the presence of antibodies as studied by Cui et al. (1992). These antibodies were directed to one or more antigens with Molecular weight such as 35, 40-45, 75, 90, or 150.

In our study we have our different set of Molecular weight antigens such as 181.38, 103.74, 82.50, 66.1053.75, 46.52 and 35.65. Thus these results are not in accordance with the published literature. Cui et al. (1995) also studied vitiligo antibodies in 83% of patients against VIT 90, VIT 75 and VIT 40 antigen and around 7% of controls also showed these antibodies. While we have not studied antibodies against VIT 90, 75 and 40. Park et al. (1996) showed autoantibodies in the vitiligo sera that reacted with vitiligo Antigen on the surface of melanocytes. While immunoblot analysis showed 44% of vitiligo sera directed to the 65kDa melanocyte antigen. We in our study have extracted all the proteins from the melanocytes and the extracted protein was run on the SDS-PAGE and then transferred on the nitrocellulose membrane to be used as antigen against which the antibody gets attached or forms a complex. Most of the authors have studied these proteins on western blot. Like Hann et al (1996) who studied antibodies to melanocytes using WB. 93% of vitiligo and 62% of controls who reacted towards 83kDa antigen. Some of antibodies were towards 45, 65 and 110kDa proteins. wherein 19% of the controls also reacted towards 45kDa protein. The heterogeneity of the antibody responses to melanocytes in vitiligo was confirmed by the presence of antibodies to at least 3 distinct antigens in one-third of the vitiligo patients but in none of the normal individuals. He did not find any difference in patients with generalized or segmental vitiligo. We also have studied antibodies towards melanocytes antibodies. We have also studied the melanocyte antibodies by western blot analysis and found to show a difference in Group I and Group II (Table 4).

Table 5 represents the number of patients showing reactivity towards the different melanocyte antigens by western blot analysis.

Out of 30 healthy controls 24 reacted towards the 66kDa protein which is normal albumin band present in the melanocyte extract. Similarly 12 healthy controls also reacted towards the 82.50 antigen. Similarly 11/49-103.742kDa, 21/49 -82.50 and 49/49 -66.097 kDa showed positive reactivity from Group II patients thus proving that these are the normal protein bands also present in the controls.

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