CHAPTER VIA

EVALUATION OF AUTOIMMUNE HYPOTHESIS IN GUJARAT VITILIGO PATIENTS

6A.1. Introduction

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes from the epidermis. The mechanism of melanocyte loss from the epidermis to cause vitiligo is not yet clearly understood (Taieb et al 2000). The three major hypotheses that are proposed to explain the pathogenesis of vitiligo are autoimmune, neurochemical, and oxidative stress hypotheses (Ortonne and Bose 1993). Vitiligo is considered to be an autoimmune disease because of its association with several other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata (Kemp et al 2001). In addition, various circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients (Kemp et al 2001; Shajil et al 2005). A recent study performed on 2624 vitiligo probands from North America and UK confirmed significant increase in the frequencies of six autoimmune disorders in vitiligo probands and their first degree relatives: vitiligo itself, autoimmune thyroid disease (particularly hypothyroidism), pernicious anemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease (Alkhateeb et al 2003). Association of these diseases with vitiligo indicates that vitiligo disorder shares common genetic etiologic link with other autoimmune diseases (Passeron and Ortonne 2005). Also the immune suppressive effect of a number of repigmenting therapies (steroids, UV radiation) indirectly supports the autoimmune mediated process of depigmentation (Ongenae et al 2003).

In the present study, plasma from vitiligo patients was examined for the reactivity with the human melanoma cell line (SK Mel 28) to find the levels of antimelanocyte antibodies in vitiligo patients compared to controls. Also plasma from vitiligo patients was examined for the reactivity with membrane proteins of human melanoma cell line SK Mel 28 by western blot to identify the pigment cell antigens defined by antibodies in Gujarat vitiligo patients.

6A.2. Materials and Methods

Blood was collected from the vitiligo patients (n = 102) visiting Sir Sayajirao Gaikwad Medical College hospital, Param Ayurvedic hospital and Sakarda and Civil hospital, Ahmedabad who were not suffering from any other disease after a written consent was obtained from them. Also blood was collected from one twenty seven age matched healthy controls.

Human melanoma cell line SK Mel 28 was obtained form NCCS, Pune and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 50 mg/l gentamycin at 37°C under 5% CO_2 in a humidified atmosphere (Hann and Kim 1995).

Enzyme linked immunosorbent assay (ELISA)

Cells were harvested by scraping and lysed by adding lysis buffer (HEPES 20 mM, EGTA 1 mM, PMSF 1 mM, $MgCl_2$ 1.5 mM, NaCl 150 mM, $CuSO_4$ 1mM, Trition -X 100 1%, Glycerol 1%). Protein estimation in the lysate was done by Lowry's method (Lowry et al 1951). Fifty µl of the cell lysate containing 1 µg protein was loaded in each well of the microtiter plate and kept at 4° C overnight. Excess antigen was discarded, washed 3 times with PBS, blocked with 1% BSA in PBS and incubated for 1 hour. Excess blocking reagent was discarded and washed 3 times with PBS. Fifty µl of plasma (1:10,000 diluted in PBS) was added to the micro titer plates and incubated for 2 hours at room temperature. Excess plasma was discarded and washed 3 times with PBS containing 0.2% Tween 20. Added 50 µl of 1: 2000 diluted secondary antibody (Rabbit anti human IgG HRP conjugate, Bangalore Genei, India) and incubated for 1 hour. Excess antibody was discarded and washed 3 times with PBS containing 0.2% Tween 20. Fifty µl substrate (TMB H_2O_2) was added and incubated for 5 minutes for the color development. The reaction was stopped by adding 200 µl of 1N H_2SO_4 as the color developed and OD was read at 405 nm.

SK Mel 28 membrane preparation

SK Mel 28 cells were harvested using 1 mM EDTA in Hank's balanced salt solution and homogenized in sucrose buffer (0.25M sucrose, 0.5mM $MgCl_2$, 50mM Tris, 1 mM PMSF, 1µM leupeptin) pH 7.4 at 4°C (Hann et al 1996). The homogenate was centrifuged at 2000x g for 10 minutes and the supernatant was collected. The supernatant was centrifuged at 1,05,000 x g for 1 hour. The resulting pellet was washed with washing buffer (0.15 M NaCl, 50 mM Tris, 1 mM EDTA pH 8.0) and resuspended in 0.5% NP40 and stored at –80° C until use.

Immunoblotting

SK Mel 28 cell membrane lysate containing 20 µg of protein was subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) under reducing conditions (2 mercaptoethanol, 10%) (Hann et al 1996) and electroblotted onto PVDF membrane. The membranes were blocked with 3% gelatin in PBS for one hour at room temperature and incubated with individual plasma (1:500 diluted in blocking buffer) overnight at 4°C. The membranes were then washed 3 times with PBS containing 0.15% Tween 20 and then incubated with HRP conjugated secondary antibody (Rabbit anti human IgG, HRP conjugate) diluted 1:1000 for 2 hours at room temperature, washed 3 times with PBS containing 0.15% Tween 20 and developed with DAB/H_2O_2 reagent. The developing reagent contained of 5 ml PBS, 2 mg DAB, 50 µl nickel chloride and 5 µl H_2O_2.

Reagents for SDS PAGE and Immunoblotting

Acrylamide monomer solution (25 ml)	
Acrylamide	7.3 g
Bis acrylamide	200 mg
Double distilled water	25 ml

Running gel buffer, pH 8.8 (4X, 50 ml)	
Tris Base (1.5 M)	9.08 g

Stacking gel buffer, pH 6.8 (4X, 50ml)	
Tris Base (0.05 M)	3 g

Tank buffer, pH 8.3 (500 ml)	
Tris Base (0.025 M)	1.52 g
Glycine	7.20 g
SDS 0.1%	5 ml from 10% SDS Stock solution

Treatment buffer (2 ml)	
Stacking gel buffer	500 µl
SDS 10%	800 µl
Glycerol	400 µl
2 Mercaptoethanol	200 µl
Bromophenol blue	4 mg
Double distilled water	200 µl

Water saturated butanol	
Butanol	50 ml
Double distilled water	5 ml

After shaking well used the top phase

Running gel, 8% (5ml)	
30% acrylamide solution	1.3 ml
Running gel buffer	1.3 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	3 µl
Double distilled water	2.3 ml

Stacking gel, 5% (4ml)	
30% acrylamide solution	670 µl
Double distilled water	2.7 ml
Stacking gel buffer	500 µl
10% SDS	40 µl
10% APS	40 µl
TEMED	4 µl

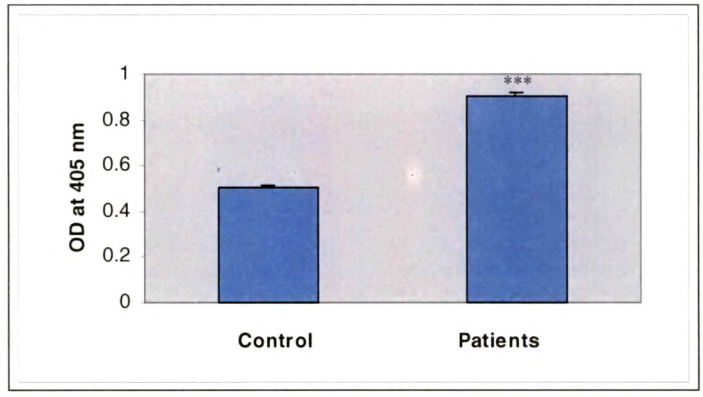
Transfer buffer (900 ml)	
Tris	2.72 g
Glycine	12.96 g
Double distilled water	720 ml
Methanol	180 ml

6A.3. Results

Autoimmune hypothesis is one of the theories to explain the pathogenesis of vitiligo. The aim of this study was to determine the presence of antimelanocyte antibodies in vitiligo patients compared to controls and also to correlate the level of antibodies in different age groups, different clinical types as well as in active and stable vitiligo.

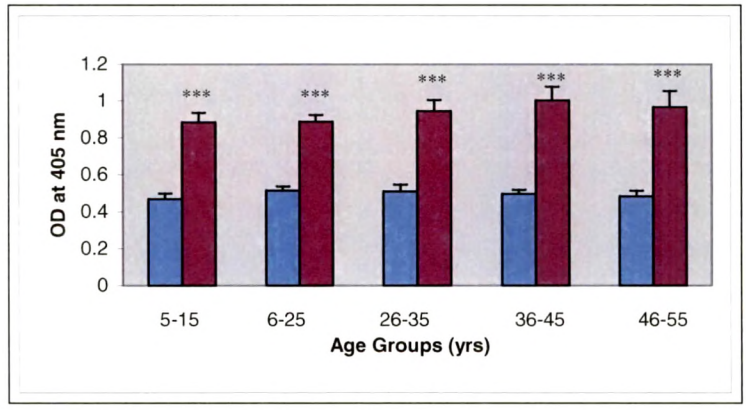
Sixty-six percent of vitiligo patients had antimelanocyte antibodies in their circulation. The levels of antimelanocyte antibodies were found to be significantly increased in vitiligo patients compared to controls (Figure 1). Vitiligo patients were divided into five age groups i.e. 5 – 15, 16 – 25, 26 – 35, 36 – 45 and 46 - 55 yrs for the analysis of antimelanocyte antibody levels to determine whether age has any relevance for the onset of the disease and the results are shown in Figure 2. All the age groups showed significant increase in the antimelanocyte antibody levels in vitiligo patients compared to controls.

Figure 1. Antimelanocyte antibody levels in vitiligo patients compared to controls#



Values are given as mean ± SE of 127 individual observations in controls; 102 individual observations vitiligo patients. ***, p<0.001

Figure 2. Antimelanocyte antibody levels in different age groups of vitiligo#

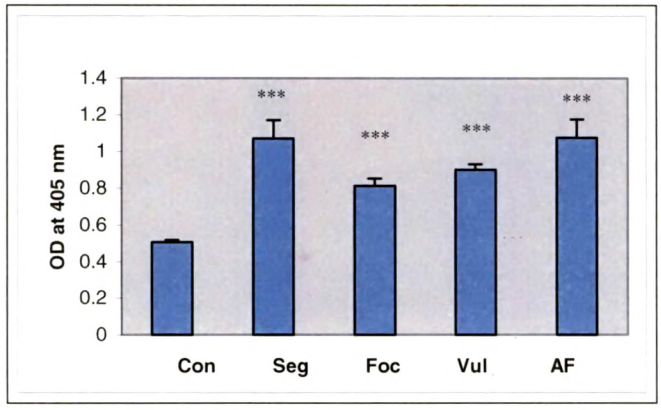


Values are given as mean ± SE of 11 and 21 individual observations in controls and patients in 5-15 age group; 51 and 36 individual observations in controls and patients in 16-25 age group; 25 and 11 individual observations in controls and patients 26-35 yrs age group and 22 and 14 individual observations in controls and patients in 36-45 age group; 13 and 9 individual observations in controls and patients in 46-55 yrs age group respectively. ***, p<0.001. The blue bars are of controls and the pink bars are of vitiligo patients.

Further, vitiligo patients were classified according to different clinical types i.e. vulgaris, focal, segmental, acrofacial and universal for the analysis of antimelanocyte

antibody levels and the results are shown in Figure 3. Different clinical types of vitiligo patients showed significant increase in the antibody levels compared to controls.

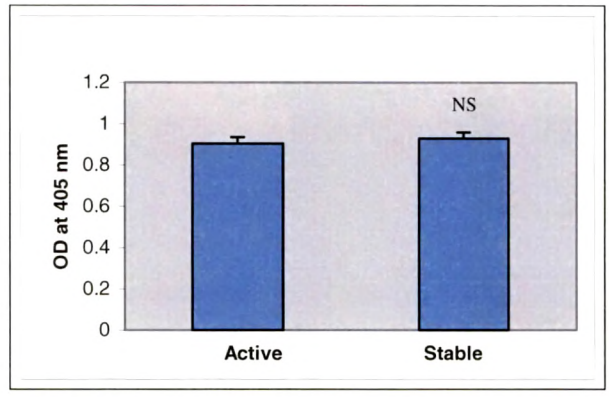
Figure 3. Antimelanocyte antibody levels in different clinical types of vitiligo#



Values are given as mean ± SE of 127 individual observations in controls; 57 individual observations vulgaris vitiligo; 18 individual observations in focal vitiligo; 8 individual observations acrofacial vitiligo and 8 individual observations in segmental vitiligo respectively. ***$p<0.001$. Con, Control; Seg, Segmental, Foc, Focal; Vul, Vulgaris, AF, Acrofacial.

Also we have compared the antimelanocyte antibody levels in active and stable vitiligo patients to find the role antimelanocyte antibodies in the progression of disorder. There was no significant change observed in the levels of antimelanocyte antibodies in active vitiligo patients compared to stable vitiligo patients. The results are shown in Figure 4.

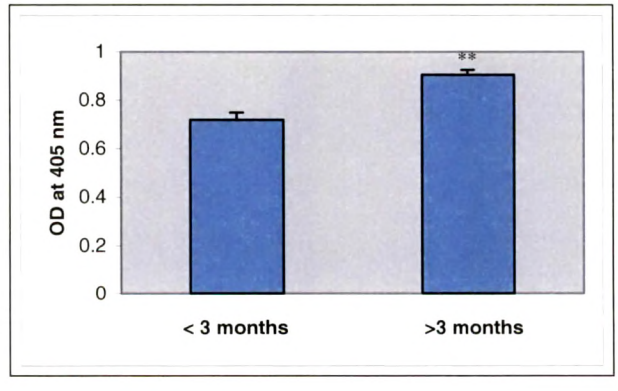
Figure 4. Antimelanocyte antibody levels in active and stable types of vitiligo#



Values are given as mean ± SE of 41 individual observations in active vitiligo; 52 individual observations in stable vitiligo. NS, non significant.

We have compared the antimelanocyte antibody levels in patients during the onset of vitiligo (< 3 months) with those suffering with vitiligo for a long duration (>3 months) to find the role of autoimmunity in precipitating vitiligo in susceptible patients. There was significant decrease ($p<0.005$) observed in the levels of antimelanocyte antibodies in <3 months patients compared to >3 months patients. The results are shown in the Figure 5.

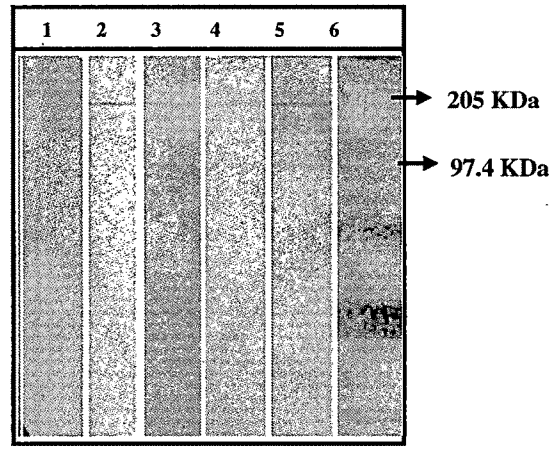
Figure 5. Antimelanocyte antibody levels based on the duration of vitiligo#



Values are given as mean ± SE of 16 individual observations in <3 months vitiligo patients; 87 individual observations in > 3 months vitiligo patients. **, $p<0.01$.

We have also performed western hybridization of membrane antigens of SK Mel 28 cells to identify the pigment cell antigens defined by antibodies and the results are shown in the Figure 6. As can be seen from Figure 6, one prominent protein band of approximately 200 kDa was obtained in vitiligo patients compared to controls.

Figure 6. Western hybridization of antibodies to melanocyte membrane antigens in vitiligo patients compared to controls



Lane 1: Control, Lane 2, 3, 4, 5 vitiligo patients, Lane 6: Molecular weight marker

6A.4. Discussion

The results of this study indicate that 66% of Gujarat vitiligo patients exhibited antimelanocyte antibodies in their circulation. Vitiligo patients showed significant increase in the levels of antimelanocyte antibodies in their circulation compared to controls (Figure 1). Naughton et al (1983a) reported that 82% of vitiligo patients showed antimelanocyte antibodies in their circulation (Naughton et al 1983a). In another study, 12/12 vitiligo patients and 0/12 controls were reported to have antimelanocyte antibodies in their circulation (Naughton et al 1983b). However, Grimes et al (1983) reported that 19% of Black vitiligo patients showed antimelanocyte antibodies in their circulation. Recently, Farrokhi et al (2005) also

showed the presence of antimelanocyte antibodies in 30.9% of Iranian vitiligo patients.

Further we have analyzed antimelanocyte antibody levels in different age groups of vitiligo to find whether age has any significant role in the precipitation of vitiligo. All age groups showed significant increase in the antimelanocyte antibody levels compared to controls (Figure 2). Harning et al (1991) reported that there is a correlation between the incidence and the level of antimelanocyte antibodies and the disease activity in vitiligo i.e. 0/14 with active vitiligo, 0/14 in inactive vitiligo and 0/19 controls were found to exhibit circulating antibodies in their circulation (Harning et al 1991). However, our present study has not shown any significant difference in the levels of antimelanocyte antibodies in active and stable vitiligo patients (Figure 4). Our study showed a significant increase in the antimelanocyte antibody levels in all clinical types of vitiligo compared to controls. However, our analysis by ANOVA suggests that there is no significant change in the level of antimelanocyte antibodies in different clinical types of vitiligo (Figure 3). There are no reports available on the antimelanocyte antibody levels in different clinical types of vitiligo. Further, to find the role of antimelanocyte antibodies during the onset of vitiligo, the patients were classified into two groups i.e. the duration of disease <3 months and duration of disease >3 months. Patients with early onset (< 3 months) of vitiligo showed a significantly decreased ($p<0.005$) level of antimelanocyte antibodies compared to patients with long duration (>3 months) of vitiligo (Figure 5). This shows that autoimmune hypothesis may not be playing a major role in the initiation of vitiligo. Several authors reported that the principal antigen recognized by these antibodies are tyrosinase, TRP1, TRP 2 and other melanocyte differentiating antigens such as gp 100/Pmel 17 (Kemp et al 1997; Kemp et al 1998a; Kemp et al 1998b).

All the vitiligo patients showed a prominent band of approximately 200 kDa in the western blot analysis. In controls no bands were observed (Figure 6). Vitiligo antibodies are usually directed against pigment cell antigens of 35 kDa, 40-45 kDa, 75

kDa, 90 kDa and 150 kDa, which are located on the surface of the cells (Cui et al 1992; Cui et al 1993). The proteins of 40-45 kDa, 75 kDa and 150 kDa are common tissue antigens while proteins of 35 kDa and 90 kDa are preferentially expressed on pigment cells (Norris et al 1988). In addition, antibodies to the melanocyte specific proteins tyrosinase, TRP1, TRP 2 (Kemp et al 1997) and Pmel 17 were also detected in the sera of vitiligo patients (Kemp et al 1998a). However no antigen of 200 kDa has been reported in vitiligo patients. The surface antigen of 200 kDa reported in this study may be a new antigen which might be playing a major role in the precipitation of vitiligo in Gujarat population.

The exact role of antimelanocyte antibodies in the pathogenesis of vitiligo is unknown. Cross-reacting antigens that are expressed either on other cells or on infecting microorganism may elicit the production of antimelanocyte antibodies (Kemp et al 2001). Alternatively, vitiligo antibodies may result from an immune response to melanocytes antigens following damage to melanocytes by other mechanisms. These antibodies may further exacerbate the condition. It is not clear that antimelanocyte antibodies are the cause or effect/result of the disease. However vitiligo antibodies have the capacity to destroy melanocytes *in vitro* by complement mediated cytotoxicity and antibody dependent cellular cytotoxicity (Norris et al 1988; Cui et al 1993). Vitiligo antibodies also have the ability to kill the melanocytes *in vivo*, when passively administered to nude mouse grafted with human skin (Gilhar et al 1995).

6A.5. References

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CHAPTER VI B

EVALUATION OF OXIDATIVE STRESS AND AUTOIMMUNE HYPOTHESES AT THE ONSET OF VITILIGO

6B.1. Introduction

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes from the epidermis. The mechanism of melanocyte loss from the epidermis to cause vitiligo is not yet clearly understood (Taieb et al 2000). The three major hypotheses that are proposed to explain the pathogenesis of vitiligo are oxidative stress, autoimmune and neurochemical hypotheses (Ortonne and Bose 1993). Vitiligo is considered to be an autoimmune disease because of its association with several other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata (Kemp et al 2001). In addition, various circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients (Kemp et al 2001). Also the immune suppressive effect of a number of repigmenting therapies (steroids, UV radiation) indirectly supports the autoimmune mediated process of depigmentation (Ongenae et al 2003). However the exact mechanism involved in vitiligo pathogenesis remains unknown.

The aim of this study was to find whether oxidative stress or autoimmunity plays a major role for the initiation of vitiligo pathogenesis in Gujarat population. Lipid peroxidation levels, the index of oxidative stress were assessed to evaluate the oxidative stress hypothesis and the levels of antimelanocyte antibodies were measured to evaluate the autoimmune hypothesis in patients with the onset of vitiligo (<3 months) and compared with the patients suffering from the disease for long duration of (>3 months).

6B.2. Materials and methods

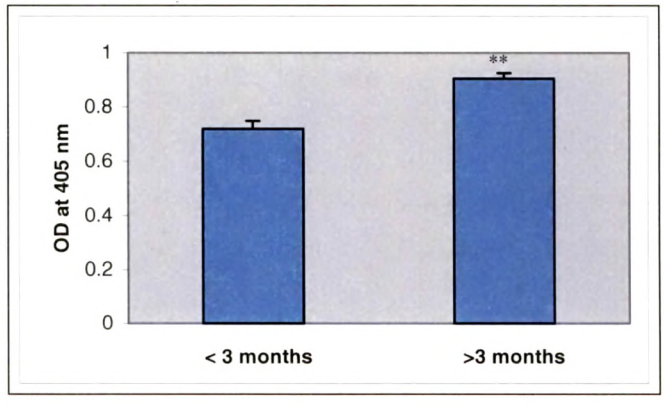
Blood was collected from the vitiligo patients (n = 102) visiting Sir Sayajirao Gaikwad Medical College hospital and Civil hospital Ahmedabad who were not suffering from any other disease after a written consent was obtained from them. Also blood was collected from one twenty seven age matched healthy controls.

Human melanoma cell line SK Mel 28 was cultured and ELISA was performed as described in chapter 6 A. Lipid peroxidation levels were estimated as described in chapter 3 A.

6B.3. Results

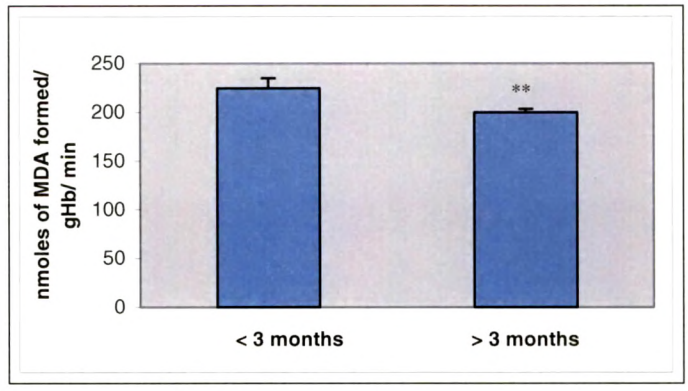
Oxidative stress and autoimmune hypotheses are the major theories that explain the pathogenesis of vitiligo. The aim of this study was to determine which hypothesis (autoimmune/ oxidative stress) is playing a major role in the initiation of vitiligo pathogenesis in Gujarat population. We have compared lipid peroxidation levels to evaluate the oxidative stress hypothesis and antimelanocyte antibody levels to evaluate autoimmune hypothesis in patients at the onset of vitiligo (< 3 months) and with those suffering from vitiligo for a long duration (>3 months). Significant decrease ($p<0.005$) in the levels of antimelanocyte antibodies was observed in <3 months patients compared to >3 months patients. On the contrary, significant increase ($p<0.0085$) in lipid peroxidation levels was observed in vitiligo patients at the onset (>3 months) compared to vitiligo patients with long duration of the disease. The results are shown in the Figure 1 & Figure 2.

Figure 1. Antimelanocyte antibody levels based on the duration of vitiligo#



Values are given as mean ± SE of 16 individual observations in vitiligo patients at the onset (< 3 months) of disease and 87 vitiligo patients with long duration of disease (> 3 months). **, $p<0.01$.

Figure 2. Erythrocyte lipid peroxidation levels based on the duration of vitiligo#



Values are given as mean ± SE of 16 individual observations in vitiligo patients at the onset (< 3 months) of disease and 87 vitiligo patients with long duration of disease (> 3 months). **, $p<0.01$.

6B.4. Discussion

There are several reports on the antimelanocyte antibody levels in vitiligo patients compared to controls. However no study has been performed to evaluate both oxidative stress and autoimmune hypotheses simultaneously at the onset of vitiligo to find out which hypothesis is playing a major role to trigger vitiligo.

Naughton et al (1983) reported that 82% of vitiligo patients showed antimelanocyte antibodies in their circulation (Naughton et al 1983a). In another study, 12/12 vitiligo patients and 0/12 controls were reported to have antimelanocyte antibodies in their circulation (Naughton et al 1983b). However, Grimes et al (1983) reported that 19% of Black vitiligo patients showed antimelanocyte antibodies in their circulation. Recently, Farrokhi et al (2005) also showed the presence of antimelanocyte antibodies in 30.9% of Iranian vitiligo patients.

Patients with early onset (<3 months) of vitiligo showed a significant decrease ($p<0.005$) in the levels of antimelanocyte antibodies compared to patients with long duration (>3 months) of vitiligo (Figure 1). Lipid peroxidation levels were significantly increased ($p<0.0085$) in vitiligo patients at the onset (>3 months) compared to vitiligo patients with long duration of the disease (Figure 2). These

results suggest that oxidative stress hypothesis plays a major role in the initiation of vitiligo rather than auto antibodies. Moreover, our results on neurochemical hypothesis also showed that significant decrease in acetylcholine esterase levels in vitiligo patients compared to controls (Shajil et al 2006). This could be due to H_2O_2 mediated oxidation of AChE, thus emphasizing the role of oxidative stress in precipitating vitiligo (Schallreuter et al 2004).

In conclusion, this study shows that oxidative stress is the initial triggering event to precipitate vitiligo in Gujarat population.

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