Mitochondrial Impairment in Peripheral Blood Mononuclear Cells During the Active Phase of Vitiligo

Maria Lucia Dell'Anna, Vittoria Maresca, Stefania Briganti, Emanuela Camera, Mario Falchi,* and Mauro Picardo

Department of Cutaneous Physiopathology of the San Gallicano Dermatological Institute, Rome, Italy; *Department of Ultrastructures, Ist. Superiore Sanità, Rome, Italy

Several hypotheses have been made about the pathogenesis of vitiligo, and some of them have considered a systemic involvement in the course of the disease. Evidence has been presented on the role of oxidative stress as the initial event in melanocyte degeneration. In accordance with this view, we determined the levels of some antioxidants, i.e., superoxide dismutase, catalase, reduced glutathione, and vitamin E, in erythrocytes and/or peripheral blood mononuclear cells from patients with active or stable vitiligo and from a control group of healthy subjects. In erythrocytes the parameters evaluated were not significantly different. On the contrary, in peripheral blood mononuclear cells, superoxide dismutase activity was increased in both groups of patients, whereas catalase activity, reduced glutathione and vitamin E levels were decreased exclusively in subjects with active disease. The imbalance of antioxidants was associated with hyperproduction of reactive oxygen species due to a mitochondrial impairment as cyclosporin A, an inhibitor of the permeability transition pores opening, significantly reduced the reactive oxygen species production. Moreover an alteration of the mitochondrial transmembrane potential and a higher percentage of apoptotic cells were observed in active vitiligo patients. Based on these results, we suggest that, in vitiligo, mitochondria might be the target of different stimuli, such as reactive oxygen species generation, cytokines production, catecholamine release, alteration of Ca^{2+} metabolism, all of which capable of inducing melanocyte degeneration. ***Key words: apoptosis/catalase/oxidative stress. J Invest Dermatol 117:908–913, 2001***

The pathogenesis of vitiligo has not been completely clarified and even the process of melanocytes disappearance from involved skin, i.e., necrosis or apoptosis, is still to be identified (Nordlund and Ortonne, 1992, 1998). There is evidence suggesting that vitiligo is a genetic disease with a high degree of familial segregation, although the molecular aspects have not been explored. An inherent melanocyte defect has been suggested at the base of the disease as melanocytes, in depigmented areas, are absent or functionally inactive and, in normal appearing skin, may present morphologic abnormalities (Nordlund and Ortonne, 1998). Nevertheless, alterations of the whole epidermis with vacuolization of keratinocytes and functional modifications of Langerhans cells in the affected areas have been reported (Nordlund and Ortonne, 1992, 1998). A possible autoimmune mechanism is also suggested by the presence of autoantibodies against melanocyte antigens in some patients, the level of which correlates with the evolution of the disease. Moreover, significant associations with some organ-specific autoimmune disorders have been reported (Naughton *et al*, 1983; Nordlund and Ortonne, 1998) and skin homing melanocyte-specific cytotoxic T lymphocytes (CTLs) were found in patients with autoimmune vitiligo, suggesting a role for CTLs in the disappearance of melanocytes (Ogg *et al*, 1998). An increase in catecholamine discharge or synthesis has been associated with disease activity (Morrone *et al*, 1992; Cucchi *et al*, 2000) indicative of a direct or indirect role of its release in the depigmentation process.

Recently, an oxidative stress has been suggested to be the initial pathogenic event in melanocyte degeneration (Schallreuter *et al*, 1994; Maresca *et al*, 1997) and evidence has been presented for H_2O_2 accumulation in the epidermis of patients with active disease (Schallreuter *et al*, 1994, 1999). Defective recycling of tetrahydrobiopterin in the phenylalanine hydroxylase reaction has been reported in vitiligo epidermis (Schallreuter *et al*, 1994), which could induce the intracellular production of H_2O_2 (Schallreuter *et al*, 1991, 1999). Moreover, in cultured melanocytes an alteration of the antioxidant pattern, with a significant reduction of catalase activity, has been associated with an increased susceptibility to an external pro-oxidant agent (Maresca *et al*, 1997), even if catalase mRNA expression was not modified (Schallreuter *et al*, 1999). Therefore, rather than a primary defect, the antioxidant imbalance seems to be a marker of the intracellular generation of reactive oxygen species (ROS). In fact, antioxidant patterns of plasma and erythrocytes are not altered in vitiligo patients (Picardo *et al*, 1994). Recently, however, a decrease in the glutathione peroxidase activity has been reported in erythrocytes of active vitiligo patients (Schallreuter *et al*, 1999). In order to evaluate better whether the

Manuscript received November 27, 2000; revised February 26, 2001; accepted for publication April 30, 2001.

Reprint requests to: Dr. Mauro Picardo, San Gallicano Dermatological Institute, Via San Gallicano 25/a, 00153 Rome, Italy. Email: picardo@ifo.it

Abbreviations: SOD, superoxide dismutase; Cat, catalase; GSH, reduced glutathione; PBMC, peripheral blood mononuclear cells; DCFH-DA, 2′,7′-dichlofluorescein diacetate; CsA, cyclosporine A; PTP, permeability transition pores; ROS, reactive oxygen species.

Table I. Antioxidant pattern alteration in RBC and PBMC from active or stable vitiligo patients

	Normal subjects (n = 40)	Stable vitiligo (n = 15)	Active vitiligo (n = 25)
RBC			
Cat (U per mg Hb)	408 ± 38.50	385 ± 58.5	370 ± 61.4
SOD (U per mg Hb)	0.55 ± 0.12	0.54 ± 0.15	0.56 ± 0.15
GSH (nmol per mg Hb)	61.5 ± 17	63 ± 7.3	67.4 ± 16
PBMC			
Cat (U per mg protein)	167.17 ± 49.01	162.62 ± 46.62	134.47 ± 33.44[a]
SOD (U per mg protein)	3.94 ± 0.17	5.95 ± 0.5	4.65 ± 0.05[a]
CuZnSOD (U per mg protein)	2.011 ± 0.03	3.96 ± 0.07[b]	3.24 ± 0.016[b]
MnSOD (U per mg protein)	1.88 ± 0.04	2.05 ± 0.15	1.44 ± 0.12
GSH (nmol per mg protein)	63.6 ± 9.7	65 ± 8.4	32.8 ± 4[a]
Vitamin E (ng per mg protein)	0.36 ± 0.12	0.21 ± 0.15	0.18 ± 0.16[c]

[a] $p < 0.001$ and [b] $p < 0.05$ *vs* normal subjects; [c] $r = -0.8$ between VitE and MnSOD.

activity of the disease is associated with a systemic oxidative stress, we measured the antioxidant pattern and the intracellular generation of ROS in red blood cells and/or peripheral blood mononuclear cells (PBMC) of patients with vitiligo.

Our findings demonstrate the presence of an imbalance of antioxidants in the peripheral blood mononuclear cells of active vitiligo patients, which is correlated to an increased intracellular ROS production. This imbalance appears to be due to a mitochondrial impairment.

MATERIALS AND METHODS

Subjects RBC and PBMC were obtained from 40 consecutive patients with nonsegmental vitiligo (13 males, 27 females, median age 42y, range 18–53; 25 in the active phase and 15 in the stable phase) and from 40 age- and sex-matched control subjects. The active or stable phase was defined on the basis of the progression or appearance of lesions in the last 3 mo and on the absence of new lesions or their progression in the last 6 mo, respectively. In 11 patients (seven active and four stable phase) thyroiditis with anti-thyreoglobulin autoantibodies was detected. No signs of autoimmune diseases were found in any other patient. A positive family history was recorded in about 25% of the patients (n = 10).

Cells PBMC were isolated by a gradient of Ficoll-Hypaque (Uppsala, Sweden) (400 × *g* for 30 min at room temperature). The PBMC layer was removed and washed twice with NaCl 0.9% (200 × *g* for 15 min at 4°C).

Enzymatic activities RBC and PBMC were lyzed in ipotonic solution (Tris 0.005 M) on ice for 1 h and centrifuged at 800 × *g* for 10 min at 4°C. Hemoglobin concentration was determined by Drabkin's method (Drabkin's reagent, Sigma, St Louis, MO) and expressed as g per 100 ml of total blood. Protein concentration in PBMC was determined in the supernatant by Bradford test and expressed as mg per ml. Superoxide dismutase (SOD) activities were evaluated by spectrophotometer (Spitz and Oberley, 1989). In this competitive inhibition assay, superoxide generated by xanthine–xanthine oxidase is detected by monitoring the reduction of nitroblue tetrazolium at 505 nm. Total SOD activity was measured at pH 7.8 in Tris–HCl 0.2 M and $Cu^{2+}Zn^{2+}$ SOD activity was measured at pH 10.2 in Tris–HCl 0.2 M. Standard curve was performed using human SOD (Sigma) at different concentrations (0.1, 0.25, 0.5, 1, 2 U per ml). One unit of activity was defined as the amount of protein that yields 50% of maximal inhibition of nitroblue tetrazolium reduction by superoxide. The results were reported as units of SOD per mg of proteins or units of SOD per mg of Hb.

Catalase activity was determined by the rate of disappearance of hydrogen peroxide (10 mM) measured at 240 nm by spectrophotometer in a phosphate buffer at pH 7.4 (Claiborne, 1985). One unit of catalase (Cat) is defined as the amount of enzyme that degrades 1 μM of H_2O_2. The results were reported as units of Cat per mg of proteins or units of Cat per mg of Hb.

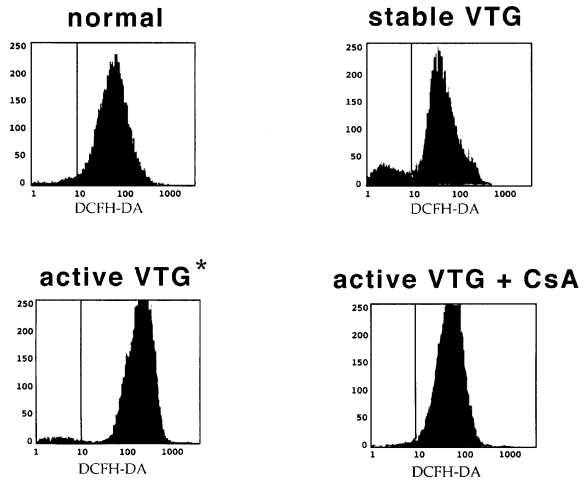
Vitamin E analysis Pellets of PBMC isolated from 15 ml of heparinized blood were extracted three times in exane/ethanol (3 : 1 vol/vol) with 125 ng of γ-tocopherol and 125 ng of δ-tocopherol as internal standards. The extracts were dried under nitrogen flux, treated with 25 μl of dry pyridine and then directly silylated with 25 μl of N,O-bis-(trimethyl-silyl)trifluoroacetamide containing 1% trimethylchlorsilane as catalyst. Tocopherols were analyzed by gas chromatography mass spectrometry with an Ultra 2 column (30 m × 0.2 μm internal diameter, 0.25 mm Hewlett Packard, Cupertino, CA) by a selected ion(s) monitoring technique. The ions selected were 237.277 and 502 for α-tocopherol, 223.263 and 488 for γ-tocopherol (Picardo *et al*, 1996). Analyses were repeated twice in each extract with a difference of less than 5%.

Reduced glutathione analysis Erythrocytes were lyzed with an equal volume of cold water in the presence of 50 mM N-ethylmaleimide (Sigma) on ice for 20 min. Stroma were removed by centrifugation at 14,000 r.p.m. (20,000 g) for 10 min whereas the supernatant was filtered by Microcon-10 membrane at 10,000 r.p.m. (10,000 g) for 5 min and then analyzed by capillary electrophoresis. PBMC were lyzed in aqueous solution of N-ethylmaleimide 10 mM on ice for 30 min. Thiosalicylic Acid (20 μM) (Sigma), as internal standard, and CH_3CN (250 μl), to precipitate the proteins, were added. The final volume was 1 ml. After centrifugation the supernatant was analyzed by liquid chromatography-mass spectrometry method (Camera *et al*, 2001).

ROS detection Intracellular ROS production was detected by 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Fluka AG, Switzerland), a substance that is oxidized to fluorescent 2′,7′-dichlorofluorescein by H_2O_2, other ROS and low molecule weight peroxides produced by the cells (Wang *et al*, 1996). PBMC (1×10^6) were incubated at 37°C for 30 min with 2.5 μM DCFH-DA in phosphate-buffered saline with calcium and magnesium and 5 mM glucose (PBS) and then immediately analyzed by flow cytometer (Cytoron Absolute, Ortho Diagnostic Systems, Raritan, NJ, USA, with excitation and emission settings of 488 and 530 nm, respectively). The median of FL-1 channel of fluorescence was used as the parameter to evaluate the intracellular content of ROS because it matches the maximal number of cells with the highest fluorescence.

Mitochondrial transmembrane potential determination JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; Molecular Probes, Eugene, OR) was dissolved at 2.5 mg per ml in N-N-dimethylformamide (Merck AG Darmstad, Germany). PBMC were washed and stained with 2.5 μg per ml of JC-1 for 15 min at 37°C, then washed in PBS and immediately analyzed by flow cytometer for green and red fluorescences (Cossarizza *et al*, 1993).

Apoptosis detection After DCFH-DA exposure, PBMC were stained with 1 μl of Annexin V Cy3 (MBL, Nagoya, Japan) and immediately analyzed by flow cytometer with emission setting at 570 nm. As Annexin V binding represents one of the very early events in the apoptotic process, the differential forward light scatter of living and apoptotic cells was also used to assess the two populations, and electronic gates (R1 and R2) were evaluated independently from Annexin V staining. Decreased forward light scatter in R2 characterizes cells at all stages of apoptosis, including late ones. Another independent method for apoptosis detection was also used in 10 patients and 10 controls, i.e., propidium iodide assay according to Nicoletti *et al* (1991). Propidium



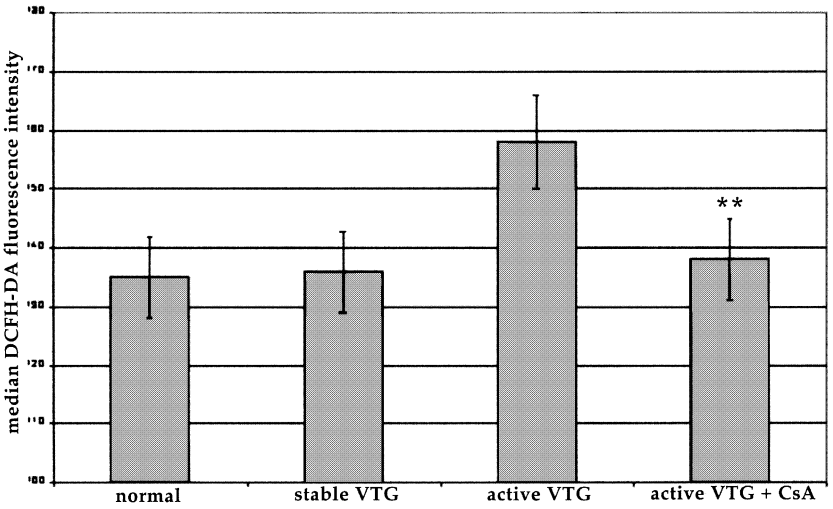


Figure 1. Hyperproduction of ROS in PBMC from active vitiligo patients. The flow cytometric analysis shows a significant higher level of DCFH-DA median intensity (FL-1 histogram, top of figure) in PBMC from active vitiligo patients (*$p < 0.0001$). The graph shows the mean ± SD of each group (n = 25 for active vitiligo; n = 40 for normal; n = 15 for stable vitiligo; n = 25 for active vitiligo pretreated with CsA). Pretreatment with CsA (1 μM) significantly reduces DCFH-DA intensity (**$p < 0.001$).

iodide (Sigma) 1 μl per ml was added to PBMC suspension after DCFH-DA staining for 20 min and then evaluated by flow cytometry.

Phenotype analysis PBMC were stained 30 min at 4°C with fluorochrome conjugated monoclonal antibodies for CD3, CD4, CD8, CD20, CD45, CD45RA, CD45R0, cutaneous leukocyte antigen (all from Pharmingen-Becton Dickinson Company, San Diego, CA), washed in PBS and immediately analyzed by flow cytometer with the gate on lymphocytes.

Cyclosporin A treatment Cyclosporin A (CsA) (Sigma) was dissolved in ethanol (stored at –20°C) and used at 1 μM immediately before any staining.

Statistical analyses Student's t and Pearson tests were utilized for statistical analyses.

RESULTS

Antioxidant imbalance in PBMC from patients with active vitiligo To evaluate whether the antioxidant imbalance during active vitiligo was systemically detectable, we evaluated SOD and Cat activity, vitamin E and GSH concentration in RBC and/or PBMC from patients with active or stable disease. In erythrocytes, the antioxidants evaluated (GSH, SOD, Cat) were not significantly different in the patient groups with respect to the values in normal subjects (**Table I**). On the contrary, statistically significant differences were observed with values from PBMC (**Table I**). SOD activity, in particular the cytosolic component ($Cu^{2+}Zn^{2+}$ SOD) was significantly ($p < 0.05$) increased in both vitiligo groups, whereas Cat activity was decreased exclusively in the cells of

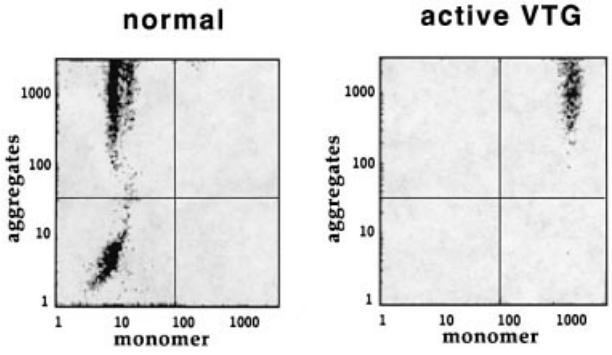


Figure 2. Mitochondrial alteration during active phase of disease. A representative flow cytometric analysis of JC-1 staining indicate a drop of transmembrane potential in PBMC from active vitiligo patients *vs* normal subjects, as indicated by the reduction of JC-1 aggregates.

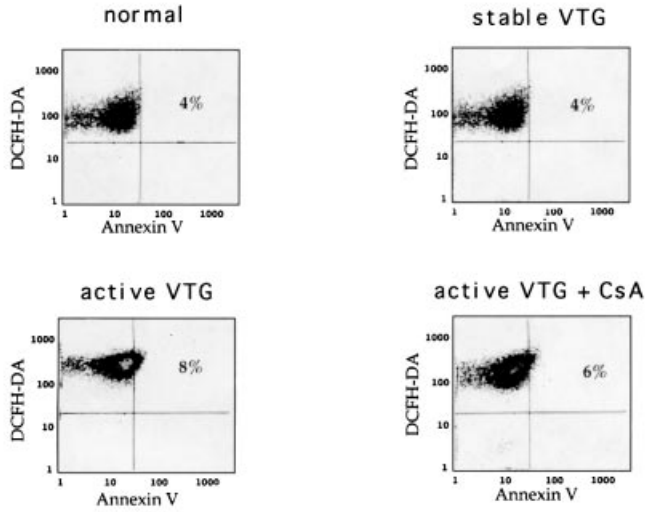
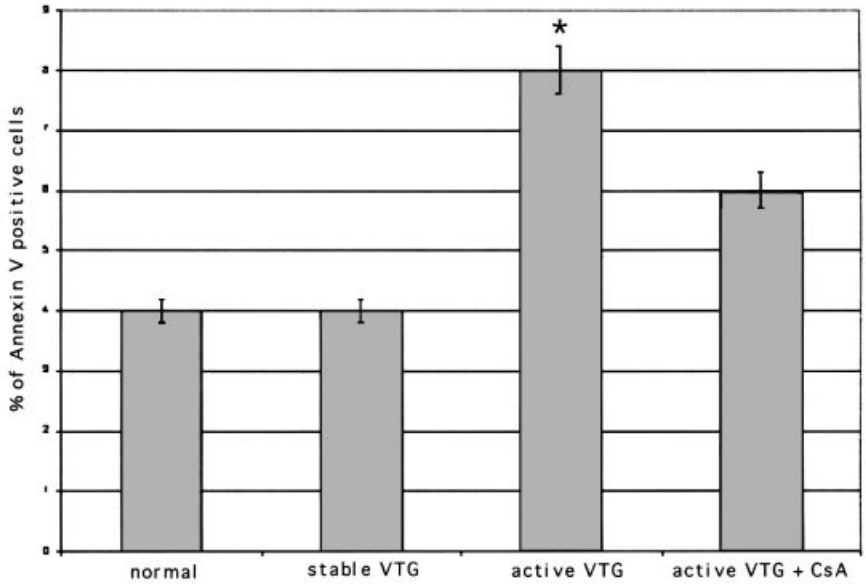


Figure 3. Apoptosis in vitiligo. A higher percentage of early apoptotic cells was detected by Annexin V^+ in PBMC from active vitiligo ($8 \pm 2.8\%$) *vs* normal or stable ($4 \pm 2.3\%$) subjects (*$p < 0.0001$). The reduction produced by CsA was not significant.



subjects with active disease ($p < 0.001$). Consequently, the U SOD/U Cat ratio, a parameter of cell susceptibility to oxidative stress (Moysan *et al*, 1993), was altered exclusively in the latter group of patients. GSH level was significantly lower ($p < 0.005$) in PBMC from active vitiligo patients and even though a wide range in the vitamin E concentration was observed (0.08–0.75 μg per mg protein) in all the groups, the mean concentration in active vitiligo was lower and inversely correlated with the Mn^{2+}SOD activity ($r = -0.8$) (**Table I**). The alteration of the antioxidant patterns detected in PBMC was similar to that previously described in melanocytes (Maresca *et al*, 1997), indicating a systemic involvement during the active phase of the disease. The observation of a normal antioxidant pattern in the RBC, however, suggests that the alterations are not a primary defect, but more likely due to a metabolic process, present in PBMC, which leads to an enhancement of intracellular ROS production.

Increased ROS generation in active vitiligo PBMC In order to determine the intracellular ROS production, DCFH-DA staining (Wang *et al*, 1996) was performed on PBMC from the same groups. Flow cytometry analysis showed a significantly higher level of fluorescence in $CD45^+$ cells from active vitiligo subjects ($p < 0.0001$) (**Fig 1**). No significant differences were observed when lymphocyte subpopulations were evaluated (CD4, CD8, CD45R0, or CD45RA).

Intracellular hyperproduction of ROS can be the result of the opening of mitochondrial permeability transition pores (PTP), which reflects in a reduction in the mitochondrial transmembrane potential ($\Delta\Psi_m$), before the cells show signs of nuclear apoptosis (chromatin condensation and DNA fragmentation) (Zamzami *et al*, 1995; Yang and Cortopassi, 1998). PTP opening is controlled by several physiologic and pharmacologic substances, including CsA, a specific short-term (up to 60 min) inhibitor acting on cyclophilin D associated with the adenine nucleotide translocator (Broekemeier and Pfeiffer, 1995). CsA (1 μM) significantly decreased DCFH-DA staining of PBMC in active vitiligo patients ($p < 0.0001$), confirming that ROS production was influenced upon PTP opening (**Fig 1**).

Alteration of mitochondrial transmembrane potential and apoptosis of PBMC from patients with active vitiligo In active vitiligo PBMC, a significant drop in the mitochondrial $\Delta\Psi_m$

was represented by a decrease in the level of JC-1 aggregates ($p < 0.005$) (**Fig 2**), whereas in the cells from the stable group the pattern of the fluorescence was similar to that observed in PBMC from normal individuals. Several reports have shown that $\Delta\Psi_m$ disruption and subsequent nuclear apoptosis are strictly connected and therefore we evaluated the percentage of apoptotic cells using Annexin V staining and forward light scatter analysis in the same cell populations (**Fig 3**). In PBMC from active vitiligo patients the percentage of Annexin V^+ cells represented $8 \pm 2.8\%$ of events gated in R1 (living cells) and 30% of those gated in R2 (apoptotic cells) (Carbonari *et al*, 1994), whereas in PBMC from stable vitiligo or normal subjects, the Annexin V^+ cells were $4 \pm 2.3\%$ in R1 and undetectable in R2 ($p < 0.0001$).

In active vitiligo patients, the R2 region comprised a higher number of cells (20% of total acquired event *vs* 2–4% in normal subjects and stable subjects). The PBMC Annexin V^+ DCFH-DA^+ in R1 region, represent cells in an early phase of apoptosis, and PBMC Annexin V^+ DCFH-DA^- in R2 presumably cells that have terminated the apoptotic program. Annexin V^+ cells were either positive or negative for cutaneous leukocyte antigen, indicating that apoptotic cells did not specifically express the skin homing receptor. A further analysis showed, in a limited group of subjects ($n = 7$), a bimodal distribution of the fluorescence intensity for DCFH-DA, dim and bright (mean of 140 and 168, respectively). The bright population was Annexin V^+, suggesting that in the early phase of apoptosis the esterases are more active, as also indicated by the negativity of DCFH-DA staining in R2. Moreover, the propidium iodide staining performed in some samples, confirmed the increase of the apoptotic process in active vitiligo (27.2 ± 11 *vs* 10.7 ± 4 in normal subjects, $p < 0.01$).

DISCUSSION

The complex of the results reported here demonstrate that, during the active phase of vitiligo, mitochondrial alterations can be detected in PBMC, which correlate with an increased ROS generation, a modification of the transmembrane potential, likely due to PTP opening, and an imbalance of antioxidant system and apoptosis. The results also confirm our previous data on a normal antioxidant pattern in RBC from patients with vitiligo (Picardo *et al*, 1994). These cells, lacking mitochondria, in fact, utilize different metabolic pathways and show a different sensitivity to pro-oxidants. Mitochondria are key organelles in the control of intracellular ROS generation as, under physiological conditions, up to 5% of the oxygen consumed in the respiratory chain is converted to ROS (Nohl and Hegner, 1978; Chance *et al*, 1979; Heales *et al*, 1999). The kinetics of superoxide production has been measured, and sites of generation have been proposed in complex I (NADH-CoQ reductase) and in complex III (ubiquinone-cytochrome c reductase) (Heales *et al*, 1999). Superoxide is removed by SOD, producing hydrogen peroxide, which in turn, under certain circumstances, reacts with free iron or copper ions, give rise to the more damaging hydroxyl radical. A variety of mitochondrial poisons increase mitochondrial superoxide production, which is associated with apoptosis (Heales *et al*, 1999).

Free radicals, oxidizing agents, modification of fatty acid pattern of the mitochondrial membrane, catecholamine release, and some cytokines, such as TNF-α, or increases in Ca^{2+} concentrations are events capable of inducing ROS production from mitochondria, through PTP opening and alteration of the electron transport. Most of these events have been previously reported as occurring in the active phase of vitiligo (Naughton *et al*, 1983; Schallreuter *et al*, 1994; Ogg *et al*, 1998). ROS production can lead to an alteration of the cellular redox state with oxidation of GSH and a decrease of the catalase activity, whereas the increase of cytoplasmic SOD activity can be a compensatory mechanism. Moreover, stimuli, such as pro-oxidants and cytokines, which are capable of reducing mitochondrial GSH or vitamin E levels can also induce alteration of the electron transport in susceptible cells.

Mitochondria – depending on cell type – display a different threshold sensitivity to pro-oxidants (75% to 25%) before inhibition of ATP synthesis occurs (Heales *et al*, 1999). High levels of polyunsaturated fatty acid at the cell membrane and low level of antioxidants, such as those present in melanocytes, are though to be responsible for a higher susceptibility to pro-oxidant agents (Yohn *et al*, 1991; Maresca *et al*, 1997; Cucchi *et al* 2000). Notably, vitiligo vulgaris has been described in the course of mitochondrial diseases, such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke like episodes (MELAS) syndrome and some cases have been reported in ataxia telangiectasia (Cohen *et al*, 1984; Taieb, 2000).

In conclusion, our results suggests that a mechanism involving different cell types is acting during the active phase of the disease (Naughton *et al*, 1983; Nordlund and Ortonne, 1992, 1998; Ogg *et al*, 1998). Given that several mechanisms are though to be at the basis of the pathogenesis of vitiligo, and that a ''convergence theory'' has been proposed (Le Poole *et al*, 1993), the mitochondrial dysfunction observed here could represent the possible target of different stimuli and the biochemical basis for the insurgence of the disease.

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