Low Catalase Levels in the Epidermis of Patients with Vitiligo

Karin U. Schallreuter, John M. Wood, and Jürgen Berger

Department of Dermatology (KUS, JMW) and Institute of Mathematics and Computer Science, University of Hamburg, Hamburg, Germany

Suction blister roofs taken from the involved and uninvolved epidermis of patients with vitiligo showed a consistent reduction in levels of catalase compared to normal healthy controls of matched photo-skin types (Fitzpatrick classification). A decrease in catalase activity is expected to increase the concentration of hydrogen peroxide in the epidermis of these patients. Hydrogen peroxide functions as a reversible inhibitor of human tyrosinase with a K_I of 8×10^{-6} M.

Also, hydrogen peroxide undergoes photochemical reduction yielding highly reactive hydroxyl radicals (OH·) and hydroxyl ions (OH^-) mainly by the Haber-Weiss reaction. Hydroxyl radicals are capable of bleaching constitutional melanin and cause membrane lysis through lipid peroxidation reactions. Hydroxyl ions increase the pH in the epidermis, and as a consequence glutathione reductase activity is increased in patients with vitiligo compared to controls. Based on these new results, together with the previously reported calcium transport defect, a new hypothesis has been formulated for the pathogenesis of vitiligo. *J Invest Dermatol 97:1081–1085, 1991*

In order to account for the pathophysiology of vitiligo, several properties associated with this depigmentation disorder must be reconciled. The onset of depigmentation requires an understanding of those mechanisms involved in a) the bleaching of constitutional pigment in the keratinocytes, b) the reversible inhibition of tyrosinase in melanocytes to prevent "de novo" melanin biosynthesis, and c) the disruption of the processes for the dispersal and distribution of melanin from melanocytes to keratinocytes. Finally, in some patients, a total loss of melanocytes can occur yielding the probability of irreversible depigmentation.

To date, those biochemical mechanisms involved in the above processes for depigmentation in vitiligo remain enigmatic. Recently there is some new evidence that vitiligo is a disease of the total epidermal unit with a major involvement of keratinocytes. Earlier studies on the histology of vitiligo revealed that both pigmented and depigmented epidermis showed increased granulation in keratinocytes [1]. Significant membrane damage has been reported for basal keratinocytes with plumping in membranes of intracellular organelles, a characteristic of lipid/membrane peroxidative damage [2]. By using $^{45}Ca^{++}$ uptake, defective calcium transport has been found in keratinocytes established from vitiliginous skin compared to control cells from the same patients' pigmented epidermis and from normal healthy keratinocytes from a matching photo-skin type [3]. A defective calcium uptake was also found in keratinocytes from tyrosinase positive albinos with Hermansky-Pudlak syndrome (HPS), highlighting the importance of calcium in the regulation of pigmentation [4]. Thioredoxin reductase (TR) has been used as a monitor for both extracellular and intracellular calcium concentration in keratinocytes and in the epidermis [5]. The activities of both plasma-membrane–associated and cytosolic TR depend on calcium concentration due to a single allosteric (EF-hands) binding site on this enzyme [5–7]. Calcium regulates intracellular redox conditions in the epidermis by controlling the activity of TR [7], and the product of TR, reduced thioredoxin, functions as an allosteric inhibitor of tyrosinase [8,9]. The importance of calcium in the regulation of pigmentation gained more strength with the recognition that amelanotic melanoma tissue contains calcium-free TR, whereas melanotic melanoma metastases contain calcium-bound enzyme [10,11]. In vitiligo membrane–associated TR activities in depigmented skin are lower than in pigmented skin, and this has been shown to be due to extracellular calcium regulation of enzyme activity rather than differences in enzyme levels [12–14].

The aim of this study has been to examine the anti-oxidant defense enzymes directly in suction blisters taken from the involved and uninvolved epidermis of patients with vitiligo compared to controls from healthy donors of matching skin types in order to assess whether a defect in the reduction of reactive oxygen intermediates [i.e., superoxide anion radical (O_2^-), hydrogen peroxide ($O_2^=$), and hydroxyl radicals (OH·)] play a major role in this depigmentation disorder.

MATERIALS AND METHODS

Enzymes Human erythrocyte catalase (CAT) and glutathione reductase (GR) were obtained from Sigma Chemical Company, St. Louis, MO. Human TR was purified by the method of Schallreuter and Wood [10,11]. Human tyrosinase was purified by a modification of the method of Yurkow and Laskin [15].

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Reprint requests to: Dr. K.U. Schallreuter, Department of Dermatology, University of Hamburg, Martinistraße 52, D2000 Hamburg 20, Germany.

Abbreviations:

CAT: catalase
GR: glutathione reductase
HPS: Hermansky-Pudlak Syndrome
ICAM-1: intercellular adhesion molecule 1
MnSOD: manganase superoxide dismutase
$O_2^=$: hydrogen peroxide
O_2^-: superoxide anion radical
OH·: hydroxyl radical
OH^-: hydroxyl ion
TNFα: tumor necrosis factor alpha
TR: thioredoxin reductase

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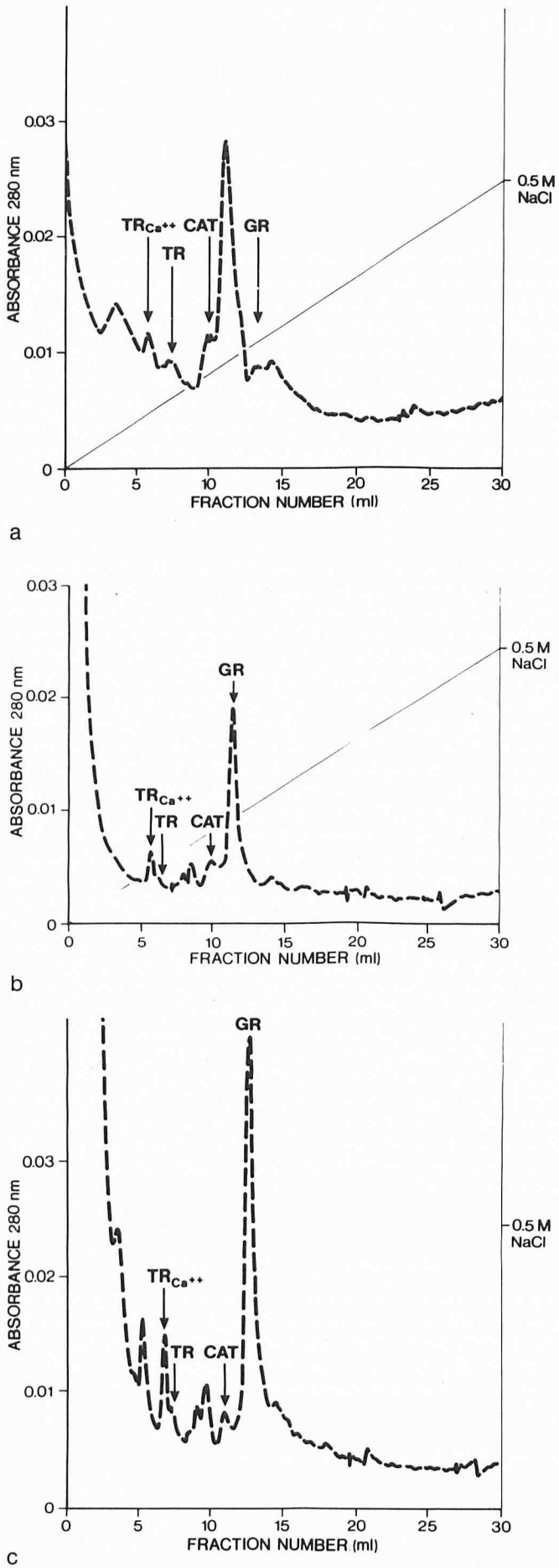

Enzyme Assays CAT was assayed by measuring oxygen evolution from hydrogen peroxide with a Clark oxygen electrode [16]. Both TR and GR were assayed by the reduction of 5,5′ dithiobis-2-nitrobenzoate (DTNB-assay) at 412 nm by the method of Luthman and Holmgren [17]. Tyrosinase was assayed by the method of Duckworth and Coleman at 280 nm [18]. Reactions with L-tyrosine were performed by following the increase in optical density at 280 nm with blanks containing identical substrate/inhibitor concentrations. Each assay contained 100 μl of tyrosinase (1.0 mg/ml).

Separation of Enzymes from Suction Blisters Epidermal suction blisters were obtained from involved (n = 12, 10 forearem, one thigh, one back) and uninvolved (n = 10, forearm) skin of patients with vitiligo (skin type III, Fitzpatrick classification), and from skin type III controls (n = 7, forearm). Patients were classified as a) vitiliginous involved, general symmetrical 8, segmental 1, and focal 3 patients; b) vitiliginous uninvolved, general symmetrical 9 and segmental 1 patient. Cell-free extracts were prepared by ultrasonication in 0.05 M tris-HCl buffer, pH 7.5, with a microprobe at the maximum setting (Heat Systems Model 350 Ultrasonicator). Cell membranes were removed by centrifugation at 5000 rpm (6,600 × g) for 30 min at 0°C. Protein concentrations were determined by the method of Kalb and Bernlohr [19]. 0.5 ml of extract (approximately 4.0 mg of protein) was applied to a mono Q HR 5/5 anion exchange column equilibrated with 0.05 M tris-HCl buffer pH 7.5 in a 0–0.5 M NaCl gradient. Enzyme standards were subjected to the same FPLC analysis prior to each extract run. CAT eluted at 0.16–0.17 M NaCl, GR at 0.22 M NaCl, and TR as two peaks: a) calcium-bound enzyme 0.10–0.105 M NaCl and b) calcium-free enzyme at 0.12 M NaCl.

Statistics The data on CAT and GR levels in suction blisters were subjected to one-way variance analysis relative to skin type III controls. A comparison between pigmented and non-pigmented value for CAT and GR were analyzed by the least-significant difference (LSD) method.

RESULTS

The soluble acidic protein fraction from the cytosol of healthy human epidermal cells (skin type I–VI, Fitzpatrick classification) contains elevated levels of the anti-oxidant enzymes CAT, GR, and TR. These enzymes are well separated by FPLC. Figure 1 shows the FPLC profiles of *(a)* skin type III control, *(b)* vitiliginous skin, and *(c)* pigmented skin from the same vitiligo patient analyzed in *(b)*. The levels of CAT, GR, and TR were standardized μg/mg of protein for 12 suction blisters from vitiliginous skin of different patients from pigmented skin of vitiligo patients (n = 10) and seven blisters from normal healthy adult controls (skin type III). The following results were obtained: i) intracellular TR levels remained similar between vitiligo patients and controls as determined previously [13]; ii) CAT levels are decreased to 39% in vitiliginous skin and 45% in the pigmented skin of patients with vitiligo compared to controls (100%) (Fig 2*a*); iii) GR levels are significantly increased in vitiliginous skin, with an even higher expression of this enzyme in pigmented skin of vitiligo patients compared to controls (Fig 2*b*).

Decreases in CAT in both involved and uninvolved vitiligo skin were highly significant, $p < 0.0001$, and similarly increases in GR yielded $p < 0.0001$. Using the LSD analysis there was no significant difference between CAT levels in involved and uninvolved epidermis in vitiligo patients. However, there is a significant difference between involved and uninvolved epidermis in these patients for GR ($p = 0.05$).

Figure 1. *(a)* FPLC of extracts prepared from a suction blister roof of a skin type III control TR, CAT, and GR peaks as indicated. *(b)* FPLC of extracts prepared from a suction blister roof of vitiliginous skin. *(c)* FPLC of extracts prepared from uninvolved skin of the same patient *(b)*.

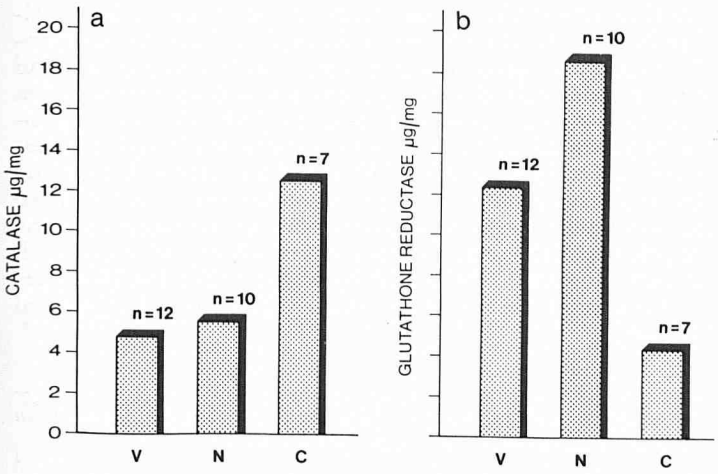

Figure 2. *(a)* Levels of CAT in vitiliginous extracts (n = 12) (V), uninvolved skin from vitiligo patients (N) (n = 10) and skin type III controls (n = 7) (C). Values are plotted as mean levels with V = 4.94 µg/mg ± 0.71 (p = 0.0001); NS = 5.64 µg/mg ± 2.0 (p = 0.0001) and C = 12.5 µg/mg ± 0.48. *(b)* Levels of GR in vitiliginous extracts (n = 12) (V) uninvolved skin from vitiligo patients (n = 10) (N) and skin type III controls (n = 7) (C). Values are plotted as mean levels with V = 12.3 µg/mg ± 2.08 (p = 0.0001), NS = 18.63 µg/mg ± 2.96 (p = 0.0001) and C = 4.3 µg/mg ± 0.46.

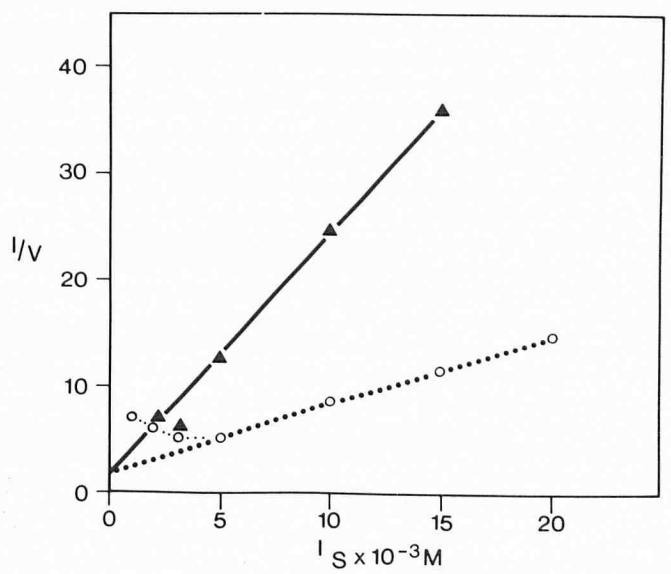

Figure 3. Lineweaver-Burke plot for the competitive inhibition of melanoma tyrosinase by hydrogen peroxide (1×10^{-5} M) using L-tyrosine as the substrate. Reactions contained different concentrations of L-tyrosine in 0.1 M HEPES buffer, pH 7.5 (total volume 1.0 ml) and 100 µl of tyrosinase (1.0 mg/ml) (○) followed by the addition of hydrogen peroxide (△). Reactions were started with L-tyrosine.

In all patients tested, a consistent decrease in CAT occurred, suggesting a defect in $O_2^=$ metabolism. The effect of $O_2^=$ on melanin biosynthesis was examined by measuring the activity of human tyrosinase in the presence and absence of $O_2^=$ when L-tyrosine was the substrate. Pre-incubation of tyrosinase with $O_2^=$, before the addition of L-tyrosine revealed competitive inhibition kinetics by $O_2^=$ with a $K_i = 8 \times 10^{-6}$ M (Fig 3). Therefore, an excessive buildup of $O_2^=$ in the epidermis could reversibly inhibit tyrosinase and prevent "de novo"melanin biosynthesis.

DISCUSSION

Human melanocytes in cell culture are especially sensitive to $O_2^=$ [20]. Recent experiments have shown that melanocytes from vitiliginous skin require the addition of CAT to the culture medium in order to grow, whereas melanocytes from normal healthy skin proliferate without addition of this enzyme [21]. Histologic examination of involved and uninvolved epidermis in vitiligo revealed evidence for peroxidative damage to both keratinocytes and melanocytes [1,2]. The data presented herein clearly show that the total epidermis of patients with vitiligo has lower than expected levels of CAT suggesting a defect in $O_2^=$ metabolism.

When one considers the major pathways for the synthesis of $O_2^=$ in human skin, the superoxide dismutases are important in generating $O_2^=$ from the disproportionation of O_2^- [22]. As a consequence, the biosynthesis of O_2^-, and its metabolism, appears to be central for the production of $O_2^=$ in the skin. In addition to the synthesis of O_2^- by UV light, the infiltration of lymphocytes causes a sudden increase in the local concentration of O_2^- through the activity of the membrane-associated lymphocyte NADPH-oxidase via the "oxygen burst" [23]. The activity of NADPH-oxidase depends on the extracellular calcium concentration with high calcium causing an increase in O_2^- production [23]. Therefore an increase in O_2^- over and above the O_2^- generated by UV light and by the melanins [24] may depend on the localized activities of infiltrating lymphocytes and their proximity to melanocytes and keratinocytes. Intercellular adhesion molecule 1 (ICAM-1) binds lymphocytes, and elevated levels of ICAM-1 have been found on keratinocytes and melanocytes of the perilesional skin in vitiligo patients by immunohistochemistry with the monoclonal antibody to ICAM-1 [25]. Furthermore, keratinocytes are known to produce tumor necrosis factor-α (TNF-α), which can increase the expression of ICAM-1 on melanocytes [25–27]. TNF-α induces manganese superoxide dismutase (MnSOD), causing a rapid increase in $O_2^=$ from O_2^- to cytotoxic levels [28]. Based on this information, together with the observed defect in calcium transport, a model for the pathophysiology of vitiligo can be proposed (Fig 4). The following sequence of events could account for depigmentation in vitiligo:

I. Keratinocytes produce TNF-α to induce ICAM-1 on melanocytes [25,27].

II. ICAM-1 binds lymphocytes to melanocytes and increases the local concentration of O_2^- by the "oxygen burst" [23].

III. Defective calcium transport in vitiliginous keratinocytes [3]

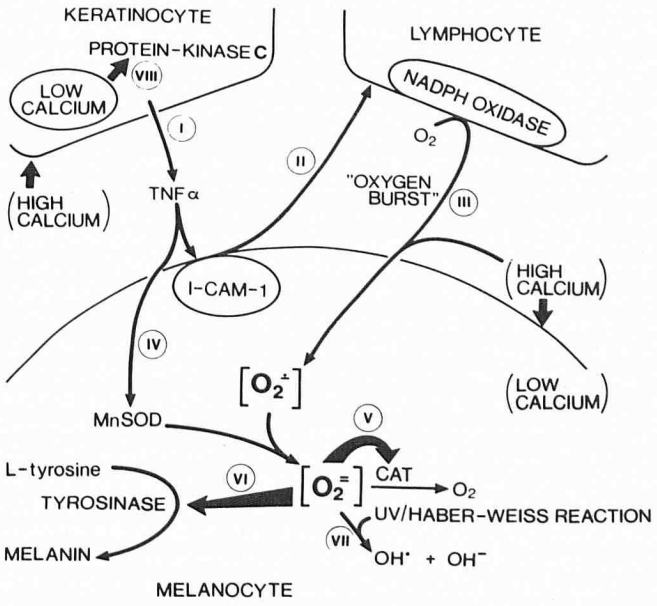

Figure 4. Proposed pathways leading to depigmentation in vitiligo (reactions I–VIII as described in the *Discussion.*) → , inhibition points.

could increase the extracellular concentration of this ion, stimulating even higher O_2^- synthesis.

IV. TNF-α from keratinocytes induced MnSOD [26]. This may cause higher local concentrations of O_2^- in both keratinocytes and melanocytes. (The induction of MnSOD by TNF-α remains to be established in keratinocytes and melanocytes although it has been shown in fibroblasts and hepatocytes.)

V. In the presence of UV light, O_2^- destroys the tetrapyrrole rings of the porphyrin prosthetic group of CAT as determined previously by Aronoff [29].

VI. As a consequence of CAT loss, O_2^- could build up sufficiently to reversibly inhibit tyrosinase ($K_1 = 8 \times 10^{-6}$M) unless it is metabolized by glutathione peroxidase.

VII. O_2^- is converted by the Haber-Weiss reaction to OH· and OH^-. OH· bleaches the constitutional melanin [29] and can cause the observed membrane damage to keratinocytes and melanocytes [1,2].

VIII. Defective calcium transport in vitiligo can be expected to interfere with the distribution of melanin from melanocytes to keratinocytes by preventing the activation of protein kinase C [30]. Cyclic-AMP modulators such as α-MSH, β-adrenergic receptors, melatonin receptors, etc., depend on the stimulation of calcium uptake by inosine-triphosphate as a prerequisite for protein kinase C activation [30]. A defect in calcium acquisition could prevent these processes [30,31].

The decrease of CAT in the epidermis of patients with vitiligo highlights the backup role of the thioproteins TR and GR. TR/thioredoxin can reduce O_2^- to water [32], and the GR/glutathione/glutathione peroxidase system can also catalyze this reaction [33]. In the absence of significant levels of CAT, the GR/GSH/glutathione peroxidase system could assume a major role in O_2^- metabolism in patients with vitiligo. The activities of TR and GR in normal healthy human epidermis have been shown to be pH dependent with high TR and low GR at an intracellular pH = 7.05 as determined by ^{31}P nuclear magnetic resonance spectroscopy [34]. Meanwhile, the dermis contains high GR and low TR with a pH range of 7.8 to 8.2 [34]. The pH optimum for TR is 6.98 and for GR 8.1 [35]. Therefore, the induction of GR in the epidermis of vitiligo patients could be caused by an increase in pH based on OH^- released in the Haber-Weiss reaction (Fig 4). Alternatively, the highly significant induction of GR in uninvolved skin compared to involved skin in vitiligo could reflect a replacement of CAT by the backup GR/GSH/glutathione peroxidase system for O_2^- reduction.

It is noteworthy that vitiliginous skin does not have any significant predisposition for actinic damage, basal or squamous cell carcinoma [36]. Therefore, the presence of TR and GR apparently provide sufficient anti-oxidant defense in these patients. In the case of HPS, there is considerable evidence for actinic damage and early onset of carcinomas of the skin. In these tyrosinase-positive albinos, TR activities are extremely low [37].

However, as with any new hypothesis, we recognize that our proposed molecular mechanism for the onset of vitiligo is based on experimental results from a number of laboratories with some features of the model well-established and with others requiring more experimental results. Our mechanism does not directly account for the suggested structural defects in melanocytes from vitiliginous skin nor does it explain the autoimmune hypothesis for vitiligo. Both of these processes could occur as a secondary response to our proposed breakdown in anti-oxidant defense. Also, epidermal suction blister extracts must primarily reflect changes in the metabolism of active oxygen intermediates (O_2^-) by keratinocytes with little contribution from melanocytes. Keratinocyte/melanocyte-derived protein in the FPLC separation (Fig 1) must be approximately 40:1. In this respect, it appears that both the involved and uninvolved epidermis of patients with vitiligo show abnormalities in anti-oxidant defense enzyme levels primarily in keratinocytes, indicating that vitiligo could be a disorder of keratinocytes. At the present time, we do not understand how these metabolic abnormalities in keratinocytes influence either the process of pigmentation or the cellular integrity of melanocytes. The recent discovery of elevated I-CAM-1, TNF-α, and IFNγ in the perilesional skin of vitiligo by monoclonal antibody techniques [25,38] suggests that cytokines may play an important role in the depigmentation process and even in the loss of melanocytes. Very recently, human melanocytes have been shown to produce TNF-α also [39]. This cytokine production is stimulated by UVB light (five- to tenfold) and the highest levels of TNF-α were induced by neurotensin (i.e., 500 times the level in keratinocytes at 10^{-8}–10^{-7} M of this neuropeptide). These in vitro results suggest a connection between the neuroendocrine system, which is activated by calcium, and TNF-α induction. Such a cascade of events supports our hypothesis in Fig 4 and provides a rationale for the neurologic etiology proposed for segmental vitiligo.

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