

Report

***In vivo* delayed-type hypersensitivity in 109 patients with vitiligo**

Stefanie C. Behrens-Williams, MD, Eva M. Peters, MD, and Karin U. Schallreuter, MD

From, Clinical and Experimental Dermatology, Department of Biomedical Sciences, University of Bradford, Bradford, UK, and Institute for Pigmentary Disorders e.V. in Association with the Ernst-Moritz-Arndt University Greifswald, Biotechnikum, Greifswald, Germany

Correspondence

Karin U. Schallreuter, MD
University of Bradford
Department of Biomedical Sciences
Clinical and Experimental Dermatology
Richmond Road
Bradford, West Yorkshire
BD7 1DP, UK

Abstract

Background Although the etiology of the depigmentation disorder vitiligo is still not completely understood, many investigators believe that an autoimmune reaction may play a major role. In this regard, T-lymphocyte-mediated immunity has been implicated frequently in the pathogenesis of the disease. Most studies have applied *in vitro* testing of cell-mediated immunity, however, rather than *in vivo* measurements. Therefore, our study was undertaken to define the cutaneous delayed-type hypersensitivity (DTH) *in vivo* reaction in association with the absence/presence of serum thyroid autoantibodies, which are a good representative marker for autoimmunity in patients with vitiligo.

Methods DTH was evaluated in the normal pigmented skin of 109 vitiligo patients (29 men and 80 women) and in the depigmented skin of 27 of this group (5 men and 22 women) using the dermal application of seven common recall antigens together with a negative control. Individuals were considered to be hypoergic if the DTH sum score was ≤ 5 mm in women or ≤ 10 mm in men, or if they responded to only one or two antigens.

Results The mean sum score was 10.2 ± 8.4 with an average of 2.3 ± 1.6 positive reactions in depigmented skin vs. a sum score of 12.4 ± 9.0 with an average number of 2.6 ± 1.6 positive reactions in normal pigmented skin. There was no statistically significant difference between depigmented and normal pigmented skin using the paired *t*-test ($P > 0.05$). Further evaluation of these data showed no significant correlation between the presence of thyroid autoantibodies as well as selected clinical parameters and an aberration in cutaneous DTH.

Conclusions In contrast to earlier reports, our *in vivo* studies of cutaneous DTH reactions revealed no clinically significant aberrant cellular immunity in this patient group. These results indicate that the immune reaction in vitiligo may be only a secondary event in the pathogenesis of the disease.

Introduction

Vitiligo is an acquired idiopathic depigmentation disorder affecting 0.5–4.0% of the world's population.¹ Although the pathogenesis of this cosmetically disfiguring disease is still not completely clarified, many investigators believe that an autoimmune reaction may play an important role.^{1–8} Impaired T-lymphocyte-mediated immunity has been strongly implicated in the pathogenesis of the disease.^{3–5,8} However, many studies used *in vitro* measurements rather than *in vivo* skin tests.

Only recently, oxidative stress has been implicated in several autoimmune diseases as the primary event, followed by an immune response.⁹ In this context, evidence has accumulated suggesting a pathogenic association of vitiligo with increased epidermal oxidative stress, caused by an increase in 6-tetrahydrobiopterin (6-BH₄), 7-BH₄ (a

potent inhibitor of phenylalanine hydroxylase), high epidermal monoamine oxidase A activity, and subsequent elevated generation of H₂O₂, which could lead to rapid oxidation of 6-BH₄ to 6-biopterin, a molecule that is known to be melanotoxic.^{10–15} Based on these observations, evidence has appeared that peroxidative damage of cells in the epidermis of vitiligo patients could be a major etiopathogenic factor in this depigmenting disorder, whereas the observed immune reaction could be a secondary event caused directly by oxidative stress.⁹

We therefore decided to employ the cutaneous delayed-type hypersensitivity (DTH) reactions in patients with vitiligo *in vivo*, as this method reflects one of the most sensitive cell-mediated immune responses. For this purpose, DTH was evaluated in normal pigmented and depigmented skin of vitiligo patients using dermal application of seven common recall antigens. As thyroid antibodies are the most common

autoantibodies in vitiligo, we included an evaluation of this humoral marker to further substantiate the possibility of an impaired immune reaction.

Patients and methods

Patients

One hundred and nine randomly chosen patients (80 women and 29 men) affected with vitiligo were studied. Cutaneous DTH was evaluated in normal pigmented skin of all 109 patients and, additionally, in depigmented skin of 27 of this group (22 women and 5 men). None of the tested patients had received phototherapy, photochemotherapy, or immunosuppressive agents including corticosteroids in the 2 months prior to the study. The diagnosis of vitiligo was based on a thorough physical examination, Wood's light examination (UVA light with a wavelength of 351 nm), and evaluation of the patient's past medical history. Documentation of clinical findings included: (a) gender; (b) assessment of skin phototype;¹⁶ (c) location and distribution of the disease; (d) age of disease onset; (e) duration of the disease; (f) associated diseases; (g) family history; and (h) disease activity. The ages at the time of testing ranged from 3 to 74 years (mean, 35.4 years $\pm$ SD 14.3). The majority of the group had skin phototype III (80%) and skin phototype II (17%); only two patients had skin phototype IV, and one patient skin phototype VI.

The localization of the hypopigmented areas established different types of vitiligo. Sixty-four per cent of all patients ($n=70$) suffered from vitiligo vulgaris, 18% ($n=20$) had acrofacial vitiligo, 15% ($n=16$) focal type vitiligo, 2% ($n=2$) segmental form, and 1% ($n=1$) universal form. These data are in agreement with other authors, where the most common type of vitiligo in all age groups is the vulgaris type with a generalized symmetrical distribution of the depigmentation pattern.¹ At the time of this investigation, 82% ($n=89$) of patients presented active disease. Twenty-nine per cent ($n=32$) of patients had a positive family history. The mean age of disease onset was 21.8 years (SD $\pm$ 13.7). The mean duration of the disease at the time the patients were examined was 13.7 years (SD $\pm$ 10.8). The study was approved by the local ethics committee and all patients provided their written informed consent.

Multitest system

The *in vivo* cutaneous DTH was determined employing the Multitest Mérieux system.¹⁷ Briefly, the system consists of a multipuncture device with eight sterile plastic-pointed heads capable of applying, simultaneously, seven preloaded standardized test antigens plus a diluent negative control (i.e. glycerin). The common recall antigens included tetanus, diphtheria, streptococcus (group C), tuberculin, *Candida albicans*, *Trichophyton mentagrophytes*, and *Proteus mirabilis*. The skin tests were read at 48 h after application by measuring two diameters expressed in millimeters (mm). For each reaction, the average diameter was calculated using the formula $a + b/c$.

Induration of 2 mm or more in diameter was considered to be positive. The total DTH score was determined by adding the mm induration values of all positive test sites. Anergy was defined as a lack of response to any antigen. Hypoergy was defined as a total DTH score ≤ 5 in women or ≤ 10 mm in men as given by the Institute Mérieux.¹⁷ Furthermore, the average induration diameter of single antigen reactions and the total number of positive reactions in each patient were documented. Individuals were also considered hypoergic if they responded to only one or two antigens.

Serum thyroid antibodies

Serum autoantibodies against thyroglobulin (TAB) and thyroid peroxidase (microsomal antigen, MAB) were measured by an indirect solid phase enzyme immunometric assay (ELISA). Serum antibody levels >250 U/L were considered to be positive.

Statistical analysis

The statistical significance of group differences was estimated with the *t*-test ($P < 0.05$ indicating a significant result). Non-paired *t*-tests were performed by analyzing men and women together in one group, as well as the separate analysis of men and women. Additionally, a paired *t*-test was conducted for all patients who had received two tests in the depigmented and normal pigmented skin. Statistical correlation analysis of *in vivo* skin test results with selected clinical parameters and absence/presence of thyroid antibodies was performed using the Pearson coefficient (statistical significance, if $r > [0.5]$).

Results

Cutaneous DTH reactions

By defining hypoergy as advised by the Institute Mérieux, with a sum score of ≤ 5 mm in women or ≤ 10 mm in men or only one or two positive reactions,¹⁷ our results revealed that cutaneous DTH in both depigmented and normal pigmented skin of all tested patients was normergic. The mean sum score for all patients was $10.2 \pm$ SD 8.4 with an average of $2.3 \pm$ SD 1.6 positive reactions in depigmented skin vs. a sum score of $12.4 \pm$ SD 9.0 with an average number of $2.6 \pm$ SD 1.6 positive reactions in normal pigmented skin (Table 1). If men and women were analyzed separately, generally lower values could be found for women in comparison to men (see Fig. 1). The average sum score of both groups, independently analyzed, confirmed again the normergic values in depigmented and normal pigmented skin of women ($8.6 \pm$ SD 7.8 vs. $12.1 \pm$ SD 8.5) and men ($16.4 \pm$ SD 8.1 vs. $13.9 \pm$ SD 9.1) (Fig. 1). The mean number of positive reactions in the depigmented skin of women was, with a value of only 2 (SD $\pm$ 1.6), borderline hypoergic, whereas the mean number of positive reactions in normal pigmented skin of

women ($2.5 \pm \text{SD } 1.5$), as well as in normal pigmented ($2.9 \pm \text{SD } 1.7$) and depigmented skin ($3 \pm \text{SD } 0.7$) of men showed normergic values for this parameter. The percentage of anergy in total was 7.3% ($n=8/109$) in normal pigmented and 14.8% ($n=4/27$) in depigmented skin.

Analyses of skin test results using paired and non-paired *t*-tests, including the analysis of men and women in one group as well as separate analysis of both genders, showed no statistically significant difference between values obtained in depigmented and normal pigmented skin. Based on the analysis of sum scores, number of positive reactions, and single antigens, *P* was >0.05 .

All patients had a negative reaction to glycerin. None of the 109 patients experienced any adverse effects to the test.

Evaluation of serum thyroid antibody levels

From the total of 109 patients, the status of thyroid tissue autoantibodies was determined in 103 patients (76 women

and 27 men). Twenty patients (17 women and 3 men) showed elevated levels of either MAB or TAB. The presence of thyroid antibodies in about 20% of our patients and the ratio of MAB ($n=14$) to TAB ($n=7$) of 2:1 are similar to the published values in the normal population.^{18,19} There was no significant correlation between cutaneous DTH and the presence of thyroid antibodies in our study.

Evaluation of a possible correlation between DTH and selected clinical parameters

Further analysis of our data established no significant correlation between (a) gender, (b) clinical subtype of vitiligo, (c) assessment of skin phototype,¹⁶ (d) age of disease onset, (e) disease activity, (f) duration of the disease, (g) associated diseases, (h) family history and an aberration of cutaneous DTH (Pearson's coefficient $r > [0.5]$).

Table 1 Mean scores of the delayed-type hypersensitivity reaction (Multitest Mérieux) in 109 patients with vitiligo (mean values $\pm$ SD)

Antigen	Women		Men		Total	
	V ($n=22$)	N ($n=80$)	V ($n=5$)	N ($n=29$)	V ($n=27$)	N ($n=109$)
Tetanus	3.3 (± 3.4)	4.9 (± 5.3)	5.7 (± 4.9)	5.2 (± 4.3)	3.9 (± 3.7)	4.7 (± 5.1)
Diphtheria	1.7 (± 1.8)	1.72 (± 2.6)	0 (± 0)	2 (± 2.6)	1.2 (± 1.7)	1.9 (± 2.7)
Streptococci	0.3 (± 1.1)	0.7 (± 1.4)	0.4 (± 0.9)	0.6 (± 1.3)	0.4 (± 1.1)	0.6 (± 1.3)
Tuberculin	1.3 (± 2.2)	1.8 (± 2.8)	5.4 (± 5.1)	2.8 (± 3.6)	2.2 (± 3.3)	2 (± 3)
<i>Candida</i>	1 (± 2.0)	1.7 (± 2.1)	1.7 (± 2.3)	1.9 (± 1.9)	1.2 (± 2.1)	1.8 (± 2.1)
<i>Trichophyton</i>	0 (± 0)	0.6 (± 1.5)	1.6 (± 1.5)	0.4 (± 1)	0.3 (± 0.9)	0.6 (± 1.5)
<i>Proteus</i>	0.9 (± 1.6)	0.7 (± 1.8)	1.6 (± 2.3)	1.2 (± 2)	1.1 (± 1.8)	0.8 (± 1.8)
Glycerin	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)

V, depigmented skin; N, normal pigmented skin.

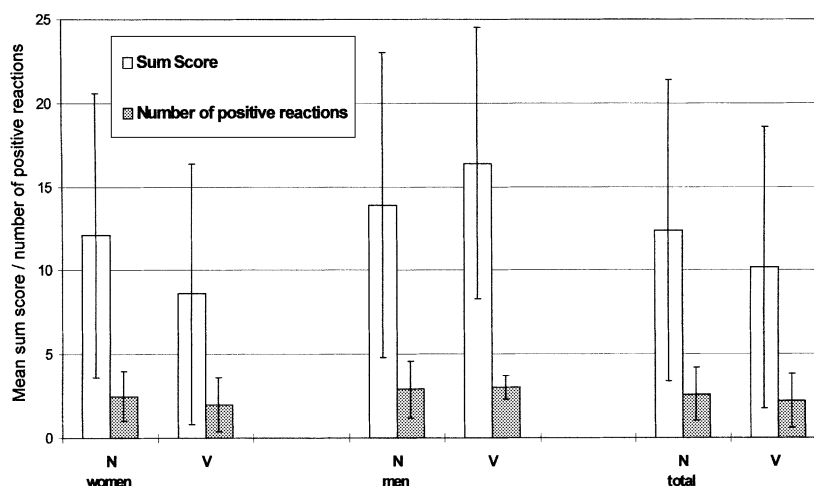


Figure 1 Delayed-type hypersensitivity reactions (Multitest Mérieux) in 109 vitiligo patients (mean sum score and mean number of positive reactions $\pm$ SD). N, normal pigmented skin; V, depigmented skin

Discussion

Various immunologic parameters have been investigated in vitiligo in the past and many abnormalities of *in vitro* testing of cell-mediated immunity have been reported.^{3,7,20–23} Our study examined the *in vivo* DTH in vitiligo, as well as its possible correlation with clinical and laboratory findings frequently reported in association with this disease. To our knowledge, this is the first report of *in vivo* DTH testing in such a large group of vitiligo patients, whereas there exist numerous studies describing a diminished DTH in patients with other diseases, such as atopic dermatitis, lichen planus, rheumatoid arthritis, malignancies, or infections.^{24–28} The fact that the vast majority of our patients were women probably simply reflects the often stronger desire of female sufferers to search for dermatologic advice and therapy concerning this cosmetically disfiguring disease.

The results of our study examining vitiligo patients indicate an overall normergic cutaneous DTH in both depigmented and normal pigmented skin. Except for one parameter, i.e. the mean number of positive reactions in depigmented skin of women at the lower limit, all other test values in men and women were unremarkable following the definition of the Institute Mérieux.¹⁷ Furthermore, there was no statistically significant difference between test results in depigmented vs. normal pigmented skin and no significant correlation between the clinical and laboratory parameters examined and an aberrant cutaneous DTH. Despite normergic reactions in these patients, however, it must be noted that our skin test values were at the lower limit and had a higher rate of anergy by comparison with healthy control groups reported in the literature.^{29–34} This fact could be due in part to socioeconomic variations, different vaccination schedules, and different rates of natural exposure to certain diseases in different populations, geographical locations, and years. Nevertheless, the lack of concurrent normal controls, as well as the rather rough grading system/norm definition given by the Institute Mérieux,¹⁷ must be considered a weakness of our data. Furthermore, these data only relate to older DTH of tested individuals as opposed to investigating the induction of new type IV skin responses.

Several hypotheses have been proposed for the pathogenesis of vitiligo: autoimmune mechanisms, neural origin, self-destruction, or a combination of all three.¹ Still, the autoimmune hypothesis seems to be currently the most favored. Various studies in the past have revealed aberrant T-lymphocyte profiles in patients with vitiligo and suggested that disturbances in cell-mediated immunity may be operative in the pathogenesis of this disorder. Conflicting results have been reported, however, indicating high as

well as low levels of CD4/CD8 ratios or no alteration at all.^{3,4,20–22,35} A clinically significant diminished contact sensitivity response in the depigmented skin of vitiligo patients has been reported previously.^{36,37} In accordance with the findings of our study, however, these authors could not show a suppressed delayed-type skin reaction to intradermal injection of tuberculin or *Candida albicans*, respectively, in vitiligo skin.^{36,37} Unfortunately, both groups only tested a small number of patients intradermally and applied only one antigen. So far, no study has been able to determine whether the immune reaction in vitiligo is the primary cause or a consequence of the disease. To date, it has been established that inflammatory cells targeted by a specific autoantigen may include the suppressor cell population, which could in turn inhibit immune reactions to other unrelated antigens. Our *in vivo* findings in 109 patients with vitiligo, however, do not support a significantly altered T-cell immune response in this disease. These data gain further support by the lack of any significant connection with the absence/presence of thyroid antibodies, which could well be a representative marker for autoimmunity in this disease. Based on our data, we conclude that the immune response in vitiligo seems to be a secondary event rather than the primary cause of the disease. This secondary reaction could be precipitated by the accumulation of H₂O₂ in association with impaired catalase and glutathione peroxidase activities in this disorder.^{9,11,38,39}

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Gender and Lupus Erythematosus I

Although the discovery by Hargraves of the 'LE cells' in 1948 has helped clinicians to identify the group of conditions we now call systemic lupus erythematosus (SLE), defining the disease continues to be a problem. Both constitutive and environmental factors are thought to be important in determining the protean manifestation of SLE.

The striking predominance of women with lupus was noted more than a hundred years ago by Erasmus Wilson (1809–1884) with the female:male ratio now estimated to be between 9–15:1. The peak incidence of lupus occurs in the 15- to 45-year old age group, during the time in life when estrogen levels are highest in women. In many genetic based murine models of lupus, including the NZB/NZW F1 and MRL/lpr/lpr mice, the females also develop more severe disease and higher titer of autoantibodies at an earlier age.

While the precise factors responsible for this gender difference remain elusive, it is known that normal females are more immunoreactive than males. For example, women have higher baseline immunoglobulin levels and produce greater and more prolonged antibody responses following immunization. Gender differences in cell-mediated responses, including accelerated allograft rejection, greater proliferative responses and relative resistance to immunotolerance in females, have all been documented. It is likely that both hormonal and non-hormonal gender-related factors contribute to the abnormal immune responses seen in lupus patients. In this review, we will examine the evidence for the role of estrogens in the pathogenesis of lupus.

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