Comparative study of vitiligo, halo nevus, and vitiligoid variant of lupus erythematosus by immunological, histological, and immunohistochemical methods*

*Estudo comparativo entre vitiligo, nevo halo e lúpus eritematoso vitiligóide por meio de métodos imunológicos, histológicos e imuno-histoquímicos**

Luiz Gonzaga C. Souza Filho[1] Evandro A. Rivitti[2] Lucy M. Miyauchi[3] Mirian N. Sotto[4]
Durvanei A. Maria[5] Shigueko S. T. Puejo[6] Venâncio A. F. Alves[7]

Abstract: BACKGROUND: There are no records of comparative studies on the immunological, histological and immunohistochemical aspects of vitiligo, halo nevus and vitiligoid variant of lupus erythematosus in the literature. The studies available present only descriptive clinical data on leucoderma that accompanies lupus erythematosus in its diverse clinical forms.

OBJECTIVES: 1- To evaluate the immunohistochemical differences between vitiligo, halo nevus and vitiligoid variant of lupus erythematosus; 2- To verify whether the depigmentation observed in the diverse clinical forms of lupus is due to post-inflammatory destruction or to specific immunological attack on melanocytes.

METHODS: 1- Detection of melanocyte antibodies: by direct and indirect immunofluorescence on nevus and melanoma cells; 2- Cytotoxicity evaluation: study of the activity of NK cells against cultivated melanoma cells; 3- Histopathological study of melanocytes and melanin: histopathology with hematoxylin-eosin, Fontana- Masson, Dopa and Dopa + silver and S-100 protein test by immunoperoxidase.

RESULTS: Vitiligo and halo nevus patients presented to antimelanocyte antibodies in 25% of cases. Patients with vitiligoid variant of lupus erythematous also presented these antibodies. The presence of risk factors favoring cellular cytotoxicity was demonstrated in vitiligo and/or halo nevus, as well as in the vitiligoid variant of lupus erythematous.. Staining with Dopa + silver nitrate was superior to traditional staining and to S-100 protein to detect melanocytes and/or melanin in depigmented lesions of vitiligo and/or halo nevus and vitiligoid variant of lupus erythematous.

CONCLUSION: The results confirm the existence of antimelanocyte antibodies in vitiligo and halo nevus. It is not possible to rule out some immunological phenomena similar to those occurring in vitiligo and halo nevus in the genesis of vitiligoid lesions in lupus erythematous. The detection of melanocytes in achromic lesions of vitiligo suggests the predominance of a functional inhibitory mechanism rather than cell destruction in the genesis of the disease.

Keywords: Allergy and Immunology; Lupus; Nevus, Pigmented; Vitiligo.

Resumo: *FUNDAMENTOS: O estudo compara o vitiligo, o nevo halo (NH) e lúpus eritematoso vitiligóide (LEV) do ponto de vista imunológico, histológico e histoquímico.*

OBJETIVOS: Avaliar diferenças imuno-histoquímicas entre essas doenças e investigar se a despigmentação do LEV deve-se à destruição pós-inflamatória ou à agressão imunológica aos melanócitos.

MÉTODOS: Foram avaliados 20 pacientes com vitiligo, 17 com vitiligo e NH, cinco com NH isolado e 15 com LEV. Detecção de anticorpos: IF direta e indireta com células névicas e de melanoma. Citotoxicidade: atividade NK contra células de melanoma. Estudo anátomo-histoquímico: exame histológico com hematoxilina e eosina, Fontana-Masson, Dopa e Dopa mais prata (D+P) e exame histoquímico com proteína S-100.

RESULTADOS: Doentes com vitiligo, NH e LEV apresentaram anticorpos antimelanócitos. Tanto no vitiligo e NH, como no LEV, demonstrou-se a presença de fatores de risco favorecedores da citotoxicidade celular. A coloração com D+P foi superior às colorações tradicionais e à proteína S-100 na detecção de melanócitos e melanina nas lesões de vitiligo, NH e LEV.

CONCLUSÕES: Demonstrou-se a existência de anticorpos antimelanócitos no vitiligo e NH. É possível que a despigmentação no LEV se deva a fenômenos imunológicos semelhantes aos do vitiligo e NH. A detecção de melanócitos nas lesões de vitiligo sugere mais inibição funcional do que destruição dessas células.

Palavras-chave: Alergia e Imunologia; Lúpus; Nevo pigmentado; Vitiligo.

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[1] PhD, Adjunct Professor of Dermatology, Department of Tropical Medicine, Universidade Federal de Pernambuco (PE).
[2] Full Professor, Department of Dermatology, Hospital das Clínicas da Universidade de Sao Paulo (SP).
[3] Biologist, Department of Dermatology, Hospital das Clínicas da Universidade de Sao Paulo (*in memoriam*) (SP).
[4] PhD, Adjunct Professor, Department of Pathology, Universidade de São Paulo (SP).
[5] Technician, Hematology Investigation Laboratory, Medical School of Universidade de São Paulo (SP).
[6] Technician, Cell Culture Laboratory of the Discipline of Oncology, Department of Internal Medicine, Universidade de São Paulo (SP).
[7] PhD, Adjunct Professor of the Department of Pathology, Universidade de São Paulo (SP).

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INTRODUCTION

Vitiligo is characterized by progressive destruction of melanocytes with subsequent decrease in or absence of melanin production. Vitiligo may possibly represent a syndrome, with different etiopathological mechanisms manifesting with a similar clinical pattern. Three etiological hypotheses stand out: the neurogenic, the autodestructive and the immunological hypotheses.[1,2]

Although etiological and pathological factors may work separately or together, the immunological hypothesis is the one providing the most consistent clinical and experimental evidences. In halo nevus, the antigenicity of melanocytes is strongly suggested by presence of a lymphocytic infiltrate, spontaneous regression of the pigmented area, with destruction of nevus cells and perinevic melanocytes leading to an achromic halo, and by the occasional presence of leucoderma in malignant melanoma. The association with autoimmune diseases is also an important argument to prove this hypothesis.

Achromic and hypochromic lesions are frequently observed in lupus erythematosus, especially in the discoid and subacute forms. Since systemic lupus erythematous (SLE) is a condition with multiple autoantibodies, the objective was to investigate, using immunological, histological and immunohistochemical methods, whether depigmentation in these clinical variations occurs exclusively due to post-inflammatory destruction or as a result of an immunological attack on melanocytes, as assumed in vitiligo and halo nevus (HN).

PATIENTS AND METHODS

We enrolled male and female patients aged over 12 years, who provided an informed consent signed by themselves or by their legal representatives, when minor.

Immunofluorescence (IF) reaction controls were skin and blood samples of healthy individuals. Sections of autologous nevi and of healthy individuals were used as reaction controls of melanocytic nevus.

The inclusion criteria were male and female patients aged 12 years or older, who agreed to participate in the study. The exclusion criteria were patients with mental disorders, autoimmune diseases or other possibly autoimmune diseases, such as diabetes mellitus, thyroid diseases, rheumatoid arthritis, alopecia areata, acquired immunodeficiency syndrome, neoplasms, patients submitted to organ transplantation or on immunosuppressants.

The study lasted two years. Skin and blood samples for immunofluorescence (IF) reactions were immediately processed for analysis. Blood samples for cytotoxicity evaluation were stored at -4ºC and read within the first 48 hours. The criteria adopted for positive tests were those standardized for each technique. IF reactions were read by three different observers and were only considered positive when the three observers agreed. It is a cross-sectional study.

LABORATORY METHODS

A) Immunological study

A1) Antimelanocyte antibody detection

Nevocellular nevus lesions and cultivated melanoma cells of patients were used as substrate. The reactions used were: 1) direct immunofluorescence (DIF), 2) indirect immunofluorescence (IIF), and 3) indirect immunofluorescence against melanoma cells in culture (IFM). The statistical method applied was Fisher's exact test.

A2) Cytotoxicity evaluation

The evaluation of NK activity of peripheral lymphocytes against melanoma cells in culture was used. The statistical method applied was Wilcoxon test.

B) Anatomical and histochemical study of the melanocytes and detection of melanin

The objective was to detect melanocytes or melanin in the healthy and achromic skin of patients with vitiligo, HN, and vitiligoid variant of lupus erythematosus (VLE). Histopathological study was performed with HE, Fontana-Masson, Dopa and Dopa plus ammoniacal silver (D+S)[3] and the histochemical study used melanocyte markers and S-100 protein with the avidine-biotin-peroxidase[4] method. The statistical method applied was Fisher's exact test.

RESULTS

1) Immunological study

The study included 42 patients (16 males and 26 females) with vitiligo and HN distributed as follows: 18 cases of disseminated vitiligo vulgaris; two localized vitiligo vulgaris; 17 cases of HN associated with vitiligo, in that, 15 were disseminated vitiligo vulgaris and two localized vitiligo; and 5 cases of HN not associated with vitiligo vulgaris. Mean age was 23.9 years. There were 15 cases of VLE, of which nine were vitiligoid variant of discoid lupus, four were subacute vitiligoid variant of lupus and two were vitiligoid variant of systemic lupus. Mean age was 30.2 years. Blood samples and specimens from healthy skin and from achromic or hypochromic skin of vitiligo, HN and lupus erythematous, as well as from nevocellular nevi were collected. Lupus cases were diagnosed based on clinical presentation and on routine laboratory studies.

Detection of antimelanocyte antibodies

The results of IF reactions in vitiligo, HN, and VLE are shown in table 1. The reaction that yielded more positive results was IIF against melanoma cells (25%) in vitiligo and HN cases, followed by DIF using nevus cells (12.5%). Positive results of IF reactions refer to the fluorescence seen on the periphery of nevus cells and in the cytoplasm of melanoma cells. Skin and blood samples of healthy individuals were used as controls. Sections of autologous nevi and nevi of healthy individuals were used as controls for the reactions with melanocytic nevus. Control results were negative.

No significant differences were seen in the fluorescences of nevus cells of vitiligo and HN, as well as VLE cases (9.3% vs. 11.1%). When melanoma cells were used, positive results were higher (25%) in vitiligo and/or HN cases, when compared to VLE patients (9%), but this difference was not significant. Fisher's exact test was used.

2) Cytotoxicity evaluation

The results of NK cell activity are shown in tables 2 and 3. Significant differences in comparison with the controls were observed both in vitiligo and HN and in VLE cases. However, no significant differences between vitiligo and HN and VLE cases were observed (40.5 vs. 54% of lymphocytotoxicity).

3) Anatomical and histochemical study of melanocytes and detection of melanin

The results of the detection of melanin and melanocytes in cases of vitiligo, NH and VLE are shown in table 4.

Table 4 shows that there were no differences in melanocyte and melanin detection in dyschromic skins in vitiligo and HN, as well as in VLE. Nevertheless, when the findings are analyzed by markers (Table 5), this detection is significantly higher in D+S staining than in traditional stainings, both in vitiligo and HN, and in VLE.

DISCUSSION

1. Immunological aspects

Antimelanocyte antibodies

The presence of antimelanocyte antibodies supports the participation of immunological mechanisms in vitiligo. There are no reports of comparisons between vitiligo and HN and lupus erythematosus as to detection of these antibodies.

Earlier reports on vitiligo were conducted with IIF using melanoma cells as substrate.[5] These antibodies were also described in patients with vitiligo and endocrine diseases using IIF with complement fixation.[6] DIF revealed IgG and C3 deposits in the basal membrane zone, thus showing evidence of the participation of immunocomplexes in the development of vitiligo.[7]

Specific immunoprecipitation (SI) with superficial radioiodinated macromolecules of human melanocytes and of melanoma cells in culture was positive in up to 100% of vitiligo cases. The antigens identified were the same as those of normal melanocytes, with molecular weight of 75, 85, and 240 kDa.[8] Naughton et al.[9] ccorrelated the presence of these antibodies to the extent of the clinical presentation. Norris et al.,[10] demonstrated antimelanocyte antibodies in active vitiligo, by using antibody dependent cellular cytotoxicity.

Antimelanocyte IgG antibodies were detected in vitiligo with enzyme immunoabsorbance and they correlated to the activity of the disease.[11] In another study, the serum of patients with vitiligo showed a higher complement-mediated cytolytic activity against human melanocytes when compared to control serum.[12]

Melanocytes around the lesions express more MHCII as well as intercellular adhesion molecule than do melanocytes located in other areas.[13] Rocha et al.[14] detected the presence of antimelanoma cells antibodies to 165, 90 and 68 kDa antigens by using SI, in familial vitiligo cases.

Kemp et al.[15] iidentified a new autoantigen

Table 1: Immunofluorescence reactions in cases of vitiligo, halo nevus and vitiligoid variant of lupus erythematosus.

	Substrates	DIF		IIF		Total	
		Positive	Negative	Positive	Negative	Positive	Negative
Vitiligo and HN	Nevus	3 (12.5%)	21 (87.55%)	1 (5.26%)	18 (94.7%)	4 (9.3%)	39 (90.6%)
	Melanoma cells	NP	NP	5 (25%)	15 (75%)	5 (25%)	15 (75%)
Lúpus	Nevus	1 (16.6%)	5 (83.33%)	0	3 (100%)	1 (11.1%)	8 (88.8%)
	Melanoma cells	NP	NP	1 (9.09%)	10 (90.9%)	1 (9.09%)	10 (90.9%)

Key: NP = not performed

TABLE 2: Evaluation of NK activity of peripheral lymphocytes against melanoma cells in 12 cases of vitiligo and halo nevus.

Patients	Lymphocytotoxicity (%) Patients % (I)	Controls % (J)
1	33.7	29.7
2	43.6	29.7
3	66.6	29.7
4	66.9	19.1
5	38.4	29.7
6	41.5	13.4
7	43.6	25.4
8	47.8	23.4
9	29.0	11.9
10	27.2	14.3
11	31.3	12.8
12	16.6	12.1
Means	40.5	20.9

Wilcoxon test showed significant "I *vs*. J.
"I": mean of every percentage of "lymphocytotoxicity" obtained from the patients.
"J": mean of every percentage of "lymphocytotoxicity " obtained from the control cases.
"I *vs*. J": comparison between "I" and "J".

TABLE 3: Evaluation of NK activity of peripheral lymphocytes against melanoma cells in six cases of vitiligoid variant of lupus erythematosus.

Patients	Lymphocytotoxicity (%) Patients % (L)	Controls % (M)
1	44,5	29,7
2	42,4	25,4
3	67,2	13,4
4	57,9	44,5
5	38,7	23,4
6	73,6	19,1
Means	54	25,9

Wilcoxon test showed significant "L vs. M".
"L": mean of every percentage of "lymphocytotoxicity" obtained from the patients.
"M": mean of every percentage of "lymphocytotoxicity" obtained from the control cases.
"L vs. M": comparison between "L" and "M".

named melanin-concentrating hormone receptor 1 (MCHR 1) and demonstrated that IgG in patients with vitiligo reacted against this receptor.

Antimelanocyte antibodies were also described in melanoma and HN with IIF using HN nevus cells as substrate.[16] Cui et al.[17] demonstrated the presence of antibodies to melanocytic antigens in vitiligo and in melanoma by means of SI. Merimsky et al.[18] demonstrated antityrosinase antibodies in vitiligo and melanoma with hypochromia, thus correlating depigmentation with antibodies. The inoculation of tyrosinase and melanoma cells in mice induced the production of antityrosinase antibodies and a lower number of metastases. Okatomo et al.[19] described high rates of IgG antibodies to tyrosinase-related protein-2 (TRP-2), expressed in normal and neoplasic melanocytes, in vitiligo, melanoma and immunotherapy-induced hypopigmentation. The highest levels were evidenced in vitiligo.

In this study, DIF and IIF using normal skin, melanocytic nevus and melanoma cells as substrate were used to detect antibodies in vitiligo, HN and VLE, with the purpose of showing antimelanocyte immunological mechanisms, which are common in diseases considered as autoimmune that progress with hypochromia or achromia. By analyzing table 1, we could verify that the detection of antimelanocyte antibodies was positive in 9.3% of vitiligo/HN cases and in 11.1% of VLE cases, when the substrate used was cellular nevus sections ($p>0.05$). When the substrate consisted of melanoma cells, results were positive in 25% of vitiligo/HN cases versus 9% of VLE cases. There is an important percentage difference in the comparison between vitiligo and LE when melanoma cells are used as substrate, but it is not significant ($p>0.05$). The results of control reactions were negative and they pointed to similar mechanisms of melanocyte destruction in the groups studied.

Cytotoxicity evaluation

NK cells may play an immunoregulatory role in the prevention of autoimmune diseases, such as SLE, and the relation between these diseases and the deficiency of NK cells is likely to exist.[20] The depletion of NK cells seems to play an important role in the development of SLE.[6] The possible existence of anti-NK cell antibodies in patients with SLE was admitted.[21] Horwitz et al.[22] demonstrated that NK cells produce tumor growth factor (TGF-beta 1), whose function is to stimulate T cells CD8(+) to inhibit antibody production. The production of this factor is decreased in SLE.

There are few studies describing this technique in vitiligo and HN. Roenigk et al.[23] suggested that melanoma anticell citotoxicity would be more important than the finding of antimelanocyte antibodies in the immunological evaluation of vitiligo. Halder et al.[24] evaluated NK activity and lymphocytic alterations using flow cytometry with monoclonal antibodies. Total and helper lymphocytes were decreased in number, whereas NK activity was very high. The study suggests an alteration in cell immunity regulation in vitiligo. Patients with vitiligo evaluated by cell-mediated natural cytotoxicity had decreased cytotoxic response against lymphoid leukemia cell lines and reduced binding abil-

TABLE 4: Histological and histochemical demonstration of melanin in melanocytes of skin affected by vitiligo, halo nevus and vitiligoid variant of lupus erythematosus

	Vitiligo and HN		Vitiligoid Variant of LE	
	Positive	Negative	Positive	Negative
HE (hematoxylin-eosin)	1 (11.1%)	8 (88.8%)	0	5 (100%)
FM (Fontana-Masson)	4 (36.3%)	7 (63.6%)	3 (42.8%)	4 (57.2%)
Dopa	3 (33.3%)	7 (66.6%)	3 (42.86%)	4 (57.14%)
Dopa + Silver	10 (100%)	0	5 (71.43%)	2 (28.57%)
S.100 (S-100 protein)	3 (60%)	2 (40%)	2 (50%)	2 (50%)

Fisher's exact test: Vitiligo vs. VLE: $p > 0.05$ in every staining.

TABLE 5: Comparison between different melanocyte and melanin markers in skin affected by vitiligo, halo nevus, and vitiligoid variant of lupus erythematosus

Markers	Positive	Negative
HE (hematoxylin-eosin)	1 (7.14%)	13 (92.86)
FM (Fontana-Masson)	7 (38.88%)	11 (61,11%)
Dopa	6 (35.29%)	11 (64.70%)
Dopa + Silver	15 (88.23%)	2 (11.76%)
S-100 (S.100 protein)	5 (55.55%)	4 (44.44%)

Fisher's exact test:
- $p < 0.05$ in comparisons between D+S and FM, and Dopa
- $p > 0.05$ in the comparison between D+S and S-100

ity of NK cells against these cell lines. When myeloid leukemia cell lines were used, the responses were normal and equivalent to those of the control group.[25]

In this study, NK activity was increased in VLE cases (Table 3) as well as in vitiligo and HN (Table 2), in comparison with the control groups. Therefore, patients with VLE may possibly present an increase in NK activity, unlike patients with SLE. However, when comparing lymphocytotoxicity results in vitiligo and HN and VLE, the lack of significant differences in the mechanisms of antimelanocytic attack occurring in vitiligo and VLE is confirmed. No significant difference in the cytotoxicity rate is verified in VLE cases (54%) (Table 3) in comparison with vitiligo/HN cases (40.5%) (Table 2) when compared to control groups. Therefore, immunological findings in the present study do not allow ruling out LE with vitiligoid lesions presenting immunological melanocytic attack mechanisms similar to the mechanisms seen in vitiligo and in HN, in addition to inflammatory destruction of the melanocytes. No reports are found in the literature comparing vitiligo and HN with lupus erythematosus as to lymphocytotoxicity.

2. Histological and histochemical aspects

Other interesting findings in the present study were related to comparing several stainings for melanocyte or melanin detection in dyschromic skin of patients with vitiligo and HN, as well as VLE (Table 4). There were no significant differences in these comparisons, reinforcing the immunological findings of this study, which point to similar mechanisms of melanocyte destruction in both groups.

The ability to detect melanocytes and melanin using different markers was also assessed both in vitiligo and HN and VLE (Table 5). Significant differences between D+S and Dopa, and between D+S and FM were observed. When D+S was compared to S-100 protein, no statistically significant differences were observed, although the percentage of positive cases had been higher with D+S. These results indicate that more sensitive staining methods are able to demonstrate melanocytes in chronic achromic lesions mostly regarded as melanocytopenic, a fact that suggests inhibition mechanisms rather than melanocyte destruction in both groups studied. Lê Poole et al.[26] used polyclonal and monoclonal antibodies and were not able to identify reaction in frozen sections of vitiligo skin. However, using laser-scanning microscopy in sections subject to dermal-epidermal split, they observed degenerated melanocytes, which can support the findings presented here.

CONCLUSIONS

Patients with vitiligo and HN had antimelanocyte antibodies detected by immunofluorescence. The best substrate was cultivated melanoma cells. There were no significant differences in the comparison between vitiligo and HN, and VLE as to the detection of these antibodies ($p>0.05$). Risk factors, possibly antibodies promoting cell cytotoxicity, were demonstrated both in patients with vitiligo and HN and in patients with VLE ($p<0.05$). With the immunological methods used, the participation of immunological mechanisms similar to those occurring in vitiligo and in HN cannot be excluded in the development of VLE lesions ($p>0.05$).

D+S staining proved to be superior to traditional stainings (Fontana-Masson and Dopa) in the detection of melanocytes and/or melanin in the cases

studied, both in vitiligo and/or HN, and in VLE ($p<0.05$). In the comparison with the histochemical method using S-100, D+S staining was superior ($p>0.05$). The detection of melanocytes in the achromic lesions of vitiligo and HN suggests predominance of functional inhibition over destruction of these cells in the development of achromia in vitiligo. However, it is possible that the areas studied had not been sufficiently affected in order to cause total disappearance of melanocytes. ❑

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MAILING ADDRESS:

Luiz Gonzaga de Castro e Souza Filho
Rua Virgílio Mota, 128 - Parnamirim
Recife PE 52060-582
Tel:/fax: 81 32685108 81 88763070
E-mail: luizgonzagadecastro@hotmail.com and lgonzaga@ufpe.br