Detection of Rheumatoid Factors in Sera and Biopsy Lesions of Vitiligo Patients

Fatemeh Vahedi Darmian[1], Soheila Joubeh[2], Mehrnoosh Doroudchi[1], Behnam Abdollahi[3], Abbas Ghaderi[1*]

Departments of [1]Immunology, [2]Dermatology and [3]Pathology, Medical School, Shiraz University of Medical Sciences, Shiraz-Iran.

ABSTRACT

Background: Vitiligo is a dermatological disorder of unknown etiology with a common incidence in southern Iran. Presence of autoantibodies to melanocyte antigens suggested an autoimmune basis of the disease. **Objective:** In this study, the presence of rheumatoid factor (RF) in sera and skin biopsies of vitiligo patients was investigated. **Methods:** The presence of RF in sera of 35 vitiligo and 32 normal individuals was assessed by an indirect ELISA assay. In addition, the presence of IgM, IgG, and IgA immunoglobulins in the biopsy lesions of patients was also investigated by Immunoperoxidase test. **Results:** IgM-RF and IgA-RF were detected in sera of 50% and 20% of patients, respectively. Five out of 35 (15%) revealed to produce both IgM and IgA rheumatoid factors. The rheumatoid factor activity of the deposited immunoglobulins at the site of lesion was confirmed by direct immunoperoxidase test. **Conclusion:** The presence of rheumatoid factors as non organ-specific autoantibodies in vitiligo provides further evidence for the autoimmune etiology of the disease and its pathological importance remains to be elucidated.

Keywords: Vitiligo, Rheumatoid Factors, ELISA, Immunoperoxidase.

INTRODUCTION

Vitiligo is characterized by the disappearance of normal skin color as a result of a selective process of melanocytes destruction. Vitiligo has been reported regardless of sex, race and geographic locality (1,2). About 30-

*Corresponding author: Dr. Abbas Ghaderi, Department of Immunology, Shiraz Medical School, Shiraz University of Medical Sciences, P.O. BOX: 71345-1798, Shiraz-Iran. Tel/Fax: 711-2334589, e-mail: ghaderia@sums.ac.ir

40% of vitiligo patients may have a familial history of the disease (2) under influence of genetic factors (3). An autoimmune etiology has been suggested based on the clinical association of the disease with other disorders that are known to be autoimmune (4). In addition, antibodies to a melanocyte enzyme, tyrosinase, which is involved in pigment production, have been detected in sera of patients with vitiligo (5,6,7). The cytotoxicity of vitiligo sera for melanocytes and the binding of IgG from these sera to the plasma membrane of viable unfixed melanocytes in vitro have already been observed (8). The association of autoimmune diseases with vitiligo is frequently reported (9,10,11,12). Among autoimmune diseases coinciding with vitiligo are diabetes mellitus (1-1.7%), Adisson's Disease (2%), and pernicious anemia (1.6-10.6%) (13,14). High levels of anti-thyroglobulin, antithyroid microsomal and antiparietal cell antibodies in vitiligo patients are also reported (3). The deposition of IgG in fixed keratinocytes and the deposition of IgG and C3 in the basement membrane zone and keratinocytes from skin lesion biopsies of vitiligo patients have also been reported (8,15).

The presence of rheumatoid factor (RF) in many organ-specific autoimmune disorders has already been reported (16,17,18). However, apart from few case reports, in which the coexistence of vitiligo and rheumatoid arthritis has been observed (12,19,20,21), the presence of RF in vitiligo has not been demonstrated so far.

In this study, the presence of IgM-RF and IgA-RF in sera of a significant number of vitiligo patients from southern Iran and the deposition of IgM, IgG and IgA at the site of vitiligo lesions is investigated. In addition, by designing a direct immunoperoxidase assay, the rheumatoid factor activity of the deposited Ig at the site of lesions is detected.

MATERIALS AND METHODS

Patients. Patient group included 35 individuals with clinical symptoms of vitiligo with an average age of 31 years. All patients were referred to our laboratory from Nemazi Medical Center, Shiraz-Iran. Patients presenting with hypopigmented patches over extensor areas with gradual extension were diagnosed as having vitiligo. Nineteen cases were male and 16 were female. Eighteen patients had a family history of vitiligo with a mean age of disease onset of 15 years compared to a 32-year age of disease onset in patients without family history of vitiligo. No nail dystrophy or alopecia was present. In 7 cases, the coexisting autoimmune disorders including rheumatoid arthritis (1 patient), diabetes mellitus (1 patient) and thyroid dysfunction (5 patients) were observed. Progressive active disease was

observed in 11 cases, 6 patients revealed generalized and one case revealed segmental pattern. Among others localized disease was observed. Control group (sex and age match) including 32 individuals were selected randomly among healthy laboratory staff.

Determination of IgM- and IgA-RF by ELISA. An indirect ELISA test for the detection of IgM and IgA rheumatoid factors was applied. Flat-bottomed microtiter ELISA plates were coated with 100 µl of a 10 µg/ml solution of human IgG in Carbonate-bicarbonate buffer, pH=9.5. Plates were incubated at 37° C for 45 min, washed 3 times in PBS-tween 20, 0.05% (pH=7.2) and blocked with a solution of 1% BSA in PBS for 1hr at 37°C. Sera from patients and controls in a dilution of 1/100 were added to the plates and incubated for further 45 minutes at 37°C. Plates were washed and incubated with goat anti-human µ or α chain specific antibodies (Sigma, St. Louise, USA). After 30 min incubation at 37°C and washing OPD substrate solution , 2 mg tablet (DAKO, Denmark) in PBS (pH=7.2), was added. The reaction was stopped by 12.5% H2SO4 solution and the absorbance was measured by an automated ELISA reader at 492 nm (Multiscan II, Finland). The mean OD obtained from normal sera plus two SD was calculated and considered as cut-off value. Therefore, all OD values equal or below cut-off were regarded as negative.

Detection of deposited IgM, IgG, and IgA in skin lesions. A Direct Immunoperoxidase test was used for detection of deposited antibodies in the skin biopsies. The frozen sections from patients biopsies were fixed using methanol, then to inactivate the endogenous peroxidase; slides were submerged in PBS (pH=7.2) containing 6% hydrogen peroxide. The dried slides were immersed in a 5% solution of goat serum for 10 minutes and then washed by stream water followed by PBS tween-20, 0.1% (pH=7.2). Fifty microliters of affinity purified HRP-conjugated goat anti-human IgM, IgG or IgA (Fc specific anti-sera, Sigma, St. Louise USA) was applied on dried slides. After 45 minutes incubation at room temperature, slides were washed and developed by addition of DAB substrate solution (10 mg of 3,5, Diaminobenzidine tetrahydrochloride and 3% hydrogen peroxide in PBS, pH=7.2).

Detection of rheumatoid factor activity of the deposited Immunoglobulin. To investigate the rheumatoid factor activity of deposited Ig at the lesions a modified direct immunoperoxidase test was performed using a highly purified human IgG preparation labeled with the enzyme horseradish peroxidase. After conjugation of this antibody with horseradish peroxidase (Sigma, St. Louise, USA) by periodate method, its specific activity was

tested by an ELISA assay. Skin frozen sections from patients were fixed by methanol and endogenous peroxidase was neutralized using 6% hydrogen peroxide in PBS (pH=7.2). A 5% goat serum was used as blocking reagent followed by addition of the peroxidase-labeled IgG at a dilution of 1/800. Slides were incubated for 45 minutes and then washed with distilled water and test was proceeded by addition of substrate solution as described above. In all experiments, the normal skin biopsies (obtained from Plastic Surgery Department, Shiraz Medical School) were used as control for the patient biopsies.

ANA test. An indirect immunofluorescent test was used to detect antinuclear antibody in patients' sera. Frozen sections of mouse liver were fixed in cold acetone for 10 min and 50µl of each patient's serum, diluted 1:20 in PBS (pH=7.2), was added to sections and incubated in wet chamber at room temperature for 60 min. A positive control serum and PBS(as negative control) were included in each test. Then slides were washed 3 times in PBS and 50µl of proper dilution of FITC-conjugated polyvalent Goat anti-Human antibody in PBS-BSA 1% was added. The slides were incubated in a wet chamber at room temperature in dark for 30 minutes. After washing, the slides' surfaces were covered by 10% Glycerol in PBS and examined by a fluorescent microscope (Zeiss, Germany).

RESULTS

IgM rheumatoid factors in sera from vitiligo and normal controls were detected by ELISA test. Control sera were used to calculate the cut off value (O.D. ± 2 S.D.) for IgA- and IgM-RF ELISA. The cut off value for IgA-RF ELISA was determined as 0.32 and for IgM-RF ELISA as 0.74 O.D. In total, 30 out of 35 (85.7%) were positive for IgM- or IgA-RF among which, 18 out of 35 (51.4%) were found to have IgM rheumatoid factor. Using isotype specific conjugated antibodies (peroxidase-conjugated goat anti-IgA antibody), 7 out of 35 (20%) of patients were shown to produce IgA-RF and 5 out of 35 (14.3%) revealed to produce both IgM and IgA-RFs (Table 1). No correlation was observed between type of the disease and the presence of RF, however, it was noted that among the patients, those who had a longer duration of the disease were more likely to produce IgM- or IgA-RF (Table 2). In addition, the presence of antinuclear antibody (ANA) in patients' sera was investigated and only one 18 year-old female subject with 2 years duration of the disease was found to be ANA positive. Deposition of Immunoglobulins at the site of lesions was investigated by a direct Immunoperoxidase assay. As indicated, deposition of IgM on the

basal layer (Fig.1a) scattered on the surface of keratinocytes, abundant deposition of IgG (Fig.1b) and a linear deposition of IgA at the basal layer (Fig.1c) were detected in a few patients. The deposition of IgM was scattered and not limited to the basal layer to form a linear pattern. By conjugation of a highly purified human IgG preparation to horseradish peroxidase, the

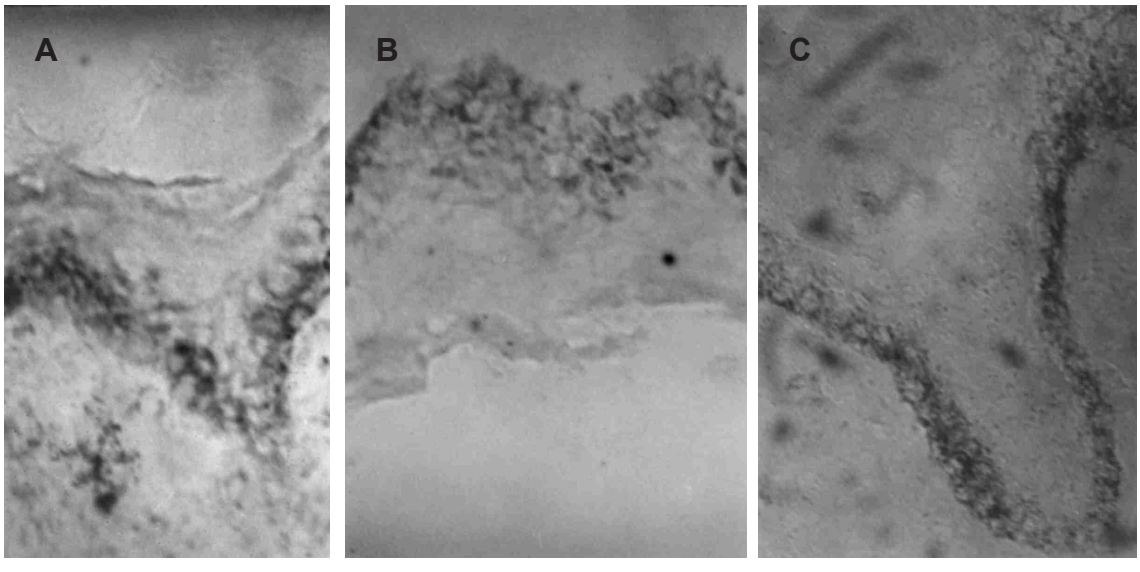

Figure 1. Direct Immunoperoxidase staining of skin biopsies of vitiligo patients for detection of deposited (a) IgM (b) IgG and (c) IgA immunoglobulins. Magnification (X400).

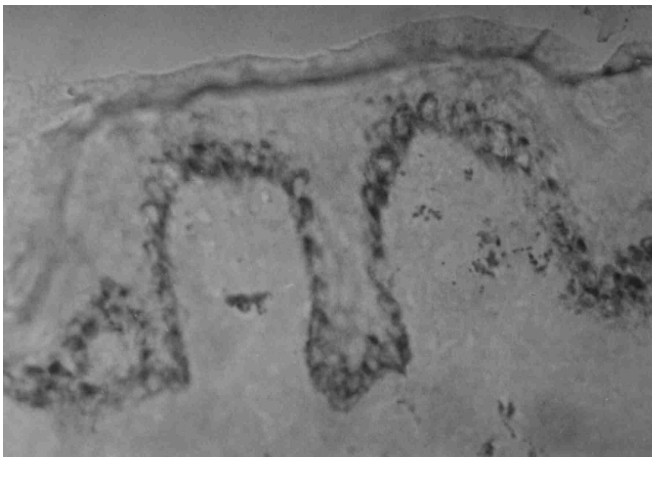

Figure 2. Determination of rheumatoid factor activity of deposited immunoglobulin in skin biopsies of vitiligo patients by direct Immunoperoxidase staining. Magnification (X400).

rheumatoid factor activity of the deposited Immunoglobulins within the lesions was tested. As it is shown in Fig 2, staining pattern in this set of experiments was restricted to the basal layer, a staining pattern identical to that shown for deposited IgA (Fig 1c).

DISCUSSION

Association of vitiligo with a range of autoimmune diseases (4,12,14) and the response of patients to cyclosporin-A and corticosteroid regimens were among the first observations suggesting an autoimmune mechanism of the pathogenesis of this dermatological disorder. Changes in immunological parameters of patients with vitiligo (22,23), an increase in the expression of HLA-DR molecules on keratinocytes and Langerhans cells of skin biopsies of patients (22), and an increased influx of activated skin-homing T cells and macrophages in the perilesional biopsies have been reported (24).

In the current work we report on the production of rheumatoid factors of IgM and IgA isotypes by vitiligo patients. There have been a few case reports on the simultaneous presence of vitiligo and rheumatoid arthritis (12,19,20,21). Recently a case of vitiligo with positive rheumatoid factor has been reported in a 67-year old male with concurrent bilateral posterior scleritis (25).

As indicated in our results 86% of patients in this study revealed to have a degree of serum level of rheumatoid factors. Immunoperoxidase analysis revealed the deposition of immunoglobulins within the skin lesion of vitiligo patients. This deposition was found to appear in a heterogeneous pattern. Since we did not examine all the patients' biopsy specimens, it was not possible to correlate the observed deposition of immunoglobulins with the RF titer in patients' sera.

Table 1. IgM and IgA rheumatoid factor positivity in patient and control groups detected by ELISA and latex agglutination tests.

	Number of Cases	IgM-RF positive latex agglutination	IgM-RF positive ELISA	IgA-RF positive ELISA	IgM and IgA positive ELISA
Vitiligo	35	11	18	7	5
Normal	32	3	4	2	0
P-value	-	P<0.001	P<0.001	P<0.05	-

In addition, we observed a strong pattern of IgA staining at the site of vitiligo lesion in comparison with IgM and IgG and the deposited immunoglobulins with RF activity revealed an identical staining pattern to that of IgA (Fig 2). The deposition of IgA within the dermis of patients with Celiac disease and dermatitis herpitoformis has already been reported (27). A case of IgA nephropathy with vitiligo symptoms has been found with deposition of IgA within the kidney but not in the skin (26). The in vitro

cytotoxic effect of vitiligo patients' sera on the cultured human melanocytes has been reported to be mediated by complement activation and antibody-dependent cellular cytotoxicity (28). However, it is not known if the appearance of immunoglobulins and particularly IgA at the vitiligo skin

Table 2. Comparison of RF positive cases according to the duration of the disease.

Group	Duration (year)	No. of IgA^+ cases	No. of IgM^+ cases	Total No. of cases
1	<1	1	1	5
2	1-4.9	1	1	9
3	5-9.9	3	10	10
4	>10	7	11	11

lesion are responsible for any in vivo tissue damage. In a study by Taher-Uz-Zaman et al. (29) circulating immune complexes accompanied by hypergammaglobulinemia were detected in vitiligo patients. Further experiments are required to disclose the exact molecular pathology of the deposited immunoglobulins at the skin lesions of vitiligo patients. It is also not clear if the presence of Immunoglobulins with RF activity at the epidermal site of skin in our patients has emerged as a result of a passive leakage to the extravascular area, or it has been produced as a result of a local immune response within the skin. Indirect immunoperoxidase staining of patients' biopsies with anti-CD19 monoclonal antibody provided a negative result (data not shown).

In this study RF was more likely to be detected in patients with a longer duration of the disease who were more prone to autoimmune dysfunction and production of organ specific and non-organ specific antibodies. This is consistent with some of the case reports presenting RF positivity in vitiligo patients who had at least 15 years duration of the disease (19). The production of non-organ specific antibodies secondary to autoimmune diseases due to polyclonal B cell activation is not unexpected and has been shown in some autoimmune disorders such as SLE (30). Therefore, it is worthwhile to investigate the prognostic value of these factors in the vitiligo associated with other autoimmune diseases.

In conclusion, the data of this study provide evidence on the production of rheumatoid factor in a significant number of vitiligo patients, which may consequently emerge in the extravascular area which, then can be detected in patients' biopsy lesion.

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