

Detection of Antibodies to Melanocytes in Vitiligo by Western Immunoblotting

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To more fully define the nature of the antibody response to melanocytes which is associated with vitiligo, a Western immunoblot assay was used to test the sera of 28 patients with vitiligo (21 with active non-segmental, and 7 with stable segmental diseases) and 26 normal individuals for antibodies to antigens in detergent extracts of melanocyte membrane fractions. Antibodies to melanocytes were found in 26 (93%) of the patients with vitiligo, and in 16 (62%) of the control individuals. Patients with vitiligo and control individuals both had antibodies to an 80~83 kD antigen. The patient with vitiligo, in addition, had antibody responses to antigens with MWs of 45, 65, and 110 kD. Antibodies to these antigens were present in 46, 25, and 31% of vitiligo patients, but in only 19%, 0%, and 0%, respectively, of the normal individuals. The heterogeneity of the antibody responses to melanocytes in vitiligo was further confirmed by the presence of antibodies to at least 3 distinct antigens in one-third of vitiligo patients but in none of the normal individuals. There was no difference in antibody response between patients with generalized and segmental vitiligo, suggesting that the pathogenesis of diseases was similar in both cases.

Key Words: Melanocyte/antibody, Western blot, vitiligo, autoimmunity

Vitiligo is a disease in which melanocytes are selectively destroyed. The cause of vitiligo remains unknown but it is believed to be an autoimmune disease. Antibodies to melanocytes are common in persons and animals in vitiligo (Naughton *et al.* 1982; Naughton *et al.* 1983; Bystryń and Naughton. 1985; Naughton *et al.* 1986a; Austin *et al.* 1992). There is a correlation between the presence and level of these antibodies and the extent (Naughton *et*

al. 1986b) and the activity of vitiligo (Harning *et al.* 1991), and these antibodies have the ability to selectively kill human melanocytes *in vitro* (Norris *et al.* 1988). The antibodies have been found to react in part to a number of melanocyte antigens with MWs of 40~45, 75, and 90 kDs which have been denominated VIT 40, VIT 75, and VIT 90, respectively (Cui *et al.* 1992; Cui *et al.* 1995), and to tyrosinase (Song *et al.* 1994). These antigens appear to be expressed on the external surface of melanocytes, as they can be selectively labelled by the lactoperoxidase technique (Cui *et al.* 1992; Cui *et al.* 1995). A recent immunoblotting study shows that autoantibodies in the sera of vitiligo patients are mostly directed to the 65 kD antigen which may originate from the melanocyte surface (Park *et al.* 1996). Thus, the antibody response to melanocytes associated with vitiligo appears to be heterogenous.

To more fully define the heterogeneity of

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the antibody response to melanocytes in vitiligo, we have in the present study, conducted a search for antibodies to melanocyte antigens in patients with vitiligo using a Western immunoblot assay. The assay was conducted using as an antigen source, extracts of the membrane fraction of melanocytes, which includes both internal and external membrane antigens.

MATERIALS AND METHODS

Sera

Sera were obtained from 28 patients with vitiligo (21 with active non-segmental and 7 with stable segmental type) and 26 normal individuals. Active vitiligo was defined as development of new lesions or extension of old lesion in the three months preceding collection of sera. None of the vitiligo patients had received any treatment for at least 3 months prior to participation in this study.

Melanocyte culture

Normal melanocytes were cultured by a modification of the method of Gilchrist *et al* (1984). Epidermal cells obtained from neonatal foreskin were grown in 5% CO₂ at 37°C in keratinocyte growth medium (Gibco BRL, Grand Island, NY, USA) supplemented with 2 mM Ca⁺⁺, 5 µg/ml insulin (Sigma, St. Louis, MO, USA), 0.4% whole bovine pituitary extract (Gibco), 2 ng/ml bovine basic fibroblast growth factor (bFGF)(Gibco), 10 ng/ml phorbol myristate acetate (PMA), 10⁻⁸M cholera toxin (Calbiochem, San Diego, CA, USA), 0.25 µg/ml amphotericin and penicillin/streptomycin. Fetal bovine serum (FBS)(Gibco) at a concentration of 10% was added for the first 2 days of the primary culture and for the first 24 hours of each subculture.

Preparation of melanocyte membrane extracts

Six to eight passaged melanocytes were harvested using 1 mM EDTA in Hank's balanced salt solution and homogenized in sucrose buffer (0.25M sucrose, 0.5 mM MgCl₂, 50 mM Tris,

1 mM PMSF, 1 µM leupeptin, pH 7.4) at 4°C in a glass homogenizer. The homogenate was centrifuged at 2,000×g for 10 minutes, the supernatant saved, and the pellet washed with sucrose buffer and centrifuged as above. The two supernatants were pooled and centrifuged at 105,000×g for 1 hour. The resulting pellet was washed in 0.15 M NaCl, 50 mM Tris, 1 mM EDTA buffer, pH 8.0, re-suspended in 0.5% NP-40 to a final concentration of 2 mg/ml, and stored at -70°C until use.

Assay of melanocyte antibodies by Western blotting

Membrane extracts were run on SDS-8% PAGE under reducing condition (2-mercaptoethanol and boiling) and electroblotted onto polyvinylidene difluoride microporous membranes. Each lane was loaded with 15 µg protein. The membranes were blocked with 5% non-fat milk in PBS (pH 7.4) for 2 hours at 25°C and reacted with 10 µl of individual sera diluted 1:100 in blocking buffer (5% non-fat milk in PBS) overnight at 4°C. The membranes were then incubated with biotinylated goat anti-human Ig G or Ig M monospecific antisera (Organon Technika, Westchester, PA, USA) diluted 1:100 for 2 hours and then with avidin-peroxidase (Organon Technika) diluted 1:100 for 1 hour, and were developed with 4-chloro-1-naphthol with intervening washings in PBS-Tween.

The level of antibody to individual antigens was estimated by quantitative densitometry using an image 1 system gel scanning system (Universal Imaging Corporation, Media, PA, USA) to measure band intensity. The level of individual antibodies was calculated by subtracting background density from band density. Total level of all antibodies in the serum was calculated from the sum of individual antibody level in that serum.

Statistical analysis

The incidence of autoantibodies in two groups were compared by Fisher's exact test and the level of antibody was compared by paired t-test. A level of 5% was taken as significant.

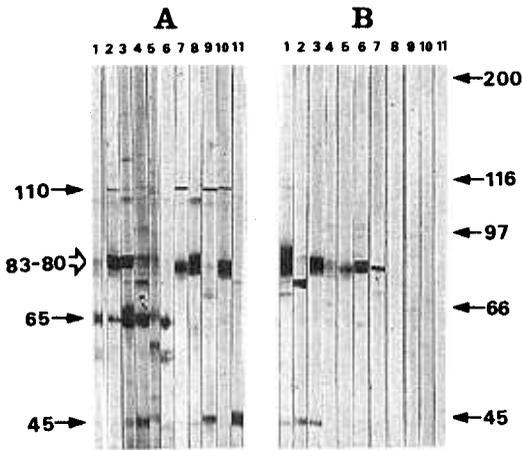


Fig. 1. Immunoblot analysis of antibodies to melanocyte membrane antigens in patients with vitiligo (A) and control individuals (B). Sera diluted 1:100 were reacted with 0.5% NP-40 extracts of melanocyte membrane. Each lane contained 15 µg protein.

RESULTS

The results of testing sera of 28 patients with vitiligo and 26 normal individuals for antibodies to melanocytes by Western immunoblotting are illustrated in Figure 1 and Table 1. Almost all patients with vitiligo and most control individuals had circulating antibodies to melanocytes. However, there were differences in the pattern and specificity of the melanocyte antibodies present in the two groups. The antibodies in patients with vitiligo were predominantly directed to one or more antigens of approximately 110 kD, 80~83 kD, 65 kD and/or 45 kDs. Antibodies to the 45, 65, and 110 kD antigens were associated with vitiligo in 46%, 25%, and 31% respectively of patients, but in only 19%, 0%, and 0% of the control individuals that was significant statistically (Fisher's exact test, $p < 0.05$). By contrast, antibodies to the 80~83 kD antigen were equally common in vitiligo and normal individuals in 75% and 62%, respectively so there was no statistically significant difference ($p > 0.05$). Antibodies to additional melanocyte antigens

Table 1. Incidence of antibodies to melanocytes in vitiligo

| Antibody Specificity | Patients with antibodies*(%) | |
|----------------------|------------------------------|-----------------------|
| | Vitiligo (n=28) | Normal control (n=26) |
| 110 kD | 9(31) [†] | 0(0) |
| 80~83 kD | 21(75) | 16(62) |
| 65 kD | 7(25) [†] | 0(0) |
| 45 kD | 13(46) [†] | 5(19) |
| any antibody | 26(93) [†] | 16(62) |

*: measured by Western immunoblotting

[†]: $p < 0.05$, compared to control group

Table 2. Incidence of antibodies to melanocytes in segmental and generalized vitiligo

| Antibody Specificity | Patients with antibodies*(%) | |
|----------------------|------------------------------|-----------------------------|
| | Segmental vitiligo (n=7) | Generalized vitiligo (n=21) |
| 110 kD | 1(14) | 8(38) [†] |
| 80~83 kD | 5(71) | 16(76) [†] |
| 65 kD | 1(14) | 6(29) [†] |
| 45 kD | 3(43) | 10(48) [†] |
| any antibody | 6(86) | 20(94) [†] |

*: measured by Western immunoblotting

[†]: $p > 0.05$, compared to patients with segmental vitiligo

with MWs of 140, 108, and 52 kD were present in 4%, 7%, and 7% of patients with vitiligo, and in 0%, 4%, and 4% of normal individuals.

The antibody response to melanocytes in vitiligo patients was generally more heterogeneous than in normal individuals. Patients with vitiligo had antibody responses to multiple antigens, whereas the response in normal individuals was directed predominantly to a single antigen, the 80~83 kD antigen.

The antibody responses of patients with segmental vitiligo and of those with generalized vitiligo were similar and patients with both types of vitiligo had increased level of antibodies to the 45, 65 and 110 kD antigens. However, the frequency of these responses,

Table 3. Level of antibodies to melanocyte antigens in vitiligo

| Antibody Specificity | Vitiligo | | Normal control | |
|----------------------|-------------------------------|-------------------------------------|-------------------------------|-----------------------|
| | No. of antibody positive sera | Antibody level ^a (range) | No. of antibody positive sera | Antibody level(range) |
| 110 kD | 9 | 42±19(21~90) | 0 | 0 |
| 80~83 kD | 21 | 115±55(33~206) ^b | 16 | 78±55(31~196) |
| 65 kD | 7 | 125±60(45~206) | 0 | 0 |
| 45 kD | 13 | 104±66(35~238) ^b | 5 | 56±33(17~105) |

^a values are mean±SD

^b P<0.05, compared to control groups

particularly to the 65 kD and 110 kD antigen, were lower in the patients with segmental vitiligo than in those with the generalized form of the disease though statistically not significant ($p>0.05$)(Table 2).

Antibody levels were estimated from the density of bands on Western immunoblots, as quantitated by computer-assisted densitometry. The results are summarized in Table 3. The analysis was restricted to patients who were antibody positive. The average level of antibody to each of the major antigens in patients with vitiligo was higher than in normal individuals.

DISCUSSION

The results of this study indicate that the antibody response to melanocytes which is associated with vitiligo is heterogeneous. In prior studies, Cui *et al.* (1992) reported that these antibodies are directed in part to melanocytes antigens of 40~45, 75, and 90 kDs, which have been denominated VIT 40, VIT 75, and VIT 90 respectively. Recent studies suggest that VIT 40 may be related or tightly bound to HLA class I molecules (Cui *et al.* 1995). None of these antigens is related to pigment cell antigens defined by commonly available monoclonal antibodies (Cui *et al.* 1995) or to tyrosinase-related protein-1 (Cui *et al.* 1995). VIT 75 may be related to tyrosinase, since its molecular weight is close to that of tyrosinase and tyrosinase has recently been reported as a target of the melanocyte antibody present

in patients with vitiligo (Song *et al.* 1994). These antigens appear to be expressed on the external surface of melanocytes as they can be labelled by the lactoperoxidase technique (Cui *et al.* 1992; Cui *et al.* 1995). In another studies, Park *et al.* (1996) reported that 44% of vitiligo sera was directed to melanocyte surface antigen with a MW of 65 kDs. In the present study, using a Western blotting assay that measures antibodies to internal as well as to external melanocyte membrane antigens, we have found that patients with vitiligo have an increased incidence of antibodies to antigens with MWs of 45, 65, and 110 kD. Antibodies to the 110 and 65 kD antigens were present in 31% and 25% respectively of patients with vitiligo, but in none of the control individuals. Antibodies to the 45 kD antigen were present in almost half of the patients with vitiligo, but in only 19% of normal individuals. Patients with vitiligo had a very high incidence of antibodies to an 80~83 kD antigen, but these do not appear to be associated with the disease, as they are present equally in normal individuals. Patients with vitiligo also had occasional antibody responses to other antigens of 52, 108, and/or 140 kDs. The heterogeneity of the antibody response to melanocytes in vitiligo is further indicated by the presence of multiple (greater than 3) distinct melanocytes antibodies in 32% of patients with vitiligo, but in none of the control individuals.

The multitude of pigment cell antibodies found in patients with vitiligo in this study differs from recent reports by Song *et al.* (1994), who reported that patients with vitiligo

had antibodies to only two pigment cell antigens—a 69 kD and a 60 kD protein and by Park *et al.* (1996), who reported that the sera of vitiligo patients have autoantibodies mostly directed to the 65 kD antigen which may originate from the melanocyte surface. These differences may reflect differences in the cell lines used as antigens source for antibody assays (melanocytes in this study compared to melanoma cells in Song's studies), or in the composition of lysing solution and the methods used to extract the antigens (from a membrane fraction in this study compared to whole cell lysates in Song's and whole cells in Park's studies).

The relation between melanocytes antigens described in this study and the previously described VIT 40, VIT 75, and VIT 90 antigens which were labelled by the lactoperoxidase technique, remains to be defined. Also remaining to be defined, is the relation between VIT 75, the 65 kD antigen defined in this study, and tyrosinase. Song *et al.* (1994) reported that 77% of patients with vitiligo have antibodies to a 69 kD protein which appears to be tyrosinase. As the size is close between VIT 75 and the 65 kD antigen, it is possible that one or the other of the latter proteins is also tyrosinase. However, other differences in the pattern of antibodies described in patients with vitiligo between the two studies raise concern about drawing conclusions based on estimated molecular weights. While both studies describe that at least one pigment cell antibody occurs with equal frequency in vitiligo and normal individuals, the identity of the antigen to which this antibody is directed differs markedly in the two studies—60 kD in theirs compared to 80~83 kD in this report.

Though not well known, it is possible that a number of antibodies directed to antigens of melanocytes may show cross reactivity with other autoantibodies to various kinds of cells or tissues such as thyroid glandular cells, gastric parietal cells or so, especially tissues closely related to melanocytes.

Antibodies to the 65 kD and 110 kD antigens were twice as common in patients with generalized than in patients with segmental vitiligo. The reason and significance of these findings

are not known. We might speculate one reason in which patients with segmental vitiligo has less active and/or extensive disease. While we cannot disprove the possibility that the pathogenesis of these two types of vitiligo differ, we believe it is unlikely since antibodies to melanocytes are also increased in segmental vitiligo, though not as often as in generalized vitiligo. Rather, the elevated incidence of antimelanocyte antibodies in segmental vitiligo suggests that the cause of the form of vitiligo also lies in an autoimmune process rather than on a neurological basis (Koga, 1977).

In conclusion, this study shows that the vitiligo patients have heterogenous antibody responses to melanocytes and we suggest that further characterization studies for these vitiligo antigens are needed.

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