

Relation Between the Incidence and Level of Pigment Cell Antibodies and Disease Activity in Vitiligo

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Patients with vitiligo often have antibodies to pigment cells. To examine whether there is a relation between the presence of such antibodies and disease activity, sera of 24 patients with vitiligo (10 with active and 14 with inactive disease) and 19 normal individuals were tested for antibodies to pigment cell surface antigens using a live cell enzyme-linked immunosorbent assay. IgG pigment cell antibodies were present in 80% (eight of 10) of patients with active vitiligo but in none of those with inactive disease or in normal individuals. The antibody level of patients with active vitiligo (mean binding index [BI] 3.3 ± 0.59) was significantly higher than in pa-

tients with inactive disease (BI 0.96 ± 0.04) or normal individuals (BI 1.0 ± 0.04 , $p < 0.001$). Antibodies present in eight patients with high titers of pigment cell antibodies reacted to three of four pigment cells but to only one of six unrelated cells. These findings indicate that a correlation exists between the incidence and level of pigment cell antibodies and the activity of vitiligo, and support the hypothesis that vitiligo is an autoimmune disease mediated by an immune reaction to pigment cells. *J Invest Dermatol* 97:1078-1080, 1991

The cause of vitiligo, a disease characterized by the selective destruction of melanocytes, is unknown but it is suspected to be of autoimmune origin [1-4]. The most convincing evidence in support of this hypothesis is that patients and animals with vitiligo often have antibodies to antigens on melanocytes or melanoma cells that can be detected by immunoprecipitation [3,5,6], Western blot techniques [7], or complement-mediated cytotoxicity [8-10]. These antibodies can be directed to antigens on the surface of the pigment cells [3,5,6], a location accessible to host immune effector mechanisms. These antibodies are suspected to be involved in the pathogenesis of the disease because they can kill pigment cells in vitro [8-10] and because there is a correlation between their presence and the extent of depigmentation [2,5]. However, it is not known whether the presence of these antibodies is related to the activity of the disease.

The present experiments were conducted to determine if there is a relation between the presence and level of pigment cell antibodies and the activity of vitiligo, using a recently developed live-cell enzyme linked immunosorbent assay (ELISA). The results confirm the presence of pigment cell antibodies in vitiligo and demonstrate that there is a relation between the incidence and level of these antibodies and the activity of the disease. This finding supports the hypothesis that vitiligo is an autoimmune disorder.

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Abbreviations:

- BI: binding index
- BSA: bovine serum albumin
- ELISA: enzyme-linked immunosorbent assay
- HBSS: Hanks' balanced salt solution
- OD: optical density
- PBS: phosphate buffered saline

MATERIALS AND METHODS

Sera Sera from 24 patients with vitiligo and 19 normal controls were studied. Of the patients with vitiligo, 10 had active and 14 had inactive disease. Active disease was defined as the appearance of new or extension of existing lesions in the 3 months prior to collection of serum. This classification was made irrespective of age, sex, treatment, or the development of repigmentation, which may sometimes occur in localized areas in patients with active disease.

Cells Normal human neonatal melanocytes were established and grown in tissue culture by the method of Eisinger [11] as previously described [3,6]. Pigmented (SK Mel 23 and SK Mel 30) and non-pigmented (SK Mel 28) melanoma cells [12], K562 leukemia, RD rhabdomyosarcoma, HCT-15 colon carcinoma, A549 lung carcinoma, and CCD-14SK fibroblast cell lines were obtained from the American Tissue Collection (Rockville, MD [13]) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO, NY). Normal lymphocytes were prepared by Ficoll-Hypaque separation.

ELISA Adherent cells (melanocytes, SK Mel 23, SK Mel 28, SK Mel 30, RD, HCT-15, A549 CCD-14SK) were gently trypsinized (0.05% trypsin, GIBCO, Grand Island, NY) for 3 min at 37°C and washed twice in Hanks' balanced salt solution (HBSS, Whittaker, MD). Cells in suspension (K562 and normal lymphocytes) were washed twice in HBSS. The wells of 96-well microtiter plates (Corning, NY) were blocked for non-specific binding by incubation with 100 μ l of a 2.0% bovine serum albumin (BSA, Sigma, St. Louis, MO) for 30 min at 37°C. 10^4 target cells in 50 μ l of HBSS were added to each well and incubated overnight with 50 μ l of diluted (1:50) sera at 4°C in a humidified chamber. The plates were then centrifuged at 1500 rpm at 4°C for 5 min, the supernatants decanted, and the plates washed 3 times with 1.0% BSA in PBS at pH 7.4 and once with PBS. Each well was then incubated with 100 μ l of a 1:1000 dilution of either protein A/alkaline phosphatase (to detect IgG antibodies), goat anti-human IgM/alkaline phosphatase,

Table I. Class of Pigment Cell Antibodies in Patients with Vitiligo

Diagnosis	Number of Patients	Pigment Cell Antibodies					
		IgG		IgM		IgA	
		Number (%) of Patients	BI ^a	Number (%) of Patients	BI	Number (%) of Patients	BI
Vitiligo, active	10	8 (80%)	3.3 ± 0.59 ^b	8 (80%)	3.5 ± 0.68 ^b	0 (0%)	0.95 ± 0.07
Vitiligo, inactive	14	0 (0%)	0.96 ± 0.04	3 (21%)	1.1 ± 0.14	2 (14%)	0.94 ± 0.13
Controls	19	0 (0%)	1.0 ± 0.04	3 (16%)	1.0 ± 0.1	2 (11%)	1.0 ± 0.13

^a BI = OD test serum - mean OD control serum.

^b Significantly greater than inactive vitiligo or controls, $p < 0.001$.

or goat anti-human IgA/alkaline phosphatase conjugates (Sigma) for 2 h at room temperature, washed 3 times with 1.0% BSA in PBS, washed once with PBS, then incubated with p-nitrophenylphosphate (Sigma) for 1 h at room temperature in the dark. Optical density (OD) was measured at 405 nm wavelength using an automated ELISA reader (Physica Inc., NY). All sera were tested in duplicate. The results are expressed as BI (mean OD test serum/OD mean control serum). Sera with a BI of greater than 1.5 were considered positive for pigment cell antibodies.

Statistical Analysis Statistical analyses were conducted using the Student *t* test and ANOVA programs of S.A.S. (SAS Institute, NC).

RESULTS

Sera from 10 patients with active vitiligo, 14 with inactive vitiligo, and 19 normal individuals were tested for the presence and level of IgG pigment cell antibodies by a modified, live-cell ELISA. The results are shown in Table I. IgG antibodies to pigmented melanoma cells (SK Mel 23) were detected in 80% (eight of 10) of patients with active disease, in 0% (0 of 14) of patients with inactive disease, and in 0% (0 of 19) of controls. The average level of pigment cell antibodies in patients with active vitiligo (BI 3.3 ± 0.59) was significantly greater than that of patients with inactive disease (BI 0.96 ± 0.05) or controls (BI 1.0 ± 0.04, $p < 0.001$). The average BI of patients with inactive disease did not differ significantly from that of controls (Table I).

The class specificity of the antibody response to pigment cells was examined using immunoglobulin class-specific conjugates. In active vitiligo, IgG and IgM pigment cell antibodies were present in 80% of patients whereas IgA antibodies were present in none (Table I). In inactive disease, no IgG pigment cell antibodies were detected, whereas IgM (21%) and IgA (14%) antibodies were only occasionally detected but in low levels (mean BI of 1.1 and 0.94, respectively). Normal individuals did not have IgG pigment cell antibodies, but they occasionally were positive for IgM (16%) or IgA (11%) class antibodies in low levels. The average level of IgG and IgM pigment cell antibodies for patients with active disease was significantly higher ($p < 0.001$) than that of either inactive disease patients or controls.

To examine the specificity of the pigment cell antibodies detected by live cell ELISA, a subset of eight sera with high levels of such antibodies was tested for reactivity against a panel of control cells. The results are illustrated in Table II. The different sera reacted with various patterns of cross-reactivity against different melanoma and control cells, suggesting that the antibodies were directed to different sets of antigens. All sera reacted to normal melanocytes and all but one sera reacted to at least two of the three melanoma cell lines tested. By contrast, five of the eight sera reacted to none or to only one of six control cell lines tested. The remaining sera reacted to two (two sera) or to three (one serum) of the control cells.

DISCUSSION

This study confirms that patients with active vitiligo have antibodies to pigment cell antigens that can be detected with a simple, live cell ELISA assay, and indicates that there is a correlation be-

tween the activity of vitiligo and the incidence and level of these antibodies.

The pigment cell antibodies in patients with active vitiligo detected by live cell ELISA are of the IgG and IgM classes. IgG pigment cell antibodies were detected in 80% of patients with active vitiligo, but in none of patients with inactive disease or normal individuals. The level of antibody (BI 3.3 ± 0.59) was significantly higher in patients with active vitiligo than either patients with inactive disease (BI 0.96 ± 0.05) or controls (BI 1.0 ± 0.04, $p < 0.001$). IgM pigment cell antibodies were also present in 80% of patients with active vitiligo and were also detected, although in much lower levels, in a few patients with inactive disease (21%) and normal controls (16%).

IgA antibodies were not detected in patients with active disease although they were occasionally found in patients with inactive vitiligo (14%) but their incidence and level was not different from that found in normal individuals. This finding conflicts with recent data presented by Aronson and Hashimoto [14] who found IgA class pigment cell antibodies in both depigmenting and repigmenting vitiligo patients. However, technical differences between the methods used for antibody detection make comparisons difficult. Our assay measures only antibodies to surface antigens, whereas the fixed cell assay used by Aronson and Hashimoto may also measure antibodies to internal antigens.

The pigment cell antibodies detected by ELISA in patients with active vitiligo appear to be directed to cell surface antigens, because the cells used as targets were live. Hence, it is unlikely that the antibodies penetrated the cells to react with internal antigens. In other studies we have shown that vitiligo antibodies react to cell-surface antigens that are labeled by the lactoperoxidase technique [3,6] or that decorate the external surface of melanocytes by indirect immunofluorescence [3]. Nonetheless, the possibility that some of the antibodies react with internal antigens has not been conclusively excluded, and must be kept in mind as some vitiligo antibodies have been reported to react to melanosomal antigens inside melanocytes [15]. The pigment cell antibodies we measured in this study are directed to multiple antigens, some of which are predominantly expressed on pigment cells. This is evidenced by serologic analysis that reveals that the antibodies in some patients react only to pigment cells (melanocytes and melanoma cells), whereas in other patients they react to these cells as well as to control cells. This finding is consistent with our earlier report that by immunoprecipitation SDS-PAGE analysis vitiligo antibodies are directed to multiple, distinct antigens on the surface of melanocytes—some of which are preferentially expressed on these cells whereas others are normal tissue antigens expressed on both pigmented and unrelated cells [16]. It is of interest that the control cell lines that was most reactive with vitiligo antibodies in the current study were K562 leukemia cells, a cell line known to express antigens such as the ganglioside GM2 [17], which is also expressed by pigment cells [18]. In addition, some of the antibodies in patients with vitiligo that react to control cells by ELISA may be directed to non-pigment cell antigens and are unrelated to the presence of the vitiligo.

In summary, there is a correlation between the presence of IgG and IgM antibodies to cell surface pigment cell antigens and disease activity in vitiligo as measured by a modified live cell ELISA assay.

Table II. Specificity of Pigment Cell Antibodies in Patients with Vitiligo

Patient Number	Reaction with Antibodies by ELISA ^a									
	Pigment Cells				Non-Pigment Cells					
	Melanocytes	SK23	SK28	SK30	K562	Rhabdosarc	Colon Carc	Lung Carc	Lymphocytes	Fibroblast
1	+	++	+	+	+	-	-	-	-	-
2	+	+	+	+	-	-	-	-	-	-
3	+	+++	-	-	+	-	-	-	-	-
4	+	+++	-	+	+	-	+	+	-	-
5	+	+	++	-	-	-	-	-	-	-
6	+	+++	++	-	+++	-	+	-	-	-
7	+	+	++	+	++	-	-	-	-	-
8	++	++	++	+	++	+	-	-	-	-
Percent patients	100	100	75	38	75	13	25	13	0	0

^a -, BI < 1.5 times greater than control mean by ELISA; +, BI 1.5-2.5 times greater than control; ++, BI 2.6-3.5 times greater than mean, +++, BI 3.6-5.4 times greater than control.

Autoantibodies are the only mechanism which have been shown to directly damage normal melanocytes *in vitro* [8-10]. Our findings also support the hypothesis that vitiligo is an autoimmune disorder and suggest that the antibodies are involved in its pathogenesis rather than being interesting but irrelevant epiphenomena.

REFERENCES

- Nordlund JJ, Lerner AB: Vitiligo: the loss of pigment in skin, hair, and eyes. *J Dermatol* 5:1-8, 1978
- Naughton GK, Reggiardo MD, Bystryjn JC: Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. *J Am Acad Dermatol* 15:978-981, 1986
- Naughton GK, Eisinger M, Bystryjn JC: Antibodies to melanocytes in vitiligo. *J Exp Med* 158:246-251, 1983
- Bystryjn JC, Naughton GK: The significance of vitiligo antibodies. *J Dermatol* 12:1-9, 1985
- Naughton GK, Mahaffey M, Bystryjn JC: Antibodies to surface antigens of pigmented cells in animals with vitiligo. *Proc Soc Exp Biol Med* 181:423-426, 1986
- Naughton GK, Eisinger M, Bystryjn JC: Detection of antibodies to melanocytes in vitiligo by specific immunoprecipitation. *J Invest Dermatol* 81:540-542, 1983
- Galbraith GM, Miller D, Emerson DL: Western blot analysis of serum antibody reactivity with human melanoma cell antigens in alopecia areata and vitiligo. *Clin Immunol Immunopath* 48:317-324, 1988
- Norris DA, Kissinger RM, Naughton GK, Bystryjn JC: Evidence for immunologic mechanisms in human vitiligo: patients' sera induce damage to human melanocytes *in vitro* by complement-mediated damage and antibody-dependent cellular toxicity. *J Invest Dermatol* 90:783-789, 1988
- Norris DA, Capin L, Muglia JJ, Osborn RL, Zerbe GO, Bystryjn JC, Tonnesen MG: Enhanced susceptibility of melanocytes to different effector mechanisms *in vitro*: potential mechanisms for postinflammatory hypopigmentation and vitiligo. *Pigment Cell Res* 1:113-123, 1988
- Abdel-Naser MB, Kruger S, Krasagakis K, Garbe C, Gollnick H, Orfanos CE: Evidence for involvement of both cell mediated and humoral immunity in generalized vitiligo (abstr). *J Invest Dermatol* 96:1024, 1991
- Eisinger M, Marko O: Selective proliferation of normal human melanocytes *in vitro* in the presence of phorbol ester and cholera toxin. *Proc Natl Acad Sci USA* 79:2018-2022, 1982
- Fogh J, Fogh JM, Orfeo T: One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59:221-226, 1977
- American Type Culture Collection: Catalog of cell lines and hybridomas, 6th ed., 1988
- Aronson PJ, Hashimoto K: Timed immunoperoxidase-gray scale cytologic analysis correlates with elevated IgA antimelanoma antibodies in vitiligo patients' sera with depigmentation (abstr). *FASEB Meeting Abstracts*, 1989, p 397
- Moellman GE, Krass P, Halaban R, et al: On the subject of serum antibodies to melanocytes in vitiligo (abstr). *J Invest Dermatol* 84:333, 1985
- Cui J, Harning R, Li J, Bystryjn JC: The antibody response to immunization to a polyvalent melanoma antigen vaccine (abstr). *Clin Res* 38:660a, 1990
- Ando I, Hoon DS, Suzuki Y, Golub SH, Irie RF: Ganglioside GM2 on the K562 cell line is recognized as a target structure by human natural killer cells. *Int J Cancer* 40:12-17, 1987
- Tsuchida T, Saxton RE, Morton DL, Irie RF: Gangliosides of human melanoma. *Cancer* 63:1166-1174, 1989