Identification of Hitherto Undefined B-Cell Epitopes by Antibodies in the Sera of Vitiligo Patients Using Phage-Display Peptide Library

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ABSTRACT

A random 12 mers phage library was used to screen a pool of immunoglobulin fractions obtained from vitiligo patients. Subsequent to panning experiments, a panel of affinity selected phage from vitiligo patients were obtained. This panel was tested using an ELISA for their reactivity with pooled sera from patients and normal controls. Among the 16 randomly selected clones, two of clones showed distinct positive reactivity with the patient's sera compared with controls. The peptides displayed by these phages expressed the following amino acid sequences: SHMPLANQYQWA and NHVQAWEQFWDS. Thus, screening with phage-displayed random peptide library of vitiligo sera can reveal peptide sequences that mimic vitiligo-related self-antigen.

Key words: B-cell Epitope, Random Peptide Library, Self-antigen, Vitiligo.

INTRODUCTION

Vitiligo is a common skin disorder characterized by areas of depigmentation resulting from loss of melanocytes in the epidermis.[1] The cause of vitiligo is not definitely known and several hypothesis have been proposed.[1,2] An immunologic hypothesis is currently advanced as a possible cause of pathogenesis in vitiligo.[3,4]

Some researchers tried to identify the autoantigens against which vitiligo antibodies react.[5-7] One of the major problems in exploring the immunological events leading to the development of this disease is insufficient information regarding the nature of the antigens to conduct a productive investigation. Thus, many studies have been hampered by the lack of such information. The technology of phage-displayed random peptide libraries (RPL) has recently become a powerful technique for elucidating protein-protein interactions.[8] This technology offers a number of advantages in the research area of autoimmune diseases.[9] Thus, the random peptide library screening method can be used to map any epitope without prior knowledge of the structure of protein antigen. Furthermore, several reports have pointed out that polyclonal antibodies could be used as screening ligates.[9,10] This implies that autoimmune patients' sera could be used as a source for isolating screening ligates, thus providing a unique advantage of linking the RPL approach to a disease-related antigenic structure directly. In this study using such technology and panning with vitiligo sera, B cell ligands that might be involved in the etiology of vitiligo were identified.

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MATERIALS AND METHODS

Patients

Sera from 34 vitiligo patients (14 men; 20 women; mean age 34 years) were collected in Dermatology department of Imam Khomeini general hospital. The clinical diagnosis of the patients was accomplished by

consultant dermatologist. Sera from 23 normal individuals (12 men; 11 women; mean age 24 years), with no history of either vitiligo or autoimmune disorders, such as Hashimoto's thyroiditis, Grave's disease, Insulin-dependent diabetes mellitus, and Alopecia areata were used as controls. All sera were kept frozen at -70°C until use.

Phage displayed random peptide libraries

In this study we used a Ph.D.-12 mers phage display library kit (New England Biolabs, Inc, Cambridge, MA). This kit is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein of M13 phage. Each clone of phage particles displayed on the surface a short chain of peptide consisting of a sequence of 12 amino acids. The library consisted of ~ 2.7×10^9 electroporated sequences, amplified once to yield ~ 55 copies of each sequence in 10 µl of the supplied phage.

Affinity purification of the phages

Sera from 34 patients and 23 normal controls were precipitated with (NH4)2SO4.[11] A solution of 100 µg/ml of precipitated immunoglobulins in 0.1 M NaHCO3 (pH 8.6) was prepared. 150 µl of this solution was added to 10 wells of microplates (Nunc Maxisorp, Denmark). The plates were incubated overnight at 4°C. After washing with TBS buffer [50 mM Tris-HCL (pH 7.5), 150 mM NaCl], the wells were filled with blocking buffer [0.1 M NaHCO3 (pH 8.6), 5 mg/ml bovine serum albumin (BSA)], and were incubated for 1 h at 4°C. The wells were washed with TBST (TBS + 0.1% [V/V] Tween 20) and incubated with UV-killed M13K07 (10^{12} phage particles/ml) in TBS/0.1% Tween 20, 1 mg/ml BSA for 4 h at 4°C. The plates were washed with TBST. Then 1.5×10^{11} plaque-forming unit (PFU)/ml from 12-mers library was allowed to react with immobilized antibodies for 4 h at 4°C. The wells were washed again with TBST and then bound phages were eluted with 0.2 M glycine-HCL buffer (pH 2.2), 1 mg/ml BSA. The eluates were transferred to microfuge tubes, neutralized with 150 µl 1 M Tris-HCL and amplified for a next round of panning. The eluate and amplified phages were titrated by infecting ER2738 bacterial cells and the number of clones was determined by plating infected bacteria on X-Gal/IPTG Luria-Bertani (LB) agar medium.[12,13] The amplified phages reapplied to antibody coated wells. This is the so-called "positive" selection process. After the second round of panning, there was a "negative" selection step in which phages unreactive specifically with vitiligo sera were removed using 100 µg/ml of $(NH_4)_2SO_4$ precipitated immunoglobulins prepared from 23 normal sera. The nonadherent phages were collected and propagated. These phages were expected to react with vitiligo but not with normal sera. The amplified eluate was enriched by three further rounds of positive selections.

Sequence determination of displayed peptides

Sixteen phage clones were selected after the sixth round of panning. They were amplified and the single-stranded DNA was extracted by plasmid DNA purification kit (MNG) and sequenced with ABI automated sequencer.

Assaying selected peptides by ELISA for target binding

96 well microtiter plates (Nunc Maxisorp, Denmark) were coated with 200 µl of 100 µg/ml of ammonium sulfate-precipitated immunoglobulin in 0.1 M $NaHCO_3$ (pH 8.6) and incubated overnight at 4°C. After washing with TBST, wells were filled with blocking buffer and incubated at 4°C, for 2 hours. The plates were washed and fourfold serial dilutions of phages that had been resuspended in TBST were deposited in ELISA microplates. After 2 h incubation at room temperature, the unbound phages were removed by washing. In the next step horseradish peoxidase (HRP)-conjugated anti-M13 antibody (Pharmacia, Sweden) diluted with blocking buffer at a ratio of 1: 5000 was added to the plates and incubated for 1 h at room temperature. Finally following another washing step the immune complexes were detected by adding 2, 2`-Azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt (Roche, Germany) as substrate. After 20 min incubation at room temperature, absorbance was read at 405 nm in an automated ELISA reader (Organon Teknika Reader 210).

Homology search

Blast P (basic local alignment search tool program) via the National Center for Biotechnology Information (NCBI) BLAST Network service was searched.

Statistical analysis

Student's t test was performed. A p value of less than 0.05 was considered significant.

RESULTS

Random peptide library was screened by panning against a pool of immunoglobulin fractions collected from 34 vitiligo patients. The amino acid sequences of randomly selected phages which were found in the present study are indicated in Table 1.

In order to select epitope mimics for antibody which is produced by patients with vitiligo, six panning rounds were performed. Microtiter plates coated with patients' sera were used to affinity select 1.5×10^{11}

Table 1. Amino acid sequences of the selected phagotopes.

Sequence											Frequency *
S	H	M	P	L	A	N	Q	Y	Q	W	A	1
N	H	V	Q	A	W	E	Q	F	W	D	S	1
W	H	D	D	N	P	F	F	F	E	A	P	4
D	H	S	P	F	Y	L	S	T	A	H	Y	1
S	L	P	P	W	Y	S	P	F	K	L	D	2
L	H	A	Y	N	P	F	L	L	S	W	P	1
K	H	Y	P	E	V	G	P	N	G	F	I	2
R	H	F	P	N	D	Y	N	P	F	L	L	1
W	H	E	A	Q	P	S	P	G	L	L	R	1
D	H	S	S	A	D	L	I	N	P	V	A	1
G	W	Y	S	P	F	Q	L	G	W	P	Q	1

*The number of times each sequence occured in the 16 phages is given in the fequency column.

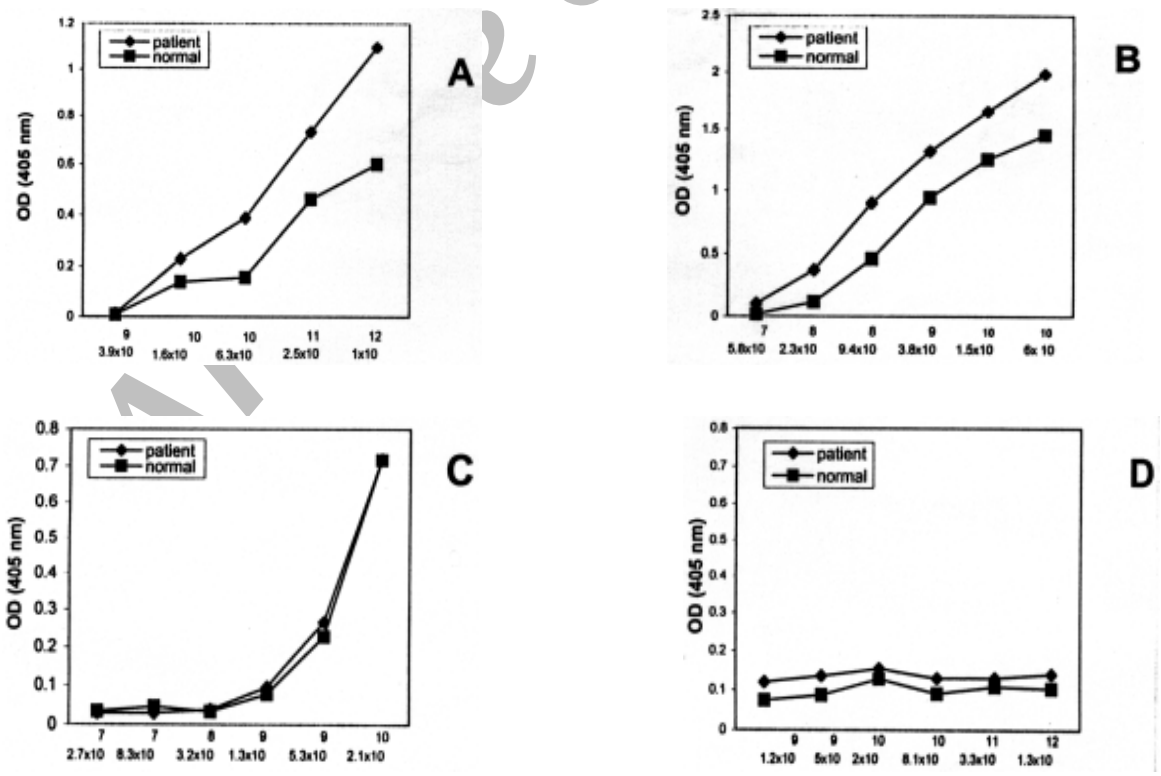

Figure 1. Titration curves. Reactivity of series of phage dilutions with pooled sera from patients and normals. (A through D) ELISA reactivities to clone 1(A); clone 2(B); clone 7(C); and wild-type (D). Each point is the mean of at least two values. Phage dilutions (PFU/200 µl) are indicated in abcissa.

library phage particles. In the first round of affinity selection, about 10^6 phage particles were recovered and after the second round the number of eluted phage increased to 10^9 (Table 2).

Table 2. Affinity selection of random peptide library with patients and normal sera.

Panning round	Phage particles		Output / Input Ratio
	Input	Output	
1	1.5×10^{11}	6.5×10^{6}	4.3×10^{-5}
2	1×10^{13}	7×10^{9}	7×10^{-4}
3	1.2×10^{13}	1.45×10^{12}	1.2×10^{-1}
4	2×10^{13}	1.5×10^{10}	7.5×10^{-4}
5	1×10^{13}	5×10^{10}	5×10^{-3}
6	2×10^{13}	2.5×10^{10}	1.25×10^{-3}

The ratio between the bound and input phages for each round indicates that phage clones reacting with serum antibodies were specifically enriched.

Furthermore, we tested the reactivity between selected phagotopes and screening ligate by ELISA. For each dilution of phage, the differences in means of OD_{405} nm reading between the vitiligo sera and the normal sera was statistically compared, and for two clones (clone 1 and clone 2) the differences were very significant ($p< 0.05$).

DISCUSSION

Autoantibodies are produced in patients with vitiligo and are heterogeneous in their epitope specificities.[14] Analysis of these antibodies might reveal information important to the understanding of the etiology and pathogenesis of the disease, because some studies have shown that anti-melanocyte antibodies are often present in the sera of vitiligo patients[15] and there is a correlation between the presence and level of these antibodies and the extent and activity of vitiligo.[16,17] For these reasons, in the present study, random peptide library was employed to identify the specific peptides which are recognized by the immune system of vitiligo patients. The results of this investigation indicated that specific ligands could be identified using vitiligo sera by an approach that does not require prior structural knowledge. Phage display has not previously been employed to identify vitiligo epitopes using polyclonal antibodies. However, this technique has been recognized as a useful tool in the identification of cell ligands for polyclonal antisera from patients with autoimmune diseases.[9] This technique allows the recognition of (auto) antigen responsible for the initiation and/or perpetuation of the immune response in these patients.[18-20] We identified two phagotopes which showed significant difference of reactivity with the sera of patients and normal controls. The epitopes reported here represent the first characterization of epitopes that bind antibodies in vitiligo sera and none of them have been described previously as possible stimulant for antibody production in vitiligo patients.

It is known that the peptide library provides a possibility to identify discontinuous epitopes consisting of residues distant in the sequence that are brought together by the folding of the polypeptide chain.[21]

Moreover, it has been suggested that majority of antibodies recognize and bind discontinuous epitopes.[22] Thus, the peptide epitopes obtained in this study may represent two discontinuous epitopes which provoke specific antibody production in susceptible persons. In addition, peptides described in this study may be part of antigenic chains in other still unknown proteins involved in vitiligo pathogenesis.

In brief, we used the combination of polyclonal autoimmune sera with the random peptide phage-displayed library which identified the repertoire of epitopes recognized by autoantibodies from vitiligo patients. Additional studies will be required to determine the significance of the peptides described in the present study.

REFERENCES

1. Castanet J, Ortonne JP. Pathophysiology of vitiligo. Clin Dermatol 1997; 15(6): 845-51.
2. Kovacs SO: Vitiligo. J Am Acad Dermatol 1998; 38(5 Pt 1): 647-66.
3. Bystryn JC: Immune mechanisms in vitiligo. Clin Dermatol 1997; 15(6): 853-61.
4. Le Poole IC, Wankowicz-Kalinska A, van den Wijngaard RM, Nickoloff BJ, Das PK. Autoimmune aspects of depigmentation in vitiligo. J Investig Dermatol Symp Proc 2004; 9(1): 68-72.
5. Cui J, Harning R, Henn M, Bystryn JC. Identification of pigment cell antigens defined by vitiligo antibodies. J Invest Dermatol 1992; 98(2): 162-5.
6. Kemp EH, Gawkrodger DJ, Watson PF, Weetman AP. Immunoprecipitation of melanogenic enzyme

autoantigens with vitiligo sera: evidence for cross-reactive autoantibodies to tyrosinase and tyrosinase-related protein-2 (TRP-2). Clin Exp Immunol 1997; 109(3): 495-500.
7. Baharav E, Merimski O, Shoenfeld Y, Zigelman R, Gilbrud B, Yecheskel G, et al. Tyrosinase as an autoantigen in patients with vitiligo. Clin Exp Immunol 1996; 105(1): 84-8.
8. Kay BK, Kasanov J, Yamabhai M. Screening phage-displayed combinatorial peptide libraries. Methods 2001; 24(3): 240-6.
9. Sioud M, Forre O, Dybwad A. Selection of ligands for polyclonal antibodies from random peptide libraries: potential identification of (auto) antigens that may trigger B and T cell responses in autoimmune diseases. Clin Immunol Immunopathol 1996; 79(2): 105-14.
10. Prezzi C, Nuzzo M, Meola A, Delmastro P, Galfre G, Cortese R, et al. Selection of antigenic and immunogenic mimics of hepatitis C virus using sera from patients. J Immunol 1996; 156(11): 4504-13.
11. Hudson L, Hay FC, editors. Practical Immunology. London: Blackwell Scientific Publications, 1991: 1-33.
12. Nicholl DST, editor. An introduction to genetic engineering. UK: Cambridge University Press, 1994: 104-21.
13. Barbas CF, Burton DR, Scott JK, Silverman GJ, editors. Phage display: A laboratory manual. New York: Cold Spring Harbor Laboratory Press, 2001: 19.1-19.41.
14. Ongenae K, Van Geel N, Naeyaert JM. Evidence for an autoimmune pathogenesis of vitiligo. Pigment Cell Res 2003; 16(2): 90-100.
15. Naughton GK, Eisinger M, Bystryn JC. Detection of antibodies to melanocytes in vitiligo by specific Immunoprecipitation. J Invest Dermatol 1983; 81(6): 540-2.
16. Naughton GK, Reggiardo D, Bystryn JC: Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. J Am Acad Dermatol 1986;15(5 Pt 1): 978-81.
17. Harning R, Cui J, Bystryn JC. Relation between the incidence and level of pigment cell antibodies and disease activity in vitiligo. J Invest Dermatol 1991; 97(6): 1078-80.
18. Rowley MJ, Scealy M, Whisstock JC, Jois JA, Wijeyewickrema LC, Mackay IR. Prediction of the immunodominant epitope of the pyruvate dehydrogenase complex E2 in primary biliary cirrhosis using phage display. J Immunol 2000; 164(6): 3413-9.
19. Lunardi C, Bason C, Leandri M, Navone R, Lestani M, Millo E, et al. Autoantibodies to inner ear and endothelial antigens in Cogan's syndrome.
Lancet 2002; 360(9337): 915-21.
20. Dybwad A, Forre O, Natvig JB, Sioud M. Structural characterization of peptides that bind synovial fluid antibodies from RA patients: a novel strategy for identification of disease-related epitopes using a random peptide library. Clin Immunol Immunopathol 1995; 75(1): 45-50.
21. Cwirla SE, Peters EA, Barrett RW, Dower WJ. Peptides on phage: a vast library of peptides for identifying ligands. Proc Natl Acad Sci U S A 1990; 87(16): 6378-82.
22. Davies DR, Padlan EA, Sheriff S. Antibody-antigen complexes. Annu Rev Biochem 1990; 59: 439-73.