Post-Panning Computer-Aided Analysis of Phagotope Collections Selected with Neurocysticercosis Patient Polyclonal Antibodies: Separation of Disease-Relevant and Irrelevant Peptide Sequences

K.G. Gazarian*[a], M.J. Rowley[b], T.G. Gazarian[c], J. Sotelo[d], E. García-Mendoza[d] and R. Hernández[a]

[a]*Department of Biotechnology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de Mexico, UNAM, México D.F. México;* [b]*Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia;* [c]*Faculty of Medicine, UNAM, México D.F. México;* [d]*Instituto Nacional de Neurología y Neurocirugía, México D.F., México*

Abstract: The homology of peptide sequences selected from a 7mer phage display library with antibodies elicited by the multicelled parasite *Taenia solium* in cerebrospinal fluid and serum of neurocysticercosis (NCC) patients and by antibodies of uninfected control patients with similar neurological complications of other ethiology (non-NCC) were analyzed using a PILEUP-Tudos sequence alignments program. The analysis generated dendrograms bearing two types of sequence clusters, those containing (1) only NCC patients-derived peptides and (2) both NCC- and control non-NCC – patient derivatives. By using ELISA, peptides that were selected by the antibodies were identified predominantly in the NCC-derived clusters. In repeated analysis in which sequences were added or removed, the first type of clusters maintained their structure, while the second type of clusters were split into many separate homology units dispersed throughout the guide tree. These results are interpreted as the ability of the analysis to segregate NCC-specific peptide sequences from other sequences. Altogether, this study demonstrates the high potential of the PILEUP-Tudos computer program to analyze phagotope collections recovered through biopanning with polyclonal antibodies elicited in patients by complex and as yet unknown multiple pathogenic antigens and to separate all phagotopes that are disease-relevant on the basis of the sequence homology.

INTRODUCTION

The phage display methodology that is based on screening peptide libraries with monoclonal antibodies has amply demonstrated its potential for epitope studies in recent years [1]. Phage clones isolated from phage libraries have been shown to contain peptide sequences that are functional mimics of the original epitope (mimotopes), and in some cases the mimotopes shared common motifs matching the epitope region that permitted the identification of the actual epitopes on antigens [reviewed: 2-4].

*Address correspondence to this author at the Department of Biotechnology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. Apartado Postal, 70228,Ciudad Universitaria 04510 México D.F.; Phone: (525)622 3821; Fax (525)622 3855; E-mail karlen@servidor.unam.mx

Polyclonal antibodies from patient sera have also been utilized to yield mimotopes for known antigens [5], including autoantigens [6-8]. Furthermore, polyclonal sera containing antibodies elicited by as yet unknown antigens also selected phagotopes which were considered likely mimotopes relevant to the disease, based on immunoassay data [9,10]. Sequences of this type of mimotopes could not be compared with the antigen sequences, but carried common motifs thought to mimic putative epitope structures that elicited the disease-related antibodies. Phage display has also been applied successfully to fluids other than serum that contain locally produced IgG. Presumed to be antibodies specific to diseases, these IgG consensus motifs are considered to mimic individual epitopes involved in immune responses and have been identified in synovial fluid in rheumatoid arthritis [9] and

1386-2073/01 $28.00+.00

cerebrospinal fluid (CSF) in multiple sclerosis [11-13].

Recognition of disease-related mimotopes in a background of unrelated sequences presented in phagotope populations recovered in phage display experiments is a critical step in evaluation of the utility of this methodology in studies of diseases. Various specific immunological procedures are used routinely for identification of disease relatedness of selected phage displayed peptides or their synthetic counterparts. Although reactivity with the selecting antibody is quite satisfactory for evaluating the importance of phagotopes, many assays yield imprecise and poorly reproducible results, and may detect only a minor part of the relevant sequences [11] or even a single though high quality mimotope [10,13].

In phage display studies, the potential of computerized sequence, statistical [13,14], database [15], and alignment analysis [7] has not yet been exploited in its entirety. In a series of recent reports, it was demonstrated that peptide sequences of different origin can be segregated in separate homology groups by a multiple sequence alignment procedure using the algorithm PILEUP together with the TUDOS matrix for scoring amino acid substitutions based on physicochemical properties [7]. In those studies, the method was successfully tested on groups of common derivation peptides selected by monoclonal antibodies [7,16] or serum of subjects with autoimmune disease in which a single epitope of known specificity was involved [7,8]. It was found that phagotopes selected by each antibody and displaying a reactivity with it align together in a single cluster with good accordance between the alignment and immunological data. In the present study, we investigated the applicability of this approach to peptides selected using polyclonal antibodies, which were induced in patients with neurocysticercosis (NCC) by an unknown set of epitopes of the tapeworm *Taenia solium.* NCC is a parasitic disease of the nervous system causing severe pathologies of CNS such as epilepsy [17].

MATERIALS AND METHODS

Cerebrospinal Fluid and Serum Samples

Cerebrospinal fluid (CSF) and serum were obtained from six NCC patients from the Institute of Neurology and Neurosurgery (Mexico City). Diagnosis of NCC in these patients was based on magnetic resonance imaging and computer tomography [18], supplemented by immunodiagnosis using CSF and serum, which are obtained routinely for diagnostic purposes [19]. Table 1 presents data on patients who provided CSF or serum. The patients with NCC were at active stages of the disease with cysts lodged in different parts of their brain, and all had antibodies

Table 1. Parasites and Anti-parasite Antobody in Patients

Patient	Brain infection (cyst location)	Anti- *T. solium* Ab*
Pt.1f	ventricular; parenchimal	2.2
Pt.2f	arachnoiditis	2.2
Pt.3f	arachnoiditis	2.2
Pt.4f	arachnoiditis	2.1
pt.5f	parenchimal	1.4
pt.5s		1.2
Pt.6f	ventricular; parenchimal	1.4
Pt.7f;s	no cysts	0.04
Pt.8	no cysts	0.04

*O.D. at 405 nm of ELISA-determined reactivity of CSF (f) or serum (s) dilutions (1:10) with total antigen purified from cysticerci lodged in infected pig muscle (see Material and Methods.)

reacting in ELISA with total parasite antigens extracted from parasitized pig cysticerci as described by Ordoñez, *et al.* [20]. CSF and serum from two patients with similar pathologies not diagnosed as NCC ("non-NCC patients") and shown to lack anti-parasite antibodies, served as controls. The immunological characteristics of CSF and sera in NCC and non-NCC patients have been described in detail elsewhere[19].

Peptide Library and Panning Procedure

A 7mer peptide library was obtained from BioLabs (now Genesis Bioventures, Vancouver, BC) (lots 1.5 and 2.0). The library consisted of $2x10^9$ different clones of the phage each bearing a unique 7-aa peptide and was amplified once by the manufacturer to give 10^2 copies of each individual clone (the final titer: $2x10^{11}$ phage particles). CSF was centrifuged at 15000xg for 30 min to remove cells. The biopanning was based on the procedures of Smith and Scott [21] and Dower and Cwirla [22] with minor modifications [23]. Briefly, 300 µl of CSF diluted with PBS (1:100) were incubated overnight at 4°C with gentle rocking in six wells of 96-well polystyrene microtitration plates (Immulon 4 flat bottom plates, Dynatech Lab Inc., USA). Wells were then washed 6 times with PBS-T (PBS-0.1% Tween 20) and blocked 1 h at 37°C with blocking buffer (PBS-1%BSA), followed by washing 5 times with PBS-T. For the affinity selection of clones, 10 µl of the 7-mer random peptide library ($2x10^{10}$ plaque-forming units, p.f.u.) were mixed with 590 µl of PBS-0.1% BSA. Then, the diluted library was distributed in six wells with immobilized and blocked CSF and incubated 2 h at 4°C while rocking to allow phage to bind. Following five-fold washing with PBS-T at 0-4°C, the wells were filled with blocking buffer, incubated while rocking 30 min at 4°C and washed 5 times with PBS-T at 4°C. Bound phage were eluted from each well by stirring with 100 µl of elution buffer (0.1 M HCl-glycine, pH 2.2). Eluates from six wells were combined and immediately neutralized by the addition of 35 µl of 2 M Tris solution (pH non-adjusted). The phage particles were quantitated by titering on log-phase *E.coli* ER2537 (BioLabs), plated on LB plates and amplified by the standard procedure. The amplified phage, affinity selected with each CSF (titer 10^{12}-10^{13} phage per ml), were used for two additional rounds of affinity purification using less amounts of CSF (1:200 and 1:300 for the second and third rounds, respectively). Phage was purified by precipitation with 0.1 M acetic acid [21], resuspended in TBS and quantitated by titration. Single-stranded DNA was prepared from individual random clones. The gp3 N-terminal region and the inserted peptide-coding oligonucleotide were sequenced by the di-deoxynucleotide method [24] using S^{35}-labelled dNTP (Amersham), –28-bp primer and the T7 sequenase (Amersham). The amino acid sequence of peptides was deduced from the nucleotide sequence data.

The Alignment Procedure

Sequences of phage-displayed peptides were aligned using the multiple sequence alignment algorithm PILEUP [25]. The 'default' matrix used by PILEUP that scores amino acids according to their evolutionary relatedness was replaced by the Tudos matrix that scores amino acid substitutions on the basis of physicochemical properties of amino acid side chains. Therefore, the Tudos matrix seemed more appropriate for the analysis of antigen-antibody interactions [26]. A low penalty of 1 was assigned for the introduction of gaps. Peptide sequences were included as often as they were selected.

ELISA

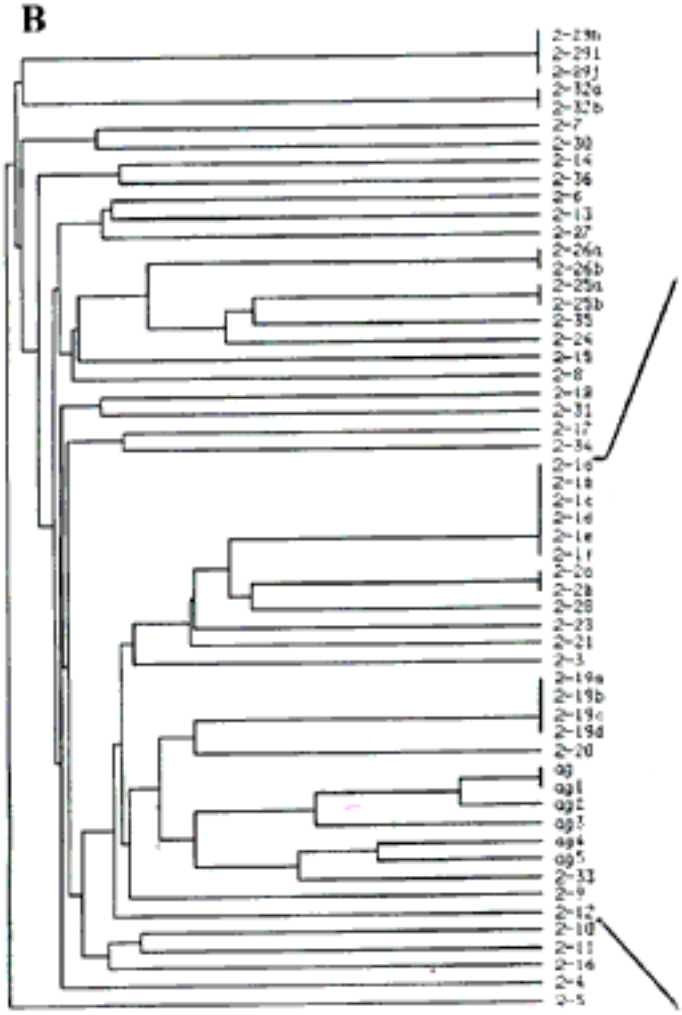
The anti-parasite Ab titers in CSF and serum of each NCC patient were determined first during diagnosis of patients and subsequently confirmed before being used in biopanning experiments. For this, 96-well microplates (Nunc–Immuno Plate, MaxiSorp F96, NUNC Brand Products, Roskilde, Denmark) were coated with 1 µg/well of total Ag extracted from pig cysticerci membranes [19,20] by overnight incubation in carbonate buffer, pH 9.5 at 4°C. Unbound Ag was pipetted out, and wells were washed 3 times with PBS-0.5% Tween, incubated 1 h at 37°C with CSF or serum diluted 1:10 with PBS-0.2%Tween-0.2%BSA, and

A

23 sequences, 6 clusters
reactive: none
no common motif matching Ag

18 sequences, 1 cluster
reactive: 13 sequences
common motif: LYGM
replaceaments: F A
W
N
Ag: $_{20}$LYGS$_{23}$

B

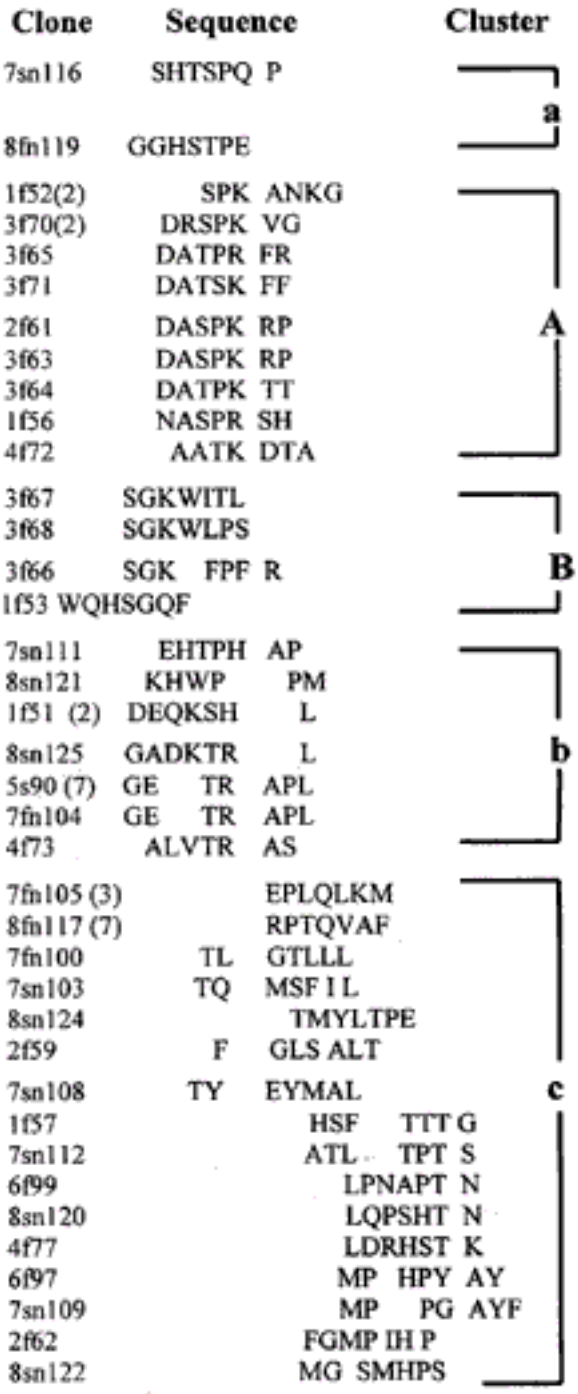


```
2-1a  .......F.R T..AISGTPQ FY........
2-1b  .......F.R T..AISGTPQ FY........
2-1c  .......F.R T..AISGTPQ FY........
2-1d  .......F.R T..AISGTPQ FY........
2-1e  .......F.R T..AISGTPQ FY........
2-1f  .......F.R T..AISGTPQ FY........
2-2a  ......SY.R T..PITGT.. LIT.......
2-2b  ......SY.R T..PITGT.. LIT.......
2-28  .....FPN.R T..PISGT.. ..TW......
2-23  .......H.R V..PISGT.Q AYK.......
2-21  ......HIAR T..PIAGT.N L.........
 2-3  .......HSH THKALAGTP. ..........
2-19a ......SLSR M..PIIGTSA ..........
2-19b ......SLSR M..PIIGTSA ..........
2-19c ......SLSR M..PIIGTSA ..........
2-19d ......SLSR M..PIIGTSA ..........
2-20  ......DI.R Q..PIIGTLH P.........
  ag  MSESHVKISR T..IIRGTSP ST........
 ag1  MSESHVKIS. .......... ..........
 ag2  ...SHVKISR T......... ..........
 ag3  ......KISR T..IIRG... ..........
 ag4  .........R T..IIRGTSP ..........
 ag5  .......... ....IRGTSP ST........
2-33  .......... S..FLRGLSP PLVH......
 2-9  .......... ...SILSTMS PHGAT.....
2-12  .......... IHFPVRGNTP V.........
```

Fig. (1). Control alignments of peptide sequences selected by simple agents.
A. The consensus motif shared by peptides selected by the mAb BNTX 18 to scorpion toxin, noxiustoxin [27].
B. Alignments of sequences selected by rabbit antiserum raised against synthetic peptide corresponding to aa1 to aa20 region of the *T. solium* paramyosin [23]. The antigen sequence divided in several overlapping fragments was included. The peptides with the consensus sequence matching the antigen region identified as putative epitope are in single cluster.

washed as indicated. The bound Ab was detected with anti-human alkaline-phosphatase conjugated Ab (Zymed Laboratories Inc. USA) diluted 1:1000, and p-nitrophenyl phosphate substrate diluted in diethanolamine buffer. After incubation 10 min at 37°C, the absorbance was read at 405 nm in an automated reader.

Reactivity of phage displayed peptides was tested by direct ELISA in which the microplate



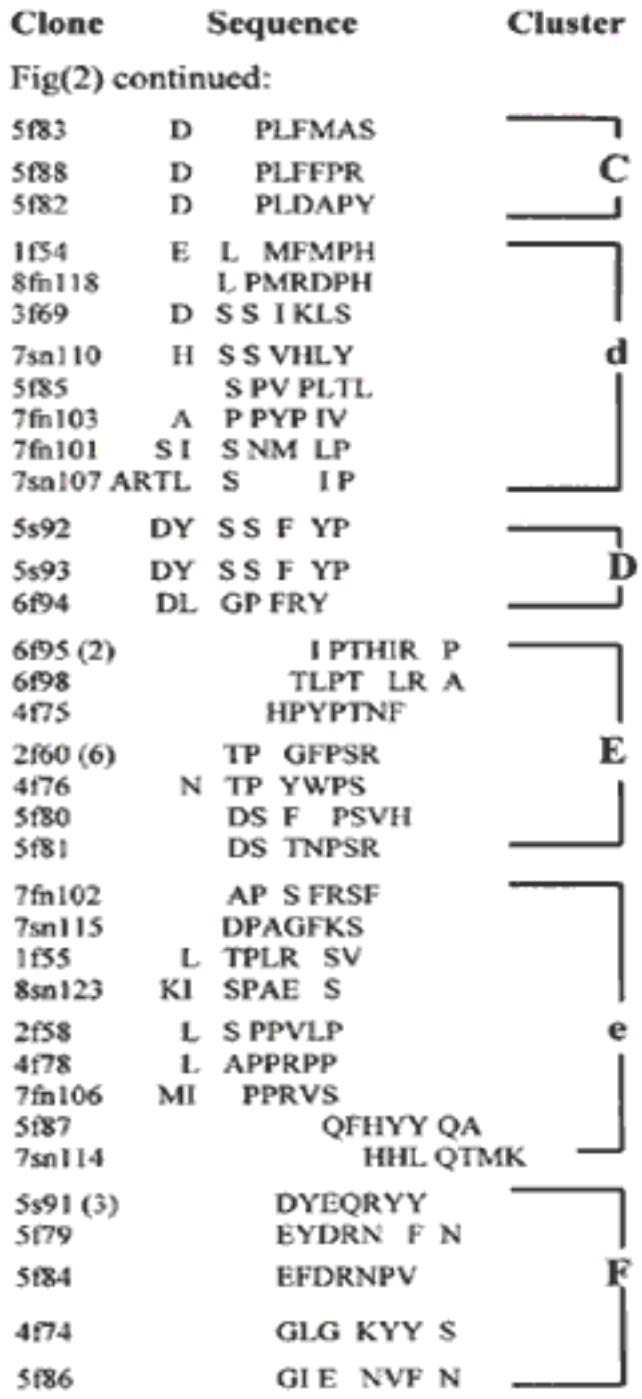


Fig. (2). First round of the alignments.
Pattern of clusters containing 73 aligned sequences found in 98 phagotopes isolated from the library panned on antibodies of NCC and non-NCC patients. Designations:
f– CSF of NCC patient; **fn** – CSF of non-NCC patient; **s** –serum of NCC patient; **sn** – serum of non-NCC patient.
a; **A**; **B**; **b**; **c**; **C**; **d**; **D**; **E**; **e**; **F** – clusters of only NCC-derived (**A**-**F**) and clusters containing NCC- and non-NCC-derived (**a**-**e**) sequenences.

was coated with the phage particles bearing selected peptides by filling each well with 100 μl of the phage suspension, 10^9 - 10^{10} pfu, in PBS-

BSA (50 ng/well), and incubating overnight at 4°C. After washing with PBS-0.3%Tween 20, the phage were incubated at 37°C for 1 h, or at 4°C for 3-12 h with CSF diluted (1:50 or 1:100) with PBS-0.2% Tween-0.2%BSA. After washing, the phage were incubated with the anti-human second Ab conjugated with alkaline phosphatase (Zymed Laboratories Inc. USA), and diluted 1:1000 in the same buffer as the CSF. The immune complexes were detected by adding p-nitrophenyl phosphate substrate diluted in diethanolamine buffer, and the absorption was measured at 405 nm. Control wells contained total antigen preparation of *T. solium* (1 μg/well), or no phage, or a phage amplified from the unselected library. Samples were also tested for reactivity by using capture ELISA, in which wells were coated with 100 μl/well of diluted (1:50) CSF (or serum) by overnight incubation at 4°C, blocked with PBS-1%BSA 1 h at 37°C, and then incubated with phage suspension (10^{10} pfu in 100 μl/well), 2 h at 4°C with gentle shaking. Next, the samples were incubated with anti-m13 Ab-HRP conjugate diluted 1:5000 in blocking buffer and ABTS as a substrate (Amersham Pharmacia Biotech. Kit). After developing for 40 min at room temperature, the color was measured at 410 nm. Positive clones showed at least a two-fold difference between standard deviation of ELISA data compared to control phage. Human IgG (Zymed Laboratories Inc. USA) was introduced to control probes to test for the possible reactivity of selected clones with the antibody Fc region.

RESULTS

Sequences derived from the biopanning of the library using CSF and serum of patients with NCC were aligned using the PILEUP program [7] to see their intrinsic homology. Seventy three sequences from ninety eight phagotope clones were analyzed. There were 64 sequences derived by biopanning with CSF from 6 NCC patients and 34 sequences derived from patients with similar neuropathology but not diagnosed as NCC who served as controls. Phage clones isolated from both CSF and serum of non-NCC patients were included to assist in identification of NCC-irrelevant sequences. The sequences were derived from independent biopanning CSF and sera of individual patients, not pools, to gain information on patient-specificity of the sequences.

Validity of the Alignment Strategy

As a preliminary step to validate the alignment strategy, alignments were carried out using peptide sequences from previous biopanning experiments [23,27] with simple selecting systems in which reactive phagotopes had been identified immunologically and by the sequence similarity with epitopes. In the first of these, a group of peptides derived from screening two phage libraries (7mer and 12mer) with the mAb BNTX18 specific to scorpion toxin, noxiustoxin [27] were aligned with the known epitope sequence. The alignment separated all mimotopes and the epitope sequence from other sequences and presented these sequences in a single cluster (Fig. (**1A**) shows the net result of this alignment). In the second alignment, we used sequences recovered by biopanning with rabbit antiserum raised against a 20aa-long synthetic peptide corresponding to the epitope of *T. solium* paramyosin [23]. A group of similar peptides that reacted with the antiserum were aligned in one cluster with the epitope sequence previously identified [23] and shown in the dendrogram in Fig. (**1B**).

Alignments Using Polyclonal Antibodies from Patients

1. Initial Round of the Alignments

The alignments derived using a monoclonal antibody or polyclonal antibodies from an animal immunized with a single peptide are relatively simple. It might be expected that the alignments of sequences selected with polyclonal antibodies induced by a multicellular parasite in patients would generate patterns that were too complex for interpretation. Fig. (**2**) shows the pattern of alignments for all sequences derived from biopanning with CSF of NCC and non-NCC patients, with serum from one NCC patient (Pt5) and sera of non-NCC-patients. In spite of the complex composition of the analyzed peptide

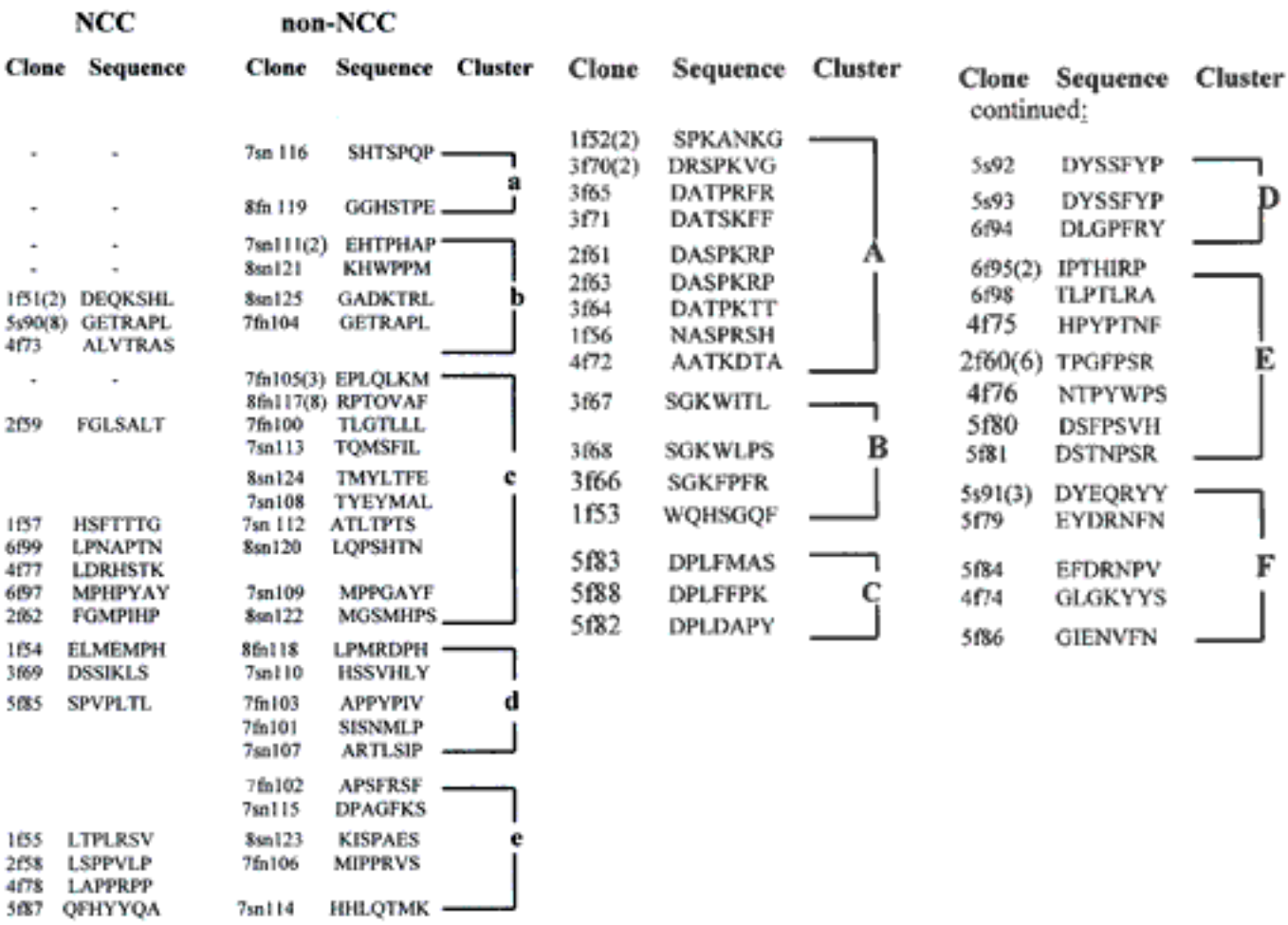


Fig. (3). Clusters of NCC-derived (A) and of the NCC + non-NCC -derived (B) sequences from Fig. (**1**).

population, the dendrogram shows a pattern that is easily interpreted. The basic homology units making up the dendrogram are first-level branches of 2 or 3 sequences displaying closest homology within the analyzed population. These branches are linked to form second level homology categories, clusters, which were families of related sequences. Clusters could be identified that contained sequences selected only by NCC-derived antibodies, only by non-NCC-derived antibodies, or sequences selected using both NCC and non-NCC samples. Clusters containing only NCC CSF-derived sequences are named **A**,**B**,**C**,**D**,**E**,**F**, and those containing both NCC CSF and non-NCC (CSF and serum)-derived sequences are named "**a** – **e**". In Fig. (**3**), these two groups of sequences are shown separately, and the distribution of the sequences in two types of clusters are shown in Table 2. The group of six NCC-sequence clusters, **A** - **F**, included 31 sequences (66%) and the clusters **a** - **e** contained 16 NCC- derived sequences that displayed homology with sequences derived from non-NCC patients. At first glance it is evident that the NCC-group clusters are characterized by a higher number of closely related sequences. In contrast, the clusters that include both NCC-derived and non-NCC-derived sequences are not so uniform. Some branches consisted of only non-NCC sequences (**a**), but in most of them NCC- and non-NCC sequences were aligned. Also, most of branches brought together CSF- and serum-derived sequences.

Table 3 shows how individual patients contributed to clusters of the NCC and non-NCC groups. There were differences in both the proportion of sequences from particular patients that aligned to NCC clusters, and also in the number of clusters in which the sequences aligned.

Table 2. Distribution of Sequences in Clusters

	Total	Number of sequences												
		In Clusters												
		A	B	C	D	E	F	(A-F)	a	b	c	d	e	(a-e)
NCC	47	9	4	3	3	7	5	31(66%)	0	3	6	3	4	16(34%)
Non-NCC	26	0	0	0	0	0	0	0	2	4	10	5	5	26
TOTAL	73	9	4	3	3	7	5	31	2	7	16	8	9	42

For example, Pt.1 yielded 3 sequences that aligned to NCC clusters A and B, and 4 sequences that aligned to non-NCC clusters, while Pt. 3 contributed 7 of 8 sequences to the NCC-clusters A and B. Pt 5 also derived mostly peptides located in NCC-clusters, but these were found in 3 other clusters (C, E, F). Cluster A, whose sequences share a single motif (DAT/SPK/R), were derived from 4 of the 6 patients. Cluster E included sequences derived from another group of four patients; and clusters F, B and D included sequences of 3, 2 and 2 patients, respectively. Cluster C was the only cluster that contained sequences from only one patient. NCC-derived sequences that occurred in branches with non-NCC-derived sequences were mostly scattered in several clusters. For example, Pt. 1 yielded four such sequences, each in a different cluster.

2. Second Round of Alignments

Since the CSF of the Pt 3 yielded a high proportion of sequences aligned to NCC-clusters (7 of 8 sequences), we sequenced an additional sample of 16 phage clones from the final eluate of this patient. The whole previous population including the 16 newly obtained sequences was then realigned. The new guide tree contained all previous clusters with the following changes (Fig. (**4**)).

(1) 11 of the 16 new sequences aligned to the 2 NCC-clusters, **A** and **B**, in which the 7

Table 3. Disttribution of Sequences Derived by Different NCC Patients in the Clusters Containing only NCC-Sequences (A-F) and in Clusters Containing Both NCC- and Non-NCC Deriived Sequences (a-e)

Selecting Agent*	Number of sequences in cluster										
	A	B	C	D	E	F	a	b	c	d	e
Pt1f	2	1						1	1	1	1
Pt2f	2				1				1		2
Pt3f	4	3								1	
Pt4f	1				2	1			2		1
Pt5f			3		2	3				1	1
Pt5s				2		1		1			
Pt6				1	2				2		
Total	9	4	3	3	7	5	0	2	6	3	5

*f - CSF; s - serum

original NCC-related sequences had aligned. **A** contained sequences isolated from 4 different NCC-patients, whereas **B** contained mostly Pt. 3 derived sequences.

(2) 3 of the new sequences aligned to cluster **b** that contained both NCC- and non-NCC patients derived sequences.

(3) 2 new sequences (**FYRAPQT** and **FYRSPSN**) formed a new NCC-type branch **G** (Fig. (**4**), asterisks).

(4) The positions of several clusters were changed to give an altered succession of clusters in the guide tree in the region of the new sequences. Thus, the positions of clusters **A** and **B** were reversed; cluster **a** shifted to a new position below **A**; and cluster **c** was split into 2 subclusters (**c1** and **c2**) which occupied new positions on 2 sides of the transformed part of guide tree

(5) The part of the guide tree that did not include new sequences retained the previous pattern.

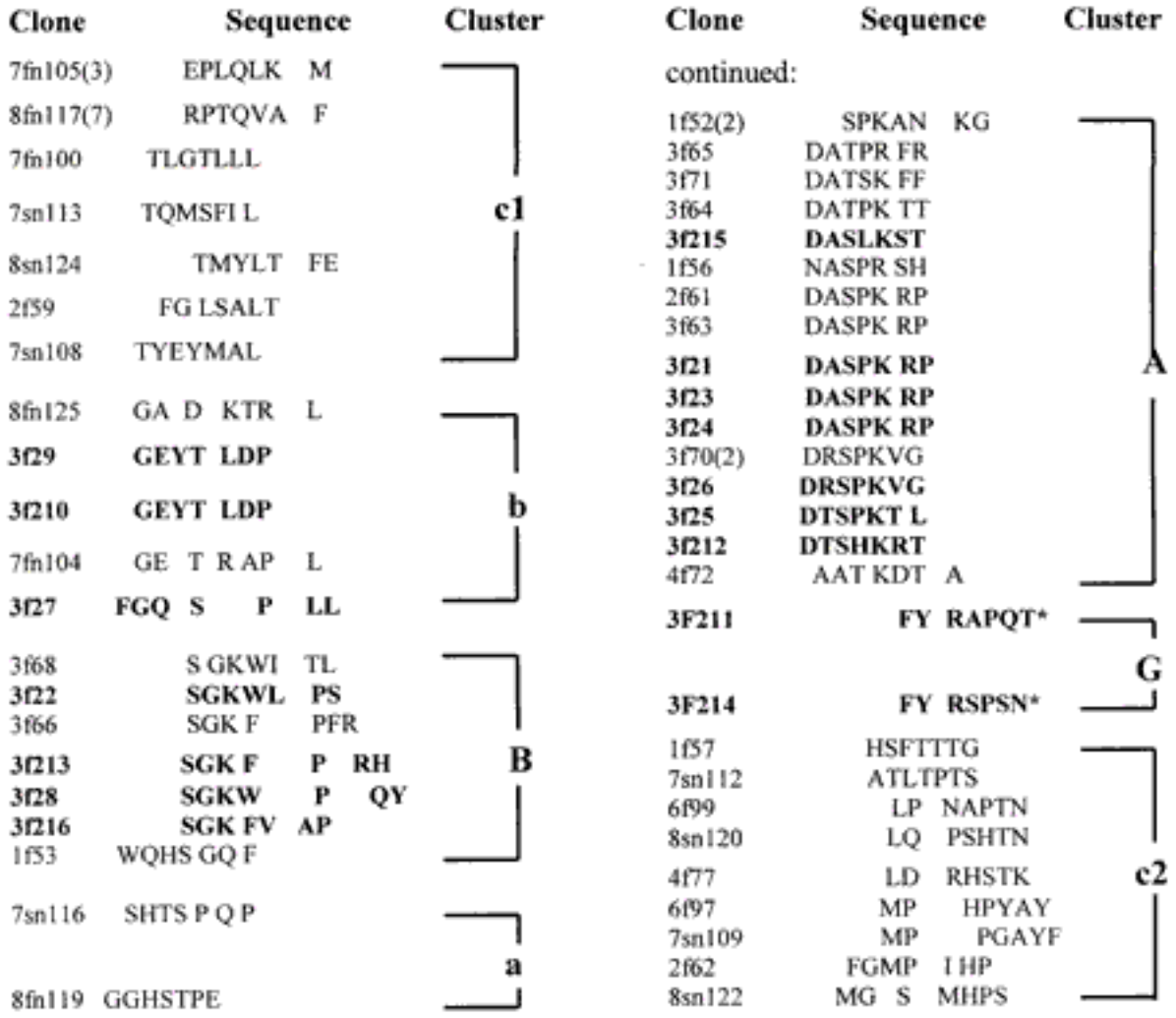


Fig. (4). Second round of the alignments.
The aligned sequences are the same as those analyzed in round 1 including 16 sequences obtained from an additional sample from the phage eluate of the Pt.3 (shown in bold). Only clusters that were altered are shown:
- all occupied other positions in the new dendrogram;
- clusters **A**; **b** and **B** acquired the newly introduced sequences;
- cluster **c** was split into two, $\mathbf{c_1}$ and $\mathbf{c_2}$,

3. Third Round of Alignments

In order to determine whether the NCC-derived sequences within the non-NCC clusters influenced the distribution of the aligned sequences in clusters, and in an attempt to reveal more sequences matching non-NCC sequences among phagotopes derived from NCC patients, the NCC-derived sequences that aligned with non-NCC sequences were removed, and the remaining sequences were realigned. The results obtained in all three rounds of alignments are shown in Fig. (**5**). The removal of the 16 non-NCC related sequences of NCC patients led to drastic changes

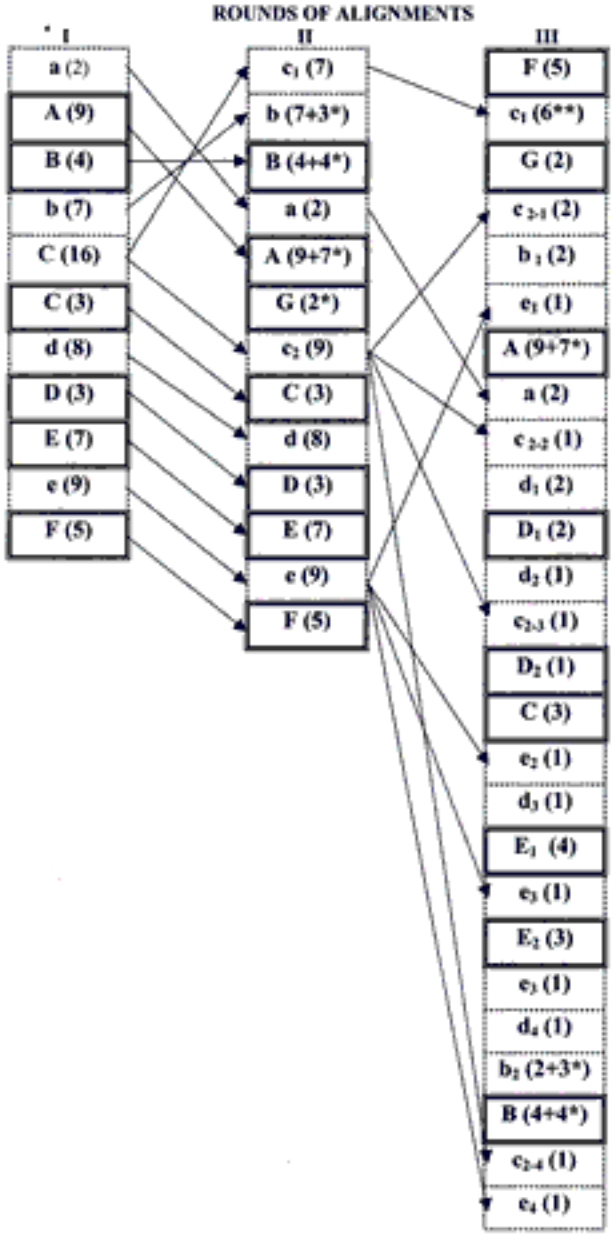


Fig. (5). Patterns of clusters in dendrograms generated by the three consecutive rounds of the alignments.
Rounds:
I– initial set of the 73 sequences found in 98 phage clones;
II– the same set including an additional sample of 16 sequences (asterisks) from the phagotopes selected by the CSF of Pt.3.
III– the sequences of the round II excepting 16 NCC-derived sequences that aligned to branches containing non-NCC-derived sequences (see Fig. **3**(**A**), left column).
A;B;C;D;E and F in continuous line boxes: NCC-derived sequence clusters; a: b; c; d; e and f (in dotted line boxes): clusters containing either both types of sequences (rounds 1 and 2) or only non-NCC-derived sequences (subindices: number of subclusters derived from splinted clusters)

in the dendrogram. In general, all NCC-related clusters retained their branch and sequence composition whereas each non-NCC cluster was split into several independent small homology units. For example, cluster **c** which was split into 2 in the 2nd round alignments, divided into 5 independent homology units dispersed throughout the guide tree; cluster **d**-divided into 4, and cluster **e** divided into 5 new units, many of which contained only a single sequence after the removal of their homology NCC-derived partners from the previous branches. Only two NCC-clusters, **E** and **D**, were slightly destabilized: the **E** formed 2 closely located clusters separated by a single **e** sequence and the same occurred with **D**. The sequences selected by the serum from Pt.5 were separated from the CSF-derived sequences.

These changes confirm that NCC-derived peptides that aligned to specific NCC-related branches contained highly related sequences, and that these clusters remained conserved during perturbations caused by changed composition of the whole aligned population. By contrast, the sequences derived from non-NCC patients were not so closely related, and the alignment and composition of clusters a - e were markedly affected by removal of NCC-derived sequences. Those clusters split to yield duplicate, triplicate or even single sequences that aligned in entirely new regions of the dendrogram.

4. ELISA of Phage Clones

The phagotopes were tested by direct ELISA for reactivity with the CSF or serum that was used to select them. In the initial assays in which the incubation with CSF or serum was at 37°C, the only peptides that gave a clearly positive result were those with the consensus motifs DASPK and SGKFxP selected by CSF from Pt 3 (Fig. (**6A**)). However, several additional clones from other patients showed reactivity in repeated determinations that was slightly but consistently higher than the background level shown by control phage. By changing the assay conditions to those used in biopanning (i.e., by incubating with antibody at 4°C), these phage clones and some additional clones from patients 1, 2, 4, and 5 became positive (Fig. (**6A**)). However, none of the phagotopes selected using CSF from patient 6 showed any reactivity. The ability of the phage clones to react with antibodies from multiple patients was also tested, using a panel of CSF from 15 patients. Only one phagotope, that containing the DASPK motif (clone 3f61, Fig. (**2**)) exhibited significant crossreactivity with one CSF (data not shown).

To test the specificity of the observed reactivity, a competition experiment was conducted by mixing the total parasite Ag with the CSF and incubating with phage immobilized on microtitre plates. No competition was observed. In contrast, there was a rise in optical density in the presence of the added Ag. Next, a reverse type competitive ELISA was used in which CSF bound to the well was allowed to react with the phage alone or mixed with the Ag. Then, the phage remaining after washing was detected using anti-m13 Ab. No competition was seen at 4 μg/well, but at 6 and 8 μg/well, there was a decrease in the reactivity of four tested phagotopes (Fig. (**6B**).

This observation and the non-reactivity of phagotopes with purified human IgG (Fig. (**6B**)) point to the involvement of the CSF Ab paratope, but not the Fc-region, in the reaction. Notably, the same follows from the fact that phagotopes did not react with CSF of many other patients.

DISCUSSION

We used the PILEUP – Tudos program [7] for homology analysis of peptide sequences selected on screening of a phage library with antibodies of NCC patients and control patients. Overall, our results show the following:

1. Biopanning random peptide phage displayed library with CSF and serum from NCC patients and non-NCC patients yielded 2 different types of sequences which could be readily distinguished by alignment using PILEUP as “NCC-related” and “NCC-unrelated” sequences;

2. In addition to NCC-related sequences derived using CSF from NCC-patients, the

same biopanning yielded "non-NCC-related" sequences (one-third of the total) that aligned with the sequences derived using non-NCC serum or CSF.

3. The proportion of NCC-related and unrelated sequences differed between patients, and for some patients most sequences were predominantly NCC-related;

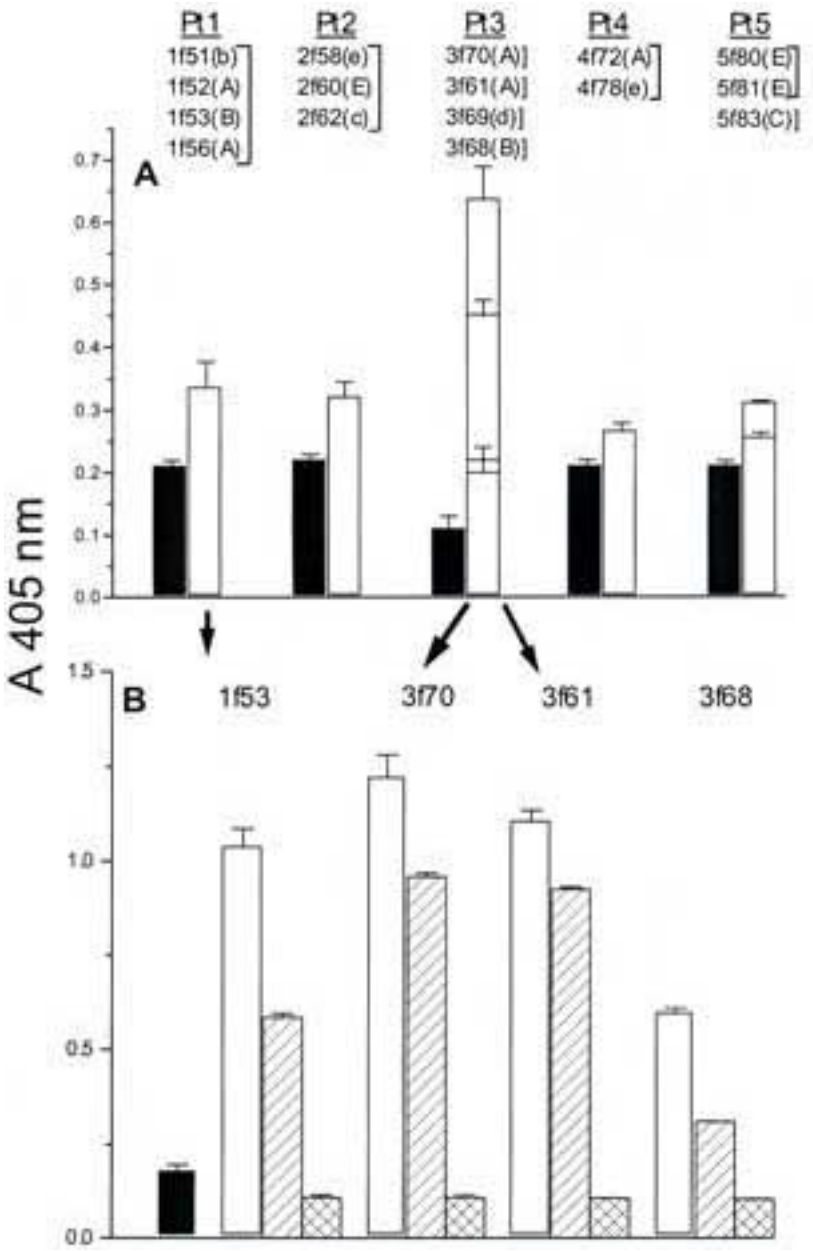


Fig. (6). Reactivity of phagotopes with CSF in ELISA

A. Reactivity of phagotopes from 5 different patients with the CSF used for selection (open bars). Phagotopes from patient 6 showed no reactivity. Phage clones and the designation of the clusters to which the peptide align (parantheses) are indicated above the columns (brackets) showing their reactivity in the same order. Results of two experiments with duplicates were used to determine the average OD and standard deviations (SD) indicated. Solid bars represent the OD observed with each CSF using irrelevant phage from the library as antigen.

B. Reactivity (mean + SD) of phagotopes of Pts 1 and 3 with immobilized CSF in a capture ELISA format without (open bar), and with human IgG (cross-hatched). Reactivity was reduced by the addition of total Ag (6 μg/well) as inhibitor (hatched). The solid bar represents the OD observed upon incubation of CSF of the corresponding patient with irrelevant phage from the library.

4. Branches of the dendrogram that contained NCC-related sequences contained sequences isolated using CSF from more than one patient.

Patient sera or other antibody-containing fluids are capable of selecting peptides from phage displayed libraries, some of which can mimic the epitopes of pathogen antigens [5,10,28] or epitopes in autoantigens [6-8;11]. Furthermore, model experiments with short synthetic peptides used as immunogens have shown that an antiserum, in spite of the diverse Ig population it contains, might select predominantly immunogen-specific peptide sequences from phage library (see [29] and Fig. (**1B**)). This suggests that in many cases particular immunogen-induced antibodies in natural fluids used in biopanning might be the main selecting agent due to their high concentration, and may select mainly peptides carrying common amino acid motifs. The multiple sequence alignment procedure (PILEUP) has been shown to discriminate such immunogen-relevant peptides from those selected by other immunogen(s) or peptides derived from binding to non-Ab substrates [7,16]. In those experiments, collections of phagotopes were used that were selected by several mAbs [16,7] and by polyclonal antibodies of patients with primary biliary cirrhosis [7]. The phagotopes were analyzed and those relevant to the mAb-elicited epitope and the autoantigen (pyruvate dehydrogenase complex, E2 subunit) were found aligned to individual clusters harboring the epitope-mimicking motif.

We extended the application of this approach to a more diverse range of antibodies elicited by as yet unknown antigenic determinants of a multicelled parasite *T. solium* causing NCC, a parasitic disease of the CNS [17,18;30]. In infected individuals, the tapeworm develops complex and variable relationships with the host immune system leading to chronic humoral and cytotoxic immune responses. Analysis by Western blot shows a wide range of antigen-specific antibodies against the parasite [19;20]. Many phagotopes obtained with Abs of NCC patients are difficult to characterize immunologically, probably because of their low concentration in CSF, the complexity of the *T.solium* antigen, and the relative insensitivity of available immunoassays. Phage display provides a technique for detecting antibodies of low affinity, or low abundance, and sequence alignment of peptide sequences using PILEUP, may reveal their intrinsic relatedness. Accordingly, we decided to align all recovered sequences derived from NCC-patients as well as phagotopes derived by non-NCC patients to see how they would be distributed in dendrograms.

In the model alignment shown in Fig. (**1**), we used phagotopes selected either by a mAb [27] or by antiserum from rabbits immunized with an immunogen carrying a single epitope [23] and confirmed the alignment of all epitope-relevant phagotopes. The alignment of sequences selected with polyclonal antibodies induced by a multicellular parasite in patients might generate too complex pattern of clustering. Therefore, we took advantage of CSF enriched in parasite-specific antibodies [19,20,30]. Earlier, CSF of patients with multiple sclerosis had been used for selecting phage libraries to find out the causative agent [11-13]. Immunological study of recovered phagotopes led to identification of only three endogenous [11] or even a single pathogenic (viral) [13] motifs involved in the immune response in multiple sclerosis. Among our peptide sequences, it was also possible to carry out a visual detection of consensus motifs, the most interesting being those derived from the Pt3. The properties of these phagotopes are consistent with their being mimotopes related to NCC. The alignment showed many other NCC-derived sequences located in the same clusters where these motifs were compactly located, thus presenting strong evidence that other sequences of the clusters are NCC-related. This is emphasized by the fact that none of the non-NCC derived sequences were aligned to this cluster. Similarly, other clusters not containing control sequences were good candidates for the NCC-related species. Altogether, this type of cluster included two-thirds of all sequences derived from NCC patients. One-third of the clusters matched control sequences in separate smaller clusters which were unrelated to the other two-thirds of the phagotopes. Moreover, the analysis identified sequences derived from several patients that

aligned together in NCC-specific branches of the dendrogram.

Two properties of peptide sequences revealed in this study are criteria for their being considered disease-related: (a) alignment to clusters devoid of sequences from non-NCC patients and (b) preservation of the cluster in subsequent alignments while non-NCC clusters are unstable (irrespective of the origin of their sequences, from NCC or non-NCC patients). This was shown by changing the composition of the peptide population which dispersed the non-NCC clusters while retaining NCC-clusters (Fig. (**5**)). Thus, after removal of the 34% of NCC-derived sequences which matched non-NCC sequences, the non-NCC clusters were entirely splintered and the fragments scattered. By contrast, the inclusion (or removal) of NCC-related sequences had minimal effect on the dendrograms, as shown by the lack of effect of the addition of 16 additional sequences from Pt3 of which 13 were NCC-related.

The behavior of the clusters of NCC–derived sequences in repeated alignments provided persuasive evidence that sequences align in clusters in accordance with their relatedness to the epitopes (Fig. **1** and [7,16]). This strongly suggests that clusters **A** - **F** contained sequences selected by individual or groups of cross-reacting antibodies elicited by the parasite antigenic determinants. The alignment of sequences derived from several patients in one cluster is of interest. For instance, in patient 3 three putative prevalent antibody specificities selected 80% of the third round eluate phagotopes. Sequences related to them by alignment were selected by CSF of 3 other patients. Consistent with this finding is the evidence that phagotopes selected with polyclonal IgG from patients with primary biliary cirrhosis have been shown to vary in their reactivity with particular preparations of anti-PDC-E2, yet align in the same region of the guide tree [7].

Immunological assay in this study, as well as in the CSF study of multiple sclerosis [11], showed that the recovered phagotopes reacted only with the CSF that derived them, indicating that CSF selects patient-specific mimotopes. The discrepancy between the alignment and immunological data may reflect the known limitations of the immunoassays to identify specific mimotopes, especially when only a single assay format is utilized (discussed in [7,8])**.** The main result described in this study with NCC is that the sequence-based analysis with the PILEUP-Tudos alignment procedure [7,16] might allow the identification of a much greater range of complex disease-specific phagotopes in phage display studies, such as NCC, where immunological tests are negative. The procedure consists of two main steps: (1) the alignment of mixed patient-derived and control subjects-derived sequences followed by (2) repeated round(s) of alignment after removal of the patient-derived sequences that matched the control sequences. In a practical sense, the combination of these with immunological data on phagotope specificity might allow effective post-panning analysis of patient-derived phagotope collections.

ACKNOWLEDGEMENTS

The technical assistance of O. Ramirez and C. Beltran is acknowledged. This work was supported by Consejo Nacional de Ciencia y Tecnología, México (25166IN) and in part by Dirección General de Asuntos de Personal Academica – Universidad Nacional Autónoma de México (IN24797).

REFERENCES

[1] Smith, G.P.; Petrenko, V.A. *Chemical Rev.*, **1997**, *97*, 391.

[2] Yip, Y.L.; Ward, R.L. *Comb. Chem. High Throughput Screen.*, **1999**, *2(3)*, 125.

[3] Wilson, D.R.; Finlay, B.B. *Can. J. Microbiol.*, **1998**, *44(4)*, 313.

[4] Rodi, D.J.; Makowski, L. Curr. Opin. Biotechnol., **1999,** 10,87

[5] Folgori, A.; Tafi, R.; Meola, A.; Felici, F.; Gafré, G.; Cortese, R.; Monaci, P.; Nicosia, A. *EMBO J.* **1994**, *13*, 2236.

[6] Sioud, M.; Dybwad, A.; Jespersen, L.; Sulleyman, S.; Natvig, J. B.; Forre, Ø.; *Clin. Exper. Immunol.*, **1994**, *98*, 520.

[7] Davies, J.M.; Scealy, M.; Cai,Y-P.; Whisstock, J.; Mackay, I.R.; Rowley, M.J. *Molec. Immunol.*, **1999**, *36*, 659.

[8] Rowley, M. J.; Scealy, M.; Whisstock, J. C.; Jois, J.A.; Wijeyewickrema, L.C.; Mackay, I.R. *J Immunol.*, **2000**, *164*, 3413.

[9] Dybwad, A.; Forre Ø.; Natvig, J.B.; Sioud, M. *Clin. Immunol. Immunopathol.*, **1995**, *75(1)*, 45.

[10] Mennuni, C.; Santini, C.; Lazzaro, D.; Dotta, F.; Farilla, L.; Fierabracci, A.; Botazzo, G.F.; Di Mario, U.; Cortese, R.; Luzzago, A. *J. Mol. Biol.*, **1997**, *268(3)*, 599.

[11] Cortese, I.; Tafi, R.; Grimaldi, L.M.E.; Martino, G.; Nicosia, A.; Cortese, R. *Proc. Natl. Acad. Sci. USA*, **1996**, *93*, 11063.

[12] Dybwad, A.; Oystein, F.; Sioud, M. *Autoimmunity*, **1997**, 25,85.

[13] Rand, K.H.; Houck,H.; Denslow, N.D.; Heilman, K.M. *J Neurol Neurosurg Psychiatry*, **1998**, 65(1),48.

[14] Rodi, D.J.; Janes, R.W.; Sanganee, H.J.; Holton,R.; Wallace, B.A.; Makowski, L. *J. Mol. Biol.*, **1999**, 285, 197.

[15] Tramontano, A.; Pizzi,E.; Felici, F.; Luzzago, A.; Nicosia, A.; Cortese, R. *Gene*, **1993**, 128, 143.

[16] Davies, J.M.; Rowley, M.J.; Mackay, I.R. *Immunol. Cell Biol.*, **1999**, 77, 483.

[17] Bittencourt, P.R.M.; Adamolekum, B.; Bharucha, N.; Carpio, A.; Cossío, O.H.; Danesi, M.A.; Dumas, M.; Fernandes, J.G.; Genton, P.; Manreza, M.L.; Meinardi, H.; Ordinario, A.; Senanayake, N.; Shakir, R.; Singhal, B.S.; Sotelo, J. *Epilepsia*, **1996**, *37(11)*, 1128.

Received 16 October, 2000 Accepted 5 January, 2001

[18] Castillo, M.; Salgado, P.; Rojas, R.; Sotelo, J.; Sanchez, J.; Mukherji, S.K. *Intern. J. Neuroradiol.*, **1996**, *2*, 168.

[19] Ostrosky-Zeichner, L.; Garcia-Mendoza, E.; Rios, C.; Sotelo, *J. Arch. Medic. Res.*, **1996**, *27 (4)*, 513.

[20] Ordoñez, G.; Medina M.T.; Sotelo J. *Neurol. Infect. Epidemiol.*, **1996**, *1*, 57.

[21] Smith, G.P.; Scott, J.K. *Methods Enzymol.*, **1993**, *217*, 228.

[22] Dower, W.J.; Cwirla, S.E. In *Peptide Antigens. A Practical Approach*; G.B. Wisdom, Ed.; IRL Press, Oxford Univ. Press: New York, **1994**; pp. 219-243.

[23] Gazarian, K.G.; Gazarian, T.G.; Solís, C. F.; Hernández, R.; Shoemaker, Ch.B.; Laclette, J.P. *Immunol. Lett.*, **2000**, *72(3)*, 191.

[24] Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor: New York, **1989**.

[25] Feng, D.-F.; Doolittle, R.F. *J. Mol. Evol.*, **1987**, *25*, 351.

[26] Davies, J.M. Immunol. *Cell Biol.*, **1997**, 75, 113.

[27] Gazarian, T.G.; Selisko, B.; Hérion, P.; Gazarian, K.G. *Molec. Immunol.*, **2001**, (in Press).

[28] Cortese, R.; Monaci, P.; Nicosia, A.; Luzzago, A.; Felici, F.; Galfré, G.; Pessi, A.; Tramontano, A.; Sollazzo, M. *Current Opinion Biotechnol.*, **1995**, *6*, 73.

[29] Dybwad, A.; Bogen,B.; Natvig, J.B.; Forre Ø.; Sioud, M. *Clin. Exper. Immunol.*, **1995**, *102*, 438.

[30] Sotelo, J.; Flisser, A. CNS Drugs, **1997**, *7(1)*, 17.