Molecular approach to find target(s) for oligoclonal bands in multiple sclerosis

Kenneth H Rand, Herbert Houck, Nancy D Denslow, Kenneth M Heilman

Abstract

Objectives—Oligoclonal bands are a characteristic finding in the CSF of patients with multiple sclerosis, yet their target antigen(s) remain unknown. The objective was to determine whether a filamentous phage peptide library could be employed to allow the oligoclonal bands to select their own target epitopes.

Methods—CSF IgG antibody from 14 patients with multiple sclerosis and 14 controls was used to select individual phage clones from a bacteriophage library containing $\times 10^7$ different hexamers expressed on its surface pH3 protein. The amino acid sequence selection was deduced by sequencing the DNA of the genetically engineered insert.

Results—In general, after three rounds of selection, CSF from both patients with multiple sclerosis and controls selected one to two consistent peptide motifs. Five out of 14 patients with multiple sclerosis, and one control, selected the amino acid sequence motif, RRPFF. Given 20 possible amino acids per position, the likelihood of five patients selecting the same linear five amino acid sequence is at most $1.6 \times 10^{-13}$ correct for the number of clones sequenced. A GenBank computer search showed that this sequence is found in the Epstein-Barr Virus nuclear antigen (EBNA-1), and a heat shock protein aB crystallin. Human serum antibodies to a synthetic peptide containing RRPFF were virtually exclusively found in patients with prior infection by Epstein-Barr virus.

Other studies have suggested a relation between Epstein-Barr virus infection and multiple sclerosis, including nearly 100% Epstein-Barr virus seropositivity among patients with multiple sclerosis and increased concentrations of antibody to EBNA in CSF of patients with multiple sclerosis. By antigen specific immunoblotting, antibodies to the RRPFF motif in the CSF were shown to correspond to a subset of oligoclonal bands in the CSF from the same patient.

Conclusion—This study shows that phage epitope display libraries may be used to select amino acid motifs which are potentially relevant to the pathogenesis of multiple sclerosis.

(J Neurol Neurosurg Psychiatry 1998;65:48–55)

Keywords: oligoclonal bands; multiple sclerosis; filamentous phage display library; EBNA-1

Introduction

OLIGOCLONAL BANDS

Cerebrospinal fluid from patients with multiple sclerosis contains monoclonal antibodies that can be identified as oligoclonal bands by isoelectric focusing (IEF). These oligoclonal bands are one of the most consistent laboratory abnormalities found, being detectable in about 95% of patients with clinically definite multiple sclerosis compared with roughly 10%–15% of controls.1–5 Oligoclonal bands are routinely found in encephalitis and meningitis caused by infectious agents such as enterovirus, herpes simplex type 1, human immunodeficiency virus, measles (as found in subacute sclerosing panencephalitis), and even bacterial infections such as Lyme borreliosis, neurosyphilis, and tuberculous meningitis.6–10 Although the oligoclonal bands in these infectious diseases can be shown to be directed towards the infecting agent, the identification of a target infectious agent or autoantigen for the oligoclonal bands in multiple sclerosis has not been accomplished.

Several studies have attempted to identify the target of the oligoclonal bands. In patients with multiple sclerosis, antibodies to myelin basic protein in CSF, serum, and brain eluates are found with variable frequency. Using agarose isoelectric focusing with transfer to nitrocellulose coated with or without myelin basic protein (antigen specific immunoblotting), Cruz et al11 found oligoclonal bands that reacted with myelin basic protein in nine of 28 (32%) of patients with multiple sclerosis, compared with none of 34 control patients with other neurological diseases. However, these bands could be clearly separated on their immunoblots from those oligoclonal bands stained by silver or by antihuman IgG.12 Likewise oligoclonal bands reactive with measles virus did not comigrate with oligoclonal bands reactive with myelin basic protein, nor with the oligoclonal bands demonstrable by direct staining methods in their patients with multiple sclerosis.13 Other studies using imprint immunofixation for measles, mumps, rubella, HSV-1, varicella, cytomegalovirus, and adenovirus similarly showed antigen specific oligoclonal bands; however, these bands did not correspond to those seen by direct staining using Coomassie blue.12 Studies with various brain components, such as lipid-proteolipid, ganglioside, myelin basic protein, and saline extracts of both normal and multiple sclerosis brain gave similar results.12 Souberbielle et al14 did not find any significant number of serum samples from multiple sclerosis or control patients which reacted with human brain vessels by western blot. Thus, although patients have various intrathecaally synthesised autoantibodies and antiviral antibodies in CSF,
none have been identified directly with the oligoclonal bands found by staining methods. On the other hand, in animal models of experimental autoimmune encephalomyelitis (EAE), oligoclonal bands are found and can be shown to react with the antigen used to induce the encephalitis.13

PEPTIDE EPITOPE LIBRARIES

In recent years, methods have been developed which permit monoclonal antibodies to select their own target epitopes. Although synthetic libraries have been developed,16 expression of random amino acid inserts into either the pIII or pVIII protein of M13 bacteriophage has been achieved by several groups.17–20 Briefly, a short nucleotide sequence is synthesised in such a way as to code for a random amino acid at each of six or more positions. This DNA is then inserted into the bacteriophage gene coding for a surface protein, with the result that each phage in the population displays a unique amino acid sequence on its surface.

Scott and Smith17 have shown that monoclonal antibodies can indeed select phage from their library, the inserts of which express epitopes closely matching the known target of these antibodies. They used two monoclonal antibodies against myohaemerythrin, the target epitope of which was known to be the linear sequence DEFKLI and found that all 33 clones isolated after three rounds of selection contained DEFXXX as the first three amino acids of the hexapeptide, and 19 of 33 contained the first four, DEFLXX. Similar consistency was also achieved by Cwirla et al18 using a monoclonal antibody to β endorphin, and by Koivunen et al20 for the RGD motif of the binding site of u,β integrin for fibronectin. Likewise, Devlin demonstrated the selection of a consistent motif for the biotin binding site of streptavidin,19 and Lenstra et al21 obtained similar results with monoclonal antibodies to the spike protein of coronavirus.21 More recently, this methodology has been applied to the identification of a serotype specific epitope of Dengue virus 1.22

In this study, CSF immunoglobulin G (IgG) was allowed to select specific amino acid sequences from a filamentous bacteriophage display library as an initial approach toward the identification of the target epitopes of the oligoclonal bands in the CSF of patients with multiple sclerosis.

Methods

PATIENTS AND CONTROLS

Permission was obtained from the Institutional Review Board at both the University of Florida and the Gainesville Veteran’s Affairs Medical Center to recruit volunteers with multiple sclerosis to undergo lumbar puncture. All volunteers underwent a complete history and neurological examination at the University of Florida Neurology clinic by one of the authors (KMH). Volunteers willing to undergo lumbar puncture were recruited from the North Florida Chapter of the Multiple Sclerosis Society and by word of mouth. A total of nine potential subjects volunteered, but three were excluded (one because of comitant autoimmune disease, one because of severe coronary artery disease, and one because of prior brain surgery). Two additional volunteers initially agreed to participate but one never followed through, and the other was told not to participate by her private physician. CSF was also obtained from the Chemistry Laboratory, Shands Hospital, Gainesville Fl, USA when oligoclonal bands were detected by agarose gel isoelectric focusing electrophoresis and Coomassie blue staining of 80 concentrations of CSF. If CSF contained oligoclonal bands, the patient’s chart was reviewed and those patients with a clinical diagnosis of multiple sclerosis were included for further study (see below). Control CSFs were also obtained from the clinical laboratory. Charts of these patients were reviewed to rule out possible multiple sclerosis but other neurological disease was not excluded.

All patients with multiple sclerosis had a clinical diagnosis of multiple sclerosis and were receiving treatment for it. Multiple sclerosis patients 1, 3, and 6 were selected because they had very intense oligoclonal bands on CSF electrophoresis and patients 2, 4, 5, and 11 were volunteers. Samples of CSF from patients 6–10 and 12–14 were obtained from the clinical laboratory but were not selected on any clinical or laboratory basis. All patients with multiple sclerosis except 1 and 6 met the criteria of Schumacher et al for a clinically definite case.23 All had oligoclonal bands when tested in the clinical laboratory, except patient 11.

CSF from 14 patients undergoing lumbar puncture for the diagnosis of other neurological conditions was also obtained from the clinical laboratory. Control patients carried the following diagnoses or conditions: unexplained seizure one, probable Guillain-Barré syndrome one, cervical disc related paraesthesias one, rule out meningitis in sepsis investigation one, CNS lymphoma one, staphylococcal endocarditis receiving vancomycin with mental status changes one, status post heart transplant with mild cerebral atrophy and recent herpes zoster one, status migrans one, and intraventricular neucytoma one. The two patients with neurosarcoid had cell counts of 13 and 16×10⁶ white blood cells/l, and one of them had a protein of 3880 mg/l and a slightly low glucose of 2.1 mmol/l. Otherwise all CSF cell counts, glucose, and protein results were normal.

PHAGE LIBRARY

The phage expression library was a 50 µl aliquot amplified from the 2×10¹⁴ clone library described by Scott and Smith15 and was generously provided by Dr George Smith, University of Missouri. This library has a titre of 4.6×10¹⁹ TU/ml and a physical particle concentration of 2.7×10¹⁰ virions/ml. Sequencing en masse showed the expected degeneracy of the 18 base pair insert, with no visible evidence of bias at any position.24 The library was propagated in K91 Kan E coli, which was also a gift from Dr George Smith.
elution, phage infected K91 Kan process repeated twice more. After the third PEG precipitation, washed, and the overnight, harvested the next day by polyethyl-
then used to infect K91 Kan, tralised in 38µl 1M Tris, pH 9.1. Phage were
volumes were combined and immediately neu-
fiveminutes, followed by a rinse with a second
incubation in 100µl 100mM HCl, pH 2.2 for
bound phage were eluted from the beads by
chloroform extraction, and ethanol precipita-
tion as described.17 Sequencing was carried out with a Sequenase™ version 2.0 sequencing kit (US Biochemical, Cleveland, OH, USA) according to the instructions provided by the manufacturer.

For multiple sclerosis patients 1–5, 7, and 8 and control patients 1–3, 20 clones/patient were sequenced. For the remaining patients only 10 clones/patient were sequenced. In some pa-
tients whose CSF was repeatedly tested, only five clones were sequenced (table 1).

<table>
<thead>
<tr>
<th>MS patient 4</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRPPFH</td>
<td>RRPFFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRPPFR</td>
<td>RRPFFR</td>
<td>RRPFFR</td>
<td></td>
</tr>
<tr>
<td>RRPPFR</td>
<td>RRPFFR</td>
<td>RRPFFR</td>
<td></td>
</tr>
<tr>
<td>RRPFFM</td>
<td>RRPFFM</td>
<td>RRPFFM</td>
<td></td>
</tr>
<tr>
<td>RRPFFL</td>
<td>RRPFFL</td>
<td>RRPFFL</td>
<td></td>
</tr>
<tr>
<td>RLVRVA</td>
<td>RLVRVA</td>
<td>RLVRVA</td>
<td></td>
</tr>
<tr>
<td>PLVRWA</td>
<td>PLVRWA</td>
<td>PLVRWA</td>
<td></td>
</tr>
<tr>
<td>ELVRVA</td>
<td>ELVRVA</td>
<td>ELVRVA</td>
<td></td>
</tr>
<tr>
<td>PLVRWA</td>
<td>PLVRWA</td>
<td>PLVRWA</td>
<td></td>
</tr>
<tr>
<td>PHAPP</td>
<td>PHAPP</td>
<td>PHAPP</td>
<td></td>
</tr>
<tr>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td></td>
</tr>
<tr>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td></td>
</tr>
<tr>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td></td>
</tr>
<tr>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td></td>
</tr>
<tr>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td>PPHAPP</td>
<td></td>
</tr>
<tr>
<td>DPWLRGL</td>
<td>RASLAI</td>
<td>AWDLIF</td>
<td></td>
</tr>
</tbody>
</table>

SELECTION OF EPTOPES FROM PEPTIDE LIBRARY
Streptavidin coated magnetic beads (Dynabeads, Dynal Inc, Lake Success, NY, USA) were washed once in 50 mM Tris pH 7.5, 150 mM NaCl and 0.05% Tween 20 (TBST). Approximately 100 µl beads (≈6–7×10^8 beads) were incubated for two to four hours on a rotating tube holder at 4°C with 10 µg biotin labelled mouse monoclonal antihuman IgG (Zymed Labs Inc, San Francisco, CA, USA cat No 05–4240) in a volume of 200 µl TBST. After washing three with TBST, 0.1 ml beads were incubated with 10 µl CSF diluted to 200 µl with TBST overnight at 4°C with mixing. Un-
blocked streptavidin binding sites were blocked with 1 mM biotin included in the TBST used for all steps from this point on. After five washes with TBST using the magnetic separator, the beads with bound CSF were incubated with 10 µl phage library in a final volume of 200 µl overnight at 4°C. After 10 washes in TBST, the bound phage were eluted from the beads by incubation in 100 µl 100 mM HCl, pH 2.2 for five minutes, followed by a rinse with a second volume of 100 µl HCl at pH 2.2. The two HCl volumes were combined and immediately neu-
tralised in 38µl 1M Tris, pH 9.1. Phage were
then used to infect K91 Kan E coli in LB broth overnight, harvested the next day by polyethyl-
ene glycol (PEG) precipitation, washed, and the process repeated twice more. After the third elution, phage infected K91 Kan E coli were plated and the colonies at the end point of the titration grown for sequencing. For the DNA, the insert was sequenced by standard methods and the amino acid sequence of the insert deduced from the DNA sequence. Except for the use of the streptavidin magnetic beads, this procedure is essentially identical to that of Scott and Smith.17

SEQUENCING
Phage clones were grown in E coli and prepared for sequencing by PEG precipitation, phenol chloroform extraction, and ethanol precipita-

ISOELECTRIC FOCUSING, BLOTTING, AND IMMUNOPEROXIDASE ASSAY
Undiluted CSF and serum samples diluted to roughly the same IgG concentration were applied to precast RESOLVE™ (pH 3–10, 85 mm×100 mm×1 mm thick) agarose isoelectric focusing gels (IsoLab Inc, Akron, OH, USA). Anode wicks were soaked in 0.5 M acetic acid and cathode wicks in 0.5 M NaOH. The gel was focused in a Hoefer Isobox isoelectric focusing flatbed unit (Hoefer Scientific Instruments, San Francisco, CA, USA) at 5°C and 15 W for 15 minutes, 10 W for 15 minutes, and 15 W until complete as determined by migration and resolution of methyl red dye loaded 1 cm from the cathode wick. Voltage was limited to 1500 V. Isoelectric point markers ranging in pI from 3.6–9.3 (Sigma, St Louis, MO, USA) were run in the outer lanes.

Immediately after the run, PI marker lanes were cut from the gel and stained with 0.1% Coomassie brilliant blue R-250 in 25% ethanol, 9% acetic acid. Serum and CSF lanes were preblotted with dry nitrocellulose (NC) paper (either 0.45 µ Biorad, Hercules, CA, USA, or 0.45 µ Schleicher and Schuell, Keene, NH, USA) for 15 seconds to remove cross reacting high molecular weight aggregates and cell fragments that can result in smeared blots.16 Proteins were then blotted on to either uncoated NC paper, hydrated in Tris bu-
ver, pH 7.4 or NC paper coated with 800 µg/ml avidin and 300 µg/ml biotinylated peptide for detection of pep-
tide specific IgG. Transfer of proteins was

SAFETY
If not assayed immediately, blots were air dried. They were rinsed briefly in phosphate buffered saline (PBS), pH 7.4, rinsed once with PBS plus 0.05% Tween-20 (PBST), then incubated with a blocking solution of 1% BSA in PBST plus 0.05% sodium azide for one hour. Bound IgG was detected by incubating blots with a 1:500 dilution of goat antihuman IgG Fc (Sigma, St Louis, MO, USA), for determination of total IgG or NC paper coated with 300 µg/ml biotinylated peptide for detection of pep-
tide specific IgG. Transfer of proteins was performed for 30 minutes as follows: NC paper was carefully placed over the gel, air bubbles were removed, and the NC paper was overlaid with one piece of “Tris buffer soaked Quick-
Draw™ extra thick blotting paper (Sigma) fol-
ed by several layers of paper towels. A glass plate was placed on top of the assembly and weighted down with a 1kg weight.

For multiple sclerosis patients 1–5, 7, and 8 and control patients 1–3, 20 clones/patient were sequenced. For the remaining patients only 10 clones/patient were sequenced. In some pa-
tients whose CSF was repeatedly tested, only five clones were sequenced (table 1).
seen. The reaction was stopped with excess water and the blots dried before analysis.

PREPARATION OF QUANTITATIVE ISOELECTRIC FOCUSING FRACTIONS

For quantitative preparation of isoelectric focusing fractions 1 ml CSF was concentrated to 100 µl and applied across the entire gel in a wide band. After focusing, the gel was cut in two vertical strips one 0.5 cm wide for staining the pl markers and oligoclonal bands and the remaining portion for "snow plowing". The gel was lined up on graph paper and narrow strips of about 2.5 mm in width were scraped from the plastic backing ("snow plowed") with a thin spatula and placed in microfuge tubes. IgG was extracted from the gel by repeated freeze-thawing in the presence of 0.5–1.0 ml 20 mM Tris, 0.3 mM NaCl, pH 7.4 with 0.02% NaN₃. This procedure produced fractions containing an estimated zero to two visualisable oligoclonal bands. Each fraction was washed and centrifuged three times in an 0.22 µm Spin X centrifuge tube (Costar, Cambridge, MD, USA) and the collected supernatant concentrated to 1 ml CSF was concentrated to 100 µl, which was used for three rounds of selection with the phage library as described above.

PEPTIDE SYNTHESIS

Peptides were prepared by solid phase methodology, using FMOC chemistry on an Applied Biosystems 322A peptide synthesiser, in the Interdisciplinary Center for Biotechnology Research at the University of Florida, Gainesville, Florida, USA.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Nunc microwell module F-16 (Nunc Roskilde, Denmark) wells were coated with 100 µl of a 20 µg/ml solution of the appropriate peptide in 100 mM carbonate buffer, pH 9.6 overnight at 4°C. Wells were then rinsed once with phosphate buffered saline containing 0.05% Tween-20 (PBST) and blocked for two hours at 37°C with PBST with 5% fetal bovine serum (PBST-FBS). Serum was diluted 1:50 in PBST and CSF was diluted 1:4 in PBST-FBS and 100 µl incubated for one hour at 37°C. Wells were then rinsed three times with PBST and incubated with 100 µl of a 1:500 dilution of mouse antihuman IgG-alkaline phosphatase (Fisher Scientific, Atlanta, GA, USA) in PBST for one hour at 37°C. Wells were rinsed three times with PBST; followed by the addition of 100 µl 1mg/ml p-nitrophenyl phosphate substrate (Sigma) in 100 mM glycine buffer, pH 9.6 for 30 minutes at room temperature (22°C). Reactions were stopped by the addition of 100 µl 1.5M NaOH and read at 405 nm. Negative control wells were coated with antigen, but received no CSF or serum.

EPSTEIN-BARR VIRUS AND CYTOMEGALOVIRUS SEROLOGY

Epstein-Barr virus serology was measured by immunofluorescence to the viral capsid antigen (VCA) using an indirect immunofluorescent assay (Gull Laboratories, Salt Lake City, UT, USA). A titre 1:10 was considered positive. Cytomegalovirus serology was measured by ELISA (Bartels, Issaquah, WA, USA). A titre was considered positive if the optical density was greater than that of the average of three low positive controls, supplied by the manufacturer.

RESULTS

CSF from both multiple sclerosis and control patients generally selected one to three dominant amino acid sequence motifs, with only minimal variation in one or two amino acids within the motif (tables 1 and 2). This pattern of consistent selection of a peptide motif is essentially identical to that described by Scott and Smith and others for monoclonal antibodies. By contrast, when the mouse monoclonal antihuman IgG that was used to bind the human CSF IgG to the magnetic beads was used to select phage, a total of 16 different peptide sequences were found, only two of which showed sequence consistency. None of the sequences selected by the mouse antihuman IgG had any similarity to those of the patients with multiple sclerosis or the control patients (data not shown).

Table 3 shows the amino acid motifs most often selected by multiple sclerosis and control patients. The linear motif RRPFEX (where X=H,R,M,L,N, or I) was found in five of 14 patients with multiple sclerosis and one of 14 controls (p=0.074, Fisher's exact probability). As there are 20 different amino acids per position in the random hexamer, the chances that any two random linear hexamer sequences selected by different subjects will have the same 5/6 linear sequence is (1/20)⁶(2) or 1.6 x 10⁻⁶. When corrected for the total number of independent clones sequenced, the probability of finding five patients with multiple sclerosis with this same linear 5/6 sequence by chance alone is at most 1.6 x 10⁻¹³ (calculation given in
Table 3  Amino acid motifs most often selected by patients with multiple sclerosis and controls

<table>
<thead>
<tr>
<th>Motif</th>
<th>Patients with multiple sclerosis</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRPFFX*</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>PWXWLX4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>LYAAFFY</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PWAAFY</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>PWTAFFH</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PWFAAFFH</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GDFVFI</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GDFVFI</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GDFVFI</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PXHFP</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>VPHFP</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PWTAFFH</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PQYFFYC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PQCFYPC</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
</table>

*X=H,R,M,L, or I; †X=P,Q,L,G, or D; ‡X=G,Q,I,Y, or D; §this sequence was found in several individual IEF pH fractions, rather than among those in the whole CSF (data not shown).

Individual pH fractions were recovered from preparative isoelectric focusing gels and used to select phage clones. Table 2 shows a representative patient. Generally only one and occasionally two motifs were found in these narrow pH fractions with motifs often overlapping adjacent fractions. Interestingly the dominant motif in patient 4 (table 2) seems to be present with some degree of variation across the entire pH gradient, a finding shared in isoelectric focusing fractions from patient 5 for the motif AVYRPP (data not shown). It is unclear why the RRPFF motif was not selected among the individual fractions despite being selected by whole CSF from patient 4 on three separate occasions. The RRPFF antibodies could have been outside the pH range of the fractions tested or among the clones from fraction 16 which were accidentally lost.

To determine whether the motifs selected from the phage library were the actual target(s) of individual oligoclonal bands, antigen specific immunoblotting was performed. Figure 9 shows the total IgG banding pattern from a representative patient (No 13). The arrows indicate 8 MS-1 specific bands which have their counterparts in the total IgG bands, certain of which appear to be on the edge of an IgG oligoclonal band suggesting more than one component to the IgG band. At least one (or more) specific band(s) has no counterpart among the total IgG bands. The specificity of these findings is supported by the observation that only a subset of the total IgG oligoclonal bands bind specifically to MS-1. We also performed antigen specific isoelectric focusing using a biotinylated 20 mer which

Table 4  Relation of serum antibody to the RRPFF containing peptide (MS-1) and Epstein-Barr virus among multiple sclerosis and control populations

<table>
<thead>
<tr>
<th>Antibody to RRPFF peptide*</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody to EBV viral capsid antigen†</td>
<td>35 (10)‡</td>
<td>21 (5)§</td>
</tr>
<tr>
<td>Negative</td>
<td>1§</td>
<td>31§</td>
</tr>
</tbody>
</table>

*Positive=OD >0.2; †VCA positive=IFA titre >1:10; ‡numbers in parentheses are patients with multiple sclerosis. All were VCA positive; §includes 10 patients who were EBV VCA negative, but CMV positive, one of whom had a low but positive (OD=0.27) titre to the RRPFF peptide.
Target(s) of oligoclonal bands

53

surveys30–32 have shown that essentially 100% of patients with multiple sclerosis were Epstein-Barr virus seropositive compared with only 13% of Epstein-Barr virus seronegative control patients with other neurological diseases. Although we remain uncertain as to why some patients with multiple sclerosis selected the RRPFF motif whereas others did not, a possible explanation could be the extremely low probability of selecting any identical sequences out of a library of over 10^7 different hexamers. Secondly, the serum ELISA data (table 4) suggest that not all people with or without multiple sclerosis are capable of making antibody to RRPFF, even if they have been previously infected by Epstein-Barr virus. Cheng et al.33 reported data consistent with this finding using consecutive 20 mer peptides spanning the entire EBNA-1 molecule. Only 28% of normal subjects had IgG antibodies reactive with the 20 mer which contained the RRPFF motif.34

Another important reason for the diversity of sequence motifs is related to the behaviour of the phage display library itself: according to Smith, who developed these methods and shared them with the phage with us for this work, if a monoclonal antibody is known to recognise a linear epitope, in his experience it “always selects a motif from the 6 mer library which closely resembles the eliciting epitope” (G Smith, personal communication, 1994). If the monoclonal antibody is known to recognise a conformational epitope, the chances are about 50% that a consistent motif (a mimotope) will be selected. Mimotopes are sequences which bear no amino acid sequence resemblance to the epitope to which an antibody is directed, but which by “chance” have a configuration which fits the antibody binding site. As most antibodies are directed at conformational epitopes, most patient oligoclonal bands would be expected to select mimotopes, which would produce the observed consistency within a given patient, but very little consistency between patients.

The nature of the oligoclonal bands themselves adds further complexity. Walsh et al.35 studied multiple sclerosis CSF by two dimensional gel electrophoresis. He found that fractions of 0.1–0.3 pH units in the isoelectric focusing gradient which seemed to contain a single oligoclonal band on isoelectric focusing, could be resolved into several separate L chain spots. On this basis, he concluded that such pH fractions generally contain one to three dominant monoclonal antibodies, or 50–70 total/patient with multiple sclerosis. Roström36 and Vartdal et al.37 also noted that in some patients more than one viral antibody was occasionally associated with the same oligoclonal band. The implication of these studies is that each of the oligoclonal bands contains multiple monoclonal antibodies within what may seem to be a single oligoclonal band by isoelectric focusing. Our studies of preparative isoelectric focusing are consistent with this view in that several pH fractions of 0.1–0.2 pH units selected more than a single motif.

Occasional MS-1 specific bands could not be aligned with a band in the total IgG oligoclonal band pattern. The finding that CSF contains oligoclonal bands below the limit of detection by conventional IgG staining has been demonstrated in the past by antigen
Rand, Houck, Denslow, et al

specific immunoblotting. Franciotta et al have shown that even in HIV infected patients with documented CNS infections due to toxoplasmosis and cytomegalovirus, oligoclonal bands specific for these agents are demonstrable by antigen specific immunoblotting in CSFs lacking oligoclonal bands by IgG staining. Thus the fact that motifs were selected by our controls and one patient with multiple sclerosis without classic oligoclonal bands is probably best explained by the relative lack of sensitivity of total IgG staining compared with antigen specific immunoblotting and by the sensitivity of the selective methodology using the phage library.

Recently, van Noort et al identified a small heat shock protein, αB crystallin, as the component of an extract of myelin from patients with multiple sclerosis, which most strongly and consistently elicited lymphocyte proliferation by lymphocytes from patients with multiple sclerosis compared with myelin from control brain tissue. Interestingly, the linear sequence RRPFα is found in α B crystallin. Van Noort et al has subsequently proposed that stress or other triggers cause the production of α B crystallin within oligodendroglia and that the α B crystallin becomes incorporated into myelin breaking the normal immunological tolerance to myelin. It is also conceivable that for some subjects infection with Epstein-Barr virus leads to immune recognition of the RRPFα which breaks the normal immunological tolerance to α B crystallin. Such a hypothesis could account for the strong epidemiological association of prior Epstein-Barr virus infection in patients with multiple sclerosis. Cortese et al have carried out a similar study of CSF from patients with multiple sclerosis using the phage display library approach. They used two different 9 mer libraries expressed on the pVIII protein, one of which was constrained by flanking cysteine disulphide bonds. They screened a large number of epitopes selected by CSF from two patients with multiple sclerosis by serological means to find those recognised by most patients with multiple sclerosis. Three different candidate amino acid motifs were ultimately tested and whereas they were recognised with equal frequency by serum from both multiple sclerosis and control patients (>50%, 5%, and 25% respectively for the three motifs studied), only four of 55 patients with multiple sclerosis had CSF which reacted with one of these three motifs. None of the three motifs showed sequence homology to any of those selected in our study. They did not test their motifs by antigen specific immunoblotting to determine whether they corresponded to specific oligoclonal bands. The differences between their findings and ours most probably arise from the use of a different phage library, which could influence the statistical likelihood of selecting related sequences. In other words the probability of selecting a mimotype could be very different between the two libraries, which could vastly decrease the chances of finding common motifs.

Dybwad et al used three different phage libraries to study CSF from one patient with multiple sclerosis and found amino acid motifs with significant linear homology to human collagen, a 68 kDa neurofilament protein, and several herpesviruses. One of the motifs they found with a hexamer library, PRPFα, where α = neutral non-polar residues and P = G or P, is strikingly similar to our MS-1 motif.

In conclusion, the phage display technique can be used to obtain amino acid sequence motifs which can be further tested for relevance to multiple sclerosis. Our initial data identified an epitope shared by Epstein-Barr virus and α B crystallin, both of which are postulated to play a part in the pathogenesis of multiple sclerosis, suggesting that this approach can yield biologically meaningful information.

We are extremely grateful to Dr George Smith, University of Missouri, Columbia, MO, USA for generously providing the phage library, a detailed manual for its use, and his many helpful discussions during the course of this work. We also gratefully acknowledge the support of the Protein Synthesis Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida Gainesville Fl, USA and the staff of the Shands Hospital Clinical Chemistry and Diagnostic Virology Laboratories. We also express a special thanks to those patients who willingly underwent lumbar puncture for their generous contribution. This work was supported by grants from the National Multiple Sclerosis Society, the Diagnostic Referral Laboratory of the Department of Pathology and Laboratory Medicine, University of Florida, Gainesville, Fl, and by the Gainesville Veteran’s Affairs Medical Center, Gainesville, Florida, USA.

Appendix: Calculation of random probability for amino acid sequence matching

(1) Calculation of the probability of a 5/6 match between any two selected sequences, and then for N = 5 patients

As there are 20 amino acids and six different positions in the phage library, the probability of a given amino acid in any position is (1/20)^5, where n = the number of positions to be filled, in this case five. The probability for a given amino acid sequence in 5/6 positions is (1/20)^5 × the number of remaining sequences that will also result in a 5/6 match, which is given by the formula: N!/[(N-K)!K!], where N=6, the total number of positions, and K=5, the number of positions to be specified. Multiplied out, (1/20)^5 × 6!/[(6-5)!5!] = 6/20, which is 1.875×10^-4. To calculate the probability that this sequence will have a 5/6 match with at least one other sequence among the remaining 13 patients with multiple sclerosis studied, we multiply this probability by the number of sequences identified in the "first" patient and then by the total number of remaining sequences. This yields 1.875×10^-4 × 13 = 0.0245. Therefore, we calculated the probability based on assuming the sequence we wish to match was found in the patient with the maximum number of chances to find it, namely 20, as that was the maximum number of clones/patient sequenced. This yields 1.875×10^-4 × 20, which is then multiplied by the number of remaining sequenced clones from the other 13 patients which could have matched the first patient. This gives 1.875×10^-4 × 20 × (169), as there were a total of 169 clones sequenced and 20 were from the first patient. Multiplied out,
this probability is 6.34 x 10^-3. For a third patient to have a sequence that matched, we multiply the probability of the match in the first two patients by the probability of a 5/6 match in general—that is, (6.34 x 10^-3) x 0.55 is the probability of sequences being matched. This gives 3.5 x 10^-4. Thus for the fourth patient, we get (1.94 x 10^-4) x 0.55 = 1.05 x 10^-4. Of the five patients, the maximum probability for all five patients is 1.55 x 10^-5.

(2) Calculation of the probability of a 4/6 match between any two selected sequences and then for N = 4 patients

In this case, we have (1/20) x (15/20) x (14/20) x (13/20) = 1.5 x 10^-5. Using the same iterative process and assumptions as above, the maximum probability for four patients sharing a 4/6 match is 7.0 x 10^-5.

Molecular approach to find target(s) for oligoclonal bands in multiple sclerosis

Kenneth H Rand, Herbert Houck, Nancy D Denslow and Kenneth M Heilman

*J Neurol Neurosurg Psychiatry* 1998 65: 48-55
doi: 10.1136/jnnp.65.1.48

Updated information and services can be found at:
http://jnnp.bmj.com/content/65/1/48

These include:

**References**
This article cites 38 articles, 12 of which you can access for free at:
http://jnnp.bmj.com/content/65/1/48#BIBL

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**
Articles on similar topics can be found in the following collections

- Immunology (including allergy) (1943)
- Multiple sclerosis (934)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/