Journal of Clinical Immunology, Vol. 25, No. 3, May 2005 (© 2005)
DOI: 10.1007/s10875-005-4083-5

Axon Reactive B Cells Clonally Expanded in the Cerebrospinal Fluid of Patients with Multiple Sclerosis

YIPING ZHANG,[1] RENG-RONG DA,[1] WENZHONG GUO,[1] HUI-MIN REN,[1] LUTZ G. HILGENBERG,[2] RAYMOND A. SOBEL,[3] WALLACE W. TOURTELLOTTE,[4] MARTIN A. SMITH,[2] MICHAEL OLEK,[1] SUDHIR GUPTA,[5] RICHARD T. ROBERTSON,[2] RASHED NAGRA,[4] STANLEY VAN DEN NOORT,[1] and YUFEN QIN[1,6]

Received: November 15, 2004; accepted: January 14, 2005

Demyelination and axonal loss have been described as the histological hallmarks of inflammatory lesions of multiple sclerosis (MS) and are the pathological correlates of persistent disability. However, the immune mechanisms underlying axonal damage in MS remain unknown. Here, we report the use of single chain-variable domain fragments (scFv) from clonally expanded cerebrospinal fluid (CSF) B cells to show the role of an anti-axon immune response in the central nervous system (CNS) in MS. The cellular and subcellular distribution of the antigen(s) recognized by these CSF-derived clonal scFv antibodies (CSFC-scFv Abs) was studied by immunochemical staining of brain tissues obtained at autopsy from patients with MS. Immunochemistry showed specific binding of CSFC-scFv Abs to axons in acute MS lesions. The stained axons showed three major types of axonal pathological changes: 1) linear axons, axonal ovoid formation, and axonal transection were seen in the myelinated white matter adjacent to the lesion; 2) accumulation of axonal ovoid formations and Wallerian degeneration were seen at the border between demyelinated lesions and the adjacent white matter; and 3) Wallerian degeneration occurred at the center and edge of acute demyelinated lesions. These findings suggest a B cell axonal specific immune response in the CNS in MS.

KEY WORDS: CSF B cell clonal expansion; CSFC-scFv antibody; axonal immunity; multiple sclerosis.

[1]Department of Neurology, University of California, Irvine, California.
[2]Department of Anatomy and Neurobiology, University of California, Irvine, California.
[3]Department of Pathology, Stanford University School of Medicine, Stanford, California.
[4]Neurology Service VA Greater Los Angeles Healthcare System Los Angeles, California.
[5]Department of Medicine, University of California, Irvine, California.
[6]To whom correspondence should be addressed at Department of Neurology, University of California Irvine, 100 Irvine Hall, Irvine, California 92697-4275; e-mail: qiny@uci.edu.

INTRODUCTION

Although early studies demonstrated axonal degeneration in multiple sceleros (MS), it has been believed that axonal loss is a secondary or bystander event of inflammatory demyelination in chronic MS lesions and occurs too late to be clinically important. Recent findings showing axonal damage in acute and chronic active MS lesions, particularly in areas of acute inflammation and demyelination, have suggested that axonal transection can begin much earlier during the course of the disease.

Infiltration of T and B lymphocytes, monocytes, and macrophages, demyelination, and axon loss are hallmarks of MS (1–4). The mechanisms of demyelination and axonal loss are uncertain. As in the animal model, experimental autoimmune encephalomyelitis, myelin protein-reactive T cells are considered to initiate the disease process (5–7). In MS, oligoclonal immunoglobulin (Ig) bands are present in the cerebrospinal fluid (CSF) of approximately 90–95% of MS patients (8–10). Sequence analyses of variable heavy (V_H) and light (V_L) chain region genes have shown dominant B-cell clonal expansion in the CSF and in lesions of patients with MS, as well as patients with clinically isolated syndrome (CIS) suggestive of MS (11–15). Recently, single cell PCR of CSF B cells derived from patients with clinically confirmed MS and patients with CIS suggestive of MS also showed intrathecal B cell clonal expansion demonstrated by dominantly expressed complementarity determining region 3 (CDR3) nucleotide sequences from both V_H and V_L genes (16, 17). These intrathecal B cells have presumably undergone antigen-driven somatic hypermutation through their antigen receptor in the germinal centers of secondary lymphoid tissue. These findings suggest a persistent antigen-driven chronic immune process by which B cells with a high-affinity for

0271-9142/05/0500-0254/0 © 2005 Springer Science+Business Media, Inc.

the central nervous system (CNS) antigen are clonally selected and expanded in the CNS of patients with MS.

Using the gene-engineering antibody synthesis technique, we have synthesized single chain-variable domain fragments (scFv) antibodies from the Ig V_H and V_L chains expressed by clonally expanded B cells in the CSF of patients with CIS suggestive of MS or with relapsing/remitting MS (RRMS). Immunochemical staining showed that these CSF-derived clonal single chain-variable domain fragment CSFC-scFv Abs bound specifically to axons in MS lesions.

MATERIALS AND METHODS

Tissues

For RNA preparation, CSF cells were obtained from each of two MS patients, one with CIS suggestive of MS (case 1) and one with RRMS (case 2). The duration of disease was 6 months and 7 years, respectively. DNA from a phage clone selected from a human IgG phage display library was used as an irrelevant scFv antibody control. The postmortem brain tissue specimens used for immunochemical staining were from four MS cases and were provided by the Neurospecimen Bank at the Neurology Service, VA Greater Los Angeles Healthcare System, University of California, Los Angeles, CA. These four MS patients included two females and two males with clinically definite MS with a mean age of 53.3 ± 5.7 years and an average duration of disease of 18.8 ± 7.6 years. The mean postmortem interval was 15.0 ± 3.5 h. The brain tissues from a normal donor and a patient with Parkinson's disease served as controls. Each specimen was fixed in 10% formalin and embedded in paraffin for immunocytochemistry.

PCR Amplification of V_H and V_L Chain Genes

Total RNA was extracted from 1.6×10^4 (case 1) and 0.6×10^4 (case 2) CSF B cells of the two MS patients using RNeasy kits (QIAGEN). First strand cDNA was synthesized using oligo d(T) as primer and avian myeloblastosis virus reverse transcriptase. DNA extracted from a phage clone was used as control. The V_H and V_L genes were amplified by PCR (18) in a final volume of 50 μL of reaction buffer [50 mM Tris–HCl, pH 9.0 at 25°C: 20 mM $(NH_4)_2SO_4$, 3.0 mM $MgCl_2$] containing 2 units of recombinant Taq Polymerase and 50 pmoles of V_H primers (13) or V_L primers (19). PCR was carried out for 35 cycles under standard conditions (denaturation 1 min at 94°C, annealing 2 min at 52–56°C, extension 1 min at 72°C). Aliquots of the PCR product were analyzed by electrophoresis in a 2% agarose gel (Sigma) containing ethidium bromide. When no product was obtained by PCR, nested PCR was performed.

Sequencing of Ig V_H and V_L Genes

PCR products were recovered and ligated into the pGEM T vector (Promega, La Jolla, CA) and transfected into *Escherichia coli* DH5a according to Hanahan (20). The double-stranded DNA template from vectors containing V_H or V_L gene inserts was sequenced using a DNA sequencer (ABI-PRISM).

Assignment of Mutations

Mutations were defined on the basis of nucleotide changes in the V_H and V_L segment identified by comparing each sequence with germline sequences (NCBI, The National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov). Variability at the joining sites of the V_H, D, and J_H or the V_L and J_L gene segments was not classed as mutation, since it could not be distinguished from N region insertion. Two nucleotide exchanges in a single codon were scored as a single replacement mutation.

Construction of scFv Genes

V_H and V_L genes of clonally expanded intrathecal B cells from both patients and a phage clone were amplified separately by PCR using V_H or V_L gene primers with a linker peptide consisting of three units of Gly_4Ser[3] (21). PCR products of V_H-linker and V_L-like genes from each case were assembled to a scFv by further PCR amplification with the sense primer 5′-GCC*GTCGAC*AAATGGCCCAGGTGAAACTGC-3′ and the antisense primer 5′-GC*CTCGAG*ACGCG GTTCCAGCGGATC-3′ to introduce a *Sal*I or *Xho*I restriction site, respectively. The fusion fragments were then ligated into the pET32-b expression vector in frame with the N-terminal His-tag sequence. The vector pET-32-b(+)-CSFC-scFv structure, after being confirmed by DNA sequencing (Fig. 3 Amino Acid V_H-linker-V_L), was transformed into AD 494(DE3)pLysS expression bacteria.

Expression, Purification and Refolding of scFv Antibodies

Competent AD494 cells transformed by scFv-ligated pET32-b were grown at 37°C in 100 mL of LB broth containing 50 μg/mL of kanamycin until the $O.D._{600}$ of

the culture reached 0.6, when IPTG was added at a final concentration of 1 mM. The cells were then shaken at 37°C for 3.5 h and harvested by centrifuged at 4,500 *g* for 10 min. The cell pellet was resuspended in 10 mL of lysis buffer containing 50 mM Tris–HCl (pH 8.0), 2 mM EDTA, and 100 μg/mL lysozyme (Novagen) and shaken at room temperature for 20 min. The samples were sonicated in ice-bath for 40 min (Biologies, Inc. Model 300V/T). Then, samples were spun at 20,000 *g* for 30 min to separate inclusion body from soluble cellular proteins. The scFv containing inclusion body was then solubilized in solubilization buffer (0.1 M Tris, pH 8.0, 6 M guanidine, 2 mM EDTA, 0.3 mM dithiothreitol) at the final concentration of 2 mg inclusion body/mL and incubated overnight at 4°C. The sample was spun at 20,000 *g* for 30 min to remove any insoluble material.

Refolding of scFv protein was initiated by a rapid 100-fold dilution of a stock scFv containing inclusion body into refolding buffer (0.1 M Tris, pH 8.0, 0.5 M L-arginine, 2 mM EDTA, 1 mM reduced glutathione and 0.2 mM oxidized glutathione). This refolded inclusion body containing buffer was dialyzed against 1 X PBS buffer pH 8.0 (AccuGENE) overnight at 4°C and further purified with BD-TALON Purification and Buffer Kits according to the procedures suggested by the manufacture (BD). The purity of ScFvs was determined by Coomassie staining of SDS-PAGE and by anti-his Western blot. Refolded scFv protein concentration was determined using Quick Start Bradford Protein Assay kit (Bio-Rad). From a stock of 0.2–0.3 mg wet inclusion body as starting material, we usually obtained 6.5–12.5 μg purified ScFv protein.

SDS-PAGE and Western Blotting

The recombinant protein was dissolved in SDS-PAGE buffer under reducing conditions and subjected to electrophoresis on 10% SDS gels, then either stained with 0.25% Coomassie brilliant blue R250 or transferred to a PVDF membrane (Roche). After blocking with 5% skim milk (Difco, USA), the membrane was incubated for 2 h at 37°C with 1:5000 dilution of anti-His_6 mAb (Novagen, California), followed by HRP-conjugated rabbit anti-mouse IgG antibody (Dako, California) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) as staining substrate.

Dot Blot Assay

Native specific binding of CDR3 epitope on refolded scFv Abs to anti-scFv- V_H-CDR3 peptide antibody, induced by immunizing a rabbit with CDR3 peptide expressed by case 2 (Penlabs Inc) was examined by a dot blot assay. Refolded scFv proteins in PBS were applied to nitrocellulose membranes (1 μg/dot). These membranes were treated as Western blot membranes. After blocking, the dots in lanes were separated and incubated with 1:500 diluted mouse monoclonal antibody to His_6 (Qiagen) and 1:500 diluted rabbit polyclonal antibody to scFv- V_H-CDR3 of case 2, for 1 h, followed by four washes and incubation with goat anti-mouse IgG (Bio-Rad) and anti-rabbit IgG antibodies. The membranes were then washed four times and developed by Opti-4CN substrate kit (Bio-Rad).

Immunohistochemistry

Paraffin brain sections (6 μm thick) were cut on a microtome, picked up on gelatinized slides, and dewaxed. Then the endogenous peroxidase activity was blocked by incubation with 1% H_2O_2 in ethanol for 30 min. An immunoperoxidase method was used to localize bound scFv antibodies; all steps were performed at room temperature. Briefly, sections were rinsed in phosphate-buffered saline containing 0.1% Tween 20 (PBS/Tween) and incubated for 30 min with 5% porcine serum, then for 2 h with biotin-conjugated scFv antibody, prepared as described previously (22). They were then washed, incubated for 1 h with peroxidase-conjugated anti-biotin antibody (Dako), then developed for 10 min with 3′ diaminobenzidine–H_2O_2 as recommended by Dako. The sections were counterstained with haematoxylin and eosin, dehydrated, and coverslipped. Negative control sections were processed with an irrelevant scFv Ab from a phage clone from a human IgG phage display library and without scFv antibody. The brain tissues from a normal donor and a patient with Parkinson's disease served as tissue controls. Table I listed antibodies used for this study.

RESULTS

Clonal Expansion of Intrathecal B Cells

The Ig V_H and V_L genes of the intrathecal cells and from a phage clone were analyzed by RT-PCR and gene sequencing. RT-PCR showed that the V_H and V_L genes expressed by intrathecal cells from MS patients and a

Table I. Primary Antibodies Used for Immunocytochemistry

Antigen	Antibody type	Target	Source
CD3	polyAb	T cells	Dako, California
CD68	mAb	Macrophages/ Microglia	Dako, California
Biotin	Peroxidase-conjugated F(ab')		Dako, California

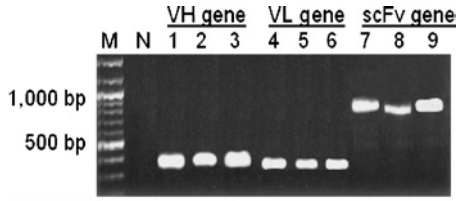


Fig. 1. Amplification of V_H, V_L, and scFv genes from intrathecal B cells and a phage clone from a human IgG phage display library. Lane M, 100 bp Marker; Lane N, negative control; Lanes 1-3, V_H for case 1 (lane 1), case 2 (lane 2) and an irrelevant phage clone (lane 3); lanes 4–6, V_L for case 1 (lane 4), case 2 (lane 5) and an irrelevant phage clone (lane 6); lanes 7–9, V_H-linker-V_L fused scFv genes for case 1 (lane 7), case 2 (lane 8) and an irrelevant phage clone (lane 9).

control phage clone (Fig. 1, lanes 1 to 6). B cell clonality was determined by analyzing the deduced amino acid sequences of CDR3 in the joining region of the V_H chain and the V_L chain, which showed clonal expansion in case 1 and case 2 (Fig. 2). The clones derived from the same patient were identical in the V_H-D-J_H regions (V_H) and in the V_H-J_H regions (V_L) in terms of sequence, with a defined V_H and D or V_L and J_L fragment usage.

V_H and V_L Family Utilization and Somatic Hypermutations

The clonal expansion of intrathecal B cells in MS raises questions about the origin and differentiation pathway of these cells. To elucidate these issues, the sequences of the V_H and V_L genes derived from the two cases were analyzed. Table II shows the V_H and V_L family and gene segment utilization of the clonal B cells. The V_H and V_L gene rearrangement used V_H 3-30 and V_L-B3 in case 1 and V_H 3-53 and V_L-L2 in case 2. The differences in sequences compared with the closest known germline V_H and V_L genes are also summarized in Table II.

In case 1, the V_H and V_L gene sequences showed the highest degree of similarity with the V_H germline sequence V_H 3-30 (27) and the V_L germline sequence V_L-B3 (28) (94 and 97% identity, respectively). The V_H gene contained 14 substitutions, 8 in the FRs (5 resulting in amino acid substitution) and 6 in the CDRs (5 resulting in amino acid substitution). The replacement (*R*) to silence (*S*) mutation ratio in the FRs and CDRs was 1.7 and 5.0, respectively. The V_L gene had six substitutions, two in the FRs and four in the CDRs, the *R*/*S* mutation ratio in the FRs and CDRs were 1.0 and 3.0, respectively. In case 2, the V_H and V_L gene sequences showed 94% identity with the V_H germline gene V_H3-53 (27) and 94% identity with the V_L germline gene V_L-L2 (29). The V_H gene showed 20 substitutions, 13 in the FRs (3 resulting in amino acid substitution) and 7 in the CDRs (6 resulting in amino acid substitution) The *R*/*S* mutation ratio in the FRs and CDRs being 0.3 and 6.0, respectively. The V_L gene showed 12 substitutions, 7 in the FRs and 5 in the CDRs. The *R*/*S* mutation ratio in the FRs and CDRs was 6.0 and 1.5, respectively. Taken together, these findings indicate that the intrathecal B cells derived from these two patients had undergone antigen-driven somatic hypermutation in the germinal centers of the secondary lymphoid tissue and had been clonally expanded in the CNS.

Case	Ig V chain	Identical clone	VDJ or VJ	
Case 1	V_H	7/8	CAKDMGYHYASGSSYFDSWGQ	(VDJ)
	V_L	9/10	CARGGYYTTPYTFGQGTKL	(VJ)
Case 2	V_H	7/8	CARGWRGVAARLAHWGQ	(VDJ)
	V_L	8/10	YYCQEYNNWPPTLTSGGGTKL	(VJ)
Phage clone	V_H	5/5	CARVGPYSWDDFPRQYYWGQ	(VDJ)
	V_L	5/5	NVGTAALWFFGRKGPWRP	(VJ)

Fig. 2. CDR3 gene-encoded amino acid sequences expressed by intrathecal B cells and a phase clone.

Construction of the scFv Fragments

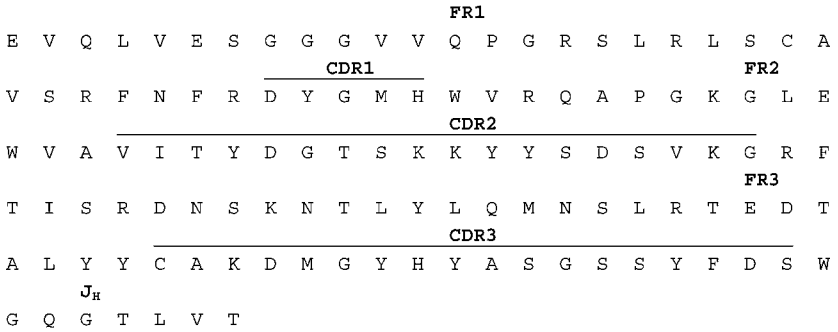
To generate the CSFC-scFv Abs, the clonally expanded V_H and V_L chain genes from each of two CSF clones and a phage clone were assembled with the linker DNA fragment (Fig. 1, lanes 7 to 9) and ligated into pET32b(+). Figure. 3 showed gene-encoded amino acid sequences of scFv-genes. The expression plasmid was then introduced into DA494 competent cells and induced by 1 mM IPTG

Table II. V_H and V_L Family Usage and Differences in the Nucleotide Sequences of Ig Variable Genes

Case	Germline V gene	Percent homology	FRs *R*/*S*	CDRs *R*/*S*	Total FRs	Total CDRs	*R*:*S* FRs *R*/*S*	*R*:*S* CDRs *R*/*S*
Case 1								
	V_H 3–30	94	5/3	5/1	8	6	1.7	5.0
	V_L B3	97	1/1	3/1	2	4	1.0	3.0
Case 2								
	V_H 3–53	93	3/10	6/1	13	7	0.3	6.0
	V_L L2	94	6/1	3/2	7	5	6.0	1.5

Case 1

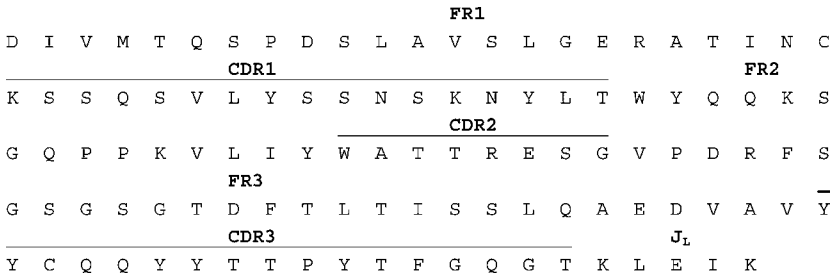
V_H3-30



Linker

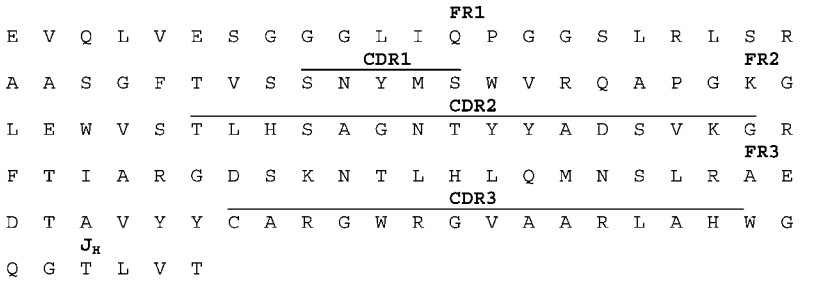
G G G G S G G G G S G G G G S

V_L-B3



Case 2

V_H3-53



Linker

G G G G S G G G G S G G G G S

V_L-L2

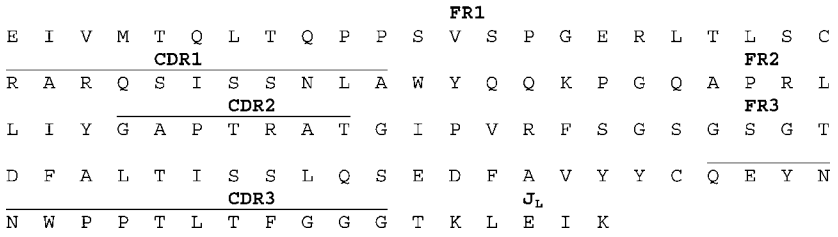


Fig. 3. Continued

Phage clone

V_H1-3

												FR1											
E	V	Q	L	V	E	S	G	A	E	V	K	K	P	G	A	S	V	K	V	S	C	Q	
								CDR1												FR2			
A	S	G	Y	R	F	S	N	F	D	I	H	W	V	R	Q	A	P	G	Q	R	F	E	
												CDR2											
W	M	G	W	I	N	P	Y	N	G	N	K	E	F	S	A	K	F	Q	D	R	V	T	
																				FR3			
I	T	A	D	T	S	A	N	T	A	Y	M	E	L	R	S	L	R	S	A	D	T	A	
												CDR3											
V	Y	Y	C	A	R	V	G	P	Y	S	W	D	D	F	P	R	Q	Y	Y	W	G	Q	
		J_H																					
G	T	L	V	T																			

Linker

G	G	G	G	S	G	G	G	G	S	G	G	G	G	S

V_L-V2-1

												FR1											
G	E	S	L	T	H	P	P	S	V	S	V	S	P	G	Q	T	A	R	N	T	C	S	
				CDR1																FR2			
G	D	K	M	G	E	K	Y	A	C	W	Y	Q	Q	K	P	G	R	T	P	S	L	V	
				CDR2																FR3			
I	Y	E	D	T	K	R	P	S	G	I	P	D	R	F	S	G	S	N	S	G	N	T	
A	T	L	T	I	N	G	T	Q	A	X	D	E	X	D	Y	F	C	Q	N	V	G	T	
				CDR3																			
A	A	L	W	F	F	G	R	K	G	P	W	R	S	N	V	E							

Fig. 3. V_H-Liker-V_L gene-encoded amino acid sequences of scFv expressed by pET32-b vector. FR, framework region; CDR, complementarity determining region.

at 37°C. As shown in Fig. 4, SDS-PAGE and Western blotting analysis showed that a protein band with an apparent molecular weight of about 47 kDa was strongly expressed after IPTG induction for 3 h (Fig. 4 lanes 2 to 4), but not without IPTG induction (Fig. 4 lane 1). After purification on an anti-His column, each scFv antibody gave a single band (Fig. 4 lanes 5 to 7), which was recognized by anti-His_6 mAb (Fig. 4 lanes 8 to 10).

Native Specific Binding of Conformational Ab to V_H-CDR3 Peptide of scFv Ab

To study whether refolded scFv molecules carry the native-conformational structure of V_H-CDR3, we generated rabbit antibody to V_H-CDR3 peptide of case 2 (RRMS). The specific binding of the refolded scFv molecule to the conformational antibody, anti-V_H-CDR3 peptide (case 2, RRMS), was examined by Western dot assay. The result showed that the scFv Ab derived from case 2 responded specifically to its corresponding anti-CDR3 antibody. No cross reactivity to CSFC-scFv-Ab of case 1 and to control scFv was observed (Fig. 5).

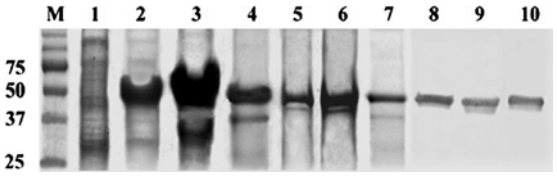


Fig. 4. SDS-PAGE and Western blotting analysis using anti-His6 mAb. Lane M, Mid-range protein molecular weight markers (kDa): 75, 50, 37, and 25; lane 1, AD494 competent cell culture supernatant without IPTG induction; lanes 2, 3 and 4 inclusion body proteins from case 1 (lane 2), case 2 (lane 3) an irrelevant phage clone (lane 4) and after 3 h IPTG induction; lanes 5, 6 and 7 anti-His_6 column purified protein from case 1 (lane 5), case 2 (lane 6) and an irrelevant phage clone (lane 7); lanes 8–10, Western blotting analysis of the protein in lanes 5–7.

Immunoreaction of CSFC-scFv Ab in the MS Brain

To study the brain antigens recognized by antibodies produced by clonally expanded CSF B cells in MS, we stained two lesions from each of four MS brains with the two CSFC-scFv Abs and an irrelevant scFv Ab generated from a phage clone selected from a human IgG phage display library. All samples used in this study fulfilled

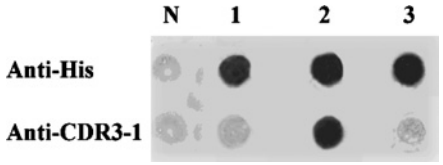


Fig. 5. The specific binding of refolded scFv molecule to the conformational antibody. Lane N, negative control applied with PBS; Lane 1 applied with scFv protein of case 1; Lane 2 applied with scFv protein of case 2 and Lane 3 applied with scFv protein of a phage clone. Anti-His, incubate with mouse antibody to His_6; Anti-CDR3, incubate with rabbit antibody to V_H-CDR3 peptide of case 2.

the neuropathological criteria of MS. The criteria is defined by plaques containing at least one area in the active stage of demyelination, as manifested by demyelination, T cell and macrophage infiltration, and reactive microglia at the border between areas with a normal and reduced density of myelinated fibers (Fig. 6 A–C). Lesion-infiltrated macrophages contained intracytoplasmic granules of myelin debris that were reactive for myelin basic protein (not shown).

Immunochemical staining revealed an abundant binding of CSFC-scFv Ab to axons within the area of an acute demyelinated lesion adjacent to normal-appearing white matter, but not to the adjacent myelin sheath. Figure 6D (immunochemical staining with scFv Ab from case 2) and Figure 6E (immunochemical staining with scFv Ab from case 1) showed CSFC-scFv-Ab positive axons in acute lesions. No immunoreactivity was seen in lesions stained with an irrelevant control scFv-Ab generated from a phage clone selected from a human IgG phage display library (Fig. 6F). Staining revealed several different patterns of axons, including terminal axons with boutons (Fig. 6G), thin linear axons (Fig. 6H), swellings (ovoids) and disjointed axons (Fig. 6I), degenerating axon terminals (Fig. 6J), massive axon degeneration (Fig. 6K), a ring of degenerated axons (Fig. 6L) and degenerated axon residuals (Fig. 6M). These axon staining patterns were observed in different lesions stained with CSFC-scFv-Abs from both patients, but not in lesions stained with an irrelevant scFv-Ab generated from a phage clone selected from a human IgG phage display library. The axon patterns presented in Fig. 6H, I, K, L, and M were seen in three lesions from MS brains of case 1 and 2. The staining pattern shown in Fig. 6J was also observed in three lesions from MS brains of cases 1, 2 and 3. The residuals of degenerated axons depicted in Fig. 6M and Fig. 6P were seen in 7 of 8 lesions from all 4 MS brains. These patterns of axonal changes may represent axonal damage during different time courses of inflammation. Based on lesion activity, CSFC-scFv Ab-stained axons revealed different axonal damage in three lesion areas. Area 1 in Fig. 6A and D showed that at the border between areas of active demyelination and normal-appearing white matter, most CSFC-scFv Ab-positive axons appeared as continuous lines (Fig. 6H) with terminal ovoid formations (Fig. 6I). Area 2 of Fig. 6A and D showed that degenerating CSFC-scFv Ab-positive axons and ovoids (Fig. 6J) were most numerous in areas of demyelination. In addition, in these areas, CSFC-scFv-positive axons revealed an accumulation of grape-like ovoid formations (Fig. 6N). Area 3 of Fig. 6A and D showed that at the center of acute lesions, where LFB staining demonstrated marked demyelination, CSFC-scFv Ab-positive axons showed Wallerian-type terminal degeneration, characterized by irregular disjointed and truncated fragments of axons and axonal ovoids with axon lines passing through the region (Fig. 6O, arrows). Few CSFC-scFv Ab-positive axon residuals were seen in end-stage lesions (Fig. 6P). No CSFC-scFv Ab immunoreactivity was seen in normal-appearing white matter distant from acute lesions (Fig. 6Q) or in brain tissues from a patient with Parkinson's disease (Fig. 6R) or a normal donor (Fig. 6S) or in the absence of CSFC-scFv Ab (Fig. 6T). These results provide evidence for B cell-mediated autoimmunity directed against axons in MS and demonstrate that CSFC-scFv Ab-positive axons show pathologic changes which correlated with lesion activity.

DISCUSSION

To study the immune mechanisms underlying axonal damage in MS, we analyzed the V_H and V_L genes expressed by intrathecal B cells derived from two patients; one with CIS and one with RRMS. The clonally expanded V_H and V_L genes from CSF B cells were fused to generate CSFC-scFv Abs, which were then tested for binding to lesions in MS brain sections. Immunochemical staining showed that these CSFC-scFv antibodies bound to the vast majority of axons and axonal ovoids in acute lesions and in the adjacent normal-appearing white matter. Clonally expanded axon-reactive B cells were found in the CSF of patients with both CIS suggestive of MS and RRMS, indicating that an immune response directed against axons can be initiated at an early stage of disease and persistently drives B cell clonal expansion during disease progression.

Early studies using a silver impregnation technique and electron microscopy demonstrated axonal degeneration in MS (23–25). Recently, immunocytochemical staining for amyloid precursor protein (ß-APP) and nonphosphorylated neurofilament (SMI-32) as well as imaging studies have provided support for axonal damage in the CNS in MS and its correlation with serious functional consequences (26–30). These findings have implications for both the diagnosis and treatment or prophylaxis of CNS damage and raise the question of whether an immune response against axons occurs in the CNS of these patients and is responsible for this damage.

The study of such immune mechanisms has been hampered by the lack of experimental systems able to analyze axonal immunoreactivity of CNS lymphocytes. Our previous studies (11, 13) showed that clonal expansion of intrathecal B cells is a common and early event in an inflammatory reaction in the CNS in MS. These findings provide the opportunity to study the antigens that initiate and persistently drive B cell clonal expansion in the CNS

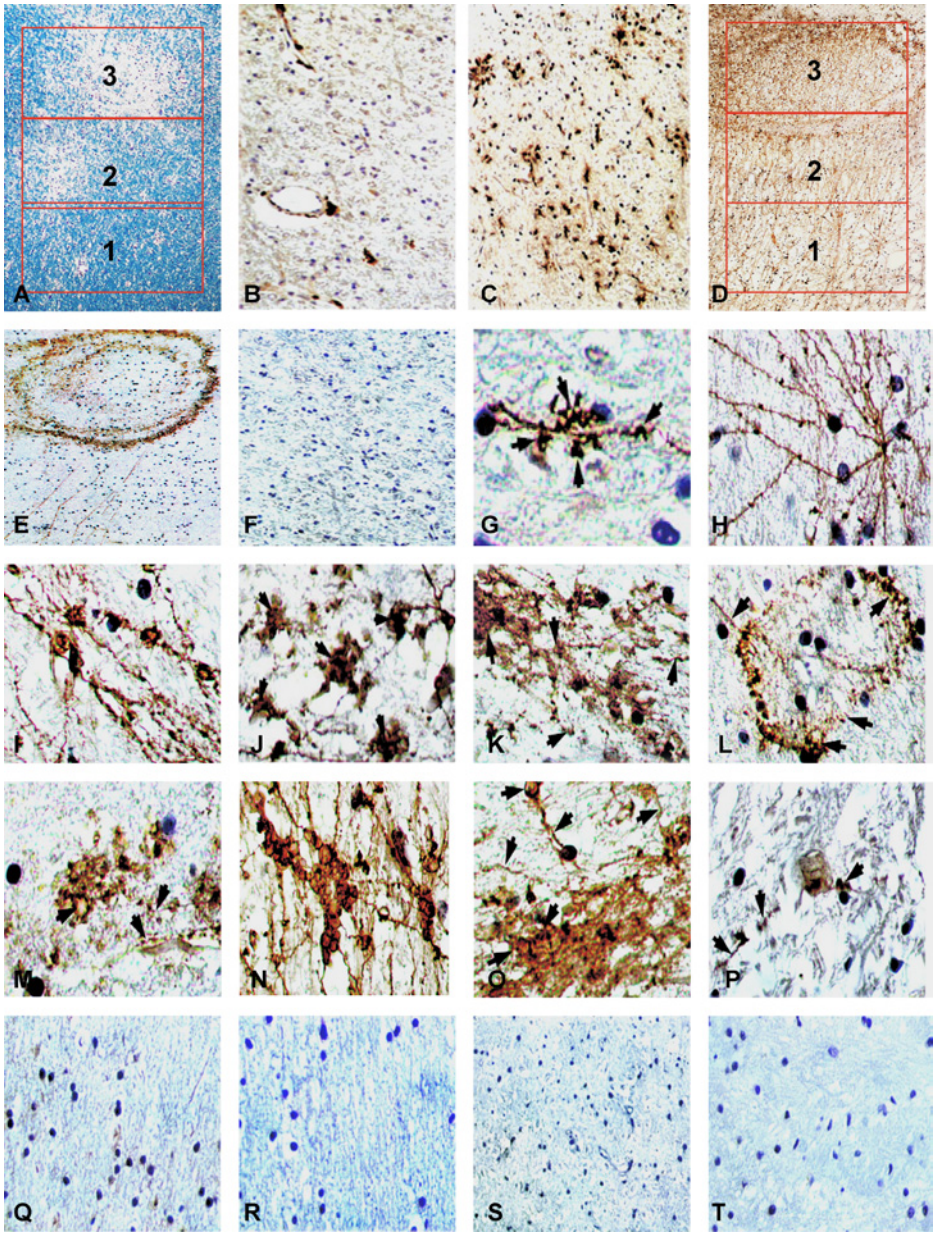


Fig. 6. CSFC-scFv Ab immunoreactivity of axons in multiple sclerosis. (A): Luxol fast blue (LFB)-hematoxylin and eosin staining showing the edge of the plaque with intact myelin shown in blue and demyelination in white (×100). (B and C): Perivascular infiltration of CD3+ T cells (B, ×200) and CD68+ macrophages (C, ×200) in sections adjacent to that shown in A. CSFC-scFv Ab-immunoreactive axons in acute lesions, stained with scFv-Ab from case 2 (D) and stained with scFv-Ab from case 1 (E) (×100). No immunoreactive axon was seen in lesions stained with a control scFv Ab generated from an irrelevant phage clone from a human IgG phage display library (F, 100×). CSFC-scFv Ab binding a terminal axon with boutons (G, arrows), thin linear axons (H), swellings (ovoids) and disjointed axons (I, arrows), a degenerating axon terminal (J, arrows), massive axon degeneration (K), a ring of degenerated axons (L) and degenerated axon residuals (M) (all ×1,000). (H and I): Axonal damage characterized by thin linear axons with ovoid formation (1,000 magnification of area 1 in D), grape-like ovoid formation (N, ×1,000 magnification of area 2 in D), and Wallerian degeneration (O, ×1,000 magnification of area 3 in D). (P): Axon residuals in end-stage lesions. (Q–T): No CSFC-scFv Ab immunoreactivity was seen in normal-appearing white matter far from acute lesions (Q, ×200), or in brain tissue from a patient with Parkinson's disease (R, ×200) or a normal donor (S, ×200) and in the absence of CSFC-scFv Ab (T) (×100).

in MS. Clonal expansion is based on immune memory, which results in an enhanced response on restimulation with the same antigen (31, 32). Memory is generated by the increase in the affinity of B cell clones for antigen resulting from the somatic mutation of Ig V genes, with the B cells undergoing a Darwinian clonal positive selection process based on their affinity for antigen held on follicular dendritic cells in germinal centers (33–36). High-affinity mutants survive, whereas low-affinity ones cannot bind antigen and undergo apoptosis (33). Under persistent antigen stimulation, memory B cell clones undergo massive proliferation and dominate a specific B cell mediated humoral immune response throughout the lifetime of the organism. In the present study, sequence analysis of the Ig CDR3 and VDJ genes showed that B cell clonal expansion occurred in the CSF in the two patients studied. When compared to the germline, the V_H and V_L genes contained somatic mutations distributed in a pattern characteristic of antigen-driven affinity maturation, i.e. the somatic mutations were highly concentrated in the CDRs or FRs, with a clustering of replacement mutations in the CDRs and only a few in the FRs. The CDR region is considered to be critical for antigen specificity (37), and somatic hypermutations in these regions might contribute to the generation of an antibody with a high-affinity for the CNS antigen. A high CDR *R*/*S* mutation ratio reflects the positive selective pressure of an antigen on those gene products which come into close contact with antigen, while a low FRs *R*/*S* mutation reflects the negative pressure for mutant selection applied to structural components that need to be conserved (38). This pattern is consistent with the notion of an antigen-driven selection of antibodies with high-affinity antigen-binding sites and was seen in both patients in the present study. The results showed that clonally expanded intrathecal B cells from the patients studied had undergone antigen-driven somatic hypermutation and affinity maturation in the germinal centers of secondary lymphoid tissue and been recruited to infiltrate and clonally expand in the CNS.

To examine antigens that drive B cell clonal expansion in the CNS, we generated CSFC-scFv Abs engineered from the V_H and V_L genes of B cells obtained from the CSF of a patient with CIS suggestive of MS and from a patient with RRMS. An irrelevant scFv-Ab generated from a clone phage selected from a human IgG phage display library was used as negative control of structure-matched scFv molecule. Native conformation of the refolded scFv-Ab was examined by a scFvb-V_H-CDR3 specific conformational antibody. Previous reports have shown that heavy chain CDR3 structure (length) has been considered as a critical parameter in the creation of an antigen-specific binding site (39, 40). Our results show that the V_H-CDR3 epitope of the scFv molecule, refolded by the method we used, specifically bound its corresponding anti-V_H-CDR3 rabbit antibody, which may play an important role in antigen specific binding. Immunochemical staining showed that the CSFC-scFv Abs produced from both patients bind axons. Immunochemical staining with an irrelevant scFv antibody from a random selected phage clone did not bind axons. Binding of ms-derived CSFC-scFv Ab to axons was seen as thin lines and as ovoids with disjointed and truncated fragments of axons, which represent axonal swellings and Wallerian degeneration. A recent study of the time-course of events following spinal cord injury in an animal model (41) showed that axonal swelling, axonal fragmentation and an increase in the spacing between axons during weeks 1–5. Wallerian degeneration followed by debris-laden phagocytes became evident during weeks 7–8. Invading macrophages remove the debris at the lesion and scar tissue forms due to astrogliosis. The morphology of scFv Abs-reactive axons suggested that the antibodies secreted by clonally expanded CSF B cells from patients with CIS or RRMS bind axons in lesions with acute inflammation, but not to chronic inactivated lesions or normal—appearing white matter distant from the acute lesions. These antibodies also do not bind to brain specimens from a patient with Parkinson's disease and a normal donor. These findings suggest that an unidentified axonal antigen(s), which may be expressed or exposed during acute inflammation, initiates and persistently drives B cell clonal expansion in the CNS of patients with CIS suggestive of MS or with RRMS.

Any type of trauma to the CNS has the potential to produce the "domino effect" type of degeneration, in which additional systems are progressively recruited into a degenerative chain reaction of transaxonal degeneration. The findings of B cell clonal expansion in the CNS of the patient with CIS suggestive of MS and that the antibodies produced are axon-reactive suggests that the axonal antigen-induced immune response may be recruited into the immune cascade at an early stage of disease. Furthermore, the presence of CSFC-scFv Ab immunoreactive axons in acute lesions of patients who had MS for more than 15 years suggest that patients with even longstanding chronic progressive MS may exhibit signs of more recent ongoing axonal degeneration and demyelination.

ACKNOWLEDGMENTS

We thank Dr. Bruce D. Trapp for comments, and Ms. Diane Guntrip, Ms. Manzx Zhon, and Mr. James S. Riehl for the brain tissue preparation and technical assistance. We are also grateful to Dr. T. Barkas for

critical review of this manuscript. This study was supported by grant RG 3156A1/1 from the National Multiple Sclerosis Society and by grants RO1 NS40534-01A1 and NS 046414 (R.A.S) from the National Institutes of Health.

REFERENCES

1. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H: Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. Ann Neurol 47:707–717, 2000
2. Compston A, Coles A: Multiple sclerosis. Lancet 359:1221–1231, 2002
3. Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M, Dean EJ, Fournel S, Fong D, Genovese MC, de Vegvar HE, Skriner K, Hirschberg DL, Morris RI, Muller S, Pruijn GJ, van Venrooij WJ, Smolen JS, Brown PO, Steinman L, Utz PJ: Autoantigen microarrays for multiplex characterization of autoantibody responses. Nat Med 8:295–301, 2002
4. Chabas D, Baranzini SE, Mitchell D, Bernard CC, Rittling SR, Denhardt DT, Sobel RA, Lock C, Karpuj M, Pedotti R, Heller R, Oksenberg JR, Steinman L: The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 294:1731–1735, 2001
5. Ben-Nun A, Wekerle H, Cohen IR: Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein. Nature 292:60–61, 1981
6. Schluesener HJ, Lider O, Sobel RA: Induction of hyperacute brain inflammation and demyelination by activated encephalitogenic T cells and a monoclonal antibody specific for a myelin/oligodendrocyte glycoprotein. Autoimmunity 2:265–273, 1989
7. Itoyama Y, Webster HD: Immunocytochemical study of myelin-associated glycoprotein (MAG) and basic protein (BP) in acute experimental allergic encephalomyelitis (EAE). J Neuroimmunol 3:351–364, 1982
8. Tourtellotte W: On cerebrospinal fluid immunoglobulin-G (IgG) quotients in multiple sclerosis and other diseases. A review and a new formula to estimate the amount of IgG synthesized per day by the central nervous system. J Neurol Sci 10:279–304, 970
9. Tibbling G, Link H, Ohman S: Principles of albumin and IgG analyses in neurological disorders. I. Establishment of reference values. Scand J Clin Lab Invest 37:385–390, 1977
10. Esiri MM: Immunoglobulin-containing cells in multiple-sclerosis plaques. Lancet 2:478, 1977
11. Qin Y, Duquette P, Zhang Y, Talbot P, Poole R, Antel J: Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. J Clin Invest 102:1045–1050, 1998
12. Owens GP, Kraus H, Burgoon MP, Smith-Jensen T, Devlin ME, Gilden DH: Restricted use of VH4 germline segments in an acute multiple sclerosis brain. Ann Neurol 43:236–243, 1998
13. Qin Y, Duquette P, Zhang Y, Olek M, Da RR, Richardson J, Antel JP, Talbot P, Cashman NR, Tourtellotte WW, Wekerle H, Van Den Noort S: Intrathecal B-cell clonal expansion, an early sign of humoral immunity, in the cerebrospinal fluid of patients with clinically isolated syndrome suggestive of multiple sclerosis. Lab Invest 83:1081–1088, 2003
14. Baranzini SE, Jeong MC, Butunoi C, Murray RS, Bernard CC, Oksenberg JR: B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. J Immunol 163:5133–5144, 1999
15. Colombo M, Dono M, Gazzola P, Roncella S, Valetto A, Chiorazzi N, Mancardi GL, Ferrarini M: Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. J Immunol 164:2782–2789, 2000
16. Ritchie AM, Gilden DH, Williamson RA, Burgoon MP, Yu X, Helm K, Corboy JR, Owens GP: Comparative analysis of the CD19+ and CD138+ cell antibody repertoires in the cerebrospinal fluid of patients with multiple sclerosis. J Immunol 173:649–656, 2004
17. Owens GP, Ritchie AM, Burgoon MP, Williamson RA, Corboy JR, Gilden DH: Single-cell repertoire analysis demonstrates that clonal expansion is a prominent feature of the B cell response in multiple sclerosis cerebrospinal fluid. J Immunol 171:2725–2733, 2003
18. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491, 1988
19. Welschof M, Terness P, Kolbinger F, Zewe M, Dubel S, Dorsam H, Hain C, Finger M, Jung M, Moldenhauer G, *et al*.: Amino acid sequence based PCR primers for amplification of rearranged human heavy and light chain immunoglobulin variable region genes. J Immunol Methods 179:203–214, 1995
20. Hanahan D: Techniques for transformation of E. coli. Washington DC, IRL, 1985, p 109
21. Tsumoto K, Nishimiya Y, Kasai N, Ueda H, Nagamune T, Ogasahara K, Yutani K, Tokuhisa K, Matsushima M, Kumagai I: Novel selection method for engineered antibodies using the mechanism of Fv fragment stabilization in the presence of antigen. Protein Eng 10:1311–1318, 1997
22. Shamsuddin AM, Harris CC: Improved enzyme immunoassays using biotin-avidin-enzyme complex. Arch Pathol Lab Med 107:514–517, 1983
23. Suzuki K, Andrews JM, Waltz JM, Terry RD: Ultrastructural studies of multiple sclerosis. Lab Invest 20:444–454, 1969
24. Ikuta F, Zimmerman HM: Distribution of plaques in seventy autopsy cases of multiple sclerosis in the United States. Neurology 26:26–28, 1976
25. Kornek B, Lassmann H: Axonal pathology in multiple sclerosis. A historical note. Brain Pathol 9:651–656, 1999
26. Matthews PM, De Stefano N, Narayanan S, Francis GS, Wolinsky JS, Antel JP, Arnold DL: Putting magnetic resonance spectroscopy studies in context: Axonal damage and disability in multiple sclerosis. Semin Neurol 18:327–336, 1998
27. Grimaud J, Barker GJ, Wang L, Lai M, MacManus DG, Webb SL, Thompson AJ, McDonald WI, Tofts PS, Miller DH: Correlation of magnetic resonance imaging parameters with clinical disability in multiple sclerosis: A preliminary study. J Neurol 246:961–967, 1999
28. Fisher E, Rudick RA, Cutter G, Baier M, Miller D, Weinstock-Guttman B, Mass MK, Dougherty DS, Simonian NA: Relationship between brain atrophy and disability: An 8-year follow-up study of multiple sclerosis patients. Mult Scler 6:373–377, 2000
29. Paolillo A, Pozzilli C, Gasperini C, Giugni E, Mainero C, Giuliani S, Tomassini V, Millefiorini E, Bastianello S: Brain atrophy in relapsing-remitting multiple sclerosis: Relationship with 'black holes,' disease duration and clinical disability. J Neurol Sci 174:85–91, 2000

30. Pelletier J, Suchet L, Witjas T, Habib M, Guttmann CR, Salamon G, Lyon-Caen O, Cherif AA: A longitudinal study of callosal atrophy and interhemispheric dysfunction in relapsing-remitting multiple sclerosis. Arch Neurol 58:105–111, 2001
31. MacLennan IC, Gray D: Antigen-driven selection of virgin and memory B cells. Immunol Rev 91:61–85, 1986
32. Berek C, Jarvis JM, Milstein C: Activation of memory and virgin B cell clones in hyperimmune animals. Eur J Immunol 17:1121–1129, 1987
33. Gray D, Skarvall H: B-cell memory is short-lived in the absence of antigen. Nature 336:70–73, 1988
34. Jacob J, Kelsoe G: In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. J Exp Med 176:679–687, 1992
35. Liu YJ, Johnson GD, Gordon J, MacLennan IC: Germinal centres in T-cell-dependent antibody responses. Immunol Today 13:17–21, 1992
36. Siekevitz M, Kocks C, Rajewsky K, Dildrop R: Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. Cell 48:757–770, 1987
37. Zhang M, Majid A, Bardwell P, Vee C, Davidson A: Rheumatoid factor specificity of a VH3-encoded antibody is dependent on the heavy chain CDR3 region and is independent of protein A binding. J Immunol 161:2284–2289, 1998
38. Jukes TH, King JL: Evolutionary nucleotide replacements in DNA. Nature 281:605–606, 1979
39. Barrios Y, Jirholt P, Ohlin M: Length of the antibody heavy chain complementarity determining region 3 as a specificity-determining factor. J Mol Recognit 17:332–338, 2004
40. Olsen RJ, Mazlo J, Koepsell SA, McKeithan TW, Hinrichs SH: Minimal structural elements of an inhibitory anti-ATF1/CREB single-chain antibody fragment (scFv41.4). Hybrid Hybridomics 22:65–77, 2003
41. Nashmi R, Fehlings MG: Changes in axonal physiology and morphology after chronic compressive injury of the rat thoracic spinal cord. Neuroscience 104:235–251, 2001