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Cross-reactivity between related sequences found in *Acinetobacter* sp., *Pseudomonas aeruginosa*, myelin basic protein and myelin oligodendrocyte glycoprotein in multiple sclerosis

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Abstract

To investigate the possible role of molecular mimicry to bacterial components in multiple sclerosis (MS) pathogenesis we examined antibody responses to mimicry peptide sequences of *Acinetobacter*, *Pseudomonas aeruginosa* and myelin components.

Antibodies to mimicry peptides from *Acinetobacter* ($p<0.001$), *P. aeruginosa* ($p<0.001$), myelin basic protein (MBP) ($p<0.001$) and myelin oligodendrocyte glycoprotein (MOG) ($p<0.001$) were significantly elevated in MS patients compared to controls.

Antisera against MBP (residues 110–124) reacted with both *Acinetobacter* and *Pseudomonas* peptides from 4- and γ-carboxymuconolactone decarboxylase, respectively. MOG (residues 43–57) antisera reacted with *Acinetobacter* peptide from 3-oxo-adipate-CoA-transferase subunit A.

The role of these bacteria in MS is unclear but demonstrates that molecular mimicry is not restricted to viruses suggesting bacterial infections could play a role in MS pathogenesis. Further work is required to evaluate the relevance of these cross-reactive antibodies to the neuropathology of MS.

Keywords: Multiple sclerosis; *Acinetobacter*; *Pseudomonas*; Myelin basic protein; Myelin oligodendrocyte glycoprotein; Bacteria

1. Introduction

Molecular mimicry has been demonstrated as a possible mechanism by which an infectious agent may induce autoimmune disease, as seen in rheumatic fever (Kaplan and Meyeserian, 1962). A role for molecular mimicry has also been suggested in the pathogenesis of multiple sclerosis (MS) (Albert and Inman, 1999) and a number of different microbial sequences, including *Pseudomonas* peptides, activate T cell clones from MS patients specific to myelin basic protein (MBP) (Wucherpfennig and Strominger, 1995a) thereby indicating that several microorganisms, both viral and bacterial may carry epitopes of myelin. MS patients have previously been related to repeat respiratory tract infections during childhood (Lamoureux et al., 1983), chronic sinusitis infection (Gay et al., 1986) and increased risk of exacerbation due to bacterial, including *Pseudomonas* (Rapp et al., 1995) and viral (Edwards et al., 1998) infection.

We have identified an amino acid homology between a sequence present in 4-carboxymuconolactone decarboxylase (CMLD) of *Acinetobacter calcoaceticus* (Ebringer et al., 1997), γ-carboxymuconolactone decarboxylase of *Pseudomonas aeruginosa* (Hughes et al., 2001) and a sequence of myelin basic protein (MBP) (residues 110–124). This MBP sequence is known to be encephalitogenic in guinea pigs (Eylar et al., 1970) and a similar epitope; MBP 101–120 induces experimental allergic encephalo-

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myelitis (EAE) in DA rats (Stepaniak et al., 1997) and strain 13 guinea pigs (Ben-Nun et al., 1981). MBP 82–100 has been demonstrated to be immunodominant in MS patients for both T cells and autoantibodies (Salvetti et al., 1993; Wucherpfennig et al., 1997). However, more recent studies have shown that T cell responses to MBP can be variable over time. Four more dominant epitopes were identified, including MBP 105–129, which was only recognized by MS patients and not controls. Generation of T cell clones from MS patients defined the specific peptide epitope as MBP 110–124 (Mazza et al., 2002).

A further sequence similarity has been discovered between an epitope of myelin oligodendrocyte glycoprotein (MOG 43–57) known to induce EAE in many mouse strains, including Biozzi ABH mice (Amor et al., 1994), and a sequence from 3-oxoadipate CoA-transferase subunit A from *A. calcoaceticus*. This sequence can also be found in the same enzyme from *Pseudomonas putida*. Interestingly, recombinant MOG reactive T cells from MS patients have been shown to respond to three main regions of MOG including MOG 34–56 (Kerlero de Rosbo et al., 1997; Correale and de los Milagros Bassani Molinas, 2003). Anti-MOG antibodies in MS patients have a highly variable epitope specificity (Haase et al., 2001) although many groups have demonstrated increased autoantibodies to MOG 35–55, especially in secondary progressive MS (Kennel De March et al., 2003). Autoantibodies to both whole MBP (Warren and Catz, 1987) and MOG (Reindl et al., 1999) have been well described in MS patients and elevated levels of antibodies to both *Acinetobacter* and *P. aeruginosa* have previously been observed in MS patients compared to stroke patients and control healthy blood donors (Hughes et al., 2001).

The aims of this study were to determine whether MS patients have elevated levels of antibodies to the mimicry peptide sequences found in *Acinetobacter*, *P. aeruginosa*, MBP and MOG when compared to stroke patients and control healthy blood donors and to assess the extent of cross-reactivity between these amino acid sequences.

2. Materials and method

2.1. Serum samples

Serum samples used were analysed in a previous study for antibodies to whole bacteria, including *Acinetobacter* and *Pseudomonas* (Hughes et al., 2001). Sera from 26 MS patients (9 males/17 females; mean age 42; range 29–55 years) were obtained from the Institute of Neurology at the Hospital for Nervous Diseases, London. Diagnosis was made according to the Poser criteria (Poser et al., 1983). In addition, serum samples were obtained from 20 patients in the Department of Geriatric Medicine at University College Hospital, who had suffered from a unilateral hemiplegia due to a cerebro-vascular accident (CVA) (10 males/10 females; mean age 80.5 years; range 69–94 years). Sera from 25 subjects attending the London Blood Donor services were used as healthy controls (12 males/13 females; mean age 40.6 years; range 22–67 years).

2.2. Peptides

MBP peptide (110–124) with amino acid sequence according to mouse MBP was synthesized. This sequence is homologous to human MBP with the exception that MBP 110–124 differs from the human sequence at position 122 (Lys to Arg). MOG peptide (43–57) was synthesized according to mouse MOG and is identical in amino acid sequence to human MOG. Bacterial mimicry peptides, *Acinetobacter* 4-CMLD, *Pseudomonas* γ-CMLD and *Acinetobacter* 3-oxoadipate CoA-transferase subunit A were also synthesized. Peptide sequences are shown in Table 1. Peptides incorporated a C-terminal amide and were purified via high-performance liquid chromatography (HPLC) (ABC Biotechnology, UK).

2.3. Induction of EAE

ABH (H-2^{dq1}) mice were obtained from stocks bred at Imperial College London, UK. Young adult mice (5 to 8 weeks old) of either sex were used. Animals were injected

Table 1
15mer peptides used in ELISA and EAE studies comparing similar sequences in mouse myelin basic protein and myelin oligodendrocyte glycoprotein to *Acinetobacter* sp. and *Pseudomonas aeruginosa* (identical amino acids in bold) [Retrieved from Swissprot]

Peptide	Source	Amino acid sequence	Amino acid position	Location
1	*Acinetobacter* sp.	QNFI**SRFAWG**EVNSR	38–52	4-carboxy-muconolactone decarboxylase
2	*Pseudomonas aeruginosa*	QEMIT**R**H**AWG**DIWTR	38–52	γ-carboxy-muconolactone decarboxylase
3	MBP	GLSL**SRF**S**WG**AEGQR	110–124	
4	*Acinetobacter* sp.	DSYVFDE**LYRAGK**IE	83–97	3-oxoadipate CoA-transferase subunit A
5	MOG	PFSRVVH**LYRNGK**DQ	43–57	
6	Human papilloma virus (type 16)	TVIQDGDMVHTGFGA	219–233	Major capsid protein L1

The human papilloma virus sequence was used as a control peptide.
A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

subcutaneously (s.c.) with either 1 mg spinal cord homogenate (SCH) or 200 μg peptide/mouse, emulsified by sonication in incomplete Freund's adjuvant (IFA), supplemented with 60 mg of mycobacteria, on days 0 and 7. The mice also received intra-peritoneal injection of 200 ng pertussis toxin, derived from *Bordetella pertussis* (Sigma, UK) dissolved in PBS. Pertussis toxin was given immediately and 24-h post immunization with antigen in complete Freund's adjuvant (CFA) as described previously (Amor et al., 1993).

Animals were weighed from day 9 onwards and checked for neurological signs: 0=normal; 1=limp tail; 2=impaired righting reflex; 3=partial hindlimb paralysis and 4=complete hindlimb paralysis. Mice showing signs of a lesser severity than typically observed were scored as 0.5 less than the indicated grade (Amor et al., 1993).

2.4. Antibody production

At various times after immunization and EAE induction, ABH mice received a further dose of 200 mg peptide in incomplete Freund's adjuvant (IFA) injected s.c. into two sites on the flanks to boost for antibody production. Serum was collected and frozen until assayed.

2.5. Preparation of MOG and MBP

Recombinant MOG (rmMOG) (amino acids 1–116) was synthesized as follows. *Escherichia coli* strain JM109 was transfected with the cDNA encoding murine MOG N-terminal 1–116 amino acids sequence ligated to pRSET A (Invitrogen) expression vector. The pRSET A vector encodes an N-terminal fusion peptide, upstream and in-frame of the MOG DNA insert. The culture was grown in SOB media containing 100 mg/ml ampicillin and 17 mg/ml chloramphenicol (temperature 37 °C, 95% O_2/5% CO_2; 220 rpm). RmMOG expression was initiated by adding 1 mM isopropyl-*b*-D-thiogalactopyranoside (IPTG) (Sigma). Affinity purification of the soluble rmMOG was achieved by TALON metal affinity resin (Clontech), which binds the 6-histidine residues present in the fusion protein. The protein–resin complex was added to the column and non-bound proteins released from the column by repeated washing. The rmMOG protein was eluted from the column and fractions containing the rmMOG, as ascertained by UV spectrophotometer, were pooled. The eluted protein was dialysed for 16 h and concentrated via Centricon Biomax-5K concentrating tubes at 4000 g.

MBP from guinea pig brain was obtained from Sigma-Aldrich.

2.6. ELISA for detection of anti-peptide antibodies in humans

For detection of anti-peptide antibodies in humans, ELISAs were carried out as previously described (Khalafpour et al., 1988). Briefly, 200 μl peptide (5 μg/ml), diluted in a 0.05 M carbonate buffer (pH 9.6), was adsorbed on to a 96-well flat-bottom polystyrene immuno-maxisorp ELISA plate (Nunc) overnight at 4 °C. Plates were washed three times for 5 min in PBS containing 0.05% (v/v) Tween 20 (Sigma) and were blocked with PBS containing 0.1% bovine serum albumin (BSA) (Sigma) for 1 h at 37 °C. The washing procedure was repeated and 200 μl of test or control serum, diluted 1 in 200 in PBS/Tween, was added to the wells in duplicate and incubated for 1 h at 37 °C. Plates were washed three times as above and 200 μl of IgM, IgG or IgA, rabbit anti-human conjugate with horseradish peroxidase (Dako), diluted 1 in 500 in PBS/Tween, was added and incubated for 1 h at 37 °C. The washing procedure was repeated and 200 μl of substrate solution, 0.5 mg/ml 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Sigma) in citrate phosphate buffer, pH 4.1, containing 0.98 mM H_2O_2 (Sigma) was added to each well. The plates were developed in the dark, at room temperature, for 25 min. The reaction was stopped by addition of 100 μl sodium fluoride (2 mg/ml) (Sigma) solution. Absorbances were measured on a micro-titre plate reader (Dynatech MR606) at 630 nm. All studies were carried out blind.

2.7. ELISA for detection of anti-MOG antibodies

For detection of anti-MOG antibodies in mice, ELISAs were carried out as previously described (Amor et al., 1993). Briefly, 50 μl rmMOG (10 μg/ml), diluted in PBS, was adsorbed on to a 96-well flat-bottom microlon ELISA plate (Greiner) overnight at 4 °C. Excess volume was removed and plates were post-coated with 200 μl PBS containing 2% BSA (Sigma) for 1 h at 37 °C. Plates were washed ten times in PBS containing 0.05% (v/v) Tween 20 (Sigma) and 100 μl of serum, diluted 1 in 100 in 1% BSA/PBS, was added to the wells and incubated for 2 h at 37 °C. Plates were washed 10 times as above and 100 μl of alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins (IgM+IgG+IgA) (Dako), diluted 1 in 2000 in 1% BSA/PBS, was added and incubated for 1 h at 37 °C. The washing procedure was repeated and 200 μl of substrate, *p*-nitrophenyl phosphatase (*p*-NPP) and Tris buffer tablet solution (Sigma), was added to each well. The plates were developed in the dark, at 37 °C, for 20 min. Absorbances were measured on a micro-titre plate reader (Dynatech MR606) at 405 nm.

2.8. ELISA for cross-reactivity studies

To examine cross-reactivity in mice, ELISAs were carried out as previously described (Tiwana et al., 1999) using a modified method. Briefly, 50 μl peptide (5 μg/ml) or MBP (25 μg/ml), diluted in a 0.05 M carbonate buffer (pH 9.6), was adsorbed on to a 96-well flat-bottom polystyrene immuno maxisorp ELISA plate (Nunc) overnight at 4 °C. Plates were washed three times for 5 min in PBS containing 0.1% (v/v) Tween 20 (Sigma) and blocked with PBS containing 0.1% bovine serum albumin (Sigma) for 1 h at

37 °C. The washing procedure was repeated and 50 μl of peptide mouse anti-sera diluted in 0.1% PBS/Tween was added to the wells in triplicate and incubated for 1 h at 37 °C. Plates were washed three times in PBS/Tween and 50 μl of peroxidase conjugated goat anti-mouse immunoglobulins (IgM+IgG+IgA) (Dako), diluted 1 in 500 in PBS/Tween, was added and incubated for 1 h at 37 °C. The washing procedure was repeated and the plates were developed as in human studies. For inhibition studies, the competitor peptides QNFI**SRF**A**WG**EVNSR (*Acinetobacter* 4-CMLD), QEMIT**R**HA**WG**DIWTR (*Pseudomonas* γ-CMLD), GLSL**SRF**S**WG**AEGQR (MBP), DSYVFDE-**LYR**A**GK**IE (*Acinetobacter* 3-oxoadipate CoA-transferase), PFSRVVH**LYR**N**GK**DQ (MOG) and human papilloma virus (HPV) control peptide (100 μg/ml) were incubated overnight at 37 °C with the antisera from mice immunized with peptides before ELISAs were carried out.

2.9. Statistical analysis

The mean optical density (O.D.) units of control groups (CVA and healthy blood donors) were compared with MS groups using a two-tail Student's *t*-test and 95% confidence limits of control groups were calculated. Pearson's correlation coefficient (*r*) was also calculated using the statistical package Prism3.0 (GraphPad Software).

3. Results

3.1. Molecular mimicry between MOG and Acinetobacter

Swissprot database was used to identify any amino acid sequence homologies present between *Acinetobacter* and myelin oligodendrocyte glycoprotein. A sequence similarity between MOG (50–55), **LYR**N**GK** and *Acinetobacter* 3-oxoadipate CoA-transferase subunit A (90–95) **LYR**A**GK** was found.

3.2. MS patients respond to bacterial peptide sequences

In order to examine antibody responses to bacterial peptides, serum was collected from MS patients and used in an ELISA. It was observed that significantly elevated levels of antibodies to the mimicry peptides were present in MS patients when compared to CVA and healthy controls (Fig. 1a and b). However, no elevation in antibodies was seen to an irrelevant peptide from HPV in MS patients compared to the control groups. No correlations were seen between antibody responses to these peptides and either age or sex of the MS patients.

3.2.1. Acinetobacter sp. 4-CMLD: QNFISRFAWGEVNSR

Significantly elevated levels of IgA antibodies to ***SRFAWG*** were observed in MS sera (0.044±0.004) when tested against control sera (0.013±0.001) (t=6.51, p<0.0001) and CVA patients (0.008±0.002) (t=6.96, p<0.0001) (Fig. 1a). Increased levels of anti-***SRFAWG*** IgG (0.10±0.007) and IgM (0.059±0.007) antibodies were also seen in MS patients in comparison to the control group (IgG: 0.012±0.003, t=9.58, p<0.0001), (IgM: 0.011±0.003, t=5.66, p<0.0001) and CVA patients (IgG: 0.02±0.006, t=7.26, p<0.0001), (IgM: 0.0005±0.0004, t=6.44, p<0.0001) (Fig. 1a). No significant differences were seen in the levels of either IgA or IgG anti-***SRFAWG*** antibodies between control subjects and CVA patients. However, there was a significant difference between the CVA and healthy blood donor controls when observing IgM antibody levels (t=3.26, p<0.01).

3.2.2. P. aeruginosa γ-CMLD: QEMITRHAWGDIWTR

Multiple sclerosis patients showed significantly increased levels of IgA antibodies to ***TRHAWG*** (0.047±0.003) compared to control sera (0.009±0.001) (t=12.07, p<0.0001) and CVA patients (0.008±0.001) (t=11.78, p<0.0001) (Fig. 1a). Significantly elevated levels of IgG (0.104±0.009) and IgM (0.051±0.007) anti-***TRHAWG*** antibodies were also observed in MS sera when tested against controls (IgG: 0.010±0.002, t=10.35, p<0.0001) (IgM: 0.005±0.001, t=6.03, p<0.0001) and CVA patients (IgG: 0.012±0.002, t=9.11, p<0.0001) (IgM: 0.001±0.001, t=5.88, p<0.0001) (Fig. 1a). No significant difference in the levels of either IgA or IgG antibodies was seen between the two control groups. However, IgM antibody levels to ***TRHAWG*** were significantly elevated in control subjects compared to CVA sera (t=2.85, p<0.01).

3.2.3. MBP 110–124: GLSLSRFSWGAEGQR

Multiple sclerosis patients showed significantly higher levels of IgA antibodies to ***SRFSWG*** (0.037±0.005) when compared to the control sera (0.01±0.004) (t=4.08, p<0.0001) and CVA patients (0.003±0.002) (t=5.33, p<0.0001) (Fig. 1a). Significantly elevated levels of IgG (0.08±0.01) and IgM (0.08±0.01) anti-***SRFSWG*** antibodies were observed in MS patients compared with healthy controls (IgG: 0.004±0.002, t=5.79, p<0.0001), (IgM: 0.004±0.002, t=6.15, p<0.0001) and CVA patients (IgG: 0.001±0.001, t=5.41, p<0.0001), (IgM: 0.012±0.008, t=4.53, p<0.0001) (Fig. 1a). When the two control groups were compared, no significant differences were shown in the levels of IgA, IgG or IgM antibodies to ***SRFSWG***.

3.2.4. Acinetobacter sp. 3-oxoadipate CoA-transferase subunit A:DSYVFDELYRAGKIE

Significantly elevated levels of IgA antibodies to ***LYRAGK*** were observed in MS sera (0.212±0.014) when tested against control sera (0.098±0.015) (t=4.66, p<0.0001) and CVA patients (0.094±0.010) (t=5.46, p<0.0001) (Fig. 1b). Increased levels of anti-***LYRAGK*** IgG (0.306±0.027) and IgM (0.257±0.012) antibodies were also seen in MS patients in comparison to the control group (IgG: 0.155±0.032, t=3.25, p<0.01), (IgM:

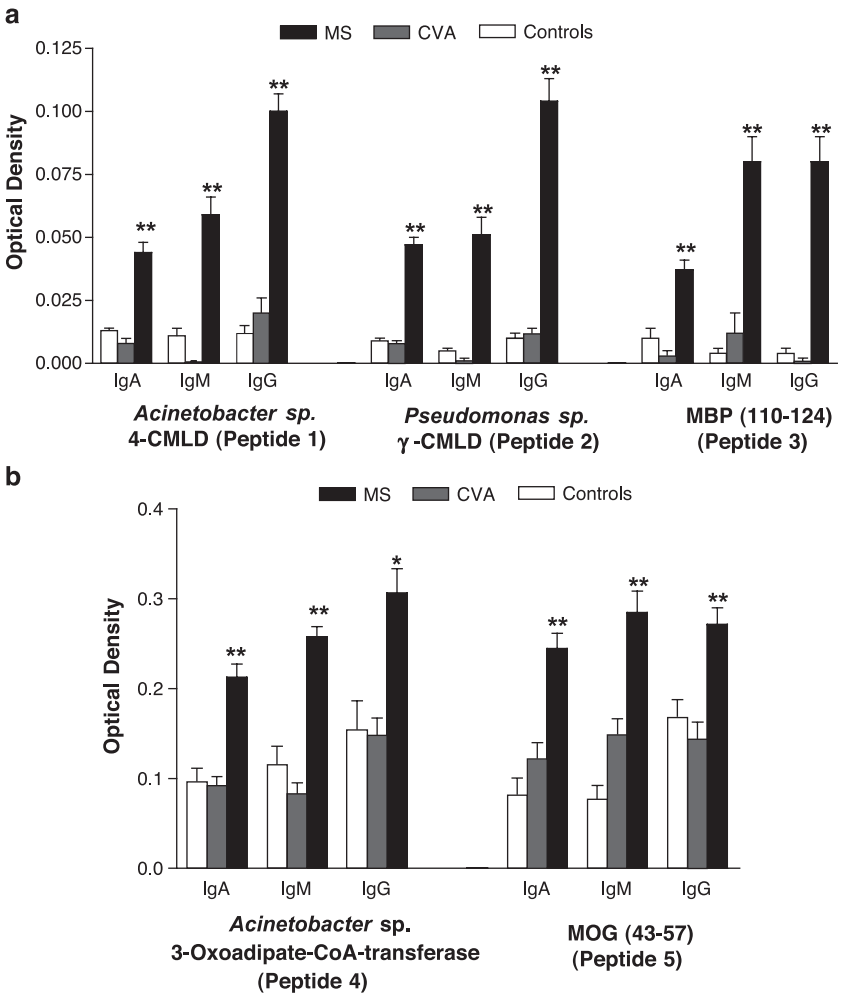

Fig. 1. Levels of IgA, IgM and IgG antibodies [mean±standard error (error bars)] to *Acinetobacter* 4-CMLD, *P. aeruginosa* γ-CMLD and MBP mimicry peptides (a) and *Acinetobacter* 3-oxoadipate CoA transferase subunit A and MOG mimicry peptides (b) in sera from 26 MS patients, 20 CVA patients and 25 healthy blood donors. **p<0.001, *p<0.01: MS compared to CVA and healthy blood donors.

0.117±0.020, t=6.26, p<0.0001) and CVA patients (IgG: 0.149±0.019, t=3.91, p<0.001), (IgM: 0.085±0.012, t=9.31, p<0.0001) (Fig. 1b). No significant differences were seen in the levels of IgA, IgM or IgG anti-***LYRAGK*** antibodies between control and CVA patients.

3.2.5. MOG 43–57: PFSRVVHLYRNGKDQ

Significantly elevated levels of IgA antibodies to ***LYRNGK*** were observed in MS sera (0.245±0.016) when tested against control sera (0.083±0.019) (t=5.65, p<0.0001) and CVA patients (0.123±0.018) (t=4.54, p<0.0001) (Fig. 1b). Increased levels of anti-***LYRNGK*** IgG (0.272±0.019) and IgM (0.285±0.024) antibodies were also seen in MS patients in comparison to the control group (IgG: 0.170±0.020, t=3.29, p<0.001), (IgM: 0.078±0.015, t=5.54, p<0.0001) and CVA patients (IgG: 0.145±0.019, t=4.34, p<0.0001), (IgM: 0.150±0.018, t=3.83, p<0.001) (Fig. 1b). No significant differences were seen in the levels of either IgA or IgG anti-***LYRNGK*** antibodies between control and CVA patients. However, there was a significant difference between the CVA and healthy blood donor controls when observing IgM antibody levels (t=2.97, p<0.01).

3.3. Dilution studies with peptide anti-sera

3.3.1. Antibodies to Acinetobacter sp.4-CMLD: QNFISRFAWGEVNSR

At the serum dilution of 1:25, total antibody levels to *Acinetobacter* peptide were significantly higher in *Acinetobacter* peptide-injected mice (0.676±0.004) as compared to healthy control mice (0.037±0.002; t=134.2, p<0.0001). A significant elevation in antibody levels to *Acinetobacter* peptide were also observed in *Pseudomonas* peptide-injected mice (0.658±0.006; t=99.9, p<0.0001), MBP peptide-injected mice (0.613±0.001; t=233.1, p<0.0001) and SCH-injected mice (0.551±0.006; t=82.6, p<0.0001) compared to healthy controls. A significantly raised antibody response was seen in all groups compared to controls up to a dilution of 1:6400 (Fig. 2a).

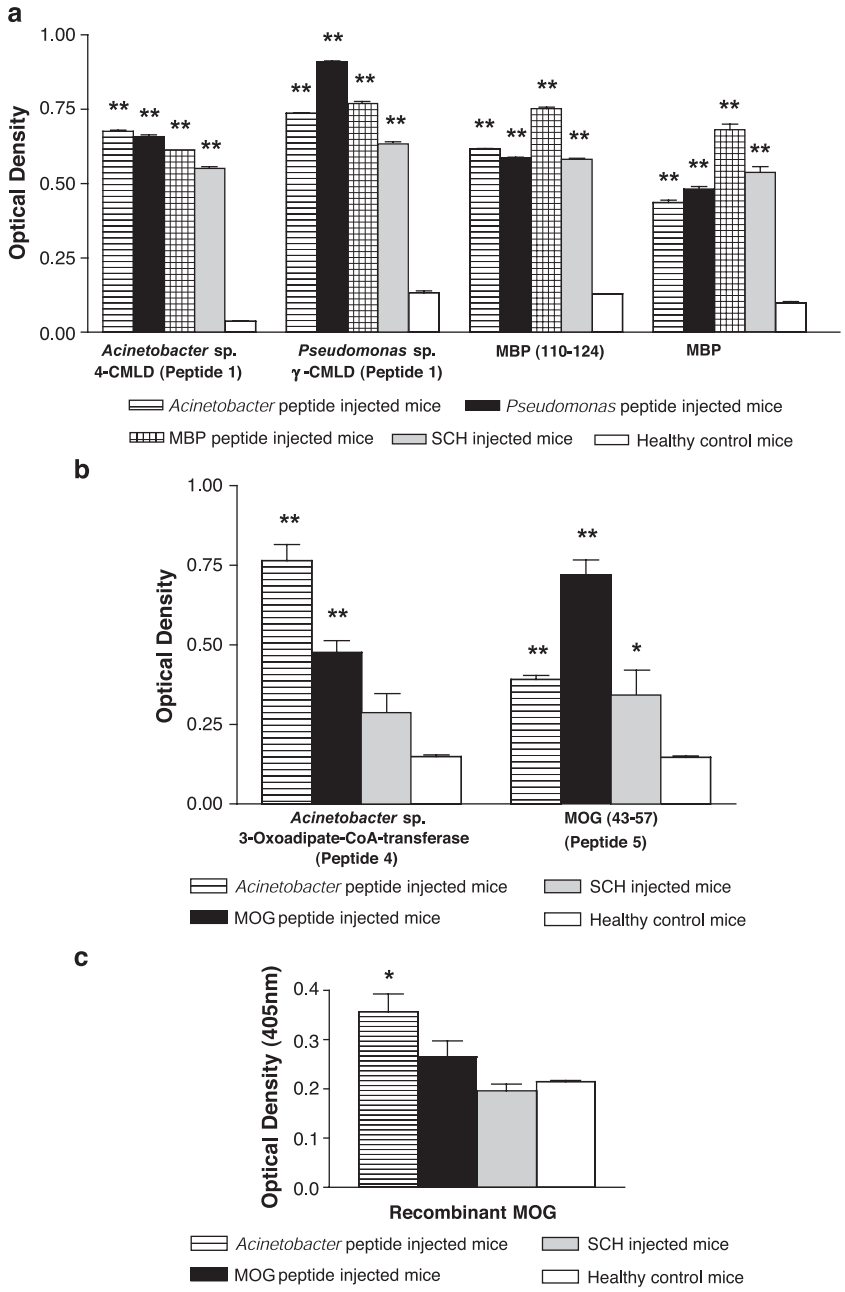

Fig. 2. Anti-peptide antiserum antibody responses by ELISA. Antisera were raised against the following peptides; *Acinetobacter* 4-CMLD, *P. aeruginosa* γ-CMLD, MBP (110–124), *Acinetobacter* 3-oxoadipate CoA transferase subunit A and MOG (43–57), as well as spinal cord homogenate. The binding of antisera and healthy control mouse serum was determined to *Acinetobacter* 4-CMLD (a), *P. aeruginosa* γ-CMLD (a), MBP (110–124) (a), MBP (a), *Acinetobacter* 3-oxoadipate CoA transferase subunit A (b), MOG (43–57) (b) and rmMOG (c). Results are shown for a dilution of 1:25. An asterisk indicates statistical significance compared to healthy controls. $^{**}p<0.001$, $^{*}p<0.05$.

3.3.2. Antibodies to *P. aeruginosa* γ-CMLD: QEMITRHAWGDIWTR

At the serum dilution of 1:25, total antibody levels to *Pseudomonas* peptide were significantly higher in *Pseudomonas* peptide-injected mice (0.910±0.003) as compared to healthy control mice (0.133±0.006; t=123.4, p<0.0001). A significant elevation in antibody levels to *Pseudomonas* peptide was also observed in *Acinetobacter* peptide-injected mice (0.736±0.002; t=103.7, p<0.0001), MBP peptide-injected mice (0.769±0.006; t=76.6, p<0.0001) and SCH-injected mice (0.634±0.005 t=69.0, p<0.0001) compared to healthy controls. A significantly raised antibody response was seen in all groups compared to controls up to a dilution of 1:6400 (Fig. 2a).

3.3.3. Antibodies to MBP 110–124: GLSLSRFSWGAEGQR

At the serum dilution of 1:25, total antibody levels to MBP peptide were significantly higher in MBP peptide-

injected mice (0.752±0.005) as compared to healthy control mice (0.129±0.001; t=116.6, p<0.0001). A significant elevation in antibody levels to MBP peptide was also observed in *Acinetobacter* peptide-injected mice (0.616±0.002; t=202.7, p<0.0001), *Pseudomonas* peptide-injected mice (0.586±0.003; t=134.4, p<0.0001) and SCH-injected mice (0.581±0.001; t=266.3, p<0.0001) compared to healthy controls. A significantly raised antibody response was seen in all groups compared to controls up to a dilution of 1:6400 (Fig. 2a).

3.3.4. Antibodies to MBP

At the serum dilution of 1:25, total antibody levels to MBP were significantly higher in MBP peptide-injected mice (0.681±0.022) as compared to healthy control mice (0.098±0.005; t=26.0, p<0.0001). A significant elevation in antibody levels to MBP was also observed in *Acinetobacter* peptide-injected mice (0.436±0.008; t=38.5, p<0.0001), *Pseudomonas* peptide-injected mice (0.481±0.009; t=39.3, p<0.0001) and SCH-injected mice (0.539±0.014; t=29.6, p<0.0001) compared to healthy controls. A significantly raised antibody response was seen in all groups compared to controls up to a dilution of 1:6400 (Fig. 2a).

3.3.5. Antibodies to Acinetobacter sp.: DSYVFDELYRAGKIE

At the serum dilution of 1:25, total antibody levels to *Acinetobacter* peptide were significantly higher in *Acinetobacter* peptide-injected mice (0.765±0.050) as compared to healthy control mice (0.149±0.005; t=13.75, p<0.0001). A significant elevation in antibody levels to *Acinetobacter* peptide was also observed in MOG peptide-injected mice (0.476±0.037; t=8.72, p<0.0001), but not SCH-injected, compared to healthy controls. A significantly raised antibody response was seen in both *Acinetobacter* and MOG peptide-injected mice compared to controls up to a dilution of 1:6400 and 1:3200, respectively (Fig. 2b).

3.3.6. Antibodies to MOG 43–57: PFSRVVHLYRNGKDQ

At the serum dilution of 1:25, total antibody levels to MOG peptide were significantly higher in MOG peptide-injected mice (0.719±0.048) as compared to healthy control mice (0.146±0.005; t=11.91, p<0.0001). A significant elevation in antibody levels to MOG peptide was also observed in *Acinetobacter* peptide-injected mice (0.391±0.013; t=18.98, p<0.0001) and SCH-injected mice (0.339±0.075; t=2.56, p<0.05) compared to healthy controls. A significantly raised antibody response was seen in both *Acinetobacter* and MOG peptide-injected mice compared to controls up to a dilution of 1:6400 (Fig. 2b).

3.3.7. Antibodies to rmMOG

At the serum dilution of 1:100, total antibody levels to rmMOG were significantly higher in *Acinetobacter* peptide-injected mice (0.357±0.036) as compared to healthy control mice (0.215±0.002; t=2.97, p<0.05). No significant difference was seen in the total antibody levels in MOG peptide-injected mice or SCH-injected mice compared to healthy control mice (Fig. 2c).

3.4. Inhibition studies

Peptide antiserum raised against *Acinetobacter* 4-CMLD peptide was inhibited by pre-incubation with 100μg of MBP 110–124 peptide per ml as well as *Acinetobacter* 4-CMLD and *Pseudomonas* γ-CMLD peptide. Similarly, antiserum against both *Pseudomonas* γ-CMLD and MBP 110–124 peptides were inhibited by pre-incubation with 100 μg/ml of each of the three peptides (Fig. 3a–c). Peptide antiserum raised against *Acinetobacter* 3-oxoadipate CoA-transferase subunit A peptide was inhibited by pre-incubation with 100μg/ml of MOG 43–57 peptide and vice versa (Fig. 3d and e).

3.4.1. Acinetobacter 4-CMLD anti-serum

Prior to incubation with MBP peptide, the anti-*Acinetobacter* antiserum had a mean antibody binding activity to *Acinetobacter* peptide of 0.676±0.004 at a dilution of 1:25 and reacted at dilutions of up to 1:6400. After incubation binding activity was reduced by 43% to 0.385±0.003 (t=53.6, p<0.0001) and reacted at dilutions of up to 1:1600. Similar results were obtained after incubation with *Pseudomonas* peptide where binding activity decreased by 37% to 0.403±0.001 (t=57.7, p<0.0001). Incubation with the irrelevant peptide from HPV did not have any effect on original binding activity (Fig. 3a).

3.4.2. Pseudomonas γ-CMLD anti-serum

Pseudomonas antiserum had a mean antibody binding activity to *Pseudomonas* peptide of 0.910±0.003 at a dilution of 1:25 and reacted at dilutions of up to 1:6400. However, after incubation with MBP peptide the binding activity was reduced by 43% to 0.519±0.006 (t=58.2, p<0.0001) and reacted at dilutions of up to 1:1600. Absorption with *Acinetobacter* peptide yielded similar results reducing binding activity by 40% to 0.545±0.005 (t=60.5, p<0.0001). Incubation with the irrelevant peptide did not have any effect on original binding activity (Fig. 3b).

3.4.3. MBP 110–124 anti-serum

Prior to incubation with *Acinetobacter* peptide, the anti-MBP antiserum had a mean antibody binding activity to MBP peptide of 0.752±0.005 at a dilution of 1:25 and reacted at dilutions of up to 1:6400. After incubation binding activity was reduced by 49% to 0.380±0.005 (t=54.0, p<0.0001) and reacted at dilutions of up to 1:3200. Similar results were obtained after incubation with *Pseudomonas* peptide where binding activity decreased by 55% to 0.338±0.005 (t=31.4, p<0.0001). Incubation with the irrelevant peptide did not have any effect on original binding activity (Fig. 3c).

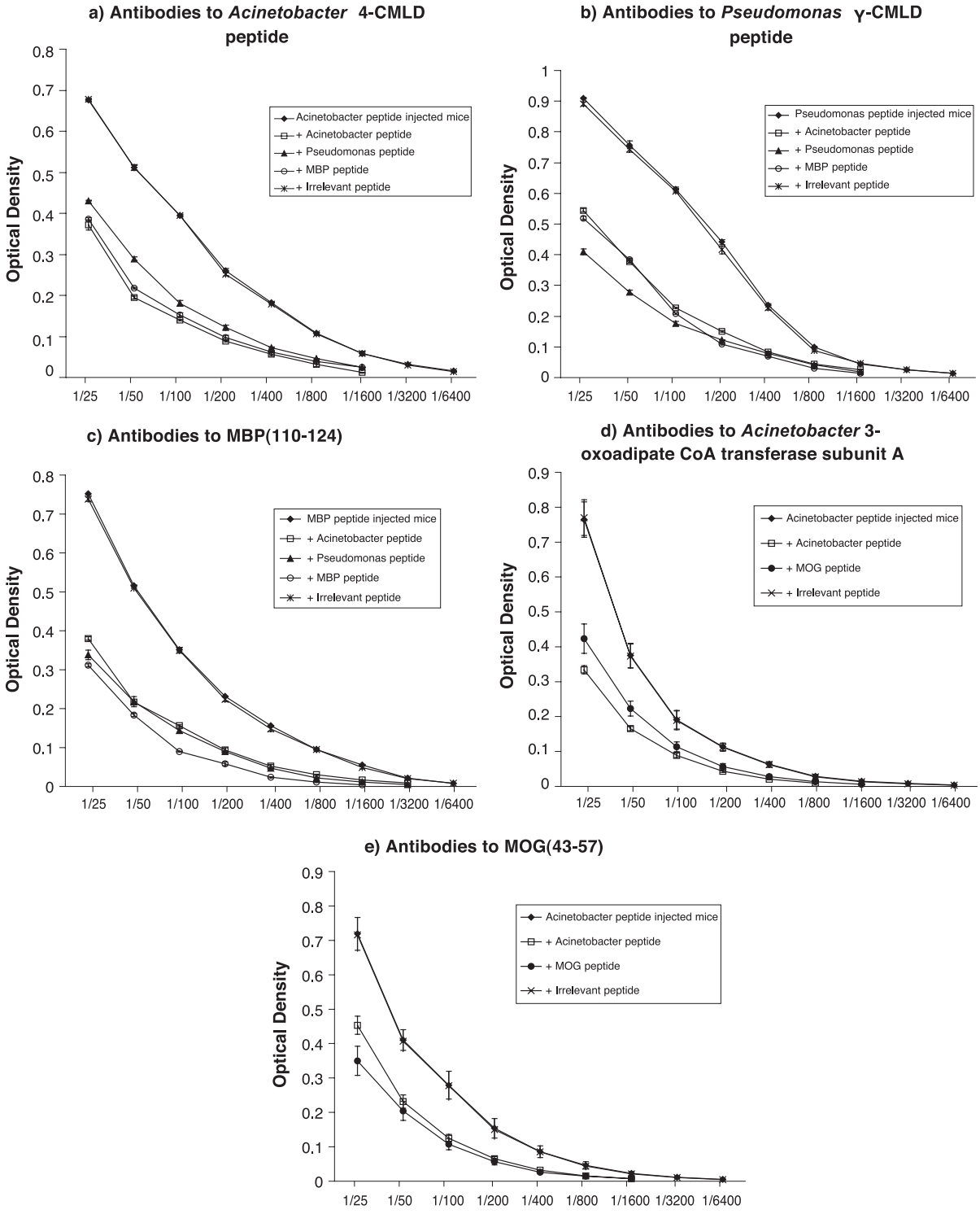

Fig. 3. Inhibition of antisera with mimicry peptides. Binding of antisera to *Acinetobacter* 4-CMLD (a), *P. aeruginosa* γ-CMLD (b), MBP (110–124) (c), *Acinetobacter* 3-oxoadipate CoA transferase subunit A (d) and MOG (43–57) (e), before and after incubation with mimicry and an irrelevant peptide.

3.4.4. Acinetobacter 3-oxoadipate CoA-transferase subunit A anti-serum

Prior to incubation with MOG peptide, the anti-*Acinetobacter* antiserum had a mean antibody binding activity to *Acinetobacter* peptide of 0.765±0.050 at a dilution of 1:25 and reacted at dilutions of up to 1:6400. After incubation binding activity was reduced by 45% to 0.423±0.042 (t=5.2, p<0.01) and reacted at dilutions of up to 1:1600. Incubation with the irrelevant peptide did not have any effect on original binding activity (Fig. 3d).

Table 2
EAE induction in peptide immunized ABH mice[a]

Antigen	No. EAE	Mean group score[b]	Mean EAE score[c]	Mean day of onset
SCH	5/5	3.9±0.2	3.9±0.2	15±1.2
Acinetobacter 4-CMLD (38–52)	0/5	N/A	N/A	N/A
Pseudomonas γ-CMLD (38–52)	0/5	N/A	N/A	N/A
MBP (110–124)	0/5	N/A	N/A	N/A
Acinetobacter 3-oxoadipate CoA-transferase (83–97)	0/5	N/A	N/A	N/A
MOG (43–57)	5/5	4.1±0.2	4.1±0.2	24.6±7.6

[a] Animals were injected with 200 μg peptide or 1 mg SCH emulsified in CFA on days 0 and 7. After administration, animals were injected i.p. with 200 ng of pertussis toxin which was repeated 24 h later. Animals received a further dose of 200 μg peptide in IFA to boost for antibody production. N/A=not applicable.

[b] Mean±S.D. of the maximum clinical score from all animals in the group.

[c] Mean±S.D. of the maximum clinical score from animals exhibiting EAE within a group.

3.4.5. MOG 43–57 anti-serum

MOG antiserum had a mean antibody binding activity to MOG peptide of 0.719±0.048 at a dilution of 1:25 and reacted at dilutions of up to 1:6400. However, after incubation with *Acinetobacter* peptide the binding activity was reduced by 37% to 0.453±0.026 (t=4.86, p<0.001) and reacted at dilutions of up to 1:1600. Incubation with the irrelevant peptide did not have any effect on original binding activity (Fig. 3e).

3.5. Induction of EAE in peptide-injected mice

Mice injected with the mimicry peptides from *Acinetobacter* 4-CMLD, *Pseudomonas* γ-CMLD and MBP 110–124 did not exhibit any clinical signs of EAE after 57 days. It was observed that mice immunized with MOG 43–57 developed weight loss and neurological signs of EAE at 24.6±7.57 days, but those injected with the *Acinetobacter* mimicry peptide of MOG did not show any clinical signs of EAE. SCH-immunized mice also developed weight loss and neurological signs of EAE at 15±1.23 days (Table 2).

4. Discussion

Elevated levels of IgA, IgM and IgG antibodies directed against peptide sequences from *Acinetobacter* 4-CMLD and 3-oxo-adipate-CoA-transferase subunit A and *P. aeruginosa* γ-CMLD were found in the sera from MS patients compared to CVA patients or healthy controls.

Current thinking suggests that both genetic and environmental factors are implicated in MS (Granieri et al., 2001) and any infectious agent involved must be ubiquitous within risk groups (Wucherpfennig and Strominger, 1995b). *Acinetobacter* and *Pseudomonas* occur frequently in the environment and could be considered as potential aetiological agents. *Acinetobacter* is a common microbe found in soil, water and on the skin and mucous membranes of animals and man. It is an opportunistic pathogen and has been associated with nosocomial infections in hospital environments, resulting in bacteremia and pneumonia (Villers et al., 1998) and occasionally chest and sinus infections (Towner, 1997). *Pseudomonas* is also an opportunistic pathogen of man, causing wound, urinary tract and upper respiratory tract infections (Singleton and Sainsbury, 1995). It is widely distributed throughout the environment and can cause transient colonization of skin and intestinal tract of humans (Salyers and Whitt, 1994). The search for an aetiological agent of MS has been focused on viruses and MS relapses have also been associated with infections, although no common microorganism has been implicated in either case (Fujinami, 2001). It has been demonstrated that 52% of MS patients have a history of repeated upper respiratory-tract infections in childhood and that as a consequence have more severe neurological symptoms and periods of exacerbation (Lamoureux et al., 1983). Increased risk of clinical relapses in MS has been associated with a number of types of infection, including urinary tract, gastrointestinal and upper respiratory tract (Buljevac et al., 2002). Most studies have suggested links with viral upper respiratory tract infections (Edwards et al., 1998; Andersen et al., 1993) although it has been demonstrated that 41% of MS patients with an exacerbation, evaluated for an infectious process, had a significant bacterial infection. These were mainly urinary tract infections caused by a number of bacteria including *Pseudomonas*. The authors hypothesized that an MS relapse could result from cross-reactivity between the infectious agent and brain antigens (Rapp et al., 1995).

A number of mechanisms have been suggested as to how an infection could initiate autoimmune disease, including molecular mimicry, determinant spreading and bystander activation (Talbot et al., 2001; Wucherpfennig, 2001). Molecular mimicry has previously been suggested as a potential pathogenic mechanism leading to the development of MS (Albert and Inman, 1999; Steinman, 2001), either through sequence or structural homology (Kohm et al., 2003). A number of microorganisms have been identified that possess amino acid sequences with homology to myelin antigens including human herpes virus 6 (HHV-6) (Tejada-Simon et al., 2003), Epstein–Barr virus, herpes simplex virus, influenza and *P. aeruginosa* (Wucherpfennig and Strominger, 1995a). We have previously identified potential molecular mimicry between *Acinetobacter*, *Pseudomonas* and brain components and demonstrated antibody responses to these bacteria in MS patients (Hughes et al., 2001). In this study, we have looked at class-specific immune responses to

peptide sequences within *Acinetobacter* and *P. aeruginosa*, which mimic the brain components MBP and MOG. Autoantibodies to myelin antigens, including MOG (Reindl et al., 1999), MBP (Warren and Catz, 1987), myelin-associated glycoprotein (Wajgt and Gorny, 1983) and gray matter neurofilaments (Newcombe et al., 1985) are well recognised in MS. T cell responses to MBP 110–124 have previously been demonstrated (Mazza et al., 2002) and we report here that this amino acid sequence can also function as a B cell epitope as increased levels of IgA, IgM and IgG antibodies to MBP 110–124 were observed in MS patients compared to controls and the CVA group. Both T cell and antibody responses to MOG 35–55 have been shown in MS patients (Kennel De March et al., 2003) and we show that IgA, IgM and IgG antibodies to a similar epitope, MOG 43–57, are elevated in MS patients, compared to CVA patients and controls.

Antibodies against the peptides from *Acinetobacter*, *Pseudomonas*, MBP and MOG were found to be significantly raised in all groups of injected mice compared to healthy control mice. Peptide antisera raised in Biozzi ABH mice against MBP (110–124) reacted with *Acinetobacter* 4-CMLD and *Pseudomonas* γ-CMLD peptides. In a reciprocal manner, antisera to both *Acinetobacter* and *Pseudomonas* peptides reacted with MBP peptide. Inhibition studies showed that incubation of MBP antisera with the bacterial peptides and bacterial antisera with MBP reduced binding to MBP and bacterial antigens, respectively. However, mice injected with MBP 110–124 or its bacterial mimics from *Acinetobacter* or *Pseudomonas* showed no signs of clinical EAE over a 57-day period. Peptide antisera raised in mice against MOG (43–57) reacted with *Acinetobacter* 3-oxoadipate-CoA-transferase subunit A peptide and in a reciprocal manner *Acinetobacter* peptide antisera reacted with MOG peptide. Inhibition studies demonstrated that incubation of MOG antisera with *Acinetobacter* peptide reduced binding to MOG and vice versa. Mice injected with MOG 43–57 demonstrated clinical signs of EAE by day 25, but no disease was observed in mice injected with the *Acinetobacter* mimicry peptide. Mice injected with SCH showed clinical signs of EAE by day 15 and demonstrated responses to both the MBP and MOG peptides suggesting that these epitopes are naturally processed when EAE is induced with whole CNS tissue. Antibodies from mice immunized with bacterial peptides also recognize the intact whole antigens, MBP and MOG, which they mimic, suggesting that these antibodies could therefore be relevant in pathogenesis. Although bacterial mimicry peptides do not directly induce EAE further work is required to assess their ability to re-induce EAE exacerbations after the initial clinical disease has resolved. To fully establish that antibacterial antibodies may play a pathogenic role, it would be necessary to passively transfer sera from bacterial peptide-immunized mice into animals with active mild EAE to assess whether any change in disease severity may be observed.

The results from this study suggest that the mimicry sequences identified between *Acinetobacter*, *Pseudomonas*, MBP and MOG could be potentially cross-reactive. It is therefore suggested that the antibody responses to the specific bacterial peptide antigens seen in MS patients could cross-react and bind to myelin constituents within the brain potentially enhancing the pathological lesions seen in MS. Further studies are needed to evaluate the role of *Acinetobacter* sp. and *P. aeruginosa* in MS.

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