

Antibodies Directed Against Rubella Virus Induce Demyelination in Aggregating Rat Brain Cell Cultures

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To link the presence of intrathecal virus-specific oligoclonal immunoglobulin G (IgG) in multiple sclerosis patients to a demyelinating activity, aggregating rat brain cell cultures were treated with antibodies directed against two viruses, namely, rubella (RV) and hepatitis B (HB). Anti-RV antibodies in the presence of complement decreased myelin basic protein concentrations in a dose-dependent manner, whereas anti-HB antibodies had no effect. A similar but less pronounced effect was observed on the enzymatic activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase, which is enriched in non-compact membranes of oligodendrocytes. These effects were comparable to those in cultures treated with antibodies directed against myelin oligodendrocyte glycoprotein (MOG), previously found to be myelinotoxic both in vitro and in vivo. Sequence homologies were found between structural glycoprotein E₂ of RV and MOG, suggesting that demyelination was due to molecular mimicry. To support the hypothesis that demyelination was caused by anti-RV IgG that recognized an MOG epitope, we found that anti-RV antibodies depleted MOG in a dose-dependent manner. Further evidence came from the demonstration that anti-RV and anti-MOG IgG colocalized on oligodendrocyte processes and that both revealed by Western blot a 28 kDa protein in CNS myelin, a molecular weight corresponding to MOG. These findings suggest that a virus such as RV exhibiting molecular mimicry with MOG can trigger an autoimmune demyelination. *J. Neurosci. Res.* 65:446–454, 2001.

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Viral infections are very common during childhood. Neurotropic viruses frequently affect the brain without any clinical evidence (Gibbs et al., 1959). B and T cells are recruited across the blood–brain barrier to eliminate the viral infection rapidly, leading ultimately to the persistence within the central nervous system (CNS) of specific memory B cells (Gerhard and Koprowski, 1977). A new inflammatory process can reactivate these cells and lead to

intrathecally released virus-specific antibodies in the absence of the pathogenic agents.

Intrathecal synthesis of virus-specific oligoclonal immunoglobulin G (IgG) is frequently observed in multiple sclerosis (MS) patients (Sindic et al., 1994). Several triggering mechanisms have been proposed, such as persisting viral infections or autoimmune manifestations, but no clear evidence has yet been found. Moreover, the relevance of these virus-specific oligoclonal IgG to the pathogenesis of MS remains obscure. High antibody titers against measles, mumps, and hepatitis B (HB) have never had any diagnostic value because similar increases were also found in patients suffering from other autoimmune diseases (Felgenhauer and Reiber, 1992). However, a study reported an immune reaction against structural glycoprotein E₂ of rubella virus (RV) specific to MS patients (Nath and Wolinsky, 1990). Moreover, neuroepidemiological observations have shown that RV infections occur at later ages for MS patients compared to normal controls (Bachmann and Kesselring, 1998).

Attempts were made to link the presence of virus-specific antibodies with the pathogenesis of demyelinating diseases. Similarity of protein sequences was found between pathogens and myelin components, a process termed “molecular mimicry.” This concept was initially proposed by Damien (1964) to describe antigen sharing between a host and a parasite. Pathogens mimic the host's environment to evade an immune attack. An unfortunate consequence is that pathogenic agents can trigger autoimmune reactions through the sharing of foreign and self-antigenic determinants. Several DNA, and more often RNA viruses, have been shown to activate T cell clones to myelin basic protein (MBP), as a result of molecular mimicry (Wucherpfenning and Strominger, 1995). A recent

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study showed that mice immunized with Semliki Forest virus (SFV) proteins developed a lymphocyte proliferation to SFV surface glycoprotein E₂ and serum antibody response that cross-react with the myelin oligodendrocyte glycoprotein (MOG), leading to CNS demyelination (Mokhtarian et al., 1999). MOG is a minor protein of myelin belonging to the immunoglobulin superfamily (Gardinier et al., 1992). MOG induces an experimental autoimmune encephalomyelitis (EAE; Adelman et al., 1995). Moreover, antibodies directed against MOG are myelinotoxic in CNS cultures (Kerlero de Rosbo et al., 1990). Their presence in lesions of chronic relapsing EAE and MS has linked them to demyelinating diseases (Genain et al., 1999).

We postulated that molecular mimicry between a viral agent and MOG could explain the relevance of virus-specific oligoclonal IgG detected in MS patients and its role in demyelinating events. Our study focused on antibodies against RV and HB. We searched for their potential demyelinating activity on a three-dimensional rat CNS culture. These CNS cultures are established and maintained in a chemically defined medium, and cell development, differentiation, and myelination occur similarly to the case in normal brain tissue (Honegger and Matthieu, 1980). Such an *in vitro* system allowed us to investigate the neurotoxicity of antibodies in the absence of complex systemic interactions leading to artifacts inherent to *in vivo* models. In this study, we identified sequence homologies between surface glycoprotein E₂ of RV and MOG. We demonstrated that antibodies directed against RV demyelinated CNS cultures in the presence of complement and found that this demyelination was probably due to a specific attachment of anti-RV IgG to MOG.

MATERIALS AND METHODS

Alignment of Peptide Sequences

A software analysis system (McVector, Oxford Molecular) was used to identify different areas of sequence homology. We focused our study on HB and on surface glycoprotein E₂ of RV, because they were found to induce specific antibodies in MS patients (Nath and Wolinsky, 1990). Their amino acid sequences were aligned with those of each of the myelin proteins, myelin basic protein (MBP), proteolipid protein (PLP), and MOG, to identify homologous stretches between them. Criteria such as partial homologies, including hydrophobicity, polarity, charge, pKa, and size and structure of the R-group, were also considered. This computer alignment was then applied to determine the percentage of homology between them.

Aggregating Brain Cell Cultures

Fetal (15 or 16 days of gestation) rat telencephalon (OFA; BRL, Füllinsdorf, Switzerland) was mechanically dissociated to prepare rotation-mediated aggregating brain cell cultures, as described previously (Honegger and Matthieu, 1980).

Antibodies

Mouse monoclonal antibody against rat MOG was derived from clone 8-18C5 (Linington et al., 1984); the IgG were

purified by affinity chromatography using the Bio-Rad Econo-Pac protein A kit (Bio-Rad, Richmond, CA). Human immunoglobulins enriched with anti-RV or anti-HB antibodies and nonspecific immunoglobulins (NS IgG; Globuman) were obtained from Berna (Berna, Berne, Switzerland). It was demonstrated that all potential remaining viruses were inactivated. The IgG fraction was purified by affinity chromatography using the Bio-Rad Econo-Pac serum IgG purification kit (Bio-Rad). Polyclonal rabbit anti-MBP antibody was produced as previously described (Bernard et al., 1981). Rabbit antiactin antibodies directed against the C-terminal actin fragment were purchased from Sigma (St. Louis, MO).

Primary MOG antibody and MBP antibody were detected using a species-specific secondary antibody either horseradish peroxidase-conjugated (Amersham Pharmacia Biotech, Piscataway, NJ) or biotin-conjugated and visualized using streptavidin-fluorescein (Amersham Pharmacia Biotech). Anti-RV antibodies were detected using an anti-human secondary antibody, either horseradish peroxidase-conjugated (Amersham Pharmacia Biotech) or directly coupled to Cy3 (Jackson ImmunoResearch, West Grove, PA).

Treatment of Brain Cell Aggregates

At culture day 27, brain cell aggregates of each culture flask were split into four equal parts, and the final volume of each flask was made up to 8 ml by addition of fresh medium. The cultures were then treated with complement alone (guinea pig serum; 25 μ l/ml of culture medium), purified anti-MOG (62.5 μ g/ml), anti-HB (125 μ g/ml), NS IgG (125 μ g/ml), and varying concentrations of anti-RV IgG (31.3 μ g/ml, 62.5 μ g/ml, 93.8 μ g/ml, 125 μ g/ml, 156.3 μ g/ml) in phosphate-buffered saline (PBS) in the presence or absence of complement. At days 29, 31, and 33, fresh medium was added in exchange of 5 ml/flask, and antibodies with or without complement were again applied to the brain cell aggregates. The cultures were harvested on day 34.

Biochemical Assays

Brain cell aggregates were washed twice with 5 ml of ice-cold PBS and homogenized in 0.5 ml of 2 mM potassium phosphate containing 1 mM EDTA, pH 6.8, using glass-glass homogenizers (Bellco, Vineland, NJ). The different homogenates were briefly sonicated and stored as aliquots for the different assays at -80°C .

Protein concentrations were measured by the Folin phenol method of Lowry et al. (1951) using bovine serum albumin as a standard. Radioimmunoassay was used to quantify the MBP content (Bürgisser, 1983).

2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNP) activity was quantified using a spectrophotometric technique with the sodium salt of adenosine-2',3'-cyclic monophosphate as substrate, as previously described (Kurihara and Tsukada, 1967). The modified method of Schier and Shuster (Wilson et al., 1972) was used to measure the choline acetyltransferase (ChAT) activity. Acetyl-[1-¹⁴C]coenzyme A was the precursor. Nonspecific activity was determined by the omission of choline in the assay mixture and allowed us to correct the activity measured in the samples.

The activity of glutamic acid decarboxylase (GAD) was assayed by a modification of the method of Wingo, described by Wilson et al. (1972), using L-[1-¹⁴C]glutamic acid as a precursor. Glutamine synthetase (GS) activity was measured by a modified radioenzymatic method of Pishak and Phillips (Patel et al., 1982) with L-[1-¹⁴C]glutamic acid as precursor and phosphoenolpyruvate/pyruvate kinase as the ATP-generating system.

Western Blot Analysis

Myelin was extracted from human and rat brain as previously reported (Matthieu et al., 1979). A solution of antiprotease (Complete Mini, Boehringer/Roche, Indianapolis, IN) was added to the homogenized brain cell aggregates, and these samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, gels were blotted onto PVDF membranes and incubated for 2 hr at 37°C with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20. The immobilized proteins were detected with the appropriate antibodies: anti-MOG (1,000-fold dilution), anti-RV (500-fold dilution), and antiactin (500-fold dilution) overnight at 4°C. The blots were then washed three times for 5 min each in TBS-Tween. This procedure was followed by a 1 hr incubation with an appropriate species-specific horseradish peroxidase-conjugated secondary antibody (10,000-fold dilution) in TBS-Tween at room temperature. The same washing procedure as described above was then applied. The signal was detected using an enhanced-chemiluminescence system (Amersham Pharmacia Biotech). The blots were exposed on BioMax MR-1 films (Kodak, Rochester, NY). The films were scanned on ImageScanner (Amersham Pharmacia Biotech), and semiquantification was performed using a software analysis system, TotalLab (Amersham Pharmacia Biotech).

Primary Cultures of Rat Oligodendrocytes

Primary cultures of rat oligodendrocytes were prepared as initially described by Labourdette et al. (1979).

Immunocytochemistry

Oligodendrocytes were grown on glass coverslips. The cells were washed twice with cold PBS and fixed for 20 min with 4% paraformaldehyde at room temperature. These fixed cultures were then blocked for 45 min with 5% goat serum and incubated overnight at 4°C with anti-MOG antibodies (200-fold dilution) in the presence or absence of anti-RV antibodies (100-fold dilution). The appropriate secondary antibody (400-fold dilution) and tertiary immunofluorescence reagents were applied for 1 hr at room temperature. The coverslips were mounted on glass slides in Lennette's medium (Lennette, 1978) and analyzed on a Leitz fluorescent microscope.

Data Analysis

Results are compared to untreated control cultures. They correspond to the mean values of nine replicate cultures performed in three independent experiments. Statistical analyses were assessed using one-way analysis of variance (ANOVA) and Dunnett posttest. Results with $P < 0.01$ were considered significant.

TABLE I. Peptide Alignments of RV Surface Glycoprotein E₂ and Human Myelin Proteins

Peptides	Sequences ^a	Identity (%)
A) E ₂ 50–62 and MOG 1–10	LAVRARPSRYPLP ... ::: :: MASLSRPS LP	61.5
B) E ₂ 76–83 and PLP 158–166	LLVWVVLIF : : . : : LTVVWLLVF	55.6
C) E ₂ 44–53 and MBP 51–60	APKTAGLAVR ::: : APKRGSGKVP	40

^aThe dots represent complete homology and partial homology, as described in Materials and Methods.

RESULTS

Sequence Homologies Between Viral Epitopes and Myelin Proteins

We focused our study on two viral agents, RV and HB. HB has been previously described to have homologous amino acid stretches with human MBP (Wucherpfenning and Strominger, 1995). Atkins et al. (1991) have shown a sequence similarity between the amino-terminus of human PLP and RV envelope E₁ protein. In this work, we analyzed structural glycoprotein E₂ of RV that was found to induce high specific antibody titers in MS patients (Nath and Wolinsky, 1990). Peptides E₂ 54–74, E₂ 140–199, and E₂ 244–263 were targeted for homology studies because they contain immunodominant sequences recognized by a group of MHC-diverse, rubella-immune human donors (Ou et al., 1992). Significant homologies were found between the amino acid sequences of E₂ 50–62 and human MOG 1–10, between E₂ 76–83 and human PLP 158–166, and between E₂ 44–53 and human MBP 51–60 (Table I). Myelin proteins are highly conserved between species, so few differences are found between human and rat myelin proteins. Because significant homologies were also found between RV E₂ glycoprotein and rat myelin proteins (results not shown), we used an *in vitro* model of rat CNS cultures as described above.

Control Cultures

Several sets of control cultures were used to evaluate the specificity of the toxic effect observed with anti-RV and anti-MOG antibodies. We first measured the toxicity of complement on aggregating brain cell cultures. In previous studies, this component was shown to have a toxic effect on oligodendrocytes (Scolding et al., 1990). In a second set of controls, we tested the effect of NS IgG with complement to assess the intrinsic toxicity of immunoglobulins. In a third set of control cultures, we tested anti-RV in the absence of complement to exclude the presence of autoreactive antibodies. The results are detailed simultaneously with test cultures in the following sections.

TABLE II. Complement-Mediated Effect of Anti-RV and Anti-MOG Antibodies on Glial and Neuronal Markers*

	Anti-MOG 62.5 μ g/ml + complement	<i>P</i> value	Anti-RV 125 μ g/ml + complement	<i>P</i> value
Total protein	104.7 \pm 7.8	NS	109.4 \pm 7.4	NS
MBP	50.7 \pm 2.2	<0.001	56.9 \pm 3.9	<0.001
CNP	60.4 \pm 1.6	<0.001	68.2 \pm 2.02	<0.001
ChAT	179.1 \pm 23.8	NS	187.97 \pm 28.8	NS
GAD	189.8 \pm 32.3	NS	181.5 \pm 32.3	NS
GS	146.5 \pm 14.1	NS	124 \pm 17.2	NS

*Data are presented in percentage of untreated control cultures (mean \pm SEM from three independent experiments, $n = 9$); they were compared to untreated controls and values, with $P < 0.01$ considered significant; NS, not significant.

Effects of Anti-MOG and Anti-RV IgG on Aggregating Brain Cell Cultures

Effect on Protein Content. The protein content was used to determine the general cytotoxicity of the different treatments on the CNS cultures. No difference was observed when we compared untreated control cultures with test cultures treated with either anti-MOG or anti-RV antibodies in the presence of complement (Table II). Furthermore, the protein content of aggregate cultures was not significantly different when treated with complement alone or NS IgG or anti-RV in the absence of complement (Table III).

Complement-Mediated Toxic Effect of Antibodies on Oligodendrocytes. We determined the toxicity of our treatments on oligodendrocytes in aggregating rat brain cell cultures by measuring the MBP content and the CNP specific activity. Based on previous research carried out in our laboratory, we used the MBP content as an index of demyelination (Kerlero de Rosbo et al., 1990). CNP activity is a more specific marker of noncompact plasmic membranes of oligodendrocytes (Brunner et al., 1989).

Anti-RV antibodies were toxic to oligodendrocytes affecting both markers, as shown in Table II. This effect was reproducible in all three experiments. The decrease in MBP varied between 40% and 70% compared to untreated control cultures. CNP specific activity diminished by about 30–40% compared to untreated control cultures. Myelin membranes seem to be more affected by the toxic effect of anti-RV antibodies, as shown by the larger decrease in MBP content. Moreover, we tested the dose-related demyelinating activity of anti-RV IgG. As depicted in Figure 1, increasing concentrations of these antibodies caused a dose-dependent pattern of demyelination. Twice the concentration of anti-RV IgG was necessary to obtain the same demyelinating effect as with anti-MOG.

Anti-MOG antibodies diminished MBP in a range of 50–80% compared with untreated control cultures (Table II). As for CNP specific activity, the toxic effect was also less drastic, with a decrease in enzyme activity by

about 40–60% compared with untreated control aggregates. In one experiment, we tested the toxicity of anti-HB antibodies on the rat CNS cultures. However, no effect on MBP or CNP could be observed (data not shown). Therefore, we did not test this particular antibody further.

Control cultures are reviewed in Table III. Aggregates treated with complement alone had few effect on MBP content, and this decrease remained nonsignificant when compared with untreated controls. The same observation was also true for the CNP specific activity. It is not clear whether this effect is specific to complement or is due to components not yet identified in the guinea pig serum.

Cultures challenged with NS IgG in the presence of complement had decreased MBP and CNP values that were similar to those observed in aggregates treated with complement alone. Therefore, NS IgG did not interact with oligodendrocytes. The toxic effect observed with anti-RV antibodies appears to be linked to their antiviral specificity.

In another set of controls, we tested the activity of anti-RV IgG without complement. As shown in Table III, no effect was observed either on MBP or on CNP with this treatment. Demyelination by anti-RV or anti-MOG IgG occurred only in the presence of complement.

Effect of Antibodies on Neurons. The effects of anti-RV, anti-HB, and anti-MOG antibodies were analyzed by measuring the specific activity of ChAT and GAD. ChAT is a marker for cholinergic neurons and GAD for GABAergic neurons. The specific activities of these two enzymes in CNS cultures have been previously used in toxicology studies as reliable indices of neuronal integrity (Monnet-Tschudi et al., 2000). Anti-RV and anti-MOG antibodies had no toxic effect on either cholinergic or GABAergic neurons (Table II). On the contrary, the values measured were higher than in untreated controls.

We examined different control cultures for the same parameters. Aggregates exposed to complement alone had also high values for ChAT and GAD specific activities (Table III). A similar observation was made for cultures treated with NS IgG and complement. However, the cultures challenged with anti-RV antibodies in the absence of complement had ChAT and GAD activities slightly higher than those of untreated controls. It seems that this protective effect could be linked to trophic factors present in the guinea pig serum used as a source of complement. This effect on neurons has been reported previously for these cultures (Kerlero de Rosbo et al., 1990).

Effect of Antibodies on Astrocytes. The toxicity to astrocytes was examined by measuring GS specific activity, a marker for astrocytes. As for neurons, anti-RV and anti-MOG antibodies had no toxic effect on astrocytes (Table II). On the contrary, they seemed to have a stimulating effect. Likewise, aggregates treated either with complement alone or with NS IgG and complement had high values of GS enzymatic activity (Table III). However,

TABLE III. Glial and Neuronal Markers in Control Experiments*

	Untreated	Complement alone ^a	Non specific IgG ^b + complement ^a	Anti-RV ^b without complement
Total protein (mg/culture)	2.7 ± 0.04	3.1 ± 0.08	3.3 ± 0.01	3.1 ± 0.02
MBP (µg/mg of total protein)	0.7 ± 0.05	0.5 ± 0.02	0.5 ± 0.02	0.7 ± 0.02
CNP (µmol of 2'-NADP/min/mg of total protein)	2.5 ± 0.5	1.9 ± 0.3	1.7 ± 0.3	2.2 ± 0.2
ChAT (pmol/min/mg of total protein)	282.8 ± 59	457.3 ± 60	470.9 ± 15.9	268.9 ± 12.2
GAD (µmol/min/mg of total protein)	1.0 ± 0.5	1.4 ± 0.1	1.6 ± 0.5	1.0 ± 0.3
GS (µmol/min/mg of total protein)	133.5 ± 1.1	194.9 ± 1.3	178.2 ± 1.1	85.3 ± 1.1

*Data are mean ± SEM values from three independent experiments, n = 9. They were compared to untreated controls, and the differences were not statistically significant ($P > 0.01$).

^aCultures were treated with guinea pig serum at a concentration of 25 µl/ml.

^bPurified nonspecific and anti-RV IgG were added to the culture flasks at a concentration of 125 µg/ml.

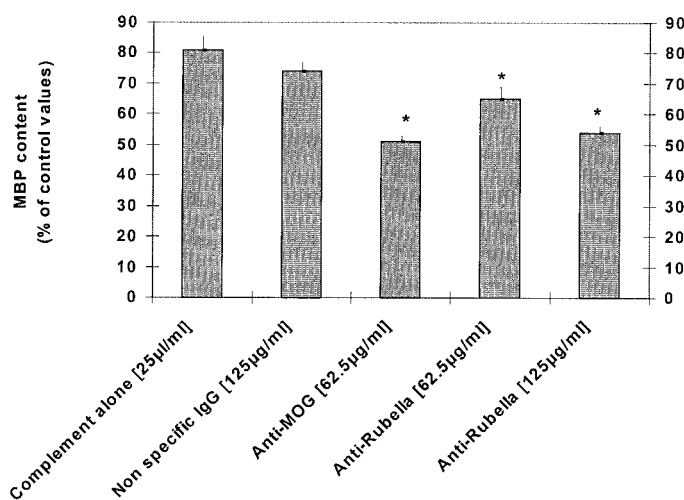


Fig. 1. Demyelination in aggregating brain cell cultures induced by various antibodies in the presence of complement. MBP levels were measured by radioimmunoassay. Data of two independent experiments (n = 6) are expressed as percentage of untreated controls (mean ± SEM). *Significant difference ($P < 0.01$) relative to untreated controls.

anti-RV IgG-treated cultures without complement had GS values similar to those of untreated controls (Table III). All these observations favor the hypothesis of the presence of trophic factors in the guinea pig serum that improved the differentiation of neurons and astrocytes.

Depletion of MOG in Rat CNS Cultures

The interaction of anti-RV antibodies with MOG was assessed using Western blot semiquantitation. Anti-

RV IgG in the presence of complement depleted MOG dramatically from myelin of aggregating brain cell cultures in a dose-dependent manner (Fig. 2). The toxic effect on oligodendrocytes was already maximal at a concentration of 93.8 µg/ml. The loss of MOG was relatively more pronounced than that of MBP with similar concentrations of anti-RV antibodies. The depletion was specific for anti-RV antibodies; it was not observed in aggregates exposed to complement alone or to NS IgG in the presence of complement (Fig. 2).

Colocalization of Anti-RV and Anti-MOG Antibodies

We used immunocytochemical and Western blot techniques to test the hypothesis that anti-RV IgG recognizes and binds to an epitope on MOG and thus induces demyelination. Primary monolayer cultures of rat oligodendrocytes were incubated with both anti-RV and anti-MOG antibodies. Anti-RV and anti-MOG antibodies colocalized on the processes of oligodendrocytes (Fig. 3). However, anti-RV IgG was less specifically restricted to the oligodendrocytic processes than anti-MOG.

To test the cross-reactivity of anti-RV antibodies with MOG, CNS and peripheral nervous system (PNS) myelin was purified and proteins were separated and blotted on a PVDF membrane. Rat PNS myelin was used as a negative control, because MOG is absent from the PNS. Anti-RV and anti-MOG IgG revealed a 28 kDa band in both human and rat CNS myelin (Fig. 4). Human MOG had a slightly higher molecular weight than rat MOG. This could be due to a difference in the glycosylation pattern of the two proteins. A nonspecific 30 kDa band was revealed with anti-RV IgG in PNS myelin. However,

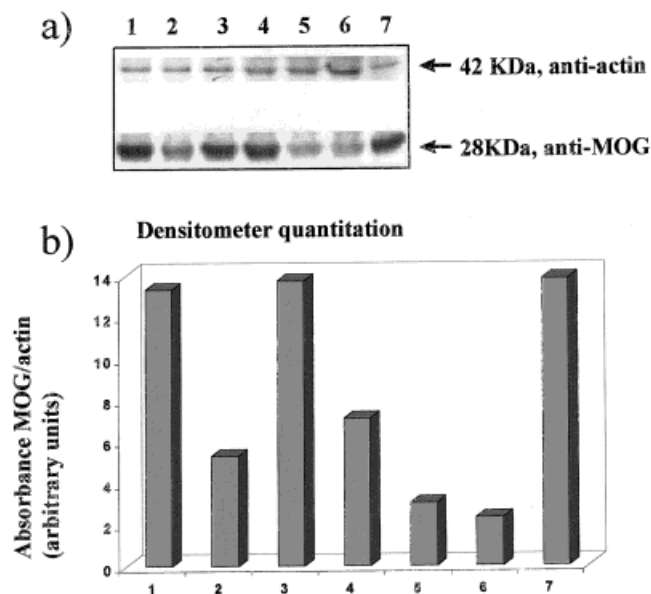


Fig. 2. Loss of MOG in aggregating brain cell cultures after treatment with increasing concentrations of purified anti-RV IgG in the presence of complement. Aggregate homogenate samples were submitted to SDS-PAGE and blotted on PVDF membranes. Blots were incubated with anti-MOG IgG (1/1,000) and with antiactin (1/500) to determine the levels of MOG in the different treated cultures. **a:** Rat CNS cultures were treated on days 27, 29, 31, and 33 with 1) complement alone (25 $\mu\text{g}/\text{ml}$), 2) anti-MOG (62.5 $\mu\text{g}/\text{ml}$), 3–6) increasing concentrations of anti-RV IgG (31.3 $\mu\text{g}/\text{ml}$, 62.5 $\mu\text{g}/\text{ml}$, 93.8 $\mu\text{g}/\text{ml}$, 125 $\mu\text{g}/\text{ml}$), 7) nonspecific IgG (125 $\mu\text{g}/\text{ml}$). In 2–7, complement was added at 25 $\mu\text{g}/\text{ml}$. **b:** Densitometer quantitation of MOG was determined on the blotted homogenates (a). Actin was used as a standard to correct for the difference in protein amounts.

incubation with either antibody gave no signal in the 28 kDa region. These observations indicate that anti-RV can recognize epitopes on MOG.

DISCUSSION

MS is an inflammatory demyelinating disease of the CNS influenced by both genetic susceptibility and unknown environmental factors. The major pathogenetic mechanism leading to specific patterns of inflammation seems to involve a class II restricted T-helper 1 immune reaction (Storch and Lassmann, 1997). These T cell-mediated immune responses induce minor demyelination in experimental models. Other immunological mechanisms must account for the widespread destruction of myelin in MS patients. Antibody involvement has been suggested by observations that intact B cell function is required for full expression of EAE (Gausas et al., 1992). Antibodies directed against very few specific CNS antigens, such as gangliosides and galactocerebroside (Roth et al., 1985), myelin-associated glycoprotein (MAG) (Sergott et al., 1988), and MOG (Kerlero de Rosbo et al., 1990), have demyelinating activities. By contrast, immunization against quantitatively important myelin proteins, MBP or

PLP, induces inflammation but little or no demyelination (Genain et al., 1995). MOG appears to be the most interesting target antigen, in that it is currently the only CNS protein that can elicit both a T cell-mediated immune reaction that induces an inflammation and a demyelinating antibody response (Adelmann et al., 1995). Moreover, in lesions of acute MS and in the marmoset model of EAE, antibodies against MOG are specifically bound to networks of disintegrating myelin sheaths (Genain et al., 1999).

The mechanisms leading to the appearance in the CNS of autoantibodies against essential antigens are uncertain. Among the various triggers potentially responsible, viruses are known to lead to autoimmune phenomena (Herrath, 2000). Neurotropic viruses recruit specific B cells in the CNS that result in long-term persistence of memory B cells (Gerhard and Koprowski, 1977). If a new inflammatory event with T-helper cells occurs in the CNS, the memory B cells can be susceptible to reactivation and various virus-specific antibodies could be locally released in the absence of the pathogenic agents. These antiviral responses may favor autoimmunity by cross-reacting with self-peptides. Molecular mimicry between microbial and self-antigens has been proposed many times in the pathogenesis of important human diseases, such as the Guillain Barré syndrome, type 1 diabetes, and rheumatic fever (Rose and Mackay, 2000). With the exception of the Guillain-Barré syndrome, the association of these clinical entities with infectious agents remains a controversial subject. Several studies linked viruses and MS. Wucherpfenning and Strominger (1995) studied the T cell reactivity of a number of viral proteins on MBP specific clones from MS patients. Mokhtarian et al. (1999) studied SFV infection in mice that induced an acute CNS viral infection, followed by demyelination after viral clearance. This autoimmune CNS disease was a consequence of molecular mimicry between SFV and MOG. The effector mechanisms involved an antibody response to the mimicked peptide. Such observations seem to favor the mimicry concept in the case of MS.

We searched for a potential viral candidate that would exhibit sufficient molecular mimicry with MOG to induce demyelinating antibodies. We focused on HB and RV, which have long been implicated in MS (Atkins et al., 1991). RV is a togavirus with a molecular biology similar to that of SFV. High titers against structural glycoprotein E₂ of RV were found to be specific for MS patients (Nath and Wolinsky, 1990). Our search for homologous amino acid stretches revealed several sequence similarities between both viruses and myelin proteins, but the most interesting sequence homology appeared to be between an epitope of structural glycoprotein E₂ of RV and MOG peptide 1–10. We found three consecutive exact amino acid homologies combined with partial homologies in the same region of both proteins. Such a cluster of complete and partial homologies is sufficient for molecular mimicry between two peptides to result in cross-proliferation (Mokhtarian et al., 1999). Further-

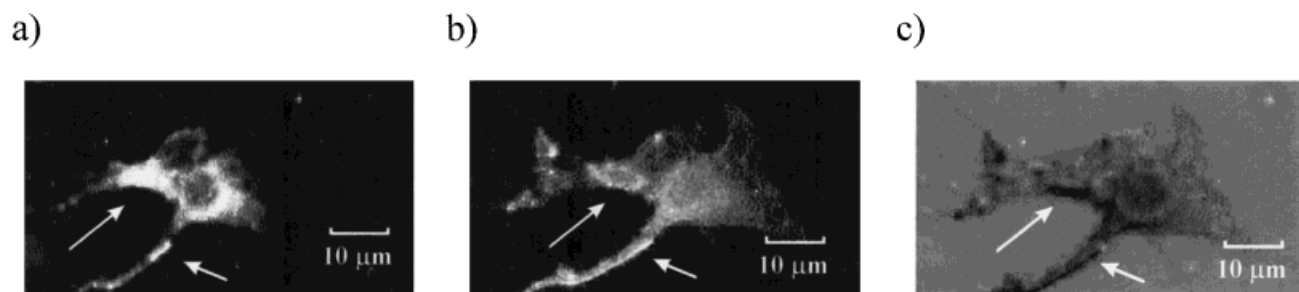


Fig. 3. Double-label immunofluorescence of anti-RV (Cy3) and anti-MOG (FITC) in the same oligodendrocyte. **a:** Localization of anti-RV IgG (100-fold dilution). **b:** Localization of anti-MOG IgG (200-fold dilution). **c:** Colocalization of both proteins visualized by image subtraction.

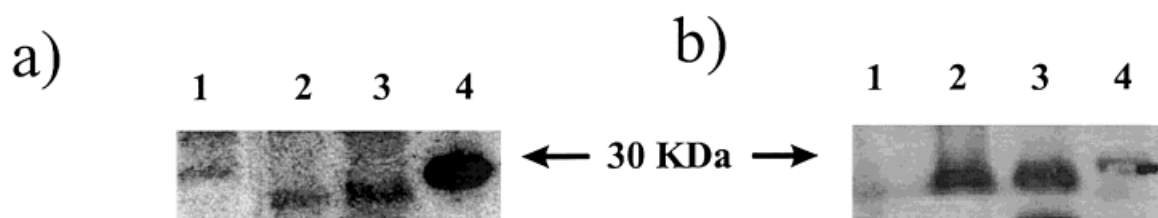


Fig. 4. Western blot of myelin purified from human and rat brain. A 28 kDa protein band was detected using anti-RV (a) and anti-MOG (b) antibodies. **a:** Incubation with anti-RV antibodies (500-fold dilution); the different lanes correspond to rat PNS myelin (1), rat CNS myelin (2), human CNS myelin (3), and molecular weight marker (4). **b:** Incubation with anti-MOG antibodies (1,000-fold dilution); the different lanes correspond to rat PNS myelin (1), rat CNS myelin (2), human CNS myelin (3), and molecular weight marker (4).

more, an important epitope has been described in MOG peptide 1–20, and demyelinating antibodies in serum of MS patients and in MOG-immunized marmosets exclusively recognize this sequence (Genain et al., 1999). The present findings reveal the existence of an epitope of glycoprotein E₂ of RV that exhibits molecular mimicry with an important epitope of MOG. Anti-RV antibodies against glycoprotein E₂ could contribute to a demyelinating activity and explain their relevance in serum of MS patients.

Aggregating brain cell cultures allowed us to focus our study on the cytotoxic effect of antiviral antibodies in the absence of complex immune interactions. Both anti-HB and anti-RV IgG were tested, but only antibodies against RV induced a dose-related demyelination in the presence of complement. In the case of HB, sequence homologies with MBP do not seem to induce demyelinating antibodies. This is consistent with the fact that HB has not been associated, clinically or epidemiologically, with MS (Fujinami and Oldstone, 1985). Furthermore, anti-MBP antibodies have no demyelinating activity (Kerlero de Rosbo et al., 1990). We confirmed the demyelinating activity in rat CNS cultures of anti-MOG antibodies in the presence of complement (Kerlero de Rosbo et al., 1990). These findings suggest that only antibodies against this CNS antigen or antibodies directed against an antigen with sufficient molecular mimicry with MOG can lead to demyelination.

Several controls were performed to test the specificity of the demyelinating activity of anti-RV and anti-MOG antibodies. We excluded the presence of autoreactive antibodies and showed that NS IgG is not intrinsically myelinotoxic, corroborating previous observations (Stangel et al., 2000). We also evaluated the toxicity of complement alone on the aggregates. In our experiments, complement did not induce a significant toxicity in CNS cultures, confirming previous studies on the same *in vitro* model (Kerlero de Rosbo et al., 1990). However, the role of complement remains controversial. Previous work has shown that rat oligodendrocytes have a unique sensitivity to complement and that exposure to syngeneic serum is able to activate the classical complement pathway in the absence of antigen–antibody interactions (Scolding et al., 1990). The surface component on oligodendrocytes responsible for the complement-mediated injuries remains elusive, but certain authors have suggested that MOG could activate an antibody-independent complement injury by binding the C1q component (Johns and Bernard, 1997). However, other studies have failed to reproduce this cytotoxicity of complement on oligodendrocytes (Ruijs et al., 1990). It seems that *in vivo* demyelination is the result of complex interactions in response to both antibody-dependent and antibody-independent complement injury to oligodendrocytes. In our study, complement alone had no significant demyelinating activity. Thus, the demyelination observed in aggregates chal-

lenged with anti-MOG or anti-RV IgG in the presence of complement was specific only for these two antibodies. The mechanism implicated in the myelin destruction in the aggregates could be linked to the generation of complement membrane attack complexes (MAC) following complement fixation and activation by myelin-specific antibodies.

The evidence of a specific injury to oligodendrocytes was further reinforced by measuring a decrease in CNP specific activity in cultures treated with anti-RV and anti-MOG in the presence of complement. This marker is more specific for the noncompact plasmic membranes of oligodendrocytes, and, as expected, the effect was less severe than for MBP, confirming that the attack was preferentially directed to the myelin sheath.

Other parameters specific for cholinergic and GABAergic neurons (ChAT and GAD, respectively) and for astrocytes (GS) were analyzed to assess the specificity of the antibodies to only one cell type, namely, the oligodendrocyte. As expected, no effect on either neurons or astrocytes was observed in cultures treated with anti-RV or anti-MOG in the presence of complement. On the contrary, all cultures treated with guinea pig serum had higher values of ChAT, GAD, and GS compared to untreated controls. These highly increased enzymatic activities in cultures exposed to serum may be attributed to the presence of trophic factors.

To support our hypothesis that demyelination was caused by the recognition of anti-RV IgG of a MOG epitope, we quantified MOG in our aggregates. MOG was depleted in a dose-dependent manner in cultures treated with increasing concentrations of anti-RV or anti-MOG in the presence of complement. This effect was more pronounced than that on MBP at similar concentrations of immunoglobulins, confirming that the primary target appears to be MOG. Further evidence of the binding of anti-MOG and anti-RV IgG on MOG came from the observation that both antibodies colocalized on oligodendrocyte processes and both revealed by Western blot a 28 kDa protein in CNS myelin, a molecular weight that corresponds to MOG (Gardinier et al., 1992). These results are strong indications that both anti-MOG and anti-RV antibodies bind specifically to this myelin antigen. Furthermore, these results support the hypothesis that an antibody response to a viral epitope exhibiting sufficient molecular mimicry with MOG can induce a demyelinating activity similar to that of anti-MOG antibodies. Depletion in MOG could be followed by a complete disintegration of the myelin membrane and a secondary loss of other myelin proteins, such as MBP. This membrane injury could in turn induce the release of sequestered self-antigens and explain the relatively broad spectrum of antigen specificity detected in the T cell repertoire of individual MS patients (Miller et al., 1995). Immune responses can spread either to new epitopes of a target antigen (intramolecular spreading) or to determinants of another antigen (intermolecular spreading; Miller et al., 1997). By the time when the autoimmune disease is di-

agnosed, the causative agent has been cleared by the host's immune system, and no viral footprints are any longer detectable. Moreover, epitope and antigenic spreading makes the search for the initiating antigen very difficult.

This study suggests that a virus such as RV can trigger an autoimmune demyelination. White matter injury can be mediated by autoantibodies that cross-react with a myelin antigen and epitope of RV. These in vitro findings should encourage further studies with MS patients to confirm the importance of a rubella infection as a triggering mechanism for the autoimmune attack on myelin. In this respect, one should consider the possibility of engineering a new rubella vaccine in which the sequence found to be similar to MOG would be deleted.

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REFERENCES

- Adelmann M, Wood J, Benzel I, Fiori P, Lassmann H, Matthieu JM, Gardinier MV, Dormmair K, Linington C. 1995. The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) induces acute demyelinating experimental autoimmune encephalomyelitis in the Lewis rat. *J Neuroimmunol* 63:17-27.
- Atkins GJ, Mooney DA, Fahy DA, Ng SH, Sheahan BJ. 1991. Multiplication of rubella and measles viruses in primary rat neural cell cultures: relevance to a postulated triggering mechanism for multiple sclerosis. *Neuropathol Appl Neurobiol* 17:299-308.
- Bachmann S, Kesselring J. 1998. Multiple sclerosis and infectious childhood diseases. *Neuroepidemiology* 17:154-160.
- Bernard CCA, Randell VB, Horvath LB, Carnegie PR, Mackay IR. 1981. Antibody to myelin basic protein in extracts of multiple sclerosis. *Immunology* 43:447-457.
- Brunner C, Lassmann H, Waehnelndt TV, Matthieu JM, Linington C. 1989. Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. *J Neurochem* 52:296-304.
- Bürgisser P. 1983. Mise au point d'un dosage radioimmunologique de la protéine basique de la myéline dans le liquide céphalo-rachidien et dans le tissu nerveux. MD thesis, Université de Lausanne, Lausanne, Switzerland.
- Damien RT. 1964. Molecular mimicry: antigen sharing by parasite and host and its consequences. *Am Nat* 98:129-149.
- Felgenhauer K, Reiber H. 1992. The diagnostic significance of antibody specificity indices in multiple sclerosis and herpes virus induced diseases of the nervous system. *Clin Invest* 70:28-37.
- Fujinami RS, Oldstone MBA. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: a mechanism for autoimmunity. *Science* 230:1043-1045.
- Gardinier MV, Amiguet P, Linington C, Matthieu JM. 1992. Myelin/oligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily. *J Neurosci Res* 33:177-187.
- Gausas J, Paterson PY, Day AD, Dal Canto MC. 1992. Intact B-cell activity is essential for complete expression of experimental allergic encephalomyelitis in Lewis rats. *Cell Immunol* 72:360-366.
- Genain CP, Nguyen MH, Letvin NL, Pearl R, Davis RL, Adelman M, Lees MB, Linington C, Hauser SL. 1995. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J Clin Invest* 96:2966-2974.

- Genain CP, Cannella B, Hauser SL, Raine CS. 1999. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med* 5:170–175.
- Gerhard W, Koprowski H. 1977. Persistence of virus-specific memory B cells in mice CNS. *Nature* 266:360–361.
- Gibbs F, Gibbs E, Carpenter P. 1959. Electroencephalographic abnormality in uncomplicated childhood diseases. *JAMA* 171:1050–1055.
- Herrath MG. 2000. Obstacles to identifying viruses that cause autoimmune disease. *J Neuroimmunol* 107:154–160.
- Honegger P, Matthieu JM. 1980. Myelination of aggregating fetal rat brain cell cultures grown in a chemically defined medium. In: Baumann N, editor. *Neurological mutations affecting myelination*. Amsterdam: Elsevier North Holland. p 481–488.
- Johns TG, Bernard CC. 1997. Binding of complement component C1q to myelin oligodendrocyte glycoprotein: a novel mechanism for regulating CNS inflammation. *Mol Immunol* 34:33–38.
- Kerlero de Rosbo N, Honegger P, Lassmann H, Matthieu JM. 1990. Demyelination induced in aggregating brain cell cultures by a monoclonal antibody against myelin/oligodendrocyte glycoprotein. *J Neurochem* 55:583–587.
- Kurihara T, Tsukada Y. 1967. The regional and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphohydrolase in the central nervous system. *J Neurochem* 14:1167–1174.
- Labourdette G, Roussel G, Ghandour MS, Nussbaum JL. 1979. Cultures from rat brain hemispheres enriched in oligodendrocyte-like cells. *Brain Res* 179:199–203.
- Lennette DA. 1978. An improved mounting medium for immunofluorescence microscopy [letter]. *Am J Clin Pathol* 69:647–648.
- Linington C, Webb M, Woodhams PL. 1984. A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody. *J Neuroimmunol* 6:387–396.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.
- Matthieu JM, Honegger P, Favrod P, Gautier E, Dolivo M. 1979. Biochemical characterization of a myelin fraction isolated from rat brain aggregating cell cultures. *J Neurochem* 32:869–881.
- Miller SD, McRae BL, Vanderlugt CL, Nikcevic KM, Pope JG, Pope L, Karpus WJ. 1995. Evolution of the T-cell repertoire during the course of experimental immune-mediated demyelinating diseases. *Immunol Rev* 144:225–244.
- Miller SD, Vanderlugt CL, Begolka WS, Pao W, Yauch RL, Neville KL. 1997. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat Med* 3:1133–1136.
- Mokhtarian F, Zhang Z, Shi Y, Gonzales E, Sobel RA. 1999. Molecular mimicry between a viral peptide and a myelin oligodendrocyte glycoprotein peptide induces autoimmune demyelinating disease in mice. *J Neuroimmunol* 95:43–54.
- Monnet-Tschudi F, Zurich MG, Schilter B, Costa LG, Honegger P. 2000. Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetyl cholinesterase and neuronal and glial markers in aggregating brain cell cultures. *Toxicol Appl Pharmacol* 165:175–183.
- Nath A, Wolinsky JS. 1990. Antibody response to rubella virus structural proteins in multiple sclerosis. *Ann Neurol* 27:533–536.
- Ou D, Chong PL, Tripet B, Gillam S. 1992. Analysis of T- and B-cell epitopes of capsid protein of rubella virus by using synthetic peptides. *J Virol* 66:1674–1681.
- Patel AJ, Hunt A, Gordon RD, Balazs R. 1982. The activities in different neural cell types of certain enzymes associated with metabolic compartmentation of glutamate. *Dev Brain Res* 4:3–11.
- Rose NR, Mackay IR. 2000. Molecular mimicry: a critical look at exemplary instances in human diseases. *Cell Mol Life Sci* 57:542–551.
- Roth GA, Roytta M, Yu RK, Raine CS, Bornstein MB. 1985. Antisera to different glycolipids induce myelin alterations in mouse spinal cord tissue cultures. *Brain Res* 339:9–18.
- Ruijs TCG, Olivier A, Antel JP. 1990. Serum cytotoxicity to human and rat oligodendrocytes in culture. *Brain Res* 42:105–112.
- Scolding NJ, Morgan BP, Campbell AK, Compston DAS. 1990. Complement mediated serum toxicity against oligodendrocytes: a comparison with other cells of the oligodendrocyte-type 2 astrocyte lineage. *J Neurol Sci* 97:155–162.
- Sergott RC, Brown MJ, Lisak RP, Miller SL. 1988. Antibody to myelin-associated glycoprotein produces central nervous system demyelination. *Neurology* 38:422–426.
- Sindic CJM, Monteyne Ph, Laterre EC. 1994. The intrathecal synthesis of virus-specific oligoclonal IgG in multiple sclerosis. *J Neuroimmunol* 54:75–80.
- Stangel M, Compston A, Scolding NJ. 2000. Oligodendroglia are protected from antibody-mediated complement injury by normal immunoglobulins (IVIg). *J Neuroimmunol* 103:195–201.
- Storch M, Lassmann H. 1997. Pathology and pathogenesis of demyelinating diseases. *Curr Opin Neurol* 10:186–192.
- Wilson SH, Schrier BK, Farber JL, Thompson EJ, Rosenberg RN, Blume AJ, Nirenberg MW. 1972. Markers for gene expression in cultured cells from the nervous system. *J Biol Chem* 247:3159–3169.
- Wucherpfenning KW, Strominger JL. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80:695–705.