Characterization of the expression and immunogenicity of the ns4b protein of human coronavirus 229E

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Abstract: Sequencing of complementary DNAs prepared from various coronaviruses has revealed open reading frames encoding putative proteins that are yet to be characterized and are so far only described as nonstructural (ns). As a first step in the elucidation of its function, we characterized the expression and immunogenicity of the ns4b gene product from strain 229E of human coronavirus (HCV-229E), a respiratory virus with a neurotropic potential. The gene was cloned and expressed in bacteria. A fusion protein of ns4b with maltose-binding protein was injected into rabbits to generate specific antibodies that were used to demonstrate the expression of ns4b in HCV-229E-infected cells using flow cytometry. Given a previously reported contiguous five amino acid shared region between ns4b and myelin basic protein, a purified recombinant histidine-tagged ns4b protein and (or) human myelin basic protein were injected into mice to evaluate whether myelin–viral protein cross-reactive antibody responses could be generated. Each immunogen induced specific but not cross-reactive antibodies. We conclude that ns4b is expressed in infected cells and is immunogenic, although this does not involve amino acids shared with a self protein, at least in the experimental conditions used.

Key words: human coronavirus 229E, nonstructural protein, ns4b protein, expression, immunogenicity.

Résumé : Le séquençage d'ADN complémentaires préparés à partir de divers coronavirus a révélé des cadres de lecture ouverts codant d'hypothétiques protéines qui ne sont pas encore caractérisées et que l'on nomme protéines non structurales (ns). Dans une première étape de la caractérisation de sa fonction, nous avons étudié l'expression et l'immunogénicité du produit du gène ns4b de la souche 229E du coronavirus humain (HCV-229E), un virus respiratoire possédant un potentiel neurotrope. Le gène a été cloné et exprimé dans des bactéries. Une protéine de fusion de ns4b avec la protéine liant le maltose a été injectée à des lapins afin de produire un antisérum spécifique qui a ensuite été utilisé pour démontrer, par cytométrie de flux, l'expression de ns4b dans des cellules infectées. Étant donné notre observation antérieure d'une séquence de cinq acides aminés partagée entre ns4b et la protéine basique de la myéline, une protéine ns4b recombinante comprenant une queue de résidus histidine et (ou) la protéine basique de la myéline humaine ont été injectées à des souris pour évaluer l'induction possible d'anticorps montrant une réaction croisée envers ces deux protéines. Chaque immunogène a induit des anticorps spécifiques qui ne présentaient pas de réactions croisées. Nous concluons que la protéine ns4b est exprimée dans les cellules infectées et qu'elle est immunogène, quoique cette réponse immunitaire ne ciblait pas les acides aminés partagés avec une protéine du soi, au moins dans les conditions expérimentales utilisées.

Mots clés : coronavirus humain 229E, protéine non structurale, protéine ns4b, expression, immunogénicité.

Received February 19, 1998. Revision received July 6, 1998. Accepted August 5, 1998.

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Introduction

Human coronaviruses (HCV) are known to cause between 15 and 35% of common colds (McIntosh 1974; Myint 1994). Coronaviruses possess a single-stranded, positive-sense RNA genome of more than 30 kb (Holmes and Lai 1996). In infected cells, six subgenomic RNAs constitute a nested set of 3′-coterminal mRNA species, of which only the 5′-unique region appears to be translated (Holmes and Lai 1996). Of those mRNAs, four encode structural proteins found in the virion: in HCV-229E infected cells, they are the 50- to 60-kDa nucleocapsid N protein associated with genomic RNA (Schreiber et al. 1989); the 21- to 25-kDa M glycoprotein associated with the viral envelope (Jouvenne et al. 1990; Raabe and Siddell 1989*a*); the 170- to 200-kDa S glycoprotein that forms viral spikes (Raabe et al. 1990); and the 9- to 12-kDa E protein, a small membrane protein with unknown functions (Holmes and Lai 1996). In addition to

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these proteins, two genes on mRNA 4 of HCV-229E have, by analogy to other coronaviruses, the potential to encode nonstructural proteins that have until now not been detected and characterized (Jouvenne et al. 1992; Raabe and Siddell 1989*b*). Interestingly, we have previously reported that the putative protein encoded by mRNA 4b of human coronavirus 229E shares five identical contiguous amino acids (LSLSR sequence) with residues 109–113 of myelin basic protein (Jouvenne et al. 1992).

In this study, we show that the ns4b protein is indeed expressed in HCV-229E-infected cells and is immunogenic in rabbits and mice, although antibodies that are produced in SJL/J mice do not appear to recognize the region of amino acid homology with myelin basic protein. HCV-229E was propagated in the L-132 human embryonic lung cell line as described previously (Jouvenne et al. 1992). Infected (multiplicity of infection, 0.01; 33°C; 27 h) and uninfected cells were lysed by freezing at –90°C overnight. The next day, the lysate was treated with guanidium isothiocyanate and the RNA was purified by ultracentrifugation on cesium chloride (Chirgwin et al. 1979).

Production of a ns4b fusion protein with the maltose-binding protein (MBP) was achieved after reverse transcriptase – polymerase chain reaction and cloning into the pMAL-c2 vector (New England Biolabs). The antisense primer 5′-AGCAGGACTCTGATTACGAGAAGG-3′, complementary to nucleotides 783–806 of the HCV-229E N protein gene (Schreiber et al. 1989), was used for cDNA synthesis. The sense primer used for cDNA amplification was 5′-CTTCAATGTAAGGATCCTTTGCT**ATG**CAAGG-3′ and the antisense primer was 5′-GATCATCCACTAGCTTGTCGACCATC**TTA**GTGG-3′. Those primers correspond to nucleotides 417–447 and 700–732, respectively, of the ns4b gene (Raabe and Siddell 1989*b*). The sense primer contains a *Bam*HI restriction site (underlined) and the antisense primer contains a *Sal*I restriction site (underlined) to allow directional cloning into pMAL-c2 and expression through the supplied initiation and termination codons (bolded). The reverse transcriptase – polymerase chain reaction was performed in two steps: 15 μg of RNA and 40 pmol of the antisense primer were mixed and heated at 65°C for 5 min and allowed to cool to 20°C in 20 min. Then, 10 μL of Moloney RT buffer 10× (500 mM Tris–HCl (pH 8), 625 mM KCl, 30 mM $MgCl_2$, 100 mM dithiothreitol), 2 μL of RNAguard™ (30 U/μL), 2 μL of 20 mM deoxynucleoside triphosphate, and 3 μL of Moloney leukemia virus reverse transcriptase (16 U/μL; Pharmacia) were added, and the mix was heated at 42°C for 90 min to allow cDNA synthesis. For polymerase chain reaction, 10 μL of cDNA was added to 25 pmol of each of the primers, 10 μL of 10× *Taq* DNA polymerase buffer (Bio-Can), 10 μL of 25 mM $MgCl_2$ (Bio-Can), and 3 μL of 20 mM deoxynucleoside triphosphate (Pharmacia). The samples were then heated at 94°C for 5 min and at 60°C for 5 more min (hot start). One microliter of *Taq* DNA polymerase (5 U/μL) was then added, and the samples went through 30 cycles of: 1 min at 94°C, 2 min at 60°C, and 2 min at 72°C, followed by 10 min at 72°C. The amplification product was cloned into the pCRII TA (Invitrogen) vector and then subcloned into pMAL-c2. The resulting plasmid was introduced into *E. coli* BL21 (DE3), which had been rendered competent by permeabilization with calcium chloride (Sambrook et al. 1989). The plasmid was introduced into bacteria by thermal shock, heating the mix at 65°C for 1 min, adding medium, and shaking the mix for 1 h at 37°C before plating on a petri dish and incubating overnight at 37°C.

Production of the MBP–ns4b fusion protein was induced in selected clones by adding 0.3 mM isopropyl β-thiogalactoside. The location of the expression of the fusion protein was first assayed, and it was found to be soluble which allowed purification under native conditions. The fusion protein was purified by affinity chromatography on an amylose resin. The soluble fraction was deposited directly on the amylose column, washed to eliminate everything that did not bind, and the protein was eluted with 10 mM maltose according to the manufacturer's instructions (New England Biolabs). An immune rabbit serum was then prepared. Approximately 300 μg of the purified MBP–ns4b fusion protein in complete Freund's adjuvant was injected subcutaneously into a 3 kg New Zealand White female rabbit, and this was followed by five injections (every 2 weeks) of fusion protein in incomplete Freund's adjuvant. For flow cytometric detection of ns4b with this immune serum, 10^6 cells infected by HCV-229E at a multiplicity of infection of 0.01 for 44 h were fixed with 1% v/v paraformaldehyde for 20 min at room temperature. Cells were washed twice with 0.05% w/v saponin in phosphate-buffered saline (PBS) and permeabilized with PBA–saponin (PBS containing 0.5% w/v bovine serum albumin, 0.05% w/v sodium azide, and 0.05% w/v saponin) on ice for 30 min. Cells were then blocked with 1.25 μg of human IgG and stained with primary rabbit antiserum to MBP–ns4b at a dilution of 1/125 on ice for 30 min. After three washes, the secondary antibody, a phycoerythrin-conjugated anti-rabbit antibody (Bio-Can), was added at a dilution of 1/100, and incubation proceeded on ice in the dark for 20 min. Cells were washed and analyzed on a Coulter XL flow cytometer. Alternative detection techniques, Western immunoblotting using Tris–tricine polyacrylamide gels (Schägger and von Jagow 1987), and radioimmunoprecipitation (Daniel and Talbot 1990) were also attempted, although without success.

A recombinant ns4b protein was also produced without a fusion partner. The ns4b gene was amplified with the sense primer 5′-CTTCAATGTAACCACACTTTCAT**ATG**CAAGG-3′ and the antisense primer 5′-CATCCACTAGCTTAAGGAACATCCTCGAGG-3′, complementary to nucleotides 417–447 and 703–732 of the ns4b gene sequence (Raabe and Siddell 1989*b*). The sense primer contains a *Nde*I restriction site (underlined) and an initiation codon (bolded) and the antisense primer contains a *Xho*I restriction site (underlined) to allow a directional cloning in the pET-21b vector (Novagen), thus getting rid of the MBP but adding a histidine tail to facilitate purification. The amplification product was cloned into the pCRII TA vector and then subcloned into pET-21b. The resulting plasmid was introduced into *E. coli* BL21 (DE3) as described above. The histidine-tailed ns4b protein was produced and purified as described for the MBP–ns4b fusion protein, except that a nickel-agarose resin was used for purification according to the manufacturer's instructions (Qiagen).

For immunogenicity studies, five groups of 35-day-old female SJL/J mice (National Cancer Institute contract to

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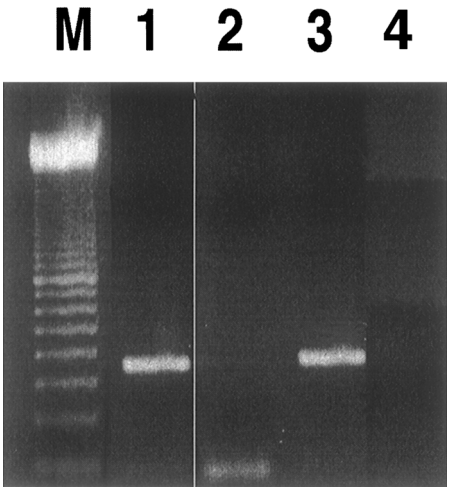
Fig. 1. Ethidium bromide-stained agarose gel showing the amplified ns4b gene. Lane M, 100 bp DNA ladder (Pharmacia); lane 1, amplification of a 316-bp amplicon for cloning into pMAL-c2; lane 2, amplification of RNA from uninfected cells; lane 3, amplification of a 313-bp amplicon for cloning into pET-21b; and lane 4, no DNA. Images were generated with an Applied Innotech IS-100 digital imaging system (San Leandro, Calif.) and formatted with Adobe Persuasion 3.0.5 software.



Charles River) were formed: group 1 received two injections of 400 μg of human myelin basic protein prepared as described previously (Talbot et al. 1996); group 2 received two injections of 200 μg of recombinant ns4b (equimolar with myelin basic protein) produced after cloning into the pET-21b vector; group 3 received one injection of 400 μg of human myelin basic protein and one injection of 200 μg of recombinant ns4b; group 4 received one injection of 200 μg of recombinant ns4b and one injection of 400 μg of human myelin basic protein; and group 5 received two injections of PBS. All injections were subcutaneous and in complete Freund's adjuvant. The first injection was at the base of the tail and the second in the flank. Sinus retroorbital bleedings were performed on days 0, 6, 13, and 28 after the second injection. Serum titers of specific antibodies were followed throughout the experiment using the enzyme-linked immunosorbent assay (ELISA) as described below. Microtiter plates were coated overnight with a preparation of protein (MBP–ns4b, ns4b, or human myelin basic protein) at 2.5 μg/mL. The plates were then blocked with PBS containing 10% v/v fetal calf serum and 0.2% v/v Tween 20. Animal sera were then added at a dilution of 1/50 and serial three- (rabbits) or five-fold (mice) dilutions were made. After a 2-h incubation at room temperature, plates were washed and the secondary antibody conjugated to peroxidase (Kirkegaard and Perry Laboratories) was added at a dilution of 1/2000. After another 2-h incubation followed by washes, the substrate preparation was added. It consisted of 0.05 M citric acid (pH 5.0), 0.1 M sodium phosphate dibasic, 2.2 M *O*-phenylene diamine, and 3 mM hydrogen peroxide. The reaction was stopped after 30 min with 1 N HCl and the plates were read at 492 nm. Antibody titers were calculated as the last serum dilution where a specific signal was observed.

The ns4b gene was successfully amplified with both sets of primers (Fig. 1), cloned into the pMAL-c2 and pET-21b vectors, and the MBP–ns4b and ns4b proteins were produced in and purified from *E. coli* (Fig. 2). The production of a larger fusion protein was deemed necessary to render the small and hydrophobic ns4b protein (Raabe and Siddell 1989*b*) more immunogenic, although we felt it was more relevant to work with the viral protein without a fusion partner (other than a short histidine tail for purification) in immunogenicity experiments. The specificity of the rabbit antibody produced against the MBP–ns4b recombinant protein was determined against MBP–ns4b produced with the pMAL-c2 vector and also against the ns4b produced in the pET-21b vector. Although antibodies were generated against the MBP fusion partner, specific anti-ns4b antibodies were produced in reasonably high titers (ELISA titers of 8 000 against ns4b and 15 000 against MBP–ns4b). However, a second rabbit produced lower specific antibody titers and two guinea pigs did not produce significant anti-ns4b antibodies (data not shown).

As shown in Fig. 3, the anti-ns4b rabbit antiserum detected a signal in HCV-229E-infected cells, unlike preimmune serum, thus demonstrating the expression of the ns4b protein in HCV-229E-infected cells. The peak displacement was estimated at a significant and reproducible 1 log, compared with 3 logs for the positive hyperimmune anti-HCV-229E serum. No signal was observed in uninfected cells (data not shown). Despite numerous attempts, we were unsuccessful in detecting the expression of the ns4b protein in HCV-229E-infected cells by Western immunoblotting or radioimmunoprecipitation (data not shown).

Having shown the expression of the ns4b protein by flow cytometry, we verified its immunogenicity in mice and tested whether the five amino acid homology with myelin basic protein could be the target of cross-reactive antibodies. SJL/J mice were chosen because of the ease with which myelin basic protein immune responses are generated, thus providing an animal model for myelin autoimmunity. Indeed, mice injected twice with human myelin basic protein developed clinical symptoms typical of experimental allergic encephalomyelitis (Krakowski and Owens 1996; data not shown). Throughout the experiment, the development of antibodies against the injected proteins was assessed to detect ns4b–myelin basic protein cross-reactive antibody responses (Fig. 4). All mice developed a good antibody response against all of the proteins they received; however, cross-reactive antibody responses were not observed and no immune-boosting effect of one protein for the other was noticeable.

Knowledge of the expression and function of every protein encoded by a virus genome is critical in understanding viral pathogenesis. Structural proteins of coronaviruses are known and well studied. However, putative nonstructural proteins are much less well characterized, and the expression of some has not even been demonstrated yet. This is the case for the protein encoded by open reading frame 4b of HCV-229E. From its predicted amino acid sequence, computer analysis showed that it should be a small hydrophobic protein with a molecular mass of 10.2 kDa, three potential phosphorylation sites, and a high content (20%) of leucines

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Fig. 2. (A) SDS–PAGE showing the production and purification of the recombinant MBP–ns4b. Samples were separated by SDS–PAGE on a 10% w/v acrylamide gel under reducing conditions and stained with Coomassie blue. Lane M, molecular mass standards (Pharmacia); lane 1, induced cells; lane 2, uninduced cells; and lane 3, purified MBP–ns4b. (B) Tris–tricine gel showing the production and purification of the recombinant ns4b protein. Samples were separated by a Tris–tricine gel on a 10% w/v acrylamide gel under reducing conditions and stained with Coomassie blue. Lane M, molecular mass standards (Bio-Rad); lane 1, induced cells; lane 2, uninduced cells; and lane 3, purified ns4b. Images were generated with an Applied Innotech IS-100 digital imaging system (San Leandro, Calif.) and formatted with Adobe Persuasion 3.0.5 software.

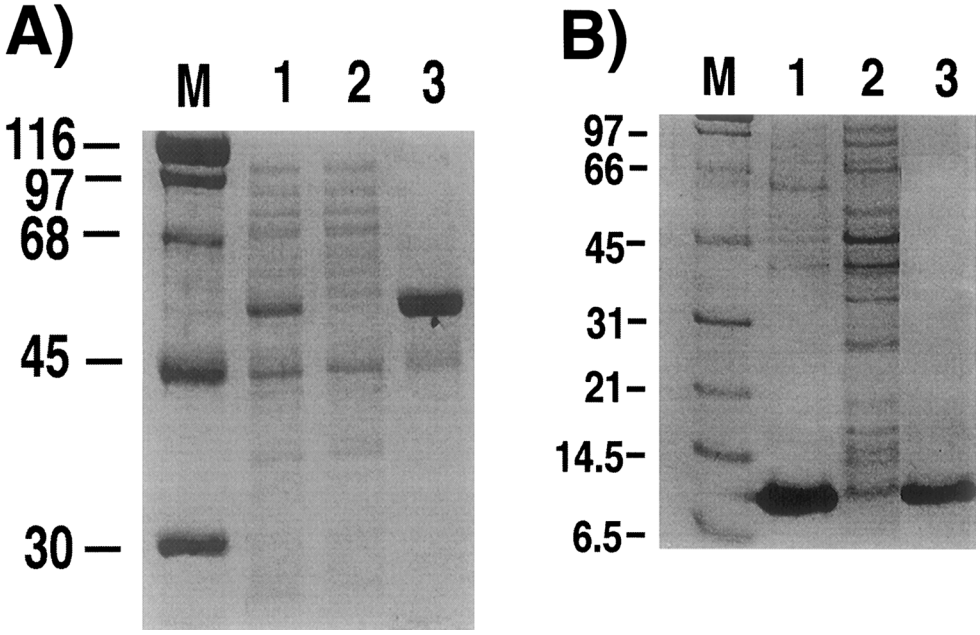
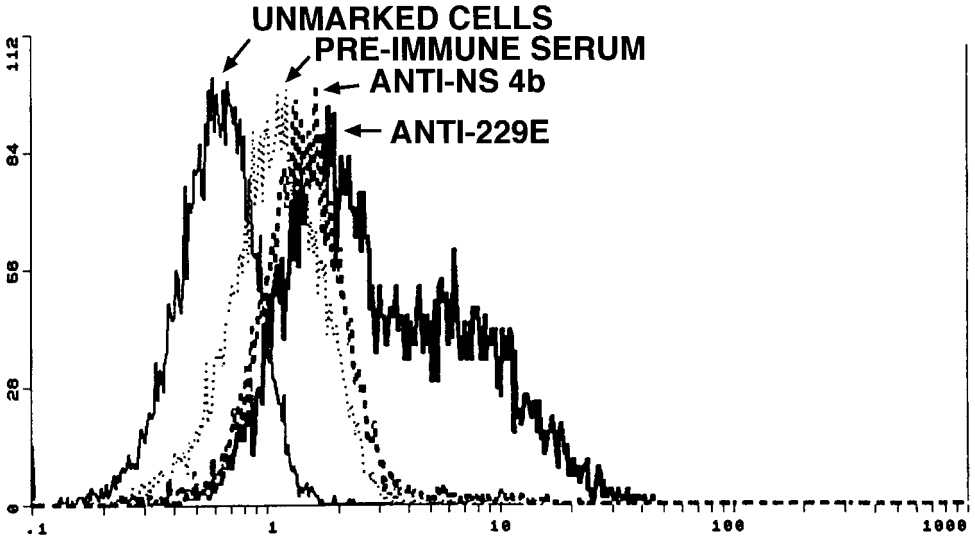


Fig. 3. Flow cytometry analysis on L-132 cells infected with HCV-229E. At 44 h postinfection, cells were fixed and labeled with different primary antibodies, and then with a phycoerythrin-conjugated anti-rabbit secondary antibody. Thin line, unmarked cells (autofluorescence); thin dotted line, preimmune rabbit serum; thick line, anti-229E polyclonal hyperimmune rabbit serum; thick dotted line, anti-ns4b monospecific rabbit serum. The *x* axis represents fluorescence intensity and the *y* axis, the number of cells.



and isoleucines (Raabe and Siddell 1989*b*). This last observation allowed comparison with two other coronaviral nonstructural proteins: one encoded by open reading frame 4 of transmissible gastroenteritis virus, and another encoded by open reading frame 5 of infectious bronchitis virus (Tung et al. 1992; Liu and Inglis 1992). The expression of the former has been demonstrated in infected cells and in association with intracellular membranes, but it has not been found in virions (Tung et al. 1992). The infectious bronchitis virus protein is also expressed in infected cells but nothing else is currently known (Liu and Inglis 1992). This led us to believe that the ns4b protein of HCV-229E would be expressed

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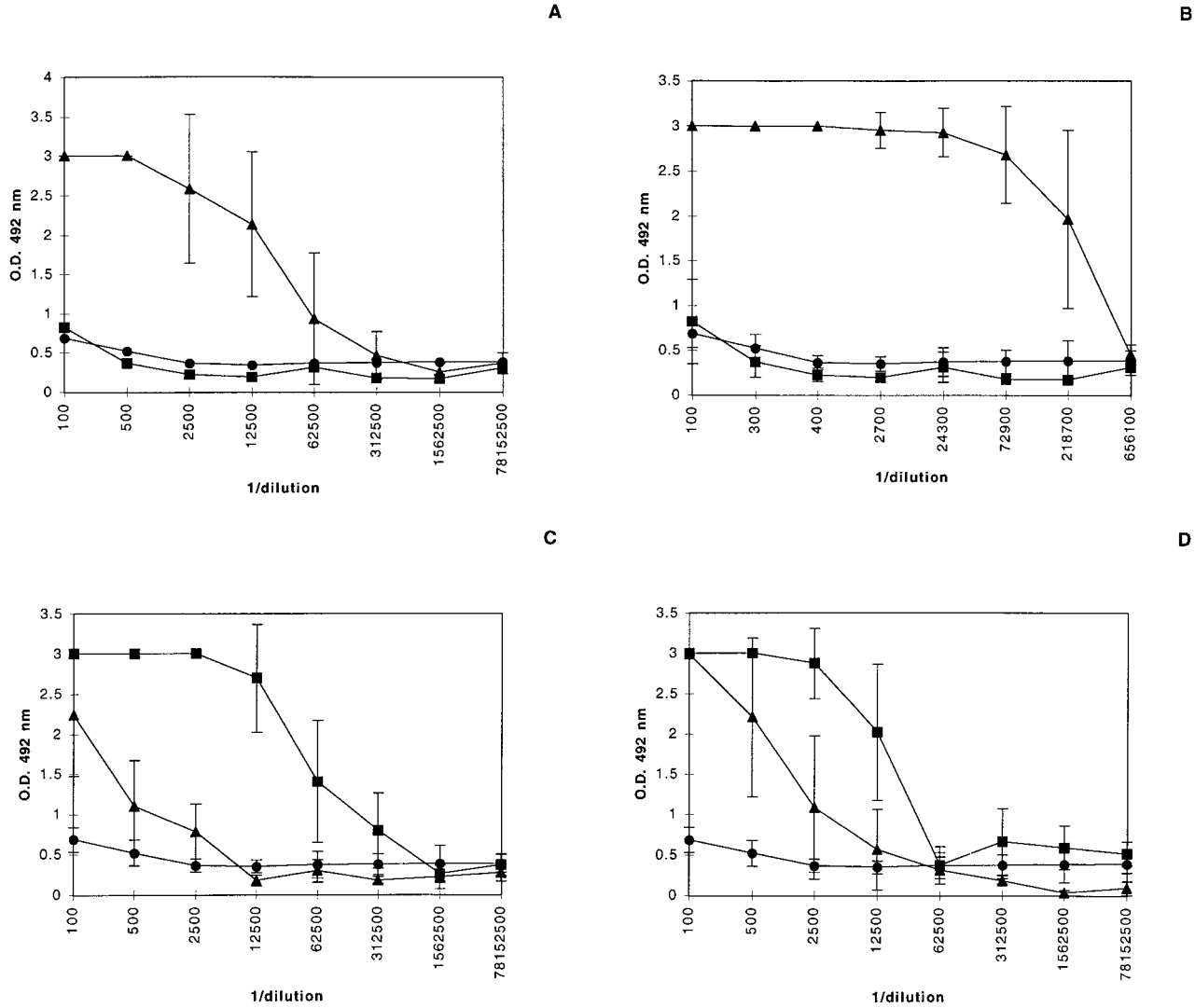
Fig. 4. Immune responses against myelin basic protein and ns4b of mice injected with various combinations of these two antigens. The two antigens were used in the ELISAs. Panel A, two ns4b injections. ▲, ns4b; ■, myelin basic protein; ●, preimmune serum. Panel B, two myelin basic protein injections. ▲, myelin basic protein; ■, ns4b; ●, preimmune serum. Panel C, one myelin basic protein injection followed by one ns4b injection. ▲, ns4b; ■, myelin basic protein; ●, preimmune serum. Panel D, one ns4b injection followed by one myelin basic protein injection. ▲, ns4b; ■, myelin basic protein; ●, preimmune serum.



in infected cells, and we have now indeed shown this expression in infected cells by flow cytometry. Our inability to detect the ns4b protein by Western immunoblotting or radioimmunoprecipitation may be the result of unpredictable factors that will be very difficult to verify, such as a short half-life or the synthesis of very low amounts that only flow cytometry can detect. Also, the predicted hydrophobicity of ns4b may make its detection by Western immunoblotting difficult by preventing its interaction with the blotting membrane and (or) a strong interaction with antibody. Since a homologous protein found in another coronavirus is associated with intracellular membranes, it is likely that ns4b is also. Its function could involve a role in virus replication or virion assembly, although this remains to be investigated.

Previous studies in our laboratory have shown that the predicted amino acid sequence of the ns4b protein shares five contiguous amino acids with residues 109–113 of myelin basic protein. Therefore, we were interested in determining whether the two proteins could induce cross-reactive antibodies. We chose the SJL/J mouse because it is highly responsive to myelin basic protein and also because human and murine myelin basic protein show an amino acid identity of 96%, the shared five amino acids being conserved (Fritz and McFarlin 1989). One of the experimental groups of mice received a first injection of myelin basic protein and a second injection of ns4b and another group received one injection of ns4b, followed by an injection of myelin basic protein. This was done to evaluate whether one protein could prime an antibody response to the other. Cross-reactive antibody responses between ns4b and myelin basic protein were not observed in the two groups of mice that were injected twice with the same protein, and immune boosting of one protein for the other was not apparent at the antibody level. Our results suggest that the myelin basic protein – ns4b shared amino acid sequence is not an immunologically relevant epitope in this mouse strain. It remains

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possible that the sequence would be recognized in other strains of mice, although it is unlikely given the high-responder status of SJL/J mice to myelin basic protein.

In summary, our results confirm the expression of the HCV-229E ns4b protein in infected cells and its immunogenicity in rabbits and mice, although a five amino acid sequence shared with myelin basic protein was not the target of a cross-reactive antibody response in a strain of mouse that is highly susceptible to the development of auto-immune myelin-specific immune responses.

Acknowledgements

We thank Marcel Desrosiers for help with flow cytometry. This work was supported by operating grant MT-9203 from the Medical Research Council of Canada to P.J.T. We thank the Fonds de la recherche en santé du Québec for a studentship to F.C. and senior scholarships to P.J.T. and T.O. We also thank the Natural Sciences and Engineering Research Council of Canada for a studentship award to M.K.

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