Production of virus-specific antiserum corresponding to sequences in the lactate dehydrogenase-elevating virus (LDV) ORF6 protein

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Abstract

The elucidation of the antigenic structure of the envelope proteins of Arteriviridae which includes lactate dehydrogenase-elevating virus (LDV) will provide further understanding of a mechanism of strict host cell specificity. To analyze the linkage between LDV envelope proteins, M/VP-2 and VP-3, which may play an important role in viral infectivity, we generated specific antibody against M/VP-2 that has not been reported in previous studies. A synthetic polypeptide corresponding to the C-terminal region of LDV strain C (LDV-C) ORF6, which encodes M/VP-2, was chemically synthesized and coupled to keyhole limpet hemocyanin (KLH). The peptide was immunogenic in rabbits and induced antibody specific for viral protein. Western blotting and immunofluorescence analysis of virion M/VP-2 in infected macrophages showed that the antibody was able to react specifically with authentic virion protein. The immunoreactive antibody against LDV M/VP-2 described in this study will be useful for further studies of the specific roles of the envelope proteins in arterivirus assembly and infectivity.
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Résumé

L'explication de la structure antigénique des protéines d'enveloppe des Arteriviridae, dont le virus de la lacticodéhydrogénase (LDV) fait partie, devrait permettre de mieux comprendre le mécanisme de la stricte spécificité des cellules hôtes. Pour analyser les liens entre les protéines d'enveloppe du LDV, à savoir M/VP-2 et VP-3, qui jouent certainement un rôle important dans le pouvoir infectant du virus, nous avons créé un anti-corps spécifique de la M/VP-2 dont il n'a pas encore été fait mention dans les études précédentes. Pour ce faire, nous avons chimiquement synthétisé un polypeptide correspondant à la zone de l'extrémité C d'un virus LDV de souche C (LDV-C), ORF6, codant M/VP-2, et nous l'avons couplé à l'hémocyanine de patelle (KLH). Ce peptide, immunogène chez le lapin, induit un anticorps spécifique à la protéine virale. Le transfert de type western et l'analyse en immunoflorescence de M/VP-2 du virion dans des macrophages infectés ont montré que l'anticorps pouvait réagir de manière spécifique avec une protéine authentique de virion. L'anticorps immunoréactif avec la M/VP-2 du LDV décrit dans cette étude va s'avérer utile dans les études qui vont être conduites sur le rôle spécifique des protéines d'enveloppe dans l'Arteriviridae et dans son pouvoir infectant.

1. Introduction

Attachment of the membrane of enveloped viruses to the plasma membrane of receptive host cells is an essential step for viral entry and infection by enveloped viruses. Analysis of the interaction between these membranes is crucial to describe the process of infection by enveloped viruses, as well as to achieve an understanding of replication and pathogenicity of these viruses.

The Arteriviridae are enveloped positive-stranded RNA viruses comprised of a variety of animal pathogens, including lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and porcine reproductive and respiratory syndrome virus (PRRSV) [1]. The ability to replicate in a variety of cell lines is characteristic of only EAV but not of LDV, SHFV, and PRRSV, which demonstrate strict host cell specificity. Only subpopulations of macrophages from the peritoneum and various tissues of mice support LDV replications [2]. Since no LDV receptors responsible for cell tropism have been identified, the underlying mechanism of the restriction of LDV susceptibility has not yet been elucidated.

LDV possesses two major envelope proteins. The smaller of the two is a 18–19 kDa nonglycosylated protein (M/VP-2; encoded by ORF6) possessing three potential adjacent transmembrane segments close to the N-terminal end, which mimic sequences present in the M protein of coronaviruses [3]. The larger protein is a primary envelope glycoprotein (VP-3; encoded by ORF5) that is generally heterogenous in size (25–40 kDa) due to varying amounts of glycosylation [4]. Li et al. [5] have postulated that LDV VP-3 may be the virus attachment protein. Their studies have shown that the neutralization epitope is located in the very short ectodomain of the primary envelope glycoprotein, and that

the epitope is associated with polylactosaminoglycan chains, which may affect the binding of neutralizing antibodies to the LDV virions.

It has been shown that M/VP-2 and VP-3 of LDV are present in virions as heterodimers that are covalently linked by disulfide bond(s), probably located between single cysteine residues in the ectodomains of the two proteins [6]. Since breakage of the disulfide bond(s) in virions resulted in a loss of viral infectivity, the linkage between M/VP-2 and VP-3 seems to be required for host cell entry, perhaps by generating the virion receptor attachment site. Further analysis of the functional properties of VP-3-M/VP-2 heterodimer envelope proteins of LDV will require antibodies directed specifically against each envelope protein. Although much effort has been invested toward the generation of several specific antibodies against LDV VP-3 [7], almost no antibodies that react consistently with virion M/VP-2 have been produced in studies using various immunological methods of analysis such as immunoprecipitation, Western blotting and immunofluorescence. It has been reported that anti-LDV antibodies raised in mice to peptides of ORF6 protein weakly immunoprecipitated in vitro-synthesized ORF6 protein, but even these antibodies did not consistently react with virion M/VP-2 [8]. As a first step to examine the biological and functional properties of LDV M/VP-2, we prepared newly generated specific antibody against LDV ORF6.

2. Materials and methods

2.1. Virus and purification

Purified LDV type C (LDV-C) used in this study were prepared using 8- and 4-week-old SJL/J mice as described previously [9].

2.2. Selection and synthesis of peptide

LDV M/VP-2 possess three potential transmembrane segments near the N-terminal end of which probably functions as an uncleaved signal sequence [8]. Considering the postulated N-terminal ectodomain of ORF6 protein (nine amino acids), which is too short to be an efficient immunogen, a suitable antigenic site for production of specific antibody was predicted as being located in the C-terminal region, which may be located on the cytoplasmic side of the membrane. For this reason, the C-terminal amino acid sequence of the LDV-C ORF6 protein was screened according to Hopp and Woods method [10] for predicting an antigenic region on the basis of hydrophilicity plots. A peptide H_2N-CVLGGKKAVSKG-COOH, corresponding to the residue sequence 151–161 in the C-terminal region of LDV-C ORF6 protein, had an extra cysteine-NH_2 residue on the N-terminus; this residue was added to allow coupling to the carrier, and was chosen and synthesized by the solid phase method based on Fmoc-chemistry [11] following carrier conjugation to keyhole limpet hemocyanin (KLH) as described in a report by Liu et al. [12].

2.3. Preparation of polyclonal antiserum

Two rabbits, #36 and #37, each received 200 μg of KLH-conjugated peptide emulsified in 1.5 ml complete Freund's adjuvant (1 ×) and in incomplete Freund's adjuvant (4 ×) at 3–4-week intervals. Ten weeks later, serum from each rabbit was independently pooled for affinity purification. Each sample of crude antiserum was purified with Protein A sepharose (HiTrap rProtein A, Amersham Biosciences, Buckinghamshire, England).

The purified antibodies #36 and #37 (Ab#36 and Ab#37) were then concentrated and dialyzed.

2.4. ELISA

Rabbit specific IgG to LDV peptide in crude and purified sera were measured by ELISA according to a previously described procedure [13]. In brief, microplates (Nunc Immuno Modules, CovaLink NH, NALGE NUNC International K.K., USA) were activated with *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, HCl (EDC) at room temperature for 2 h, coated with viral peptide or peptide 15-9 corresponding to a major allergen of Japanese cedar pollen [14] (10 μg/ml in phosphate buffered saline (PBS)) overnight at 4 °C. After blocking with 0.1% casein-PBS, pre-immune serum obtained from rabbit #36 and #37 (pre-immune #36 and #37) or anti-peptide serum Ab#36 and #37 diluted with 10% fetal calf serum in PBS were incubated at room temperature (r.t.) for 1 h, and were incubated with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 (Zymed, San Francisco, CA, USA) at r.t. for 1 h. The reaction was visualized using Orthophenylenediamine dihydrochloride as substrate. After the enzyme reaction was stopped with 4N sulfuric acid, the absorbance at 492 nm was measured with a colorimetric microplate reader (Flow Laboratories, McLean, VA).

2.5. Western blotting

For Western blot analysis, virion protein extracted from LDV-infected SJL/J mouse sera were prepared as described previously [9,15]. The pelleted virus from infected sera was lysed in lysis buffer composed of 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 1.0% Triton X-100 and Complete (Roche Diagnostics, Mannheim, Germany) as protease inhibitor cocktail. Lysate of LDV virions was separated by SDS–polyacrylamide gels (SDS–PAGE) on 12.5% polyacrylamide gels and was electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The blot was probed with Ab#36 or #37 (diluted 1:100) as mentioned above, then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (diluted 1:10,000), and developed with the enhanced chemiluminescence (ECL) Western blotting analysis system (Amersham Biosciences, Buckinghamshire, England).

2.6. Immunofluorescence analysis

To examine the intracellular localization of LDV M/VP-2 in infected cells, immunofluorescence analysis was performed as described previously [7,16], but with a few modifications. In brief, LDV-C-infected macrophages derived from 4-week-old SJL/J were harvested at 8–10 h post-infection, respectively, and the cells were fixed for 10 min with 2% paraformaldehyde in PBS at 4 °C. After the fixed cells were treated for 10 min in 0.25% Triton X-100 for permeabilization, they were incubated with Ab#36 (diluted 1:100) or pre-immune #36 IgG purified with Protein A sepharose (diluted 1:100) at 4 °C overnight. After a washing with PBS, Alexa Fluor 448 goat anti-rabbit IgG (H + L) (diluted 1:400) (Molecular Probes Co. Ltd, Eugene, USA) were incubated with the cells for 1 h at room temperature. The cells were washed again with PBS, and mounted preparations were observed on LSM510 laser-scanning confocal microscope equipped with an Axiovert-100 M (Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. ELISA

Specificity of the rabbit IgG to LDV ORF6 peptide was assessed by ELISA. As shown in Fig. 1A, both Ab#36 and #37 reacted specifically with LDV ORF6 peptide.
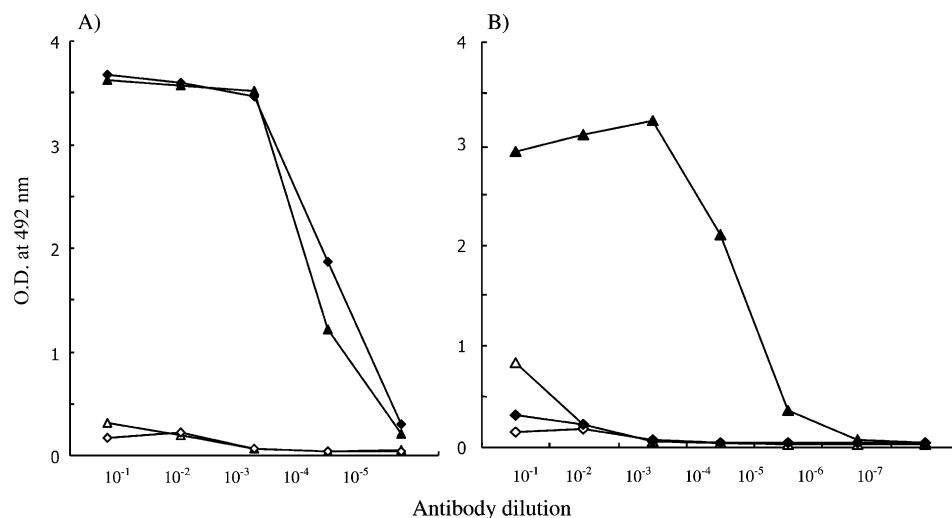

Fig. 1. Titration curves of rabbit pre-immune sera and Ab#36 and #37 in ELISA using LDV-C ORF6 peptide as an antigen are shown in (A). Corresponding data using peptide 15-9 against major allergen of Japanese cedar pollen as an antigen are depicted in (B). (A) Pre-immune sera #36 (△), Pre-immune sera #37 (◇), Ab#36 (▲), Ab#37 (◆). (B) Pre-immune sera #36 probed with LDV peptide (△), Pre-immune sera #36 against peptide 15-9 (◇), antibody # 36 against LDV-C ORF6 peptide (▲), antibody #36 against peptide 15-9 (◆).
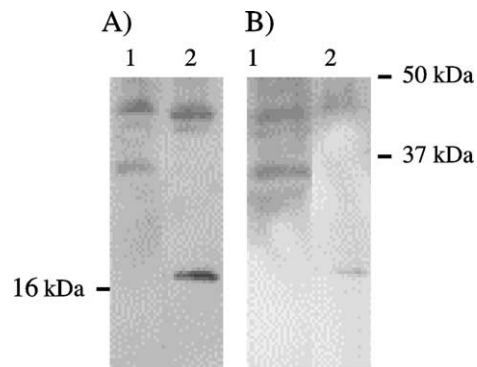
Fig. 2. Western blotting analysis with anti-peptide antibodies to the LDV-C ORF6 protein, Ab#36 (A) and Ab#37 (B). Samples were loaded from normal SJL/J mouse sera (lane 1), LDV virion protein (lane 2).

Neither pre-immune #36 nor #37 reacted with LDV peptide. In addition, it was revealed that Ab#36 did not react with a peptide 15-9 against major allergen of Japanese cedar pollen (Fig. 1B). Also Ab#37 did not recognize a peptide 15-9 (data not shown).

3.2. Western blotting

As shown in Fig. 2, Ab#36 clearly reacted with virion M/VP-2, whereas a reaction of Ab#37 was very weak (Fig. 2). The 16 kDa protein, which may represent virion M/VP-2, was detected, although the molecular mass of the detected virion protein was slightly smaller than that of authentic 18–19 kDa virion M/VP-2 reported previously [4].

3.3. Immunofluorescence analysis

Since Ab#36 was clearly capable of reacting with virion M/VP-2 in Western blotting, only Ab#36 was used for the immunofluorescence analysis, which revealed that Ab#36 reacted with virion M/VP-2 in infected macrophages. As shown in Fig. 3C, clear
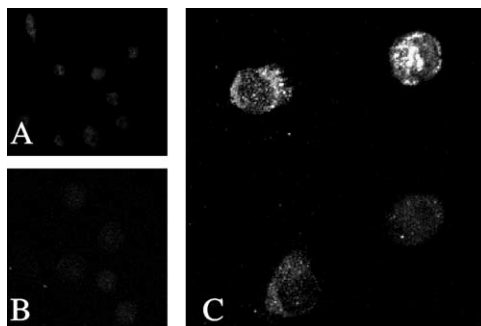

Fig. 3. Immunofluorescence analysis with Ab#36. LDV-infected macrophages were probed with pre-immune #36 IgG (A) and Ab#36 (C). Normal macrophages were probed with Ab#36 (B). Magnification, × 640.

fluorescence was distributed throughout the cytoplasm of the infected macrophages. The frequency of antigen-positive cells was approximately 5–7%, which was similar to the results obtained from immunostaining using mouse hyperimmune serum by Inada and Mims [17]. It was indicated that no fluorescence was detected in infected macrophages probed with pre-immune #36 IgG (Fig. 3A) and in normal macrophages probed with Ab#36 (Fig. 3B).

4. Discussion

The results of this study suggest that antibodies raised against a peptide corresponding to the C-terminal region of LDV ORF6 protein are able to specifically recognize LDV virion structural envelope protein M/VP-2, as determined by in Western blotting and immunofluorescence analysis. However, in spite of successful production of the specific antibody against virion M/VP-2 achieved in this study, obtaining specific antibodies against M/VP-2 has been very difficult. Other researchers have reported similar difficulties; for example, Coutelier et al. [18] were unable to detect an anti-M/VP-2 response in infected mice, despite a strong response to VP-3. Moreover, in another study, antisera consistently reactive to virion M/VP-2 were not obtained by immunization of animals with bovine serum albumin (BSA)-conjugated synthetic peptides against the C-terminal region of ORF6 protein, which was the same antigenic site chosen in this study [8]. Failure in generating such antibodies may have been due to the immunization methods. It has been reported that KLH and BSA may activate cell mediators such as antigen-presenting cells by different mechanisms due to their respective sizes and their capacities to concentrate in lymphoid centers [19]. Previous study has also shown that peptides coupled with KLH could mimic epitopes present on the native protein structure more accurately than they could those coupled to BSA, with the result that peptides coupled to KLH more efficiently stimulated the immune response [20]. In the same study, it was also reported that plasma raised from peptide coupled to KLH reacted with MHV spike glycoprotein, as shown by Western blotting, whereas the plasma raised from BSA-conjugated peptide reacted only with carrier protein or with spike glycoprotein and carrier protein. A more likely explanation for the difficulty in production of anti-peptide antisera against LDV ORF6 protein may be that linear epitopes do not form a significant part of the antigenic structure, and that conformation-dependent epitopes predominate.

In this report, we have generated antibody only directed to the C-terminal region of the endodomain of LDV M/VP-2 which is not expressed on the virion surface. Although antibodies which react with M/VP-2 on virion surface are necessary for studying virus adsorption to host cell membranes, no such specific antibodies have been available to date. As a next step to generate specific antibodies against M/VP-2, our studies on expression of recombinant M/VP-2 for immunogen are in progress.

In conclusion, we first prepared specific antibody for immunological analysis of LDV envelope protein, M/VP-2. This antibody may prove to be a very useful tool for analysis of the biological and functional properties of the envelope proteins of *Arteriviridae*.

Acknowledgements

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