

Baculovirus expression of parvovirus B19 (B19V) NS1: utility in confirming recent infection

O. Ennis, A. Corcoran, K. Kavanagh, B.P. Mahon, S. Doyle *

Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland

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Abstract

Background: The presence of anti-parvovirus B19 (B19V) IgM against viral capsid proteins (VP1 and VP2) has long been used to detect recent infection. The utility of antibodies directed against B19V NS1 protein has received less attention as a serological indicator of recent infection, although anti-B19V NS1 IgG has been associated with persistent infection. **Objectives:** To elucidate the role of anti-B19V NS1 antibody detection in recent infection, full-length B19V NS1 was expressed and purified. The resultant antigen was used to develop both Western blot assays and microplate ELISA for the detection of NS1 antibodies. **Study design:** Serum specimens were obtained from individuals recently infected with B19V (children ($n = 16$), adults ($n = 40$)) and from 17 individuals with no evidence of recent B19V infection. All specimens were screened for anti-B19V NS1 IgG and IgM. **Results:** It was observed that 68.8% (11/16) of children recently infected with B19V were anti-B19V NS1 IgG seropositive. Furthermore, 27.5% (11/40) anti-B19V VP2 IgM positive specimens also contained anti-B19V NS1 IgM when tested by ELISA, while no reactivity was observed following Western blot analysis, possibly due to the absence of conformational epitopes. **Conclusions:** Anti-B19V NS1 IgM detection may have utility in the confirmation of recent infection with B19V. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Baculovirus; ELISA; Epitopes; Immunocompromised; Pregnancy

1. Introduction

Since its discovery in 1975, parvovirus B19 (B19V) has been identified as the causative agent of a number of clinical conditions in humans (Anderson et al., 1985; Kurtzman et al., 1987). The spectrum of symptoms caused by B19V, in-

cluding rash, arthralgia and transient aplastic crisis, are generally self-limiting in healthy individuals. However, serious complications due to viral infection may arise in certain populations including pregnant women, immunocompromised patients and individuals suffering from sickle cell disease or other blood-related disorders (Jordan, 1996; Török, 1997).

The B19V capsid is composed of two structural proteins, VP1 (83 kDa) and VP2 (53 kDa), with VP2 comprising approximately 95% of total cap-

* Corresponding author. Tel.: + 353-1-7083858; fax: + 353-1-7083845.

E-mail address: sean.doyle@may.ie (S. Doyle).

sid protein (Ozawa and Young, 1987). Both of these antigens have been expressed in eukaryotic expression systems in order to assess their potential use as diagnostic reagents for B19V infection (Brown et al., 1990). This work has culminated in the recent demonstration of the absolute requirement for intact capsid structure for optimal detection of anti-B19V VP2 IgG (Kerr et al., 1999).

B19V NS1 had received much less attention as a marker of B19V infection until the proposal that anti-B19V NS1 IgG may be a marker of persistent infection (von Poblitzki et al., 1995a,b). Recent studies have demonstrated a range of 21.7–36% NS1 IgG seropositivity in control subjects previously infected with B19V (Venturoli et al., 1998; Jones et al., 1999; Hemauer et al., 2000). An anti-B19V NS1 IgG seropositivity of 12.5% (5/40) has been observed in recently infected individuals, and a similarly low rate of anti-B19V NS1 IgG positivity has been reported in acutely infected immunocompetent individuals (5/43; 11%) (Jones et al., 1999; Hemauer et al., 2000). Most of these studies on the immune response to B19V NS1 have focused on the diagnostic utility of anti-B19V NS1 IgG as a marker of specific B19V-related disease or persistent infection, whereby the level of anti-B19V NS1 IgG positivity may be as high as 61% (24/39) and 80% (4/5), respectively, in cases of B19V infection during pregnancy or chronic B19V infection in immunocompetent individuals (Hemauer et al., 2000). Interestingly, all of these studies have been performed using *E. coli* expressed NS1 fragments as the detection antigen. Furthermore, no evidence of anti-B19V NS1 IgM has been found from specimens taken during recent B19V infection (Hemauer et al., 2000). A limited evaluation of baculovirus-expressed NS1 for B19V serological studies has been reported, whereby 12% (3/25) of specimens obtained from arthropathy patients were anti-B19V NS1 IgG positive by Western blot analysis (Hicks et al., 1996). These authors also noted that an anti-B19V IgG positive serum pool tested negative for anti-B19V NS1 IgM, as expected. In this study, we sought to determine the utility of the full-length B19V NS1 protein

produced in the baculovirus expression system for the determination of recent infection with B19V via the analysis of anti-B19V NS1 IgG and IgM, respectively.

2. Materials and methods

2.1. Clinical specimens

Three groups of sera were used in this study. (i) Specimens ($n = 19$) were obtained from children (age range: 7–11 years) within 3 months of a school-based outbreak of B19V infection, (ii) anti-B19V IgM negative sera ($n = 17$) were obtained from volunteer donors exhibiting no signs of recent B19V infection, and (iii) specimens ($n = 40$) that were positive for anti-B19V VP2 IgM. All sera were screened for anti-B19V IgG and IgM by US FDA cleared in-vitro diagnostic immunoassays (Biotrin, Dublin, Ireland).

2.2. Construction of recombinant baculovirus encoding NS1

B19V DNA template suitable for PCR was obtained from a B19V viremic specimen. Primers based on the B19V sequence to include sequences complementary to the 5' and 3' ends of the entire 2 kb NS1 open reading frame were selected. The 5'-primer (NSnt:GGTATGAATTCTACTTGTTAACATCC) included an *Eco*RI site and the 3'-primer (NSc:CAAATTTATCATCATCACTTCTGCAGCATTTGCC) included a *Pst*I site in order to facilitate the subsequent cloning of the PCR product into the baculovirus transfer vector, pBacPAK9 (Clontech, Palo Alto, CA) using standard molecular biology techniques. *Spodoptera frugiperda* (Sf₉) insect cells were co-transfected with recombinant transfer vector and *Bsu*36 I-linearised BacPAK-6 baculovirus using Bacfectin[®] liposomal preparation (Clontech). Well plates were infected with the co-transfection mixtures and the TC-100 supernatant was harvested after 5 days' incubation at 27°C. A pure clone of a recombinant baculovirus was identified by plaque assay.

2.3. Antigen expression and purification

Recombinant baculovirus encoding the NS1 gene was used to infect Sf9 cells in monolayer culture at a multiplicity of infection of 2.1. Infected cells (5×10^8) were harvested 4 days post-infection. SDS-PAGE and Western Blot were used to confirm the presence of recombinant B19V NS1. The cells were then lysed in the presence of protease inhibitors (0.1 mM phenyl methyl sulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ of pepstatin and 2 $\mu\text{g}/\text{ml}$ of leupeptin) by the addition of phosphate-buffered saline-0.5%(w/v) sodium deoxycholate. After centrifugation ($3000 \times g$ for 5 min), the insoluble fraction that contained recombinant NS1 and nuclei was resuspended in 25 mM Hepes, 5 mM MgSO_4 , pH 7.4, and subjected to DNase (Sigma, Poole, UK) treatment (final concentration: 10 $\mu\text{g}/\text{ml}$) for 1 h at room temperature. Following a brief centrifugation and wash step ($3000 \times g$ for 3 min), the insoluble NS1 was resuspended in 0.5–1.0 ml of 6 M guanidinium thiocyanate at a concentration of 4–5 mg/ml and stored at -20°C .

2.4. B19V NS1 IgG Western blot

Purified NS1 was subjected to SDS-PAGE by layering 26 μg of NS1 across the stacking gel in a final volume of 250 μl of solubilisation buffer (Laemmili, 1970). Electrotransfer onto nitrocellulose was carried out according to standard protocols except that 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid (CAPS), pH 10, containing 10% (v/v) methanol was used to facilitate electrotransfer. After electrotransfer and membrane blocking in 5% (w/v) milk powder/phosphate-buffered saline-0.05% (v/v) Tween-20[®] (PBST), the nitrocellulose sheets were cut into 4 mm wide strips (1 μg NS1/strip). Specimens were diluted 1/100 in 1% (w/v) milk powder/PBST, added to individual strips and incubated for 3 h at room temperature. After a washing step, horse-radish peroxidase (HRP)-conjugated anti-human IgG (Dako, Glostrup, Denmark) was added to the membrane and incubated for 1 h at room temperature. Bound antibody-conjugate complex was detected by

the addition of H_2O_2 and diaminobenzidine (Sigma, Poole, UK). The band intensity was determined visually, and a positive result was indicated by the appearance of a band corresponding to the position of NS1 (77 kDa).

2.5. B19V NS1-IgM enzyme immunoassay

Prior to testing all specimens were adsorbed using anti-human IgG adsorbent (Incstar, Stillwater, MN) by adding 10 μl of serum to 150 μl of adsorbent. After a brief centrifugation step ($15000 \times g$ for 2 min), the entire supernatant was added to 850 μl of PBST (final dilution: 1/100) and mixed gently. Diluted specimens were then added to microwells that had been previously coated with recombinant NS1 at 0.7 $\mu\text{g}/\text{ml}$ in 50 mM sodium carbonate pH 9.6. Following a 1 h incubation at room temperature and a wash step, HRP-labelled rabbit anti-human IgM (Sigma, Poole, UK) was added for 30 min to detect any anti-B19V NS1 IgM present. This complex was then detected by addition of tetramethyl benzidine substrate. Sulphuric acid (1 N) addition terminated the reaction after 10 min, followed by absorbance measurement at 450/630 nm. The immunoassay cut-off value was determined by obtaining the mean absorbance \pm 2S.D. ($0.183 \pm (2(0.077)) = 0.338$) of a panel of anti-B19V VP2 IgM negative specimens. Test specimens with an index value (specimen/cut-off value ratio) greater than 1.0 were deemed positive for anti-B19V NS1 IgM.

2.6. B19V NS1 IgM Western blot

Conditions for the detection of anti-B19V NS1 IgM were identical to those described for anti-B19V NS1 IgG detection, except that all specimens were pre-treated as described for the B19V NS1 IgM enzyme immunoassay (above) and detected using HRP-labelled rabbit anti-human IgM (Sigma, Poole, UK).

3. Results

A single clone termed pBac11.9 was identified that contained an insert of the appropriate size.

Sequence analysis of pBac11.9 from the 5'- and 3'-termini using an ABI 310 Genetic analyser (PE Applied Biosystems, Foster City, CA) confirmed that the inserted PCR fragment encodes the full-length open reading frame for B19V NS1 (data not shown). For subsequent recombinant protein work, passage two viral stock was used to infect Sf₉ cells. SDS-PAGE and Western blot characterisation of infected cell lysates and purified NS1 is shown in Fig. 1. It can be seen that protein of the expected size (77 kDa) is obtained, which is strongly immunoreactive with human IgG [anti-B19V NS1]. No reactivity was observed against uninfected insect cells, although there is evidence that limited NS1 degradation has occurred during purification, as confirmed by the presence of low M_r immunoreactive fragments. After expression, it was observed that the recombinant NS1 protein was highly insoluble and required 6 M guanidinium thiocyanate and 20 mM dithiothreitol (DTT) to facilitate solubilisation, but the protein remained soluble following dialysis or dissolution, at low $\mu\text{g/ml}$ concentrations, into non-denaturing buffers (e.g. 50 mM sodium carbonate pH 9.4). Protein quantitation (Bio-Rad, Munich, Germany) indicated a yield of 1 mg of B19V NS1 per 10⁷ infected Sf₉ cells.

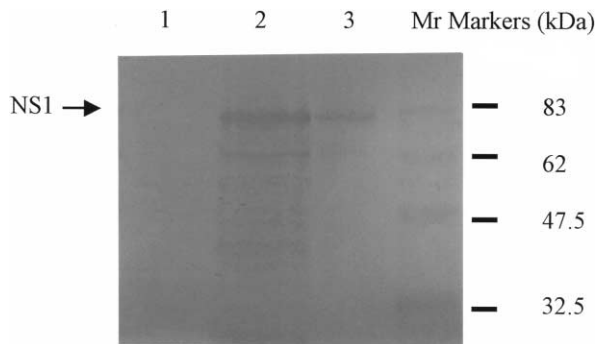


Fig. 1. Western blot analysis of baculovirus-expressed B19V NS1. No immunoreactivity is observed in uninfected insect cell lysate (1). Immunoreactivity is observed at the expected M_r (77 kDa) in both NS1 recombinant-baculovirus infected insect cell lysate (2) and purified NS1 (3). Antigen visualisation was carried out using an anti-B19V NS1 IgG positive specimen, as described in Table 1.

Table 1

Anti-B19V IgG and IgM antibody reactivity against baculovirus-expressed VP2 and NS1 antigens

	Anti-B19V NS1 IgG status	
	Positive	Negative
Anti-B19V VP2 IgG positive (16)	10 (62.5%)	6 (37.5%)
Anti-B19V VP2 IgG negative (3)	0 (0%)	3 (100%)
	Anti-B19V NS1 IgM status	
	Positive	Negative
Anti-B19V VP2 IgM positive (40)	11 (27.5%)	29 (72.5%)
Anti-B19V VP2 IgM negative (17)	1 (5.9%)	16 (93%)

When the sera of 16 children who had been infected with B19V 3 months earlier were examined by Western blot, it was found that 11/16 (68.8%) contained anti-B19V NS1 IgG, while 100% contained IgG against B19V VP2. No anti-B19V NS1 or VP2 IgG was detected in the sera of three seronegative children who had not been infected during the outbreak.

When tested by B19V NS1 IgM ELISA, 11/40 (27.5%) anti-B19V VP2 IgM positive specimens also exhibited anti-B19V NS1 IgM positivity (Table 1). Subsequent Western blot analysis of 10 specimens (five anti-B19V NS1 IgM positive and five anti-B19V NS1 IgM negative by ELISA) produced a negative test result (data not shown). Only one specimen obtained from either clinical laboratories or laboratory volunteers with or without previous exposure to B19V tested anti-B19V NS1 IgM positive (Table 1). Eleven of these specimens were anti-B19V VP2 IgG positive.

4. Discussion

Here, we report the cloning and expression of the full-length open reading frame encoding the NS1 protein of B19V in the baculovirus expres-

sion system. The potential diagnostic utility of the resultant purified NS1 protein has been explored, and it is clear that the antigen facilitates the detection of both anti-B19V NS1 IgG and IgM following infection with B19V. This finding offers the possibility of using anti-B19V NS1 IgM to aid confirmation of recent B19V infection in parallel with anti-B19V VP2 IgM positivity and PCR testing.

The high-level expression and purification of intact B19V NS1 protein were achieved. No evidence of NS1 cytotoxicity was observed, as has been reported for other recombinant expression systems used to produce the NS1 protein (von Poblitzki et al., 1995a,b). This is most likely due to the late protein expression obtained for any gene under the control of the baculovirus *polh* promoter in infected insect cells. The yield of purified NS1 was between 2 and 10 mg per 10^8 cells, which is within the expected range for the baculovirus expression system (O'Reilly et al., 1994).

Initial reports suggested that the presence of anti-B19V NS1 IgG was a strong indicator of persistent viral infection (von Poblitzki et al., 1995a,b). However, recent work in this area has indicated that there may not be a significant difference between anti-B19V NS1 IgG prevalence between individuals with either chronic or uncomplicated past B19V infection (Jones et al., 1999). Interestingly, the high level of anti-B19V NS1 IgG positivity in recently infected children observed in the present study (68.8%) is similar to that seen for recently infected pregnant women (61%) (Hemauer et al., 2000). Taken together, these data suggest that levels of anti-B19V NS1 IgG may peak within months after infection due to NS1 presence during viral replication and then wane as the virus is completely cleared.

Although a number of groups have undertaken the expression of B19V NS1 in bacterial and baculovirus expression systems, there has been no report to date on the successful detection of anti-B19V NS1 IgM in specimens obtained from recently infected individuals (von Poblitzki et al., 1995a,b; Hicks et al., 1996; Venturoli, 1998; Hemauer et al., 2000). It is conceivable that the absence of relevant conformational epitopes on

the NS1 antigens (or NS1 sub-fragments) previously employed resulted in the observed false negative results. Alternatively, the attempted detection of anti-B19V NS1 IgM in pooled specimens may also have led to the conclusion that anti-B19V NS1 IgM is not present in previously infected individuals (Hicks et al., 1996). We propose that the dilution of denatured and reduced NS1 into high-pH buffer prior to microplate coating facilitates the reformation of relevant conformational epitopes that are subsequently recognised by anti-B19V NS1 IgM present in sera obtained from certain individuals recently infected with B19V. Evidence for this comes from the results obtained following Western blot analysis of anti-B19V NS1 IgM positive specimens, whereby SDS denaturation and DTT presence apparently lead to the disruption of conformationally relevant epitopes with concomitant false negative detection of anti-B19V NS1 IgM. Since it has been previously shown that all specimens that are anti-B19V VP2 IgM positive by microplate ELISA also test positive by Western blot (Kerr et al., 1999), which used a similar method for antigen electrotransfer onto nitrocellulose, it is unlikely that methodological differences account for the observed difference with respect to anti-B19V NS1 IgM detection between microplate ELISA and Western blot immunoassay. It is notable that no specimen tested was anti-B19V VP2 IgM negative and anti-B19V NS1 IgM positive, which is not unexpected given the antigenic nature of viral capsid proteins. A single specimen taken from an individual with no recent indication of B19V infection (Group (ii)) did test anti-B19V NS1 IgM positive, but since this specimen also exhibited strong IgG reactivity against B19V VP2, it is likely to represent a false positive reaction in the B19V NS1 IgM immunoassay.

It should now be possible to further utilise baculovirus-expressed recombinant NS1 to determine the seroprevalence of anti-B19V NS1 IgG in selected population groups, particularly in recently infected, immunocompetent individuals to confirm the high prevalence of anti-B19V NS1 IgG in recent infection. Significantly, Kaikkonen et al. (1999) have recently identified a specific heptapeptide epitope in B19V VP2 which is recog-

nised by patients with acute or recent infection. Thus, it is tempting to speculate that anti-B19V NS1 IgG reactivity against specific B19V NS1 epitopes could be used, in association with antibody detection of linear epitopes on B19V VP2, as a marker of recent infection. Finally, the association between anti-B19V NS1 IgM detection and infection with specific isolates of B19V and putative associated pathogenicities, in both immunocompetent and immunocompromised individuals, also merits further investigation.

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