Antibodies to parvovirus B19 NS-1 protein in infected individuals

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Human parvovirus B19 is the aetiological agent of the common childhood disease erythema infectiosum (fifth disease). The infection is usually benign and self-limiting, but in adults cases of severe arthritis which may persist for years have been reported. Neutralizing antibodies directed against the structural proteins are usually produced shortly after the infection. The immune response against the third major protein, the non-structural protein NS-1, of parvovirus B19 has not been characterized so far. We cloned and expressed the full-length NS-1 protein and fragments thereof in Escherichia coli. The purified recombinant proteins were used to investigate the presence of antibodies to the NS-1 protein in sera from patients with parvovirus B19 infection. Specific antibodies could be detected in sera from patients suffering from severe parvovirus B19-associated arthritis using Western blot analysis and an ELISA. Sera from patients with acute or past infection without complications did not contain detectable levels of immunoglobulins to NS-1. The use of subfragments of the NS-1 protein allowed localization of the antigenic domains in the carboxy-terminal region of the protein.

Introduction

Parvovirus B19 was first discovered in the sera of healthy blood donors in 1975 (Cossart et al., 1975). It has since been identified as the causative agent of erythema infectiosum or fifth disease, a common childhood exanthema (Anderson et al., 1983). Besides this usually harmless illness, the virus can cause a series of more severe symptoms. Infection of the immunocompromised or patients suffering from haemolytic anaemia can lead to aplastic crisis that may become life-threatening (Pattison et al., 1981; Serjeant et al., 1993). A frequent complication in adults is an acute arthritis which usually disappears after some weeks but sometimes persists for years (Reid et al., 1985; White et al., 1985; Cohen et al., 1986; Naides et al., 1990; Ueno et al., 1993). Soon after infection, antibodies directed against the structural proteins of parvovirus B19 appear. The major capsid protein, the 58 kDa VP2, and the minor capsid protein, the 83 kDa VP1, both contain neutralizing epitopes (Sato et al., 1991a, b; Yoshimoto et al., 1991; Saikawa et al., 1993). These two proteins are encoded in the right-hand half of the viral single-stranded DNA genome in the same reading frame (Cotmore et al., 1986). VP1 is composed of the whole amino acid sequence of the smaller VP2 and has 227 additional amino acids at its amino terminus (Ozawa & Young, 1987; Cotmore et al., 1986). After the onset of neutralizing antibody production, the virus is rapidly cleared from the circulation. In some cases of parvovirus B19 infection, persistence of the virus has been reported (Kurtzman et al., 1989a; Pont et al., 1992; Cassinotti et al., 1993). Persistently infected patients often fail to produce neutralizing antibodies. In particular the response directed to the VP1-specific parts of the structural proteins seems to be absent in these cases (Kurtzman et al., 1989a, b). More recently, the use of PCR in diagnosis of B19 infection has been established (Koch & Adler, 1990; Cassinotti et al., 1993; Foto et al., 1993; Patou et al., 1993). This very sensitive assay has led to the discovery that parvovirus B19 may persist for longer intervals in a greater proportion of infected people than was previously recognized (Cassinotti et al., 1993). In contrast to the rather well characterized antibody response to the structural proteins, the immunogenicity of the third major parvovirus B19 protein, NS-1, has not been assessed. We have cloned and expressed the non-structural protein NS-1 and fragments thereof in Escherichia coli and developed an ELISA using the purified recombinant protein as antigen. Subsequently we investigated the presence of NS-1-specific antibodies in sera of patients with acute or past B19 infection and different outcomes of infection. Immunoglobulins to NS-1 were detected and appeared to be associated preferen-
tially with B19 virus-associated arthritis, possibly reflecting persistent viral infection. The specificity of these antibodies in our patients mapped to the carboxy-terminal part of the protein as determined using overlapping protein fragments of NS-1.

Methods

Sera. Samples were collected at the Department for Internal Medicine, University of Regensburg Hospital and the St Joseph Hospital, Olsberg, Germany and were tested in the Diagnostic Virology Department of our Institute (Table 1). Routine tests for the presence of specific antibodies against parvovirus B19 structural proteins were done using an assay based on recombinant B19 capsids (Virotech). The use of such capsids has been shown to give results comparable to tests using native virus as antigen (Salimans et al., 1992). Positive IgM was confirmed by Western Blot. Some of the patients suffered from severe B19-associated arthritis that persisted for more than 6 weeks, as judged from the coincidence of the onset of symptoms with appearance of parvovirus B19 IgM.

Antisera. For generation of antisera directed against the full-length NS-1 protein, rabbits were immunized with partially purified protein preparations emulsified in Titermax-Adjuvans (Serva, Heidelberg, FRG). All sera reacted specifically with the complete NS-1 protein and with all fragments in Western blot analysis (data not shown). A rabbit serum against a β-galactosidase-NS-1 fusion protein (NS-88) was kindly provided by Dr P. Beard (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland).

Construction of expression plasmids. Primer sequences were designed using the published parvovirus B19 genome sequence (Shade et al., 1986) and synthesized using a Millipore Expediti nuclei acid synthesizing system. For insertion into plasmid pQE40 in frame with the poly-histidine linker, unique restriction enzyme recognition sites were introduced at the 5’- or 3’-ends. Primers NS-nl (nt 435–354) and NS-cr (nt 2431–2451) were used for the amplification of the complete NS-1 open reading frame (ORF). Primers NS-nl and NS-ab (nt 864–884) were used for the NS/A fragment. Subfragments NS/B, NS/C, NS/D, NS/E and NS/F were amplified using oligonucleotides NS-bf (nt 886–904) and NS-bb (nt 1315–1330) for NS/B, NS-bf and NS-nr (nt 1618–1638) for NS/C, NS-df (nt 1349–1365) and NS-db (nt 1913–1930) for NS/D, NS-el (1642–1660) and NS-cr (nt 2431–2451) for amplification of NS/E, and NS-ff (nt 1915–1929) and NS-cr for NS/F, respectively. For the amplification of the VP1 and VP2 ORFs pQ-dv (nt 2443–2461) and pQ-v2 (nt 3125–3144) were used as upstream primers and pQ-v2r (nt 4778–4795) as downstream primer for both

Table 1. Characteristics of the sera used in this study

<table>
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<th>Anti-NS-1</th>
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* The presence of IgM was taken as indicative of acute parvovirus B19 infection.
† CP, chronic polyarthritis.
‡ RA, rheumatoid arthritis.
Antibodies to parvovirus B19 NS-1

Fig. 1. Schematic representation of the expression vectors for the recombinant proteins. The appropriate parts of the B19 genome were amplified with PCR primers and inserted into plasmid pQE40. From plasmid pQE40NS-1 the full-length protein is synthesized after induction with IPTG. The fragments comprise overlapping parts of the NS-1 protein for the characterization of the antibody response. All constructs contain a poly-histidine linker composed of six consecutive histidine residues at the amino terminus (HIS). Protein NS/A was expressed as a fusion protein with dihydrofolate reductase (DHFR). The positions of amino acid stretches with homology to consensus motifs are given as shaded boxes (NLS, nuclear localization sequence; ATP, ATPase-like consensus sequence). The structural proteins are expressed as fusion proteins from the plasmids pQE40VP1 and pQE40VP2. The numbers indicate the first and last amino acid coded by the respective insert according to the sequence published by Shade et al. (1986).

Expression and purification of recombinant proteins. LB medium containing 100 µg/ml ampicillin and 30 µg/ml kanamycin was inoculated with the bacteria and incubated at 37 °C. For the production of full-size NS-1 it was necessary to lower the growth temperature to 25 °C to obtain high levels of expression. Expression of the recombinant protein was induced by addition of 1 mM-IPTG for at least 3 h of culture. The bacteria were harvested by centrifugation and checked for the presence of protein by SDS-PAGE followed by staining with Coomassie Brilliant Blue or by analysis on Western blot using NS-1-specific polyclonal rabbit sera or convalescent phase patient sera specific for structural B19 virus proteins. The cells were lysed in sodium phosphate buffer pH 8.0 containing 8 M-urea followed by sonification. The cellular debris was pelleted by centrifugation and the supernatants were loaded on a Ni-resin column (Diagen). The column was washed with sodium phosphate buffer pH 6.3 and 5.9 containing 8 M-urea and eluted with 0.2 M-imidazole for the NS-1 protein or sodium phosphate buffer pH 4.5/8.0 M-urea for the structural proteins. Fractions were tested for the recombinant proteins by SDS-PAGE and subsequent Coomassie Brilliant Blue staining, the fractions containing the recombinant protein were further separated on a preparative SDS-PAGE column using a Prep-Cell device (Bio-Rad). The fractions from the column were photometrically monitored for their protein content and specifically tested for the B19 virus proteins as described above.

ELISA. Microtitre plates (Greiner ELISA F) were coated with various amounts of recombinant protein in 100 µl 0.2 M-carbonate buffer/0.15 M-NaCl at pH 9.2 at 37 °C overnight. ELISAs were done essentially as described previously (Haist et al., 1992). Briefly, 100 µl of serum diluted in PBS containing 3 % FCS/2 % Tween-20 was added per well and incubated at 37 °C. After washing, 100 µl of second antibody diluted 1 : 1000 in PBS/3 % FCS/2 % Tween 20 was added. For the detection of human antibodies, peroxidase-conjugated rabbit antibodies to human γ-chains and peroxidase-conjugated rabbit antibodies to human μ-chains were used (both purchased from DAKO). After colour development using o-phenylenediamine as substrate, the reaction was stopped with 0.5 M-H₂SO₄ and the A₄₀₂ was measured.

Results

Expression of recombinant proteins in E. coli

The non-structural protein NS-1 of parvovirus B19 has been described as cytotoxic when expressed in eukaryotic cells (Ozawa et al., 1988). This may be a result of the functional activities ascribed to this protein in parvovirus replication. To circumvent these problems, we decided to express the protein in the prokaryotic host E. coli. Since toxic effects in bacteria could not be excluded and to facilitate subsequent purification of the recombinant protein, we used the pQE expression system (Diagen). Induction under standard conditions resulted in low protein yields partly due to poor growth of the bacteria after addition of IPTG. This problem could be overcome by reducing the growth temperature to 25 °C. Under those conditions, bacteria produced the NS-1 protein fused to six amino-terminal histidines, displaying a
molecular mass of 71 kDa (Fig. 2a). The protein could be purified from inclusion bodies by solution in urea-containing buffer and adsorption to a Ni-matrix and subsequent SDS-PAGE. The identity of the protein was proven by Western blot analysis with NS-1-specific rabbit antiserum (Fig. 2c). Similar expression problems were encountered with the production of the NS/A fragment. This protein was only expressed in high

Fig. 2. Expression and purification of NS-1 protein and subfragments. (a) E. coli strain SG13009(pREP4) containing plasmid pQE40 NS-1 was induced by addition of 1 mM-IPTG. Lysates of the cells were separated by SDS-PAGE. Coomassie Blue staining after SDS-PAGE showed a band of 71 kDa (lane labelled + IPTG) not present before induction (lane labelled −IPTG) corresponding to the complete NS-1 protein with the amino terminal poly-histidine linker. The molecular mass of the standard proteins are shown on the left. (b) SDS-PAGE of the subfragments of NS-1 protein after induction with IPTG. Proteins of 40 kDa (NS/A), 17 kDa (NS/B), 28 kDa (NS/C), 22 kDa (NS/D), 30 kDa (NS/E) and 21 kDa (NS/F) corresponding to the expected molecular masses of the fragments were expressed after induction. A lysate of non-induced cells containing plasmid pQE40 is shown at the right (lane labelled −IPTG). (c) Western blot analysis after purification of NS-1 proteins by affinity chromatography and preparative SDS-PAGE with NS-1-specific rabbit sera. Bands of the correct molecular masses of the full-length NS-1 protein or subfragments NS/A, NS/B, NS/C, NS/D, NS/E and NS/F were recognized. The DHFR expressed without a NS-1 fusion part did not show any reactivity.

Fig. 3. Western blot analysis using patients’ sera for recognition of the recombinant NS-1 proteins. Three examples with different history of infection are shown. The serum of patient Ra (acute infection and arthritis) recognized the structural proteins (lanes VP1 and VP2) in combination with NS-1 protein (lane NS-1). The serum of patient 377 (past infection) contained antibodies against only the structural proteins. A control serum from an uninfected person (patient 382) did not recognize any of the B19 proteins. All sera were diluted 1:200 and incubated overnight at room temperature on Western blots. Peroxidase-coupled rabbit antibodies to human γ-chains were used for detection.
Antibodies to parvovirus B19 NS-1

Fig. 4. Reactivity of patient sera with NS-1 protein. Microtitre plates were coated with 50 ng/well recombinant protein overnight and incubated for 3 h with the sera diluted 1:100. (a) IgG response to NS-1 protein. Sera are as described in Table 1. Reactivity was only seen with sera 2, 10 and Ra from patients with severe parvovirus B19-associated arthritis. (b) IgM response to NS-1. None of the sera showed any reactivity. Sera were judged as positive when the A492 was at least twice the mean value of the negative controls (P/N > 2.0), the cutoff is shown in (a) and (b) by a dotted line.

Detection of antibodies to NS-1 proteins after B19 infection

After preparative SDS–PAGE we used the purified protein to investigate the presence of NS-1-specific antibodies in sera of patients with acute or past parvovirus B19 infection. Thirty serum samples (Table 1) were tested for their reactivities with the non-structural protein NS-1 and the structural proteins VP1 and VP2.

According to the results of Western blot analysis, the sera could be divided into three groups of reaction patterns (Table 1 and Fig. 3). First, samples obtained from persons without incidence of acute or past parvovirus B19 infection never showed reactivity in this assay. The second group consisted of serum samples from individuals with acute or past B19 virus infection without reported complications (e.g. arthralgia or prolonged viral persistence). Sera of these persons showed the presence of specific antibodies to the structural proteins. Sera derived from patients with acute parvovirus B19 infection contained both IgM and IgG to VP1 and VP2, whereas sera from individuals with past infection contained only IgG to the structural proteins. None of the samples displayed reactivity against the non-structural protein NS-1. The third group consisted of
sera that reacted with the structural proteins as well as with the NS-1 protein (Fig. 3 and Table 1). All three samples showing this antibody pattern had IgG to all viral proteins tested including NS-1. When we looked for the presence of specific IgM to VP1, VP2 and NS-1 proteins in these samples all of them showed reactivities to VP1 and VP2, whereas IgM directed to NS-1 proteins could not be detected.

With regard to clinical manifestations of the infection, all three cases in the latter group came to the hospital because of a sudden onset of severe arthralgia or arthritis. None of the patients displayed the exanthema on the cheeks or along the inner sides of arms and legs typical for infection with parvovirus B19. Initially they were diagnosed as having early rheumatoid arthritis or chronic polyarthritis. The onset of illness correlated with the appearance of the specific IgM and IgG response to parvovirus B19 which led to the diagnosis of a parvovirus B19-associated arthritis. No IgM antibodies to rubella virus, coxsackievirus, Epstein–Barr virus or influenza virus were found. Sera 2 and 10 were also negative for rheumatoid factor whereas serum Ra reacted in a latex haemagglutination test. Using the sera in single round PCR analysis with subsequent hybridization (using which we were able to detect about $5 \times 10^4$ viral particles per ml) parvovirus B19 DNA could not be detected (data not shown).

**NS-1 protein ELISA and antibody titre determination**

In order to quantify the antibody response to parvovirus B19 NS-1 protein and to screen large numbers of serum samples for the presence of NS-1-specific antibodies, we established an ELISA using the recombinant protein as antigen. The suitability of the recombinant NS-1 protein for this purpose was evaluated using NS-1-specific rabbit serum. An amount of 50 ng/well of NS-1 protein and incubation with a 100-fold dilution of sera gave best results. Sera were judged to be reactive when the absorbance was at least twice the $A_{492}$ of the mean value of the negative controls ($P/N > 2$; Fig. 4). As in the results obtained by immunoblot analysis, IgM antibodies against the NS-1 protein could not be detected (Fig. 4b). In an ELISA specific for IgG, sera that had been found previously to contain NS-1-specific antibodies in Western blots also reacted with the NS-1 protein when tested in ELISA (Fig. 4a). All samples positive for IgG showed values of at least five times the $A_{492}$ of the negative controls. None of the sera derived from the control groups, including samples from uninfected individuals as...
well as sera derived from patients with acute or past B19 virus infection showed significant specific reaction to the recombinant NS-1 protein. This led us to believe that the ELISA was sensitive and highly specific for the detection of anti-NS-1 antibodies. The content of specific antibodies in reactive sera was determined by endpoint dilution to be 512 for serum Ra and 1024 for the other two sera, numbers 2 and 10.

Localization of antigenic regions in NS-1 protein

For characterization of the antibody reaction to different parts of the NS-1 protein, we cloned partially overlapping fragments of the NS-1 gene in the vector pQE40 and expressed the encoded protein parts in E. coli (Fig. 1). We chose the constructs so that they should allow differentiation of the antigenicity of the individual protein parts. Since constructs containing the first 150 amino acids were not efficiently expressed as a non-fusion protein, we added the DHFR sequences to the amino terminus of NS/A. The resulting product never gave elevated background reactivities in our assays which led us to believe that the DHFR is not critical for the specificity of our tests. The part of the amino terminus initially intended to overlap with NS/C was separately expressed as the non-fusion protein NS/B. We tested the IgG antibody pattern against the NS-1 fragments for all samples that gave positive reactions with the full-length protein. In the sera from patients 2 and 10, reactivity was found to be specifically directed to the proteins NS/E and NS/F but not to the other four fragments (Fig. 5). With respect to the overlapping nature of the NS-1 protein fragments, it became obvious that the terminal 179 amino acids of the NS-1 protein corresponding to NS/F are the target of the antibody response in these two individuals. The serum from patient Ra reacted with the region between amino acids 305 and 403 represented by NS/D (Fig. 5). This may be concluded from the observation that fragments NS/C and NS/D gave high \( A_{492} \) values in the ELISA whereas no response to either NS/A, NS/B or NS/E was found. The amino-terminal part of NS-1 represented by the proteins NS/A and NS/B was not recognized by any of the patients’ sera. Therefore we conclude that the most antigenic part of the NS-1 protein is located in the carboxy-terminal half of the protein.

Discussion

The antigenic properties of the non-structural protein NS-1 of parvovirus B19 have not been characterized so far. To address this question, we have cloned, expressed and purified the parvovirus B19 NS-1 and used it for the screening of patients’ sera for the presence of specific antibodies. Efficient expression in E. coli was found to be difficult for the complete protein and the NS/A fragment. A common feature of these two proteins is the presence of the amino terminus of NS-1. This led us to speculate that inhibitory DNA sequences might be present in these constructs. However, analysis of the DNA sequence revealed no elements known to interfere with efficient expression of foreign proteins in E. coli. Another aspect is that the poor production of the complete protein together with rapid degradation points to toxic effects of NS-1 protein in bacteria. Since NS-1 has been described as a transcriptional transactivator and is, in analogy with other parvoviral non-structural proteins, proposed to have DNA-nicking and helicase activities, these functions may render the protein toxic even in prokaryotic cells (Doerig et al., 1989). The fact that we found the protein localized in inclusion bodies does not necessarily exclude the possibility that functional protein is present in the cytoplasm at levels sufficient to exert the inhibitory effect we observed. The most likely toxic effects of the NS-1 protein in E. coli correspond well to similar findings in eukaryotic cells (Ozawa et al., 1988).

When the purified proteins were used as antigens in Western blot and ELISA, the majority of sera from parvovirus B19-infected individuals did not contain specific antibodies. This may be explained by the life cycle of the virus. During replication in the erythroid progenitor cells, only low amounts of the non-structural protein are synthesized (Cotmore et al., 1986; Ozawa & Young, 1987). Accordingly, only small amounts of NS-1 antigen may be released by cell death. This may be insufficient to prime a strong and long-lasting immune response detectable in persons with past parvovirus infection. However, sera from three persons showed a strong IgG response to the NS-1 protein. All had an acute parvovirus B19 infection as shown by the presence of high IgM titres specific for the structural proteins VP1 and VP2 and suffered from severe arthritis. In such cases, immunoglobulins giving non-specific reactions in diagnostic tests, e.g. rheumatoid factor, are frequently observed. Since we were unable to detect parvovirus B19 DNA by PCR, we tested for the presence of antibodies to other viruses and rheumatoid factor as indicators of non-specific stimulation of antibody production. However, in two patients neither IgM to other viruses tested for nor rheumatoid factor was found. In one case (patient Ra), rheumatoid factor was detected by latex agglutination test. These findings together with our ability to localize the target of the immune response and the fact that no reactivity with NS-1 was found in other sera known to contain rheumatoid factor, led us to believe, that we indeed observed an NS-1-specific immune response. The PCR data need to be interpreted carefully because the number of viral particles in the serum might be below the
sensitivity threshold. Even in this case, B19 DNA might be present in other tissues, e.g. bone marrow cells (Foto et al., 1993).

The different immunological recognition of the NS-1 protein may reflect an atypical course of the infection with parvovirus B19. In other viral infections, the appearance of antibodies to non-structural proteins has been correlated with chronically active infection of the host. This has been shown for hepatitis B virus and in Epstein-Barr virus infections (Hoofnagle et al., 1981; Miller et al., 1987). Persistent or at least prolonged infection has been also described in immunocompromised patients and patients suffering from chronic B19 virus-associated arthritis (Kurtzman et al., 1989b; Foto et al., 1993). In the latter case, viral persistence was demonstrated by the detection of DNA in bone marrow samples from patients with arthritis over periods of months to years. From these experiments it did not become clear whether the parvovirus B19 genome is actively expressed or not. The permanent presence of virus might lead to infection of non-permissive cells. It has been shown that the viral receptor, erythrocyte P antigen, is present on a variety of human tissues (Brown et al., 1993). In non-permissive cells the expression pattern of the parvovirus B19 genome is shifted to the preferential production of NS-1 protein (Liu et al., 1992). After cell death caused by the toxic properties of NS-1, the protein might be released in large amounts and become accessible to the humoral immune system, leading to synthesis of detectable amounts of NS-1-specific antibody as observed in our patients. This phenomenon could be related to the HLA haplotype of the patients. A correlation between B19-associated arthritis and HLA DR4 or B27 has been proposed (Klouda et al., 1986; Jawad, 1993).

In contrast to IgG, present in titres of about 1000, IgM directed to the NS-1 protein could not be detected. Since the IgM response is usually stimulated by complex protein particles, the NS-1 protein, which is produced only in small amounts during infection is not a part of the B19 virion, might be rather inefficient in stimulating IgM antibody production (Cotmore et al., 1986). Additionally, our samples from patients with acute parvovirus B19 infection were collected about 5 to 6 weeks after infection. By this time, the NS-1 specific IgM titres may have declined below the threshold of the detection system.

The question of whether antibodies against the parvovirus B19 NS-1 protein represent a marker for altered forms of viral replication or directly contribute to the development of disease remains to be investigated. On the basis of our data we propose that the detection of specific antibodies may prove useful for the diagnosis of B19 virus-associated arthritis.

The authors thank Wolfgang Igl, Institut für Medizinische Mikrobiologie, Regensburg for providing B19 virus antibody positive sera and for many helpful and critical discussions. We also are indebted to Dr Traudi Böltz for providing the sera from the St Joseph's Hospital, Olsberg, Germany. The polyclonal rabbit antiserum N88 was kindly provided by Peter Beard, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland and the B19 virus genomic DNA clone by J. Clewley, Virus Reference Laboratory, Colindale, London, UK.

References


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(Received 28 September 1994; Accepted 11 November 1994)