

Immunoglobulin A responses to Puumala hantavirus

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Puumala hantavirus (PUUV) causes nephropathia epidemica (NE), a form of haemorrhagic fever with renal syndrome that occurs in northern and central Europe. The immunoglobulin A (IgA) response in NE patients was studied. The levels of total serum IgA in acute-phase samples from NE patients were found to be significantly elevated when compared with the levels in healthy controls. ELISAs for detection of the IgA1 and IgA2 responses against each PUUV structural protein (N, G1 and G2) were developed and evaluated. Sequential sera from NE patients (acute, convalescent, 2-year) and 10–20 year NE-convalescent sera were examined. Most patients developed detectable levels of IgA1 against N and G2, while the G1 responses were low or undetectable. Seven of nine 10–20 year sera contained virus-specific IgA1, which may indicate the prolonged presence of viral antigens after the initial infection. PEPSCAN analysis revealed several IgA-reactive antigenic regions in the N protein. Serum IgA and IgG was purified by affinity chromatography and examined by a virus-neutralization assay. Three of five sera from acute-phase NE patients contained neutralizing IgA1. The diagnostic potential of the PUUV-specific IgA1 response was evaluated. The N and G2 assays showed specificities of 100% with sensitivities of 91 and 84%, respectively, compared with an IgM μ -capture ELISA. Several NE patients, clinically diagnosed for acute PUUV infection, with borderline or undetectable levels of PUUV-specific IgM, were found to be highly positive for the presence of PUUV N-specific serum IgA1, proving the diagnostic value of IgA analysis as a complement to detection of IgM.

Introduction

Puumala virus (PUUV) is one of several serologically distinct members of the genus *Hantavirus*, family *Bunyaviridae*. PUUV is the aetiological agent of nephropathia epidemica (NE), a disease that occurs in Scandinavia, Finland, western Russia and central Europe (Lundkvist & Niklasson, 1994; Plyusnin *et al.*, 1996). NE belongs to a group of diseases commonly known as haemorrhagic fever with renal syndrome (HFRS), characterized by fever, renal dysfunction and, in some cases, haemorrhagic manifestations (Kanerva *et al.*, 1998).

HFRS caused by PUUV is generally milder than HFRS caused by Dobrava or Hantaan hantaviruses and rarely results in haemorrhages. Although the mortality of PUUV infections is low (< 0.2%), the virus causes significant morbidity in northern and eastern Europe; Russia reports around 5000,

Finland 1000, Sweden 300 and Norway 50 PUUV cases each year. Sporadic outbreaks are observed in central Europe and we have recently reported major outbreaks in Bosnia and Belgium with hundreds of cases (Lundkvist *et al.*, 1997; Heyman *et al.*, 1999).

The PUUV virion consists of four structural proteins: the RNA polymerase, two envelope glycoproteins (G1 and G2) and a nucleocapsid protein (N) (Schmaljohn, 1996). Both glycoproteins have been shown to express epitopes that are recognized as targets for neutralizing antibodies (Lundkvist & Niklasson, 1992).

The significance of a specific immunoglobulin M (IgM) response during acute NE and its outstanding value for serodiagnosis have been described by several authors (Zöller *et al.*, 1993; Elgh *et al.*, 1996; Brus-Sjölander *et al.*, 1997). We have previously investigated the kinetics of the IgM, total IgG and IgG subclass responses to the different structural components of PUUV (Lundkvist *et al.*, 1993a, b, 1995). In addition to the highly virus-neutralizing IgG response in NE-convalescent serum, IgM has been suggested to have a significant

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neutralizing activity during the acute course of NE (Hörning *et al.*, 1992). In contrast, knowledge of the IgA responses to hantavirus infections is still limited and only a few studies have been reported (Elgh *et al.*, 1996, 1998; Groen *et al.*, 1994; Patnaik *et al.*, 1999).

At a production rate of 66 mg/kg/day, secretory and systemic IgA is quantitatively the most important immunoglobulin in the development of mucosal immunity and plays a major role in protecting mucosal surfaces. Neutralizing IgAs have been reported against several viruses, e.g. influenza, polio and Sendai viruses (Wang, 1986; Page *et al.*, 1988; Mazanec *et al.*, 1987). It has also been reported that monoclonal IgA alone was capable of protecting mice against influenza by passive transfer (Renegar & Small, 1991). Antigen-specific IgA responses may therefore provide an important reduction in levels of the infectious pathogen at the first line of defence, the mucosal surface. Furthermore, IgA may play a role in the pathology of hantavirus infection, e.g. by immune complex formation. This study describes the characterization of the IgA response in NE patients and an evaluation of the diagnostic value of PUUV IgA-specific assays.

Methods

■ **Sera.** Five groups of sera were included in the study. (i) Single acute-phase sera from 100 NE patients, previously confirmed for PUUV antibodies in routine diagnosis at the Swedish Institute for Infectious Disease Control (SIIDC), Stockholm, Sweden, by a μ -capture IgM ELISA based on native viral antigen (Lundkvist *et al.*, 1995; Brus-Sjölander *et al.*, 1997). (ii) Single sera from eight clinically diagnosed NE patients, previously examined as PUUV IgM negative or with borderline levels of PUUV-specific IgM in routine diagnosis at SIIDC. (iii) Twenty-eight sequentially drawn sera from 10 NE patients hospitalized and confirmed serologically for NE. Eight of the 10 patients contributed three serum samples; one sample drawn 1–13 days after onset of illness (acute-phase sample), one sample drawn 16–41 days after onset of illness (convalescent sample) and one sample drawn 2 years after onset of illness (2-year sample). From two of the patients, only a convalescent sample and a 2-year sample were available. In addition, nine sera from NE convalescents, drawn 10–20 years post-onset of illness, were analysed. (iv) Eight sera from acute-phase ($n = 5$) or convalescent ($n = 3$) NE patients, included in a reference serum panel authorized by SIIDC and Umeå University Hospital (Å. Lundkvist and C. Ahlm, unpublished data). (v) Sixty-seven sera from a normal population in a non-endemic area of Sweden, shown previously to be negative for antibodies to PUUV (Hörning *et al.*, 1992), were used to estimate cut-off levels or specificities of the assays.

■ **Radial immuno-diffusion (RID) assay.** Total serum IgA was determined by RID assay as described by the manufacturer (The Binding Site). Briefly, 10 μ l of the serum samples or calibrators was applied to the gel plate. The plate was incubated at room temperature for 18 h before measurement of radial diffusion. The IgA concentrations of the samples were estimated by comparison of the diffusion diameters with those of the calibrators containing known amounts of IgA. Statistical analyses were performed with Student's unpaired *t*-test and the Mann-Whitney U-test.

■ **Reagents for ELISA.** Antigen preparations (detergent-treated cell lysates of Vero E6 cells infected with PUUV strain Sotkamo) containing

the structural proteins of PUUV, N, G1 and G2, were prepared as described elsewhere (Lundkvist & Niklasson, 1992). Bank vole (*Clethrionomys glareolus*) monoclonal antibodies (MAbs) 1C12, 5A2 and 5B7, specific for N, G1 and G2, respectively, were prepared and purified on protein G columns as described elsewhere (Lundkvist *et al.*, 1991; Lundkvist & Niklasson, 1992). Biotin-labelled mouse MAbs (Southern Biotechnology Associates) were used for detection of human IgA1 and IgA2, followed by streptavidin-conjugated peroxidase (Sigma).

■ **ELISAs for detection of IgA subclasses against the structural proteins of PUUV.** Three series of ELISAs were employed, one for each structural protein of the PUUV virion. For each series, a bank vole MAb specific for the appropriate viral protein (N, G1 or G2) was used for antigen binding. Affinity-purified MAbs were diluted to 10 μ g/ml in 0.05 M sodium carbonate, pH 9.6, and adsorbed to 96-well microtitre plates (Costar) overnight at 4 °C. Non-saturated binding sites were blocked overnight at 4 °C with 3% BSA in PBS.

The following reagents were diluted in ELISA buffer (PBS, 0.05% Tween 20, 0.5% BSA), added to wells and incubated for 1 h at 37 °C and the plates were washed five times with 0.9% NaCl with 0.05% Tween 20 between each step. After incubation with viral antigen and negative-control antigen (ELISA buffer), serum samples diluted 1:100 were added in duplicate to wells with antigen and to wells with negative-control antigen, followed by biotin-labelled MAbs specific for the two subclasses of human IgA. Streptavidin-peroxidase was added and wells were incubated for 45 min at 37 °C followed by addition of tetramethylbenzidine (TMB) substrate (Sigma).

On all plates, one acute-phase serum was used as an internal standard. The mean absorbance of the standard duplicate was recalculated to 1.000 on each plate and the mean value of the sample duplicates was then adjusted correspondingly. The results were calculated as follows: the mean absorbance of the serum duplicates with virus antigen was reduced by the background mean absorbance obtained with negative-control antigen.

■ **Epitope mapping (PEPSCAN).** The PEPSCAN method (Geysen *et al.*, 1987), designed for identification of linear B cell antigenic sites, was used to locate antibody-reactive peptides contained within the sequence of N protein of PUUV strain Sotkamo (Vapalahti *et al.*, 1992). In total, 86 peptides (10-mer overlapping peptides covering the complete N protein by shifts of 5 amino acids; Lundkvist *et al.*, 1995) were examined. Antibody reactivities with PEPSCAN peptides were measured by ELISA, as described previously (Geysen *et al.*, 1987), using sera diluted 1:100. Bound antibodies were detected with peroxidase-conjugated goat anti-human IgA (DAKO) and TMB substrate according to the manufacturer's instructions (Sigma).

■ **Purification of IgA.** IgA1 was purified from acute-phase NE patient serum on Jacalin (*Artocarpus integrifolia*) (Sigma), as described previously (Johansen *et al.*, 1994). IgG was affinity-purified on protein A-Sepharose as described by the manufacturer (Pharmacia).

■ **SDS-PAGE and immunoblotting.** The purity of the affinity-purified IgA1 and IgG fractions was examined by SDS-PAGE and immunoblotting. Serum, IgA1 and IgG fraction samples were mixed with SDS sample buffer and applied to 4–20% gradient SDS-polyacrylamide gels. Gels were stained with Coomassie blue or transferred to a nitrocellulose sheet for immunoblotting analysis, which was performed essentially as described previously (Johansen *et al.*, 1994). The nitrocellulose membrane was cut into strips, which were blocked with 5% milk powder in PBS (assay buffer) for 2 h at room temperature. Alkaline phosphatase-conjugated rabbit anti-human IgA and IgM (DAKO) or goat anti-human IgG (Sigma), diluted 1:1000 in assay buffer, were added

and the strips were incubated for 4 h at room temperature. After four washes with PBS with 0.05% Tween 20, the reactions were developed with BCIP/NBT tablets (Sigma) dissolved in distilled water.

■ **Neutralization assay.** Neutralizing activity of serum or purified fractions of serum against PUUV (strain Sotkamo; Vapalahti *et al.*, 1992) was analysed by focus-reduction neutralization test (FRNT) (Lundkvist *et al.*, 1997). Briefly, samples were serially diluted and mixed with an equal volume containing 30–70 focus-forming units of virus per 100 µl. The mixtures were incubated for 1 h and inoculated into wells of 6-well plates containing confluent Vero E6 cell monolayers. After adsorption for 1 h, the wells were overlaid with agarose and incubated for 12 days. PUUV-specific polyclonal rabbit antisera followed by peroxidase-labelled goat antibodies and TMB substrate were used for detection of virus-infected cells. An 80% reduction in the number of foci was used as the criterion for virus neutralization titres.

Results

Total IgA

Total IgA was determined by RID assay for 49 sera drawn from acute-phase NE patients. For comparison, the serum IgA levels were determined of 40 patients suffering from other acute virus infections (varicella-zoster virus, cytomegalovirus, influenza A virus or measles virus) and of 53 healthy individuals. Large variation in total IgA could be detected in all groups (Table 1). The levels of total IgA in NE patients varied between 766 and 6990 mg/l (mean 2617 mg/l) and were significantly elevated ($P = 0.018$, *t*-test) compared with the levels in healthy individuals. There were no significant differences between the levels in the NE patient group when compared to the other patient groups.

Kinetics of IgA responses

IgA1. The individual absorbances of sera drawn on three occasions from 10 patients monitored for 2 years are shown in Fig. 1. All 10 serially bled NE patients developed IgA1 against the PUUV N protein (Fig. 1*a*) and nine of the patients also had detectable levels of IgA1 to the G2 protein (Fig. 1*c*), while the response to the G1 protein was undetectable in most cases (Fig. 1*b*). The development of IgA1 antibodies to N and G2 showed

similar patterns; the highest absorbances were found in the acute-phase sera, lower for the convalescent sera and lowest for the 2-year and 10-year sera. However, the rate of decrease in mean absorbance between the acute-phase sera and convalescent sera was greater for anti-G2 than for anti-N IgA1. Interestingly, low levels of N- and G2-specific IgA1 were detected in six and five samples, respectively, of nine sera drawn 10–20 years after the disease (Fig. 2).

IgA2. Most serum samples were negative for PUUV-specific IgA2. Only two of 10 serially bled patients developed low levels of N-specific IgA2, while none of the patients had detectable IgA2 levels against the G1 or G2 proteins.

NE-negative sera

The mean absorbances of 27 negative sera were calculated separately for each viral protein and IgA subclass. The mean absorbance plus 3 SD was used as the cut-off value. The cut-off values of the six assays varied between 0.035 and 0.216.

Epitope mapping

In total, 86 overlapping 10-mer peptides of PUUV (strain Sotkamo), covering the whole N protein in 5-amino-acid shifts, were used for epitope mapping by the PEPSCAN method. Several linear B cell antigenic sites were identified over the entire N protein when a pool of NE sera, drawn from 10 acute-phase patients, was analysed. The results are shown in Fig. 3 as absorbances, with the reactivities of a pool from 10 non-exposed donors subtracted. The majority of the antigenic regions detected were located in the C-terminal part of the protein, represented by major peaks at amino acids 256–265, 351–360 and 411–420.

Neutralizing activities of IgA and IgG fractions compared with total serum

Total IgA1 and IgG fractions were purified by affinity chromatography from five sera obtained from acute-phase NE patients and three sera drawn from NE convalescents. The purity of each fraction was analysed by SDS-PAGE (Fig. 4) and the results indicated that there were no significant amounts of contaminating proteins in either the IgA1 or the IgG fractions. In addition, acute-phase sera and the corresponding purified IgA1 and IgG fractions were analysed by immunoblotting. The results confirmed the SDS-PAGE data: IgA was detected only in the purified IgA1 fraction and the serum sample and IgG was detected only in the purified IgG fraction and the serum sample, while IgM was detected only in the serum samples (not shown).

The fractions were analysed by IgM, IgG (Brus-Sjölander *et al.*, 1997) and IgA1 ELISAs as well as by FRNT and compared

Table 1. Total serum IgA as determined by RID assay

Levels of IgA are given as mg/l serum.

Sera	Range	Mean
NE ($n = 49$)	766–6990	2617
Measles virus ($n = 10$)	1260–6610	2668
Cytomegalovirus ($n = 10$)	1550–4620	2606
Influenza A virus ($n = 10$)	766–3770	2252
Varicella-zoster virus ($n = 10$)	766–3020	1704
Healthy individuals ($n = 53$)	809–4130	2106

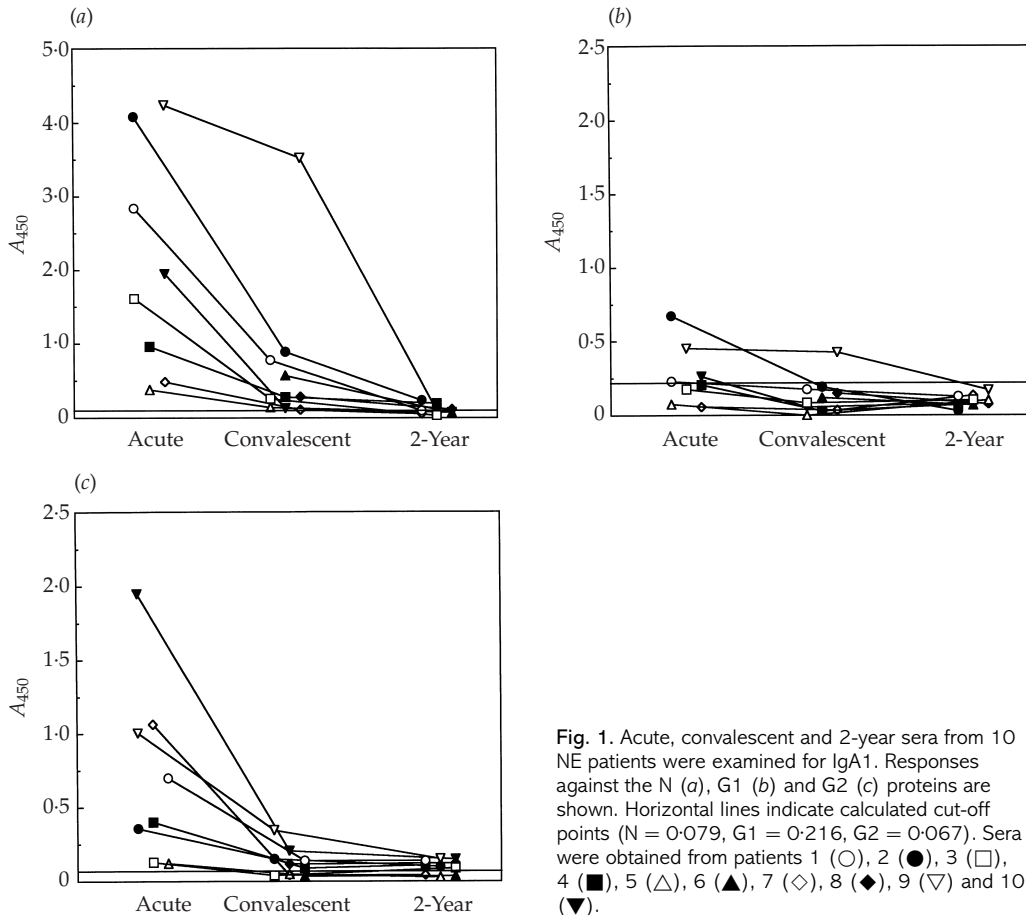


Fig. 1. Acute, convalescent and 2-year sera from 10 NE patients were examined for IgA1. Responses against the N (a), G1 (b) and G2 (c) proteins are shown. Horizontal lines indicate calculated cut-off points ($N = 0.079$, $G1 = 0.216$, $G2 = 0.067$). Sera were obtained from patients 1 (○), 2 (●), 3 (□), 4 (■), 5 (△), 6 (▲), 7 (◇), 8 (◆), 9 (▽) and 10 (▼).

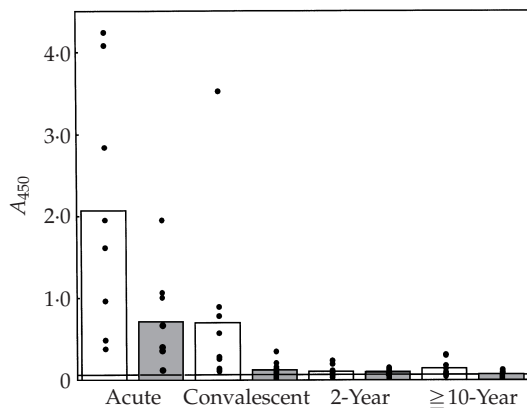


Fig. 2. Acute, convalescent and 2-year sera from 10 NE patients together with sera drawn 10–20 years post-NE infection from nine patients were examined for IgA1 antibodies directed against N (open bars) and G2 (shaded bars) proteins. The bars express the mean absorbance at different intervals post-onset of disease. Dots indicate individual values. Lines indicate calculated cut-off points ($N = 0.079$, $G2 = 0.067$).

with the titres of the original sera (Table 2). The results showed that four of five acute-phase sera had detectable levels of N- and G2-specific IgA1 and that three of five IgA1 fractions from

acute-phase sera had low but significant levels of neutralizing IgA1. There was a good correlation between neutralizing IgA1 and the IgA1 ELISA data for the G2 protein; i.e. three of four ELISA IgA1-positive fractions from acute-phase sera also showed neutralizing activity while none of the four ELISA IgA1-negative sera (one acute-phase and three convalescent) did. However, one acute-phase serum fraction had a significant level of G2-reactive IgA1 without detectable levels of neutralizing IgA1.

Diagnostic value of PUUV IgA1

The IgA1 anti-N and -G2 assays were evaluated by use of serum samples from 100 patients with acute NE. In comparison with the routine SIIDC μ -capture IgM ELISA, which uses native PUUV N antigen (Brus-Sjölander *et al.*, 1997), 91% of the PUUV IgM-positive sera also displayed positive results in the IgA1 anti-N assay, while 84% were IgA1 anti-G2 positive. None of 67 negative-control sera reacted positively in the two assays (specificity 100%).

Eight sera that contained low or undetectable levels of PUUV-specific IgM but were from patients with clinical symptoms of acute-phase NE were analysed for N-specific

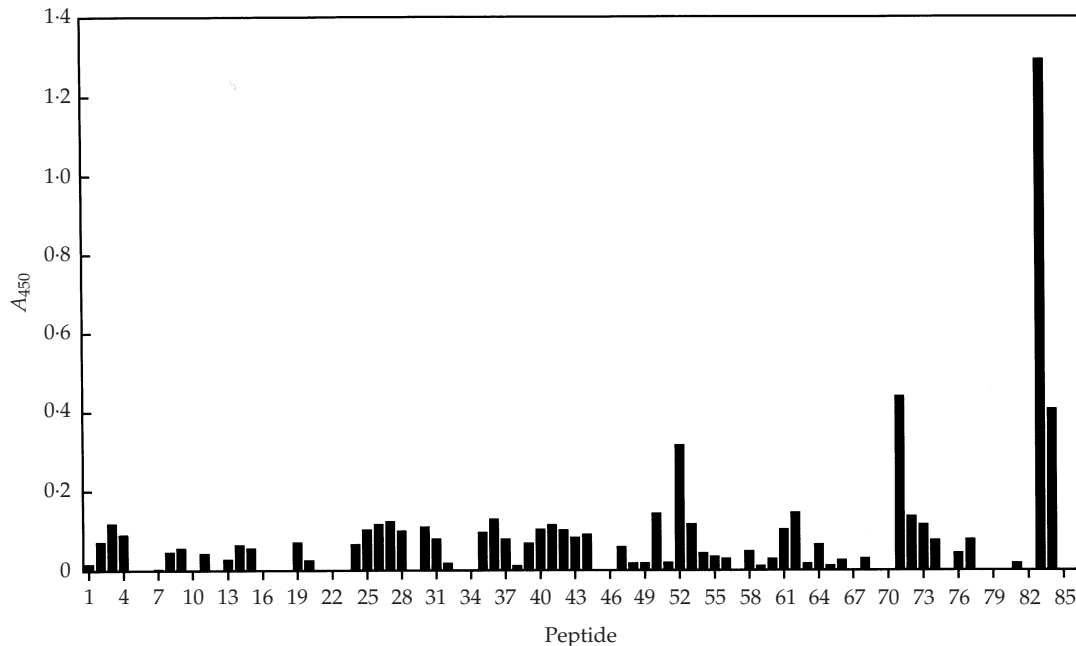


Fig. 3. PEPSCAN analysis of antibody reactivity to overlapping decamer peptides spanning PUUV N. Each peptide overlaps the following peptide by five amino acids. The first bar corresponds to amino acids 1–10, the second to residues 6–15 and so on. Each bar displays the antibody reactivity obtained with pooled serum samples from 10 acute-phase NE patients after subtraction of the reactivity of pooled serum samples from 10 non-exposed donors.

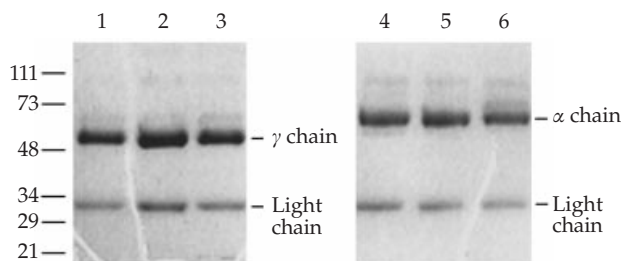


Fig. 4. SDS-PAGE-separated and Coomassie blue-stained purified IgG and IgA1 fractions from acute-phase NE patient sera. Lanes 1–3 represent IgG fractions and lanes 4–6 represent IgA1 fractions from three patients. The migration of molecular mass standards is indicated in kDa on the left.

IgA1 (Table 3). All eight sera reacted as positive; four sera with high absorbances (0.777–1.540) and four sera with reactivities slightly above the cut-off value (0.100–0.157; cut-off = 0.079). Since two of these sera were negative for PUUV-specific IgM, the results clearly revealed the value of IgA1 detection as a complement to the IgM assay.

Discussion

This paper describes the kinetics, specificity and antiviral activity of serum IgA during and after PUUV infection in man. During the initial development of PUUV IgA-specific assays, we evaluated several different strategies and also several commercial reagents from various suppliers. The results revealed that ELISAs based on α -chain capture and assays

based on direct coating of the antigen had significantly lower sensitivities than the system selected, which was based on MAb-capture of the antigens followed by α -chain-specific conjugates. In addition, by amplification of the specific signals via the highly efficient binding between biotin and streptavidin, we were able to increase the assay sensitivities greatly. In the system selected, no increases in the assay sensitivity were obtained by adsorption of potentially inhibitory virus-specific IgG prior to IgA testing.

The patterns of the IgA1 responses against the different viral components were reminiscent of the findings for IgM in a previous study (Lundkvist *et al.*, 1993a). Like the measurement of PUUV-specific serum IgM, as well as IgG3 (Lundkvist *et al.*, 1993b; Groen *et al.*, 1994), detection of specific IgA proved to be an adequate method for determination of the stage of infection: a significant decrease was seen already in the early convalescent samples (drawn 16–41 days after onset of disease). Similar observations have been made for other virus infections that primarily infect mucosal surfaces, e.g. measles, rota- and enteroviruses (Friedman *et al.*, 1989; Coulson *et al.*, 1990; Pozzetto *et al.*, 1990).

Our finding of the presence of IgA1 in several of the 2-year and 10-year NE convalescent samples is noteworthy, because of the suggested role of persistent IgA as a marker for prolonged antigenic stimulation (Friedman & Eichler, 1991; Nilsen *et al.*, 1991). Hantaviruses persist, usually for life, in their natural rodent carriers and PUUV has been detected 1 year after experimental infection (Bogdanov *et al.*, 1987). In a

Table 2. Antibody specificity/neutralizing activity of total serum or affinity-purified IgG/IgA1 fractions from PUUV-infected patients

Results of ELISA and FRNT are shown. Values are end-point titres. Sera were from acute-phase and convalescent NE patients as shown. Lowest serum titres: IgM ELISA, 1:200; IgG ELISA, 1:400; IgA ELISA, 1:100; FRNT, 1:20. –, Negative; NT, not tested.

Serum ...Cut-off value	ELISA				FRNT		
	IgM anti-N 0·150	IgG anti-N 0·100	IgA1 anti-N 0·079	IgA1 anti-G2 0·067	Total serum –	IgG fraction –	IgA1 fraction –
Acute							
A	800	6 400	2 500	500	640	80	40
B	200	6 400	–	–	160	40	–
C	3 200	400	12 500	2 500	320	80	20
D	800	1 600	500	500	640	40	–
E	3 200	25 600	500	500	NT	80	40
Convalescent							
F	–	25 600	–	–	640	≥ 320	–
G	–	102 400	–	–	1 280	≥ 320	–
H	–	25 600	–	–	640	80	–

Table 3. PUUV N-specific IgA1 in sera with low IgM levels

Cut-off values were 0·079 (IgA1), 0·150 (IgM) and 0·100 (IgG).
p.o., Post-onset of disease; –, below the level of detection.

Sample	Days p.o.	IgA1 (A_{450})	IgM (A_{450})	IgG (A_{405})
1	2	1·349	–	0·294
2	9	0·134	0·165	0·723
3	14	0·791	0·303	0·718
4	11	0·157	0·286	0·495
5	11	0·100	0·218	0·652
6	10	0·777	0·257	0·619
7	21	0·125	–	0·238
8	8	1·540	0·311	–

previous study, we noted elevated levels of PUUV-specific IgG4, especially directed to G2, in 2-year and 10–20-year NE-convalescent sera. Since virus-specific IgG4 has previously been demonstrated in virus infections that tend to persist in the host, e.g. hepatitis B, herpes simplex and varicella-zoster viruses (Asano *et al.*, 1987, 1988; McBride & Ward, 1987; Sällberg *et al.*, 1990), we thereby speculated on prolonged/persistent antigenic stimulation during/after PUUV infection (Lundkvist *et al.*, 1993b). In line with this, PUUV N and G2 proteins and RNA have recently been reported to persist in experimentally infected cynomolgus macaques for 7, 10 and at least 30 weeks, respectively (Groen *et al.*, 1995). Although the kinetics of the human IgA response to PUUV might support the presence of a prolonged or repeated antigenic stimulation, the question of whether viral RNA and/or viral antigens actually remain in the human body for an extended period after

the initial PUUV infection is still unclear. In several attempts, using highly sensitive RT–PCR, PUUV RNA has been found in only a minority of patient samples and only within the first days after onset of disease (Hörling *et al.*, 1995; Plyusnin *et al.*, 1997, 1999). Several other explanations for long-term memory preservation and antibody synthesis have been proposed, e.g. that viral antigens persist in the form of antigen–antibody complexes on the surface of follicular dendritic cells (Tew *et al.*, 1980), that continuing antibody synthesis is stimulated by idiotype–anti-idiotype interactions (Morris *et al.*, 1985) and that B and/or T cell memory may be maintained by cross-reactive stimulation (Beverley, 1990). However, the detection of virus-specific IgA1 in 2-year and 10–20-year convalescent samples may not be unique to PUUV infection; it may also be explained, at least partially, by the comparatively higher sensitivities of the PUUV assays. Extended studies, e.g. by direct analysis of the presence of viral RNA and antigens in biopsy material from convalescents or from experimentally infected monkeys, are needed for a better understanding of this issue.

Mapping of epitopes in the PUUV N protein revealed several antigenic regions recognized by the human IgA response. At least six regions were detected by a pool of 10 acute-phase NE sera, with pronounced activity against three regions in the C-terminal part of the protein. This pattern does not match completely the pattern of the total IgG response, which has been shown previously to be more equally distributed over the whole protein (Lundkvist *et al.*, 1995; Vapalahti *et al.*, 1995).

Our previous work has suggested the presence of high levels of neutralizing serum IgM during the acute phase of NE (Lundkvist *et al.*, 1993a). In that study, five acute-phase sera, all

with substantial levels of IgM directed to both envelope proteins, maintained their neutralizing activity after IgG depletion. However, no attention was paid to virus-specific serum IgA. In the present study, we purified IgA1 and IgG and studied the respective neutralizing activities separately. Interestingly, we found significant levels of neutralizing IgA1 in three of four PUUV IgA-positive sera, which also shows the importance of the IgA response in terms of virus inhibition. When the neutralizing activities of the IgA and IgG fractions were compared with the neutralizing activity of the original serum, the results suggested that the major neutralizing activity was not caused by these two Ig classes. Thus, the results are in line with our previous data, indicating that the major antibody-mediated virus neutralization during the acute stage of NE is caused by PUUV-specific IgM, supported in some cases by IgA and/or IgG. In line with this, maternal IgA and IgG have been shown to protect infant rats from lethal doses of Seoul virus, after transfer either *in utero* or by breastfeeding (Dohmae *et al.*, 1993; Dohmae & Nishimune, 1998). Since hantaviruses are mainly spread via aerosol and primarily infect via the respiratory tract, the presence of neutralizing IgA is interesting in terms of the recovery from the acute infection and also for long-term immunity in convalescents.

Secretory IgA (S-IgA) responses are unique to mucosal surfaces and secretions are mainly induced by infection or immunization (e.g. intranasal, oral, rectal or vaginal) via the mucosal route. In humans, serum IgA is predominantly detected as a monomer, whereas dimeric or polymeric S-IgA is found in mucosal secretions. Transport of S-IgA across epithelial surfaces to external secretions, where antigen-specific S-IgA interacts with potential pathogens and inhibits their interaction with the host, may be the most important mechanism provided by S-IgA. S-IgA antibodies are particularly effective in virus neutralization, probably mainly because of the presence of multiple antigen-binding sites. Mazanec *et al.* (1992) have hypothesized that IgA may interfere with virus replication by binding to newly synthesized viral proteins within infected cells and may thereby neutralize microbial pathogens intracellularly. Antigen-specific S-IgA responses may provide an important reduction in levels of the infectious pathogen at the first line of defence, the mucosal surface. Further studies on S-IgA during clinical disease and after recovery will be needed to understand better the mechanisms and importance of PUUV-specific IgA.

Two previous studies have evaluated the diagnostic value of PUUV-specific serum IgA (Groen *et al.*, 1994; Elgh *et al.*, 1996). In the first study, all acute-phase patient samples examined were found to be positive for PUUV-specific IgA, although a comparatively small number of sera (18) were analysed. In contrast, in the latter study, where a larger number (108) of patient sera were analysed, the detection of IgA by recombinant N ELISA was regarded as being of minor clinical relevance, mainly because of the low sensitivity (63.0%) of the assay. The discrepancies were suggested to be caused by

differences in the size of the serum panels or by the use of recombinant instead of native viral antigen (Elgh *et al.*, 1996). In a more recent study, 13/17 (76%) sera drawn 2–8 days post-onset of disease were found to be positive by an IgA ELISA based on truncated recombinant PUUV N, while 17/17 (100%) of the sera drawn at days 5–15 were positive for PUUV-specific IgA (Elgh *et al.*, 1998). In line with our data, one IgA-positive serum was completely negative for virus-specific IgM and IgG (Elgh *et al.*, 1998).

In the present study, we found initially that the sensitivity of different IgA ELISA protocols varied to a large extent. By the selected combination of MAb-captured antigen and biotin–streptavidin amplification, we obtained highly specific and sensitive assays. The highest sensitivity (93%, $n = 100$) was shown for the anti-N IgA1 ELISA, probably indicating that the majority of the PUUV-specific IgA1 response is directed to N. When eight sera from NE patients with undetectable or borderline levels of IgM were analysed, all sera were found to be positive for PUUV-specific IgA1. Thus, our study clearly indicates a diagnostic value for IgA1 as a complement to IgM and IgG detection.

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