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Anti-endothelial cell antibodies in nephropathia epidemica and other viral diseases

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SUMMARY

Increased capillary permeability is a central feature of the severe forms of haemorrhagic fever with renal syndrome (HFRS) and occurs also, though less frequently, in nephropathia epidemica (NE), one of the milder forms of this syndrome, caused by Puumala virus. We therefore searched for anti-endothelial cell antibodies (AECA) in patients with NE and in those with other presumed or serologically proven acute viral illnesses. By enzyme immunoassay, using human umbilical vein endothelial cells (HUVEC) as the substrate, IgG class AECA were detected significantly more frequently in patients with NE and with influenza A than in Red Cross blood donors. A lesser degree of reactivity could be shown with a human alveolar cell carcinoma line and with human and mouse embryonic fibroblasts. Pretreatment of HUVEC with interferon-gamma (IFN-γ), but not with IL-1 or tumour necrosis factor-alpha (TNF-α), increased their ability to bind IgG of sera from patients with NE and acute febrile illnesses. We conclude that, although AECA can be demonstrated in NE, they occur also in other acute viral illnesses and, unless cytopathic by a mechanism not shared by the AECA of these other illnesses, are unlikely to be causally related to the capillary leak in HFRS.

Keywords anti-endothelial cell antibodies IL-1 interferon-gamma nephropathia epidemica tumour necrosis factor-alpha viral diseases

INTRODUCTION

Nephropathia epidemica (NE) is an acute illness caused by Puumala virus which belongs to the Hantavirus genus of the family Bunyaviridae [1,2]. NE is a member of the haemorrhagic fever with renal syndrome (HFRS) group [3] and is usually mild although a few instances of fatal shock have been reported from Finland [4]. The clinical picture is characterized by a high fever, malaise, headache and back and abdominal pain followed on the third day by proteinuria, microscopic haematuria and oliguria. Resolution and polyuria begin in the second week. The blood pressure usually remains normal although both hypertension and hypotension have been described [5]. Myopia is rare but pathognomonic [6]. Renal biopsy shows an interstitial nephritis with haemorrhages into the interstitium but an acute glomerulonephritis has also been described [5]. Deposits of IgG, IgM and complement have been reported [5,7] but are patchy and rare and serum levels of complement are usually normal. Thus far, attempts to demonstrate the virus in renal biopsy specimens or to grow it from the urine, have been unsuccessful [8].

Increased vascular permeability is a central feature in all forms of HFRS. It is, however, uncertain whether it is due to viral invasion of endothelial cells, to the action of virus-induced cytokines such as interferon-gamma (INF-γ), IL-1 or tumour necrosis factor-alpha (TNF-α) or to other, as yet unrecognized mechanisms. Though hantaviruses infect human endothelial cells *in vitro* [9], cytopathic changes are not seen.

The pathogenesis of NE is thus still unresolved. Since many human diseases, characterized by vascular damage, are associated with antibodies to endothelial cells (AECA), e.g. systemic lupus erythematosus (SLE), systemic sclerosis, rheumatoid vasculitis, microscopic polyarteritis and Wegener's granulomatosis [10–15], we have sought these antibodies in patients with acute NE and in patients with other acute, febrile and clinically infectious diseases as control subjects. We have also attempted to determine whether, in acute NE, IgG binding to human umbilical vein endothelial cells (HUVEC) is increased if the cells are grown in the presence of IL-1, IFN-γ or TNF-α, which might conceivably increase the expression of potential antigens.

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PATIENTS AND METHODS

Patients and control subjects

Sera from the following categories of subjects were tested:

17 patients with acute NE in whom the diagnosis had been verified serologically by demonstrating the presence of low-

avidity IgG antibody to Puumala virus by an indirect immunofluorescence test using Vero E6 cells infected with Puumala virus. The demonstration of this type of antibody is based on treatment of the Vero E6 cell layers with 8 M urea which causes a reduction in the titre of low-avidity antibody but does not affect the level of high-avidity antibody [16];
26 patients with acute febrile illnesses in whom the test for recent NE was negative. Six of the patients in this group had evidence of past infection as shown by the presence of high-avidity specific antibody;
99 blood donors from the Finnish Red Cross Blood Transfusion Service, kindly provided by Dr T. Krusius. These sera were not tested for NE but the serological prevalence of past NE infection in Finnish blood donors is known to be approximately 5%;
nine patients with serologically verified influenza A;
19 patients with serologically verified influenza B;
21 patients with serologically verified Epstein–Barr virus (EBV) infection.

Paired samples, taken 7–10 days apart, were available from the patients with influenza and single samples from the patients with infectious mononucleosis.

AECA assay

AECA were sought by a cellular enzyme immunoassay [17], similar to that described by Rosenbaum *et al.* [18]. Briefly, HUVEC were harvested by collagenase digestion [19], grown to confluence in gelatin-coated flasks in RPMI 1640 with 20% fetal calf serum (FCS), 2 mM L-glutamine, 25 μg/ml endothelial cell growth supplement (Sigma, no. E2759), 30 U/ml heparin and 80 μg/ml gentamycin. At this stage, the cells were detached by exposure to trypsin (0·2% in 0·05% EDTA, GIBCO) and either subcultured or pooled and transferred to 96-well gelatin-coated Costar flat-bottomed plates at a density of $1–2 \times 10^4$ cells/well. Cells were used between the second and fifth passages. When confluent, the cells were fixed for 5 min in 0·2% glutaraldehyde and washed 3×3 min in PBS under sterile conditions.

For the assay, the plates were blocked for at least 1 h with 10% FCS in PBS. The coded test sera were diluted 1:200 in 0·1% bovine serum albumin (BSA)/PBS and 100 μl/well were incubated in the wells in triplicate for 2 h at room temperature. After 3×3 min washes in BSA/PBS, 100 μl/well of alkaline phosphatase-conjugated swine anti-human IgG (Orion Diagnostica, Espoo, Finland), diluted 1:200 were added. After a further 1 h incubation at room temperature and 3×3 min washes in BSA/PBS, 100 μl/well of 2 mg/ml of disodium *p*-nitro-phenylphosphate in diethanolamine buffer (Orion Diagnostica) were added. The plates were incubated for 1 h at 37°C and the absorbances were read at 405 nm in a Multiskan MCC/340 plate reader (Labsystems, Helsinki, Finland) connected to an ELISA+ software program (Meddata Inc., New York, NY).

A standard curve of five dilutions of a high positive serum, another positive control and a panel of five normal control sera were included on each plate. Results were expressed as arbitrary U/ml taking the binding of the strongest dilution (1:250) of the high positive serum as representing 100 U/ml and those exceeding the mean and 3 s.d. of the normal control panel were regarded as positive. The high positive serum was from a patient with juvenile seronegative but antinuclear antibody-positive rheumatoid arthritis. Using this as a standard, we have found AECA in 30 of 48 patients with SLE with titres to a maximum of 210 U/ml. The inter-assay co-efficient of variation of the assay was 10·6% ($n=32$).

A selection of the sera was also tested in an identical manner against the following non-endothelial cell lines: A549 (alveolar cell carcinoma [20]), NIH MRC 5 (human lung fibroblast-like) and NIH 3T3 (mouse embryo fibroblast). Cytokines were not used in these cultures.

Fc receptors were sought on unfixed and ethanol-fixed HUVEC grown without or with cytokines using MoAbs to human FcRI, FcRII and FcRIII (32.2, IV.3 and 3G8, Medarex Inc, West Lebanon, NH) followed by alkaline-phosphatase conjugated anti-mouse IgG (Dako-APAP). Sera were also tested in parallel before and after treatment with PEG 6000 to remove immune complexes as described by Breedveld *et al.* [21].

Pretreatment of HUVEC with cytokines

Endothelial cells were grown in culture flasks and/or microtitre plates in the presence of cytokines as described by Leung *et al.* [22,23]: recombinant IFN-γ (a generous gift from Dr M. Hurme) 200 U/ml for 96 h; ultrapure consensus IL-1 (Endogen, Boston, MA) 10 U/ml for 4 h or recombinant TNF-α (Genzyme, Boston, MA) 50 U/ml for 4 h. Parallel cultures were incubated in an identical manner but without cytokines. At the end of the incubation periods the cells were fixed with glutaraldehyde as already described and tested so that both the sham-incubated and the cytokine-incubated cultures were run in the same assay.

Statistical analysis

Statistical significance of differences between groups was tested by a two-tailed Student's *t*-test, either paired or unpaired as indicated by the composition of the groups.

RESULTS

Sera from patients with acute NE showed significantly greater binding of IgG to unstimulated HUVEC than did the sera from patients with acute febrile illnesses (Table 1). If the six patients with high avidity IgG antibody to Puumala virus were separated from the other patients with acute febrile illnesses and the two groups were considered separately, the difference to the patients with acute NE remained significant for the former six ($P=0·014$) but not for the latter ($P=0·089$). When compared with the 99 Red Cross donors, the sera from patients with acute NE, but not from those with acute febrile illnesses, again showed significantly greater IgG binding to untreated HUVEC (Table 1).

Incubation of HUVEC with cytokines before use in the assay resulted in augmented binding of IgG by the sera from patients with acute NE and acute febrile illnesses when tested against cells grown in the presence of IFN-γ (Table 1) but not when the sera were tested against HUVEC cultured with IL-1 or TNF-α.

AECA were also found in the paired sera from four of nine patients with influenza A with titres which were higher than those found in NE, in the first sample from one patient and both samples from another patient with influenza B and in one of the 21 single samples from patients with EBV infection.

The proportion of positive results against unstimulated HUVEC was significantly higher than the Red Cross donors only in acute NE and influenza A ($P=0·022$ and 0·0003 respectively by Fisher's exact test).

Table 1. IgG binding to native and cytokine-stimulated human umbilical vein endothelial cells (HUVEC) (units/ml, mean and s.d.) of sera from patients with nephropathia epidemica (NE), acute febrile illnesses and specified viral infections

Patient group	*n*	Unstimulated VEC	VEC IFN$^-$	VEC IFN$^+$	VEC IL-1$^-$	VEC IL-1$^+$	VEC TNF$^-$	VEC TNF$^+$
Acute NE	17 (3)*	13·3††‡	9·2	15·0§	21·7	17·2¶	10·5	11·3
		3·6	5·6	5·7	8·4	7·8	5·9	6·3
Acute febrile illnesses	26 (3)	10·4	8·9	12·9**	18·1	14·4††	9·8	9·7
		3·6	7·5	8·1	8·0	7·3	7·2	7·4
Red Cross donors	99 (2)	9·4	ND	ND	ND	ND	ND	ND
		5·9						
Influenza A	9 (4)	21·6	ND	ND	ND	ND	ND	ND
		28·3						
Influenza B	19 (2)	6·3	ND	ND	ND	ND	ND	ND
		3·5						
Infectious mononucleosis	20 (1)	11·9	ND	ND	ND	ND	ND	ND
		5·5						

* Number in parentheses denotes number of positive subjects in group.
†$P=0·05$ compared with miscellaneous febrile diseases by two-tailed unpaired Student's *t*-test.
‡$P=0·007$ compared with Red Cross donors by two-tailed unpaired Student's *t*-test.
§$P=0·0028$ compared with corresponding control by two-tailed paired Student's *t*-test.
¶$P=0·049$ compared with corresponding control by two-tailed paired Student's *t*-test.
**$P=0·0029$ compared with corresponding control by two-tailed paired Student's *t*-test.
†† $P=0·02$ compared with corresponding control by two-tailed paired Student's *t*-test.
ND, Not done.

Pretreatment of sera with PEG 6000 before testing did not affect the AECA titre. For acute NE the titres (mean ± s.d.) before *versus* after absorption were 13·2 ± 11·9 *versus* 11·8 ± 8·1 and for influenza A 24·5 ± 23·9 *versus* 23·8 ± 21·8. Regression coefficients were $r=0·814$ for acute NE and 0·916 for influenza A.

We were unable to demonstrate FcR on HUVEC grown without or with IFN-γ by immunostaining with MoAb to the three types of human leucocyte FcγR although leucocytes included as controls gave positive staining.

IgG bound also to non-endothelial cells but the degree of binding was substantially lower than that to HUVEC. Thus, the binding of acute NE sera to A549, MRC5 and 3T3 cells was 35%, 40% and 44% of the binding to HUVEC and for influenza A sera 56%, 52% and 69%.

Dilution curves of the positive control serum (five separate assays) and a negative control, and of sera from the different groups of patients are shown in Fig. 1. It can be seen that the titres of the second samples of the patients with influenza A and influenza B were higher than those of the first samples.

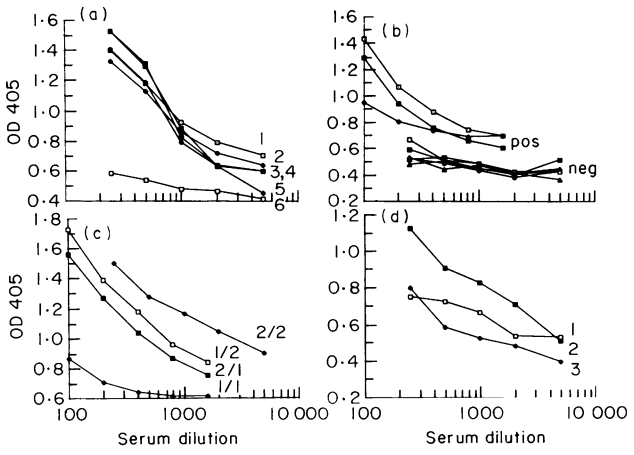

Fig. 1. Dilution curves of positive and negative sera as follows: (a) high positive control serum in five separate assays (1–5) and a negative control (6); (b) three positive and five negative sera from patients with acute nephropathia epidemica (NE); (c) paired positive sera from two patients with influenza A (1/1 and 1/2 are first and second specimens from patient 1, 2/1 and 2/2 the corresponding specimens from patient 2); (d) paired positive sera from a patient with influenza B (1 and 2) and a single positive serum from a patient with glandular fever (3).

DISCUSSION

This study showed the presence of AECA in a proportion of patients with NE but also in patients with defined viral diseases and those with undiagnosed acute febrile and presumably viral illnesses. To our knowledge, AECA have not previously been reported in acute viral diseases although their presence is not unexpected. Penttinen *et al.* [24] found rheumatoid factors, circulating immune complexes and increased levels of IgM in all 18 patients with NE; the levels of IgG and IgA increased during the clinical disease but remained normal. The transient appearance of antibodies directed against self-antigens such as smooth muscle [25] and negatively charged phospholipids [26] is well recognized in viral illnesses. However, in our series it seems unlikely that the binding was due to immune complexes since we were unable to detect FcγR of the three types normally found on human myeloid cells and monocytes on HUVEC grown without or with IFN-γ. Moreover, treatment of the sera with PEG 6000

did not significantly reduce the amount of IgG bound. It is, however, worth noting that the IgG did not bind exclusively to HUVEC but also, although to a lesser extent, to a human alveolar carcinoma line and to human and murine embryonic fibroblasts.

Like previous workers [22,27], we were able to show increased binding of IgG to HUVEC grown in the presence of IFN-γ which is known to up-regulate the production of several cell surface molecules [28] but we found no such effect of IL-1 or TNF-α despite using the cytokines at the recommended concentrations. We are uncertain whether the difference is a real one or whether it is due to technical factors.

AECA of the IgM class have been shown to mediate complement-dependent lysis of HUVEC in Kawasaki disease and the haemolytic-uraemic syndrome [22,23,29]. However, in more recent work, Savage *et al.* [27] were unable to show damage to HUVEC mediated either by complement or through ADCC in most patients with Wegener's granulomatosis or microscopic polyarteritis and AECA. We did not seek evidence for a cytopathic effect of the AECA in the patients with NE and, having moreover sought only IgG class AECA, are unable to speculate on whether they may play a part in causing the vascular permeability which is sometimes such a prominent feature of this condition. However, their presence also in other serologically defined acute viral diseases such as influenza A and B, in which increased vascular permeability is not clinically evident, suggests they do not play a part in its causation unless they are cytopathic by a mechanism which is not shared by the AECA of these other viral illnesses. Increased virus-induced secretion of cytokines such as IL-2, which is known to cause a capillary leak syndrome when used in therapeutic doses [30], may provide an alternative explanation worth probing.

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