

Detection of Specific Serum Immunoglobulin M in Nephropathia Epidemica (Scandinavian Epidemic Nephropathy) by a Biotin-Avidin-Amplified Immunofluorescence Method

BO SETTERGREN,^{1*} PER JUTO,² AND GÖRAN WADELL²

Departments of Infectious Diseases¹ and Virology,² University of Umeå, S-901 85 Umeå, Sweden

Received 17 November 1986/Accepted 16 February 1987

A biotin-avidin-amplified indirect immunofluorescence method was used to demonstrate specific serum immunoglobulin M (IgM) antibodies in nephropathia epidemica, the Scandinavian type of hemorrhagic fever with renal syndrome. The antigen in the test was the cross-reacting agent of Korean hemorrhagic fever, Hantaan virus. Sixty-two serum samples from 15 patients with clinically typical nephropathia epidemica were analyzed. Eleven patients had specific IgM in one or more serum samples. The IgM could be demonstrated from day 2 up to day 37, and all patients had detectable specific IgM within 15 days after the onset of disease. In 49 control serum samples, no specific IgM could be detected, indicating a high specificity for the method. The findings demonstrate that the biotin-avidin-amplified immunofluorescence IgM assay is a useful tool in the diagnosis of early nephropathia epidemica disease.

The causative agents of nephropathia epidemica (NE) and Korean hemorrhagic fever (KHF) are antigenically closely related. This relationship has been used to study the serological response in NE by the immunofluorescence method (3-6, 9). With this technique, specific cross-reactive anti-KHF/NE serum immunoglobulin G (IgG) antibodies were successfully demonstrated when cells infected with the causative agent of KHF (Hantaan virus) and lung sections from bank voles persistently infected with the NE virus were used as antigens. In contrast, no specific serum IgM was found when lung sections from bank voles persistently infected with the NE virus were used (2). However, when serum samples were fractionated by ultracentrifugation and KHF virus-infected cells were used as antigen, specific IgM to KHF/NE could be demonstrated (10).

In this paper an IgM-specific method is described for the serological diagnosis of NE, consisting of an indirect fluorescent-antibody technique (IFAT) amplified by the biotin-avidin system. A total of 62 serum samples were collected from 15 patients with NE (11 males and 4 females; ages, 26 to 68 years [mean, 45.7 years]). The patients lived in the counties of Västerbotten and Jämtland, areas where NE is highly endemic. They all had typical NE symptoms: fever, abdominal or back pain or both, headache, and signs of impaired renal function, e.g., raised serum creatinine, polyuria, and proteinuria. One patient had a more severe disease, with disseminated intravascular coagulation, which has previously been reported (8). From each patient, two to seven serum samples were drawn from day 2 up to 13 months after the onset of NE. A significant rise in titer of anti-KHF/NE IgG antibodies was demonstrated in sera from all 15 patients after 11 to 73 days (median, 21 days), as measured by IFAT as described below (data not shown). Forty-nine healthy blood donors from the same geographic areas as the NE patients were randomly chosen as controls.

Hantaan virus was obtained from the American Type Culture Collection, Rockville, Md. The virus strain was derived from the fifth Apodemus lung passage of KHF virus 76-118 further passaged nine times in cell line A549 (5).

Vero E-6 cells (CRL 1586) obtained from the American Type Culture Collection were grown in 25-ml plastic flasks (Nunc, Roskilde, Denmark) in Eagle minimal essential medium supplemented with 10% fetal calf serum, 1% glutamine, 100 IU of penicillin G per ml, and 100 µg of streptomycin per ml. The medium and calf serum were purchased from Flow Laboratories, Glasgow, Scotland. Fluorescein-labeled rabbit anti-human IgG (F 202; Dakopatt, Copenhagen, Denmark), biotin-labeled goat anti-human IgM (Ba 3020; Vector Laboratories, Sunnyvale, Calif.), and fluorescein-labeled avidin D (A 2001; Vector) were used in the IFAT.

Hantaan virus was adsorbed to Vero E-6 cell cultures at room temperature for 2 h. After adsorption, the inoculum was diluted with growth medium and incubated for 7 days at 37°C in a 5% CO₂ atmosphere. The infected cells were then trypsinized and passaged into two new similar plastic flasks. Viral antigen was demonstrable in the cells by IFAT after five to seven passages. To increase the antigen yield, cultures were incubated for 12 days before the final harvest. The Hantaan virus-infected cells were then trypsinized, washed twice in phosphate-buffered saline (PBS), and seeded at an appropriate density, as judged by inspection with a light microscope, onto spot slide glasses. The slides were air dried, treated with 100% cold acetone (-20°C) for 10 min, and stored at -70°C until use.

The serum samples were screened for the presence of anti-KHF/NE IgG by IFAT (3, 5). Briefly, sera diluted 1/20 in PBS were added to KHF spot slides and incubated for 30 min at 37°C in a moist chamber, followed by rinsing for 10 min in PBS. Fluorescein-conjugated anti-human IgG was added at a 1/40 dilution and incubated for 30 min at 37°C. After being washed, the slides were counterstained with 0.003% Ewan blue for 2 min, rinsed briefly in deionized water, and mounted by use of 90% glycerol in PBS and cover slips. Serum samples that demonstrated reactivity at the 1/20 dilution were endpoint titrated in twofold dilutions steps.

To eliminate interference of rheumatoid factors, all serum samples were treated with latex RF reagent (Rapitex; Behringwerke AG, Marburg, Federal Republic of Germany) before being tested. This was done by mixing 5 µl of serum with 35 µl of PBS and 160 µl of the latex bead solution. The

* Corresponding author.

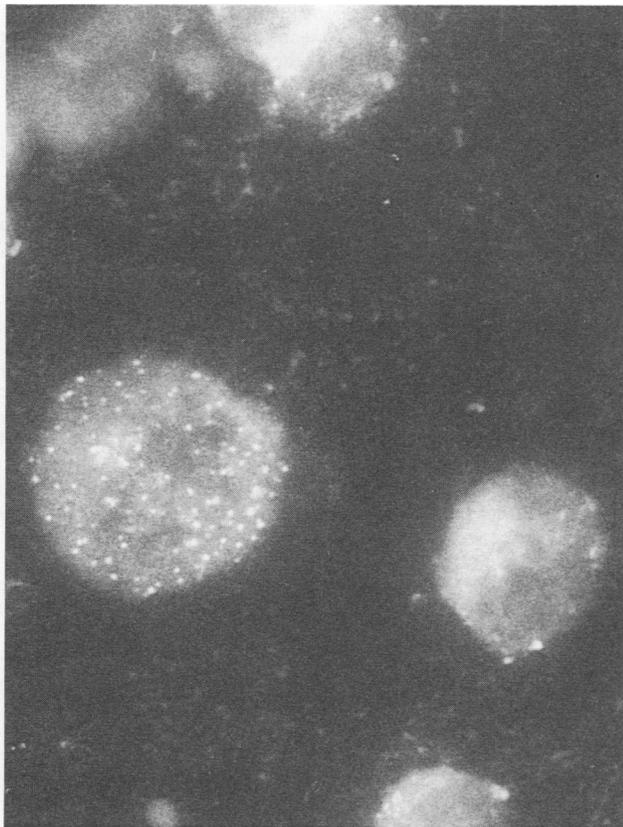


FIG. 1. Fluorescence pattern of anti-KHF/NE IgM antibodies reacting with Hantaan virus-infected Vero E-6 cells stained by the biotin-avidin-amplified IFAT. The antiserum in the test was obtained from a IFAT patient with NE 6 days after the onset of disease.

mixture was incubated for 25 min at room temperature and centrifuged at $2,300 \times g$ for 10 min. The supernatants, containing sera diluted 1/40, were incubated on KHF spot slides overnight at 37°C in a moist chamber. After the slides were washed with PBS, biotin-labeled anti-human IgM (final dilution, 1/300) was added and incubated for 30 min at 37°C, followed by washing. Fluorescein-conjugated avidin (final dilution, 1/300) was then allowed to react on the slides for 5 min at room temperature. After a final wash, the slides were counterstained, rinsed in deionized water, and mounted. Serum samples that showed a positive reaction at the 1/40 dilution were endpoint titrated in twofold dilution steps. The slides were examined in a Leitz Ortholux II fluorescence microscope with a 10× ocular lens and a 40× objective lens. The endpoint was defined as the highest titer at which a bright cytoplasmic, particulate fluorescence could be seen (Fig. 1). Negative and positive control sera were included in all experiments, and all sera were studied under code.

Of 15 patients, 11 had specific IgM to KHF/NE in one or more of their serum samples (Fig. 2). In these 11 patients, the specific IgM was demonstrable within 15 days after the onset of disease and in one patient as early as 2 days after onset. Onset of disease was defined as the first day of fever. The maximal duration of detectable specific IgM was 5 weeks, and the highest IgM titer demonstrated was 1/320.

Four patients had no demonstrable specific IgM. All serum samples from these patients had been freeze-thawed three times or more. To determine whether this procedure had degraded preexisting IgM antibodies, three serum samples with high titers (1/160 to 1/320) were freeze-thawed repeatedly. After the first freeze-thaw, titers dropped to 1/80, and after the third freeze-thaw, no specific KHF NE IgM antibodies could be detected (data not shown). Anti-KHF/NE IgM or IgG antibodies were not detected in any of the 49 control serum samples.

The specific serum IgM response in NE has been little studied. In one study, in which lung sections from NE virus-infected bank voles (*Clethrionomys glareolus*) were used as antigen, no specific IgM could be demonstrated by

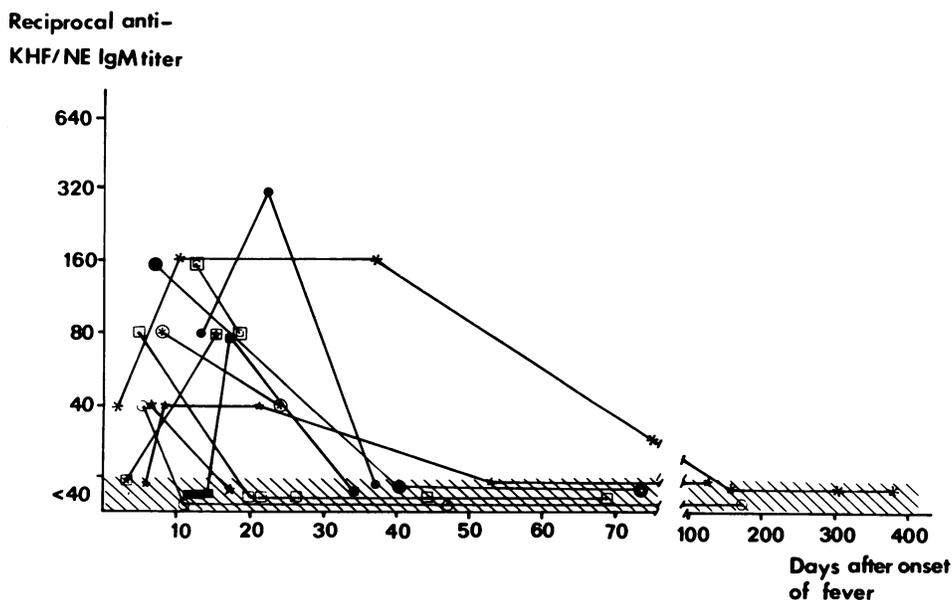


FIG. 2. Serum anti-KHF/NE IgM antibody titers in 11 patients with NE.

IFAT (2). Traavik et al. (10) found, by using IFAT with the KHF virus as antigen, specific KHF/NE IgM in sera that had been obtained early and fractionated by sucrose density gradient centrifugation. All fractionated sera with demonstrable specific IgM were obtained less than 40 days after the onset of disease. Presumably, the levels of circulating specific IgM are low in NE. One reason for this could be the deposition of antibodies in immune complexes, as previously reported (7). Sensitive methods are therefore needed for the detection of specific IgM.

The successful demonstration of specific anti-KHF/NE IgM antibodies in 11 of our 15 patients could be explained by the following factors. First, the IFAT used was amplified by the biotin-avidin system, which has been reported to increase sensitivity at least five times (1). The mechanism behind the amplifying effect of the biotin-avidin system is the extraordinarily high affinity between biotin and avidin ($K_d = 10^{-15}$), but it is also a result of the multiple binding sites of avidin for biotin (1, 11). Second, sensitivity was increased by using a prolonged serum incubation period (overnight at 37°C). In a previous study (2) in which unfractionated sera were used, the incubation time was 3 h at room temperature. Third, rheumatoid factor formation often occurs in NE (7). The interference of rheumatoid factors leads to nonspecific IgM positivity in the IFAT in the presence of specific IgG antibodies. This interference was eliminated in the present study by preadsorption of sera with latex RF reagent. Fourth, as expected, repeated freeze-thaws degraded the anti-KHF/NE IgM antibodies. The lack of demonstrable specific IgM in four of our patients could possibly have been due to such a process since all their serum samples had been freeze-thawed several times.

We found no specific anti-KHF/NE IgM antibodies in any of the 49 control serum samples, which indicates a high specificity for the IFAT-biotin-avidin system method. This has also been documented previously (1, 11).

In conclusion, the biotin-avidin-amplified immunofluorescence technique is a sensitive and reliable method for the detection of specific serum IgM against KHF/NE, provided serum samples are not repeatedly freeze-thawed.

This work was supported by grants from the Swedish Medical Research Council (MFR-6560) and the Swedish National Defense Research Institute (FOA-4-83-942).

We are grateful for the excellent technical assistance of Kristina Lindman and Lena Rova. The secretarial assistance of Katrine Isaksson is also gratefully acknowledged.

LITERATURE CITED

1. Berman, J. W., and R. S. Basch. 1980. Amplification of the biotin-avidin immunofluorescence technique. *J. Immunol. Methods* **36**:335-338.
2. Brummer-Korvenkontio, M., A. Vaheri, T. Hovi, C.-H. von Bonsdorff, J. Vuorimies, T. Manni, K. Penttinen, N. Oker-Blom, and J. Lähdevirta. 1980. Nephropathia epidemica: detection of antigen in bank voles and serologic diagnosis of human infection. *J. Infect. Dis.* **141**:131-134.
3. French, G. R., R. S. Foulke, O. A. Brand, G. A. Eddy, H. W. Lee, and P. W. Lee. 1981. Korean hemorrhagic fever: propagation of the etiologic agent in a cell line of human origin. *Science* **211**:1046-1048.
4. Friman, G., G. R. French, L. Hambræus, W. R. Beisel, and N.-G. Ilbäck. 1981. Serum antibodies reactant with Korean hemorrhagic fever agent in Scandinavian epidemic (endemic) nephropathy (nephropathia epidemica) demonstrated by immunofluorescence utilizing an in vitro antigen source. *Scand. J. Infect. Dis.* **13**:89-93.
5. Lee, H. W., P. W. Lee, and K. Johnson. 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* **137**:298-308.
6. Lee, H. W., P. W. Lee, J. Lähdevirta, and M. Brummer-Korvenkontio. 1979. Aetiological relation between Korean hemorrhagic fever and nephropathia epidemica. *Lancet* **i**:186-187.
7. Penttinen, K., J. Lähdevirta, R. Kekomäki, B. Ziola, A. Salmi, A. Hautanen, P. Lindström, A. Vaheri, M. Brummer-Korvenkontio, and O. Wager. 1981. Circulating immune complexes, immunocglutinins and rheumatoid factors in nephropathia epidemica. *J. Infect. Dis.* **143**:15-21.
8. Settergren, B., S. R. Norrby, U. Näslund, and G. Wadell. 1983. Case of epidemic nephropathy associated with disseminated intravascular coagulation. *Lancet* **ii**:1419.
9. Svedmyr, A., P. W. Lee, D. C. Gajdusek, C. J. Gibbs, and K. Nyström. 1980. Antigenic differentiation of the viruses causing Korean hemorrhagic fever and epidemic (endemic) nephropathy of Scandinavia. *Lancet* **ii**:315-316.
10. Traavik, T., R. Mehl, B. P. Berdal, S. Lund, and J. M. Dalrymple. 1983. Nephropathia epidemica in Norway: description of serological response in human disease and implication of rodent reservoirs. *Scand. J. Infect. Dis.* **15**:11-16.
11. Wilchek, M., and E. A. Bayer. 1984. The biotin-avidin complex in immunology. *Immunol. Today* **5**:39-43.