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Immunoglobulin G Subclass Responses against the Structural Components of Puumala Virus

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Sera from sequentially bled nephropathia epidemica patients (acute-phase, convalescent-phase, and 2-year sera) and sera from 10 to 20 years postinfection were examined by immunoglobulin G (IgG) subclass-specific ELISAs for reactivities against each of the Puumala virus structural proteins (N, G1, and G2). IgG1 was found to be the dominating antiviral subclass, and most of the patients had IgG1 directed to all three structural proteins at all times with a continuous increase over time. IgG2 to the three viral proteins was detected in very low amounts in only a few of the samples. All of the serially bled patients developed IgG3 against each of the proteins, but the responses against the different viral components varied; anti-N- and anti-G1-specific IgG3 showed a peak in the convalescent-phase samples, while the anti-G2 IgG3 response was highest in the acute-phase sera. Eight of ten serially bled patients developed specific IgG4. The IgG4 responses showed similar patterns against all three viral proteins with very low optical density values in the acute- and convalescent-phase samples, followed by higher optical densities in the sera drawn 2 years after infection. Seven of nine 10-year sera contained virus-specific IgG4. These results support the theory concerning the persistence of Puumala virus or viral antigens several years after infection.

Puumala (PUU) virus is one of several serologically distinct members of the genus *Hantavirus*, family *Bunyaviridae* (16, 24). PUU virus or closely related virus strains are the etiologic agents of nephropathia epidemica (NE), an illness that occurs in Scandinavia, Finland, western Russia, and central Europe (3, 7, 19). NE belongs to a group of diseases commonly known as hemorrhagic fever with renal syndrome, characterized by fever, renal dysfunction, and, in severe cases, major hemorrhagic manifestations (18, 24). In recent years several fatal cases of NE have also been documented (9).

PUU virus was originally isolated in 1983 (13, 25). The PUU virion consists of four structural proteins: a large protein (L), two envelope glycoproteins (G1 and G2), and a nucleocapsid protein (N) (4). Both glycoproteins have been shown to express epitopes recognized as targets for neutralizing antibodies (12).

High levels of immunoglobulin G (IgG) antibodies against PUU virus in convalescent-phase and very late convalescent-phase sera have been described by several authors (2, 3, 14, 17). In addition, it has been shown that sera drawn 10 to 20 years after PUU virus infection contained very high titers of neutralizing antibodies (5). It has accordingly been suggested that virus or viral antigens might persist after the acute disease, giving continuous stimulation of the immune system (14).

In a previous study, enzyme-linked immunoassays (ELISAs) for detection of the IgG and IgM responses against the individual structural proteins of PUU virus were designed and evaluated (11). It was found that the development of IgG antibodies to the different viral proteins during the acute disease and convalescence followed different patterns for N, G1, and G2. The IgG antibody response initially showed a higher rate of increase to N than to the glycoproteins. This was followed by a period with the opposite pattern, in which the anti-glycoprotein antibodies showed a higher increase, reaching very high levels in the 2-year specimens.

Previous studies have shown that different patterns of the IgG subclass responses are seen in persistent viral infections compared with transient viral infections (8). The possibility of studying antibodies specific for the individual structural proteins of the PUU virion and the question of whether antigen persists in the body prompted us to follow the development of IgG subclasses 1 to 4 in NE patients. Sequentially drawn sera and sera from 10 to 20 years postinfection were examined.

MATERIALS AND METHODS

Sera. Three groups of sera were analyzed. (i) Twenty-nine sequentially drawn serum samples from 10 patients hospitalized for NE, previously serologically confirmed by IgM ELISA, were included (14). Nine of the 10 patients each contributed three serum samples: one sample drawn 1 to 13 days after onset of illness (acute-phase sample); one sample drawn 16 to 41 days after onset of illness (convalescent-phase sample); and one sample drawn 2 years after onset of illness (2-year sample). From one of the patients only a convalescent-phase sample and a 2-year sample were available.

(ii) Single serum specimens from 16 patients with serologically confirmed PUU virus infection were also available. Four of these samples were acute-phase samples and three samples were convalescent samples, as defined above. The remaining nine serum samples were drawn 10 to 20 years after onset of illness (10-year samples; kindly provided by Kurt Nyström, Umeå Hospital).

(iii) Twenty-four serum samples from a normal population in an area of Sweden where NE is not endemic, previously

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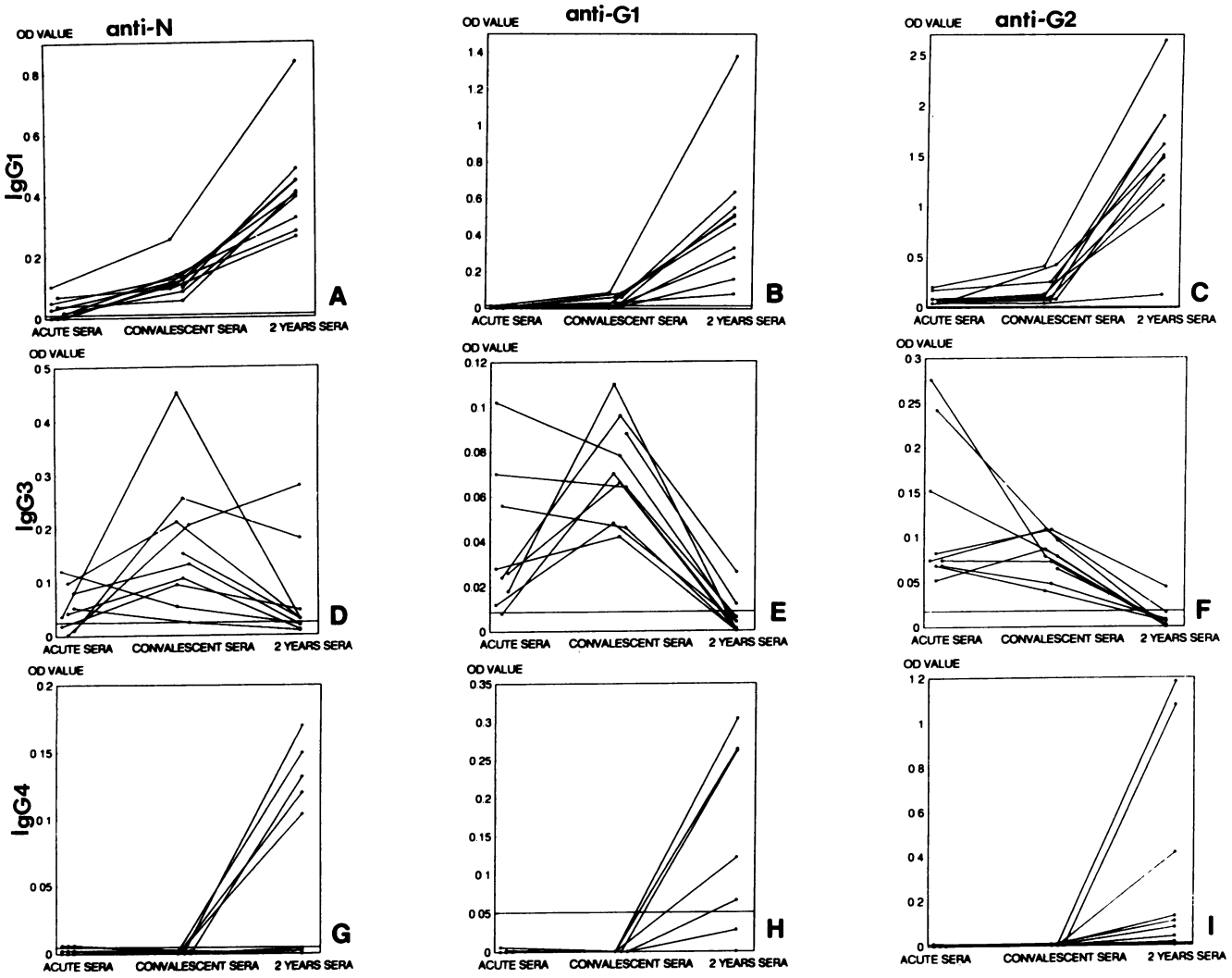

FIG. 1. Acute-phase, convalescent-phase, and 2-year sera from 10 NE patients were examined for IgG subclass specificities. (A to C) IgG1 responses; (D to F) IgG3 responses; (G to I) IgG4 responses; (A, D, and G) anti-N responses; (B, E, and H) anti-G1 responses; (C, F, and I) anti-G2 responses. Calculated cutoff points are indicated by horizontal lines.

shown to be negative for antibodies to PUU virus, were used to estimate cutoff levels of the assays.

Reagents for ELISA. Antigen preparations (detergent-treated cell lysates of strain 83-223L-infected Vero E6 cells) containing the structural proteins of PUU virus, N, G1, and G2, were prepared as described elsewhere (11). 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 (10, 12). Biotin-labelled mouse MAbs (Zymed, San Francisco, Calif.) were used for detection of the human IgG subclasses, followed by alkaline phosphatase-conjugated extravidin (extravidin-ALP; Sigma, St. Louis, Mo.). The MAbs used were as follows: anti-IgG1, clone HP6069; anti-IgG2, clone HP6002; anti-IgG3, clone HP6047; and anti-IgG4, clone HP6025 (15). To determine optimal dilutions of the reagents, microtiter plates (Costar, Cambridge, Mass.) were coated with purified human myeloma IgG1, IgG2, IgG3, and IgG4 (Sigma). Serial twofold dilutions of subclass-specific MAbs and extravidin-ALP were added in a chessboard manner. Optimal dilutions were found to be 1/1,000 for anti-IgG1 and anti-IgG2, 1/3,000 for anti-IgG3 and anti-IgG4, and 1/10,000 for extravidin-ALP.

ELISAs for detection of IgG subclasses against the different structural proteins of PUU virus. Three series of ELISAs were employed, one for each structural protein of the PUU 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 μg of MAb per ml in coating buffer (0.05 M sodium carbonate, pH 9.6) and adsorbed to 96-well microtiter plates (Costar) at 4°C overnight. Nonsaturated binding sites were blocked by 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4°C overnight. All of the following reagents were diluted in ELISA buffer (PBS with 0.05% Tween 20 and 0.5% BSA), and the plates were washed five times with 0.9% NaCl–0.05% Tween 20 for each step. Viral antigen and negative control antigen (ELISA buffer) were incubated for 1 h at 37°C. Serum samples diluted 1/100 were added in duplicate to wells with antigen and to wells with negative

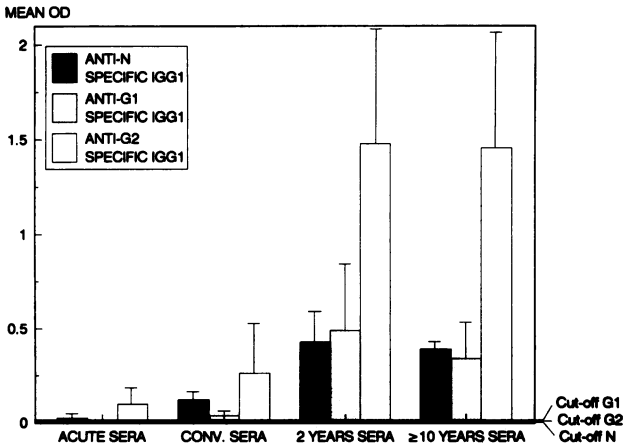

FIG. 2. Acute-phase, convalescent-phase, and 2-year sera from 17 NE patients together with sera from 9 patients drawn 10 to 20 years post-NE infection were examined for IgG1 antibodies directed to N, G1, and G2. The bars express the mean ODs at different intervals post-onset of disease. Calculated cutoff points are indicated by lines. Standard errors of the mean are indicated by bars.

control antigen and incubated for 1 h at 37°C, followed by biotin-labeled MAbs specific for each of the four subclasses of human IgG for 1.5 h at 37°C. Extravidin-ALP was incubated for 1 h at 37°C, followed by *p*-nitrophenyl phosphate (Sigma) in 10% diethanolamine. The plates were read when the positive serum control reached an optical density (OD) value of approximately 2 (ca. 60 min).

Two kinds of positive controls were used. (i) One row of each plate was coated with human myeloma IgG1, IgG2, IgG3, or IgG4. No antigen or serum was added to this row, only biotin-labelled IgG subclass-specific MAbs and extravidin-ALP. This control assured that the two latter reagents functioned properly. (ii) One convalescent-phase NE serum sample with a high titer of anti-PUU virus-specific IgG was used to indicate that MAb coating and antigen binding functioned properly. Alkaline phosphatase-labelled goat antibodies to human IgG (γ-chain specific; Sigma), diluted 1/2,000, were used to detect specific total IgG in this serum. The control was used as an internal standard on all plates. The mean OD value of the standard duplicate was recalculated to 2,000 on each plate, and the mean values of the serum samples were then adjusted correspondingly. The results were calculated as the mean OD value of the duplicate serum samples with virus antigen, reduced by the mean value obtained with negative control antigen.

RESULTS

Patterns of IgG subclass responses. (i) IgG1. All the serially bled patients developed IgG1 against all three structural proteins (Fig. 1A to C). The development of IgG1-specific antibodies (as measured by mean ODs) to the three different viral proteins showed similar patterns (Fig. 2). The lowest ODs were found for the acute-phase sera; higher ODs were found for the convalescent-phase sera, and the highest ODs were found for the 2-year and 10-year sera. The rate of increase in mean OD values between convalescent-phase sera and 2-year sera was greater for anti-G1- and anti-G2- than for anti-N-specific IgG1.

(ii) IgG2. Most serum samples were negative for PUU virus-specific IgG2. Eight of 26 patients developed low levels of N-specific IgG2, six patients were weakly positive against G1, and only four patients had detectable levels against G2. None of the positive sera had ODs of more than 0.070.

(iii) IgG3. All of the serially bled patients developed IgG3 against all three structural proteins (Fig. 1D to F).

As can be seen in Fig. 3, the development of antibodies to N and G1 showed a pattern different from that of G2-specific IgG3. The mean ODs for N and G1 were highest for the convalescent-phase sera and low to very low in the acute-phase, 2-year, and 10-year serum groups. The mean ODs for G2-specific IgG3 were highest for the acute-phase sera, somewhat lower for the convalescent-phase sera, and very low for the 2-year and 10-year sera.

(iv) IgG4. Not all of the serially bled patients developed IgG4 (Fig. 1G to I). Five out of 10 patients developed antibodies to N and G1, while eight of 10 developed G2-specific IgG4. The results showed a similar pattern for all three viral proteins (Fig. 4). The highest mean OD was found in the 2-year sera. Seven of nine specimens collected 10 to 20 years postinfection were also positive, but with lower mean ODs than those of 2-year specimens. In contrast, only a few of the acute-phase and convalescent-phase samples were found positive, and these specimens had very low OD values.

(v) Negative sera. The mean ODs of the 24 negative sera were calculated separately for each viral protein and IgG subclass. The mean ODs (+3 standard deviations) were used as cutoff values. The cutoff values of the 12 different assays, indicated in the figures, varied between 0.003 and 0.051.

DISCUSSION

This paper describes, for the first time for any hantavirus, the development and specificities of the IgG subclasses during and after PUU virus infection in humans. The use of bank vole MAbs for indirect binding of the different viral components to the solid phase, and the use of biotinylated mouse MAbs for specific detection of the IgG subclasses, proved a convenient technique for determination of quantitative variations of antibody reactivity (i.e., OD variations). The method excluded purification or labelling steps of each

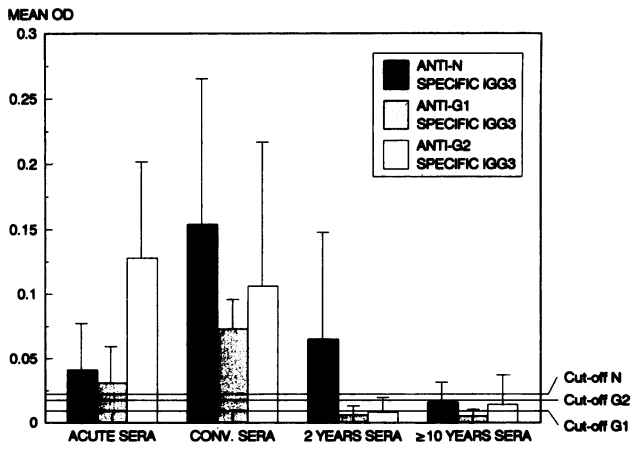

FIG. 3. Acute-phase, convalescent-phase, and 2-year sera from 17 NE patients together with sera from 9 patients drawn 10 to 20 years post-NE infection were examined for IgG3 antibodies directed to N, G1, and G2. The bars express the mean ODs at different intervals post-onset of disease. Calculated cutoff points are indicated by lines. Standard errors of the mean are indicated by bars.

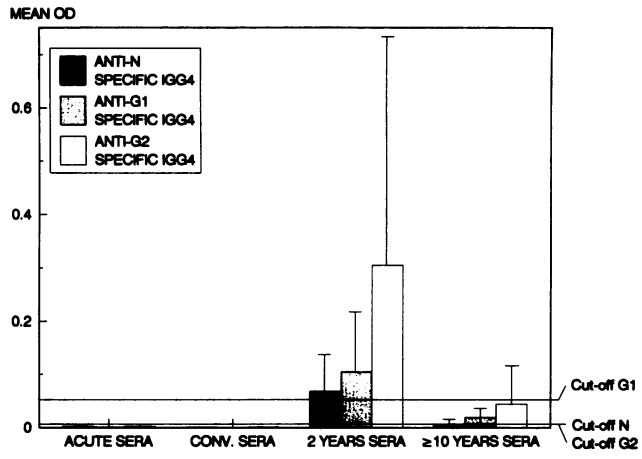

FIG. 4. Acute-phase, convalescent-phase, and 2-year sera from 17 NE patients together with sera from 9 patients drawn 10 to 20 years post-NE infection were examined for IgG4 antibodies directed to N, G1, and G2. The bars express the mean ODs at different intervals post-onset of disease. Calculated cutoff points are indicated by lines. Standard errors of the mean are indicated by bars.

viral protein, thus circumventing the difficulties associated with the poor growth of PUU virus in cell culture.

It has previously been shown that IgG1 and IgG3 are the main IgG subclasses found during viral infections (8, 20). The present study showed that this is true also for NE. All of the serially bled patients developed antibodies of the IgG1 and IgG3 subclasses against all three structural proteins. The patterns of the IgG1 responses were similar to the findings for total IgG in a previous study (11). This is not surprising, since IgG1 constitutes approximately 65% of the total IgG in normal serum and the polyclonal conjugate used in the assays for detection of total IgG was directed mainly to IgG1.

IgG2 antibodies are known to be of limited importance in several viral infections (8). Our data are in line with this finding, since IgG2 directed against the structural proteins of PUU virus was detected only in a few cases and none of the positive samples gave high ODs. Wagner and coworkers (23) reported high titers of IgG2 directed against the G glycoprotein, but not against the F glycoprotein, of respiratory syncytial virus. The results were explained by the main reactivity of IgG2 with carbohydrate moities and the high content of O-linked carbohydrates in the G glycoprotein of respiratory syncytial virus. However, 10 of 45 serum samples examined in the present study had low but significant levels of IgG2 directed to N, a nonglycosylated protein.

The patterns of the IgG3 responses against the different viral components were reminiscent of the findings for IgM in a previous study (11). However, our finding of IgG3 in several of the 2-year and 10-year samples is notable because of the suggested role of IgG3 as a marker for continuous antigenic stimulation (8, 22).

The results concerning IgG4 are most interesting. IgG4 responses to other viruses are not known to be associated with any specific pattern (6, 23), although repeated antigenic stimulation is thought to result in a more IgG4-restricted response (1, 21). The detection of specific IgG4 in several NE convalescent-phase serum samples, most prominent in the 2-year samples but also found in seven of nine samples drawn >10 years after infection, suggests persistence and/or reactivation of PUU virus or viral antigens several years after the infection. Whether the proposed persistence has any biological or medical significance remains to be elucidated.

REFERENCES

1. **Aalberse, R. C., R. Van der Gaag, and J. Leeuwen.** 1983. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. J. Immunol. **130:** 722–726.
2. **Brummer-Korvenkontio, M., H. Henttonen, and A. Vaheri.** 1982. Hemorrhagic fever with renal syndrome in Finland: ecology and virology of nephropathia epidemica. Scand. J. Infect. Dis. Suppl. **36:**88–91.
3. **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 serological diagnosis of human infection. J. Infect. Dis. **141:**131–134.
4. **Elliott, R. M.** 1990. Molecular biology of the Bunyaviridae. J. Gen. Virol. **71:**501–522.
5. **Hörling, J., Å. Lundkvist, and B. Niklasson.** 1992. Antibodies to Puumala virus in humans determined by neutralization test. J. Virol. Methods **39:**139–147.
6. **Khalife, J., B. Guy, M. Capron, M.-P. Kieny, J.-C. Ameisen, L. Montagnier, J.-P. Lecocq, and A. Capron.** 1988. Isotypic restriction of the antibody response to human immunodeficiency virus. AIDS Res. Hum. Retroviruses **1:**3–9.
7. **Lähdevirta, J.** 1971. Nephropathia epidemica in Finland. A clinical, histological and epidemiological study. Ann. Clin. Res. 3, suppl. 8.
8. **Linde, A., V.-A. Sundqvist, T. Mathiesen, and B. Wahren.** 1988. IgG subclasses to subviral components. Monogr. Allergy **23:**27–32.
9. **Linderholm, M., B. Settergren, C. Ahlm, L.-Å. Burman, S. Träff, U. Bäcklund, and P. Juto.** 1991. A Swedish fatal case of nephropathia epidemica. Scand. J. Infect. Dis. **23:**501–502.
10. **Lundkvist, Å., A. Fatouros, and B. Niklasson.** 1991. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. J. Gen. Virol. **72:**2097–2103.
11. **Lundkvist, Å., J. Hörling, and B. Niklasson.** The humoral response to Puumala virus infection (nephropathia epidemica) investigated by viral protein specific immunoassays. Arch. Virol., in press.
12. **Lundkvist, Å., and B. Niklasson.** 1992. Bank vole monoclonal antibodies against Puumala virus envelope glycoproteins; identification of epitopes involved in neutralization. Arch. Virol. **126:**93–105.
13. **Niklasson, B., and J. LeDuc.** 1984. Isolation of the nephropathia epidemica agent in Sweden. Lancet **i:**1012–1013.
14. **Niklasson, B., E. Tkachenko, A. P. Ivanov, G. Van der Groen, D. Wiger, H. K. Andersen, J. LeDuc, T. Kjellson, and K. Nyström.** 1990. Haemorrhagic fever with renal syndrome: evaluation of ELISA for detection of Puumala-virus-specific IgG and IgM. Res. Virol. **141:**637–648.
15. **Sällberg, M., H. Norder, O. Weiland, and L. Magnius.** 1989. Immunoglobulin isotypes of anti-HBc and anti-HBe and hepatitis B virus (HBV) DNA elimination in acute hepatitis B. J. Med. Virol. **29:**296–302.
16. **Schmaljohn, C. S., S. E. Hasty, J. M. Dalrymple, J. W. Leduc, H. W. Lee, C. H. Von Bonsdorff, M. Brummer-Korvenkontio, A. Vaheri, T. F. Tsai, H. L. Regnery, D. Goldgaber, and P. W. Lee.** 1985. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science **227:**1041–1044.
17. **Settergren, B., C. Ahlm, P. Juto, and B. Niklasson.** 1991. Specific Puumala virus IgG half a century after haemorrhagic fever with renal syndrome. Lancet **338:**66.
18. **Settergren, B., P. Juto, B. Trollfors, G. Wadell, and S. R. Norrby.** 1988. Hemorrhagic complications and other clinical findings in nephropathia epidemica in Sweden: a study of 355 serologically verified cases. J. Infect. Dis. **157:**380–382.
19. **Sheshberadaran, H., B. Niklasson, and E. A. Tkachenko.** 1988.

Antigenic relationship between hantaviruses analysed by immunoprecipitation. J. Gen. Virol. **69**:2645–2651.
20. **Skvaril, F.** 1986. IgG subclasses in viral infections. Monogr. Allergy **19**:134–143.
21. **Sundqvist, V.-A., A. Linde, and B. Wahren.** 1984. Virus-specific immunoglobulin G subclasses in herpes simplex and varicella-zoster infections. J. Clin. Microbiol. **20**:94–98.
22. **van Loon, A. M., F. W. A. Heessen, and J. T. M. Van der Logt.** 1987. Antibody isotype response after human cytomegalovirus infection. J. Virol. Methods **15**:101–107.
23. **Wagner, D. K., D. L. Nelson, E. E. Walsh, C. B. Reimer, F. W. Henderson, and B. R. Murphy.** 1987. Differential immunoglobulin G subclass antibody titers to respiratory syncytial virus F and G glycoproteins in adults. J. Clin. Microbiol. **25**:748–750.
24. **Yanagihara, R., and D. C. Gajdusek.** 1988. Hemorrhagic fever with renal syndrome: a historical perspective and review of recent advances, p. 151–188. *In* J. H. S. Gear (ed.), CRC handbook on viral and rickettsial hemorrhagic fevers. CRC Press, Boca Raton, Fla.
25. **Yanagihara, R., D. Goldgaber, P. W. Lee, H. L. Amyx, D. C. Gajdusek, C. J. Gibbs, and A. Svedmyr.** 1984. Propagation of nephropathia epidemica virus in cell culture. Lancet **i**:1013.