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THE USE OF RED CELLS WITH FUSED SEMLIKI FOREST VIRUS ENVELOPE PROTEINS IN ANTIBODY DETERMINATIONS BY HEMOLYSIS IN GEL

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Chicken red blood cells with fused Semliki Forest virus (SFV) proteins on the cell membrane were used in the hemolysis-in-gel (HIG) plates. Optimally the plate contained a 1.5 mm thick gel of 1% agarose with 1% red cells and 1 unit/ml gel of complement. 400 ng of SFV was fused to the red cells in 1 ml of the gel (about 20 virions fused to one red cell). Five μl of inactivated (56°C, 30 min) serum samples were pipetted into wells in the gel. After 20 h of incubation at 37°C the diameters of the hemolytic zones were directly proportional to the logarithm of the serum dilution. This made it possible to calculate the antibody titers for the samples using an experimental formula $T = 10^{\phi/k}$ (T is the titer, ϕ the diameter in mm and k a variable coefficient, which had to be determined for each batch of the plates using a standard serum). Using regression and residual analyses, the formula was shown to fit the experimental results. The fusion-based HIG could be read after as early as 2 h of incubation. The specificity of the test was studied using antisera against Western equine encephalomyelitis, Eastern equine encephalomyelitis, Chikungunya and Uruma viruses, which all gave negative results in the SFV HIG test. Antisera against SFV E1 and E2 proteins were positive, but anti-E3 serum was negative when measured in the SFV HIG test.

hemolysis in gel Semliki Forest virus

INTRODUCTION

Semliki Forest virus (SFV) can hemolyse and fuse red blood cells (Väänänen and Kääriäinen, 1979, 1980). These phenomena result from the fusion of the viral envelope with the red cell membrane (Väänänen et al., 1981). Red cells with fused SFV envelope proteins in their membrane have been used for antibody determinations by complement-dependent hemolysis and radioimmunoassay (Väänänen et al., 1981). It was thus tempting to study their use also in the hemolysis-in-gel test.

The hemolysis-in-gel (HIG) test was first used for antibody measurements by Schild et al. (1975). Since then the method has been used to measure antibodies against other viruses such as mumps (Väänänen et al., 1976), parainfluenza 3 (Probert and Russel, 1975), rubella (Väänänen and Vaheri, 1979), corona (Riski et al., 1977) and togaviruses (Gaidamovich et al., 1980). In the examples mentioned, the virus antigen is attached to the red cell membrane by hemagglutination or by coupling with some chemical, such as chromium or periodate. The orientation of the viral antigens on the red cell surface after

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such treatments has not been studied. It is also uncertain how much of the viral antigens are close enough to the red cell membrane to cause the complement-dependent lysis of the cell.

The fusion of the SFV envelope with the red cell membrane causes the envelope protein spikes to become integral parts of the red cell membrane. The orientation of the spike proteins should in this case present the best possible targets for the complement-mediated immune hemolysis reaction.

This report describes the use of red cells with fused SFV envelope proteins for the preparation of HIG plates. Optimal concentrations for the reagents as well as the specificity of the SFV HIG test are described.

MATERIALS AND METHODS

Viruses

Cultivation and purification of SFV and ^{35}S-labeled SFV were performed as previously described (Väänänen and Kääriäinen, 1979, 1980).

The unlabeled SFV and the radioactive SFV were mixed before each experiment. Protein determinations (Lowry et al., 1951) and radioactive measurements were carried out from this mixture. The radioactivity per μg protein varied from mixture to mixture with a range from 14,000 c.p.m. to 23,000 c.p.m. per μg protein.

Red cells with fused SFV envelope proteins

Fusion of SFV with red cell membranes was performed as previously described (Väänänen et al., 1981). Briefly: purified SFV was adsorbed to red cells from one-day-old chickens in a 20% suspension at pH 5.8 and 0°C for 10 min. The cells were washed with phosphate-buffered saline (PBS, pH 7.2), supplemented with 0.5% bovine serum albumin (BSA), and resuspended in the same buffer to a concentration of 0.3%. The virus was fused to the red cells by incubating the suspension in a water bath at 37°C for 30 min, after which the cells were washed with the same buffer, resuspended in the buffer to a 10% suspension and immediately used for preparing the HIG plates. The amount of fused SFV protein was monitored by counting the radioactivity in the red cells. The cell counts were carried out manually in a Bürker cell counting chamber.

Antisera

Antisera against SFV and Sindbis virus envelope protein complexes were raised in rabbits as previously described (Saraste et al., 1980). Antisera against the purified SFV envelope proteins (Kalkkinen, 1980) were raised by injecting rabbits first with 150 μg of E1, 200 μg of E2, and 46 μg of E3 in complete Freund's adjuvant subcutaneously in the neck and hip regions. The immunization was continued by injecting four times at one

month intervals with half of the amount of the antigens. Blood was collected from the ear veins and the antibody titrations were carried out by radioimmunoprecipitation (Väänänen et al., 1981).

The specific rabbit antisera against Eastern equine encephalomyelitis, Western equine encephalomyelitis, Chikungunya (two strains) and Uruma viruses were kindly supplied by Dr. J. Porterfield.

Preparation of the HIG plates

Red cells with fused SFV envelope proteins as a 10% suspension in Dulbecco's PBS (Dulbecco and Vogt, 1954) supplemented with 0.2% BSA were warmed to 47°C. Guinea pig serum was added to give a final complement concentration in the gel of 1 unit/ml (the titer of the complement was 50 units/ml). Seven volumes of 1% agarose (Indubiose A 37, Industrie Biologique Française) in PBS at 47°C was added and the mixture was poured on small polystyrene dishes to form a 1.5 mm thick layer. Wells with a diameter of 2.2 mm were punched in the solidified gel.

Performance of the HIG test

The sera were heat-inactivated at 56°C for 30 min, diluted in Dulbecco's PBS with 0.2% BSA, if needed, and pipetted in a 5 μl volume into the wells of the HIG plates. The plates were tightly covered, and after an incubation period of 20 h at 37°C the diameters of the hemolytic zones were measured with an ordinary mm ruler. For graphic illustrations the diameters were plotted against the logarithm of the corresponding serum dilution.

RESULTS

When the HIG plates were prepared as described in the Materials and Methods, and inactivated serum samples were incubated in the plates at 37°C for 20 h, the result shown in Fig. 1 was obtained. The diameters of the hemolytic zones were easy to measure. In the following experiments the result was considered positive if the zone was completely hemolysed. The result was not accepted if the hemolysis was incomplete or the edges of the zone were too diffuse for accurate measurement. A negative result was read only if even a faint zone of hemolysis was not obtained.

The diameters of the hemolytic zones were measured with an accuracy of 0.5 mm. The results from the same sample when measured in different plates with the same antigen concentration varied within 1 mm.

The optimal concentration of complement in the HIG plate was found to be 1 unit/ml. In other words, guinea pig serum with a titer of 50 units/ml in the conventional CF titration against the buffer, had to be added to the gel in a final dilution of 1 in 50. A 2 units/ml concentration of complement caused hemolysis in the whole plate within 1–2

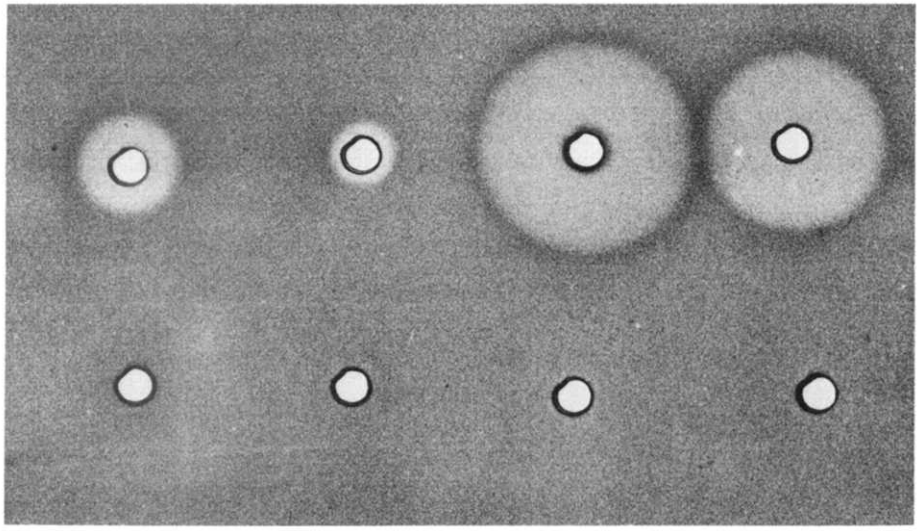

Fig. 1. An example of the hemolytic zones in a SFV HIG plate. Top row: positive sera with HIG titers 32, 10, 4217 and 100. Bottom row: negative sera.

days, whereas half a unit was insufficient to cause complete hemolysis within the hemolytic zones.

The effect of red cell concentration

The effect of red cell concentration was studied using an antigen concentration of 3.5 $\mu g/10^6$ cells. The cells were diluted to give a final concentration in the gel of 0.5%, 1% and 2%. There was no great difference in the hemolytic zones obtained with 0.5% as compared with those obtained with 2% red cells. The difference was about 2 mm for all serum dilutions.

The lowest concentration of the red cells (0.5%) gave very pale plates and the hemolytic zones were difficult to measure. On the other hand, 2% red cells gave a dark red plate and good contrast, but the hemolytic zones were not totally transparent, possibly due to the large amount of red cell stroma in the gel. A red cell concentration of 1% in the gel gave a good background and sharp edged, transparent hemolytic zones, and was used for the subsequent studies.

The effect of the antigen concentration on the red cells

[^{35}S]Methionine-labeled SFV was fused to one day old chicken red cells (see Materials and Methods) in concentrations of 1, 2 and 5 ng/10^6 cells. Dilutions of heat-inactivated rabbit antiserum to SFV were incubated in the plates at 37°C for 20 h (Fig. 2).

With diluted serum the lower antigen concentrations gave larger zones of hemolysis.

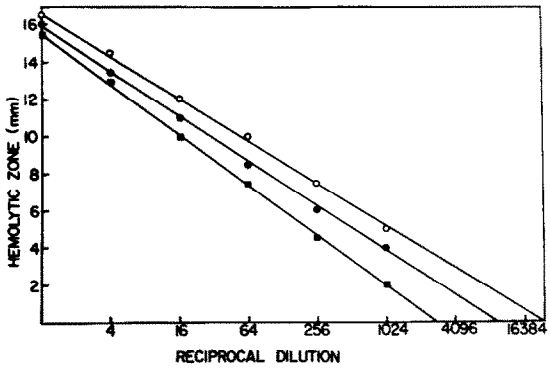

Fig. 2. The effect of antigen concentration in the red cells on the size of the hemolytic zone. The lines are regression lines drawn according to the mean diameters of five series of experiments with each antigen concentration: o——o, 1 ng; •——•, 2 ng; and ▪——▪, 5 ng/10^6 red cells.

However, antigen concentrations below 1 ng/10^6 cells always resulted in incomplete zones of hemolysis. All the antigen concentrations tested resulted in a straight line when the diameter (mm) was plotted against the logarithm of the serum dilutions (Fig. 2). A concentration of 2 ng/10^6 cells was chosen for use in the HIG plates.

The linear relationship obtained made it possible to determine the antibody titers of the samples. The lines in Fig. 2 are regression lines from five different test series with each antigen concentration. Residual analysis of the individual test results showed that they followed the normal distribution in relation to the regression line. The formula of the line is $y = \phi - k \log 1/D$ (ϕ corresponding to the diameter in mm, D the dilution and k the slope of the line). The titer of a sample was determined to be the reciprocal of the extrapolated dilution corresponding to a zone diameter of 0 mm (the intersection of the straight line with the x-axis). Solving the equation mentioned above, the titer of a sample could be calculated as: $T = 1/D = 10^{\phi/k}$.

The coefficient k was dependent on the antigen concentration (Fig. 2), but it was also affected by the condition of the red cells. Some batches gave a different slope for the ϕ versus log D line (data not shown). Therefore the value for k had to be determined for each batch of the plates using a line drawn through the results of three dilutions. The value of k for the calculation of the titers in Tables 1 and 2 was determined for the straight line of the 2 ng/10^6 red cells in Fig. 2 to be 4 as follows: the value for k is in principle y/x. For the straight line mentioned, at the intersection with y-axis $y = \phi = 16$, and at the intersection with x-axis $x = \log 1/D = \log 10{,}000 = 4$, and thus $k = y/x = 4$.

The effect of the serum diluent was studied using either normal rabbit serum or PBS + 0.2% BSA as a diluent. The only difference between these two diluents was that the normal serum seemed to inhibit the formation of hemolytic zones in the lowest antibody concentrations.

The effect of incubation time

HIG plates with two antigen concentrations (2 ng and 5 ng/10^6 cells) were incubated with serial dilutions of anti-SFV serum at 37°C (Fig. 3). The hemolytic zones were measured at different times. After 2 h of incubation the hemolytic zones were clear and measurable in all serum dilutions. The diameter of the hemolytic zone increased with a slightly diminishing rate until it reached the maximal size in about 26 h. There was no significant difference in the diameters of the hemolytic zones between the two antigen concentrations during the first 6 h (Fig. 3). The diameters at that time were already about half the maximal size, suggesting that shorter incubation times were adequate for routine tests.

The specificity and sensitivity of the SFV HIG test

The specificity of the new SFV HIG was studied using specific antisera against two closely related viruses – Vereeniging and TH35 viruses, two strains of Chikungunya virus (Porterfield, 1961), as well as with antisera against representatives from other subgroups of alphaviruses viz. Eastern equine encephalomyelitis, Western equine encephalomyelitis and Uruma viruses. Hyperimmune serum against Sindbis virus was also tested. None of the antisera gave hemolytic zones, suggesting a highly specific reaction of the fused SFV proteins in the HIG plate test.

The sensitivity of the HIG test for measuring antibodies against SFV was studied by

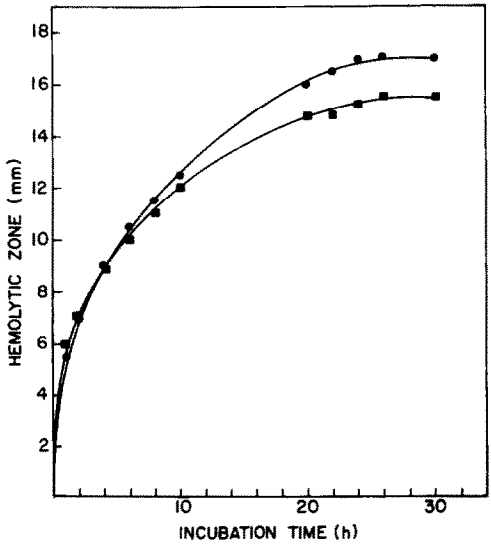

Fig. 3. The effect of the incubation time on the size of the hemolytic zone: ●——●, 2 ng; ■——■, 5 ng/10^6 red cells.

TABLE 1

The HIG titers of antisera against SFV proteins and Sindbis virus as compared to results obtained with other techniques

Antiserum	Homologous HIG titer[a]	HI titer	IP50 titer[b]
Anti-SFV (K3)	5623	320	20000
Anti-Sindbis	0	320 (160)[c]	2000[c]
Anti-SFV E1	316	<20	640
Anti-SFV E2	1000	20	640
Anti-SFV E3	0	20	5120
Normal rabbit	0	<5	<10

[a] HIG titer calculated from $T = 10^{\phi/k}, k = 4$.
[b] Immunoprecipitation titers (IP50) as described by Väänänen et al. (1981).
[c] SFV as the antigen.

TABLE 2

Positive results from an immunity survey among individuals who have been working with SFV

Serum	HIG titer[a]	HI titer	HLI titer[b]
1 I	18	5	80
II	32	NT[c]	NT
2 I	56	10	32
II	56	NT	NT
3 I	6	<5	<10
II	18	5	NT
4 I	10	<5	14
II	10	NT	NT
5	42	10	NT
6	42	10	64
7	32	10	90
8	32	5	32
Anti-SFV (K4)	5623	1280	6654
Normal rabbit	0	<5	<10

[a] HIG titer calculated from $T = 10^{\phi/k}$ with $k = 4$.
[b] Hemolysis inhibition (HLI) titer as described by Väänänen and Kääriäinen (1979).
[c] NT = not tested.

comparing the antibody titers obtained for some sera using other techniques (Table 1). The immunoprecipitation titers (IP50) in Table 1 show reactivity of the sera with SFV, even though these sera were apparently negative in the HIG test. The titers obtained in the HIG were remarkably higher than the HI titers. Anti-Sindbis serum and anti-SFV E3 serum were negative in the HIG, although they had a high titer in the immunoprecipitation

test. The very low HI titer of anti-E1 serum was probably due to the fact that the antiserum was raised against the SDS-denatured protein.

The SFV HIG plates were also used for measuring antibodies in sera from 15 individuals who have been working with SFV. From five of them, sera taken three years earlier were also available. Seven of the persons were negative in all tests. In Table 2 the positive HIG results are compared with the HI titers and hemolysis inhibition titers of the sera. The antibody titers obtained were within the detection limit of the HI test, but the HIG test revealed a clearcut difference between the positive and negative results, the smallest measurable diameter of 3 mm corresponding to a titer of 6. In two cases, an increase in titer between the sera taken 3 years apart was recorded. The results demonstrated the practical usefulness of the HIG plate for serological surveys.

DISCUSSION

Fusion of the SFV envelope with the red cell membrane results in a configuration in which the envelope proteins can react with antibodies (Väänänen et al., 1981). This property was utilized in the preparation of the hemolysis in gel (HIG) plates from SFV virus. Conventionally the viral antigens have been attached to the red cells by the agglutination or by fixation with some chemical such as Cr^{3+} or periodate (Schild et al., 1975; Probert and Russel, 1975; Väänänen et al., 1976; Riski et al., 1977; Gaidamovich et al., 1980). The viral envelope proteins are the main target for the antibodies. The conventional method of preparing the HIG plates results in the attachment of the virus particles to the red cell membrane by some of the envelope protein molecules. Most of the envelope proteins are probably too far from the red cell surface to result in complement-mediated lysis of the cell when antibodies are added. When SFV is fused with the red cell membrane, the envelope proteins become integral parts of the membrane (Väänänen et al., 1981). Every envelope protein molecule has thus the possibility of causing cell lysis with the antibodies.

It was shown that the minimum amount of SFV needed in the HIG plate was 1 ng of SFV/10^6 red cells. This corresponds to about 10 virions or 1800 spikes per one red cell (assuming the molecular weight of the SFV to be 56×10^6 according to Garoff et al. (1980) and 180 spikes per virion as communicated personally by Söderlund, 1981). Increasing the antigen concentration resulted in a slight decrease in the diameter of the hemolytic zone (Fig. 2), but the highest functional antigen concentration was not determined.

When the development of the hemolytic zone was followed, it was noticed that after 1 h of incubation at 37°C hemolysis was already visible around the wells and after 2 h all positive samples showed at least a small ring of hemolysis. The relationship between the diameter of the hemolytic zone and the logarithm of the antibody dilution was experimentally found to be linear after an incubation time of 20 h. The straight regression line obtained from the results of five different test series was also tested by residual analysis to determine whether the individual experimental results were in accordance

with the regression line; this was the case, making it possible to solve the equation of the regression line for the extrapolated titer at which the diameter would be zero. This resulted in a formula for the titer $T = 10^{\phi/k}$ for calculating the titers directly from the diameter of the hemolysis zone. The factor k in the formula was dependent on the concentration of the antigen in the plate (Fig. 2), but it was also affected by the condition of the red cells in a manner that was not studied further. The k thus remained as a kind of 'behaviour factor' of the plates which had to be determined for each batch of plates using a line drawn through the results from three different serum dilutions.

Because the antigen concentration had only a minimal effect on the diameter of the hemolytic zone during the first hours of incubation (Fig. 3), it was tempting to read the results after 4 h of incubation. However, the small differences in the diameters made the determination of the titer inaccurate. Thus 20 h was considered to be the optimal incubation time.

In the HI test many alphaviruses cross-react, even rather distantly related viruses such as SFV and Sindbis virus (Clarke and Casals, 1958; Porterfield, 1980; Väänänen et al., 1981). The specificity of the SFV HIG test was studied using antisera against close relatives of SFV as well as against representatives of other subgroups of the alphaviruses such as Eastern equine encephalomyelitis, Western equine encephalomyelitis and Uruma viruses. The antisera against other alphaviruses did not react in the SFV HIG plate, and even antisera against the closest relatives of SFV, the Vereeniging and TH35 strains of Chikungunya virus (Porterfield, 1961), did not cause hemolysis. These results suggest a high degree of specificity of the HIG test, resembling that of the neutralization test (Porterfield, 1961). The sensitivity of the SFV HIG test was greater than that of the HI test (see Tables 1 and 2). The HI test gave results at its technical limit in the serological survey (Table 2), whereas the HIG test easily pointed out the positive sera.

The HLI (hemolysis inhibition) titers of the sera in Table 2 also were higher than their HI titers, suggesting that the HLI test was more sensitive for measuring low-titer antibodies. It is a similar procedure to the HI test, and the sera must be pretreated to remove the non-specific inhibitors. Furthermore, the hemolytic activity of the antigen in the HLI test is difficult to adjust, since it is dependent on handling and purification procedures (Väänänen and Kääriäinen, 1979). The HLI test is therefore more complicated than the HIG test.

The non-specific inhibitors of hemagglutination do not affect the result of antibody antigen reactions on the surface of the red cell (Väänänen et al., 1981). This rendered the HIG test also easier to perform than the HI test in which the removal of the non-specific inhibitors and agglutinins makes the test laborious. The HI test was not even suitable for measuring the antibody titers in antisera against purified SFV envelope proteins, though the sera reacted well in the HIG test and immunoprecipitation (Table 1). The low reactivity of these antisera in the HI test could be due to the fact that the antisera were raised against SDS-denatured proteins, but this did not disturb the reactions in the HIG test. On the other hand, anti-SFV E3 serum having a high immunoprecipitating titer was negative in the HIG test. This may reflect the fact that E3 is a peripheral rather than integral membrane protein (Simons et al., 1978).

The specificity obtained in the use of red cells with fused SFV antigens for the preparation of the HIG plates would suggest the usefulness of this technique with other enveloped viruses also. Cell fusion activity of influenza- and rhabdoviruses (White et al., 1981), paramyxoviruses (Scheid and Choppin, 1974), and coronaviruses (Storz et al., 1981) have already been reported. Experiments for making HIG plates by fusing these viruses with the red cell membrane are in progress.

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REFERENCES

Clarke, D.H. and J. Casals, 1958, Am. J. Trop. Med. Hyg. 7, 561.
Dulbecco, R. and M. Vogt, 1954, J. Exp. Med. 99, 167.
Gaidamovich, S.Y., E.E. Melnikova, P. Väänänen and K. Penttinen, 1980, Arch. Virol. 64, 339.
Garoff, H., A.M. Frischauf, K. Simons, H. Lehrach and H. Delius, 1980, Nature 288, 236.
Kalkkinen, N., 1980, FEBS Lett. 115, 163.
Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, J. Biol. Chem. 193, 265.
Porterfield, J.S., 1961, Bull. W.H.O. 24, 735.
Porterfield, J.S., 1980, in: The Togaviruses. Biology, Structure, Replication, ed. R.W. Schlesinger (Academic Press, New York) p. 13.
Probert, M. and S.M. Russel, 1975, J. Clin. Microbiol. 2, 157.
Riski, H., T. Hovi, P. Väänänen and K. Penttinen, 1977, Scand. J. Infect. Dis. 9, 75.
Saraste, J., C.-H. von Bonsdorff, K. Hashimoto, L. Kääriänen and S. Keränen, 1980, Virology 100, 229.
Scheid, A. and P.W. Choppin, 1974, Virology 57, 475.
Schild, G.C., J.S. Oxford and J.L. Virelizier, 1975, in: Immunity to Influenza, Developments in Biological Standardisation, Vol. 28 (S. Karger, Basel) p. 253.
Simons, K., H. Garoff, A. Helenius and A. Ziemiecki, 1978, in: Frontiers of Physicochemical Biology, ed. B. Pullmann (Academic Press, New York) p. 387.
Storz, J., R. Rott and G. Kaluza, 1981, Infect. Immun. 31, 1214.
Väänänen, P. and L. Kääriäinen, 1979, J. Gen. Virol. 43, 593.
Väänänen, P. and A. Vaheri, 1979, J. Med. Virol. 3, 245.
Väänänen, P. and L. Kääriäinen, 1980, J. Gen. Virol. 46, 467.
Väänänen, P., T. Hovi, E.-P. Helle and K. Penttinen, 1976, Arch. Virol. 52, 91.
Väänänen, P., C.G. Gahmberg and L. Kääriäinen, 1981, Virology 110, 366.
White, J., K. Matlin and A. Helenius, 1981, J. Cell. Biol. (submitted).