A New Quantitative Method for Rabies Virus by Detection of Nucleoprotein in Virion Using ELISA

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ABSTRACT. We have developed a new quantitative method for rabies virus (RV) detection using enzyme-linked immunosorbent assay (ELISA). The method named N-ELISA was based on the quantitation of nucleoprotein (N) in RV virions captured by RV-specific polyclonal antibodies on an ELISA plate. Both infective and defective interfering (DI) particles of RV could be detected by this method. When viruses were propagated in a medium of pH 7.4 adjusted with 7% $NaHCO_3$, N-ELISA could detect them with titers of more than 10^6 pfu/m*l*, though the result did not correlate highly with that of the infectivity assay. The reason for this was considered to be that RVs included spikeless and damaged particles which were produced under conditions of low or high pH. However, in the time course of virus yield, titers of N-ELISA correlated well with those of the infectivity assay.—KEY WORDS: ELISA, G protein, N protein, rabies virus.

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Rabies virus (RV) belongs to the Lyssavirus genus in the family Rhabdoviridae. Quantitative methods of RV detection can be divided into infectivity assays and antigen assays. Examples of the former are plaque assay [2, 15, 24], $TCID_{50}$ method, rapid fluorescent antigen test (RFAT) [1] and immunoperoxidase test [17]. Antigen assays include hemagglutination test [7], hemadsorption test [16], single-radial-immunodiffusion and enzyme-linked immunosorbent assay (ELISA) [5, 13, 18, 20, 23, 25]. ELISA in particular is an easy to use and highly sensitive method.

Several investigators have developed ELISA methods for the quantitation of RV glycoprotein (G) [13, 25]. These methods have been used to test *in vitro* the potency of rabies vaccines, though they are not equivalent to quantitation of the RV virion. The reasons for this might relate to G protein and have been proposed as follows: (1) spikeless particles are produced under conditions of low pH, and a soluble-form of G (Gs) is shed into the medium [4, 11, 19]; (2) conformational changes of G protein are induced by change of pH, temperature or detergents [3, 6].

ELISA methods of RV nucleoprotein (N) detection have been used for quantitation of free-N protein or ribonucleocapsid (RNP) in assays of rabies vaccines [18, 20–23]. N protein is the major component of RNP with 1,325 molecules per virion [3]. Conformational change of N protein under natural conditions has not been reported.

The aim of this study was to develop a quantitative ELISA method of N protein detection in RV which could be used as a monitoring assay in the manufacturing process of rabies vaccine for animals.

MATERIALS AND METHODS

Virus and cell: Rabies virus (RV) RC-HL strain, a sole seed virus for production of rabies vaccines for animals in Japan, was used [8]. The virus was propagated in HmLu-1 cells of hamster lung origin.

HmLu-1 cells were cultured in Eagle's minimum essential medium (Eagle's MEM) supplemented with 5% fetal calf serum (FCS).

Virus propagation: RV was inoculated on HmLu-1 cells at a m.o.i. (multiplicity of infection) of 0.01 pfu/cell. After adsorption for 1 hr at 37°C, the cells were added Eagle's MEM of pH 7.4 supplemented with 1% FCS. The pH of the medium was adjusted with 7% $NaHCO_3$. The cells were cultured at 34°C without CO_2.

In the test of virus-growth, the pH of the medium was controlled by addition of 20 mM HEPES buffer (pH 6.5, 7.0 or 7.5) into Eagle's MEM supplemented with 1% FCS and 1.4 mM $NaHCO_3$. After adsorption of the virus, the cells were added to each medium and incubated at 34°C in air. Ten m*l* aliquots of culture fluids were sampled every day for 6 days, and their pH was measured. The samples were concentrated to 10 μl by ultracentrifugation (100,000 × g, 1 hr, 4°C).

Purification of RV: Purified RV was prepared according to the method of Kawai *et al.* [10]. The fractions with infective RV were collected as purified virion and then dialyzed against PBS.

Antibodies: To prepare an anti-N serum, N protein derived from purified RV was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The band of N protein in the gel was cut, and then homogenized in PBS. The homogenate was emulsified with oil-adjuvant ISA70 (Seppic, France) at a ratio of 3:7. Three guinea pigs were injected intramuscularly three times with the emulsion at three week-intervals. One week after final injection, the animals were bled to collect sera. Antibody titers of the sera measured by the indirect fluorescent antibody method were 1,280 to 2,560.

To prepare RV-specific antiserum, two rabbits were immunized with an emulsion of purified RV and oil-adjuvant ISA70 three times at three week-intervals. The serum obtained showed a neutralizing antibody titer of

12,800.

The mouse anti-G monoclonal antibody (MAb13-10) used in this study was supplied by Dr. N. Minamoto (Department of Veterinary Public Health, Gifu University, Gifu, Japan) [14].

Spikeless particles produced under conditions of low pH showed low-molecular-peptide (Gm) in the transmembrane and cytoplasmic regions of the envelope [11]. Rabbit anti-Gm peptide serum binding to the C-terminal cytoplasmic domain of G protein was kindly provided by Dr. A. Kawai (Kyoto University, Kyoto, Japan) [19].

Western blot analysis: SDS-PAGE and western blotting were carried out as described previously [9]. Proteins resolved in 10% gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, U.S.A.), and the protein bands were visualized on X-ray films using an ECL western blotting detection system (Amersham, UK).

Plaque assay: Infective RV was titrated by plaque assay on HmLu-1 cells. The overlay medium used was Eagle's MEM supplemented with 1% FCS and 1% methyl cellulose #4000 (Nacalai Tesque, Japan). After cultivation for 5 days at 37°C, the infected cells were fixed with methanol and stained with 0.2 (w/v)% crystal violet in 20 (v/v)% ethanol. Unstained areas were enumerated as plaque forming units (PFU).

Interference focus-forming assay: To quantify defective interfering (DI) particles in samples, an interference focus-forming assay was carried out on HmLu-1 cells by a slightly modified method of Kawai and Matsumoto [12]. The remaining cells were stained with 0.2% crystal violet in 20% ethanol. Foci excluding very small ones were counted as focus forming units (FFU).

G-ELISA: This method was carried out according to the protocol of Gamoh *et al.* [5] except that the samples used were culture supernatants and were not gel filtrated to eliminate Gs protein.

N-ELISA: The procedure for N-ELISA is shown in Fig. 1. The rabbit RV-specific antiserum for N-ELISA was diluted 1/1,000 with 0.05 M carbonate buffer (pH 9.5). The diluted antiserum could capture the same amount of RV as virions captured in G-ELISA. On the other hand, the N protein-specific antiserum was diluted to the concentration that gave the highest specific titer in N-ELISA.

The wells of a polystyrene ELISA plate (Greiner, Germany) were coated with 0.1 m*l* of the diluted RV-specific antiserum and incubated for 1 hr at 37°C. In all steps except the final one, the plate was incubated for 1 hr at 37°C. The serum-coated plate was blocked with 0.3 (w/v)% bovine serum albumin (BSA) and 5 (w/v)% sucrose in PBS. One hundred microliter aliquots of each RV sample were added into duplicate wells, and the plate was incubated. Then the plate was washed three times with PBS containing 0.05% tween 20 (T-PBS), and 100 μl each of glutaraldehyde or paraformaldehyde diluted with PBS at an appropriate concentration was added. After incubation, the plate was washed five times and 100 μl aliquots of 100 mM glycine were added into all wells to block free-aldehyde residues.

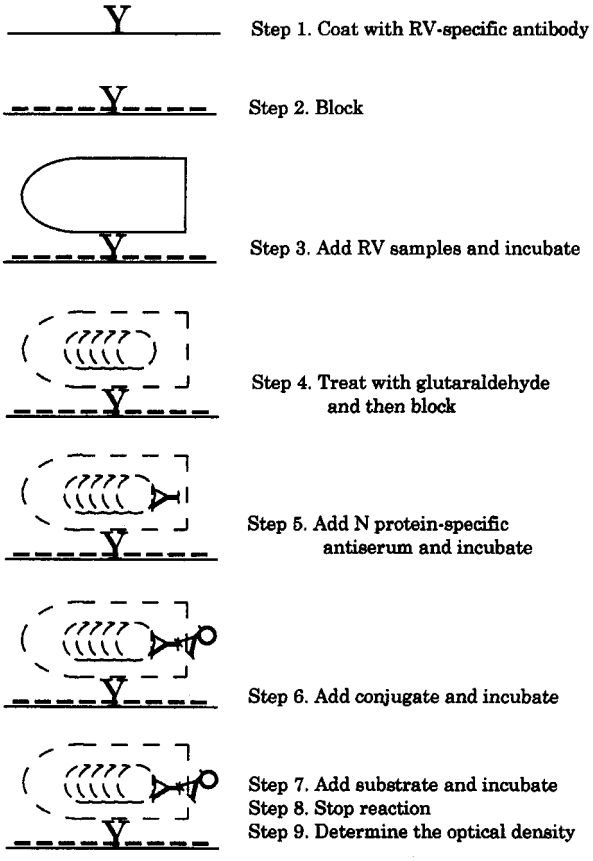

Fig. 1. Procedure for N-ELISA.

After incubation and subsequent five washes, 100 μl of a guinea pig anti-N serum diluted 1/100 with T-PBS containing 1% BSA and 1% normal rabbit serum (T-PBS/BSA/NR) was added to each well. The plate was incubated again. After five washes, 100 μl of peroxidase-conjugated anti-guinea pig IgG antibody diluted 1/10,000 with T-PBS/BSA/NR was added to all the wells and incubated continuously. After five washes, 100 μl of substrate solution [0.065 (w/v)% o-phenylenediamine dihydrochloride (WAKO, Japan) and 0.006 (v/v)% H_2O_2 in citrate-phosphate buffer, pH5.0] was added to each well, and the plate was incubated for 1 hr at 25°C. Fifty microliters of 2 N H_2SO_4 was added to the wells to stop the reaction. The ELISA titer was determined as the absorbance at a wavelength of 492 nm.

RESULTS

Characterization of anti-N protein serum: The specificity of anti-N serum and anti-G MAb 13–10 was examined by western blotting. The structural proteins except L protein of RV were observed in SDS-polyacrylamide gel by Coomassie brilliant blue (CBB) staining (Fig. 2, lane 1). Guinea pig anti-N protein serum only reacted to N protein with molecular mass of 56 kDa, but not with G protein (Fig. 2, lane 2). On the other hand, mouse anti-G MAb only

reacted to G protein with molecular mass of 67 kDa (Fig. 2, lane 3).

Effect of chemical reagents on detection of N protein: The effect of chemical reagents was investigated for reactivity of N protein-specific antiserum against N protein in virion. Glutaraldehyde and paraformaldehyde were used at a concentration of 0, 0.25, 0.5, 1, 2 and 4 (v/v)%, respectively. When RV captured on a plate was treated with glutaraldehyde, the N-ELISA titer rose gradually with increasing concentration of the reagent. The maximum titer was 0.54 at a concentration of 4 (v/v)%. On the other hand, ELISA titer at the concentration of 0 to 4 (v/v)% of paraformaldehyde was less than 0.1 (Fig. 3). On the basis of these results, 4 (v/v)% glutaraldehyde was used throughout the experiment.

Detection of DI and infective particle by N-ELISA: Culture fluids containing RV were fractionated by sucrose gradient ultracentrifugation. Samples were diluted 200-fold for G-ELISA to distinguish DI particles from infective particles, but were undiluted for N-ELISA. The results are shown in Fig. 4. DI particles were observed with a maximum titer of 7×10^3 FFU/m*l* in fraction No. 5. Infective particles were observed in fraction No. 17 with maximum titer of 1.71×10^9 pfu/m*l*. Two absorbance peaks were observed in the same fractions by both ELISA methods.

Correlation between plaque assay and ELISAs: RVs were propagated in Eagle's MEM of pH 7.4 adjusted with 7% $NaHCO_3$. Culture fluids were sampled every day after inoculation. Twenty three samples were quantified by plaque assay, N-ELISA and G-ELISA, and the correlation coefficients were calculated. They showed an infective titer higher than 10^6 pfu/m*l*. Samples diluted 4-fold for G-ELISA and undiluted for N-ELISA were employed. The correlation coefficient between the titers of plaque assay and N-ELISA was 0.767 (Fig. 5). The value obtained between plaque assay and G-ELISA was 0.547 (Fig. 5).

Influence of pH of culture medium on the quantitation by N-ELISA: When RV was propagated at low pH, Gs protein was shed into the medium and Gm peptide stuck to the virus envelope [19]. It was investigated whether the growth conditions of the virus influenced the result of N-ELISA titers. Samples were undiluted for N-ELISA, and diluted 4-fold for G-ELISA. Additionally, the presence of G protein and Gm peptide in virion was investigated by western blotting using anti-Gm antiserum.

Figure 6 (panels A and D) shows the virus growth curve obtained under the condition of pH 6.5. RVs showed a titer of 3.35×10^5 pfu/m*l* on day 2 and 7.0×10^6 pfu/m*l* on day 6. The titers of N- and G-ELISA were less than 0.25 for 6 days after virus inoculation. Gm peptide was detected from day 4 to day 6 (Fig.6, panel D).

Figure 6 (panels B and E) shows the results obtained at pH 7.0. RVs showed a virus titer of 3.5×10^7 pfu/m*l* on day 3 and 7.3×10^7 pfu/m*l* on day 6 in maximum. The titers of N-ELISA gradually rose and reached 0.83 on day 4, and then they hardly changed until day 6. The titers of G-ELISA were 3-fold higher than those of N-ELISA. The maximum G-ELISA titer was 2.53 on day 5. Gm peptide was detected in samples collected from day 3 to day 6 (Fig. 6, panel E).

Figure 6 (panels C and F) shows the results obtained at pH 7.5. RVs showed an infectivity titer of 9.5×10^4 pfu/m*l*

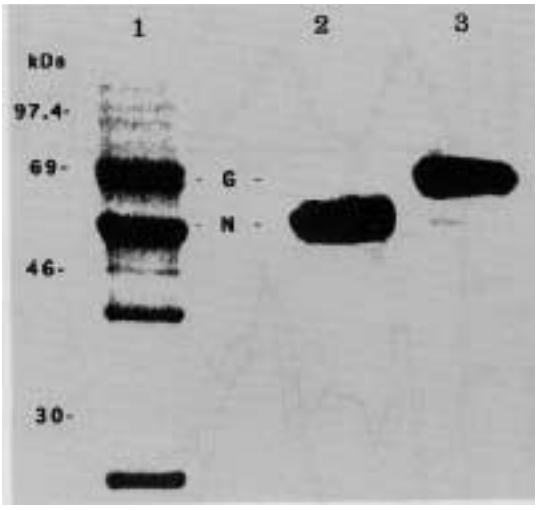

Fig. 2. Characterization of N protein-specific antiserum prepared by immunization with gel-extracted N protein. Purified RVs were solubilized and then resolved by 10% SDS-PAGE. One half of the gel was stained with Coomassie brilliant blue (CBB) solution (lane 1) and the other half was subjected to western blotting (lane 2; N protein-specific antiserum, lane 3; anti-G protein MAb). The numbers on the left indicate molecular mass.

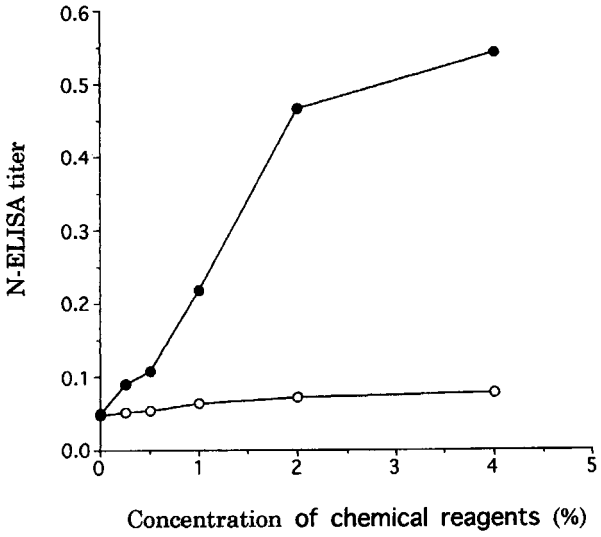

Fig. 3. Effect of chemical reagents on detection of N protein by N-ELISA. RVs captured on an ELISA plate were treated with glutaraldehyde (●) or paraformaldehyde (○) at a concentration of 0 to 4 (v/v)%. N-ELISA was carried out as described in MATERIALS AND METHODS.

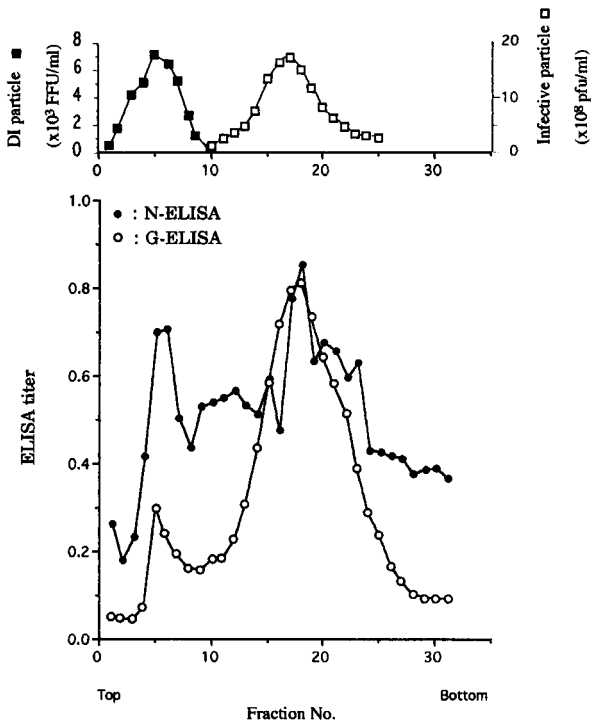

Fig. 4. Analysis of RV culture fluids fractionated by ultracentrifugation. RV culture fluids were precipitated with PEG, and then ultracentrifuged in a 10–45 (w/v)% sucrose linear gradient. The samples were fractionated by pumping out from the bottom, and were quantified by N-ELISA (●), G-ELISA (○), focus-forming assay (■) and plaque assay (□).

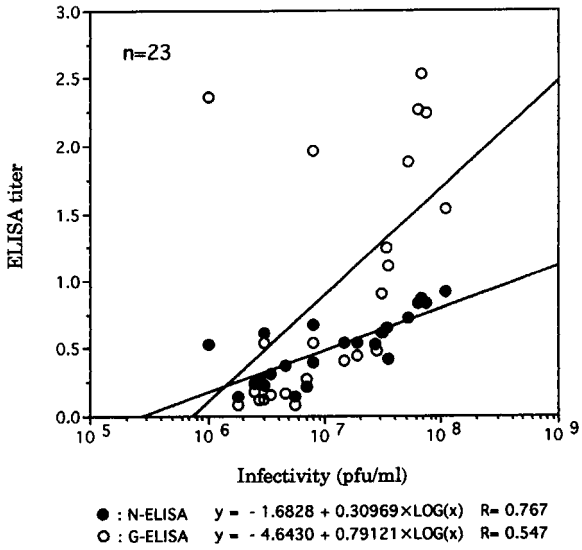

Fig. 5. Correlation between titers of plaque assay and by N-ELISA (●) or G-ELISA (○). RVs were propagated in a medium of pH 7.4 adjusted with 7% $NaHCO_3$ without CO_2.

on day 1, and reached a maximum titer of 1.1×10^8 pfu/m*l* on day 3. After that, the titer decreased day by day, and was 1.0×10^6 pfu/m*l* on day 6. The time course of N-ELISA titers showed a similar pattern to the result of the plaque assay. The N-ELISA titers were 0.395 (day 1), 0.921 (day 3) and 0.592 (day 6). The titers of G-ELISA increased day by day from 0.056 to 2.362. Gm peptide was not detected throughout the experiment.

DISCUSSION

RVs have been quantitated by plaque assay, the immunoperoxidase method, hemagglutination test, single-radial-immunodiffusion, RFAT and ELISA. We attempted to develop a quantitative method for RV by a capture ELISA for detection of N protein in virion, which could be used for quality control in the manufacturing process of rabies vaccines for animals. This method was based on an immunoelectron microscopic method (personal communication by Dr. A. Kawai) for detecting an inner structural protein of RV by treatment with paraformaldehyde.

N-ELISA was able to detect N protein in RV virion captured by RV-specific antiserum on a plate. In western blot analysis, N protein-specific antiserum was proven to be specific and reacted only with N protein (Fig. 2). The N-ELISA titers gradually increased with the rise of glutaraldehyde concentration (Fig. 3), indicating that N protein-specific antiserum bound with N protein in the RV virion. However, this method using paraformaldehyde was not able to detect the N protein in the virion, suggesting that treatment with the reagent did not make holes in the envelope of RV. N protein-specific antiserum did not, therefore, permeate into the virion. After fixation with paraformaldehyde, a treatment with Triton X-100, acetone or methanol might increase permeability of the envelope to the antiserum.

Both infective and DI particles could be detected by N-ELISA and G-ELISA alike. The results from ultracentrifuge analysis (Fig. 4) and the time course of the RVs propagated at pH 7.5 (Fig. 6, panel C) indicated that the infective and DI particles could be detected by N-ELISA, but not free-N protein nor RNP complex. The reason was thought to be that free-N protein and RNP complex were small in quantity or were removed by washing after step 3 or 4 of the procedure (Fig. 1).

In ability to detect RV, N-ELISA was inferior to G-ELISA because samples could not be diluted for N-ELISA. If the antiserum reacted with all the N proteins of a virion, then the N-ELISA titer should be similar to G-ELISA titer. However, differences in the results from the theoretical values suggested that the antiserum was bound to a part of the N protein molecule of the virion. It was considered that N protein-specific antiserum did not sufficiently permeate into the virion, or the antigenicity of N protein was changed by treatment with glutaraldehyde.

Almost all the virions of RV were considered to have been detected by the N-ELISA, even if some variation or destruction had occurred in the epitopes on G proteins. However, the correlation between the results of N-ELISA

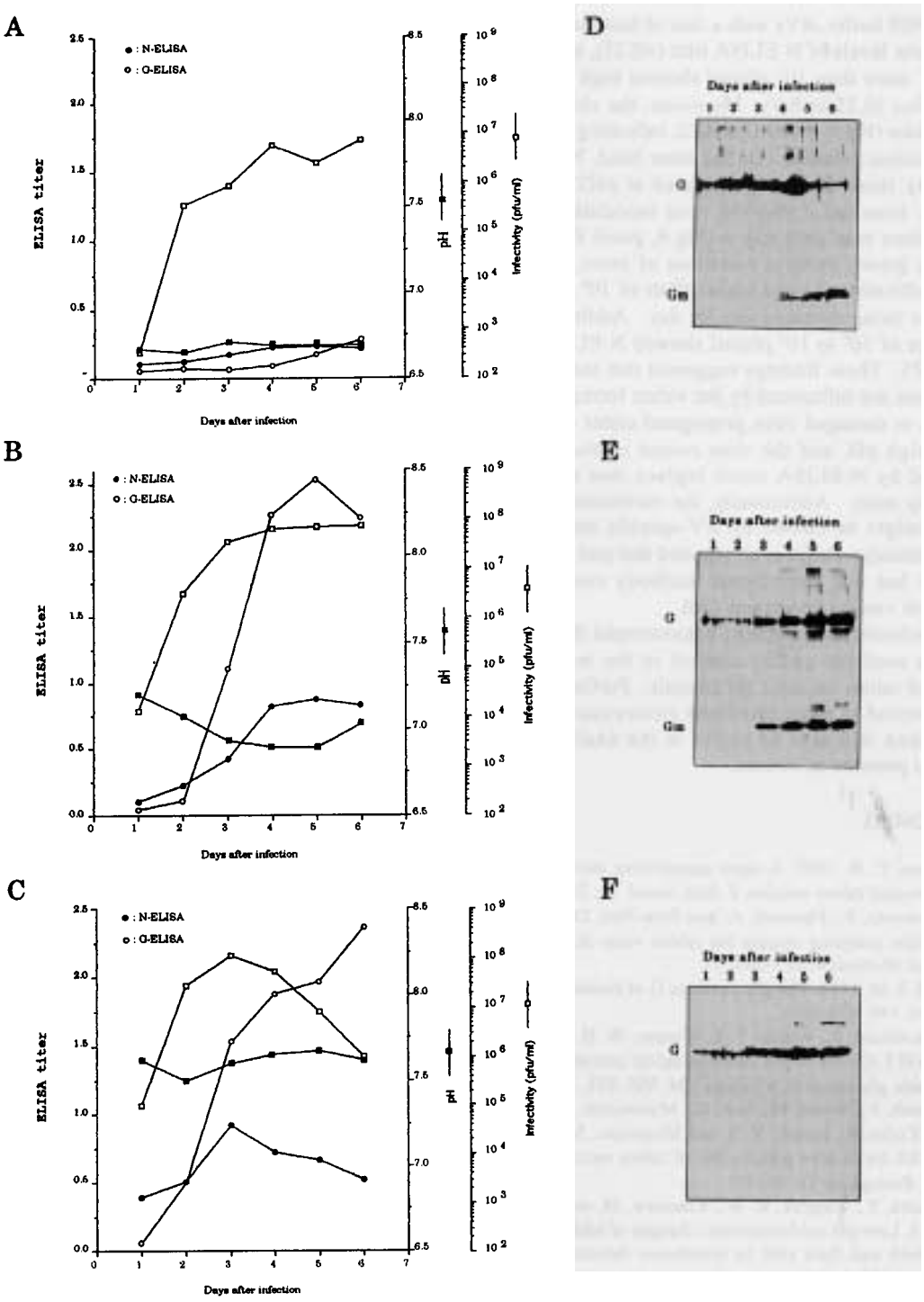

Fig. 6. Influence of medium pH on titers quantified by three methods. RVs were cultured at different pHs (panel A; pH 6.5, panel B; pH 7.0 and panel C; pH 7.5) in air. The RVs sampled every day were measured by plaque assay (□), N-ELISA (●) and G-ELISA (○). Medium pH (■) was also measured every day. Additionally, the same RV samples were pelleted down, and analyzed for the presence of G protein and Gm peptide in virion by western blotting using anti-Gm antiserum (panel D; pH 6.5, panel E; pH 7.0 and panel F; pH 7.5).

and plaque assay was not always high (r=0.767, Fig. 5). To investigate this, the influence of the pH of the culture medium on the result of N-ELISA was investigated. When RVs were propagated in medium of pH 6.5 and 7.0 adjusted

with HEPES buffer, RVs with a titer of less than 10^7 pfu/m*l* showed low levels of N-ELISA titer (<0.25), and RVs with a titer of more than 10^7 pfu/m*l* showed high levels of N-ELISA titer (0.25 to 0.9). Moreover, the virions showed Gm peptides (Fig.6, panel D and E), indicating that the RVs were spikeless particles. On the other hand, N-ELISA and infectivity titers of RVs propagated at pH7.5 decreased gradually from day 4 after the virus inoculation, while G-ELISA titers rose until day 6 (Fig.6, panel F), indicating that RVs grown under a condition of more than pH 7.5 during cultivation showed higher titers of 10^8 pfu/m*l* while they were being damaged day by day. Additionally, RVs with titers of 10^6 to 10^7 pfu/m*l* showed N-ELISA titers of 0.5 to 0.75. These findings suggested that the result of N-ELISA was not influenced by the virion formation, such as spikeless or damaged virus propagated under conditions of low or high pH, and the time course of the virus yield examined by N-ELISA could replace that examined by infectivity assay. Additionally, the correlation of the time courses might be caused by RV-specific antiserum as a coated antibody. Perrin *et al.* reported that anti-G polyclonal antibody but not monoclonal antibody could detect G proteins in various conditions [20].

In conclusion, these results demonstrated that N-ELISA could be used for quality control in the manufacturing process of rabies vaccines for animals. Further application of this method to other enveloped viruses can be expected. The method will also be useful in the analysis of inner structural proteins of viruses.

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