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Protein G 효소표지면역반응법에 의한 Sendai Virus 항체 검출

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Detection of Antibodies to Sendai Virus Using protein G-based ELISA

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Abstract

We have established an enzyme-linked immunosorbent assay(ELISA) using enzyme labeled protein G(Protein G-ELISA) to detect Sendai virus-specific antibodies in laboratory animals. Best results were obtained at pH 6.0 of diluent and at 1 to 10 dilution of sera. Antigen concentration for solid phase matrix was 5μg/mℓ and the conjugate of horseradish peroxidase (HRP) and protein G was used at 1 to 100 dilution. We, also, investigated the sensitivity and specificity of protein G and protein A. Protein G showed relatively high O. D. values than protein A in general. Furthermore, only Protein G-ELISA could detect the Sendai virus specific antibodies in rat, though both methods were effective in other species, mouse and guinea pig. No siginificant nonspecific reation was observed at our experimental conditions in both methods.

Introduction

Nonspecific positive reactions are caused by enzyme-labeled immunoglobulin.[4)] ELISA using protein A, a component of the cell wall *of Staphylococcus, aureus,*[5)] can reduce nonspecific reactions prominantly.[18)] Furthermore, they have detected antibodies of guinea pig as well as mouse without changing enzyme conjugate by Protein A-ELISA. Protein A is used in a wide variety of immunological techneques but do not react with goat and rat IgG[12)] and weakly with mouse IgG1.[2)]

The surface protein named protein G, which was first purified from group C or group G streptococci by Bjorck and Kronvall(1984) has been demonstrated to have more affinity than protein A to immunoglobulins lacking reactivity against protein A.[1,14)] Protein G binds to IgG not only of mice, guinea pigs but also of rats, and since protein G is available as an enzyme conjugate of ELISA for the diagnosis of Sendai virus infections in those three important laboratory animals.

The present paper describes the establishment of a simple ELISA method based on HRPO-labeled protein G for

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the serological diagnosis of Sendai virus infection not only in mouse, guinea pig and rat.

Materials and Methods

Sendai virus : Sendai virus was grown in the chorioallantoic fluid of 9-day-old embryonated hen eggs for 44～48 hrs. at 37℃. Titration of hemagglutination titration was carried out using 0.2% chicken red blood cells(approximately 3000 HA titer). Purification of Sendai virus was done as follows. The virus-containing chorioallantoic fluid was subjected to centrifugation at 8000xg for 30 mins. to remove cell debris and chicken red blood cells. The supernatant was layered on 7㎖ 30% and 5㎖ 60% sucrose (w/v) and centrifuged for 90 mins. At 5℃, 100,000xg(SW-28 rotor, Beckmann). The virus layer was collected and diluted 1 : 3 with saline and was purified by a linear sucrose gradient from 30% to 60% (w/v) centrifugation for 180 mins. At 5℃, 100,000xg. The visible virus layer was removed and aliquots of purified virus were stored at -70℃. Protein amount of the purified virus was determined by the microkjeldahl method.[6)]

Test sera : Sendai virus antibody positive mouse, rat and guinea pig sera were pooled and divided to 0.1㎖, respectively. Sendai virus antibody negative animal sera were obtained by exsanguinating the specific pathogen free and viral antibody free animals (SPF & VAF) purchased from Charles River company of Japan (mice ; ICR ; rats ; Sprague dawley, Guinea pigs ; Hartley). Aliquots of the sera were stored -20℃.

HRP Labeling : Protein A (Sigma Chemical Co., St. Louis) 5㎎ and recombinant protein G (Pharmacia Fine Chemicals, Uppsala, Sweden) 5㎎ were conjugated with HRP(Type Ⅵ, Sigma Chemical Co., St. Louis) 5㎎ each by the method of Nakane(1975). To separate conjugated protein A and protein G from free materials, preparative liquid chromatography system(WATERS, Delta 3,000 system, U.S.A.) was used, Protein A-HRP and protein G-HRP samples were applied to a TSK gel 4000SW column and a TSK gel 2000SW column in 0.02 M phosphate buffer, pH 6.5 respectively. The elution speed was 4㎖ min. The eluted proteins were monitored by a UV-monitor. Each fractions were tested for the peroxidase activity and IgG binding activity of the conjugates.

ELISA plate coating : Wells of micro ELISA plates(Nunc-Immuno Module, Polysorb U16, Denmark) were coated with various concentrations of purified Sendai virus in 100㎕ of 50mM carbonate buffer, pH 9.6 for 16 hrs. at 4℃. During the incubation, plates were sealed with adhesive tape. After washing 2 times with each 250㎕ phosphate buffered saline (PBS) the wells were filled with 200㎕ of PBS containing 0.5% of bovine serum albumin(Sigma Chemical Co., St. Louis) or gelatin(Merck, Germany) for 2 hrs. at 4℃. The plates were washed and stored in desicator at 4℃.

ELISA : For the protein G-ELISA, 100㎕ of positive control and negative control sera were added and the plates left for 1 hr. at room temperature. After four washes, each 100㎕ of conjugate diluted in buffer contaitning gelatin and Tween 20, was incubated for 1 hr. at room temperature. After washing four times 100㎕ of substrate [2.2'-AZINO-bis (3-ETHYLBENZTHIAZOLINE-6-SULFONIC ACID) 0.2㎎/㎖ in 0.1 M citrate-phosphate buffer, pH4.0 containing 0.04% H_2O_2] was added per well and the plates incubated in dark at room temperature. After 30 mins. incubation, 100㎕ of buffer containing 0.005% NaN_3 was added to each wells to stop the enzyme reaction and the absorbance was measured at 405 nm with reference at 492 nm. For Protein A-ELISA, PBS containing 0.5% BSA and 0.05% Tween 20 was used as conjugate diluting buffer.

Results

Determination of optimal antigen concentration : The optimal concentration of Sendai virus in Protein G-ELISA was determined by checkerboard titration. Wells were coated with 0.2, 1, 5, or 25㎍/㎖ of antigen solution and

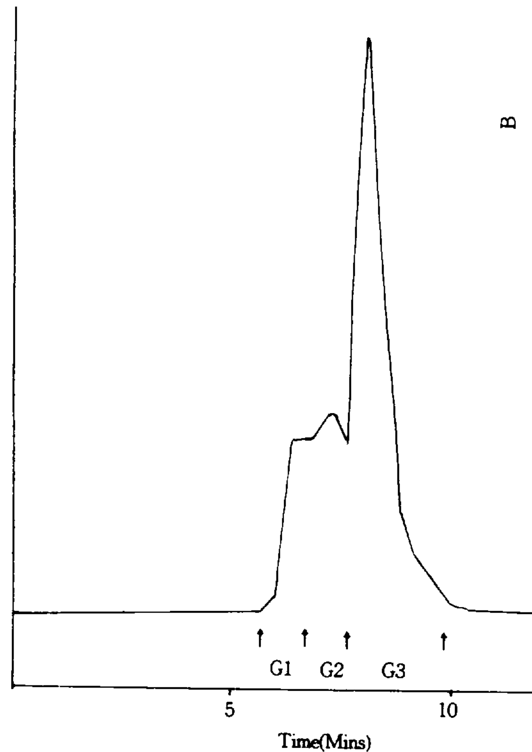


Fig. 1. Preparative liquid chromatography of HRPO labeled protein G on TSK 2000 SW column. (Eluent : 0.02 M phosphate buffer, pH 6.5; Speed : 4mℓ/min.; UV detector was used at 280nm)

reacted with mouse positive control serum serially diluted two fold from 1 : 10 to 1 : 1280. Representative reciprocal relation to serm dilution was observed at 5 μg/mℓ of the Sendai virus(Fig. 1).

Determination of optimal concentration of Protein G-HRP : The optimal concentration of protein G-HRP was also determinated by checkerboard titration. Wells coated with 5 μg/mℓ of Sendai virus antigen were filled with mouse positive control serum used for determination of the optimal protein G-HRP conjugate concentration. After the antigen had reacted with the antiserum, three dilations of G2 fraction from TWK column(Fig. 2) from 1 : 50 to 1 : 200 were tested for their binding activity. The clear linearity was shown at dilution of conjugate and nonspecific reaction was not observed(Fig. 3).

Determination of optimal dilution of test sera : Mouse sera were assayed in serial five fold dilutions. The positive control serum produced a clear dose response curve compare to negative control which showed marginal increment of O. D_{405}($\triangle$ 0.015)(Fig. 4). Although nonspecific activity was not observed after 1 : 5 dilutions, 1 : 10 dilution was used for the rest of the experiments.

Determination of optimal conditions of diluent : The effects of pH and carrier proteins in diluent bufer are shown in Fig. 5. The maximum specific binding between antibody and protein G was at pH 6.0 of diluent with gelatin. At this condition the nonspecific binding was not increased.

Determination of stopping solution : For quenching of the colorization after completion of the assay, 100 microliter of 1.25% sodium fluoride (Matsuda *et al.*, 1988) or 0.005% sodium azide were added each wells. Both the two solutions inhibited colorization reaction rapidly. In the case of sodium fluoride, colorization was progressed slowly with the lapse of time, but sodium azide stopped the development of colorization almost completely(Fig. 6).

Sensitivity and specificity of Protein G-ELISA : Sendai virus antibody titers of mouse positive serum were deter-

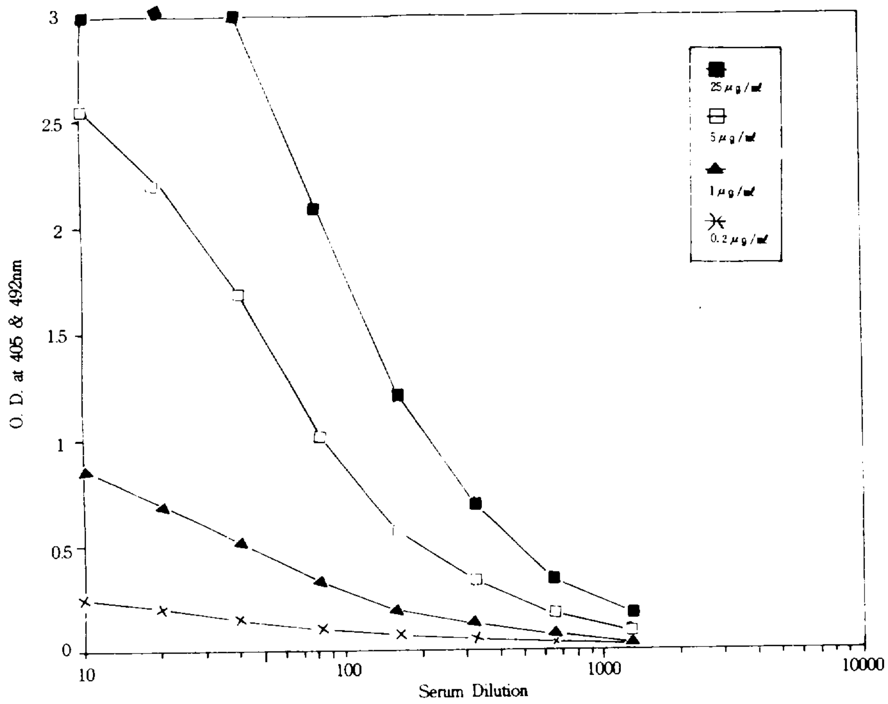


Fig. 2. Determination of optimal concentration of Sendai virus antigen for ELISA plate coating.

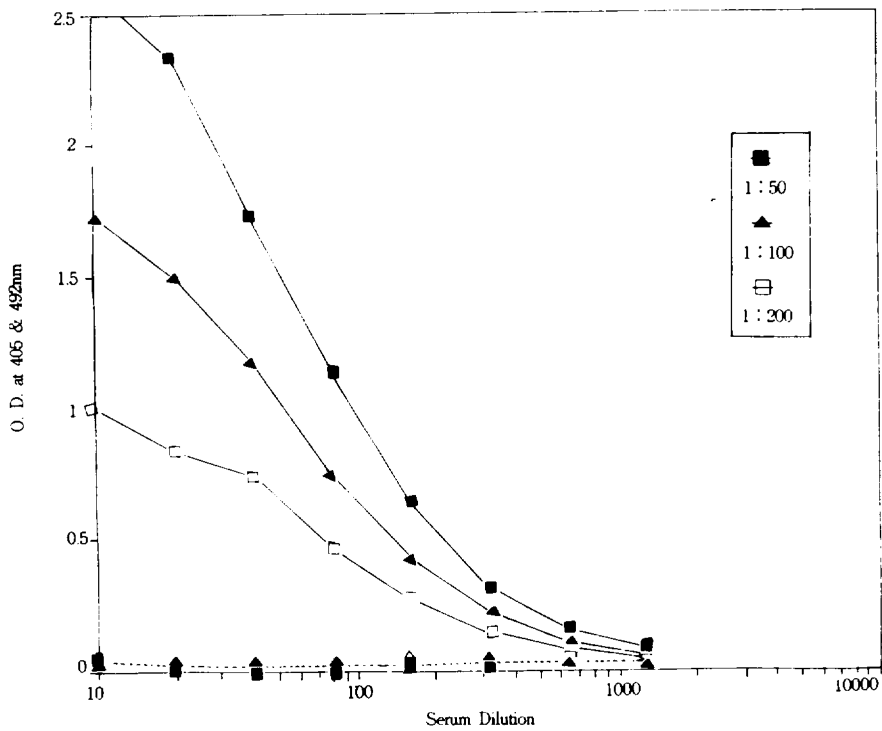


Fig. 3. Determination of optimal dilution of HRPO labeled protein G for Protein G-ELISA Sendai virus antibody positive(solid line) and negative(dotted line) mouse sera were tested at serial dilutions with diluent.

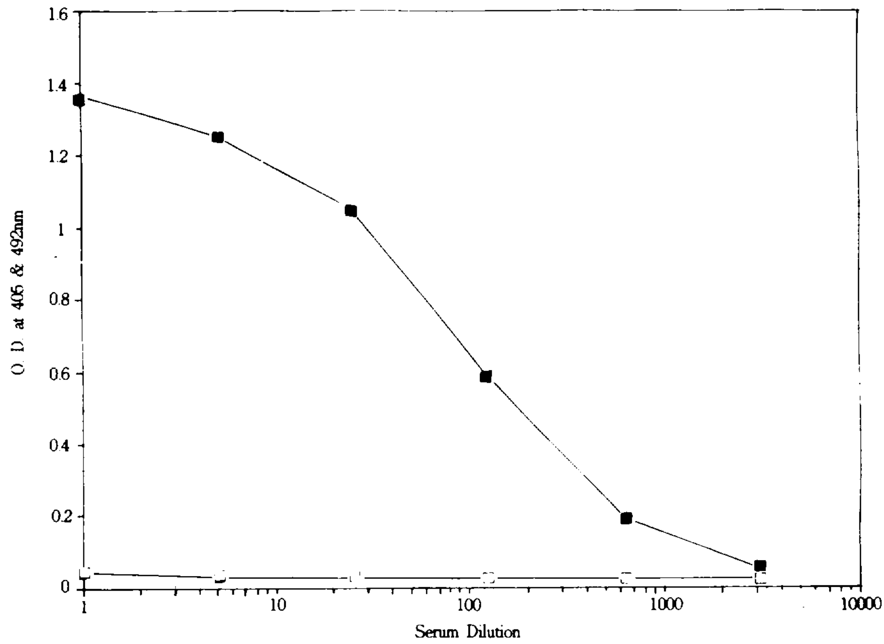


Fig. 4. Determination of optimal dilution of test serum for Protein G-ELISA. Sendai virus antibody positive(closed squares)and negative(open squares)mouse sera were tested at serial dilutions.

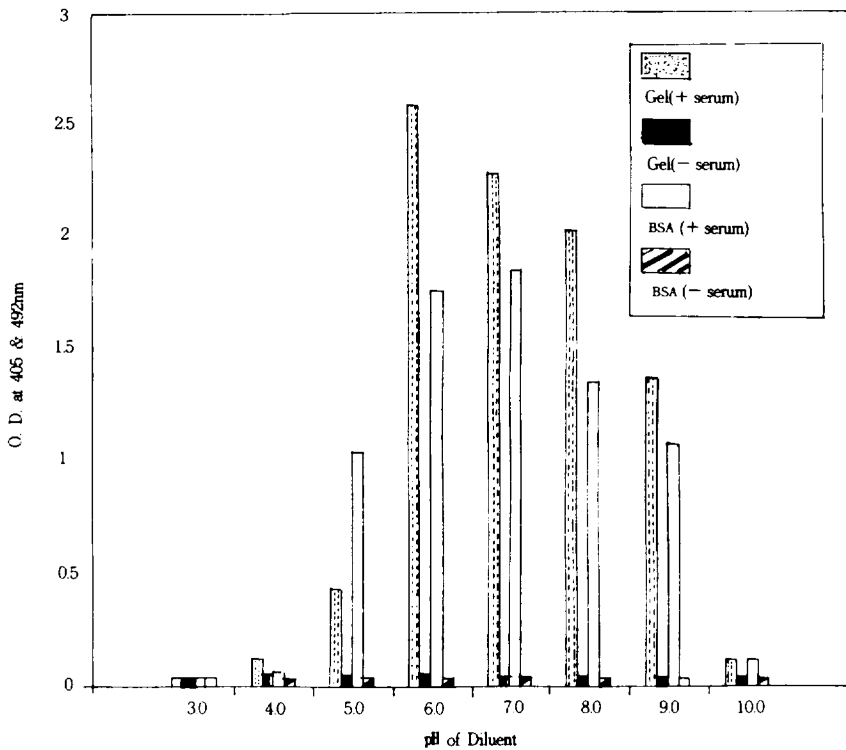


Fig. 5. Effects of pH and carrier proteins of the diluent on specific and nonspecific reactions of Protein G-ELISA. Sendai virus antibody positive(+ve)and negative(−ve) mouse sera were tested at 1 : 10 dilution.

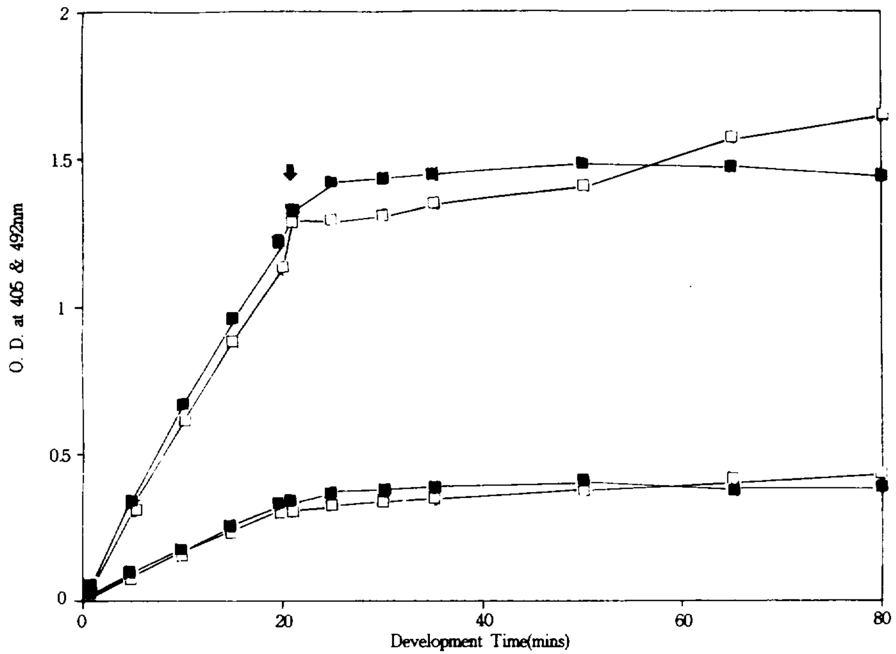


Fig. 6. Stopping effects of 0.005% sodium azide(closed squares)and 0.3% sodium fluoride(open squares) on HRPO reaction of two Sendai virus antibody positive mouse sera. The arrow indicates the addition of stopping solutions.

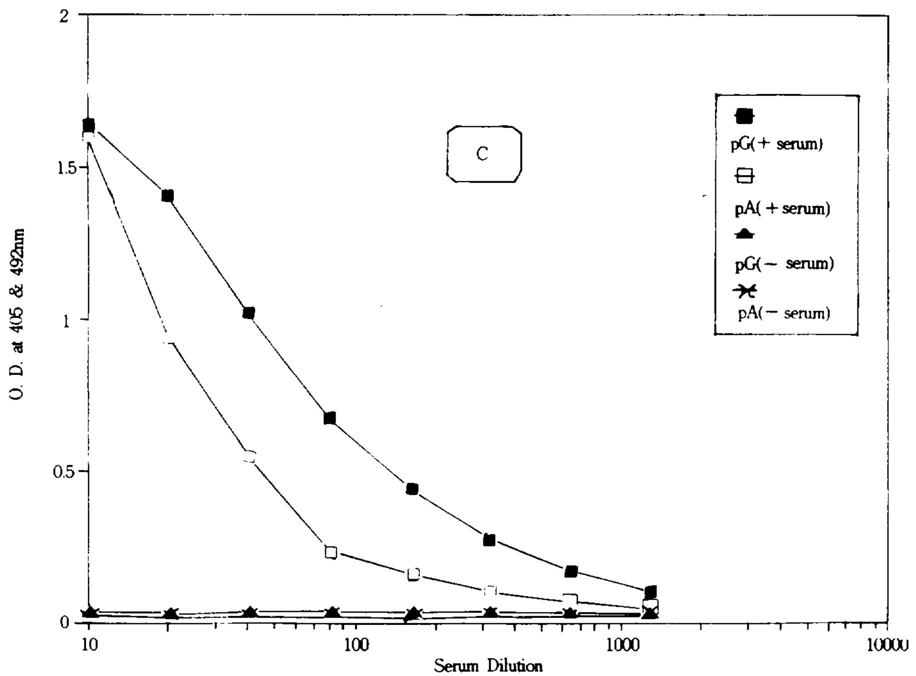
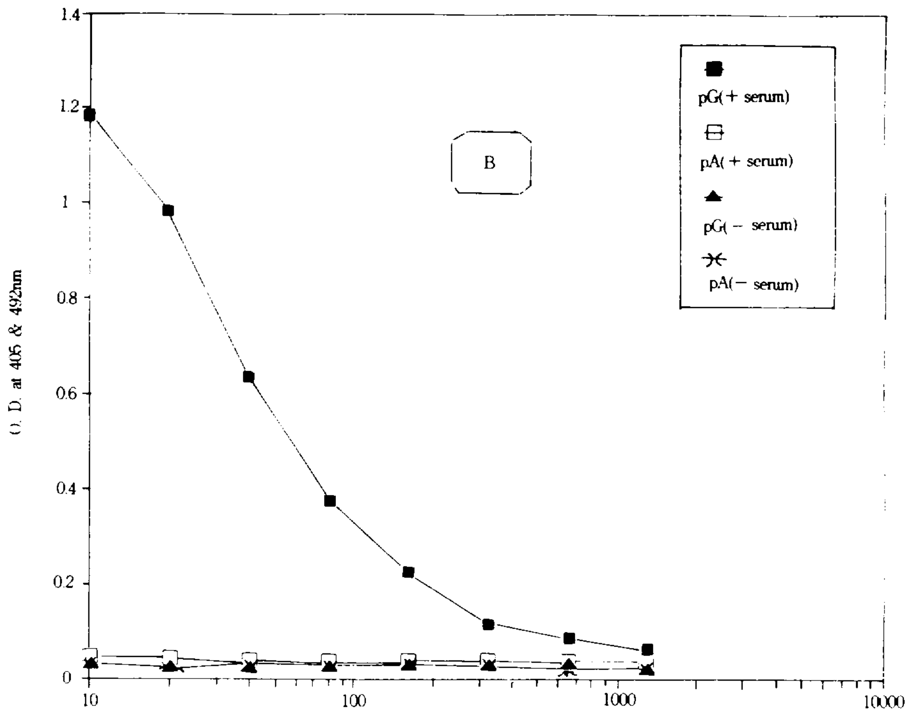
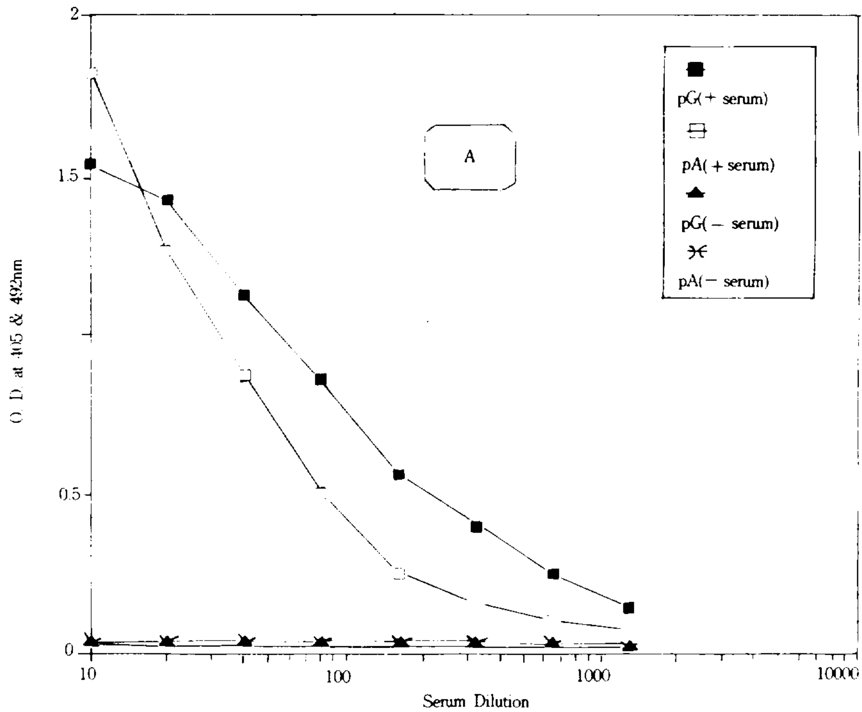


Fig. 7. Comparison of Protein G-ELISA and Protein A-ELISA with mouse(A), rat(B) and guinea pig(C) sera. (pG : Protein g-ELISA, pA : protein A-ELISA, +serum : Sendai virus antibody positive serum, −serum : Sendai virus antibody negative serum)


B
pG(+ serum)
pA(+ serum)
pG(− serum)
pA(− serum)
O. D. at 405 & 492nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
100
1000
10000


A
pG(+ serum)
pA(+ serum)
pG(− serum)
pA(− serum)
O. D. at 405 & 492nm
2
1.5
0.5
0
10
100
1000
10000
Serum Dilution

mined more than 1 : 1280 by protein G-ELISA and by Protein A-ELISA. The O. D. value in Protein G-ELISA, however, was rather higher than that of Protein A-ELISA in overall. Significant nonspecific reactions were not found in both methods(Fig. 7-A). There was no difference in binding profile of protein G to antibody from guinea pig and mouse, respectively(Fig. 7-A and 7-C). As we expected the positive control serum of rat showed specific dose response curve in Protein G-ELISA, but no specific reactions were observed in Protein A-ELISA(Fig. 7-B).

Discussion

Sendai virus is one of the important respiratory virus of mice, rats and guinea pigs.[8,10,11,16,17] Since Sendai virus can be an indicator of microbiological contamination in laboratory rodents. Sensitive and specific diagnostic method are necessary for early diagnosis of Sendai virus infection. All strains of Sendai virus are considered antigenically homologous and of a single serotype,[3] though Sendai virus in antigenically distinct from all other viruses known to infect laboratory rodents.

In ELISA, enzyme labeled second antibodies to antibodies of respective animals are necessary. However, to eliminate this inconvenience, HRP labeled protein G, which has affinity to IgG Fc region of mouse, rat and guinea pig, was used as an enzyme conjugate. We investigated optimal conditions of Protein G-ELISA system for the diagnosis of Sendai virus infection in Lab. animals.

ABTS in rather slowly developing chromogen in comparison with OPD, and in general the O. D. values are checked as soon as the assays are terminated. Therefore, there are few reports on stopping reagents for ABTS as chromogen.

Specific blocking of color development in ELISA is, however, necessary for accurate determination of test results, especially in large number of samples. Therefore, inhibitor of peroxidatic acitivity such as sodium fluoride(0.3%)[9] or sodium azide(0.005%),[13] distilled water[7] have been used for terminator. Distilled water could not block color developement(data not shown), and there was a marginal but constant colorization in sodium fluoride. Based on these observations, we think sodium azide is the suitable reagent for stopping color reaction in the ELISA with peroxidase.

The strongest binding of protein G to human, rabbit and mouse IgG is reported to occur at pH 4 and 5.[1] In this report the highest binding occured at pH 6 and pH 7 of diluents with gelatin and BSA as carrier protein, respectively. Although it is not clear at present about this discrepancy, the molecular size and conformational change of protein G by conjugation with HRP and the carrier proteins in reaction buffer may have influence on antibody-Protein G interaction.

We have optimized ELISA method for diagnosis of Sendai virus infection in mouse, guinea pig, and rat, by one step conjugation of commercially available protein G and HRP. Because of its simplicity, sensitivity and ease we think our system, especially, is for the detection of Sendai virus infection in rat.

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