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THE EFFECT OF VIRUS PARTICLE SIZE ON CHEMILUMINESCENCE INDUCTION BY INFLUENZA AND SENDAI VIRUSES IN MOUSE SPLEEN CELLS

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Suspensions of orthomyxo- and paramyxoviruses are composed of pleomorphic particles ranging from large filaments to small spheres. Influenza and Sendai viruses were separated according to size by gel filtration and the induction of luminol-dependent chemiluminescence (CL) by particles of similar size was studied in suspensions of mouse spleen cells known to contain phagocytes. CL reflects the generation by the cells of reactive oxygen species. CL induction decreased with particle size for both viruses. Compared with small spheres, large influenza filaments were approximately 10 times as efficient in activating cellular light emission while the ratio between large and small Sendai viruses was 3:1. Small Sendai virus particles were also less efficient in lysing red cells and had lower neuraminidase activity. By contrast, with influenza virus, only neuraminidase and not the hemolytic activity decreased with the virus size. When influenza virus filaments were broken into smaller particles by sonication, the capacity to induce chemiluminescence dropped markedly while the hemolytic and hemagglutinating activities increased and neuraminidase activity remained unaltered. These results suggest that the presentation of influenza virus hemagglutinin and neuraminidase glycoproteins in a large particle, leading to extensive receptor crosslinking, may be an important factor in the efficient activation of CL by filamentous influenza virus. We suggest that radical generation as reflected in cellular CL may relate to the toxic *in vivo* effects that contribute to the pathogenesis of influenza and infections with paramyxoviruses.

KEY WORDS: Reactive oxygen, pathogenesis, virus infection, chemiluminescence, virus receptor.

INTRODUCTION

Sendai and influenza viruses induce luminol-dependent chemiluminescence in mouse spleen cells[1-3] and the latter have been shown to activate CL in human polymorphonuclear leukocytes.[4-6] We have demonstrated earlier that the mechanism of CL induction depends on the interaction between the cell membrane and the virus glycoproteins that protrude from the virus envelope as spike-like knobs.[2] In Sendai virus, CL seems to be mediated by cell surface receptors that bind the hemagglutinin-neuraminidase and fusion glycoproteins, respectively.[3]

While the measurement of virus-induced CL is a useful phenomenon to study early virus-cell interactions, its biological significance is not understood. CL reflects the generation of reactive oxygen species by phagocytic cells. The sensitivity of CL measurement can be enhanced by the use of indicators such as luminol or lucigenin (reviewed in[7]). The luminol-dependent CL generated by spleen and thymus cells has

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been demonstrated to originate from phagocytes present in the cell suspensions (for a review see[8]). Some of these species (e.g. ·OH) are highly toxic and have been shown to play a role in killing microorganisms as well as exerting harmful effects on host tissue.[9] Influenza- and paramyxoviruses are known to have toxic effects *in vivo*.[10-14] The toxicity of influenza virus has been shown to depend to the size of the virus, large filamentous particles being more toxic compared with small spheres.[15] Freshly isolated influenza viruses are predominantly of filamentous phenotype[16,17] but change to a more spherical appearance after some 12–15 egg passages.[18,19] Both filaments and spheres are infectious.[20]

In this paper, we report that the size of influenza and Sendai viruses plays an important role in their ability to induce CL. In particular, large filamentous influenza viruses are approximately 10 times more efficient in CL induction than small spherical particles. In Sendai virus, the CL inducing, hemolytic and neuraminidase activities decrease with the virus size. In contrast, the induction of CL by filamentous influenza virus is unrelated to its hemolytic and neuraminidase activities but can be reduced by breaking up the filaments into smaller particles. This suggests that the more extensive cross-linking of virus receptors by filamentous virus may be an important factor in CL induction by influenza virus. Our observations are in line with our previous suggestion that reactive oxygen species may have a role in the pathogenesis of viral diseases.[21,22]

MATERIALS AND METHODS

Growth and Purification of Viruses

Sendai virus, influenza virus (strains A/RI5$^-$, H_2N_2, and A/Port Chalmers, H_3N_2) were grown in the allantoic cavity of 11 day old embryonated eggs at 37°C. Allantoic fluids were harvested after 48–72 hours incubation and freed from debris by centrifugation at 4000 × g for 15 min. The viruses were then pelleted at 12,000 RPM in a Sorvall GSA rotor for 2 hours (Sendai virus) and 2.5 hours (influenza virus), respectively. Resuspended pellets were further purified on a 10–60% continuous sucrose gradient in a Beckman SW 27 rotor at 24,000 RPM for 60 minutes. Virus bands were collected and diluted with PBS (phosphate buffered saline) before pelleting in the same rotor for 60 min. at 25,000 RPM for 1 hour. Virus pellets were then resuspended with PBS and used in gel filtration experiments within 3 days.

Gel Filtration

Gel filtration was performed at 4–6°C in Pharmacia K 15/90 or K 16/100 chromatography columns using Biogel A-150 m coarse gels (Bio-Rad Laboratories, Richmond, Cal., USA). Bed height was 85 and 95 cm, respectively. Typically, 3×10^5 to 1.3×10^6 hemagglutinating units (HAU) of virus in 1–1.5 ml was loaded onto the column and chromatography carried out at a flow rate of 5 ml/h for 24 hrs. Fractions (0.9–1.1 ml) were collected and assayed as described below.

Hemagglutination

Virus was titrated by doubling dilution in 100 μl saline in plastic microtiter trays

(Greiner Plastics, West Germany). Hemagglutinin was detected by the addition of 10 ml of 5% (v/v) chicken red blood cells. The highest dilution showing agglutination was taken as 1 HAU.

Assays

To determine infectivity, dilutions of influenza virus were injected in 11 days old embryonated chicken eggs and allantoic fluid was assayed for hemagglutinin 48 hrs later. Protein was assayed using Coomassie Blue reagent supplied by Bio-Rad Laboratories. Hemolytic activity was assayed photometrically (λ = 540 nm) by measuring the release of hemoglobin from 0.5% (v/v) chicken red cells. Red cells were suspended in PBS (pH 7.3) and acetate buffer (pH 5.0) for Sendai and influenza virus, respectively. Neuraminidase activity was assayed as described using Fetuin as the substrate.[23]

Electron Microscopy

The virus particles were adsorbed by the drop method to parlodion-coated grids for 45–60 seconds and stained with 2% sodium phosphotungstate, pH 6.7, for 20 seconds. The results of the gel filtration were assessed by visually rating according to particle size of code-named samples taken from fractions eluted from the column. In addition, the particle size distribution of influenza viruses before and after gel filtration was determined using morphometric procedures. Electron micrographs were taken and the area enclosed by the contours of the particles was measured using a MOP AM-02 analyzer (Kontron, Zürich). As a size standard, Latex particles of defined diameter were employed (Balzers Union, Balzers, Principality of Liechtenstein).

Chemiluminescence Measurement

Spleen cells suspensions were prepared as described previously.[1] Briefly, spleens from 6–12 weeks old C57 Bl/6 mice were passed through a steel mesh immersed in Hanks' balanced salt solution (HBSS, buffered with 20 mM HEPES instead of bicarbonate and supplemented with 10% fetal calf serum and 5 mM glucose). After removing dead and red cells on a Ficoll-Hypaque cushion,[24] cells were suspended at 16 $\times$ 10^6/ml in HBSS without fetal calf serum and 5 μl of 4% (w/v) bovine serum albumin saturated with luminol (5-amino-2,3-dihydro-1,4-phtalazinedione, Sigma) was added per ml (final concentration of luminol 5–10 μM).

The cells were then transferred into Pico (Packard, Downers Grove, Ill., USA) glass scintillation vials (0.75 ml/vial) and dark adapted for 1 h at 37°C. CL was measured in a MR 300 liquid scintillation spectrometer (Kontron, Zürich) using the tritium channel with the dead time correction switched off. Modifications of the liquid scintillation spectrometer included a heating device for the sample compartment to maintain the cell suspensions at 37°C and program alterations to allow the automatic repeated measurements of up to 10 samples at fixed time intervals. Readings were recorded on line with a Hewlett-Packard HP 85A computer. Analysis of the data included dead time correction and the plotting of light emission curves calculated from replicate samples. Unstimulated cell suspensions were routinely run in parallel with virus-stimulated suspensions.

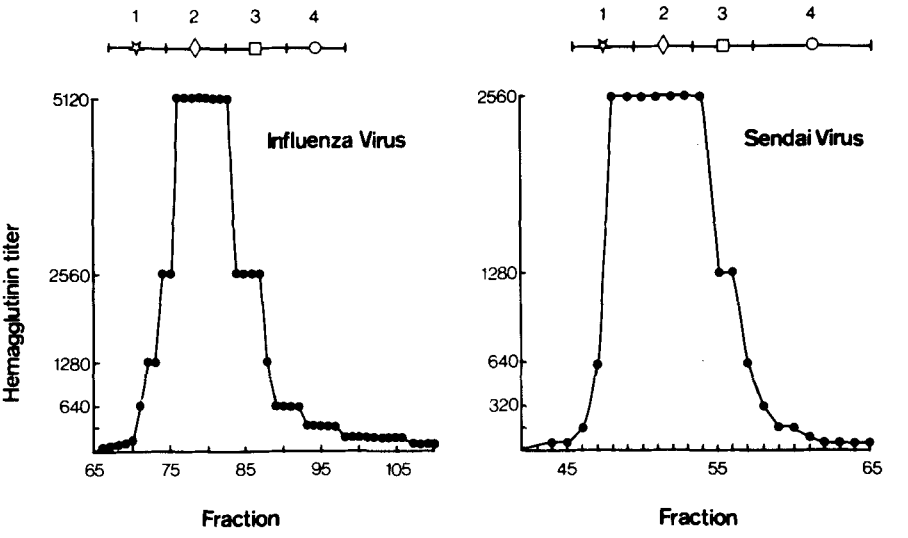

FIGURE 1 Gel chromatography of influenza and Sendai viruses. Influenza and Sendai viruses purified by sucrose gradient centrifugation were chromotographed at 4–6°C on Biogel A-150m. One ml of influenza virus (1.2×10^6 HAU and 1.5 ml of Sendai virus (6.1×10^5 HAU) was applied to a Pharmacia K 16/100 column. Bed height was 95 cm and flow rate 5 ml/h. Fractions of 1 ml were collected and assayed for hemagglutinin. Symbols on top of the profiles (☆, ◇, □, ○) refer to the virus pools used in the experiments shown in Figures 4 and 5.

RESULTS

Separation of viruses according to Size

Virus suspensions obtained from sucrose gradient centrifugation were subjected to gel filtration on a Biogel A-150 m column. Figure 1 shows the gel fractionation profiles of influenza and Sendai viruses as monitored in the hemagglutination assay. Both viruses eluted in a broad peak, the 1.5 ml sample being diluted in some 40–60 ml of eluate. Samples were taken from individual fractions and were negatively stained for examination in the electron microscope. It can be seen in Figure 2 that influenza viruses eluted from the column according to their size, large filamentous viruses being enriched in the earliest fractions.

To assess quantitatively the particle size distribution, a morphometric analysis was carried out on influenza viruses which had been subjected to gel filtration. Figure 3 shows that unfractionated influenza virus contained a high proportion of small particles, 71% of the viruses had a contour area of less than 0.034 μm^2. In contrast, fraction #65 that eluted early from the gel filtration column contained a high proportion of large particles, 81% of the viruses were over 0.034 μm^2. The proportion of large particles decreased; in fraction #80, only 27% of the viruses were over 0.034 μm^2. Finally, fraction #95 contained very few large viruses, 94% being less than 0.034 μm^2. Visual inspection of Sendai viruses filtrated on Biogel A-150 m revealed a pattern similar to that observed with influenza virus.

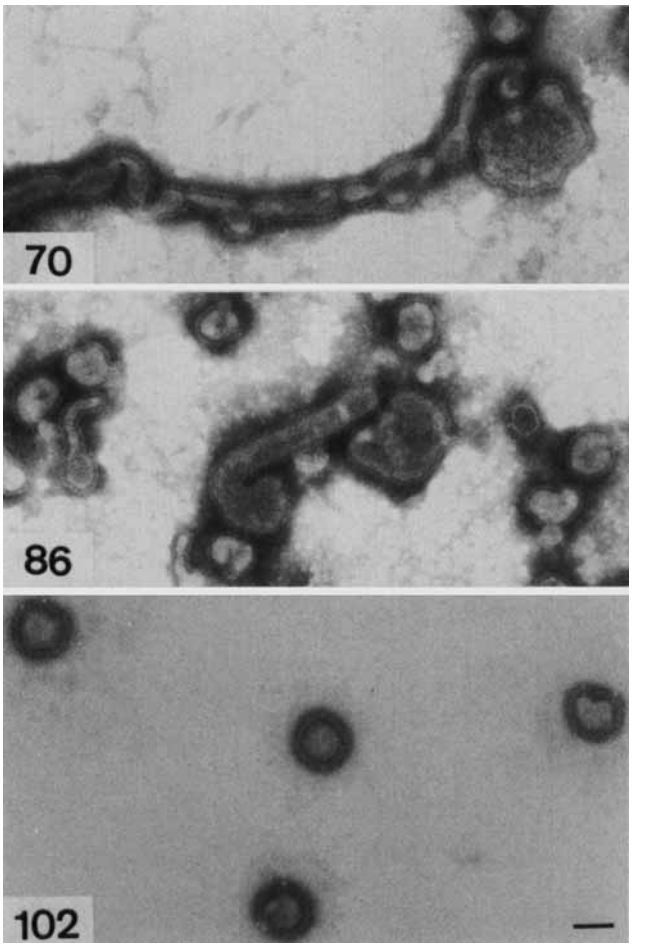

FIGURE 2 Electron micrographs of influenza virus obtained from gel chromatography were negatively stained. Numbers refer to fractions shown in Figure 1. The bar indicates 100 nm. The figure shows influenza virus of filamentous (Fraction #70), filamentous-spherical (Fraction #86) and spherical shape (Fraction #102).

CL Induction by "Small" and "Large" Influenza and Sendai Viruses

The fractions containing virus of different size were divided into four pools and concentrated by centrifugation (see Figure 1 for pool distribution). To compare CL induction by similar numbers of filamentous and spherical particles, the hemagglutinin titer of virus pools #1–4 was determined and a fixed amount of hemagglutinin was added to mouse spleen cells. Figure 4 shows that large influenza viruses (pool #1, ☆) were markedly more active in CL induction than small spherical viruses, the total light emission induced by filaments being approximately 10 times that stimulated by

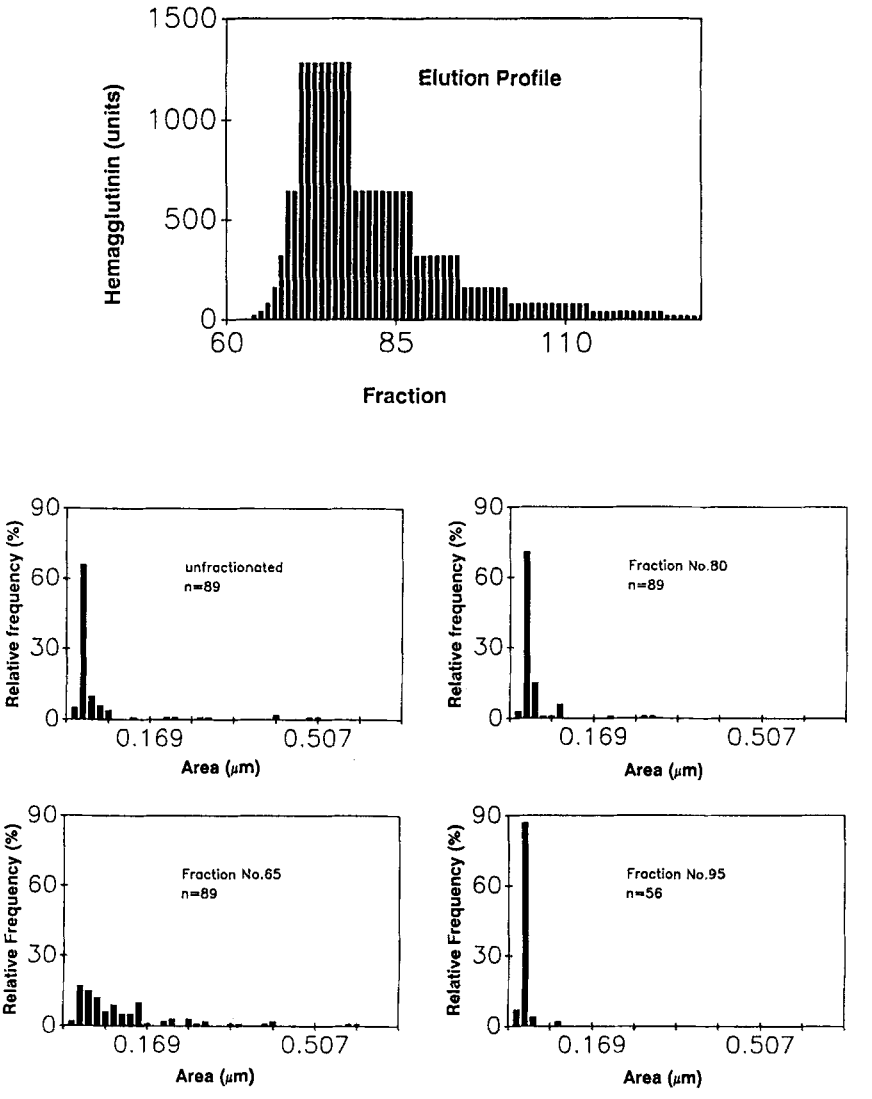

FIGURE 3 Particle size distribution of influenza virus subjected to gel filtration. Influenza virus was purified, chromatographed and processed for electron microscopy as described in Materials and Methods. Photographs were taken at a fixed magnification and the area enclosed by the contours of virus particles was measured using an image analyzer. The areas measured were divided in 35 size classes ranging from 0–0.59 μm^2.

spheres (pool #4, ○). Similar differences were observed when influenza virus strain A/Port Chalmers (H_3N_2) was used (not shown). The largest Sendai virus particles were also most active in CL induction (Figure 4). However, the difference between the various size classes was less than that seen with influenza virus, the ratio between pools #1 (☆) and #4 (○) being 1:0.31.

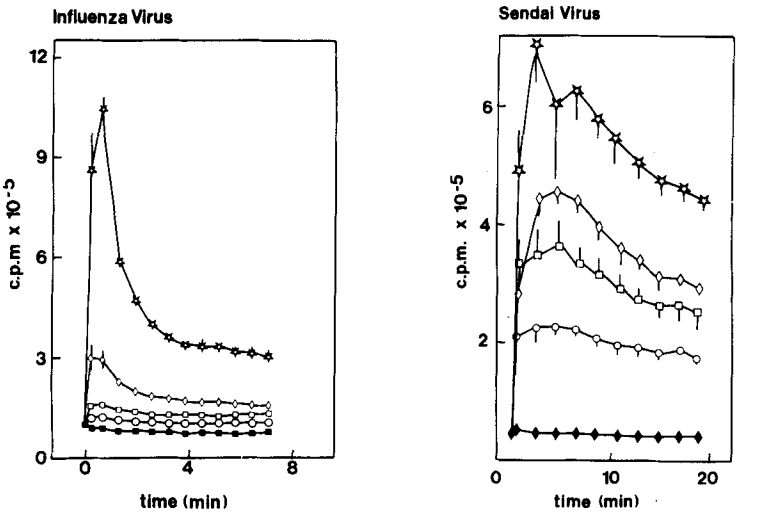

FIGURE 4 Chemiluminescence induced by influenza and Sendai virus in mouse spleen cells. Virus purified by gel filtration was pooled as indicated in Figure 1 and concentrated by centrifugation. CL was measured in a Liquid Scintillation Spectrometer as indicated in Materials and Methods. A virus dose of 80 HAU and 25 HAU/10^6 cells was used for influenza and Sendai virus, respectively. Zero time counts represent CL measured before addition of virus to the cells. Triplicate samples were run of each virus pool. Bars represent standard deviation. Symbols ☆, ◇, □, ○ refer to virus pools as indicated in Figure 1, ■, ◆ unstimulated controls.

Correlation between CL Induction and Hemolytic and Neuraminidase Activities

Sendai virus causes hemolysis at pH 7 and it has been demonstrated that influenza viruses induce hemolysis between pH 5–6.0.[25,26] We have shown earlier that the burst of CL evoked by Sendai virus correlates with the hemolytic activity of the virus.[2] As both hemolysis and CL are manifestations of the envelope glycoproteins, we investigated the relationship between the two phenomena. In addition, we determined the neuraminidase activity, which in influenza virus is manifested by a separate glycoprotein (N), while in Sendai virus this is expressed by one glycoprotein (HN), together with the hemagglutinating activity.

Figure 5 summarizes these experiments and compares the CL, hemolytic and neuraminidase activities with one another. For ease of comparison, the activities of pool #1 of each virus are taken as 100%. With Sendai virus, CL induction, hemolytic and neuraminidase activities decreased with the size of the viruses, the hemolytic activity decreased to a lesser extent than CL induction and neuraminidase activity. In contrast to Sendai virus, the hemolytic activity of influenza virus remained high even in pools #3 and #4 containing mainly small spherical viruses. In addition, neuraminidase activity decreased to a lesser extent than did CL induction.

Correlation between Hemagglutinating Activity, Protein Content and Infectivity in Influenza Virus

The comparison of the various biological activities of pooled virus fractions shown in Figures 4 and 5 had been based on identical amounts of hemagglutinin as estimated

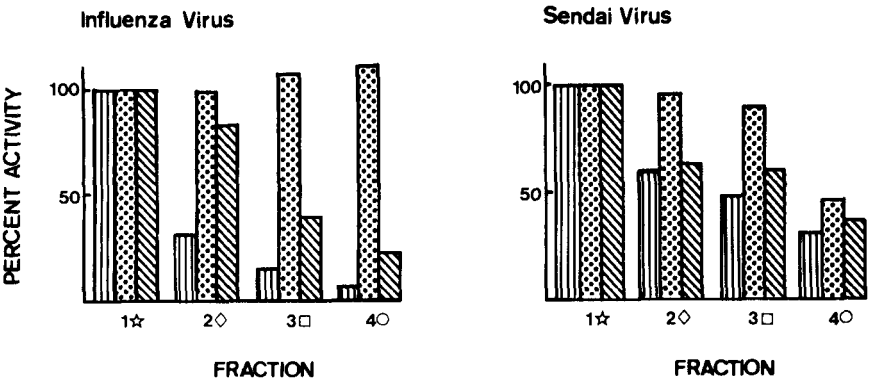

FIGURE 5 Chemiluminescence, hemolytic and neuraminidase activities of pooled influenza and Sendai virus fractions. Fractions obtained from gel filtration were concentrated by centrifugation and identical numbers of hemagglutinating units were assayed for CL, hemolytic and neuraminidase activities as indicated in Materials and Methods. Symbols ☆, ◇, □, ○ refer to virus pools as indicated in Figur 1. ■, chemiluminescence; ▨, hemolysis; ■, neuraminidase. CL refers to integrated light emission over 7.25 (influenza virus) and 19 minutes (Sendai virus). For ease of comparison, the activities of fraction 1(☆) are taken as 100 percent.

in the hemagglutination assay. Table I compares hemagglutinin of pooled fractions of influenza virus with protein content and infectivity. Protein per hemagglutinating unit was highest in pool #1, the ratio being 1:0.24. In contrast to protein content, infectivity in pools #1–4 was similar, being approximately 1/3 of influenza virus not subjected to gel filtration.

Effect of Sonication of Influenza Virus on Glycoprotein Related Activities

The previous experiment had shown that the protein content of filamentous influenza virus exceeds that of spherical particles having the same hemagglutination titer. To investigate CL induction by differently-sized influenza virus particles having the same protein content, pool #1, containing predominantly filaments, was subjected to sonication under conditions that resulted in breaking up the filaments into smaller particles (electron micrographs not shown). Table II shows that sonicated influenza

TABLE I
Protein/hemagglutinin and infectivity/hemagglutinin of influenza virus obtained from gel filitration.

Sample	Protein (ng/HAU)	Infectivity (EID_{50} / HAU)
before gel filtration	5.37	1.5×10^5
Pool #1	14.60	5.6×10^4
Pool #2	9.47	5.6×10^4
Pool #3	5.15	5.3×10^4
Pool #4	3.55	5.0×10^4

Protein was determined using the Bio-Rad protein assay with bovine gamma globulin as the standard. Infectivity of influenza virus was determined by inoculation of the allantoic cavity of 11 day old embryonated chicken eggs and assaying for hemagglutinin 48 hours post-infection.

HAU = hemagglutinating unit; EID_{50} = egg infectious $dose_{50}$.

TABLE II
Effect of sonication on biological activities of filamentous influenza virus

Biological activity	Percent of untreated control
Chemiluminescence	54
Hemagglutinin	300
Neuraminidase	100
Hemolysis	150

Filamentous influenza virus was obtained by gel filtration. Sonication and assay details are given in Materials and Methods. Sonication had no effect on the kinetics of CL and integrated light emission curves (7.25 min.) are therefore compared.

virus was less active in CL induction than the untreated control while the neuraminidase activity was not changed. In contrast, both the hemagglutination titer and the hemolytic activity were increased by this treatment.

DISCUSSION

The comparison of the membrane glycoprotein-related biological properties of small and large viruses depended on a method of preparing populations of predominantly large and small particles from the heterogeneous virus preparation. In contrast to sucrose gradient centrifugation, gel filtration fulfilled this criterion in a reproducible way.

Visual rating of the particle size of code-named influenza virus fractions correlated with the position of these fractions in the elution profile in three separate experiments. It became evident in these experiments that gel filtration did not completely free the filaments of spheres although it was possible to obtain pure populations of small spheres with this method (compare fractions #70 and #102 in Figure 2). This was further substantiated by the results of the morphometric analysis (Figure 3). However, the average size of the virus particles clearly decreased with increasing retention time (Figure 3). The estimation of the protein/HAU and infectivitiy/HAU ratios supplied additional evidence for this morphological difference. Thus, the infectivity/HAU ratio remained the same while the protein/HAU ratio decreased (Table I). Since the hemagglutinin titer reflects the number of virus particles,[27,28] these observations indicate that particles eluting late were smaller than those eluting early. Furthermore, the similar infectivity/HAU ratio argues against the possibility that the fractions containing small spheres contained significant amounts of free hemagglutinin originating from disrupted virus particles.

The glycoprotein-related biological activities of small and large influenza and Sendai viruses were compared using identical amounts of hemagglutinin as determined in the hemagglutination assay. The rationale for using the parameter was based on the view that the hemagglutinin titer reflects the number of virus particles.[27,28] This view was also supported by the increase in hemagglutinin titer observed after sonication (Table II). It could be argued that the difference in CL induction between filamentous and spherical particles observed reflects the simple fact that the latter contained less protein per hemagglutinating unit (Table I). However, this explanation is unlikely to be correct. Firstly, the difference in CL induction between filaments and spheres exceeds that of the protein content by more than twofold. Secondly, CL

induction by a fixed amount of viral protein was markedly decreased when the filaments were broken up into smaller particles (Table II). Since CL by influenza and paramyxoviruses is triggered by an interaction between the envelope glycoproteins and the cell membrane,[1-3] we compared CL with the other biological activities of the envelope spikes. It became obvious from these experiments that, together with lower CL induction, small Sendai viruses had also low hemolytic and neuraminidase activities (Figure 5). In contrast, with influenza virus, CL induction, but not the hemolytic activity was decreased in small virus particles. It is important to note that unlike Sendai-, the hemolytic activity of influenza virus, reflecting envelope-membrane fusion, is manifested at low pH only.[25,26] This suggests that the mechanism of CL induction by influenza virus is unrelated to a fusion event. The same conclusion can also be drawn from the effect of sonication, which increased the hemolytic while decreasing the CL inducing activities of influenza virus (Table II). Moreover, neuraminidase activity was not altered by sonication, arguing against a simple correlation between enzyme activity and CL induction. We suggest that the extent of cross-linking of cell surface receptors (multipoint binding) may be an important factor in the high CL inducing activity of filamentous virus particles. For spatial reasons, the probability of extensive cross linkages forming between the receptors is higher with a large virus than with a small one. The sonication experiment (Table II) suggests that extensive receptor cross-links resulting from the binding of a few large virus particles may trigger the CL response more efficiently than less extensive receptor cross-links induced by a large number of small virus particles. In addition, the lipid bilayer that forms the virus envelope has been demonstrated to be more fluid in filamentous influenza virus than in the spherical type.[29] It remains to be seen whether different membrane fluidity also plays a role in the difference in CL induction between spherical and filamentous influenza virus.

Finally, the observation that filamentous virus particles are more efficient in triggering CL also sheds an interesting light on the role of CL, and hence reactive oxygen, in the pathogenesis of virus disease. It is well known that influenza and paramyxoviruses exert toxic effects, some of which relate to the release from inflammatory cells of pyrogens.[10-14] Interestingly, filamentous influenza particles, observed mainly in virus freshly isolated from patients[16,17] are markedly more toxic[15] than spheres which predominate after prolonged passage in embryonated chicken eggs,[18,19] and the experiments reported in this paper show that they are also more active in CL induction. It seems conceivable that part of the virus toxicity as well as the inactivating effect of leukocytes on myxoviruses[30] may relate to the triggering of reactive oxygen generation that is reflected in CL. In fact, Sendai virus has recently been demonstrated to enhance cell-mediated toxicity,[31] and radicals have earlier been shown to inactivate viruses.[32] Moreover, in a mouse model of influenza, we have obtained evidence for oxidative stress *in vivo*[33] and very recently, pyran-conjugated SOD has been shown to protect mice from the lethal effect of infection with influenza virus.[34]

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