Phagocytosis and ingestion of influenza virus by human polymorphonuclear leucocytes *in vitro*: electronmicroscopy studies

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Summary. Interaction of human polymorphonuclear leucocytes (PMNL) and influenza virus (IFV) was studied *in vitro*. At 0°C, the viral particles were bound extensively to the surface of the PMNL membrane with a ratio of about 1000 virus particles to a single PMNL. The binding was sensitive to neuraminidase, suggesting attachment through sialo-compound receptors. At 37°C, the virus particles disappeared rapidly from the cell surface, about half of them being eluted and the remainder being endocytosed into the cytoplasmic vesicles. Immuno-gold electronmicroscopy suggests that the virus particles are ingested into phagosomal vesicles and lysed.

Introduction

Polymorphonuclear leucocytes (PMNL) provide the primary host defence against bacterial infection, by means of phagocytosis, lysosomal enzymes and the oxygen-dependent bactericidal system (Weissmann *et al.*, 1971, 1973; Amano *et al.*, 1975; Babior, 1978; Allen *et al.*, 1981). Their role in virus infection, however, is not well established, although there have been several studies *in vitro* and *in vivo*. In the case of influenza virus (IFV), *in-vitro* studies have demonstrated PMNL damage, with suppression of chemotactic and phagocytic activities (Schlesinger *et al.*, 1976; Ruutu *et al.*, 1977; Kiyotani *et al.*, 1985; Abramson *et al.*, 1986; Moore and Mills, 1987). Indeed, IFV infection is sometimes followed by secondary bacterial infection, attributed to suppression of PMNL functions (Young *et al.*, 1972; Fischer and Walker, 1979). On the other hand, there is evidence, from *in-vivo* studies, for an early protective role for these cells against IFV infection (Fujisawa *et al.*, 1987; Tsuru *et al.*, 1987), and activation of the oxygen-dependent microbicidal system, in the presence of IFV, is a possible mechanism for inactivation of the virus (Sweet and Smith, 1980; Mills *et al.*, 1981).

In an attempt to elucidate more precisely the role of PMNL in virus infection, we have studied the interaction of human PMNL and IFV by electronmicroscopy.

Materials and methods

Virus

Influenza virus, strain A/PR/8/34 (H0N1), was purified from the infected chorioallantoic fluid of embryonated hens' eggs by differential and sucrose-gradient centrifugation.

Determination of plaque-forming units (pfu) of virus

This was done in the canine kidney cell line (MDCK), by the method of Tobita *et al.* (1975).

Polymorphonuclear leucocytes

These were prepared as described previously (Suzuki *et al.*, 1983). Briefly, a suspension of PMNL and red blood cells was obtained from heparinised (20 IU/ml) peripheral blood of healthy adult volunteers by the method of Böyum (1968), with Lymphoprep (Nyegaard Co., Norway). The red blood cells were sedimented with dextran solution 1.5% w/v (mol. wt c. 200 000; Nakarai Chemicals Ltd, Japan) and removed with lysis buffer. The PMNL were then washed twice with Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS), by centrifugation at 350g for 10 min at 20°C, and suspended in Hanks's balanced salt solution (HBSS).

Preparation of PMNL-IFV complexes

For the electronmicroscopy studies, purified IFV—60 000 haemagglutinating (HA) units, equivalent to c. 10¹¹ pfu—were mixed with 4 × 10⁷ PMNL in 40 ml of

HBSS at 0°C. After 45 min at 0°C, non-adsorbed virus was removed by centrifugation at 350 *g* for 5 min at 4°C. The pellets of PMNL-IFV complexes were washed with cold PBS once and resuspended in one-twentieth of the original volume (2.0 ml) of cold HBSS. The suspension was then placed, in 0.5-ml amounts, into Eppendorf microtubes and incubated at 37°C. For virus-release experiment, a reduced virus (10 000 HA) and PMNL (4×10^7) were mixed to increase the sensitivity of titration of the virus, and the complexes were resuspended in one-eighth of the original volume (5 ml) of HBSS.

Assay of virus liberated from the PMNL surface

(1) Duplicate sets of virus-PMNL complexes were prepared and to one set bacterial neuraminidase (Boehringer Mannheim, Germany) was added to a concentration of 100 µg/ml. At the end of each period of subsequent incubation, the suspensions were centrifuged at 1800*g* for 5 min at 4°C and the supernates were titrated for virus HA activity, with chicken erythrocytes, in a cold room.

(2) The total amount of virus within the complexes, before incubation, was determined by solubilising the complexes with Triton X-100 1% and then estimating viral neuraminidase activity. This was done by mixing equal volumes of the solubilised suspension and the neuraminidase substrate, fetuin (Gibco Laboratories, USA), 1.0% in 0.2 M phosphate buffer (pH 5.9), and incubating for 30 min at 37°C; released sialic acid was determined by the method of Aminoff (1961).

Preparation for electronmicroscopy

Samples of the PMNL-IFV complex suspensions were pre-fixed with a mixture of glutaraldehyde 2.5% and paraformaldehyde 2.0% in HBSS. After washing with PBS, by centrifugation at 8000*g* for 2 min at 4°C, they were post-fixed with OsO₄ 1% in HBSS. Part of each sample was dehydrated and embedded in epoxy resin; ultrathin sections were cut, stained with uranylacetate 4% and examined with a Hitachi H-500 transmission electronmicroscope (TEM) at 75 kV. The rest of the sample was dehydrated, dried by the critical point method, coated with platinum-palladium by an ion-cater, and examined with a Hitachi S-700 scanning electronmicroscope (SEM).

Immuno-gold electronmicroscopy

PMNL-IFV complexes were incubated at 37°C for 30 min and then fixed with glutaraldehyde 2.5%-paraformaldehyde 2.0%, dehydrated, and embedded in Lowicryl K 4M resin with UV-irradiation. Thin sections were cut, and were treated with 5 HI (haemagglutination inhibition) units of rabbit anti-PR8 IgG—purified through protein A-Sepharose (Pharmacia, Sweden)—followed by anti-rabbit IgG conjugated to 10-nm-diameter colloidal gold (EY Laboratories Inc., USA) by the method of Roth (1982).

Results

Binding of IFV to PMNL

Serial 10-fold dilutions of a suspension of IFV, from 1.8×10^7 to 1.8×10^2 pfu, were added to an equal volume of a suspension of PMNL (2×10^6 cells/ml) at 0°C. The mixtures were centrifuged at 1800*g* for 5 min at 4°C and the amount of unadsorbed virus in each supernate was determined (table I). Adsorption of more than 80% of added virus was attained with virus concentrations of 9.0×10^4 pfu and lower, but the adsorption level decreased to 30–50% with higher virus concentrations.

SEM examination of PMNL-IFV complexes

An excess amount of IFV was added to human PMNL at 0°C and the mixture was allowed to stand at 0°C for 45 min. The PMNL were then recovered by centrifugation, fixed and examined by SEM (fig. 1). Fig. 1a shows extensive attachment of virus particles to the surface of the cell membrane. About 1000 particles were found to bind to a single PMNL (fig. 2). When the PMNL-IFV complexes were incubated at 37°C, the countable virus particles on the cell surface rapidly decreased (figs. 1b–d); after 10 min, the number of adsorbed virus particles decreased by about 80%, and by greater than 99% after 30 min (fig. 2).

Nature of the association between PMNL and IFV

Because IFV adsorbed to the cell surface of red blood cells and respiratory epithelial cells detaches when incubated at 37°C (Hirst, 1942, 1943), we examined whether this was also so with PMNL. Mixtures of virus and cells were incubated (see

Table I. Adsorption of IFV to PMNL at various virus doses

Amount of virus (pfu/0.1 ml)		Adsorption ratio (%)
added*	unadsorbed	
9.0×10^6	6.0×10^6	33.3
9.0×10^5	4.2×10^5	53.3
9.0×10^4	1.9×10^4	80.0
9.0×10^3	1.3×10^3	85.6
9.0×10^2	0.6×10^2	93.3
9.0×10^1	1.1×10^1	87.8

* Each dilution of virus was mixed with an equal volume of PMNL suspension (2×10^6 cells/ml).

Fig. 1. Scanning electronmicrograph (SEM) of PMNL mixed with IFV, showing loss of adsorbed virus particles after incubation at 37°C for (a) 0 min, (b) 2 min, (c) 10 min and (d) 30 min. Scale = 1 μm.

Fig. 2. Number of virus particles bound per PMNL cell surface after incubation at 37°C. Counts are the average from 15 cells examined by SEM. Particles were counted on the upper cell surface and the number was doubled to give an estimate for the whole cell surface. Bars represent SD.

Fig. 3. Release of IFV from PMNL surface after incubation at 37°C, with (●—●) or without (○—○) added bacterial neuraminidase.

Materials and methods) and, after centrifugation, the supernates were titrated for virus HA. The HA titre in the supernate increased with the time of incubation, to reach a maximal level after 20 min (fig. 3). Bacterial neuraminidase added to the virus-cell mixture did not increase the amount of HA released during the incubation period (fig. 3).

The total amount of virus present within the

Table II. Release of IFV from PMNL-virus complexes after incubation at 37°C

Period of incubation (min)	Sample tested for neuraminidase activity	Neuraminidase activity (adsorption at 549 nm)	Virus release (%)
0	supernate	0.045	25.0
5	supernate	0.060	33.3
10	supernate	0.080	44.4
30	supernate	0.090	50.0
—	solubilised complex*	0.180	100.0

* Without incubation.

PMNL-virus complexes was determined by solubilisation of the complexes with Triton X-100 and assay of viral neuraminidase activity; the HA technique does not measure cell-bound virus activity (table II). When non-solubilised virus-cell mixtures were incubated at 37°C and the supernates tested for neuraminidase activity, only about 44% of the total available neuraminidase activity was released into the supernate after 10 min, with only a slight further release, to 50%, after 30 min. This contrasted with less than 1% of virus particles visible by SEM on the PMNL after incubation for 30 min (figs. 1 and 2).

Phagocytosis of virus by PMNL

To investigate the possible internalisation of IFV within the cells, we examined the PMNL-IFV complexes by TEM. Fig. 4a shows that, before incubation, virus particles were observed only on the surface of PMNL. When the complexes were incubated at 37°C for 2 min, endocytosed virus particles were clearly demonstrable within cytoplasmic vesicles, with many virus particles remain-

Fig. 4. Transmission electronmicrograph (TEM) of PMNL mixed with IFV, showing the presence of intact (single arrows) and deformed (double arrow) particles within cytoplasmic vacuoles, after incubation at 37°C for (a) 0 min, (b) 2 min, (c) 10 min and (d) 30 min. Scale = 1 μ m.

ing at the cell surface, which agreed with the results of SEM observation. On further incubation for 10–30 min, small phagosomes with a few virus particles (figs. 4b, c) and larger phagosomes containing many virus particles (fig. 4c) or a single particle (single arrow in fig. 4d) were seen. As indicated by the double arrow in fig. 4d, fuzzy particles, as if being digested or degraded in the phagosomal vesicle, were observed together with intact virus particles.

Detection of viral antigen within the phagosome vesicles

When the ultrathin sections of PMNL-IFV complexes, without incubation, were treated with anti-IFV IgG and then with gold-labelled anti-IgG, colloidal gold grains were observed associated with virus particles on the PMNL surface membrane but not in the cytoplasm (fig. 5a), indicating viral antigen-specific binding of the colloidal gold. On the other hand, when the complexes were incubated at 37°C for 10 min, many gold grains were seen associated with both intact and deformed virus particles within the phagosomal vesicles of the cytoplasm (fig. 5b).

Discussion

As expected from previous reports (Abramson *et*

al., 1986; Moore and Mills, 1987), IFV bound extensively to human PMNL *in vitro*. The binding of virus was more than 80–90% efficient, but failed to reach 100% even with the highest dilution of virus. This does not necessarily mean weak binding activity, as similar incomplete adsorption was also observed with MDCK cells, which readily support the replication of IFV (data not shown). Effective adsorption was confirmed by SEM, which demonstrated approximately 1000 virus particles on the surface of each PMNL (fig. 2).

SEM observation also showed that after incubation at 37°C the virus particles on the cell surface rapidly disappeared. This could be due either to phagocytosis of the virus particles by the PMNL or to their release from the cell surface by their own specific receptor-destroying enzyme. Virus release undoubtedly occurs, and the close correspondence between the amounts of virus HA released on incubation in the presence and absence of bacterial-derived neuraminidase strongly suggests that the virus binds to the PMNL surface through a sialo-compound receptor specific for IFV. However, no more than half the total virus amount associated with cells, as estimated after solubilisation of the virus-cell complexes, could be liberated. This appeared to contradict the SEM findings, namely that fewer than 1% of the virus remained on the cell

Fig. 5. Immuno-gold electronmicrograph of PMNL mixed with IFV, after incubation at 37°C for (a) 0 min and (b) 10 min, showing intact (single arrows) and fuzzy (double arrow) virus particles. Scale = 1 μ m.

surface, suggesting that IFV was taken into the cells. Indeed, this was confirmed by the TEM results. The loss of IFV particles from the PMNL surface, on incubation, is, therefore, due to the mutually competitive activities of phagocytosis and receptor-destroying release.

The TEM experiments showed that the phagocytic activity of the PMNL was temperature-dependent, no virus particles being detected within the phagosomes after standing at 0°C. This agrees with the electronmicroscopy observations of Abramson *et al.* (1986). At 37°C, virus particles rapidly appeared in the phagosomes within the first 2 min of incubation and phagocytosis progressed thereafter. The virus particles in the phagosomes looked intact, with rigid conformation, during the first 2–10 min of incubation, but became fuzzy in appearance after 30 min. This suggests that the phagocytosed viruses are subsequently digested by lysosomal enzymes.

In host defence, macrophages are known to respond to virus infection, but, as they frequently support the growth of viruses, they are not always defensive against invading viruses (Mogensen, 1979; Inada and Mims, 1984; Nagai *et al.*, 1985;

Rowson and Mahy, 1985; Rossiter and Wardley, 1985; Cardoso *et al.*, 1986; Domke-Opitz *et al.*, 1986; Koenig *et al.*, 1986). On the other hand, studies on the role of PMNL in virus infection are very few. There are several reports that the biological functions of PMNL are suppressed in the presence of IFV (Schlesinger *et al.*, 1976; Ruutu *et al.*, 1977; Kiyotani *et al.*, 1985; Abramson *et al.*, 1986; Moore and Mills, 1987). More recently, a protective role has been suggested for PMNL in the early stages of IFV infection (Fujisawa *et al.*, 1987; Tsuru *et al.*, 1987). In these studies, however, the evidence for protection was indirect, based on the use of γ -irradiated or carageenan-treated mice. Our electronmicroscopy study has directly demonstrated, *in vitro*, that PMNL extensively adsorb and then phagocytose IFV, possibly into phagosomal vesicles. Moreover, we have unpublished data that PMNL are non-permissive for growth of IFV. Therefore, these present observations suggest a possible role for PMNL as a virus-killing factor in the early stages of virus infection. We are currently studying the inactivation of IFV by PMNL by biochemical approaches.

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