Influenza virus pyrogenicity: central role of structural orientation of virion components and involvement of viral lipid and glycoproteins

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Ultraviolet light-inactivated, non-infectious influenza virus is pyrogenic; virion components are probably responsible for this pyrogenicity. To try to identify the pyrogenic component, influenza virions were disrupted with either bromelain or sodium deoxycholate (DOC). Treatment of infectious virions with bromelain, under conditions that removed the surface glycoproteins (spikes), destroyed their pyrogenicity. The supernatant, containing non-aggregated and modified glycoproteins, was also non-pyrogenic. Disruption of virions with DOC considerably reduced pyrogenicity; however, some was retained by the sub-viral cores. Viral nucleoprotein and matrix protein, purified from the supernatant, were non-pyrogenic. Aggregated stellate clusters of surface glycoproteins separated on sucrose gradients were pyrogenic in half of numerous tests performed with different batches of material. Treatment of virus with ether resulted in complete loss of pyrogenicity. Liposomes made from extracted viral lipid were non-pyrogenic. In contrast, virosomes made from the viral lipid and the aggregated stellate clusters of surface glycoproteins were pyrogenic. Hence, optimum pyrogenicity depends upon the integrity of the virus particle, but haemagglutinin and/or neuraminidase appear essential, and lipid may be involved.

Introduction

In humans, fever and other constitutional symptoms of influenza (headache, myalgia, listlessness, nausea, shivering, anorexia and depression) result from the action of endogenous pyrogen (EP) produced by phagocytes in response to infection (Rawlins & Cranston, 1973; Scott et al., 1981; Dinarello & Wolff, 1982; Gander, 1982). Influenza viruses vary in their ability to cause these constitutional effects. Recent H1N1 isolates produced milder illness and lower fevers compared to H3N2 viruses (Kung et al., 1978; Wright et al., 1981; Frank et al., 1985). Similarly, in ferrets, infection with an old [A/Puerto Rico/8/34 (PR8; H1N1)] and two recent [A/USSR/90/77 (A/USSR), A/Fiji/15899 (A/Fiji)] H1N1 human isolates produced less fever than did two virulent H3N2 clones, 7a and 64c, of the reassortant virus, A/Puerto Rico/8/34–A/England/939/69 (Toms et al., 1977; Sweet et al., 1979; Matsuyama et al., 1980; Coates et al., 1986). In such intranasally infected ferrets, fever results from EP liberated by virus–phagocyte interaction in the upper respiratory tract (URT) (Sweet et al., 1979). Clones 7a and 64c induced more EP from inflammatory phagocytes than did PR/8, A/USSR or A/Fiji (Tinsley et al., 1987). The nature of the EP induced by influenza virus is unclear but levels of EP do not correlate with levels of the known pyrogenic cytokines, interleukin (IL)-1, IL-6 or tumour necrosis factor (TNF) (Jakeman et al., 1991).

Since fever results from virus–phagocyte interaction in the URT, the different levels of fever elicited by the various strains could result from variation in (i) the amount of virus in the URT, (ii) the number of phagocytes in the URT, (iii) the pyrogenicity of the strains, i.e. their capacity to release EP from phagocytes or (iv) a combination of these factors. The recent H1N1 isolates, A/USSR and A/Fiji, elicited low fevers despite levels of virus and phagocytes as high as those for the virulent clones 7a and 64c, which elicited high fevers, suggesting that the former pair was less pyrogenic than the latter pair. Intracardial inoculation of ferrets with large quantities of purified influenza virus produced a rapid febrile response that was significantly lower for the two H1N1 viruses than for the two H3N2 viruses (Coates et al., 1986). Ultraviolet light-inactivated virus, which was shown to be non-infectious, also produced fever but at a lower level than did infectious virus (Coates et al., 1986). Again the febrile response was less for the two H1N1 viruses than for the two H3N2 viruses. This work
suggested that virion components were EP-inducing and that these varied in nature and/or amount between the H1N1 and H3N2 viruses.

There are two approaches to identifying the viral pyrogenic components and investigating any inter-virus differences: disruption and fractionation of virions and generation and examination of reassortants. This paper describes attempts to identify the pyrogenic components of the virulent H3N2 clone 64c by fractionation of virus disrupted by enzymatic action or detergent treatment. Fractions from disrupted virus were assayed for protein content, haemagglutinating activity and pyrogenicity. The structure of the virion components in the fractions was examined by electron microscopy and the protein composition of the fractions was determined using SDS-PAGE and Western blotting with monoclonal antibodies (MAbs).

**Methods**

**Viruses and virus assay.** The H3N2 clone 64c of the A/Puerto Rico/8/34–A/England/939/69 reassortant virus system has been described previously (Sweet et al., 1974a,b; Matsuyama et al., 1980). Seed stocks were prepared and titrated in eggs or allantois-on-shell cultures (egg-bits) as described previously (Sweet et al., 1974a); titres are expressed as 50% egg infectious dose (EID₅₀) or 50% egg-bit infectious dose.

**Preparation of purified influenza virus.** Twelve-day-old hens' eggs in batches of 100 were inoculated intra-allantoically with 10⁻⁷ EID₅₀ per egg of clone 64c seed stock. After 48 h incubation at 35 °C, the eggs were chilled for at least 4 h, allantoic fluid was harvested and the debris removed by low-speed centrifugation (1000 g, 4 °C, 30 min). The fluid was then centrifuged at 80000 g for 1.5 h at 4 °C and the virus pellets were pooled and resuspended in a volume not greater than 1 ml of PBSA by incubation at 4 °C overnight. Aliquots (0.5 ml) of the supernatant containing the liberated surface glycoproteins were layered onto pre-formed 12 ml linear 20 to 60% (w/v) sucrose gradients and centrifuged at 160000 g for 18 h at 4 °C. One ml fractions were removed sequentially from the top of the centrifuge tubes and dialysed against PBSA for 3 days with three changes of buffer or in a micro-dialyser 500 (Pierce & Warriner) for 6 h with three changes of buffer. Fractions were stored at −70 °C for subsequent analysis.

**Disruption of purified virus with detergent and separation of viral components.** Purified viral pellets in 1 ml of PBSA were treated with an equal volume of 5% (w/v) sodium deoxycholate (DOC) and incubated at 37 °C for 5 min (Liew et al., 1979). Some of the treated mixture was dialysed against three changes of PBSA over 3 days, the protein concentration was determined and the mixture was stored at −70 °C prior to the pyrogenicity assay. The remainder was centrifuged at 100000 g for 40 min to pellet intact sub-viral cores (SVCs) (Liew et al., 1979) which were washed with PBSA before being resuspended in approximately 1 ml of PBSA by incubation at 4 °C overnight. Aliquots (0.5 ml) of the supernatant containing the liberated surface glycoproteins were layered onto pre-formed 12 ml linear 20 to 60% (w/v) sucrose gradients and centrifuged at 160000 g for 18 h at 4 °C. One ml fractions were removed sequentially from the top of the centrifuge tubes and dialysed against PBSA for 3 days with three changes of buffer or in a micro-dialyser 500 (Pierce & Warriner) for 6 h with three changes of buffer. Fractions were stored at −70 °C for subsequent analysis.

**Treatment of virus with ether.** The method was essentially that of Siegert & Braune (1963). Virus preparations were diluted to 1 mg viral protein/ml in PBSA and an equal volume of diethyl ether (May and Baker) was added in an atmosphere of nitrogen gas. The mixture was shaken vigorously for 3 h at 4 °C when the ether was removed by evaporation under a stream of nitrogen.

**Extraction of viral lipids and preparation of liposomes.** Purified viral particles were resuspended in deionized distilled water (DDW), frozen by immersion in liquid nitrogen and dried under vacuum (4 K modulyo freeze dryer; Edwards High Vacuum Industries). Lipid was extracted by a modification of the methods of Folch et al. (1957) and Liew et al. (1979). Lyophilized virus particles were resuspended in 10-fold their original wet volume of chloriform/methanol (2:1 v/v). The specific gravity of the suspension was lowered by the addition of 0.2 volumes methanol and the residual solid material was removed by centrifugation at 100000 g for 45 min at 4 °C. The supernatant (first lipid extract) was transferred to a Universal bottle. The pellet was then treated with 10 ml chloriform/methanol (1:2 v/v) to extract glycolipid species and centrifuged as above. The supernatant (second lipid extract) was stored in a separate Universal bottle and the pellet resuspended in approximately 1 ml of PBSA by incubation at 4 °C overnight.

The first lipid extract was purified further by washing with the 'pure solvent upper phase' (Folch et al., 1957). This was prepared by mixing chloriform/methanol and a solution of CaCl₂ (0.5 g/l in DDW) in the proportions 8:4:3. After centrifugation at 300 g for 10 min at 20 °C, a biphasic system was obtained with chloriform/methanol/CaCl₂ partitioning in the upper phase in the ratio 86:14:1. The upper phase was removed. After restoring the chloriform/methanol ratio in the first crude lipid extract to 2:1 by the addition of chloriform, 0.2 volume of a solution of CaCl₂ (0.5 g/l) was added. The mixture was stirred thoroughly, then centrifuged (300 g, 10 min, 20 °C) to separate the two
Fig. 1. Transmission electron micrograph of (a) whole untreated purified virus, (b) virus treated with 5 units of bromelain for 16 h at 35 °C and (c) virus treated with two consecutive incubations with 5 units of bromelain for 16 h at 35 °C. Bar markers represent 50 nm.

Table 1. Mean fevers in ferrets elicited by u.v.-inactivated virus and spikeless virions and supernatants obtained after two treatments with bromelain or after one treatment with DOC

<table>
<thead>
<tr>
<th>Ferret dose</th>
<th>Mean HA titre (HA/μg)</th>
<th>Protein (μg/kg)</th>
<th>Composition</th>
<th>Mean fever index (°C) No.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td>Viral proteins</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>U.v.-inactivated virus</td>
<td>36 (8)†</td>
<td>200</td>
<td>HA, NA, NP, M</td>
<td>Whole virus</td>
</tr>
<tr>
<td>Bromelain-treated virus§</td>
<td>&lt;0·03</td>
<td>250</td>
<td>NP, M</td>
<td>Spikeless</td>
</tr>
<tr>
<td>Spikeless virions</td>
<td>&lt;0·2</td>
<td>300</td>
<td>HA, NA</td>
<td>ND¶</td>
</tr>
<tr>
<td>Supernatant</td>
<td>&lt;0·03</td>
<td>250</td>
<td>NP, M</td>
<td>Spikeless</td>
</tr>
<tr>
<td>DOC-disrupted virus</td>
<td>4·6 (3)</td>
<td>200</td>
<td>HA, NA, NP, M</td>
<td>ND</td>
</tr>
<tr>
<td>Unseparated preparation</td>
<td>3·2 (1)</td>
<td>200–300</td>
<td>NP, M</td>
<td>Small bald particles</td>
</tr>
<tr>
<td>SVCS**</td>
<td>96·0 (28)</td>
<td>200–300</td>
<td>HA, NA, M</td>
<td>Stellate clusters</td>
</tr>
<tr>
<td>Liberated glycoproteins**</td>
<td>&lt;0·1</td>
<td>130–180</td>
<td>NP</td>
<td>ND</td>
</tr>
<tr>
<td>Purified NP</td>
<td>&lt;0·05</td>
<td>240–250</td>
<td>M</td>
<td>ND</td>
</tr>
<tr>
<td>Purified M protein</td>
<td>&lt;0·05</td>
<td>240–250</td>
<td>M</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Number of preparations examined.
† S.E.M. in parentheses.
‡ Mean fever indices, S.E.M. in parentheses, were calculated mathematically from the areas under the fever curves for individual animals.
§ Infectious virus was treated with two doses of bromelain, the bromelain was removed by dialysis and then the preparation was centrifuged to produce spikeless virions and supernatants.
¶ Where no rise in rectal temperatures occurred, the fever index was given a value of zero.

** After removal of SVCs by centrifugation, glycoproteins were purified and fractionated from the supernatant by sucrose gradient centrifugation.

phases. As much of the upper phase as possible was removed with a Pasteur pipette and discarded. The pure solvent upper phase (1·5 ml) was allowed to flow gently down the side of the bottle so that it collected on top of the lower phase without any mixing. The bottle was rotated during this procedure to facilitate removal of residual upper phase with a Pasteur pipette as before. This was repeated twice.

The washed first lipid extract was then pooled with the second crude lipid extract and dried under vacuum in a rotary evaporator at 32 °C. The lipid was resuspended in about 2 ml PBSA by vigorous shaking with 3·5 to 4·0 mm glass beads. It was then ultrasonicated for 1 h at a frequency of 50 Hz in a sonicating waterbath to generate liposomes (Gregoriadis et al., 1971; Almeida et al., 1975). Doses of liposomes inoculated into ferrets contained from 6·8- to 10·6-fold more lipid than did the standard dose (200 μg/kg) of whole virus.

Preparation of virosomes. Virosomes were prepared from viral lipid and haemagglutinin/neuraminidase (HA/NA) or nucleoprotein (NP) preparations using a modification of the method described by Almeida et al. (1975). A volume of less than 1 ml of the relevant dialysed sucrose density gradient fraction, previously shown to contain only NP by SDS-PAGE (see Results), was added to dried viral lipid (see above), which was then resuspended by vigorous shaking with 3·5 to 4·0 mm
glass beads. NP virosomes were formed by sonicating this suspension in a water bath for 1 h at 50 Hz. The doses of NP virosomes inoculated into ferrets contained 3.5-fold more NP than did the standard dose (200 μg/kg) of whole virus and from 14- to 18-fold more lipid.

HA/NA virosomes were formed by adding less than 1 ml of dialysed gradient fractions containing high HA activity, previously shown by SDS–PAGE to be free of NP, to a pellet of liposomes prepared as described above. Virosomes with external HA/NA spikes were formed by sonicating the suspension in a water bath for 15 min at 50 Hz. After centrifugation (100000 g, 5 °C, 45 min), the supernatant was removed and the pellet resuspended in the original volume of PBSA. HA/NA virosomes were inoculated into ferrets at doses containing 2.3- to 3.8-fold more HA/NA than the standard dose (200 μg/kg) of whole virus and from 8- to 30-fold more lipid.

**MAbs.** MAbs X-31 MC21 (anti-HA), X-31 NC92 (anti-NA) and aal01 (anti-NP) were kindly supplied by Dr Alan Douglas, World Influenza Centre, NIMR, London, U.K., and M2781/4 [anti-matrix(M) protein] by Professor Nigel Dimmock, Department of Biological Sciences, Warwick University, U.K.

**PAGE and Western blot analysis.** Samples containing virions or virion components were heated at 100 °C for 10 min after addition of an equal volume of 0.06 M-Tris–HCl pH 6.8 containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) sucrose and 0.002% (w/v) bromophenol blue. They were then analysed by SDS–PAGE. Discontinuous vertical slab gels were used according to the method of Laemmli (1970) in which the resolving gel contained 7.5% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.39 M-Tris–HCl pH 8.8. Usually 10 μl of a sample containing 75 μg protein/ml was loaded on each lane of the gels. Occasionally, two to three times this amount was loaded to show up particular proteins. All lanes on the same gel were loaded with the same amount of protein. Following electrophoresis at 200 V for 45 min at 4 °C, resolved proteins were either stained with Page Blue [0.0552% w/v Page Blue 83 (electran; BDH), 45% v/v methanol, 5% v/v acetic acid] or transferred to nitrocellulose filters at 0–1 A overnight with a Bio-Rad blotting cassette in a Bio-Rad trans-blot cell filled with blotting buffer (0.025 M-Tris, 0.19 M-glycine, 80% methanol). After drying at 20 °C for 1 h, filters were treated for 30 min at 20 °C with 3% (w/v) BSA in PBSA. Blotting was then carried out at 20 °C for 90 min with relevant IgG MAbs (20-480 units/ml) in freshly prepared PAT buffer (0.1% BSA, 0.1% v/v Tween-20 in PBSA). The filter was rinsed with three successive amounts of PBSA at room temperature (5 min each) and then treated for 90 min at room temperature with 20 ml of peroxidase-labelled goat anti-mouse IgG (Nordic Immunological Laboratories) diluted 1/1000 in PAT buffer. After washing three times in PBSA, bands were developed by incubation with 40 ml peroxidase substrate (0.6% w/v 4-chloro-1-naphthol, 6% methanol, 0.1% hydrogen peroxide in PBSA).

**Electron microscopy.** Specimens of virus or virion components, diluted to a protein concentration of about 1 mg/ml in PBSA, were added to colloidian- or formvar-coated electron microscope support grids, negatively stained with 1% (w/v) uranyl acetate pH 6.8 or 4% (w/v) sodium tungstosilicate and examined in a JEOL JEM-1200 EX transmission electron microscope at 80 kV.

![Fig. 2. SDS–PAGE analysis of viral polypeptides in (a) spikeless viral particles and (b) supernatants. Lanes 2 and 3 in (a) and (b) are preparations from two different batches of purified virus treated with two consecutive incubations of 5 units of bromelain for 16 h at 35 °C. Lanes 1 in (a) and (b) contain whole untreated purified virus and M, markers respectively.](image-url)
Fig. 3. Western blot analysis of viral polypeptides in (a) spikeless viral particles and (b) surface glycoproteins of two batches of purified virus (lanes 1, 3, 5, batch 1; lanes 2, 4, 6, batch 2) treated with two consecutive incubations with 5 units of bromelain for 16 h at 35 °C. Lanes 1 and 2 were incubated with anti-NA, lanes 3 and 4 with anti-HA and lanes 5 and 6 with anti-NP MAb.

Table 2. Mean fevers in ferrets elicited by u.v.-inactivated virus, ether-treated virus, virus lipid (liposomes), residual solid material, NP–virosomes and HA/NA–virosomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ferret dose (µg/kg)</th>
<th>Protein Mean HA titre (HA/µg)</th>
<th>Composition</th>
<th>Mean fever index (°Ch)</th>
<th>No.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.v.-inactivated virus</td>
<td>200</td>
<td>36-0 (8)†</td>
<td>HA, NA, NP, M Whole virus</td>
<td>6-0 (0-8)‡</td>
<td>6</td>
</tr>
<tr>
<td>Ether-treated virus</td>
<td>250</td>
<td>&lt;0-3</td>
<td>HA, NA, NP, M Clusters, reassociated</td>
<td>0-1 (0-1)</td>
<td>2</td>
</tr>
<tr>
<td>Virus lipid (liposomes)§</td>
<td>0</td>
<td>0</td>
<td>None        Liposomes</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Residual solid material</td>
<td>250</td>
<td>&lt;0-13</td>
<td>HA, NA, NP, M Debris, small particles</td>
<td>0-7 (0-3)</td>
<td>2</td>
</tr>
<tr>
<td>NP–virosomes</td>
<td>180</td>
<td>?</td>
<td>NP          Liposomes</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>HA/NA–virosomes</td>
<td>100</td>
<td>0-09</td>
<td>HA, NA      Liposomes</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>0-12</td>
<td>HA, NA      Liposomes</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>0-18</td>
<td>HA, NA      Liposomes</td>
<td>3-2 (1-6)</td>
<td>2</td>
</tr>
</tbody>
</table>

* Number of preparations examined.
† S.E.M. in parentheses.
‡ Mean fever indices, S.E.M. in parentheses, were calculated mathematically from the areas under curves for individual animals.
§ Lipid was extracted from u.v.-inactivated virus with methanol/chloroform and reconstituted into liposomes; residual debris was also examined. Viral lipid was reconstituted with viral NP and HA/NA prepared from DOC-disrupted virus.
‖ Where no rise in rectal temperatures occurred, the fever index was given a value of zero.

Measurement of pyrogenicity. Adult male ferrets were inoculated with virus or virion components by cardiac puncture under ether anaesthesia and rectal temperatures were monitored as previously described (Coates et al., 1986). Fever was considered significant if the rectal temperatures increased by >0-4 °C above the mean pre-inoculation temperature (determined as described by Toms et al., 1977). The rise was plotted against time post-inoculation and the level of fever (fever index) was assessed by determining mathematically the area under the curve >0-4 °C (expressed as °Ch). The significance of the difference between the mean fever levels for different virus strains was tested using Student's t-test for independent samples.

Prevention and checks on contamination with endotoxin. Glassware was sterilized by heating in an oven at 160 °C for 2 h, and solutions and centrifuge tubes were autoclaved at 103 kPa for 2 h (heat-labile media were filter-sterilized). Non-pyrogenic sterile plastic ware, syringes and
hypodermic needles were used. To confirm the absence of endotoxin in test samples and reagents, they were heated at 90 °C for 30 min and tested in ferrets for the absence of pyrogenicity.

Results

Removal of glycoproteins with bromelain

Preliminary experiments showed that two treatments with bromelain for 16 h each were required to remove the glycoprotein spikes (Fig. 1). After two treatments with bromelain >95% of the particles were spikeless. SDS-PAGE and immunoblotting with MAbs confirmed that the spikeless virions were devoid of NA and HA but contained NP (Fig. 2a, 3a) and some M protein (Fig. 2a). The supernatants contained both NA and HA and low level contamination by other proteins (Fig. 2b, 3b). Both the spikeless virions and the liberated glycoproteins were non-pyrogenic (Table 1).

Removal of glycoproteins with DOC

Virions were treated with DOC to liberate glycoproteins as stellate clusters (Laver & Valentine, 1969). First, the treated virus was tested for pyrogenicity without separation of the SVCs from the glycoprotein-containing supernatant. The pyrogenicity of the unseparated mixture of treated components was considerably reduced compared with whole virus (Table 1). The separated SVCs were devoid of glycoprotein spikes (Fig. 4a). SDS-PAGE (Fig. 5a) and Western blotting (Fig. 5b) showed that the SVCs contained predominantly NP but sometimes low levels (<10% of total protein) of HA, NA and M protein. The supernatants from the SVCs yielded sucrose gradient fractions that contained aggregated stellate clusters as shown by electron microscopy (Fig. 4b). They contained high haemagglutinating activity (fractions 5 to 7; Fig. 6) and showed on SDS-PAGE not only HA but NA, M protein and low levels of NP (Fig. 6). The mean febrile responses to both the SVCs and the stellate glycoprotein clusters were small compared with the original inactivated virus (Table 1). The febrile responses were, however, very variable. Twelve batches of SVCs were injected into a total of 20 ferrets and the fever indices varied from zero (10 ferrets) to over 2.0 °Ch (four ferrets). Eight batches of sucrose gradient-separated stellate glycoprotein clusters were injected into a total of 19 ferrets and the fever indices varied from zero (nine ferrets) to over 2.0 °Ch (three ferrets). The response variability of animals to both SVCs and stellate glycoproteins probably reflects the low pyrogenicity of such samples and individual animal variation since the samples did not appear to differ in composition from batch to batch.

NP (fractions 10 and 11; Fig. 6) and M protein (fractions 3 and 4; Fig. 6) purified on sucrose density gradients were not pyrogenic (Table 1).

Tests for pyrogenicity on ether-treated virus, liposomes of extracted viral lipid and virosomes containing glycoproteins or NP

To examine the possibility that lipid may be important in pyrogenicity, ether-treated virus was examined, as were liposomes containing viral lipid. Although ether treat-
ment produced a gross loss of pyrogenicity (Table 2), indicating that lipid may be involved, virosomes made from lipid extracted from purified virions and containing no contaminating viral proteins or infectious virus were non-pyrogenic (Table 2), even though inoculum doses contained approximately six- to 11-fold more lipid than occurs in a dose of 200 μg/kg of whole virus. The material remaining after extraction of the lipid, which contained

Fig. 5. (a) SDS–PAGE and (b) Western blot analysis of viral polypeptides in SVCs of two batches of purified virus incubated with 5% DOC for 5 min at 37 °C. Lanes 2 and 3 in (a) are two different batches of purified virus as are lanes 1, 3, 5 and 7 (batch 1) and 2, 4, 6 and 8 (batch 2) in (b). Lane 1 in (a) contains Mr markers. Lanes 1 and 2 in (b) were incubated with anti-HA, lanes 3 and 4 with anti-NA, lanes 5 and 6 with anti-NP and lanes 7 and 8 with anti-M protein MAbs, respectively.

Fig. 6. SDS–PAGE analysis of fractions from a sucrose gradient. Lanes 2 to 11 represent fractions taken from the top (fraction 1, low density) to the bottom (fraction 10, high density); lanes 1 and 12 contain Mr markers. Fractions 5 to 7 were generally used as HA/NA-enriched fractions, fractions 2 and 3 for purified M protein and fractions 10 and 11 for purified NP.
all four major viral proteins as shown by SDS–PAGE and immunoblotting, was slightly pyrogenic (Table 2). NP, prepared uncontaminated with other viral proteins, was non-pyrogenic when reconstituted into liposomes with viral lipid to produce virosomes (Table 2). Again virosomes were injected at doses containing 3.5-fold more NP and from 14- to 18-fold more lipid than occurs in 200 μg/kg of whole virus. Virosomes formed from viral lipid and the glycoprotein fraction of DOC-disrupted virus showed typical spikes embedded in lipid when examined by electron microscopy (Fig. 7). Although the original glycoprotein fraction contained M protein (Table 1, Fig. 6), the virosomes contained only HA and NA with no detectable M protein (Fig. 8). Low doses of these HA/NA virosomes (100 to 140 μg/kg) were not pyrogenic but higher doses (225 μg/kg) showed significant pyrogenicity. The latter dose contained 3.8-fold more HA/NA and 21-fold more lipid than occurs in the standard dose of 200 μg/kg of whole virus.

**Discussion**

Previous attempts to identify the influenza virus component involved in pyrogenicity have yielded conflicting results. Limited heating destroyed infectivity without affecting pyrogenicity (Wagner *et al.*, 1949) indicating, as in our studies, that virion components are involved. Physical and chemical treatments of myxo- and paramyxoviruses have suggested that the HA (Kanoh & Kawasaki, 1966), lipid (Siegert & Braune, 1964) and carbohydrate (Atkins & Bodel, 1974) and other host-
derived components of the virion (Brand & Liew, 1983) may be involved in pyrogenicity. After surveying previous work, Dinarello & Wolff (1982) concluded that lipid associated with the carbohydrate-containing HA may be responsible for pyrogenicity.

The most important conclusion from our studies is that pyrogenicity depends upon the structural orientation of virion components. Disruption of intact virions by treatment with bromelain, DOC or ether produces a gross reduction in pyrogenicity. Hence, any subsequent studies on individual components released by disruption were severely handicapped because they either had low inherent pyrogenicity or were being inappropriately presented. This in turn makes it very difficult to ascertain which components might be involved in the pyrogenicity of intact virus. Nevertheless, there were indications that the surface glycoproteins (HA/NA) may be important (together with lipid), which agrees with conclusions from previous work.

Treatment with bromelain removed all surface glycoproteins and rendered virus non-pyrogenic (Table 1). This indicates that one or both of the glycoproteins is involved in pyrogenicity either because they are pyrogens or because they are involved in cell uptake of virus particles containing the pyrogen. Monomeric (non-aggregated) and modified HA and NA, released by digestion with bromelain, were not pyrogenic (Table 1). The surface glycoproteins were then tested for pyrogenicity individually as aggregated spikes. Disruption of the virus with DOC, separation of the supernatant from SVCs and sucrose gradient fractionation of the latter produced aggregated stellar structures (Fig. 4b) containing HA, NA and M protein (Fig. 6). These structures showed some pyrogenicity in a significant number of the many tests conducted (nine of 19). The SVCs retained some pyrogenicity (10 positive tests in a total of 20) presumably because the pyrogenic complexes were not completely disrupted. It was probable that the pyrogenicity of the aggregated stellate structures was not due to their M protein content since purified M protein was not pyrogenic (Table 1).

The destruction of pyrogenicity by treatment of virus with ether (Table 2) was in accordance with previous reports (Siegert & Braune, 1964) and indicated that lipid may play some role in pyrogenicity. However, liposomes prepared from extracted viral lipid were not pyrogenic (Table 2) indicating that its role in pyrogenicity may be entirely structural. The low pyrogenicity of the residue remaining after lipid extraction was not unexpected. The important result was the pyrogenicity of the high dose of virosomes made from virus lipid and the aggregated stellate clusters (Table 2). These virosomes had spikes, as seen by electron microscopy (Fig. 7), and contained HA and NA but not M protein (Fig. 8).

These results are similar to those obtained in attempts to identify the viral component(s) that evoke interferon (IFN) and other cytokines. Influenza virus [A/USSR/053/74 (H3N2)] subjected to various chemical and physical treatments could induce IFN in mice provided the surface antigens, particularly NA, were preserved (Chomik, 1988a, b; Chomik & Slusarczyk, 1988). Purified M protein, ribonucleoprotein and polymerases did not induce IFN. Virus which had been chemically or enzymatically treated to destroy the viral lipid envelope did not induce IFN even though they contained functional glycoproteins (Chomik, 1988b). In parallel with our work, Chomick (1988b) showed that isolated lipid-free HA and NA were unable to induce IFN. On the other hand, it has been shown that purified NA increases the level of IL-1 and TNF secreted by mouse peritoneal macrophages both in vivo and in vitro (Houde & Arora, 1989, 1990). Interestingly, IL-1, TNF and INF are all EPs.

Although the observation that non-infectious, u.v.-inactivated virus is pyrogenic has suggested that the latter is due to virion components, it is possible that some pyrogenicity results from limited replication events. Double-stranded RNA, a product of viral replication extracted from lungs of mice infected with influenza virus, induced fever when inoculated intracerebrally into rabbits whereas dsRNA from the lungs of uninfected mice did not (Majde et al., 1991). Also, the ability of heat-inactivated influenza virus to induce IFN has been attributed to the production of dsRNA, either as a result of abortive replication or low level incorporation (not exceeding 1 to 2%) of positive-stranded RNA into virions such that dsRNA, the suggested inducer of IFN, may be released into cells at high viral multiplicity (Tyler & Fields, 1985). In the present work, there is no evidence for this additional mechanism of production of fever. The SVCs containing viral RNA were not pyrogenic but this may be because they were unable to enter host cells efficiently owing to the absence of HA.

The overall implication of the work described here is that it is difficult to investigate either the nature of the pyrogenic components of influenza virus or the reasons for differences in pyrogenicity of strains by fractionation studies. As soon as the virus is disrupted the majority of the pyrogenicity disappears. This points the way to the use of reassortants to achieve the required objective.

References


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