Ligands for Antibody to M-Protein are Exposed at the Surface of Influenza Virions: Effect of Proteolytic Treatment on their Activity

By MONIQUE REGINSTER, LUC JOASSIN AND PATRICIA FONTAINE-DELCAMBE

Université de Liège, Laboratoire de Microbiologie générale et médicale, Institut de Pathologie, B-4000, Liège, Belgium

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SUMMARY

Antiserum to pure M-protein extracted from PR8 virions neutralized the infectivity and inhibited the haemagglutinating activity of various influenza A virions. It agglutinated concentrated suspensions of these virions and fixed complement in their presence. Antibodies to M-protein were readily absorbed by intact virions or by spikeless particles obtained after proteolytic treatment, giving clear evidence that M-protein is exposed at the surface of the virus envelope. The data suggest that when antibodies to M-protein occupy specific ligands exposed at the surface of the virion they interfere with sites critical for infectivity and haemagglutinating activity.

INTRODUCTION

Previously, it was found that spikeless influenza A particles obtained after a proteolytic digestion display a type-specific antigenicity which was attributed to the unmasking of a superficial antigen (Reginster, 1965a, b; Reginster, 1968). This finding was confirmed and it was discovered that the M-protein content of the spikeless particles was half that of the intact virions. These particles contained a 13000 mol. wt. polypeptide unknown in intact virions and fixed complement in the presence of an antiserum directed against the M-protein (Reginster et al. 1975/76). In this paper, we report data from experiments initially devised to assess the presence of an M-protein related antigen at the surface of the spikeless particles. Preliminary results have been reported (Reginster et al. 1978).

METHODS

Viruses. Influenza A viruses, PR8 (H9N1), Singapore-1957 (H2N2), X31 (H3N2) strain, swine influenza (HswIN1) and influenza B, Lee strain, grown in eggs were concentrated and purified as described by Reginster & Nermut (1976).

M-protein. This was purified using hydroxyapatite fractionation and affinity chromatography after disruption of PR8 virions by mean of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol (ME; Joassin & Reginster, 1978).

Antisera. M-protein antiserum was prepared in rabbit. One 0.1 mg dose of M-protein in 0.6 ml phosphate-buffered saline (PBS) mixed with 1 ml of complete Freund's adjuvant was administered by multiple intradermal injections of about 0.1 ml. Another identical dose of M-protein was given in the same manner 4 weeks later and the rabbit was bled after 2 more weeks. Other antisera have been described elsewhere (Reginster et al. 1975/76).

Double immunodiffusion tests. Double diffusion tests were performed in Noble agar slides containing 1.5% (w/v) Noble agar in veronal buffer. The slides were kept at room temperature in a humidified chamber. Optimal precipitation using sodium sarcosyl sulphate.
(SSS; 1%, (w/v) disrupted virions usually occurred within 24 h. Precipitation lines were revealed by staining with 0.5% Coomassie blue (Sigma, R 250).

Haemagglutination inhibition, complement fixation tests and neuraminidase measurements. These were performed as previously described by Reginster et al. (1975/76).

Infectivity neutralization tests. These were carried out by inoculating pieces of chorioallantoic membrane on shell with standard volume of serum–virus mixtures. Serum activity was estimated from neutralization by twofold serum dilutions of 100 or 1000 ID<sub>50</sub> virus challenge dose after 1 h contact at 0 °C (Reginster, 1968) and from reduction of infectivity obtained by incubating (1 h, 0 °C) 10000 ID<sub>50</sub> of virus with serum diluted one in two.

Protein determinations. These were made according to Lowry et al. (1951).

Labelling of PR8 structural proteins with <sup>125</sup>I. To 0.5 ml virus suspension containing 1.5 mg virus protein, 2% (w/v) SDS and 2% (v/v) ME were added. The solubilized virus material was kept for 2 min in a boiling water bath and dialysed against PBS containing 0.1% (w/v) SDS for 48 h. Solubilized proteins were labelled according to the method of Greenwood et al. (1963). To 200 µl of the dialysed mixture, 100 µg chloramine-T were added in a vial containing 1 mCi <sup>125</sup>I. After 30 s contact at room temperature, the unfixed <sup>125</sup>I was neutralized by adding 200 µg sodium metabisulphite. The preparation was further dialysed against PBS, 0.05% (w/v) SDS for 6 days at room temperature with several changes of buffer.

Radioimmunoprecipitation. One 50 µl sample of iodinated virus proteins was mixed with 50 µl M-protein antiserum, kept at 37 °C for 2 h and then at 4 °C for 48 h. The immunoprecipitate was collected after centrifugation at 3000 g at 4 °C, washed twice with PBS and the final pellet was used for slab gel electrophoresis.

Polyacrylamide gel electrophoresis. Virus suspensions as well as <sup>125</sup>I-immunoprecipitates were heated at 100 °C for 2 min in 2% (w/v) SDS with 2% (v/v) ME and electrophoresed either as described by Reginster et al. (1975/76) through 7 cm cylindrical running gels or vertical slab gels. Cylindrical gels were stained with Coomassie blue (Serva; G-250) and radioactive material on the slabs was visualized by autoradiography.

Autoradiography. The slab gel was put on a Whatman 3 MM paper filter and dried under vacuum. A medical X-ray film (Kodak, no-screen) was exposed to the dried gel at room temperature for 24 h and then developed for 1 min at 20 °C with Agfa–Gevaert G-150.

RESULTS

Specificity of the M-protein antiserum

In the complement fixation test, the M-protein antiserum maximum active dilution was 1/160 in the presence of as little as 65 ng of pure M-protein. In double diffusion tests, one single continuous precipitation line was observed when wells of pure M-protein alternating with wells containing SSS-disrupted PR8, Singapore or X31 virions surrounded the antiserum well. No reaction took place between the antiserum and disrupted Lee virions, assessing the type-specificity of the antiserum (Fig. 1a).

Immunoprecipitation of <sup>125</sup>I-labelled PR8 structural proteins using the M-protein antiserum was monitored by polyacrylamide gel electrophoresis and by autoradiography. Precipitation of M-protein only could be demonstrated in this way (Fig. 1).

Activity of the M-protein antiserum toward various influenza A virions

The antiserum diluted 1/10 readily agglutinated purified PR8, Singapore or X31 virions at the concentration of 0.5 mg protein/ml. It proved active in haemagglutination inhibition, infectivity neutralization and complement fixation tests toward PR8, Singapore, X31 and swine influenza viruses. The activity of the serum did not differ significantly from one strain...
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Fig. 1. (a) Electrophoresis pattern of SDS–ME-disrupted PR8 virions showing the distribution of the major polypeptides. Cylindrical gel with Coomassie blue staining. (b) and (c) Autoradiography of slab gel electrophoresis. (b) 125I-labelled virus polypeptides. Load: 150,000 ct/min. (c) Immuno-precipitate obtained after contact of M-protein antiserum and 125I-labelled virus proteins. Load: 50,000 ct/min.

...to another either in haemagglutination inhibition or in complement fixation tests but, in infectivity neutralization, its activity was consistently higher towards Singapore virus than towards the other three strains (Table 1). Pre-contact of Singapore virus (10,000 ID50) with M-protein antiserum (1/2 dilution) reduced by 10 times its infectivity. Under the same conditions the infectivity of PR8, X31 and swine viruses was not impaired. Higher neutralizing activity towards Singapore virus could not be attributed to using Singapore-infected allantoic fluid with a particularly high ratio of infectivity to haemagglutinating activity since the number of ID50/number of haemagglutinating units (Reginster, 1965b) per ml was 100, 250, 2340 and 10 for PR8, Singapore, X31 and swine infected allantoic fluids, respectively. Differences in reactivity of the various influenza strains towards the antiserum were noticeable in complement fixation tests as shown by the minimum virus protein concentration required. The serum maximum activity (Table 1) was observed by using 0.75, 3.25, 0.75 and 7.00 μg protein of PR8, Singapore, X31 and swine virions, respectively. The antiserum activity towards Lee virus was below detection level in all the above mentioned tests (Table 1) as was antiserum inhibition towards PR8 neuraminidase.

When previously absorbed with PR8 virions (contact for 45 min at room temperature of...
Table 1. Activity of the anti M-protein antiserum towards various influenza A virions*

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Haemagglutination inhibition</th>
<th>Infectivity neutralization</th>
<th>Complement fixation</th>
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<td>PR8</td>
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<td>Singapore</td>
<td>1/80</td>
<td>1/320</td>
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<td>X31</td>
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<td>Lee</td>
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* Viruses were in the form of infected allantoic fluid for haemagglutination inhibition and infectivity neutralization. Purified virions were used for complement fixation tests.

† Neutralized virus challenge doses were 100 ID₅₀ in 1 and 1000 ID₅₀ in 2 (no haemagglutination in the eight wells containing one fragment of chorioallantoic membrane on the shell, pre-incubated with the virus-serum mixture).

Fig. 2. Double radial diffusion tests. Central well: M-protein. Peripheral wells: m, antiserum to M-protein; m/PR8, antiserum to M-protein absorbed with PR8 virions; m/PR8C, antiserum to M-protein absorbed with caseinase C-treated PR8 particles; pr8c, antiserum to caseinase C-treated PR8 particles (for details, see Methods).

The undiluted serum with an equal volume of PR8 suspension containing 5 mg virus protein/ml, the M-protein antiserum did not precipitate M-protein in double diffusion tests (Fig. 2). The effect of pre-contact with PR8 virions on the antiserum activity towards the M-protein was also evaluated as follows. Virions pelleted from 1 ml PR8 suspension containing 0.5 mg virus protein/ml were resuspended in 1 ml of serum dilute 1/10. After 30 min at room temperature, the mixture was centrifuged at 4000g for 10 min. The supernatant was collected and absorbed again as described. Three such absorptions were necessary to reduce by 75% the activity of the M-protein antiserum toward M-protein in complement fixation tests.

Activity of the M-protein antiserum towards spikeless particles obtained after proteolytic treatment

PR8 spikeless particles resulting from the proteolytic action of caseinase C (Reginster, 1965b, 1968; Reginster et al. 1975/76) reacted with the M-protein antiserum as shown by complement fixation tests and by absorption of M-protein precipitating antibody. Spikeless particles were half as active in the complement fixation tests as intact virions since the M-protein antiserum could detect specific ligands when the amount of spikeless particles used
was that obtained after caseinase C treatment of 1.5 µg protein of PR8 virions, whereas it could detect specific ligands when 0.75 µg protein of PR8 virions was used as mentioned above. Maximum active serum dilution towards intact PR8 spikeless particles was half that towards intact PR8 virions. However, spikeless particles were more efficient than intact virions in absorbing anti-M-protein antibody. One single absorption with spikeless particles obtained after caseinase C treatment of 0.5 mg protein of PR8 virions/ml serum diluted 1/10 reduced by 75% the activity of the M-protein antiserum toward M-protein in complement fixation tests. As mentioned above, three absorptions with 0.5 mg protein of intact virions/ml serum diluted 1/10 were necessary to obtain such a reduction of the antisera activity. Accordingly, absorption of the serum with PR8 spikeless particles [contact for 45 min at room temperature of serum diluted 1/2 with particles pelleted from an equal volume of PR8 suspension (5 mg virus protein/ml) pre-incubated with caseinase C] abolished its ability to precipitate M-protein in double diffusion tests (Fig. 2).

**DISCUSSION**

Successful absorption of antibody to M-protein by PR8 virions unequivocally demonstrates that M-protein specific ligands are exposed at the surface of the virion. This fact might be related to observations showing that unidentified type-specific determinants are exposed at the surface of either the influenza infected cells or the influenza virus particles. Indeed, pre-infection (Schulman & Kilbourne, 1965; Folc'h & Werner, 1978) or immunization with inactivated virus (McLaren & Potter, 1974) could provide intratypic cross-protection which has been recently attributed to cell mediated reactivity (Folc'h & Werner, 1978). Influenza viruses could generate T cells displaying type-specific reactivity in vitro (Effros et al. 1977; Zweerink et al. 1977) and accelerated virus clearance in lungs of influenza-infected mice was observed in animals immunized with M-protein (Webster & Hinshaw, 1977).

Cross-reactivity of our M-protein serum cannot be attributed to host-specific antibody since it does not extend to egg-grown B Lee virus. The data practically excludes the fact that neutralizing activity of our serum is due to antibodies which recognize virus haemagglutinin. Indeed one heterologous virus was better neutralized than the homologous strain and the same virus was also the only one which proved sensitive to ‘contact’ neutralization. Therefore, antibody to M-protein itself should be regarded as capable of inhibiting haemagglutinating activity and neutralizing infectivity of influenza virion, implying that specific ligands are accessible at the surface of undamaged particles. Cross-neutralization as well as the fact that M-protein antisera controls rather than neutralizes PR8, X31 and swine virus infectivity suggest that antibodies are interfering by steric hindrance with sites critical for the expression of these properties and that ligands for M-protein antibodies have a closer relationship with sites critical for Singapore infectivity. In this connection, it is worth recalling that Singapore virus is sensitive to amantadine, a drug which can prevent any expression of the virus genome (Skehel et al. 1977) and that amantadine sensitivity of influenza viruses is determined by the M-protein gene (Hay et al. 1979). Great differences in the reactivity of the various influenza virions towards the anti-M-protein serum, which were revealed by minimum virus protein concentration required for optimal complement fixation, could indicate differences in accessibility of specific ligands at the surface of the virions.

The fact that anti-M-protein sera prepared by others (Schild, 1972; Oxford & Schild, 1976; Webster & Hinshaw, 1977) do not interfere with properties involving the surface of the virion could depend on the quality of the M-protein preparation used as immunogen. Future work will probably help to define native M-protein. However, it is already clear that in contrast with the M-protein preparation obtained by electrophoresis on cellulose acetate
of detergent disrupted virions (Schild, 1972), our M-protein (Jossain & Reginster, 1978) cannot be regarded as a lipoprotein (Stuart-Harris & Schild, 1976).

A substantial part of the M-protein is not protected from proteolysis by the lipids of the envelope (Reginster et al. 1975/76). This finding is confirmed by the discovery reported here that the caseinase C treatment leaves an M-protein derived antigen at the surface of the spikeless particles. The best candidate for this function is the 13,000 mol. wt. polypeptide which increases as M-protein decreases under caseinase C treatment (Reginster et al. 1975/76). Consistent with this suggestion, caseinase C reduces the activity of the PR8 particles towards anti-M-protein serum as tested by complement fixation tests. Since caseinase C also removes all the glycoproteins of the virions, enhanced efficacy of spikeless particles in absorbing M-protein antibody could be interpreted in terms of better accessibility of the specific ligands.

Type-specific antibody elicited by spikeless particles (Reginster, 1965a, b, 1966, 1968; Reginster et al. 1975/76) does not precipitate M-protein in double diffusion tests nor does it fix complement in its presence. This suggests that the M-protein related antigen detected by reaction with M-protein antibody, not unlike M-protein at the surface of intact virions, does not elicit antibodies, or that the M-protein related antigen is immunogenic but elicited antibody cannot be detected by means of native M-protein antigen. However, one cannot exclude the possibility that the immunogen revealed by caseinase C treatment is a third type-specific component unrelated to M or N proteins, possibly derived from the hydrophobic end of the spikes as already suggested (Reginster et al. 1975/76).

Our findings do not comply with the current model of influenza virus envelope (Laver, 1973; Schulze, 1973) which arose from lack of evidence for the presence of M-protein at the surface of the virion and from persistence of this protein in the virus particles recovered after various proteolytic treatment. Evidence now available strongly suggests that M-protein antigen is a genuine superficial constituent with obscured immunogenicity. More data are needed to understand how M-protein, although exposed at the surface of the virion, is partially insusceptible to proteolysis (Reginster et al. 1975/76) and unequivocally protected by lipids (Reginster & Nermut, 1976).

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