Immunogenic Properties of the Small Chain HA$_2$ of the Haemagglutinin of Influenza Viruses

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SUMMARY

The small chain of influenza virus haemagglutinin, HA$_2$, was isolated by a selective enzymic removal of HA$_1$, or by preparative SDS–polyacrylamide gel electrophoresis. Anti-HA$_2$ specific antisera and monoclonal antibodies were subtype-specific in immunodiffusion tests and radioimmunoassays. These antibodies did not inhibit haemagglutination or haemolysis, did not prevent virus release, did not neutralize infectivity, and HA$_2$ did not induce a protective immunity. HA$_2$-specific antigenic determinants could not be demonstrated on the surface of infected cells. Lymphocytes from pre-immunized mice could not be stimulated by HA$_2$ to exert a cytotoxic effect.

INTRODUCTION

The haemagglutinin (HA) of influenza viruses has a high antigenic variability and is important for the induction of protective immunity (for review, see Ennis, 1982). According to crystallographic analysis, the HA is anchored in the lipid membrane by HA$_2$, the smaller of the two structural polypeptides, while the larger HA$_1$ has a rather globular shape and is exposed at the surface of the virus particle (Wilson et al., 1981). Most studies of the antigenic structure and variability of the HA have therefore been focused on the HA$_1$ moiety, where four major antigenic sites have been described (Wiley et al., 1981).

When the amino acid sequence of the HA polypeptide of several influenza virus strains was compared, it became evident that some regions of the HA$_2$ polypeptide are highly conserved (for reviews, see Ward, 1981; Palese & Young, 1982; Webster et al., 1982). It was therefore of interest to determine the potential contributions of these relatively invariant regions to the antigenic variability of the haemagglutinin and to consider the possibility that they might induce an immune response reaching beyond the known range of subtype specificities. The type-specific cell-mediated cytotoxic reactions (Braciale, 1977; Effros et al., 1977; Zweerink et al., 1977) seemed to be of particular importance in this context. This type-specific killing of infected cells by T lymphocytes (for review, see Sherman et al., 1983) had been attributed to reactions against the matrix (M) protein (Biddison et al., 1977; Ada & Yap, 1977). Inhibition studies with monoclonal antibodies, however, raised doubts that the M protein is recognized by cytotoxic cross-reactive T cells (Hackett et al., 1980) and suggested that the antigenic target site is located on the haemagglutinin (Askonas & Webster, 1980). In view of these studies, HA$_2$ with its conserved regions was a candidate for this cross-reactive target antigen.

We therefore set out to define the serological position and variation of HA$_2$ with a specific antiserum and monoclonal antibodies, to examine the influence of these antibodies on virus replication, to establish HA$_2$-specific cytotoxic lymphocytes, and to outline possible antigenic targets for cytotoxic T-cells and antibodies on the surface of infected cells and on virus particles.
**METHODS**

**Viruses.** The strain A/FPV/Rostock/34 (H7N1) was used for the preparation of HA₂. Virus was grown in embryonated chicken eggs and purified through a sucrose gradient. All other strains used are indicated in the text and were prepared according to the same procedure.

**Preparation of HA₂.** HA₂ had mainly been produced by centrifugation in a gradient of guanidine hydrochloride (Laver, 1971) or by gel filtration after dissociation of the virus with SDS (Bucher et al., 1976). Since it was our aim to study the biological effect of an anti-HA₂-specific immune response, we tried to include procedures requiring the least denaturing conditions for the isolation of HA₂. One procedure employed was based on the observation by Knight (1980) and Roth et al. (1980) that the small chain of the HA is resistant to an acid protease.

To 9 ml of a purified virus suspension 1 ml of a 1 M-sodium acetate buffer pH 4.5 was added in which 100 mg of the acid protease from *Aspergillus saitoi* (Sigma, type VIII) had been dissolved. The mixture was incubated for 1 h at 37 °C and centrifuged briefly (2000 rev/min for 5 min) to sediment aggregates which appeared during incubation. The supernatant which had lost virtually all its haemagglutinating activity by the digestion was centrifuged at 20000 rev/min for 1 h, and the virus pellet was washed twice with phosphate-buffered saline (PBS). The final pellet was resuspended in 25 ml of a 3% solution of octylglucoside (OG) in PBS, stirred for about 30 min at room temperature, and the viral cores were sedimented at 20000 rev/min for 1 h. The small amount of residual neuraminidase which was in the supernatant as well as HA₂ was removed by passing the solution through an affinity column of sulphamic acid-conjugated Sepharose as described previously (Bosch et al., 1980). The flow-through in 0.1 M-acetate buffer pH 5.5 contained HA₂ in pure form (HA₂-AP) as judged by SDS-gel electrophoresis (not shown). It was used for immunizing a rabbit after the detergent had been removed by dialysis.

HA₂ was separated from HA₁ by preparative SDS-polyacrylamide gel electrophoresis. HA was released from purified virus suspensions by treatment with 1.5% OG. The detergent was removed by dialysis against PBS containing 2 mM-EDTA. HA was adsorbed onto chicken erythrocytes fixed with glutaraldehyde (Bing et al., 1967) in an ice-bath. The red cells were washed with ice-cold PBS-EDTA, and the HA was finally eluted with 3 M-KCl in 0.02 M-Tris-HCl pH 8.2, at room temperature. The eluate which contained virtually all of the original HA activity was concentrated by pressure dialysis (Amicon, membrane PM 30) and dialysed against sample buffer (0.01 M-Tris-HCl pH 8.0, 1 mM-EDTA). Four to 5 ml of the sample which usually had an HA titre between 2⁻¹⁰ and 2⁻¹⁸ was dissociated with 1% SDS and reduced with 1% 2-mercaptoethanol at room temperature. An equal volume of 2% agarose (without electroendosmosis) was melted in sample buffer which contained 8 M-urea, and after the agarose had cooled to 37 °C it was mixed with the sample. The mixture was applied on top of a column of polyacrylamide gel in a LKB ‘Uniphor’ apparatus for preparative electrophoresis. The mode of application of the sample proved to be essential for a good and reproducible separation of the two subunits. When agarose was liquefied in urea it remained soluble enough at 37 °C or even at room temperature to be mixed with the dissociated and reduced HA and to be applied to the top of the polyacrylamide gel column. After cooling the column to the working temperature of 10 °C in the apparatus, the agarose solidified enclosing the sample in a firm matrix. The separating gel consisted of 50 ml of 7.5% polyacrylamide, and the spacer gel was 10 ml in a Laemmli buffer system. After the sample had solidified in the cooled column, the Tris-glycine electrode buffer was added and a constant current of 50 mA was applied. The first peak appearing behind the bromophenol blue front during the continuous elution was concentrated again, redialysed against the sample buffer. 1% SDS and 2-mercaptoethanol were added again and applied as in the first run to a second column containing 30 ml of a 10% polyacrylamide separating gel and 10 ml of spacer gel. Material appearing more slowly and containing mostly the HA₁ was electrophoresed again through 50 ml of a 7.5% gel. Depending on the amount of material available after the first separation, the peaks of several runs were combined for the second step. SDS was finally removed from the preparations by extensive dialysis against 0.01 M-Tris-HCl pH 7.2.

**Production of antibodies.** About 500 to 600 µg of HA₁ and HA₂ incorporated in Freund’s complete adjuvant were injected subcutaneously into a rabbit. The animals received one or more booster injections about 3 weeks later. Blood samples were drawn 5 to 7 days after the last injection. Two chickens were injected intramuscularly according to the same schedule. In order to exclude any interference with antibodies against host components, possibly directed against the carbohydrate side-chains (Jackson et al., 1981), the sera were passed through a column of Sepharose coated with an extract from normal chorioallantoic membranes and allantoic fluid.

The antiserum raised against the HA₁ prepared by acid protease digestion precipitated NP antigen. The immunosorbent for the removal of these antibodies was prepared by conjugating NP antigen to agarose which had been obtained from FPV-infected chorioallantoic membranes by an antibody-mediated affinity chromatography as previously described (Becht & Malole, 1975).

**Production of monoclonal antibodies.** BALB/c mice were immunized intraperitoneally twice with a 2 week interval with 50 µg HA₁-AP in Freund’s incomplete adjuvant and a booster was given intravenously 2 weeks later. Three days later, spleen cells were fused with P3X63 Ag8 myeloma cells (Köhler & Milstein, 1975) using polyethylene glycol (PEG) 1500. Growth of hybridomas was visible after 1 week. Supernatants were screened for antibodies against HA₁ by radioimmunoassay, and positive hybridomas were cloned in soft agar and used for ascites production.
Immunogenicity of influenza HA₂

Serological procedures. HA- and neuraminidase-inhibition tests followed standard procedures. The influence of antibodies on virus release was tested by incorporating the antisera into the culture medium or the agar overlay at a dilution of 1:10 and 1:20 after the infecting virus had been allowed to adsorb to chick embryo fibroblasts for 30 min. Double immunodiffusion tests (ID) were carried out in 1% agarose in PBS containing 0.5 mM KCl and 3% PEG 6000 which had been added to the molten agar at about 80 °C. In this medium, rabbit and chicken antisera could be tested simultaneously.

Inhibition of haemolysis by anti-HA₂. Haemolysis of chick erythrocytes was carried out at pH 5.5 as described previously (Huang et al., 1981). A 0.1 ml amount of anti-HA₂ serum undiluted and in dilutions of 1:2 to 1:8 in saline was mixed in a series of tubes with 0.1 ml of purified virus suspension (HA titre 2 -10). A control tube contained saline only. The serum-virus mixture was kept at room temperature for 1 h, and 2 ml of a 1% suspension of chick erythrocytes and 0.5 ml of a 0.5 M-sodium acetate buffer (pH 5.5) were added to each tube. After an incubation period of 15 min in a water-bath at 37 °C the tubes were centrifuged at 2000 rev/min for 5 min, and the haemoglobin liberated into the supernatant was measured by absorbance at 540 nm.

Radioimmunoassay. A solid-phase radioimmunoassay (RIA) was performed in microtitre plates with single polystyrol wells (Remove-a-well, Dynatech Inc.). Antigens were bound to the surface of the wells at pH 9-8 overnight at 4 °C. Residual binding sites on the surface of the wells were saturated with PBS–Tween and the monoclonal antibody had been used in saturating amounts. The wells were washed twice and incubated with washed and the bound radioactivity was determined in a gamma spectrometer. With rabbit antisera, in the RIA previously (Huang et al., 1981). A 0.1 ml amount of anti-HA₂ serum undiluted and in dilutions of 1:2 to 1:8 in saline was mixed in a series of tubes with 0.1 ml of purified virus suspension (HA titre 2 -10). A control tube contained saline only. The serum-virus mixture was kept at room temperature for 1 h, and 2 ml of a 1% suspension of chick erythrocytes and 0.5 ml of a 0.5 M-sodium acetate buffer (pH 5.5) were added to each tube. After an incubation period of 15 min in a water-bath at 37 °C the tubes were centrifuged at 2000 rev/min for 5 min, and the haemoglobin liberated into the supernatant was measured by absorbance at 540 nm.

Preparation of HA₂ : specificity of anti-HA₂ antibodies

Treatment of virus particles with an acidic protease destroyed the HA₁, leaving the HA₂ chain intact in accordance with results obtained with an enzyme preparation from Aspergillus niger (Knight, 1980; Roth et al., 1980). The size of the HA₂ molecule (HA₂-AP), according to its migration distance in an acrylamide gel, was not affected by the digestion. There was some neuraminidase activity left which could be removed by affinity chromatography. Treatment of the resulting virus particles with OG liberated the HA₂ from the lipid envelope. The detergent also released some NP which gave rise to contaminating antibodies in the anti-HA₂ serum. These anti-NP antibodies could be removed with an NP immunosorbent prepared with an antigen isolated from infected chorioallantoic membranes. The destruction of HA₁ must have been specific and very effective, because the antiserum did not react with the isolated HA₁ in ID or RIA.

This HA₁ was prepared, as was HA₂, by an electrophoretic procedure in an SDS–polyacrylamide gel. Fig. 1 shows the absorbance profile of the continuous elution from the 7.5% polyacrylamide gel. There was a symmetrical peak for HA₂ after a second run through a 10% gel (Fig. 1c). The longer migration distance in a 7.5% gel produced a broader peak for HA₁ (Fig.
Fig. 1. Preparative electrophoresis of FPV HA$_1$ and HA$_2$ in an SDS-polyacrylamide gel. HA was isolated with octylglucoside as described in the text. (a) Elution from a 7-5% gel (50 ml), constant current of 50 mA at 10 °C. (b) HA$_1$ from (a) in a second run, conditions as in (a). (c) HA$_2$ in a second run through a 10% gel (30 ml). Bromophenol blue (BB) was used as a marker.

The analytical gel confirmed HA$_2$ to be pure and clearly separated from HA$_1$. The HA$_2$ preparation (HA$_2$-PAGE) remained in solution after excess SDS had been removed by dialysis.

The antiserum raised against HA$_2$ prepared by either procedure proved to be monospecific; it did not cross-react in ID with HA$_1$ (Fig. 2) or with the other viral antigens NP or M. Absence of neuraminidase inhibition when tested made certain that the HA$_2$ preparation was not contaminated with neuraminidase. The antiserum also formed a good precipitation line when it diffused against the original undissociated HA prepared by treatment of virus particles with OG which had been removed by dialysis in order to avoid any possible interference of non-specific precipitates or turbidities caused by the detergent in the agar gel.

On the other hand, the isolated HA$_2$ was readily precipitated by a convalescent serum from a bird which had survived an active infection with FPV.

Precipitates also developed with the HAs of strains known to belong to the same subtype (Webster et al., 1981). The intensity of the precipitation lines was considerably less in the case of turkey/Wisconsin or the seal virus. No trace of reaction could be noted with any other of the type A strains listed in Table 1.

These results were supported by the specific binding of monoclonal anti-HA$_2$ in an RIA. A total of 34 hybridomas could be isolated which were positive with the HA$_2$-AP used for immunization. Among these, 18 clones were HA$_2$-specific (Table 2, group I), two clones reacted with HA$_2$-AP only and not with HA$_2$-PAGE (Table 2, group II), and 14 reacted with a host
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Fig. 2. Double-immunodiffusion of anti-HA₁ and anti-HA₂. The antigens used in this test and for the preparation of antisera were isolated as outlined in Fig. 1. SDS was removed by extensive dialysis against 0.01 M-Tris-HCl pH 7.2.

Table 1. Immunodiffusion with anti-HA₂ serum and isolated haemagglutinin from different strains of influenza A viruses

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/FPV/Rostock/34 (H7N1)</td>
<td>++ +</td>
</tr>
<tr>
<td>A/turkey/Oregon/71 (H7N1)</td>
<td>++</td>
</tr>
<tr>
<td>A/seal/Mass/1/80 (H7N7)</td>
<td>+</td>
</tr>
<tr>
<td>A/Victoria/5/75 (H3N2)</td>
<td>-</td>
</tr>
<tr>
<td>A/chick/Germany/49 (H1N7)</td>
<td>-</td>
</tr>
<tr>
<td>A/USSR/90/77 (H1N1)</td>
<td>-</td>
</tr>
</tbody>
</table>

* The antigen wells contained HA prepared by disrupting virus particles with 1.5% octylglucoside. After sedimentation of the cores, the supernatant was dialysed against PBS (HA titre about 2⁻¹⁵). The 1% agarose gel contained 0.5 M-KCl and 3% PEG 6000. The final evaluation was carried out after 48 h at room temperature.

component which was even present in the purified HA₂ preparations (Table 2, group III). The hybridomas listed in Table 2 are representative examples of these three types of reaction.

Like the polyclonal rabbit serum, the HA₂-specific antibody only reacted with virus of subtype H7.

Effect of anti-HA₂ serum on haemagglutination, haemolysis, and on virus replication

Anti-HA₂ rabbit serum did not inhibit haemagglutination by whole virus. HA was clearly inhibited, however, when isolated HA was employed in the inhibition test. Inhibition titres were 2⁻³ with an anti-HA₂ rabbit serum and 2⁻⁵ in a serum produced in a chicken. The haemagglutinin used had been stripped from the virus particles by OG treatment, and the detergent had been removed by dialysis. The HA titre of the isolated haemagglutinin corresponded approximately to the titre of the original virus preparation. Anti-HA₂ serum did
Table 2. *Reactivity pattern of monoclonal antibodies against HA2-AP with various antigens* 

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>HA2-AP</th>
<th>HA2-PAGE</th>
<th>HA1-PAGE</th>
<th>FPV-AP</th>
<th>FPV</th>
<th>FM-1</th>
<th>NDV</th>
<th>Allantoic fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>3700</td>
<td>5643</td>
<td>382</td>
<td>5272</td>
<td>2747</td>
<td>99</td>
<td>137</td>
<td>79</td>
</tr>
<tr>
<td>I-2</td>
<td>3069</td>
<td>4288</td>
<td>766</td>
<td>3445</td>
<td>1433</td>
<td>398</td>
<td>271</td>
<td>118</td>
</tr>
<tr>
<td>II-1</td>
<td>3090</td>
<td>131</td>
<td>40</td>
<td>3708</td>
<td>2213</td>
<td>94</td>
<td>135</td>
<td>178</td>
</tr>
<tr>
<td>II-2</td>
<td>2350</td>
<td>110</td>
<td>156</td>
<td>3210</td>
<td>2099</td>
<td>158</td>
<td>118</td>
<td>102</td>
</tr>
<tr>
<td>III-1</td>
<td>2427</td>
<td>1141</td>
<td>2889</td>
<td>3920</td>
<td>342</td>
<td>94</td>
<td>135</td>
<td>178</td>
</tr>
<tr>
<td>III-2</td>
<td>3049</td>
<td>2634</td>
<td>3186</td>
<td>4107</td>
<td>3913</td>
<td>4637</td>
<td>5391</td>
<td>2295</td>
</tr>
<tr>
<td>P3X63.Ag8</td>
<td>112</td>
<td>80</td>
<td>123</td>
<td>87</td>
<td>59</td>
<td>117</td>
<td>95</td>
<td>70</td>
</tr>
</tbody>
</table>

*The microtitre wells were coated with HA2, purified by the acidic protease procedure (HA2-AP) or by polyacrylamide gel electrophoresis (HA2-PAGE). FPV-AP was undisrupted, washed virus pellet after protease treatment; FPV, FM-1 (A/FM/1/47, H1N1) and NDV (Newcastle disease virus) were whole virus. Allantoic fluid was taken from uninfected embryonated eggs.

Table 3. *Summary of effects measured with anti-HA2 antibodies*

<table>
<thead>
<tr>
<th>Inhibition of</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>haemagglutination, virus</td>
<td>—</td>
</tr>
<tr>
<td>haemagglutination, HA</td>
<td>+</td>
</tr>
<tr>
<td>haemolysis</td>
<td>—</td>
</tr>
<tr>
<td>virus release</td>
<td>—</td>
</tr>
<tr>
<td>Neutralization</td>
<td>—</td>
</tr>
</tbody>
</table>

not interfere significantly with the haemolytic activity of FPV; the extinction values hardly differed from the saline control.

In accordance with the absence of HA inhibition, infectivity of FPV was not neutralized by anti-HA2 serum. When the serum was present in the culture medium of FPV-infected cells there was no reduction in virus yield measured as HA activity of the medium 8 h after infection. When the serum was incorporated into the agar overlay of a plaque assay, plaques had the same average diameter as in control cultures containing normal serum. This means that anti-HA2 serum has no neutralizing effect and it does not inhibit virus release. Table 3 summarizes the lack of influence of anti-HA2 on the various biological parameters tested. All these results were identical when anti-HA2 serum was employed which had been produced by immunizing rabbits with HA2 prepared by SDS–PAGE or by digestion of the virus with the acid protease.

The same results were obtained with monoclonal anti-HA2. Not even the concentrated antibodies in ascitic fluid were capable of inhibiting haemagglutination or haemolysis, or of neutralizing the virus.

*Failure to demonstrate HA2 on the surface of infected cells*

As shown in Fig. 3, uninfected cells were neither labelled with anti-HA, an antiserum prepared against whole haemagglutinin (a), nor with anti-HA2 (b). The surface of FPV-infected cells and budding virus were readily labelled with anti-HA serum (c), whereas virtually no label was found on these cells after incubation with anti-HA2 serum (d); only a few limited spots on virus particles contained some label. Since the cultures were pre-fixed with glutaraldehyde, neither IgG nor the ferritin-conjugated secondary antibody were engulfed by the cells.

A panel of eight monoclonal antibodies which were RIA-positive against HA2 did not bind to FPV-infected cells of the permanent human T cell line CEM to a greater extent than to cells infected with the unrelated NDV. The higher amount of radioactivity retained with a monoclonal antibody directed against a cellular surface antigen served as a positive control for the procedure (Table 4). Positive haemadsorption had shown that HA is exposed at the surface of these cells.
Fig. 3. Electron micrographs of immunoferritin-labelled BHK cell surface. (a, b) Uninfected cell incubated with anti-HA serum (a) or anti-HA$_2$ serum (b). (c, d) FPV-infected cell incubated with anti-HA serum (c) or anti-HA$_2$ serum (d). After incubation with primary antibody, all cells were treated with ferritin-conjugated secondary antibody (see Methods). Note the intense labelling of FPV-infected cells and virions with anti-HA serum (c) whereas very little label is observed with anti-HA$_2$ serum exclusively on virus particles (d). In all cases primary rabbit antisera were diluted 1:50 and the goat anti-rabbit ferritin conjugate 1:25 with PBS. Bar marker represents 100 nm.
Table 4. Lack of binding of anti-HA2 monoclonal antibodies to FPV-infected cells

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Ct/min bound to CEM cells infected with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPV</td>
</tr>
<tr>
<td>I-1</td>
<td>646</td>
</tr>
<tr>
<td>I-2</td>
<td>511</td>
</tr>
<tr>
<td>I-3</td>
<td>758</td>
</tr>
<tr>
<td>I-4</td>
<td>470</td>
</tr>
<tr>
<td>I-5</td>
<td>496</td>
</tr>
<tr>
<td>II-1</td>
<td>710</td>
</tr>
<tr>
<td>III-1</td>
<td>292</td>
</tr>
<tr>
<td>III-2</td>
<td>726</td>
</tr>
<tr>
<td>W6/32†</td>
<td>4056</td>
</tr>
<tr>
<td>P3X63.Ag8‡</td>
<td>280</td>
</tr>
</tbody>
</table>

* About 10⁷ cells of the human cell line CEM were washed 6 h after infection with fowl plague virus or Newcastle disease virus, incubated with 0.5 ml of monoclonal antibodies on ice, washed again, and bound antibodies were measured by treating the cells with 125I-labelled rabbit anti-mouse IgG antibodies. I to III: different anti-HA2 monoclonal antibodies (see Table 2).
† Monoclonal antibody against HLA as a positive control.
‡ Supernatant from plasmacytoma used for fusion.

Table 5. Cytotoxicity of FPV immune mouse spleen cells restimulated with FPV or HA2-AP

<table>
<thead>
<tr>
<th>Antigen added to culture</th>
<th>Percent specific lysis of P815 cells infected with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPV</td>
</tr>
<tr>
<td></td>
<td>40:1†</td>
</tr>
<tr>
<td>None</td>
<td>4.0</td>
</tr>
<tr>
<td>HA2-AP</td>
<td>3.0</td>
</tr>
<tr>
<td>FPV, u.v.-inactivated</td>
<td>26.3</td>
</tr>
</tbody>
</table>

* Six weeks after infection with FPV, DBA/2 spleen cells were incubated for 5 days and then tested for cytotoxicity.
† Effector:target cell ratio.

**Failure to induce cytotoxic T lymphocytes with HA2**

HA2-AP added to the culture medium of spleen cells from a mouse which had been pre-immunized by an infection with FPV 6 weeks previously was not capable of stimulating a cytotoxic effect. U.v.-inactivated whole virus added to a parallel culture generated virus-specific cytotoxic T cells (Table 5). There was no significant lysis of NDV-infected target cells which served as negative controls.

**Absence of a protective immunity in chickens vaccinated with HA2**

Two chickens which had received two intramuscular injections of about 500 µg HA2-PAGE incorporated in Freund’s complete adjuvant within 3 weeks died after challenge with 1 ml of egg-grown virus diluted 10⁻⁴-fold. Blood samples drawn immediately before the challenge infection contained antibodies which precipitated HA2 specifically in ID. Control animals injected with whole HA were fully protected against the lethal infection, as expected (Rott, 1977).

**DISCUSSION**

The selective digestion of virus particles with acid protease avoids the application of any denaturing agents. In this procedure, HA2 remains in situ in the virus envelope before it is released by the mild detergent octylglucoside which normally preserves the entire haemagglutinating activity of a virus preparation. Although it cannot be totally excluded that the amino-terminal region of HA2 is not attacked by the protease, the polyacrylamide gel pattern shows that larger pieces are not cleaved from the HA2 polypeptide.
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The preparative PAGE employed for the separation of HA₂ from HA₁ and any residual neuraminidase which had remained adherent to the HA proved to be convenient and effective after the technique had been optimized for the LKB ‘Uniphor’. The fact that isolated HA₂ still reacted with a convalescent serum proves that the denaturing effect was limited and that at least an important part of its original antigenic domain was retained.

Antiserum against HA₂ showed subtype specificity in immunodiffusion tests, in accordance with results obtained by Brown et al. (1980). Although the reactions were not quantified, the weaker intensity of the precipitin lines obtained with heterologous strains can be regarded as rough estimates that the antigenic variation of HA₂ follows the antigenic drift within this subtype established by haemagglutination-inhibition tests. The results were confirmed with monoclonal antibodies whose reactions were also limited to the subtype. Although it could be clearly established by the present experiments that HA₂ is not type A-specific, a larger variety of strains must be examined before it can be firmly established that the antigenic variation of HA₂ is confined to the subtype of the respective haemagglutinin.

The number of antigenic regions and their location on the HA₂ molecule cannot be estimated at present. There is evidence from a competitive RIA that several antigenic domains are expressed on the small HA chain. When individual monoclonal antibodies were labelled and mixed with others, these antibodies did not compete with each other for the respective antigenic determinants (data not shown). Since neither with the polyclonal anti-HA₂ antisera nor with monoclonal antibodies could any reactivity be observed beyond the particular subtype H7, it does not seem to be justified to attribute a major antigenic role to this region (Atassi & Webster, 1983).

One difference was found between the HA₂ remaining after digestion with the protease and HA₂, prepared by PAGE, which is listed in Table 2. Most likely the monoclonal antibodies of group II are directed against an antigenic domain which was irreversibly denatured by treatment with SDS, while it remained intact after HA₁ had been removed by the enzyme. Since the latter purification procedure started with whole virus, it cannot be excluded that a further split product of another unknown viral component is hidden in this HA₂ preparation and gave rise to these monoclonal antibodies.

The observation that anti-HA₂ antibodies do not inhibit haemagglutination and fusion-haemolysis and that they do not neutralize can be explained by the three-dimensional structure of the HA (Wilson et al., 1981), according to which the smaller HA₂ chain is covered by the globular HA₁ and is not accessible to the antibodies. The importance of the spatial arrangement of the antigenic sites of the HA is underlined by our finding that haemagglutination by isolated HA is readily inhibited by anti-HA₂ serum. This means that antigenic determinants of the HA₂ become accessible even when the receptor-binding region remains intact. Some unfolding of the viral envelope which may have occurred during the coating of the RIA solid phase with virus particles at a high pH probably allowed sufficient access of monoclonal antibodies to antigenic determinants of HA₂ so that the binding values of Table 2 could be measured.

Our failure to demonstrate HA₂ on the surface of the infected cells indicates that during the process of insertion of the HA into the plasma membrane or during virus budding, HA₂-specific antigenic determinants do not become exposed at the cell surface. The lack of HA₂-specific antigenic sites which became evident in the electron micrographs of Fig. 3 was supported in experiments with labelled monoclonal antibodies where the degree of binding did not exceed background levels.

Since HA₂ is not exposed at the surface of infected cells, anti-HA₂ specific antibodies do not find a point of attachment for the inhibition of virus release. This also furnishes an explanation for the failure to stimulate a cell-dependent cytotoxic effect with HA₂. Even if cytotoxic lymphocytes were to be generated by HA₂ the infected cells cannot be recognized as targets by the T cells. It is highly improbable, therefore, that the type-specific cytotoxic responses described in numerous reports (see Sherman et al., 1983) are determined by antigenic domains of the HA₂. Our results rather support conclusions indicating that the antigen responsible for this cytotoxic effect is independent of the HA (Townsend & Skehel, 1982; Bennink et al., 1982).

They are in contrast to a recent report (Wabuke-Bunoti & Fan, 1983) that a residue of 21
amino acids of HA$_2$ is recognized at the surface of the HA by cytotoxic lymphocytes. Since a cyanogen bromide cleavage peptide was employed in these experiments and no serological analysis was carried out, these discrepancies can hardly be evaluated.

The lack of any protective immunity in animals immunized with the whole HA$_2$ polypeptide underlines that neither the humoral nor cell-mediated immune response against the smaller HA chain plays a significant role in protection or recovery from an infection. Since antibodies against HA$_2$ can readily be demonstrated in convalescent sera of chickens, the failure to leave a protective immunity is not due to a lack of immunogenicity of HA$_2$ synthesized in the infected cell; it must be explained by the absence of an HA$_2$-specific target on the surface of the infected cell and on the virus particle.

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REFERENCES


Immunogenicity of influenza HA


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