Evidence for a New Type-specific Structural Antigen of the Influenza Virus Particle

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Three virus-coded structural antigens of the influenza virus have been previously described. The ribonucleoprotein (RNP) antigen, identified as the 'soluble' antigen of influenza by Hoyle & Fairbrother (1937), is located internally in the virus particle and is antigenically invariant and type-specific, providing the basis for the division of influenza viruses into types A, B and C. The haemagglutinin and neuraminidase antigens occur at the virus surface and are immunologically and morphologically (Laver & Valentine, 1969) distinct proteins which show a considerable degree of antigenic variation. In addition, Dimmock & Watson (1969) have presented evidence for a non-structural antigen induced by the influenza virus which appeared to be present in the nucleoli of cells early after infection with influenza. This report describes studies on an additional antigen of the virus which appears to be located internally in the particle.

Skehel & Schild (1970) reported 7 species of polypeptide of mol. wt ranging from 94,000 to 25,000 which were identified by polyacrylamide gel electrophoresis of purified influenza virus particles. The smallest of these polypeptides (polypeptide no. 7, Skehel & Schild, 1971) of mol. wt 25,000 was abundant in the particle (approximately 30% of total virus protein) and appeared to be located internally since it was a major component of subviral particles (virus 'cores') obtained after the removal of the haemagglutinin and neuraminidase proteins from virus particles (Skehel, 1971). The antigen described in this paper appears to correspond with this low mol. wt internal component of the virus particle and in this paper will be referred to as the internal protein (IP) antigen.

X-31 virus (Kilbourne, 1969), a high yielding recombinant influenza virus antigenically identical to A2/Hong Kong/68 virus, was cultivated in embryonated eggs, and purified as described by Skehel & Schild (1971). Virus concentrates were prepared which contained $10^6$ to $10^5$ haemagglutinating units/ml. and 10 to 15 mg./ml. of protein (Lowry et al. 1951). To obtain preparations containing polypeptide no. 7, concentrates of X-31 virus were disrupted by the addition of sodium dodecyl sulphate (1% final concentration) at pH 6.6 and the protein components separated by electrophoresis on cellulose acetate paper strips using a modification of the technique described by Laver (1964) with a pH 6.6 buffer of the following composition: Na$_2$HPO$_4$:NaH$_2$PO$_4$, 0.05 M; EDTA, 0.001 M; sodium dodecyl sulphate 0.5% w/v. The separation of the structural components of X-31 virus under these conditions will be described (G. C. Schild, in preparation). The protein band which migrated fastest towards the anode was identified by polyacrylamide gel electrophoresis as described by Skehel & Schild (1971) and was found to contain polypeptides of species no. 7. A further protein band which moved relatively slowly towards the anode was composed essentially of polypeptides of species no. 5 of mol. wt 53,000 and was identified (Skehel & Schild, 1971) as corresponding to the RNP antigen. To obtain the IP antigen for the present study the protein from the fastest migrating band from cellulose acetate strips was eluted in 0.15 M-NaCl and dialysed over a period of two weeks against several changes of saline in order to remove sodium dodecyl sulphate. Finally the eluates were concentrated by vacuum dialysis and the resulting preparations adjusted to contain approximately 400 μg./ml. of...
protein. Antisera to X-31 IP antigen were prepared in rabbits and guinea pigs immunized by three injections, at two weekly intervals, each containing 40 μg. of antigen together with Freund's complete adjuvant. Serum samples collected 4 weeks after the final dose of antigen contained high levels of antibody to the antigen as detected in immuno-double-diffusion and complement-fixation tests. Attempts to prepare antibodies against antigen prepared in the same manner but without prolonged dialysis were unsuccessful, possibly because of the presence of sodium dodecyl sulphate which may be reversibly bound to the protein molecules and render the protein non-antigenic.

Antisera to X-31 IP contained no demonstrable HI activity with X-31 virus (1:5 serum dilution treated with receptor destroying enzyme) and also failed to react in neuraminidase-inhibition tests (Webster & Pereira, 1968). No virus neutralizing activity was detected after incubating 50 to 100 EID₅₀ of X-31 virus for 3 hr at 25°C with 1:5 dilutions of sera. In complement-fixation tests (Bradstreet & Taylor, 1962) anti-IP sera for X-31 virus reacted to high titres with preparations of isolated IP antigen (serum end-point titre 1:3200 at an optimal antigen concentration of 0.001 mg. protein/ml.) or with concentrated, purified X-31 virus disrupted by the addition of a detergent, Nonidet-P₄₀ (serum end-point titre 1:6400, optimal antigen concentration 0.005 mg. protein/ml.). Only low levels of complement fixation were detected with the same preparation of influenza virus which was not treated with detergent (serum end-point 1:400, optimal antigen concentration 0.1 mg. protein/ml.). The CF activity detected with undisrupted virus probably represented the presence in the virus concentrate of a small proportion of damaged or incomplete particles in which the IP was exposed. Detailed comparisons of influenza A viruses from human and non-human sources and influenza B viruses in complement-fixation tests (G. C. Schild, in preparation) enabled the conclusion that the IP antigen was type-specific for influenza A and B viruses but antigenically distinct from the RNP antigen. Similar conclusions were obtained from the immuno-precipitin tests described in this paper.

In immuno-double-diffusion tests (Schild & Pereira, 1969) using concentrates of X-31 virus disrupted with sodium sarcosyl sulphate (Geigy NL97; 1% final concentration) anti-
sera to X-31 IP produced in rabbits and guinea pigs gave a single, dense precipitin line which was first seen after 2 hr and which formed near the antibody-containing well. The intensity of the precipitates given by anti-IP antiserum (Fig. 1) suggested that the antigen was abundant in virus particles. IP antigen was also detected in virus 'cores' prepared as described by Skehel (1971). The precipitin reactions of the antiserum to IP were compared with those given by antiserum against influenza A ribonucleoprotein and influenza A2 neuraminidase (Schild & Pereira, 1969) and A2/HONG KONG/68 haemagglutinin (Schild, Winters & Brand, 1972b; Schild et al. 1972a). The precipitin line given by antiserum to X-31 IP did not show reactions of identity with monospecific antisera to the other virus antigens (Fig. 1). When a mixture of antiserum to RNP protein and IP were used (see Fig. 1) two clearly separated lines were formed. The line corresponding to RNP formed nearer to the virus-containing well and several hours later than that corresponding to the IP antigen indicating a slower rate of diffusion of the RNP antigen. These findings were confirmed for a wide range of antiserum concentrations (1:1, 1:40). When the same batch of antisera were tested against concentrates of an avian influenza A virus ('N'/CHICKEN/GERMANY/49) known to contain haemagglutinin and neuraminidase antigens distinct from that of X-31 virus only the anti-IP and anti-RNP sera reacted (Fig. 2). Rabbit antiserum against influenza host (carbohydrate) antigen (Haukenes, Harboe & Mortensson-Egnund, 1966) gave a precipitin line in tests with X-31 virus which did not show reactions of complete or partial identity with the lines given by antiserum to IP, RNP, neuraminidase or haemagglutinin.

To study the antigenic specificity of their IP antigens, concentrates of a number of influenza A viruses of human, avian and procine origin, were disrupted with detergent and used in precipitin tests. The strains examined were: human isolates; A0/PR8/34, A0/BEL/42, A1/EM1/47, A2/SINGAPORE/1/57, A2/ENGLAND/12/64, A2/HONG KONG/1/68, A2/ENGLAND/878/69; avian isolates; FPV/DUTCH/27, 'N'/CHICKEN/GERMANY/49 (DINTER); and swine, SHOPE S15 (1930). All the influenza A viruses tested gave precipitin lines with antiserum to X-31 IP which showed reactions of identity for each strain (representative tests are shown in Fig. 3). When a mixture of anti-IP and anti-RNP sera were used (Fig. 4) two precipitin lines were formed for all influenza A strains tested showing that the IP and RNP antigens shared the same type-specificity for influenza A viruses. In contrast, antiserum to influenza A RNP gave only a single precipitin line (Fig. 5). Influenza B strains (B/LEE/40, B/ENGLAND/5/66 and B/VICTORIA/9826/70 failed to react with antiserum to X-31 IP.

Rabbit antiserum was prepared against the internal protein antigen derived from influenza B/VICTORIA/70 virus using a method identical to that employed for X-31. This serum gave strong precipitin reactions with detergent-disrupted influenza B viruses but failed to react with influenza A strains. In tests with B/LEE/40, B/ENGLAND/5/66 and B/VICTORIA/70 viruses placed in adjacent wells in the gel a continuous precipitin line was produced indicating that the influenza B viruses share an antigenically common IP antigen which was antigenically distinct from that of influenza A viruses.

Although the IP antigen appears to be a major antigenic component of the influenza virus and is probably present in larger amounts than other antigenic components, its structural and functional role in the virus particle is at present uncertain. Recent studies (G. C. Schild, unpublished) have indicated that antibody to IP antigens appears in human and animal sera as a result of natural influenza infections and studies are in progress to determine the possible biological and epidemiological significance of such antibody.

Since the IP antigens, at least in the influenza viruses so far studied, like the ribonucleoprotein antigens, are type-specific their identification in influenza A and B viruses does not complicate the current system of classification of influenza viruses (see WHO
Group on Nomenclature of Influenza Viruses, 1971). However, the possibility of antigenic variation in the IP antigens of influenza A or B viruses should not be overlooked in future investigations.

*Note added in proof:* During the preparation of this manuscript the term MP (membrane or matrix protein) was suggested by the Subcommittee on Influenza of the National Institutes of Allergy and Infectious Diseases, Bethesda, U.S.A. to describe the influenza virus component corresponding to polypeptide number 7.

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**REFERENCES**


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