A Comparison of Proteins among Various Influenza B Virus Strains by One-Dimensional Peptide Mapping

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SUMMARY
The major virus-specific proteins (HA, NA, NP, NS1 and M) of five different isolates of influenza B virus (B/Lee/40, B/Osaka/2/70, B/Yamagata/1/73, B/Aomori/1/76 and B/Yamagata/26/77) were compared by limited proteolysis with Staphylococcus aureus V8 protease and subsequent polyacrylamide gel electrophoresis. The peptide patterns of matrix (M) proteins from all five strains were virtually identical. The nucleoproteins (NP) as well as the non-structural proteins (NS1) were also very similar among strains although the peptides of B/Lee/40 could be distinguished from those of the strains isolated from 1970 to 1977. In contrast, the peptides from haemagglutinin (HA) glycoproteins were largely different even among the strains isolated later than 1970. It therefore appears that the HA glycoproteins of influenza B virus are more changeable than any of the non-glycosylated proteins. Furthermore, it was found that the maps of HA1 were markedly different among strains while the maps of HA2 were very similar, which suggests that the structural changes in the HA polypeptide occur preferentially in the HA1 portion. The neuraminidase (NA) glycoproteins also showed strain-dependent differences in their mapping patterns.

INTRODUCTION
Influenza B virus, like the influenza A virus, consists of the two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) and the five internal non-glycosylated proteins, nucleoprotein (NP), matrix (M) protein and three P proteins (P1, P2, P3) (Almond et al., 1979). The HA glycoproteins may be present in virions as two cleavage products, HA1 and HA2. In addition to these structural proteins, two non-structural proteins, NS1 and NS2, can be detected in infected cells (Racaniello & Palese, 1978; Almond et al., 1979; Lamb & Choppin, 1979).

It is well known that the surface glycoproteins of influenza A viruses undergo two types of antigenic variation, major antigenic shift and antigenic drift (for review, see Webster & Laver, 1975). The previous serological studies have shown that influenza B viruses also undergo antigenic drift, but unlike influenza A viruses they do not undergo antigenic shift (Chakraverty, 1971, 1972; Schild et al., 1973). However, little is known about the biochemical basis for the antigenic drift of influenza B virus. The internal non-glycosylated proteins of influenza B virus may be also changeable in structure, as has been observed with influenza A virus (Laver & Downie, 1976; Brand et al., 1977; Dimmock et al., 1977, 1980). Racaniello & Palese (1979) have compared P1, NP, NS1 and M proteins of the B/Lee/40 and B/Maryland/59 strains by gel electrophoresis of peptides produced after partial protease digestion, and have demonstrated that the maps of P1, NP and NS1 are different between these two strains.
The present studies were undertaken to analyse the structural variation in the glycoproteins as well as in the non-glycosylated proteins of influenza B virus in more detail. For this purpose, we compared the HA, NA, NP, NS1, and M proteins of five different influenza B virus strains which included the earliest isolate, Lee/40 and the four isolates during the period of 1970 to 1977 from Japan, utilizing one-dimensional mapping of the peptides produced after partial protease digestion.

METHODS

Virus and cells. The following influenza B virus strains were used: B/Lee/40 (Lee/40), B/Osaka/2/70 (Osaka/70), B/Yamagata/1/73 (Yamagata/73), B/Aomori/1/76 (Aomori/76) and B/Yamagata/26/77 (Yamagata/77). Virus stocks of all strains were grown in the allantoic sac of 10-day-old embryonated hens' eggs inoculated with $10^4$ to $10^5$ diluted seed stock. Allantoic fluids were harvested after incubation for 48 h at 34 °C and stored at −80 °C until used. For purification of viruses from allantoic harvests, cellular debris was removed by low-speed centrifugation, which was followed by pelleting of virus at 30000 rev/min for 45 min in an IEC type A 192 rotor. The resultant pellet was suspended in phosphate-buffered saline lacking Ca$^{2+}$ and Mg$^{2+}$, pH 7.2 (PBS) and further purified by banding in a potassium tartrate gradient as described previously (Compans et al., 1970; Landsberger et al., 1971). The MDCK line of canine kidney cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% bovine serum.

Isotopic labelling of infected cells. Monolayers of MDCK cells on 3 cm plastic Petri dishes were inoculated with virus stock at a multiplicity of 20 to 50 EID$_{50}$/cell. After an adsorption period of 1 h, unadsorbed inoculum was removed and serum-free MEM was added. At 7 h after infection, cells were thoroughly washed with Hanks' balanced salt solution and labelled for 1 h in the solution containing $[^{35}\text{S}]$methionine. The concentrations of $[^{35}\text{S}]$methionine used were 5 μCi/ml for normal labelling of cells and 100 μCi/ml for samples for peptide mapping. At the end of labelling, cells were washed with cold PBS and scraped in this buffer. After pelleting by low-speed centrifugation, cells were suspended either in 0.0625 M-tris-HCl pH 6.8 for analysis by polyacrylamide gel electrophoresis or in 0.025 M-tris–HCl pH 8 containing 0.5% Triton X-100, 0.5% sodium deoxycholate and 0.005 M-NaCl for immunoprecipitation.

Immunoprecipitation. The washed cell pellet was disrupted in 500 μl 0.025 M-tris–HCl pH 8 containing 0.5% Triton X-100, 0.5% sodium deoxycholate and 0.005 M-NaCl at 0 °C for 30 min. It was then centrifuged in a Fischer microfuge for 5 min, and the resultant supernatant was used for immunoprecipitation. To the lysate, 10 μl of anti-rabbit serum against egg-grown Lee/40 virions (20 480 HIU/ml) was added, and the mixture was incubated at 37 °C for 30 min. This was followed by addition of 200 μl of a suspension of protein A-bearing Staphylococcus aureus (10%, v/v) prepared as described by Kessler (1975). After incubation for 30 min at room temperature, the bacteria were pelleted by centrifugation in the microfuge for 3 min. The pellet was washed twice with 0.05 M-tris–HCl pH 8 containing 0.1 M-NaCl and 0.1% Triton X-100 and then twice with the buffer lacking Triton X-100. The final pellet was suspended in 0.0625 M-tris–HCl pH 6.8 and boiled for 3 min in the presence of 2% SDS and 5% 2-mercaptoethanol. After centrifugation for 3 min, the supernatant was analysed by gel electrophoresis.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). High resolution polyacrylamide slab-gel electrophoresis was performed according to the method of Laemmli (1970). Unless otherwise noted, 13% gels (acylamide/bisacrylamide, 218:5:1) containing 4 M-urea with a 3.6% stacking gel (acylamide/bisacrylamide, 30:0.8) were used for both analytical and preparative purposes. For fluorography, gels were treated with 2,5-diphenyloxazole as
Protein variations of influenza B viruses

described by Bonner & Laskey (1974) and then dried under vacuum at 60 °C. Dried gels were exposed to Kodak X-Omat RP film at −80 °C.

Peptide mapping by limited proteolysis in SDS and subsequent gel electrophoresis. This was done essentially according to the method of Cleveland et al. (1977). To analyse the peptides of HA, NA, NP, NS₁ and M, the proteins synthesized in infected MDCK cells were used. Influenza B virus proteins synthesized in infected cells were labelled with [³⁵S]methionine and separated on 13% gels containing 4 M-urea as described above. The relevant bands were excised from dried gels by using an autoradiogram as template. Gel chips were rehydrated in a buffer consisting of 0-0625 M-tris–HCl pH 6-8, 0.1% SDS and 0-001 M-EDTA for 15 min at room temperature and then inserted into the wells of a 15% acrylamide gel containing an acrylamide/bisacrylamide ratio of 30:0-8. They were overlaid with 10 μl of the buffer containing 330 μg/ml bovine serum albumin and 20% glycerol, and further overlaid with 20 μl of buffer containing 20 μg/ml S. aureus V8 protease, 0-0002% bromophenol blue and 10% glycerol (Burge & Huang, 1979). Samples were allowed to enter the stacking gel (3-6% gel with acrylamide/bisacrylamide ratio of 30:0-8). When the dye reached the bottom of the stacking gel, the current was turned off. After digestion for 30 min, the current was turned on again. At the end of electrophoresis, gels were processed for fluorography as described above.

For analysis of peptides of HA₁ and HA₂, the glycoproteins were isolated after gel electrophoresis of purified virions grown in eggs. The bands corresponding to HA₁ and HA₂ were removed from wet gels after brief staining with Coomassie Brilliant Blue, and the proteins were electrophoretically eluted from gel pieces into dialysis tubes. Proteins were partially concentrated by dialysis against water under vacuum and then precipitated by addition of 9 vol. n-butanol. The precipitate was washed with ether to remove residual n-butanol and dissolved in 0-0625 M-tris–HCl pH 6-8 containing 0.1% SDS, 0-001 M-EDTA, 10% glycerol and 0-0001% bromophenol blue. After addition of V8 protease at an enzyme/substrate ratio of 1:5, samples were applied to 20% gel with the acrylamide/bisacrylamide ratio of 30:0-4. Digestion of proteins and subsequent gel electrophoresis of the resultant peptide fragments were carried out as described above. Mapping patterns were visualized by staining with 2% Coomassie Brilliant Blue dissolved in 50% methanol.

Preparation of antiserum against Lee/40 strain. A rabbit was intravenously injected with 1 mg purified virus grown in eggs. This was followed by four subcutaneous injections at weekly intervals, each containing 1 mg purified virus in 1 ml with an equal volume of Freund's complete adjuvant. The animal was bled on the 10th day after the last immunization.

Isotope and chemicals. L-[³⁵S]methionine (800 to 1200 Ci/mmol) was obtained from CEA, Gif-Sur-Yvette, France. Chemicals for SDS–PAGE were obtained from Nakarai Chemicals, Tokyo, Japan. S. aureus V8 protease was obtained from Miles Laboratories, Elkhart, Ind., U.S.A.

RESULTS

Electrophoretic mobility of virus proteins synthesized in infected cells

To compare the mobility of virus-specific polypeptides among influenza B strains, MDCK cells infected with each of five strains were labelled with [³⁵S]methionine and then subjected to SDS–PAGE. As shown in Fig. 1 (a) HA, NA, NP, NS₁ and M proteins were resolved with any of the strains analysed. In addition to these proteins, the presence of a protein designated NP_c₁ in infected cells was observed. This protein has been identified to be a cleaved form of the NP polypeptide (Nakamura & Homma, 1981). It should be noted that while NP and M proteins of all influenza B strains co-migrated, the mobility of HA, NA and NS₁ proteins was distinct among the strains. The strain-dependent difference in mobility was particularly
Fig. 1. Polypeptides synthesized in MDCK cells infected with five different strains of influenza B virus. The MDCK cells infected with various influenza B virus strains were labelled with [35S]methionine for 1 h at 7 h after infection. (a) The whole cells were directly analysed by SDS–PAGE. (b) The cells were subjected to immunoprecipitation with anti-Lee/40 serum and the resultant precipitates were analysed by SDS–PAGE. In these and all subsequent figures the following abbreviations were used: UC, uninfected cells; L, B/Lee/40; O, B/Osaka/2/70; Y-3, B/Yamagata/1/73; A, B/Aomori/1/76; Y-7, B/Yamagata/26/77.

As described above, three non-glycosylated proteins, NP, NS1 and M, were detectable in cells infected with influenza B viruses. Although there was no difference in the electrophoretic mobility of NP and M among influenza B strains, the NS1 protein of Lee/40 strain was found
Protein variations of influenza B viruses

Fig. 2. Peptide mapping of NP, NS1, and M proteins of various influenza B strains by limited proteolysis with V8 protease. The NP and M proteins were isolated by gel electrophoresis of the immunoprecipitates obtained after treatment of infected cells with anti-Lee/40 serum. The NS1 proteins were isolated after gel electrophoresis of whole infected cells. (a) NP; arrows indicate the peptides unique to Lee/40 strain. (b) NS1. (c) M.

to migrate faster than those of the other strains, which may suggest a change in structure of NS1 protein of influenza B virus. To investigate the structural variation of non-glycosylated proteins in more detail, peptide fragments obtained after limited proteolysis with V8 protease were compared among influenza B strains. For this purpose, either NP and M or NS1 protein was isolated after gel electrophoresis of immunoprecipitates or whole cells respectively.

The peptide patterns of NP polypeptides of various influenza B strains are shown in Fig. 2(a). The results indicate that NP of the Osaka/70, Yamagata/73, Aomori/76 and Yamagata/77 strains generated identical sets of peptides. The NP of Lee/40 also produced similar peptides, although a few peptides absent in the other strains were found. Fig. 2(c) presents the results of peptide mapping of M protein. The data indicate that M proteins of all influenza B strains generated essentially identical peptides. Fig. 2(b) shows the mapping patterns of NS1 protein. As was expected from the difference in the mobility of NS1 protein (see Fig. 1 a), the pattern of Lee/40 strain differed in several peptides from those of the other strains while the peptides of the four strains isolated from 1970 to 1977 were indistinguishable from each other.

The results described above suggest that structures of the non-glycosylated proteins of influenza B virus are highly conserved. This was particularly evident with four isolates during the period 1970 to 1977: there was no difference in the peptides of NP, NS1, and M among these strains. The structural similarity of NP, NS1, and M proteins among influenza B virus strains was further confirmed by using α-chymotrypsin for protein digestion instead of V8 protease. The peptides produced from NP and M proteins after α-chymotryptic digestion were identical among all the strains tested. The maps of NS1 protein were also indistinguishable among the four isolates between 1970 and 1977 although the Lee/40 strain showed a pattern different from the other strains (data not shown).
Fig. 3. A comparison of HA and NA glycoproteins of various influenza B strains by partial proteolysis with V8 protease. The HA and NA glycoproteins were isolated after gel electrophoresis of the immunoprecipitates from infected MDCK cells treated with anti-Lee/40 serum. (a) HA; (b) NA.

Peptide mapping of glycoproteins

To compare the peptides of HA and NA among influenza B virus strains, the glycoproteins were isolated by SDS–PAGE of the immunoprecipitates prepared by treatment of infected cells with anti-Lee/40 serum. After limited proteolysis of isolated glycoproteins with V8 protease, the resultant peptides were mapped by SDS–PAGE. The peptide patterns of HA glycoproteins are demonstrated in Fig. 3 (a). The results showed that the peptides produced from HA differed among strains to greater degrees than those from any of non-glycosylated proteins, although the Aomori/76 and Yamagata/77 strains produced the same peptides. The Lee/40 strain was found to lack several fragments detected in the Aomori/76 and Yamagata/77 strains. The HA glycoproteins of Osaka/70 and Yamagata/73 generated similar peptides. This was particularly evident with smaller peptide fragments while the larger peptides were apparently different.

Fig. 3 (b) demonstrates the peptide patterns of NA glycoproteins. The results showed that the peptide patterns of NA glycoproteins also differed depending on virus strain. The peptides of Lee/40 strain were easily distinguishable from those of the other strains. Although the patterns of the four strains isolated from 1970 to 1977 were similar, the Osaka/70 and Yamagata/73 strains appeared to be slightly different from Aomori/76 and Yamagata/77.

To see if the differences observed in the peptides of HA glycoproteins are due to the differences in the structure of either HA1 or HA2 or both, we attempted to compare the peptides of HA1 and HA2 among influenza B virus strains. For isolation of HA1 and HA2, purified virions grown in eggs were used. When the electrophoretic mobility of virus glycoproteins was compared among various influenza B strains, the mobility of HA1 was different depending on the virus strain whereas HA2 of all the strains migrated identically (data not shown). Fig. 4 demonstrates the peptide patterns of HA1 and HA2 isolated from three influenza B strains, Osaka/70, Yamagata/73 and Yamagata/77. The results indicate
Fig. 4. Peptide mapping of HA\textsubscript{1} and HA\textsubscript{2} of various influenza B strains by limited proteolysis with V8 protease. The HA\textsubscript{1} and HA\textsubscript{2} glycoproteins were isolated after gel electrophoresis of purified virions grown in eggs on 12\% gels as described in Methods. The isolated glycoproteins were partially digested with V8 protease, and the resultant peptide fragments were mapped on 20\% gels with an acrylamide/bisacrylamide ratio of 30:0.4. (a) HA\textsubscript{1}; (b) HA\textsubscript{2}.

that all the peptides generated from HA\textsubscript{2} were common to all virus strains tested (Fig. 4\textit{b}). In contrast, the peptide patterns of HA\textsubscript{1} clearly differed among these strains (Fig. 4\textit{a}). It therefore appears that the structural changes in HA polypeptides have occurred preferentially in the HA\textsubscript{1} portion.

**Discussion**

In previous studies, variation among influenza B virus strains has been investigated utilizing serological techniques (Chakraverty, 1971, 1972; Schild \textit{et al.}, 1973). The studies using these techniques were generally limited to the examination of variation in the surface glycoproteins, HA and NA. The recent advancements in techniques of polyacrylamide gel electrophoresis of proteins enabled a comparison of the electrophoretic mobility of virus-specific polypeptides among influenza B virus strains. Racaniello & Palese (1978, 1979) have demonstrated the migrational differences of NS\textsubscript{1} and M proteins between the B/Lee/40 and B/Maryland/59 strains. Almond \textit{et al.} (1979) have compared the proteins of four influenza B strains by SDS–PAGE, and have demonstrated that the mobility of NS\textsubscript{1}, NP and HA proteins are different among strains. In the present paper, we have also found migrational differences in HA, NA and NS\textsubscript{1} proteins. These observations suggest the possibility that structural changes can occur not only in the surface glycoproteins but also in the internal non-glycosylated proteins of influenza B virus. Although the differences in the mobility of three P proteins have not yet been described, these proteins may also be changeable since it has been demonstrated that all eight RNA segments of the B/Lee/40 strain migrate...
differently from those of the B/HK/8/73 or the B/Maryland/59 strain (Ueda et al., 1978; Racaniello & Palese, 1979).

An alternative approach for investigating the variation of non-glycosylated proteins may be the analysis of peptides produced after protease digestion. Racaniello & Palese (1979) have compared P, NP, NS, and M proteins of two influenza B virus strains, Lee/40 and Maryland/59, by limited proteolysis with various proteases and subsequent gel electrophoresis. The results have shown that the mapping patterns of P, NP and NS proteins are different between these two strains whereas the patterns of M protein are identical, which indicates that at least the P, NP and NS proteins of influenza B virus are changeable in their structure. To extend these observations, we compared three non-glycosylated proteins, NP, NS, and M between the Lee/40 strain and the four strains isolated more recently, utilizing the technique similar to that employed by Racaniello & Palese (1979). The results also demonstrated that the NP and NS proteins of the Lee/40 strain were different from those of the other strains. However, the peptide patterns of these proteins were identical among the four isolates between 1970 and 1977. Since the maps of HA and NA were found to be different even among these isolates, it appears that structures of the non-glycosylated proteins of influenza B virus are less changeable than those of the surface glycoproteins. The relative conservation of the non-glycosylated proteins can be understood since unlike the surface glycoproteins, the internal non-glycosylated proteins are not subjected to selective immunological pressure. An alternative explanation may be that the structural requirements may be more stringent for the non-glycosylated proteins than the glycoproteins. The extent of variation may differ even among the non-glycosylated proteins. Data of Racaniello & Palese (1979) and ours showed that the peptides from M protein were indistinguishable among the influenza B strains tested, which suggests that the structure of this protein may be more stable than those of P, NP and NS proteins. A similar conclusion has been obtained with influenza A virus from various approaches (Laver & Downie, 1976; Brand et al., 1977; Dimmock et al., 1977, 1980; Erickson & Kilbourne, 1980).

A number of studies have been done to elucidate the molecular basis for antigenic variation of HA polypeptides of influenza A virus. In the case of influenza B virus, however, there have been few reports in which chemical properties of HA polypeptides were compared among strains. The present paper demonstrated that the peptides produced after partial digestion of HA polypeptides with V8 protease were different among strains to a higher degree than any of the other virus-specific polypeptides. Furthermore, the findings that the migrational differences among influenza B strains could be observed in HA, but not in HA, and that the peptides of HA were apparently different among strains whereas no difference was detectable in the peptides of HA, suggest that the HA proteins are more changeable than the HA proteins. This has also been observed for influenza A virus by various approaches including analysis of amino acid composition (Erickson & Kilbourne, 1980), peptide mapping and amino acid sequencing (Bucher et al., 1976; Skehel & Waterfield, 1975; Laver et al., 1980). The observations that the HA proteins are more highly conserved than the HA proteins may be accounted for if, unlike the HA proteins, the HA proteins may not be under antibody pressure since HA has been found to be antigenically silent (Brand & Skehel, 1972; Jackson et al., 1979).

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Protein variations of influenza B viruses


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