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Coronavirus IBV Glycopolypeptides: Size of Their Polypeptide Moieties and Nature of Their Oligosaccharides

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SUMMARY

Analysis of differentially radiolabelled avian infectious bronchitis virus (IBV) indicated that the matrix (M) polypeptides of mol. wt. 23 × 10^3 (23K), 26K, 28K, 30K and 34K (M23 to M34) which have been shown to give the same peptide maps, differed in their degree of glycosylation; M23 was not glycosylated while glycosylation increased with increasing mol. wt. from M26 to M34. Both glucosamine and mannose were components of M26 to M34 but [^{3}H]fucose appeared to be associated mainly with M34. Endo-β-*N*-acetylglucosaminidase H removed oligosaccharides from M28 and M30 but not M26 and M34, to give a polypeptide of 23K. The surface projection glycopolypeptides S1 (90K) and S2 (84K) incorporated ^{3}H-labelled glucosamine and mannose but not fucose and had oligosaccharides removed by endoglycosidase H. The mol. wt. of the resultant polypeptides varied among experiments; the lowest mol. wt. observed were 64K and 61K. These results indicate (i) that the polypeptide moieties of the S polypeptides are approximately 64K and 61K, and 23K for the M polypeptide, (ii) that the oligosaccharides of the S and M polypeptides are of the high-mannose type and are linked to the polypeptides by *N*-glycosidic linkages, and (iii) that the M glycoprotein of IBV differs from that of murine coronaviruses and bovine coronavirus L9 which have *O*-linked oligosaccharides.

Avian infectious bronchitis virus (IBV) appears to have three protein structural elements: surface projections (S), a nucleocapsid (N) protein and a matrix/membrane (M) protein (Cavanagh, 1981). Two of these, S and M, are glycosylated (Cavanagh, 1981; Lomniczi & Morser, 1981; Wadey & Westaway, 1981; Stern *et al.*, 1982). My studies of IBV have indicated that the surface projections are associated with two glycopolypeptides, S1 (90K; K = 1000 daltons) and S2 (84K), and that the M protein was associated with two glycopolypeptides of 30K and 28K (Cavanagh, 1981); S1 had previously been assigned a mol. wt. of 94K. Stern *et al.*, (1982) have shown that several polypeptides of IBV, analogous to polypeptides of 34K, 30K/28K, 26K and 23K (M34 to M23) in our studies, all have the same peptide maps. Little is known about the oligosaccharides of the S and M polypeptides. In contrast, it is known from studies with tunicamycin, that the S (or E2) polypeptide(s) of murine and bovine L9 coronaviruses have *N*-glycosidically linked oligosaccharides while the M (or E1) glycoprotein has *O*-glycosidically linked oligosaccharides (Niemann & Klenk, 1981; Holmes *et al.*, 1981; Rottier *et al.*, 1981; Siddell *et al.*, 1981*a*; Cheley & Anderson, 1981). The glycosylation of the IBV glycopolypeptides has been studied to determine the nature of their oligosaccharides and the mol. wt. of their polypeptide moieties.

Working stocks of IBV-M41 and IBV-D41 (Darbyshire *et al.*, 1979) were prepared in embryonated chicken eggs (Cavanagh, 1981). Virus was radiolabelled in pairs of de-embryonated chicken eggs (Cavanagh, 1981). Each egg received 125 μCi [^{35}S]methionine (sp. act. > 800 Ci/mmol) and one of the following: 165 μCi of a mixture of 15 ^{3}H-labelled amino acids (code TRK 440); 125 μCi D-[6-^{3}H]glucosamine hydrochloride (sp. act. 20 to 40 Ci/mmol); 125 μCi D-[2-^{3}H]mannose (sp. act. 10 to 20 Ci/mmol), all purchased from Amersham

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International. Virus was purified by ammonium sulphate precipitation and isopycnic centrifugation in a sucrose gradient (Cavanagh, 1981). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in tubes with a 10% acrylamide separation gel (Cavanagh, 1981). Samples were dissociated at room temperature with 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. After the gels had been sliced the slices were dried and digested with 30% (w/v) hydrogen peroxide at about 80 °C for 2 to 3 h, prior to scintillation counting. The following enzymes were used: endo-β-*N*-acetylglucosaminidase H (endoglycosidase H) from *Streptomyces plicatus* (Miles Laboratories, U. K.); endo-β-*N*-acetylglucosaminidase D (endoglycosidase D) from *Diplococcus pneumoniae* (Miles Laboratories); β-*N*-acetylglucosaminidase from jack beans (Sigma); β-galactosidase from *Escherichia coli* (Sigma; grade VIII); neuraminidase from *Clostridium perfringens* (Sigma, type X). The buffer used was 50 mM-citrate–phosphate buffer pH 6·0. Sucrose solution, containing virus, was adjusted to pH 6·0 by addition of 100 mM-citric acid and divided into aliquots of 200 µl. Enzymes were used at the following final concentrations: endoglycosidase H (25 mU/ml) with or without 0·1% SDS, endoglycosidase D (50 mU/ml), neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase at 50, 100 and 100 mU/ml respectively. In one experiment phenylmethylsulphonyl fluoride (PMSF; Sigma) in isopropanol was added to 1 mM; the final reaction volume was 260 µl. After incubation at 37 °C for 40 h the pH was adjusted to approximately 7 by addition of 0·5 M-NaOH plus phenol red. The samples were kept at − 20 °C prior to analysis by SDS–PAGE.

With the exception of M23, which is normally present in very small amounts, the M polypeptides of IBV were glycosylated, as shown by the incorporation of [^{3}H]glucosamine (Fig. 1) and [^{3}H]mannose (Fig. 2); M23 had previously been assigned a mol. wt. of 21K (Cavanagh, 1981). Analysis in five polyacrylamide gels of IBV-D41 labelled with 15 ^{3}H-labelled amino acids and [^{35}S]methionine showed that the ^{3}H/^{35}S d/min (disintegrations per min) ratios for M23, M26, M28, M30 and M34 were 1·9, 1·9, 1·8, 1·9 and 1·9 respectively. Similar analysis of IBV-D41 labelled with [^{3}H]glucosamine and [^{35}S]methionine gave ^{3}H/^{35}S d/min ratios for M23, M26, M28, M30 and M34 of 1·2, 2·2, 3·2, 4·1 and 6·3 respectively. These data support the view of Stern *et al.* (1982) that M23 to M34 have the same polypeptide moiety but differ in their degree of glycosylation, this increasing with increasing mol. wt.

A virion polypeptide of 170K, said to be non-glycosylated, has been described for IBV-Beaudette (Lomniczi & Morser, 1981). This polypeptide was variably present in these IBV preparations, contains mannose (Fig. 2) and glucosamine (not shown), and is an aggregate of S2 and some S1 (D. Cavanagh, unpublished results). Both S1 and S2 contained mannose (Fig. 2) in addition to glucosamine (Fig. 1). Analysis of virus grown in the presence of [^{3}H]fucose and [^{35}S]methionine indicated that no fucose was associated with S1 or S2, in contrast to the equivalent E2 glycopolypeptides of mouse hepatitis virus (MHV; Sturman *et al.*, 1980), but that some fucose might be associated with the higher mol. wt. M glycopolypeptides, chiefly M34.

N-linked oligosaccharides are of two types, 'high-mannose' and 'complex' (Hubbard & Ivatt, 1981) which can be removed from the polypeptide by endoglycosidases H and D respectively (Tarentino & Maley, 1974; Tarentino *et al.*, 1974; Koide & Muramatsu, 1974). The specificity of endoglycosidase H is such that if a polypeptide had only high-mannose oligosaccharides, endoglycosidase H would be expected to yield a polypeptide with some residual *N*-acetyl-glucosamine but no mannose. Such polypeptides were generated from S1, S2, M30 and M28. Endoglycosidase H removed oligosaccharides from both S1 and S2 of virus doubly labelled with [^{35}S]methionine and [^{3}H]glucosamine (Fig. 1) or [^{3}H]mannose (Fig. 2). The products of hydrolysis were generally heterogeneous and their mol. wt. varied among experiments. Mean mol. wt. values of 71K ± 7K and 64K ± 3K were obtained from eight experiments. The smallest products, with mol. wt. of 64K and 61K, were obtained in the experiment shown in Fig. 2. Endoglycosidase H also decreased the ^{3}H/^{35}S ratio of gp170 (Fig. 2) but not to as great an extent as with S1 or S2; it is possible that some of the oligosaccharides are not sufficiently exposed for the enzyme to hydrolyse them. A polypeptide of mol. wt. 58K, which we usually detect only as a slight shoulder on the N peak, was present in greater amount in the virus preparation of Fig. 1. Endoglycosidase H converted this to a polypeptide of mol. wt. 48K with a decreased [^{3}H]glucosamine content (Fig. 1). In the experiments shown in Fig. 1 and 2, the

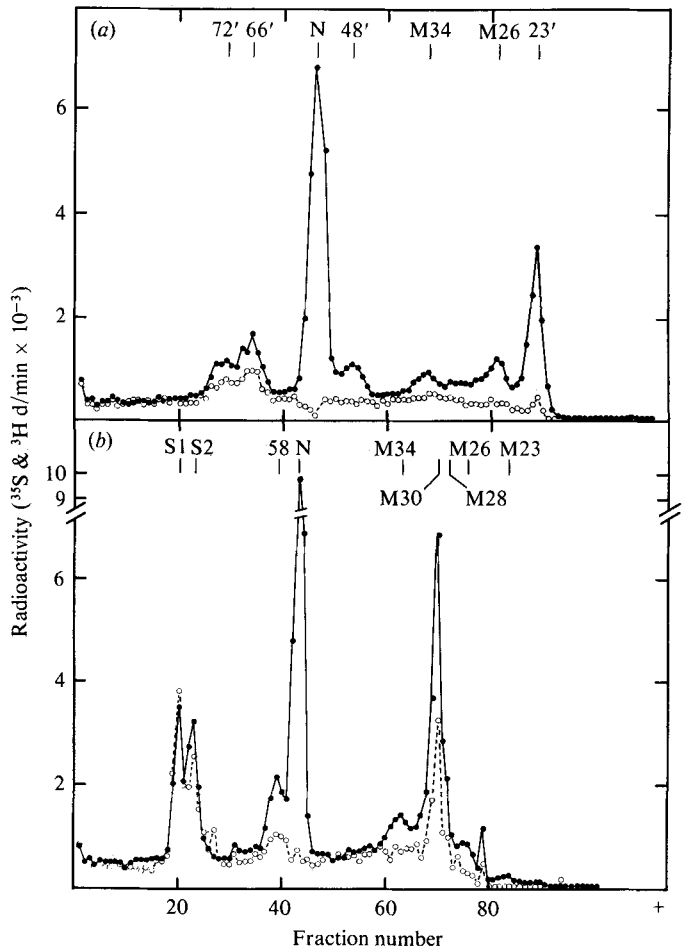

Fig. 1. SDS–PAGE of IBV-M41 labelled with [^{35}S]methionine (●) and [^{3}H]glucosamine (○) after incubation at 37 °C for 40 h at pH 6·0 with (*a*) 25 mU/ml endoglycosidase H and (*b*) no enzyme. 72′, 66′, 48′ and 23′ refer to the mol. wt. (× 10^{-3}) of polypeptides which are the products of hydrolysis by endoglycosidase H.

endoglycosidase H concentration was 25 mU/ml. Enzyme concentrations of up to 125 mU/ml did not decrease the size of S1 and S2 to less than in Fig. 2. Endoglycosidase D had no detectable effect on the surface projection polypeptides by itself or with the exoglycosidases neuraminidase, β-galactosidase and *N*-acetylglucosaminidase (Koide & Muramatsu, 1974) without or with (Fig. 2) endoglycosidase H. These results indicate that the oligosaccharides of S1 and S2 are *N*-glycosidically linked and are probably of the high-mannose type.

In all experiments the effect of endoglycosidase H on the M glycopolypeptides of IBV-M41 was the same: most of M30 and M28 was converted to a polypeptide of 23K with little or no [^{3}H]glucosamine (Fig. 1) and no [^{3}H]mannose (Fig. 2*a*, *b*), while M34 and M26 were not affected. Endoglycosidase D had no detectable effect on any of the M polypeptides. These results indicate that the oligosaccharides of M30 and M28 are *N*-glycosidically linked, high-mannose side-chains, and that the oligosaccharides of M34 and M26 are different from those of M30 and M28. This is in contrast to MHV and bovine coronavirus L9 in which the matrix glycopolypeptide has exclusively *O*-linked oligosaccharides and lacks mannose. That the hydrolysis of S1, S2, M30 and M28 was due to endoglycosidase H and not to possible protease contamination was indicated by (i) the lack of hydrolysis of N, M34 and M26, (ii) the finding

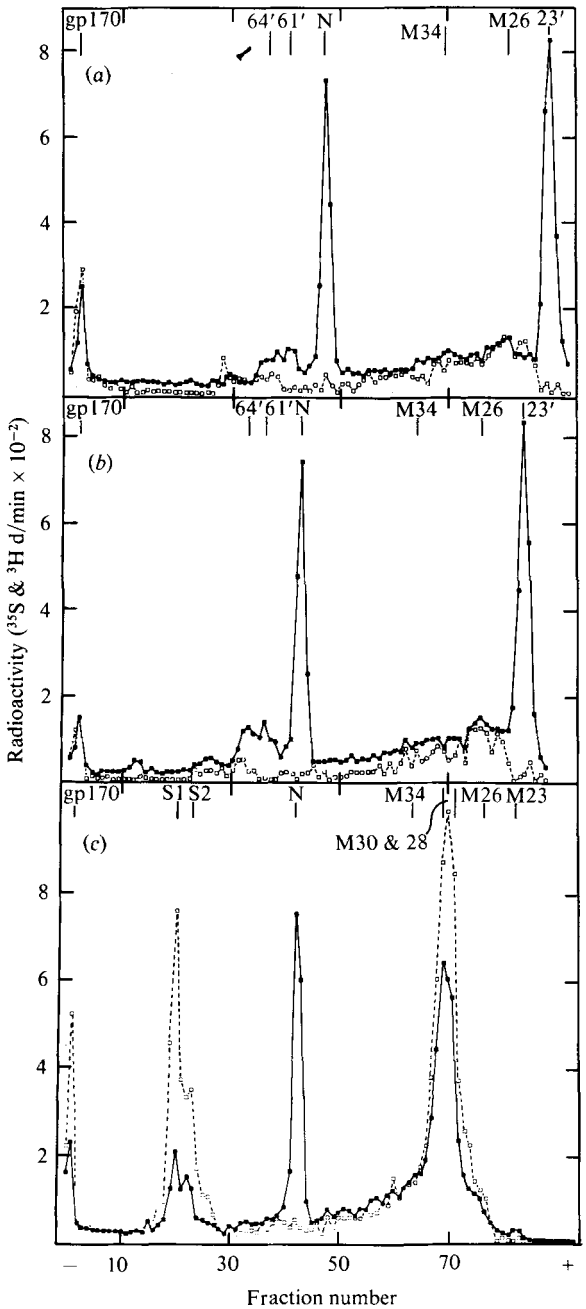

Fig. 2. SDS–PAGE of IBV-M41 labelled with [^{35}S]methionine (■) and [^{3}H]mannose (□) after incubation at 37 °C for 40 h at pH 6·0 with (*a*) 25 mU/ml endoglycosidase H, (*b*) a mixture of 25 mU/ml endoglycosidase H, 50 mU/ml endoglycosidase D, 50 mU/ml neuraminidase, 100 mU/ml β-*N*-acetylglucosaminidase and 100 mU/ml β-*N*-acetylglucosaminidase, and (*c*) no enzyme. All samples contained 1 mM-PMSF. 64′, 61′ and 23′ refer to the mol. wt. (× 10^{-3}) of polypeptides which are the products of hydrolysis by endoglycosidase H.

that there was no difference in the amount of radiolabel travelling at or near the electrophoretic front after the electrophoresis of enzyme-treated and control virus, and (iii) the failure of the protease inhibitor PMSF to affect the hydrolysis of S1, S2, M30 and M28 by endoglycosidase H.

The results of these differential radiolabelling and endoglycosidase experiments, coupled with the proteolysis data of Stern *et al.* (1982), indicate that the polypeptide moiety of the IBV matrix protein has a mol. wt. of 23K, which is similar to the non-glycosylated matrix polypeptide of MHV (Macnaughton, 1980; Siddell *et al.*, 1981*a*; Holmes *et al.*, 1981; Rottier *et al.*, 1981) and human coronavirus (HCV; Macnaughton, 1980). However, the extent of glycosylation is much greater with the M polypeptides of IBV (the oligosaccharides of M34 and M30 accounting for approximately 32% and 23% of the mol. wt. respectively) than with MHV or HCV, where the glycosylated forms have mol. wt. of only 2K or 3K higher than the non-glycosylated polypeptide.

Work with MHV and tunicamycin has shown that the surface projection glycopolypeptide(s) is derived from a precursor polypeptide. Siddell *et al.* (1981*a*, *b*) with the JHM strain, and Rottier *et al.* (1981) with the A59 strain have indicated that the polypeptide moiety of the surface projection precursor has a mol. wt. of about 120K and 110K respectively. My data suggest that the non-glycosylated IBV surface projection precursor would have a mol. wt. of about 125K, this being the combined mol. wt. of the surface projection polypeptides after endoglycosidase H hydrolysis.

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