BINDING OF ANTIBODIES THAT STRONGLY NEUTRALISE INFECTIOUS BRONCHITIS VIRUS IS DEPENDENT ON THE GLYCOSYLATION OF THE VIRAL PEPLOMER PROTEIN

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INTRODUCTION

Most of the biological properties of infectious bronchitis virus (IBV) are associated with the S_1 glycoprotein (1) that together with the S_2 glycoprotein forms the peplomer protein (2,3). Both glycoproteins bear N-linked oligosaccharides (4).

In an earlier study, we mapped the antigenic domains of the peplomer protein topographically and functionally using a competitive binding assay. This article summarises the results of the competitive binding experiments which will be described in detail elsewhere (Koch et al., submitted).

In the present article, we studied whether the binding of MAbs that strongly neutralise IBV are dependent on glycosylation of the peplomer protein. We conclude from the results of these experiments and the biochemical characteristics of the MAbs that the antigenicity of antigenic domains on IBV that induce virus-neutralising antibodies depends on the tertiary structure of the peplomer glycoproteins.

MATERIALS AND METHODS

Virus, cell culture, and monoclonal antibodies

IBV strain D207 was used throughout this study. The growth and purification of virus, cell culture conditions, production of MAbs, and techniques used for the biochemical and biological characterisation of MAbs have been described earlier (5, Koch et al, submitted).

Isolation of the S_1 subunit of the peplomer

The glycoprotein S_1 was isolated from allantoic fluid by precipitating proteins with polyethylene glycol 6000 followed by affinity chromatography with MAb CVI-IBVS_1-48.3 coupled to Sepharose.

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Deglycosylation of virus and S_1 glycoprotein

Virus was deglycosylated with endoglycosidase F that contained glycopeptidase F, glycopeptidase-free endoglycosidase F, or endoglycosidase H (all purchased from Boehringer Mannheim GmbH, FRG). Samples of 240 μg of IBV or of 3-4 μg of purified S_1 in 100 μl Tris-HCl buffer pH 7.2 (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA) were incubated for 44 h at 37 ^{o}C with 50 mU of either contaminated or purified endoglycosidase F, or with 10 mU of endoglycosidase H. At hours 16 and 44 of incubation, samples were collected and their reactivity with MAbs was tested in enzyme immunoassays and Western blotting.

Enzyme immunoassay

An indirect enzyme immunoassay (EIA) and a double-antibody sandwich (DAS) EIA were used to test whether MAbs against IBV reacted with endoglycosidase-treated IBV samples. The indirect EIA has been described earlier (5, Koch et al, submitted). The DAS-EIA was performed as follows. Microtiter plates were coated with 1 μg of the immunoglobulin fraction of various MAbs directed against viral proteins. MAb CVI-IBVS_1-69.3, which is directed against S_1, was labelled with peroxidase and used as conjugate.

Western blotting

Polyacrylamide gel electrophoresis was performed as described before (Koch et al, submitted) and Western blotting was performed by the method of (6). Blots were stained with Concanavalin A (Con A) (Pharmacia) and horseradish peroxidase to detect glycoproteins (7).

RESULTS

Antigenic topography of the peplomer

The antigenic map of the peplomer protein was determined by measuring the competitive binding of labelled and unlabelled MAbs. This approach was based on the premise that if two epitopes are either identical, overlapping, or adjacent, the binding of MAb to one of the epitopes will sterically block the binding of MAb to the second epitope and *vice versa*. In

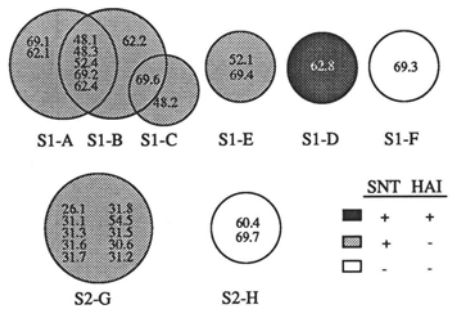

Fig. 1. Antigenic map of the peplomer protein of IBV. Circles represent antigenic domains of the peplomer protein. MAbs that define domains are indicated within circles. Biological functions of MAbs are indicated by hatched lines.

contrast, no blocking will occur if epitopes are sufficiently distant from each other. The results of the competitive binding studies are described and discussed in detail in another paper (Koch et al, submitted). Eight antigenic domains were identified on the peplomer protein, six on S_1 (S_1-A, -B, -C, -D, -E, and F) and two on S_2 (S_2-G and -H) (Fig. 1).

The MAbs with strong neutralising activity were directed against domains S_1-A, -B, -C, -D, and -E; one (MAb 31.1) was directed against domain S_2-G. Only domain S_1-D was associated with haemagglutination.

Effect of deglycosylation

To determine the nucleotide sequences encoding for the antigenic domains, we inserted peplomer gene fragments into a prokaryotic expression vector, pEX. Bacteria transfected with the vector produced hybrid proteins consisting of β-galactosidase and polypeptide fragments of the peplomer. Polypeptides were thus synthesised that together covered the whole peplomer protein (8,9). Only MAbs directed against the domains S_1-F and S_2-G bound to some of the hybrid proteins (MAbs directed against domain S_2-H were not tested) . Since proteins synthesised by bacteria are rarely glycosylated, we questioned whether the binding of MAbs directed against other domains than these three were dependent on protein glycosylation. We therefore investigated whether MAbs directed against these antigenic domains also bound to deglycosylated virus and deglycosylated isolated S_1 glycoprotein.

IBV and purified S_1 were deglycosylated by incubating with a endoglycosidase F that contained glycopeptidase F, glycopeptidase-free endoglycosidase F, or endoglycosidase H. The enzyme-treated virus or protein samples were analysed by Western blotting. In untreated virus samples, only one 94K protein was stained by MAb 69.3 (directed against S_1). In contrast, in endoglycosidase-treated virus, proteins with apparent MW ranging from 65,000-92,000 were stained by MAb 69.3 (Fig. 2). Likewise, in untreated virus, MAb 26.1 (directed against S_2) stained a 275K protein and

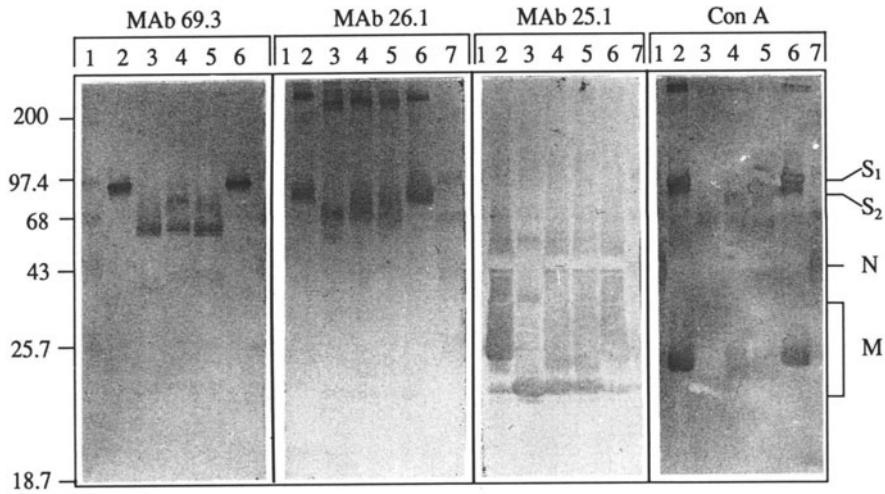

Fig. 2. Western blotting of endoglycosidase-treated IBV. Virus was incubated with endoglycosidase F containing glycopeptidase F (lane 3), purified endoglycosidase F (lane 4), endoglycosidase H (lane 5), or with buffer only (lane 2) at 37 °C for 44 h. Virus that was not incubated at 37 °C was electrophoresed in lane 6. Blots were stained with MAb directed against S_1 (MAb 69.3), against S_2 (MAb 26.1), or against the matrix protein (MAb 25.1) and stained for glycoproteins with Con A and peroxidase. Molecular weight markers were electrophoresed in lanes 1 and 7.

a 88K protein, whereas in treated virus, this MAb stained proteins with MW ranging from 61,000-92,000 and from 200,000-250,000. MAb 25.1 (directed against matrix protein) stained a 22K protein and proteins with a MW ranging from 24,000-36,000 in untreated virus. The amount of the latter proteins was decreased compared to the amount the 22K protein in enzyme-treated samples. Enzyme treatment of IBV had no affect on the MW of its nucleoprotein (data not shown). Con A stained all proteins that were stained by MAbs 69.3, 26.1, and 25.1 except the 200K-250K, 65K, 61k, and the 22K proteins.

When purified S_1 was treated with endoglycosidases, only a 65K protein was stained by MAb 69.3 in Western blots. Proteins with MWs ranging from 73,000-92,000 were very weakly stained by Con A only in virus samples which were treated with purified endoglycosidase F (Fig. 3, right panel).

Effect of deglycosylation on the binding of MAbs

Whether MAbs directed against IBV were also able to bind to deglycosylated virus was tested in an indirect EIA (Table 1) and in the DAS-EIA (data not shown). The binding of MAbs to coated, enzyme-treated virus was reduced compared to untreated virus, in particular, when treated with endoglycosidase F containing glycopeptidase F. Reduced binding resulted in a lower optical density. Incubating virus at 37 °C reduced the binding of MAb 62.8 to the virus even without enzyme.

The reactivity of MAbs with affinity-purified S_1 was determined in the DAS-EIA. All S_1 samples were diluted 1:240, except those that were added to wells coated with MAb 62.8. The latter samples were diluted 1:24, since at higher dilutions, MAb 62.8 did not bind to untreated S_1. Binding of S_1 was strongly reduced after deglycosylation. The binding was severely

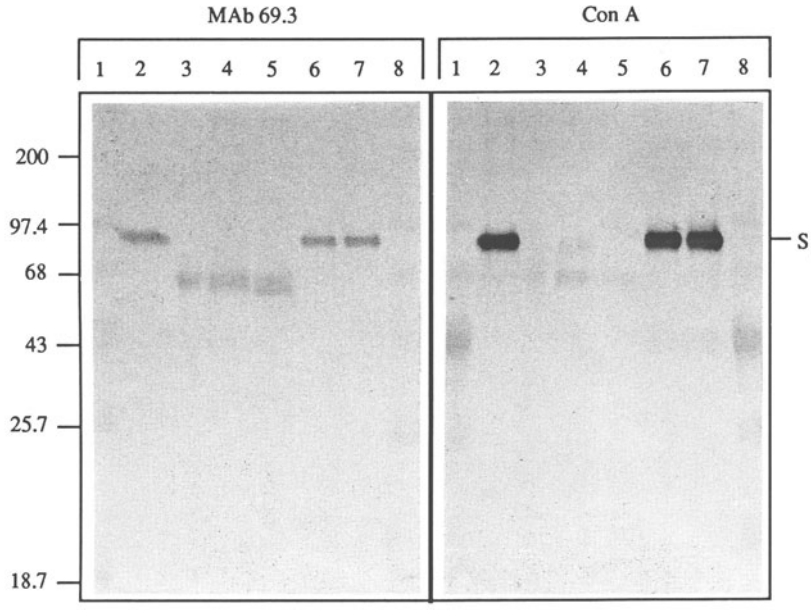

Fig. 3. Western blotting of endoglycosidase-treated S_1. Glycoprotein S_1 was incubated with endoglycosidase F and glycopeptidase F (lane 3); purified endoglycosidase F (lane 4); endoglycosidase H (lane 5); or with buffer only (lane 2). Untreated glycoprotein S_1 was electrophoresed in lane 6 and 7. Blots were stained by incubation with MAb directed against S_1 (left panel) and for glycoproteins with Con A and peroxidase (right panel). Molecular weight markers were electrophoresed in lanes 1 and 8.

Table 1. Effect of deglycosylation on the binding of MAbs directed against IBV proteins.

Treatment[a]	Optical densities[b] of IBV samples bound by monoclonal antibodies														
	69.1	62.1	48.1	48.3	52.4	69.2	62.4	62.2	48.2	62.8	52.1	69.3	26.1	25.1	26.2
endo F/ Glycopept. F	0.3	0.5	0.3	0.3	0.4	0.3	0.7	0.9	0.3	0.7	0.8	2.1	1.9	2.5	2.8
endo F	0.6	1.2	0.7	0.6	0.9	0.8	1.2	1.5	0.7	0.7	0.8	2.3	2.1	2.5	2.8
endo H	0.2	0.4	0.3	0.3	0.3	0.3	0.4	0.8	0.2	0.8	0.8	1.4	2.1	2.5	2.8
buffer	1.6	2.0	1.5	1.6	1.7	1.7	2.1	2.2	1.7	0.5	0.2	2.9	2.3	2.5	2.7
untreated	1.3	1.8	1.3	1.3	1.5	1.4	1.9	2.1	1.5	2.0	0.6	2.6	2.4	2.7	3.0

a. Virus strain D207 was incubated for 44 h at 37 0C with 50 mU of endoglycosidase F containing glycopeptidase, 50 mU of glycopeptidase-free endoglycosidase F, 10 mU of endoglycosidase H, or with buffer. Virus that was not incubated at 37 0C was used as positive control. Wells of microtitre plates were coated with 1 ug of treated or untreated samples and then incubated with monoclonal antibodies.

b. Wave length of 450 nm after incubation with optimal dilution of monoclonal antibodies.

Table 2. Effect of deglycosylation on the binding of MAbs directed against glycoprotein S_1.

Treatment[a]	Optical densities[b] of IBV samples bound by monoclonal antibodies:										
	69.1	62.1	48.1	48.3	52.4	69.2	62.4	62.2	48.2	62.8	52.1
endo F/ Glycopept. F	0.1[b]	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.1	0.4	0.2
endo F	0.3	0.8	0.2	0.3	0.5	0.3	0.7	1.0	0.3	1.1	0.4
endo H	0.2	0.4	0.2	0.2	0.3	0.2	0.4	0.6	0.2	1.0	0.4
buffer	3.0	3.0	2.4	2.4	3.0	3.0	3.0	3.0	3.0	0.8	2.2
untreated	2.1	3.0	1.6	1.6	2.6	2.4	2.8	3.0	2.3	0.7	0.6

a. Affinity purified S_1 of IBV strain D207 was incubated for 44 h at 37 °C with 50 mU of endoglycosidase F containing glycopeptidase F, 50 mU of glycopeptidase-free endoglycosidase F, 10 mU of endoglycosidase H, or buffer only. Wells of microtitre plates were coated with 1 μg of purified MAb and incubated with 1:240 diluted, enzyme-treated or untreated samples except for wells coated with MAbs 62.8 which was incubated with 1:24 diluted samples. Untreated S_1 was used as positive control. The plates were stained by incubation with peroxidase-labelled MAb 69.3 and substrate solution.

b. Optical density measured at a wave length of 450 nm.

reduced when S_1 was incubated with endoglycosidase F that contained glycopeptidase and with glycopeptidase H; binding was least reduced when S_1 was incubated with glycopeptidase-free endoglycosidase F (Table 2).

DISCUSSION

MAbs that had high neutralising titres bound to microtitre plates coated with virus and with affinity-purified S_1. In addition, when used as catching antibodies in the DAS-EIA, these MAbs bound S_1 in solution. However, when either virus or purified S_1 was deglycosylated by incubating it with endoglycosidases, the reaction of the MAbs with S_1 was severely reduced. Thus, binding of neutralising MAbs to the IBV peplomer is dependent on glycosylation of the virus.

Deglycosylation of IBV lowered the MW of its glycoproteins (Fig. 2). Like Stern (4) and Cavanagh (10), we found that IBV contains only N-linked oligosaccharides, to which type of oligosaccharides enzymes used for deglycosylation are specific. As a result of deglycosylation, the MW of S_1 and S_2 were reduced. Since the 65K protein was stained by a MAb directed against S_1, but not by Con A (Fig. 2 and 3), this protein was concluded to be the unglycosylated form of S_1 and out of similar reasoning the 61K protein the unglycosylated form of S_2. After oligosaccharides were removed from S_1 by endoglycosidase H, the MW of S_1 was estimated to be 64,000 (10) and MW of the total peplomer was calculated on basis of the nucleotide sequence to be 127,000 (11). Deglycosylation of S_1 was incomplete, because various proteins with MW ranging between 73,000 and 92,000 were both stained with MAb 69.3 and Con A. Deglycosylation may have been incomplete because we incubated S_1 with endoglycosidase under nonreducing and nondenaturating conditions. Protein conformation may have prevented the enzymes coming in contact with the oligosaccharides. Also, we may have used too

little enzyme; it should be noted, however, that we used higher concentration (90 mU/ml) than Stern et al. (4) (32.5 mU/ml) and Cavanagh et al. (10) (25 mU/ml), and thus this possibility is highly unlikely. Nevertheless, these investigators detected complete deglycosylation using radio-labelled IBV.

We detected that IBV was best deglycosylated by endoglycosidase F containing glycopeptidase F. According to Tarrentino et al. (12), glycopeptidase F hydrolyses N-linked oligosaccharides directly from the polypeptide chain at the glycosylamine linking site. In contrast, endoglycosidase F and H cleave the oligosaccharide chain in such a way that a single N-acetylglucosamine residue bound to asparagine remains in the protein. Endoglycosidase F and H cleave different oligosaccharide substrates. Endoglycosidase F cleaves N-linked high mannose and complex oligosaccharides (13), whereas endoglycosidase H cleaves N-linked high-mannose and hybrid type of oligosaccharides (14). The fact that endoglycosidase H deglycosylated better than glycopeptidase F-free endoglycosidase F suggests that the oligosaccharides on the IBV peplomer protein are mainly of the hybrid type.

Deglycosylation reduces the binding of neutralising MAbs to virus and to purified S_1 (Tables 1 and 2). Results of the Western blotting tests showed that less antibody was bound after oligosaccharides were removed from virus or from S_1 by treatment with a endoglycosidase F contaminated with glycopeptidase F than after treatment with glycopeptidase F-free endoglycosidase or with endoglycosidase H. In both immunoassays, the binding that was detected, particularly when IBV was treated with endoglycosidase F, was probably caused by small amounts of glycoproteins, which remained after deglycosylation.

The effect of deglycosylation on the binding of MAb 62.8 could not be determined, since the binding of this MAb was reduced merely by incubation at 37 ^{0}C. The binding of MAb 62.8 was reduced by 50% when endoglycopeptidase F contaminated with glycopeptidase F but was unreduced when both other enzymes were used to deglycosylate S_1. Reduced binding cannot be explained by proteolytic degradation of deglycosylated S_1, because a single 65K protein stained by a MAb directed against S_1, was detected by Western blotting. MAbs may be directed against the carbohydrate unit itself, but this possibility is highly unlikely, first, because oligosaccharide are immunogenically weak and second, because the MAbs would then have cross-reacted with different IBV serotypes and with other avian viruses.

We conclude that the binding of neutralising MAbs to antigenic domains on IBV is dependent on glycosylation. Because the oligosaccharide unit of glycoprotein is important for the tertiary structure of proteins (15), removing this unit by deglycosylation probably results in changes of the protein conformation. Antigenic domains S_1-A, -B, -C, -D, and -E are probably dependent on protein conformation. In contrast, the antigenic domains S_1-F and S_2-G do not depend on protein conformation, since binding of MAbs directed against these domains was not affected by deglycosylation (Table 1, Fig. 2, and data not shown). Results of the Western blotting tests supported these conclusions; antigenic domains S_1-A, -B, -C, -D, and -E were destroyed under the denaturating conditions, whereas antigenic domains S_1-F and S_2-G were conserved.

The results of this study have important implications for vaccine development. In producing a vaccine, whether inactivated attenuated, subunit, or peptide vaccine, our results indicate that the conformation of the viral proteins must be conserved or mimicked. In producing a peptide vaccine, Geysen et al. (16) have described a procedure for mimicking the conformation of an epitope with a synthetic peptide. They have improved the binding of MAbs to a synthetic peptide by systematically replacing its amino acids. In producing an inactivated attenuated vaccine, any change in conformation caused by inactivation should be avoided. In production of viral sub-units, eukaryotic expression systems should be preferred to prokaryotic expression systems to ensure the glycosylation of subunits.

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