Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions

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Monoclonal antibodies (MAbs) directed against structural proteins of infectious bronchitis virus (IBV) were produced to analyse the antigenic structure of this virus. Competitive binding of enzyme-labelled and unlabelled MAbs to IBV peplomer protein was analysed in an antibody binding assay to test the relatedness of the epitopes defined by the MAbs. Based on the competition groups, eight epitope clusters were defined (S-A to S-H); six of these clusters (S1-A to S1-F) were located on the S1 subunit and two (S2-G and S2-H) on the S2 subunit of the peplomer protein. Epitope clusters S1-A and S1-B overlapped extensively. The biological activities of the MAbs were determined and correlated to the epitope clusters. Monoclonal antibodies directed against epitope clusters S1-A to S1-E and one MAb directed against cluster S2-G moderately to strongly neutralized IBV at titres higher than 2 log₁₀, whereas the remaining MAbs, directed against S1 and S2, neutralized at titres lower than 2 log₁₀. One MAb, directed against cluster S1-D, inhibited the agglutination of chicken erythrocytes.

Introduction

Infectious bronchitis is a highly contagious disease of chickens. The disease is caused by infectious bronchitis virus (IBV), the type species of the family Coronaviridae, and is economically important to the poultry industry.

The structural proteins of IBV are a phosphorylated nucleoprotein with an Mr of 56000 (56K), a membrane protein that is heterogeneously glycosylated with an Mr ranging from 23000 to 36000 and two glycoproteins, S1 and S2, with an Mr of 92000 and 84000 respectively (Lanser & Howard, 1980; Cavanagh, 1981; Lomniczi & Morser, 1981; Wadie & Westaway, 1981; Stern & Sefton, 1982). The spikes or peplomers of IBV are formed from two or three copies each of S1 and S2 (Cavanagh, 1983a). The S2 subunit is anchored in the virus membrane by a small hydrophobic transmembrane segment and forms the stalk of the peplomer, whereas the S1 subunit, which is not covalently bound to S2, forms the bulb (Cavanagh, 1983a, b; De Groot et al., 1987). The S1 subunit performs several biological functions. Monoclonal antibodies (MAbs) directed against S1 neutralize the virus and inhibit haemagglutination (HA); it is therefore assumed that both functions are properties of S1 (Mockett et al., 1984). In addition, virus that lacks S1 fails to induce neutralizing and HA-inhibiting antibodies (Cavanagh & Davis, 1986). Thus, the S1 subunit probably interacts with the cell virus receptor and induces membrane fusion (Cavanagh et al., 1986). However, the antigenic structure of the IBV peplomer protein has not been thoroughly analysed topographically and functionally (Mockett et al., 1984; Niesters et al., 1987).

In the present study, we analysed the antigenic structure of the IBV peplomer protein using MAbs. To test the relatedness of epitopes defined by the MAbs, we analysed the competitive binding of labelled and unlabelled MAbs to IBV antigens in an antibody binding assay (ABA). Epitope clusters were then correlated to biological activities of the virus, such as infectivity and HA, by testing the ability of the MAbs to neutralize virus and inhibit HA.

Methods

Virus and cell culture. The following IBV strains were obtained from the Poultry Health Institute (Doorn, The Netherlands) (Davelaar et al., 1984): IBV strain D207 at the 110th, strain D389 at the 102nd, strain D274 at the 52nd, and strain D312 at the 64th passage in chicken embryos. IBV strain H120 at the 120th and strain B801 at the 9th passage in chicken embryos were obtained from Laboratory F. De Zeeuw (De Bilt, The Netherlands). Strain D207 was adapted to grow in primary chicken embryo kidney (CEK) cells. Cells were grown in M199 culture medium (Gibco Europe) supplemented with 10% foetal calf serum, 1-4 mm-HEPES, 10% trycose phosphate broth (Flow), vitamins, 5000 units/ml sodium benzytrapenicillin, 5000 μg/ml streptomycin sulphate (Pharmachemie) and 5 μg/ml pimafucin (Gist Brocades).

Production of MAbs directed against IBV structural proteins. MAbs directed against IBV strains were produced as described before (Koch et al., 1985). Culture supernatants were screened for antibodies in an
ABA on polystyrene plates (Greiner). Plates were coated with purified IBV or with allantoic fluids of embryonated eggs that were either uninfected or infected with Newcastle disease virus. Only the hybrid cell lines that produced antibodies directed against IBV were selected and cloned by limiting dilution. In addition, culture supernatants were screened for IBV-neutralizing antibodies. Ascites fluid containing immunoglobulin solutions at a wavelength of 280 nm. The extinction concentrations were estimated by measuring the absorbance of most proteins with caprylic acid (Russo et al., 1981). Protein concentrations were estimated by measuring the absorbance of immunoglobulin solutions at a wavelength of 280 nm. The extinction coefficient of a 1% immunoglobulin solution was assumed to be 13.8. Immunoglobulin isotypes were determined by a double radial immunodiffusion assay using a panel of rabbit antisera specific for mouse isotypes (Nordic Immunochemicals).

**Micronutralization test.** The microneutralization test was performed essentially as described earlier (Blore & Skeel, 1981; Koch et al., 1985). Ascites fluid was incubated at 56°C for 30 min to inactivate complement. Instead of chicken polyclonal antibodies, however, MAb CV1-IBV-26.2, directed against IBV nucleoprotein, was used to detect virus-infected cell cultures.

**Virus purification.** Allantoic fluid of embryonated eggs infected with IBV strain D207 was harvested at 16 to 24 h after infection. The allantoic fluid was clarified and then concentrated by overnight centrifugation at 10000 g. The virus pellet was recovered in TNE (10 mM-Tris-HCl pH 7.4, 0.1 M-NaCl, 1 mM-EDTA). Virions were further purified by centrifuging at 100000 g for 16 h through discontinuous sucrose gradients consisting of 32%, 39%, 46%, 53% and 65% (w/v) sucrose in TNE. The opaque virus band was collected and diluted with TNE. Virions were subsequently pelleted by centrifugation at 100000 g for 1 h. Virions were suspended in TNE and stored at -70°C. Virus was labelled with [35S]methionine (Amersham), essentially as described by Stern & Setton (1982).

Radioimmunoprecipitation (RIP) test. The culture fluid of infected and uninfected CEK cells was homogenized with a syringe, centrifuged for 5 min in a Biofuge (Heraeus Christ) and the pellet discarded. The culture fluid was diluted with 1 vol. of TNE: pH 7.4 supplemented with 2% Nonidet P40, 1% sodium deoxycholate, and 0.2% SDS (RIP buffer) and incubated on a reciprocating shaker at 4°C for 1 h to dissociate virus. Immune complexes were prepared by mixing 1 μl of MAb (in centrifuged ascites fluid) and 250 μl of dissociated virions. The mixture was incubated on a reciprocating shaker at 4°C for 1 h. Immune complexes were adsorbed to 20 μl Protein A-Sepharose CL-4B (Pharmacia), which had been shaken with 1% bovine serum albumin at 4°C for 1 h. The Sepharose beads were washed three times with 0.9 ml of RIP buffer and suspended in 35 μl of electrophoresis sample buffer.

Polyacrylamide gel electrophoresis (PAGE). Purified virus or immune complexes were analysed by PAGE on 15% acrylamide-0.09% bisacrylamide gels (Stern et al., 1982). Viral proteins were visualized using Coomassie blue staining or fluorography.

**Antibody binding assay (ABA).** Polystyrene microtitre plates (Dynatech M29 A, Cooke) were coated overnight at 4°C with 1 to 4 μg per well of purified IBV diluted in 0.05 M-sodium bicarbonate buffer pH 9.6. The plates were stored in this buffer at 4°C until use, usually within 6 months. Monoclonal antibodies in ascites fluid were serially diluted in phosphate-buffered saline supplemented with 0.07 M-NaCl, 0.05% Tween 80, and 4% normal horse serum, and incubated for 1 h at 4°C. The plates were incubated with rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase for 90 min at 4°C and were stained at room temperature with 10 μg/ml 3,3',5,5'-tetramethylbenzidine and 0.003% H2O2 dissolved in 0.1 M-acetate buffer pH 5.5.

**Table 1. Characterization of monoclonal antibodies directed against IBV strain D207**

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Strain used for immunization</th>
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<th>Ig isotype</th>
<th>log10 titre</th>
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<tr>
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<td>M</td>
<td>IgG2b</td>
<td>&lt;1-0</td>
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* S1 and S2 are subunits of peplomer protein, N is nucleoprotein and M is the membrane protein of IBV.
† SN, log10 of reciprocal of dilution that neutralized 100 TCID50 IBV strain D207 in the microneutralization test.
‡ HAI, log10 of reciprocal of dilution that inhibited the agglutination of 8 haemagglutinating units of IBV D207.
§ ABA, reciprocal of dilution of ascites fluid that produced 50% of the maximal absorbance in an enzyme immunoassay on microtitre plates coated with IBV strain D207.
¶ ND, Not determined.

Peroxidase activity was quantified by measuring the absorbance at a wavelength of 450 nm in a Titertek Multiskan (Flow).

In the competitive ABA, binding of peroxidase-labelled MAb, the optimal dilution of which had been determined in the ABA, was inhibited by the addition of purified unlabelled MAbS in various concentrations. The plates were incubated for 1 h at 4°C, washed and stained with tetramethylbenzidine. The A450 of the reaction mixture was measured. The ratio of the absorbance that resulted from the binding of labelled antibody in the presence of and in the absence of unlabelled MAbS was calculated. The inhibition of the binding of labelled MAb was calculated as 1-0 minus this ratio and expressed as a percentage.

**Haemagglutination inhibition test.** Haemagglutinating antigen was prepared as described by Lashagari & Newman (1982), except that we used 10⁴ EID50 of IBV strain D207. Virus concentrated from allantoic fluid was mixed with 5 ml of polyspholipase C type 1 (crude culture supernatant of bacterium Clostridium perfringens type C, strain DS298, which was a gift from Dr W. Hesselink, Intervet International,
Results

Production and characterization of MAbs

Table 1 lists the MAbs that reacted with IBV strain D207 and were used in the present study. The table also shows the virus strains used for immunization, the specificity of the MAbs for structural proteins, and immunoglobulin isotypes.

Protein specificity of the MAbs was determined by immunoprecipitation of 35S-labelled IBV proteins from CEK cell culture fluid. Several MAbs precipitated a protein with an $M_r$ of 94000 (Fig. 1, lane 1 and lanes 6 to 10) from cultures infected with strain D207. The $M_r$ of this protein corresponds to the $M_r$ reported for the S1 subunit. Monoclonal antibody 25.1 precipitated proteins with a heterogeneous $M_r$ (Fig. 1, lane 3), characteristic of membrane protein. Monoclonal antibody 26.2 precipitated proteins with $M_r$ values of 49000, 43000 and 39000 (Fig. 1, lane 4). The $M_r$ of the 49K protein is similar to the $M_r$ reported for nucleoprotein. Monoclonal antibody 69.7 did not precipitate any protein.

Since most proteins with an $M_r$ greater than 150000 were immunoprecipitated from the culture fluids of both infected and uninfected CEK cells (Fig. 2), cellular proteins may have been precipitated non-specifically. However, MAbs 26.1 and 30.6 precipitated a protein with an $M_r$ of approximately 200000 to 300000 (p250) (Fig. 1, lanes 2 and 5) that was not precipitated from uninfected cultures. When the culture medium of CEK cells infected with the IBV strain H120 was used for immunoprecipitation, MAb 26.1, like several other MAbs, precipitated both p250 and a protein with an $M_r$ of 84000 (Fig. 3, lanes 2 to 9), which is identical to the $M_r$ of the S2 subunit.

Competitive ABA

Four types of competitive binding curves were found: curves showing more than 80% inhibition, curves...
showing no inhibition, curves showing moderate inhibition (Fig. 4 and 5) and curves showing enhanced binding of labelled MAb in the presence of various concentrations of unlabelled MAbs (Fig. 5). When concentrations of unlabelled MAbs were between 0.1 and 10 μg/ml, unlabelled MAbs inhibited more than 80% of the binding of the labelled MAbs. The binding of labelled MAbs was sometimes enhanced by unlabelled MAbs (Fig. 5). Only MAbs that inhibited more than 50% of the absorbance produced by labelled MAbs were considered to inhibit binding.

Figures 6 and 7 show the competitive binding of all combinations of labelled and unlabelled MAbs directed against the S1 and S2 subunits. Some MAbs directed against S2 had been raised against strain H120 but, because they cross-reacted with strain D207, they were used to analyse epitopes of strain D207 topographically and functionally. Reciprocal inhibition of MAb binding was considered to be the result of identical or overlapping epitopes. The MAbs identified eight epitope clusters on the peplomer protein, six on S1 (S1-A, S1-B, S1-C, S1-D, S1-E, and S1-F), and two on S2 (S2-G and S2-H) (Fig. 8). Monoclonal antibodies directed against S1 did not compete with MAbs directed against S2 (data not shown).

The differences between the inhibition patterns of individual MAbs indicated that the MAbs were unique.

### Biological activity

The biological activity of MAbs directed against the peplomer protein was determined in vitro by using microneutralization and HA inhibition tests.

Because the titre of a virus batch varied between different tests, we determined the neutralization titre at two different virus doses. The neutralization titre at a virus dose of 100 TCID₅₀ was then determined by graphical interpolation.

The neutralization titres of the MAbs directed against the peplomer proteins varied from 1·0 log₁₀ to more than 4·9 log₁₀ (Table 1). Only MAbs directed against S1 (except for MAb 69·3) and MAb 31·1, directed against S2, neutralized the virus at significant titres. The MAbs directed against S1 had similar titres in the ABA and the neutralization test, whereas the titres of MAbs directed against S2 differed by about 2 log₁₀ to 3 log₁₀ in both tests.

Monoclonal antibody 62·8 inhibited HA at dilutions
Antigenic topography of IBV

Fig. 5. Competitive antibody binding assays. Binding of optimally diluted peroxidase-labelled MAb 54.5 was inhibited by the addition of purified MAbs directed against S2 in various concentrations. Unlabelled MAbs were 31.7 (○), 31.3 (□), 54.5 (△), 26.1 (▽), 60.4 (●) and 69.7 (■).

Fig. 6. Epitope mapping of S1 by competitive antibody binding. Assays were performed as described in the legend of Fig. 4. The figure shows the competitive binding of all possible combinations of labelled and unlabelled MAbs directed against the S1 subunit. □, less than 50% inhibition at 100 μg/ml; ■, greater than 50% inhibition at 30 μg/ml; ■, greater than 50% inhibition at 10 μg/ml; ■, greater than 50% inhibition at 1 μg/ml.

Fig. 7. Epitope mapping of S2 by competitive antibody binding assays. Assays were performed as described in the legend of Fig. 5. The figure shows the results of competitive binding of all possible combinations of labelled and unlabelled MAbs directed against the S2 subunit of the IBV peplomer protein. □, less than 50% inhibition at 100 μg/ml; ■, greater than 50% inhibition at 30 μg/ml; ■, greater than 50% inhibition at 10 μg/ml; ■, greater than 50% inhibition at 1 μg/ml.

Fig. 8. Topography and functional analysis of the IBV peplomer protein. Titres of MAbs higher than 2 log_{10} in the neutralization test (NT) and haemagglutination inhibition test (HAI) are indicated by + and titres lower than 2 log_{10} by −. ± indicates that not all MAbs in the cluster have neutralizing titres higher than 2 log_{10}.

up to 2.8 log_{10}, whereas the other MAbs did not inhibit HA. The MAbs specific for the membrane protein and nucleoprotein did not neutralize virus or inhibit HA.
Discussion

Monoclonal antibodies directed against structural proteins of different IBV strains were produced. Epitopes were analysed topographically and functionally, using 25 MAbs that bound to strain D207 and were directed against the S1 and S2 peplomer subunits. Competitive ABAs were used for the topographical analysis.

The Mr of the proteins precipitated by the various MAbs corresponded to the published Mr of structural proteins of IBV (Cavanagh, 1981; Lomniczi & Morser, 1981; Wadey & Westaway, 1981; Stern et al., 1982). In addition to a nucleoprotein with an Mr of 49 000, MAb 26.2 precipitated a 45K protein and small amounts of a 39K protein. Using limited proteolysis and two-dimensional electrophoresis, Lomniczi & Morser (1981) detected a 43K protein, which they identified as cellular actin, a nucleoprotein degraded and non-phosphorylated nucleoprotein. MAb 26.2 did not precipitate such proteins from uninfected CEK cultures and we therefore conclude that the 45K and 39K proteins are degraded and non-phosphorylated nucleoprotein.

Monoclonal antibodies specific for the peplomer protein were classified into two groups: those directed against the S1 subunit (Fig. 1, lanes 1 and lanes 6 to 10) and those directed against the S2 subunit (Fig. 3, lane 2 and lanes 5 to 9). The S2 subunit tends to aggregate (Stern & Sefton, 1982), which may explain why p250 was sometimes detected. When supernatants of cultures infected with strain H120 instead of strain D207 were used, MAb 26.1 precipitated more 84K protein than p250 (compare lanes 2 of Fig. 1 and 3). Thus, the extent to which the S2 subunit aggregates can vary between IBV strains. We could not determine the specificity of MAb 69.7 by using the RIP test or the Western blotting technique (results not shown). Since this MAb competed reciprocally with MAb 60.4 (Fig. 7), we assumed that it is directed against the S2 subunit.

Several investigators have used ABAs to analyse antigens topographically (Stone & Nowinski, 1980; Jackson et al., 1982; Breschkin et al., 1981). Nevertheless, for various reasons, some MAbs compete non-reciprocally (Stone & Nowinski, 1989; Breschkin et al., 1981; Jackson et al., 1982; Lubeck & Gerhard, 1982). Therefore, Fig. 8 shows only epitope clusters defined by MAbs that competed reciprocally.

All MAbs directed against the S1 subunit of the peplomer protein, except MAb 69.3, neutralized IBV at titres above 2 log_{10}. In contrast, all MAbs directed against the S2 subunit, except MAb 31.1, neutralized IBV at titres below 2 log_{10}. The titre of MAb 31.1 was 2 log_{10} lower in the ABA than in the neutralization test. This finding suggests that the mechanism by which MAb 31.1 neutralizes virus is different from that of the other MAbs. Only MAb 62.8, which defined S1-E, inhibited the ability of strain D207 to haemagglutinate. Our results suggest that clusters S1-A to S1-E and S2-G are located close to sites where the virus binds to cell membranes or mediates membrane fusion. Cluster S1-D may be located close to the site where IBV binds to erythrocytes.

Using competitive ABAs, Niesters et al. (1987) defined five epitopes on the S1 subunit of IBV strain M41. Of the MAbs that they used, only MAb 13 neutralized virus and inhibited HA. Because this MAb and two MAbs (A38 and A13) produced by Mockett et al. (1984) are specific for strain M41, it is impossible to test whether these MAbs compete with MAbs directed against strain D207. The biological activities of MAbs 13, A38, A13 and 62.8 are similar, however, and these MAbs may bind to similar epitope clusters on two different strains. Monoclonal antibodies 52.4, 69.1, 69.4 and 69.3 bind to both strain D207 and strain M41 and do not inhibit the binding of labelled MAbs 13 and A38 and vice versa (data not shown).

Kusters et al. (1989) produced fusion proteins of fragments of the peplomer protein with synthetic peptides of three to 12 residues with overlapping sequences of the peplomer protein. These were used to locate epitope clusters on the amino acid sequence of the peplomer protein. Epitopes defined by MAbs 26.1, 30.6, 31.7 and 54.5 could be located on 20 amino acid residues at the N terminus of the S2 subunit (Lenstra et al., 1989; Kusters, 1989; Kusters et al., 1989). These results confirm that the binding of labelled MAbs is reciprocally inhibited by unlabelled MAbs because epitopes are identical or overlapping. Epitopes defined by strongly neutralizing MAbs could not be located using the fusion proteins. Therefore, we are presently sequencing genome RNA isolated from virus mutants that escape neutralization by these MAbs. Epitopes can be located on the S1 gene by comparing amino acid sequences derived from the nucleotide sequences of the mutant and parent peplomer genes.

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References


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