Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide

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Neutralizing monoclonal antibodies directed against five antigenic sites on the spike (S) S1 glycopolypeptide of avian infectious bronchitis virus (IBV) were used to select neutralization-resistant variants of the virus. By comparing the nucleotide sequence of such variants with the sequence of the IBV parent strain, we located five antigenic sites on the amino acid sequence of the S1 glycopolypeptide. The variants had mutations within three regions corresponding to amino acid residues 24 to 61, 132 to 149 and 291 to 398 of the S1 glycopolypeptide. The location of three overlapping antigenic sites on the IBV spike protein was similar to the location of antigenic sites on the spike protein of other coronaviruses.

Introduction

Avian infectious bronchitis virus (IBV), a coronavirus, causes a highly contagious disease in chickens; the clinical signs of disease occur mainly in the respiratory tract.

The spike protein (S) of IBV is synthesized as a 180K glycoprotein. This protein is proteolytically cleaved, yielding a 92K glycoprotein (S1) and an 84K glycoprotein (S2) (Stern & Sefton, 1982; Stern et al., 1982; Cavanagh, 1983a). The S2 subunit, which is anchored in the viral membrane by a small hydrophobic transmembrane segment, forms the stalk of the spike, whereas the S1 subunit forms the bulbous part (Cavanagh, 1983a, b; Cavanagh & Davis, 1986; de Groot et al., 1987a).

S1 induces virus-neutralizing antibodies in chickens (Cavanagh et al., 1986). Strongly neutralizing monoclonal antibodies (MAbs) and haemagglutination-inhibiting MAbs are also directed against S1 (Mockett et al., 1984; Niesters et al., 1987; Koch et al., 1990). Thus, biological functions of IBV such as virus binding to cells and erythrocytes and membrane fusion are considered to be properties of S1.

In an earlier study, we analysed MAbs by competitive binding tests and defined eight antigenic sites on the S protein of IBV strain D207 (Koch et al., 1990). Six antigenic sites are located on S1 (S1-A to S1-F) and two sites on S2 (S2-G and S2-H). Only MAbs directed against sites S1-A to S1-E and only one out of nine MAbs directed against S2-G neutralized IBV at titres higher than 10^2.

To locate the antigenic sites on the amino acid sequence of the spike protein, we produced fusion proteins that contain fragments of S by using the prokaryotic expression system, pEX (Kusters et al., 1989a; Lenstra et al., 1989). In addition, we determined the reactivity of MAbs with all synthetic peptides with a length of three to nine amino acids and with overlapping sequences derived from the amino acid sequence of the N terminus of S2 (Kusters et al., 1989a). The antigenic site S1-F was found to be located within a region of 47 amino acids of the S1 subunit C terminus, whereas S2-G was located within a region of 20 amino acids of the S2 subunit N terminus (Kusters et al., 1989a). However, antigenicity of epitopes expressed by fusion proteins and by synthetic peptides depends on the primary structure only (conformation-independent) and not on the native structure of the protein antigen (conformation-dependent) (Lenstra et al., 1990). As the MAbs that define the antigenic sites S1-A to S1-E did not react with fusion proteins, these antigenic sites were considered to be conformation-dependent.

The purpose of the present study was to locate conformation-dependent sites. We selected variants of IBV strain D207 that resisted neutralization by MAbs directed against sites S1-A to S1-E. Direct RNA sequence analysis was used to compare the S1 sequence of MAAb-resistant variants to the S1 sequence of the parent strain. Amino acid substitutions were located in the regions 24 to 61, 132 to 149 and 291 to 398 of the amino acid sequence.
Methods

Virus. IBV strain D207 was obtained from the Poultry Health Service (Doorn, The Netherlands) (Davelaar et al., 1984). Strain D207 had been passaged 60 times on embryonated eggs. Virus was grown in embryonated eggs or on monolayers of primary chicken embryo kidney (CEK) cells as described by Koch et al. (1990).

MAbs. MAbs directed against the S1 glycoprotein were produced and characterized as described elsewhere (Koch et al., 1990).

Double antibody sandwich enzyme immunoassay (DAS-EIA). The DAS-EIA was performed as described (Koch & Kant, 1990). Briefly, microtiter plates were coated with MAbs directed against antigenic sites S1-A to S1-E. The plates were incubated with allantoic fluid of embryonated eggs infected with various IBV strains. MAb CVI-69.3, which is directed against S1-F (Koch et al., 1990), was conjugated to horseradish peroxidase and used to detect virus binding.

Selection of MAb-resistant variants. MAb-resistant variants were selected as follows. Equal volumes (100 μl) of ascites fluid containing MAb diluted 1:10 and 10-fold dilutions of virus were mixed and incubated for 1 h at room temperature. To prevent the dissociation of immune complexes in the mixture after injection into embryonated eggs, we inoculated the allantoic cavity of 10-day-old chicken embryos twice: first with 100 μl of the undiluted ascites fluid and then with the mixture. The allantoic fluid of eggs that were still viable after 7 days of incubation at 38 °C was harvested. Variants resistant to MAbs were grown by single passaging in embryonated eggs and cloned by limiting dilution in CEK cell cultures. After harvesting of the culture supernatants, cells were assayed for IBV infection by immunoperoxidase staining with a conjugated MAb directed against IBV nucleoprotein as described (Koch et al., 1990). Stocks of the cloned MAb-resistant variant were obtained by passaging it on embryonated eggs. All variants were cloned three times unless otherwise indicated. The MAb-resistant variants were serologically characterized by using the DAS-EIA (Koch & Kant, 1990).

RNA isolation. Virus was concentrated from allantoic fluid by centrifugation overnight at 10000 g. The virus pellet was recovered in 20 mM-Tris-HCl pH 7.4 containing 0-1 M-NaCl, 1 mM-PMSF and 1 mM-EDTA, sonicated, and centrifuged at 1500 g for 3 min. Virus was disrupted by adding dithiothreitol to a final concentration of 100 mM and SDS to a final concentration of 1% (w/v) and then incubated for 15 min at 37 °C. Disrupted virus was extracted twice with phenol-chloroform (50:50, v/v), once with chloroform-isooctyl alcohol (24:1, v/v) and once with ether. Finally, RNA was precipitated by adding ethanol to a final concentration of 70% (v/v).

Direct RNA sequencing. RNA was sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedures essentially as described (Sanger et al., 1977; Air, 1979; de Bordes et al., 1986; Fichot & Girard, 1990). To prime cDNA synthesis on the viral genomic RNA, we used 10 synthetic oligonucleotides consisting of 23 to 24 bases complementary to the S sequence of IBV strain D207 at intervals of about 150 bases (Kusters et al., 1989b).

Results

Antigenic characterization of MAb-resistant variants of IBV

Eleven MAbs (69.1, 62.1, 52.4, 48.1, 48.3, 62.4, 62.2, 48.2, 62.8, 52.1 and 69.4) directed against antigenic sites S1-A to S1-E were used to select 25 MAb-resistant variants. The reactivity of the MAb-resistant variants was determined with a panel of 13 MAbs directed against S1 in a DAS-EIA (Fig. 1) and compared to the reactivity of the D207 parental strain. Although MAb-resistant variants 52.4V1, 52.4V2 and 52.4V4, 48.1V1 and 48.3V1, 48.2V1 to 48.2V3, and 62.8V1 to 62.8V4 did not differ serologically, these variants were included in Fig. 1 because their amino acid sequences differed (Table 1).

MAb-resistant variants that were selected by MAbs directed against antigenic sites D and E reacted with all MAbs except the selecting MAb. Some MAb-resistant variants that were selected by MAbs directed against sites A to C, however, did not react with the selecting MAb or with certain other MAbs directed against these antigenic sites. The epitopes of these MAbs probably have amino acids in common. Because none of the MAb-resistant variants was neutralized by the selecting MAb (data not shown) or reacted with the selecting MAb in the DAS-EIA they are probably true variants.

Sequence analysis

The nucleotide sequence of the S gene of strain D207 was determined earlier by sequencing cloned cDNA fragments (Kusters et al., 1989b). To check whether the published sequence represents the consensus sequence of D207 stock or merely the sequence of a minor D207
Table 1. Amino acid changes in MAb-resistant variants of IBV strain D207

<table>
<thead>
<tr>
<th>MAb-resistant variant</th>
<th>Antigenic site</th>
<th>Phenotype*</th>
<th>Amino acid change†</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.1V§</td>
<td>A</td>
<td>1</td>
<td>334 S→N</td>
</tr>
<tr>
<td>69.1V§</td>
<td>A</td>
<td>1</td>
<td>325 D→Y</td>
</tr>
<tr>
<td>69.1V2</td>
<td>A</td>
<td>2</td>
<td>323 A→D, 476 T→I</td>
</tr>
<tr>
<td>69.1V3</td>
<td>A</td>
<td>1</td>
<td>332 H→R</td>
</tr>
<tr>
<td>52.4V1</td>
<td>A/B</td>
<td>1</td>
<td>333 P→Q, 56 S→F</td>
</tr>
<tr>
<td>52.4V2</td>
<td>A/B</td>
<td>1</td>
<td>333 P→Q, 224 V→L</td>
</tr>
<tr>
<td>69.1V1§</td>
<td>A</td>
<td>2</td>
<td>325 D→Y</td>
</tr>
<tr>
<td>52.4V3</td>
<td>A/B</td>
<td>1</td>
<td>333 P→Q, 420 R→T</td>
</tr>
<tr>
<td>52.4V4</td>
<td>A/B</td>
<td>1</td>
<td>333 P→Q, 420 R→T, 435 T→I</td>
</tr>
<tr>
<td>48.1V</td>
<td>A/B</td>
<td>2</td>
<td>387 S→T</td>
</tr>
<tr>
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<td>A/B</td>
<td>1</td>
<td>387 S→A</td>
</tr>
<tr>
<td>48.3V2</td>
<td>A/B</td>
<td>1</td>
<td>325 D→Y</td>
</tr>
<tr>
<td>48.3V3</td>
<td>A/B</td>
<td>2</td>
<td>361 Q→R</td>
</tr>
<tr>
<td>62.4V1</td>
<td>A/B</td>
<td>1</td>
<td>394 R→S</td>
</tr>
<tr>
<td>62.4V3</td>
<td>A/B</td>
<td>2</td>
<td>398 T→K, 78 A→V</td>
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<tr>
<td>62.2V</td>
<td>B</td>
<td>1</td>
<td>291 G→E</td>
</tr>
<tr>
<td>48.2V1</td>
<td>C</td>
<td>1</td>
<td>299 Y→H</td>
</tr>
<tr>
<td>48.2V2</td>
<td>C</td>
<td>1</td>
<td>299 Y→H, 302 S→N</td>
</tr>
<tr>
<td>48.2V3</td>
<td>C</td>
<td>1</td>
<td>299 Y→H, 311 L→F</td>
</tr>
<tr>
<td>48.1 + 48.2V¶</td>
<td>A/B + C</td>
<td>1</td>
<td>361 Q→R, 299 Y→H, 302 S→N</td>
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<tr>
<td>62.8V1§</td>
<td>D</td>
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<td>60 G→D</td>
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<td>D</td>
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<td>60 G→V</td>
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<td>60 G→S</td>
</tr>
<tr>
<td>62.8V4§</td>
<td>D</td>
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<td>E</td>
<td>1</td>
<td>132 H→Y</td>
</tr>
<tr>
<td>69.4V¶</td>
<td>E</td>
<td>1</td>
<td>149 L→S</td>
</tr>
</tbody>
</table>

* Compared to the parental strain D207, clones and MAb-resistant variants had different amino acid residues at positions 48 and 117. See Results.
† Amino acid residues are numbered according to the alignment presented by Kusters et al. (1989b).
§ For code of MAb-resistant variants see legend of Fig. 1.
¶ Variant selected using a mixture of MAbs 48.1 and 48.2.
† Variant 69.4V was cloned twice by limiting dilution.

variants had different amino acid residues at positions 48 and 117. See Results.

We sequenced the entire S1 gene of strain D207 and of two of its subclones. The nucleotide sequence of strain D207 was identical to the published sequence (Kusters et al., 1989b). The sequence of one of our D207 subclones, however, was identical to the published sequence of a cDNA clone of D207 (Kusters et al., 1989b). This sequence differed at three base positions, two of which resulted in amino acids substitutions; these were Glu instead of Asp at position 48 and Lys instead of Asn at position 117 (numbering of the immature protein signal peptide included, Kusters et al., 1989b, Fig. 1). Because only four of the 20 subcloned MAb-resistant variants had the same amino acid substitutions as this particular D207 clone, the sequence of this subclone represents a minor variant of strain D207. We have tentatively designated the four MAb-resistant variants with the sequence of the minor variant as phenotype 2 and the other variants as phenotype 1 (Table 1). Because the minor variant and the parent strain reacted identically with the MAbs, we concluded that amino acid residues 48 and 117 do not contribute to the binding of MAbs to S1.

All of the MAb-resistant variants analysed contained one or more nucleotide substitutions causing amino acid changes. The complete nucleotide sequences of the S1 subunit of variants selected by MAbs directed against five antigenic sites were determined. The differences in amino acids of the MAb-resistant variants and the D207 strain are listed in Table 1. Although several MAb-resistant variants had more than one amino acid substitution, these amino acids were not necessarily part of the epitope. Since Pro at position 333 was substituted by Gln in all MAb-resistant variants of 52.4, and since Tyr at position 299 was substituted by His in all variants of 48.2, we think that these residues are needed for these MAbs to bind to IBV. Other substitutions (e.g. Thr at position 476 in 69.1V2, Val at 224, Ser at 56, Arg at 420 and Thr at 435 in variants of MAb 52.4; Ala at 78 in 62.4V4; Ser at 302 in 48.2V2; Leu at 311 in 48V3; Asn at 24 in 62.8V4) may also be needed for the selecting MAbs to bind to the virus. Amino acids Val at position 224 in 52.4V2 and Ala at position 78 in 62.4V2 were substituted by similar amino acids. Because Thr is substituted by Ile at position 435 in 52.4V4, a potential glycosylation site was lost.

Fig. 2 shows the location of the antigenic sites on the amino acid sequence of S1. We included mainly single amino acid substitutions in the figure, but multiple substitutions were also included when they occurred in more variants. The relative order of antigenic sites A, B and C is uncertain. TM, transmembrane anchor.
were substituted in MAb-resistant variants selected by MAbs specific for antigenic site S1-D. Residues 132 and 149 were substituted for MAb-resistant variants selected by MAbs specific for site S1-E. Amino acids in the region of residues 291 to 398 were substituted in variants that were selected by MAbs specific for the overlapping antigenic sites A, B and C.

Discussion

In an earlier report, we described the antigenic topography of the IBV spike protein (Koch et al., 1990). Expression products of S gene fragments and synthetic peptides were used to map two of the eight antigenic sites on the amino acid sequence (Kusters et al., 1989a). Epitopes within these sites are, therefore, mainly conformation-independent. In the present study, we used strongly neutralizing MAbs to locate conformation-dependent epitopes.

When MAb-resistant variants have two or three amino acid substitutions, it is difficult to determine which of the substituted residues form part of the epitope. In all variants of MAb 52.4, however, Pro was always substituted by Gln; and in variants of MAb 48.2, Tyr was substituted by His. The other substitutions in these variants might be merely coincidental, or they may, in some way, compensate for conformation changes induced in S1 by other substitutions. These explanations may also account for the substitutions at position 78 in 62.4V2 and position 24 in 62.8V4. In mapping antigenic sites (Fig. 2), we assumed that the substituted residues form part of the epitope. However, the substituted residues may affect the folding of the protein, causing the conformation of the protein to change at distant parts. We were not able to confirm our result by using other techniques, because our neutralizing MAbs did not bind to relevant synthetic peptides in a Pepsan analysis (Geyser et al., 1984) or to prokaryotic expression products of S1 gene fragments (Kusters et al., 1989a). This failure is caused by conformation dependence of the antigenic sites (Koch et al., 1990; Koch & Kant, 1990; Lenstra et al., 1990). The amino acids composing the epitopes of some MAbs are widely separated on the sequence map (Fig. 2), a finding which agrees with these epitopes being conformation-dependent. Residues that contribute to the binding of MAb 48.3, for example, were located in the region from residue 325 to residue 387. In addition, variants resistant to neutralization by MAb 62.4 did not bind to MAbs 62.2 or 69.6 (Fig. 1), although amino acids that contribute to binding of MAbs 62.4 and 62.2 were separated by 103 to 107 residues (Table 1). Residues that are so far apart can contribute to antibody binding only when they are spatially close in the native protein. Moreover, epitopes that are composed of amino acids located on different peptide chains have also been described (Thomas et al., 1988).

Comparison of the amino acid sequences of S1 of the Beaudette and M41 strains of IBV showed that amino acid differences clustered within the regions of residues 56 to 69 (hypervariable region I) and of residues 117 to 133 (hypervariable region II) of the immature protein (Niesters et al., 1986). Cavanagh et al. (1988) compared seven strains of the Massachusetts serotype and found that nearly one-third of the 32 amino acid differences were within hypervariable regions I and II. These authors suggested that amino acids within these hypervariable regions were part of neutralization epitopes. However, the existence of hypervariable regions alone does not prove that they are immunogenic. By sequencing MAb-resistant variants, Cavanagh et al. (1988) were able to show that the location of a neutralization epitope on strain M41 coincided with hypervariable region I. The epitope defined by MAb 62.8 (antigenic site S1-D) in the region of residues 24 to 61 is adjacent to residue 63; when this residue was mutated, it prevented neutralization of IBV strain M41 by two neutralizing MAbs (Cavanagh et al., 1988). These two MAbs reacted specifically with strain M41; this finding is consistent with the deletion of amino acid residue 63 in strain D207. We now present evidence that another antigenic site (S1-E) coincides with hypervariable region II. The location of the overlapping antigenic sites S1-A to S1-C did not coincide with a hypervariable region. However, comparing the S1 sequence of strains that belong to three different serotypes, Kusters et al. (1989b) concluded that most of the amino acid substitutions were found in the region of the first 300 N-terminal residues of S1; they also suggested that serotype determinants are located in this region. They were not able, however, to predict more precisely the location of these determinants. When the S1 sequences of six strains of related serotypes were compared, most differences were found between residues 43 to 144 and residues 285 to 325 (Cavanagh et al., 1992).

The antigenic structure and location of neutralization epitopes have been reported for two other coronaviruses: transmissible gastroenteritis virus (TGEV) (Correa et al., 1990; Delmas et al., 1990) and mouse hepatitis virus (MHV) (Parker et al., 1989). It is difficult to compare neutralization epitopes of these coronaviruses directly with those of IBV, however, since the sequence identity of IBV S1 and the N-terminal part of S of the other viruses is low (de Groot et al., 1987b). Nevertheless, sequences of feline infectious peritonitis virus (FIPV), MHV and IBV have been aligned (de Groot et al., 1987b) and several common sequence motifs were apparent. Although the antigenic structure of FIPV has not been
reported, FIPV and TGEV sequences can be easily exchanged in the alignment, since 94% of 1173 C-terminal residues of the sequences of FIPV S and of TGEV S are identical (Jacobs et al., 1987). Most of the antigenic sites of coronaviruses that induce neutralizing antibodies are located on the N-terminal part of S of TGEV (Correa et al., 1990; Delmas et al., 1990), IBV (Koch et al., 1990), MHV (Parker et al., 1989) and bovine coronavirus (Parker et al., 1990). The location of antigenic site A (Correa et al., 1990), the overlapping sites A/B of TGEV (Delmas et al., 1990) and antigenic sites of S1-A to S1-C of IBV are very similar. Remarkably, these antigenic sites of TGEV and IBV are located about 60 residues away from the N terminus of a short region of high similarity among all coronaviruses (de Groot et al., 1987b; Parker et al., 1990). MAb-resistant variants of MHV have large deletions of 142 to 159 residues, all of which end 33 to 56 residues from this region. The deleted sequences probably contain neutralization epitopes (Parker et al., 1990). Thus, although the alignment of the MHV S sequence with the FIPV and IBV sequences is only tentative (de Groot et al., 1986) in the above region, all three coronaviruses may have neutralization epitopes in highly similar locations. We speculate that the locations are similar because three-dimensional structures are conserved in these coronaviruses.

The stock of IBV strain D207 was heterogeneous, since we obtained two different subclones of strain D207 by limiting dilution. These subclones probably represented different variants of IBV D207. The main variant has Glu at position 48 and Lys at 117 (phenotype 1); this sequence was detected by direct RNA sequencing of uncloned IBV D207 and most of the MAb-resistant variants. Kusters et al. (1989b) also detected these mutations in independent cDNA clones of D207 S1. Because most cDNA clones had Glu at position 48 and because they found Lys at 117 by direct RNA sequencing, they concluded this to be the sequence of the main variant. Since the reactivity of both of the D207 clones with the panel of MAbs was identical, we concluded that both amino acid residues are extraneous to neutralization epitopes. We did not test the virulence of the MAb-resistant variants and both D207 clones. In vitro, clones of strain D207 grew differently in embryonated eggs and in CEK cells (data not shown). Differences in neurovirulence have been reported for MAb-resistant variants of MHV (Wege et al., 1988; Fleming et al., 1986) and have been found to be associated with deletions in the N-terminal half of S (Parker et al., 1989).

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