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Epitopes on the Peplomer Protein of Infectious Bronchitis Virus Strain M41 as Defined by Monoclonal Antibodies

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Sixteen monoclonal antibodies (Mcabs) were prepared against infectious bronchitis virus strain M41, all of them reacting with the peplomer protein. One of them, Mcab 13, was able to neutralize the virus and to inhibit hemagglutination. Competition binding assays allowed the definition of five epitopes, designated as A, B, C, D, and E, of which epitopes A and B are overlapping. Furthermore, the binding of Mcab 13 (epitope E) could be enhanced by the addition of Mcabs from group B, C, and D. A dot immunoblot assay was used to analyze the effect of denaturation on antibody recognition of the epitopes. Only the binding of Mcab 13 was affected, indicating that the epitope involved in neutralization and hemagglutination is conformation dependent. The epitopes A to D were highly conserved among IBV strains, while epitope E was specific for strains M41 and D3896. In this last strain, however, this epitope was not involved in neutralization.

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INTRODUCTION

Avian infectious bronchitis virus (IBV) is the etiological agent of a highly contagious respiratory disease of young chickens. The virus also causes a marked and rapid decline of egg production and quality in laying hens (Darbyshire, 1981).

IBV belongs to the coronaviruses, a group of positive-stranded enveloped RNA viruses. The virion contains three major structural proteins: the peplomer or spike (S), nucleocapsid (N), and matrix (M) proteins (Cavanagh, 1981; Stern and Sefton, 1982). The M protein is present as polypeptide p23 and the glycosylated forms gp28, gp31, and gp36. The N protein has an apparent mol wt of 51 kDa (Macnaughton and Madge, 1977).

The characteristic surface projections of the virus are made up by the peplomer protein; the peplomer is composed of two molecules of each of the glycopolypeptides S1 (gp90) and S2 (gp84). Both contain high-mannose, N-linked oligosaccharides. The S2 protein is anchored in the membrane while S1 is attached to S2 via a loose noncovalent association (Cavanagh, 1983a, b).

The peplomer protein is believed to be responsible for the induction of neutralizing antibodies and a protective immune response (Mockett et al., 1984; Cavanagh et al., 1986).

In this study we describe the production of monoclonal antibodies (Mcabs) directed against the peplomer protein of IBV strain M41. To map the epitopes we performed reciprocal competitive binding assays. The Mcabs were used in a dot immunoblotting assay to analyze the sensitivity to denaturation of the epitopes responsible for antibody attachment. Furthermore, the variability of these epitopes in different IBV strains was analyzed.

MATERIALS AND METHODS

Virus growth and purification

All IBV strains were obtained from the Poultry Health Institute, Doorn, The Netherlands. Purified stocks were prepared from cloned virus (Niesters et al., 1986).

Immunization of mice

Gradient-purified M41 virus was disrupted in 1% Triton X-100 and diluted to 400 μg/ml in TESV buffer (0.02 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 M NaCl). Balb/c mice (from CPB, TNO, Zeist, The Netherlands) were primed intramuscularly with 100 μg virus in 0.5 ml complete Freund’s adjuvant and boosted twice at weekly intervals with the same amount of antigen, in
incomplete Freund's adjuvant. At least 6 weeks after the initial immunization and 3 days before the mice were sacrificed for the preparation of hybridomas, they were boosted intravenously with 10 μg purified intact virus (0.1 ml in TESV).

Production of mouse hybridoma cell lines

Hybridoma cell lines were prepared and cloned as previously described (Osterhaus et al., 1981). Immune ascitic fluid was made in Balb/c mice primed with pris-tane (2,6,10,14-tetramethylpentadecane; Janssen Chimica, Belgium; Goding, 1980).

Serological screening methods

Hybridoma culture media and mouse body fluids were screened for antibodies in an ELISA. Microtiter plates (Inotech) were coated (3 hr at 37°C followed by overnight incubation at 4°C) with 0.5 μg virus in 100 μl 0.15 M NaCl per well. Plates were washed four times with washing fluid (0.15 M NaCl, 0.05% Tween 20 (v/v)). All subsequent steps were performed at 37°C. Fifty microliters of V-buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 0.1% BSA (w/v), 0.05% Tween 20 (v/v), 10% newborn calf serum) and 50 μl hybridoma supernatant or dilutions of ascitic fluid in V buffer were added to each well and incubated for 2 hr. After washing four times with washing fluid, peroxi-dase-conjugated rabbit anti-mouse IgG (H + L) (Nordic Immunological Laboratories) was added and incubated for another 2 hr. The strips were washed with phosphate buffered saline (PBS, pH 7.4). Binding was visualized by incubation with a freshly prepared substrate solution containing PBS, 0.6 mg/ml 3,3'-diaminobenzidinetetrahydrochloride (DAB, Serva), and 0.3% H2O2. The reaction was stopped by washing the strips with PBS (Roos et al., 1982).

Radioiodination of viral surface glycoproteins

Gradient-purified virus was labeled using Iodogen (Pierce) to a specific activity of 1.9 μCi/μg (Markwell and Fox, 1978).

Immunoprecipitation, virus neutralization, and hemagglutination inhibition

Approximately 100,000 cpm 125I-labeled M41 virion proteins in 25 μl were immunoprecipitated with 2 μl ascitic fluid (Van der Zeijst et al., 1983). Immunoprecipitates were analyzed by electrophoresis in 15% SDS–polyacrylamide gels (Rottier et al., 1981).

Virus neutralization was carried out according to Kusters et al. (1987). Hemagglutination inhibition tests were performed with 8 hemagglutinating units in microtiter plates as described by Alexander et al. (1976).

Coupling of horseradish peroxidase (HRPO) to monoclonal antibodies

Immunoglobulin fractions were isolated from ascites fluids by precipitation at 4°C for 2 hr with an equal volume of saturated ammonium sulfate (pH 7.0). After overnight dialysis against 0.01 M sodium carbonate buffer (pH 9.5), antibodies were coupled to horseradish peroxidase (HRPO; Boehringer-Mannheim) by the periodate method (Wilson and Nakane, 1978) and stored at −70°C.

Antibody blocking assay

Before carrying out the actual competition binding assays, the amount of binding in the ELISA was deter-
mined for all Mcabs (peroxidase conjugated and non-
conjugated). A series of twofold dilutions was reacted
with antigen-coated plates. The resulting dose–re-
sponse curve yielded the level of maximum binding (a
measure for the relative avidity) and the Mcab con-
centration at which 50% binding occurred.

For the competition assays, virus-coated plates
were incubated for 1 hr at 37° with 200 µl per well of
PBS containing 0.2% Tween 20 and 10% newborn calf
serum to saturate all binding sites on the plastic. The
solution was removed and the plates were washed
twice with washing fluid. A mixture of the peroxi-
dase-conjugated Mcab (at twice the concentration
giving half maximal binding) and the unconjugated,
competing antibody at various concentrations from
the linear part of the dose response curve, were
added simultaneously. Dilutions were made in PBS
containing 1% newborn calf serum. The mixture was
incubated for 1 hr at 37°. The plates were then
washed four times with washing fluid and binding of
the peroxidase-conjugated Mcab was visualized as
described above. Nonspecific binding to plates with-
out antigen was taken to represent background. Bind-
ing of each conjugate to viral antigen in the absence of
competitor represented 100% binding (0% inhibition),
while the binding of the conjugate in the presence of
excess of the same unlabeled Mcab represented 0%-
binding (100% competition).

To define epitope groups, competition was consid-
ered to be positive only if it occurred symmetrically,
i.e., when similar results were obtained when the anti-
body was used as competitor and as peroxidase-con-
jugated antibody. The percentage of competition
usually reached a plateau. Competition was rated as
strong if it was more than 60%, and significant if it was
more than 35%.

RESULTS

Production and characterization of monoclonal
antibodies

Our purpose was to generate and subsequently an-
alyze Mcabs directed against the surface glycopro-
teins of IBV strain M41. To obtain predominantly
Mcabs to surface proteins, immunized mice were
boosted with intact sucrose-gradient-purified virions
by intravenous injection a few days before the fusion.
Spleen cells from two hyperimmunized mice were
fused in independent experiments. The hybridoma cell
lines secreting antiviral Mcabs were screened with
the aid of an ELISA. The amount of viral antigen which
provided maximum antibody binding and minimum
background with polyvalent rabbit anti-M41 sera had
been determined previously (0.5 µg sucrose-purified
intact virions per well). These conditions were used
throughout. Sixteen Mcabs directed against the pe-
plomer protein were selected for further analysis
(Table 1). Their polypeptide specificities had been
characterized by immunoprecipitation of 125I-
radiolabeled purified virions; typical results are shown in Fig.
1. No cross-reactivity was detected with allantoic fluid
from uninfected eggs, both by ELISA and immunopre-
cipitation of 125I-radiolabeled allantoic fluid. Cellular
proteins, such as actin, transferrin, and ovalbumin,
sometimes coprecipitated with the viral polypeptides
(results not shown). These proteins may associate
nonspecifically with the virion (Cavanagh, 1981; Lom-
nici and Morser, 1981; Wadep and Westaway, 1981).

Characterization of the isotypes of the Mcabs
showed that most antibodies belonged to the IgG3
(eight) or IgG1 (six) subclass, whereas only two (Mcab
6 and 13) were from the IgG2 subclass. Of all the
Mcabs only one, Mcab 13, was able to neutralize virus
infectivity in ovo as well as to inhibit hemagglutination
significantly. This Mcab did not neutralize the other
IBV strains listed in Table 2.

A direct relationship exists between the avidity and
the maximal amount of Mcab bound to a given amount
of antigen (Frankel and Gerhard, 1979; Bruck et al.,
1982; Van Drunen Littel-van den Hurk et al., 1985).

TABLE 1

| Hybridoma cell line | Isotype specificity | Neutralization titer[a] | Hemagglutination inhibition titer[b] |
|---------------------|--------------------|------------------------|-------------------------------------|
| 1                   | IgG3               |                        |                                     |
| 2                   | IgG3               |                        |                                     |
| 3                   | IgG1               |                        |                                     |
| 4                   | IgG1               |                        |                                     |
| 5                   | IgG3               |                        |                                     |
| 6                   | IgG2               |                        |                                     |
| 7                   | IgG3               |                        |                                     |
| 8                   | IgG3               |                        |                                     |
| 9                   | IgG1               |                        |                                     |
| 10                  | IgG3               |                        |                                     |
| 11                  | IgG3               |                        |                                     |
| 12                  | IgG3               |                        |                                     |
| 13                  | IgG2               | 630,000                | 2560                                |
| 14                  | IgG1               |                        |                                     |
| 15                  | IgG1               |                        |                                     |
| 16                  | IgG1               |                        |                                     |

[a] Neutralization assays were performed with 100 EID50 per egg
and serial 10-fold dilutions of the ascitic fluid. Only significant neu-
tralization (> 100) is indicated.

[b] Inhibition was considered to be positive if the titer was 160 or
more.
FIG. 1. Specificity of IBV-M41 monoclonal antibodies for the peplomer protein. 125I-radiolabeled M41 virions were disintegrated and the proteins were immunoprecipitated with ascitic fluids of the monoclonal antibodies. The immunoprecipitates were electrophoresed. Migration positions of the viral structural proteins are shown on the left. Markers: lanes 1 and 7, [35S]methionine-labeled IBV-M42; lane 2, 125I-iodine-labeled M41. Immunoprecipitates: lane 3, rabbit anti-M41 hyperimmune serum; lane 4, rabbit preserum; lane 5, Mcab 6; lane 6, Mcab 13.

and avidities were estimated using the plateau level absorbance values; they varied between 0.45 and 2.0. Mcabs 14, 15, and 16 did not reach a plateau level within the range studied. Mcab 13 behaved in a remarkable way: at concentrations up to 0.5 μg protein per well, this Mcab was able to saturate those viral antigen coated, while at concentrations higher than 2 μg per well, more antibodies could be bound (Fig. 2B).

Mapping of epitopes

Experiments were made to determine whether the Mcabs can be divided into clusters recognizing the same immunogenic region of the peplomer protein. All preparations except Mcabs 14, 15, and 16 retained their binding capacity after coupling to horseradish peroxidase. The avidity of the latter Mcabs apparently is too low in the ELISA. The remaining 13 Mcabs were used in the antibody competition assay. Dose-response curves for the peroxidase-conjugated (not shown) and the nonconjugated antibodies (Fig. 2) were plotted to determine the proper concentrations for the blocking assay. The relative avidity as well as the kinetics of self-competition were determined. Each antibody was used both as a competitor and as a peroxidase-conjugated preparation. Mcabs were considered to recognize the same epitope if use of reciprocal antibody preparations prevented binding. The percentage of competition was normalized to 100% in the homologous reaction. Five epitopes were found, which were designated A, B, C, D, and F. Epitopes A and B overlap, while a one-way competition was observed between Mcabs from group C and those of group D and between Mcab 1 (group A) and those of groups B and C (Fig. 3, Table 3).

Besides competition, enhancement was also observed. The binding of peroxidase-conjugated Mcab 13 could be enhanced by the binding of Mcabs 4, 8, 9, 10, and 12 (Table 3, Fig. 4). Furthermore, the binding of peroxidase-conjugated Mcab 5 could be enhanced by Mcabs 9 and 12; the binding of peroxidase-conjugated Mcab 6 was enhanced by Mcab 12 (Table 3). Enhancement was not observed in the reciprocal assays. Pairs of Mcabs which induced more than 50% enhancement are designated by arrows in Table 3.

Cross-reactivity of Mcabs among IBV strains

The Mcabs were tested for their cross-reactivity with other IBV strains in an indirect ELISA (Table 2). All

| VIRUS STRAIN | MCAB |
|--------------|------|
| M41          | 1    |
| M42          | 2    |
| H2            | 3    |
| HVX           | 4    |
| D97           | 5    |
| D71           | 6    |
| D144          | 7    |
| D446          | 8    |
| DB8           | 9    |
| D85           | 10   |
| D189          | 11   |
| D120          | 12   |
| D134          | 13   |

Note. Twice the amount of monoclonal antibody giving maximal binding to IBV-M41 was used. Binding to the heterologous virus is expressed as percentage of the A405 obtained with IBV-M41. Empty squares: 50 to 100%; crossed squares: 25 to 50%; full squares less than 25%.
FIG. 2. Avidity of the monoclonal antibodies to the IBV-M41 peplomer protein. Serial twofold dilutions of antibody were incubated in the wells of microtiter plates containing absorbed purified IBV-M41 virions. Binding to the viral antigen was detected by the addition of peroxidase-labeled rabbit anti-mouse IgG and the resulting A405 was read and used to rate Mcabs for avidity. The other properties of the monoclonal antibodies are summarized in Table 1.

strains belong to Dutch neutralization serotypes. The origin and serological relationships of the strains has been described before (Davelaar et al., 1984; Kusters et al., 1987). The M42 (Beaudette) strain is a laboratory strain of the same serotype as M41. The Mcabs 1–12 of the epitopes A to D recognized most strains. An exception is Mcab 10 (epitope D) which failed to react with the M42 strain as well with D207, D212, D274, and D1466, while Mcab 6 and Mcab 12 did not recognize strains M42 and D207, respectively. Epitope E was only present in strains M41 and D3896; however, Mcab 13 did not neutralize D3896 (results not shown).

DISCUSSION

In this paper we describe properties of Mcabs directed against the peplomer protein of IBV strain M41. Probably due to the immunization protocol in which mice were boosted intravenously with intact purified virus a few days before the fusion, only antipeplomer protein, Mcabs were obtained. Similar observations have been made with influenza virus (Gerhard et al., 1980), vesicular stomatitis virus (Lefrancois and Lylee, 1982a, b), measles virus (Giraudon and Wild, 1981), and Newcastle disease virus (Iorio and Bratt, 1983).

Although the Mcabs do precipitate the peplomer protein S1, we cannot exclude that this is the result of its association with S2, although the latter protein could never be precipitated as a clear band (Fig. 1). The Mcabs did not recognize (glyco)proteins of the allantoic fluid in the ELISA, the dot blot analysis (see Fig. 5), or after immunoprecipitation of radiolabeled allantoic fluid. This indicates that the Mcabs are not directed to oligosaccharide side chains of the peplomer protein (Jackson et al., 1981). All 13 antibodies were used in a blocking assay to identify the number of epitopes on the peplomer protein. The definition of epitopes by this assay is based on the assumption that a Mcab binding to a specific
site hinders the attachment of another antibody to the same or a proximal site (Stone and Nowinski, 1980). However, two antibodies directed to the same site will compete in a manner related to their relative avidities and concentrations. In practice, a spectrum of relative interference efficiencies will be obtained (Stone and Nowinski, 1980; Lefrancois and Lyles, 1982a, b.) Two-way competition is significant for the identification of Mcabs recognizing the same epitope, even when the degree of competition is low. Five epitopes (A to E) could be delineated on the peplomer protein, epitopes A and B overlapped. Furthermore there was extensive one-way competition between epitopes C and D and to a lesser extent between epitopes A and C. This does not necessarily indicate overlap of the epitopes, but may also be the result of conformational changes in the peplomeric protein induced by a Mcab against one epitope, leading to a reduced binding of a Mcab to another epitope.

Cooperative effects among antibodies directed against different epitopes were observed. Binding of Mcab 13 in the ELISA was enhanced by Mcabs 4, 8, 9, 10, and 12. These Mcabs, however, did not enhance neutralization or hemagglutination inhibition (HI). But
Table 3

Summary of Competition Immunassays between Monoclonal Antibodies to IBV-M41 S Protein

| COMPETING McAb | PEROXIDASE-CONJUGATED McAb |
|----------------|---------------------------|
| 1              | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| ++             | + | ++| + | + | ++| + | ++| + | ++| + | ++| ++ |
| +              | ++| + | + | ++| + | + | ++| + | ++| + | ++| ++ |
| ++             | + | ++| + | + | ++| + | ++| + | + | ++| ++| ++ |
| +              | + | ++| + | + | ++| + | ++| + | ++| + | ++| ++ |
| ++             | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |
| +              | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |
| ++             | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |
| +              | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |
| ++             | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |
| +              | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |
| ++             | + | ++| + | ++| + | + | ++| + | ++| + | ++| ++ |

Note. Competition more than 60%, ++; competition between 35 and 60%, +; enhancement more than 50%, †.

Neutralization and HI assays are not accurate enough to detect a twofold increase in titer. The binding of Mcab 5 and 6 was enhanced by Mcabs 9 and 12 and by Mcab 12, respectively. Enhancement of binding has been reported for Mcabs against VSV (Lefrancois and Lyles, 1982a,b), yellow fever virus (Schlesinger et al., 1984), Semliki Forest virus (Boere et al., 1984), and recently with another coronavirus, TGEV (Delmas et al., 1986). It might be related to a more advantageous conformation of the epitope involved, thereby increasing the binding of the antibody. Enhancement was always a one-way process.

Only epitope E is involved in both neutralization and hemagglutination inhibition. The corresponding Mcab 13 did not neutralize other IBV strains, but in the ELISA it reacted with one other IBV strain, D3896 (see Table 2). Two neutralizing Mcabs directed to S1 of IBV-M41 from Mockett et al. (1984) were also strain specific. Apparently, neutralization epitopes are not shared by IBV strains. In contrast, Jimenez et al. (1986) found that the critical epitopes involved in neutralization of TGEV were highly conserved.

The epitopes A to D were not involved in any known biological function. From Tables 2 and 3 we can deduce that the epitopes A–C are conserved. Only four Mcabs 6, 10, 12, and 13 differentiated between IBV strains. Conservation of epitopes is not unexpected in view of the conserved amino acid sequences of IBV strains (Niesters et al., 1986; J. G. Kusters et al., manuscript in preparation).

With the use of the dot immunoblotting assay, we were able to characterize the role of the conformation of the protein in antibody attachment. When the tertiary structure of the protein is disrupted by SDS and 2-mercaptoethanol, attachment of only Mcab 13 is abolished, indicating that Mcab 13 requires the native structure of the protein for recognition, whereas epitopes A to D are conformation independent (Fig. 5). Epitope E was also sensitive to SDS or NP-40/2-mercaptoethanol alone (data not shown). Talbot et al. (1984) found that two out of three neutralization relevant epitopes in MHV-JHM were completely sensitive to SDS denaturation, and one was partially sensitive. In the case of TGEV all six critical determinants were
control 2 wg allantoic fluid of uninfected eggs was applied. The
serum as described under Materials and Methods. As negative
cated.

twice maximal binding. Rabbit anti-M41 pre- and hyperimmune
monoclonals and the competition groups they belong to are indi-
tation. Antibodies to one of these were able to protect
which three were involved in neutralization. One of
these three determinants was conserved among the
strains tested. The nonneutralizing antibodies recog-
ized all strains except MHV strain S. This strain was
recognized only by one set of nonneutralizing antibod-
ies. Delmas et al. (1986) showed that there are four
sensitive to denaturation by SDS and 2-mercaptoeth-
ol (Jimenez et al., 1986).

It is not yet possible to define the number of epi-
topes involved in neutralization of IBV. Both our Mcab
13 and the Mcabs described by Mockett et al. (1984)
neutralize no other IBV strains than M41. In contrast,
polycional chicken antiserum against M41 does neu-
tralize several IBV strains, but this does not imply that
more than one epitope on S1 is involved in neutraliza-
tion, as suggested by Mockett et al. (1984). Polycional
antisera to one epitope contain a number of antibody
species which could show cross-neutralization not
observed with just one Mcab species. Recently, Koch
et al. (1986) found that some Mcabs to S2 were able to
neutralize IBV. However, this was only observed with
high concentrations of antibody and might be a con-
sequence of steric hindrance by the excess of anti-
body. Additional high-titer neutralizing antibodies
against IBV strains are needed to answer this ques-
tion.

Wege et al. (1984) identified six antigenic determi-
nants on the peplomorphic protein of MHV-JHM. Two
overlapping determinants were involved in neutraliza-
tion. Antibodies to one of these were able to protect
mice in vivo against infection. Talbot and Buchmeier
(1986) identified five epitopes on MHV strain 4 of
which three were involved in neutralization. One of
these three determinants was conserved among the
strains tested. The nonneutralizing antibodies recog-
nized all strains except MHV strain S. This strain was
recognized only by one set of nonneutralizing antibod-
ies. Delmas et al. (1986) showed that there are four
major and three minor antigenic determinants on the
TGE virus peplomorphic protein, of which two are in-
volved in neutralization.

It will be of interest to map our Mcabs on the peplo-
meric protein. We have cloned and sequenced the
protein (Niesters et al., 1986), so the epitopes can be
mapped both by the expression of small fragments of
recombinant DNA clones (Stanley and Luzio, 1984)
and by the PEPSCAN method (Geysen et al., 1984).
Mapping of the epitopes recognized by Mcab 13 by
these methods is probably difficult, since the results of
the dot immunoblot assay indicate that several distinct
parts of the protein form the neutralization epitope. In
this respect mapping of the Mcabs which enhance
Mcab 13 might give useful information. A direct ap-
proach to map the neutralization epitope recognized
by Mcab 13 is to isolate mutants escaping neutraliza-
tion.

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