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A highly conserved epitope on the spike protein of infectious bronchitis virus

L. Wang[1], R. L. Parr[2], D. J. King[3], and E. W. Collisson[1]

Departments of [1]Veterinary Pathobiology and [2]Medical Microbiology and Immunology, Texas A&M University, College Station, Texas, [3]Southeast Poultry Research Laboratory, U.S.D.A. Agricultural Research Service, Athens, Georgia, U.S.A.

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Summary. The predicted amino acid sequence and secondary structures of S1 of the spike protein (S) of infectious bronchitis viral (IBV) strains from Europe, the U.S.A., and Japan were compared. An antigenic determinant that was highly conserved in both the primary amino acid sequence and secondary structure of all strains was identified between amino acid positions 240 to 255. A synthesized peptide corresponding to this region was found to react with all polyclonal antisera examined from various IBV strains and with one monoclonal antibody (MAb), 9B1B6, out of nine known to react with the S of Gray. The specificity of the interaction with MAb 9B1B6 was confirmed by competitive ELISA using bound and unbound peptide. Interestingly, the previously described epitope for 9B1B6 had been characterized as cross-reactive with several strains of IBV, as conformation-independent but reacting only with intact whole S, and as associated with the functional integrity of other epitopes, including neutralizing epitopes on the S protein. The apparent critical functional and structural nature of this highly immunogenic determinant suggests a potential contribution in developing protective, cross-reactive subunit vaccines to IBV.

Introduction

Infectious bronchitis virus (IBV), a highly contagious respiratory pathogen of poultry, represents a serotypically diverse group of viruses [10]. The potential of IBV for genetic variation undoubtedly plays an important role in the occurence of antigenically distinct, virulent viruses that are often responsible for outbreaks of bronchitis in vaccinated flocks. It has also been shown that this remarkable evolution of IBV depends on both point mutations and recombination events and that the latter have commonly involved Mass vaccine-like strains [7, 20, 36, 37]. Although recombination between genes of murine hepatitis viruses had been experimentally produced under laboratory conditions [24, 27, 28], IBV was

the first coronavirus in which recombination has been suggested to generate new naturally occurring strains [20, 25, 36, 37].

The IBV particle has three major and one minor structural proteins [2, 10]. Antibodies are known to be readily induced to the structural proteins; the spike (S), membrane (M) and nucleocapsid (N) [32]. Whereas antibodies to N are strongly cross-reactive among strains, epitopes on the S and M proteins have been shown to be more variable [32]. The S protein contains determinants that dictate serotype and that induce neutralizing antibody and protection [4, 5, 18, 19, 29, 31]. This protein is synthesized as a large protein that is post-translationally processed to the S1 and S2 subunits. The primary neutralization epitopes of S are found on the outer globular-like S1, whereas minor neutralizing epitopes are found on the S2 which anchors the complex into the viral envelope, that is the bilipid membrane [5, 18]. A hypervariable region (HVR) within the S1 is probably associated with serotype determination of a strain [6, 10, 26, 30].

Monoclonal antibodies (MAb) specific for S have been used to identify both conformation-dependent epitopes, including S1 neutralizing epitopes, and conformation-independent epitopes [19, 29, 31]. However, certain non-neutralizing monoclonal antibodies react with S determinants that are both stable under denaturing conditions and conserved on various serologically distinct strains. The cross-reactive nature of these amino acid sequences could make them valuable if they should contribute or enhance protective immunity.

The purpose of this study was to characterize the amino acid sequence of the S1 in order to identify highly conserved, potentially immunogenic regions. The antigenic nature of a highly conserved determinant lying downstream of the HVR was examined using serotype-specific polyclonal antibody and S-specific MAb.

Materials and methods

Protein sequences and computer analysis

The S1 predicted amino acid sequences were determined from the previously determined nucleotide sequences. The secondary structures were predicted and compared by computer analysis based on the algorithms of Chou and Fasman [9]. The antigenicity indices were determined from the values of hydrophilicity [17], flexibility [23], surface probability [13], and glycosylation (GCG system 7.3).

Anti-IBV polyclonal and monoclonal antibodies

The chicken polyclonal antisera were collected after a single, intranasal/ocular inoculation [16]. The preparation of monoclonal antibodies specific for the spike protein of the IBV Gray strain and both positive and negative mouse ascites have been previously described [31].

Immunoblot assays

Modifications of the western blot procedure described by Parr and Collisson [31] were used for immunoblot assays. Briefly, immobilon-P membranes (Millipore Corp, Bedford, MA) were soaked in methanol then TBS (Tris buffered saline solution, pH 7.5) before placing on

the dot blot or slot blot apparatus (BioRad Laboratories, Richmond, CA). The Gray virus prepared as described [32] or peptide (synthesized and purified by Biosynthesis, Inc. Denton, TX) were added to the wells at concentrations of 1 μg and 5 ng per milliliter, respectively. Two hundred microliters of the virus or peptide were added to each well and filtered through the membrane by vacuum. The membranes were treated with diluent containing 3% bovine serum albumin (Kirkegard & Perry Labs, Gaithersburg, MA) and reacted with either polyclonal or monoclonal primary antibody, or normal serum diluted 1:10 in diluent. The membranes were removed from the apparatus, washed with TTBS (Tween-20 and TBS) before blocking again with diluent and adding secondary antibody consisting of either alkaline phosphatase-conjugated goat anti-chicken or anti-mouse (heavy and light chains) antibodies. The substrate, NBT/BCIP (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MA), was used to detect antigen-antibody complexes.

ELISA

Similarly prepared antigens were used to coat wells overnight at 4 °C in 96-well plates with concentrations used above in immunoblot assays for virus and, unless otherwise specified, for peptide [31]. After blocking for nonspecific binding with diluent (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MA) and incubating at 37 °C, specific viral antibodies or normal antibody controls were added (150 ng/ml and 100 μl/well) for one additional hour at 37 °C. The plates were then washed five times with PBS containing 0.05% Tween 20. The secondary antibodies, either goat anti-mouse-HRPO labeled antibody or goat anti-chicken-HRPO labeled antibody (0.5 mg/ml), were added at a 1:500 dilution for one hour at 37 °C. After thorough washing, the antigen-antibody complexes were detected with a mixture of the substrate ABTS (Kirkegaard and Perry laboratories, Inc., Gaithersburg, MA) and hydrogen peroxide. The color intensity was read with an ELISA reader at OD 490 (Dynatech Laboratories Incorp, Alexandria, VA; Manual, Kirkegard and Perry laboratories, Inc., Gaithersburg, MA). Those ratios of sample to background greater than or equal to 2 were considered positive [31].

The competitive binding assay was similar to the ELISA, except that the concentration of bound peptide was varied and the primary antibody was reacted with free or unbound peptide for 1 h at room temperature, before adding to the 96-well microtiter plate containing bound peptide [31]. The background OD in the absence of antibody were substracted from all values before determining the percentage binding with or without unbound peptide inhibitor. The maximum or 100% binding was the OD minus background in the absence of unbound peptide and the percent binding was calculated from the OD resulting from maximum binding.

Results

Primary and secondary structural analyses

The origins of the S1 gene of 24 strains of IBV isolated from Europe, Japan, and the United States that have been sequenced in our laboratory and other laboratories throughout the world (Genbank, NCBI, Bethesda, MD) are shown in Table 1. All strains represent distinct isolates. Mass42, also included in the following but not shown in the table, is a laboratory derivative of Beaudette [30]. The S1 gene of these sequences were compared and analyzed in order to identify secondary structures and determined putative conserved features of the protein. Using Ark99 as the reference strain, the conserved and variable regions, derived

Table 1. Information concerning IBV strains used in these studies

Strains[a]	Isolation [Ref.]	Geographic region
Beaudette (1936)	Beaudette et al. [1]	U.S.A.
M41 (1941)	Van Roekel et al. [34]	U.S.A.
Conn46 (1951)	Jungherr et al. [22]	U.S.A.
Iowa609 (1958)	Hofstad, [14]	U.S.A.
Gray (1962)	Winterfield et al., [38]	U.S.A.
Holte (1962)	Winterfield et al., [38]	Canada
SE17 (1969)	Hopkins [15]	U.S.A.
Ark99 (1973)	Johnson et al. [21]	U.S.A.
PP14 (1992)	Wang et al. [35]	U.S.A.
MM (1977)	Cavanagh et al. [6]	U.S.A.
H52 (1955)	Cavanagh et al. [6]	European
H120 (1955)	Cavanagh et al. [6]	European
UK-82 (1982)	Unknown	European
HV2 (1974)	Darbyshire et al. [11]	European
HVI140 (1968)	Darbyshire et al. [11]	European
D207 (1982)	Davelaar et al. [12]	European
D1466 (1978)	Davelaar et al. [72]	European
6/82 (1982)	Cook. [8]	European
UK-84 (1984)	Unknown	European
D3896 (1978)	Davelaar et al. [12]	European
UK-86 (1986)	Unknown	European
KB8523 (1983)	Sutou et al. [33]	Japan

[a]The years during which these strains were isolated are shown in parenthesis

from the predicted amino acid sequences, were superimposed onto a schematic of the computer generated secondary structure that included α-helices, β-pleated sheets, random coils and β-turn regions (Fig. 1). Based on the amino acid comparisons of these IBV strains, the S1 protein was divided into highly conserved (C1 to C4), with more than 80% identity among groups, and variable (V1 to V3), tolerating more than 20% variation. The HVR, also referred to as V1, extended from residue 50 to 150 and demonstrated less than 50% identity among groups. The V1 could also be further subdivided into 2 hypervariable domains (HV1 and HV2), separated by several internal conserved amino acids residues. The overall conservation of the secondary structure is illustrated in Fig. 2 in which the structures of S1 from 14 strains are compared. A highly conserved β-turn occupying a prominent position was predicted in C2 of the S1 from all 14 strains (Fig. 3). A region which included this β-turn between two β-pleated sheets was also predicted to have several highly antigenic sites.

Group-specific peptide on the S1

The amino acid sequence of residues 201 to 300 from the 24 strains was compared in detail in order to determine the degree of conservation of this common

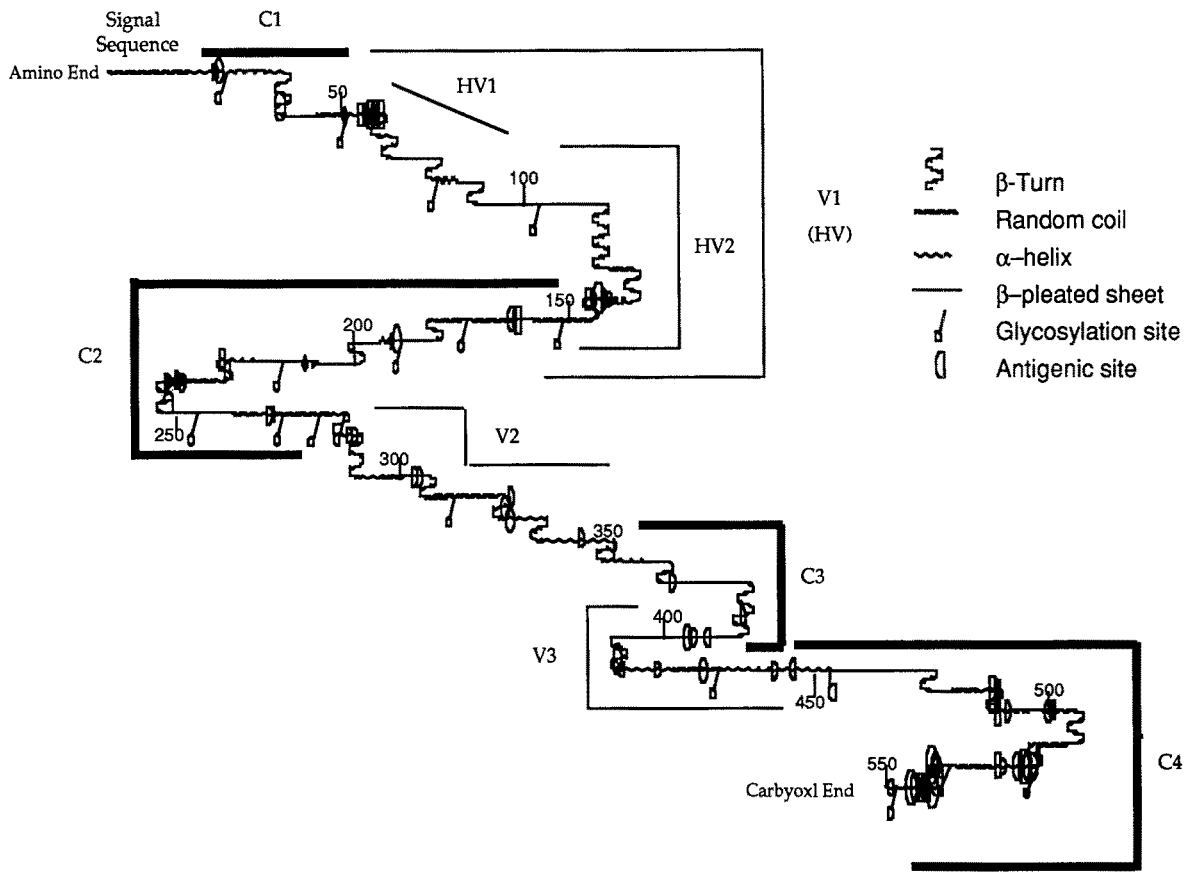

Fig. 1. Predicted variable and conserved primary sequences of S1 shown with the secondary structure of IBV strain Ark99. The conserved regions are indicated by C and variable regions are indicated by V and HV indicates the hypervariable regions. The numbers indicate the amino acid position within the S1

secondary structure lying within C2 (Fig. 4a). The primary amino acid sequence of a site that incorporated the conserved β-turn was found to be nearly totally conserved. A region within this site from 240 to 255, GlnTyrAsnTyrGlyAsnPheSerAspGlyPheTryProPheThrAsn (underscored in Fig. 4a) was also found to have a high antigenicity index (Fig. 4b). The only variations among the 24 strains were at position 248 and 251 where the serine was substituted by threonine and phenylalanine was substituted by leucine, respectively, in the Dutch strains, and at 255 where the threonine was substituted for by a isoleucine in the Mass 41 strain.

The conservation of the primary and secondary structures suggested that this site might serve a critical role in either maintaining conformational integrity or biological function. In addition, the high antigenicity index suggested that the peptide could be valuable in inducing broad based immunity to IBV. The P240 peptide corresponding to the above amino acid sequence was synthesized in order to evaluate actual antigenicity of the site. The peptide was used to determine

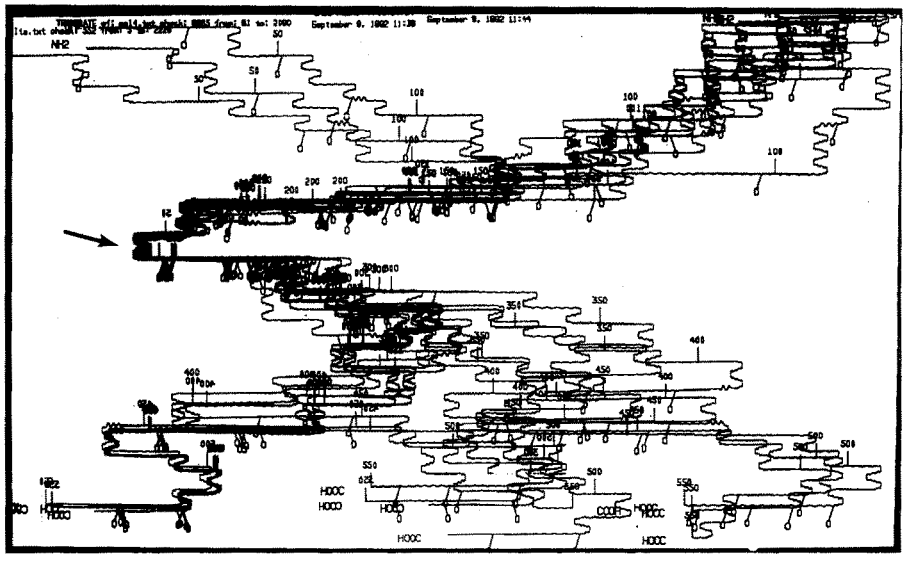

Fig. 2. Superimposed predicted secondary structure of the S1 from 14 strains of IBV. The numbers indicate amino acid position and the arrow points to the highly conserved β-turn

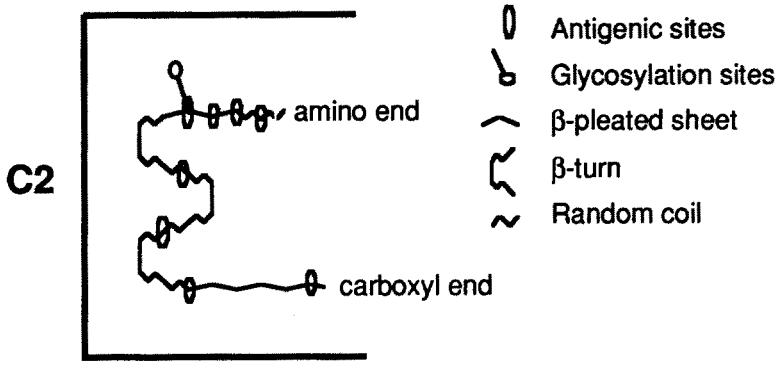

Fig. 3. Secondary structure of residues 240 to 255 in the conserved region 2 (C2) of Ark99 in Fig. 1

potential interactions with polyclonal antibody specific for eight strains of IBV (Table 2). This peptide reacted in a slot blot assay with primary chick antibody specific for Mass41, Holl52, JMK, SE17, Ark99, F188, Gray and Holte strains of IBV as determined by viral neutralization assays ([16], unpubl. obs., D. J. King). Antibody specific for ILTV (infectious laryngotracheitis virus) or NDV (Newcastle disease virus) did not react with the peptide nor did uninoculated control chicken sera. The polyclonal antisera specific for IBV strains, included PP14, also reacted with the P240 in an ELISA assay. Therefore, the sequence was not only conserved among strains in its primary sequence and predicted secondary structure but also in its functional immunogenicity.

a.

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          201                                                                                                     300
V1397     TVNGLKRRI KDTDV     ___ VE  V  DN K R          _T  L  VSNNV NDSVV  DVI TT YGK N_I_  ___S P AG N A FI
D1466     SVNGLKRRI KDTDV     ___ VE  V  D    R          _T  L  VSYNV NNSVV   VI TT YGK N_I_  ___  P AG N A FI
Holte            R  QT V      ___VH                      ___    T_DTI  D  F           LA T___ I_Q_R D  TG  DTFV
Iowa                         I___                        ___    T_DTI  D  V      I     V T___ S_QS  P  TGN DTFV
Gray                K T       ___                        ___    T_GTI  DR      T      L  T___ R__SA P  NG  DTFI
Sel7                K T       ___           I            ___    T_YTI  DR             L  N___ F_QSD L  SG  DTFV
Ark99     S                   ___ H      DT              ___    T_T_I  D              L  T___ S__S  P  TG  DSFI
Pp14      S                   ___ H      DT              ___    T_T_I  D        I     L  T___ S__S  P  TG  DSFI
6-82                          ___           T            ___    T___   E              LE T___ S_VSNAT  TG    T
UK-82                         ___           T            ___    T___   E       S      LDVT___ S_VSNAT  TG   HT
UK-84                         ___           T            ___    T___   E       S      LE T___ S_VSNAT  TG    T
UK-86                         ___           T            ___    T___   E       S      LE T___ S_VSNAT  TG    T
D207                          ___           T            ___    T___   E              LE T___ S_VSNAT  TG    T
D3896                         ___           T            ___    T___   E              LE T___ S_VSN    TG   HT
Conn                          ___                        ___    T___             I    LK ___  ___      L
H52                   R       ___                        ___    T___                     ___  ___
H120                  R       ___                        ___    T___                     ___  ___
Kb8523     V          R       ___                        ___    T___                     ___  ___
HV2                           ___                        ___    T___                     ___  ___
HVI140                R       ___                        ___    T___                     ___  ___               P
MM                            ___                        ___     ___                     Y S
Beau                          ___                        ___    T___                  C    I
M42                           ___                        ___    T___           T      C    I
M41       AGGPITYKVMREVKALAYFVNGTAQDVILCDGSPRGLLACQYNTGNFSDGFYPFINSSLVKQKFIVYRENSVNTTFTLHNFTFHNETGANPNPSGVQNIQ
                                                      ΛΛΛΛΛΛΛΛΛΛΛΛΛΛΛΛΛΛΛ
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b.

Fig. 4. Amino acid sequence of the S1 from position 199 to 300 using M41 as the reference strain (**a**). Only differences are listed. Putative glycosylation sites are represented by — and P240 is underscored with ΛΛΛ. The antigenicity profile is shown below (**b**). Positive units indicate increasing antigenic potential and the negative units represent decreasing antigenic potential

Table 2. Slot blot assay and ELISA of P250 reactions with anti-IBV polyclonal antibody

Antibody specificity	Slot blot	ELISA
Gray	+	+
Mass41	+	+
Holl52	+	+
Holte	+	+
JMK	+	+
SE17	+	+
Ark99	+	+
F188	+	+
PP14	ND	+
NDV	–	ND
ILTV	–	ND
Medium	–	–

+ Reacted with the peptide
– No reaction with the peptide
ND Not done

Interaction with an S-specific MAb

The binding properties of several MAbs generated to the Gray S had been previously examined [31]. The relative binding avidities, neutralization potentials and competitive interactions of these MAbs, and therefore, their corresponding epitopes, had been well characterized. The interactions of nine of these MAbs with P240 were examined. In a slot blot assay, only the MAb 9B1B6 consistently bound to the peptide (Fig. 5). The reaction with 9B1B6 produced a more intense

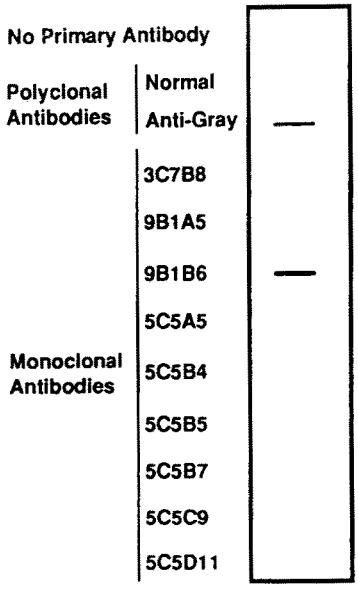

Fig. 5. P240 reacted with the MAb 9B1B6 and polyclonal anti-Gray antibody in a slot blot assay

Table 3. Dot blot assay and ELISA of P250 reactions with monoclonal antibodies generated to the Gray strain

Antibodies	Dot blot[a]	ELISA[b]			
		10^3	10^2	10^1	10^0
Polyclonal					
Mouse anti-Gray	+	++	+	–	–
Normal mouse	–	–	–	–	–
Monoclonal					
5C5A5	–	–	–	–	–
5C5B4	–	–	–	–	–
5C5B5	+/–	–	–	–	–
5C5B7	–	–	ND	ND	ND
5C5C9	–	–	–	–	–
5C5D11	–	–	–	–	–
9B1A5	–	–	–	–	–
3C7B8	–	–	ND	ND	ND
9B1B6	+	++++	+++	+	+

[a]Reaction with 100 μg of peptide
[b]Amount of peptide in μg that was added to each well

band than the polyclonal anti-Gray antibody. The specific interaction with P240 was confirmed in an ELISA and a dot blot assay (Table 3). With the exception of a weak reaction with 5C5B5 in the dot blot assay, the peptide reacted with only 9B1B6 and the polyclonal mouse antibody and the intensity of the observed bands decreased with decreasing concentrations of 9B1B6. Therefore, the peptide generated to this highly conserved region appeared to correspond to the epitope, or a part of the epitope, that interacted with the MAB 9B1B6.

Consistent with the above results, 9B1B6 had been shown to identify a conformation-independent and group-specific epitope [31]. In order to confirm the specific interaction of P240 with 9B1B6, unbound peptide was used to competitively inhibit the reaction of bound peptide with 9B1B6. Varying concentrations of 9B1B6, maximum or 100% binding was determined in the absence of unbound peptide. The unbound peptide did inhibit the interaction between the MAb and the bound P240, and the inhibition occurred in a dose-dependent manner (Fig. 6). The inhibition profile was very similar with the four concentrations of bound peptide shown. Lower concentrations of bound peptide used were below the level of sensitivity of the assay. Therefore, the peptide corresponding to a highly conserved region, consisting mostly of a β-turn common to most, if not all, S1 proteins, specifically reacted with 9B1B16, a MAb that defined a conserved and conformation-independent epitope.

Discussion

Because S, especially the S1, has been implicated in the binding of the IBV particle to the host cell membrane, it is a logical target antigen for developing

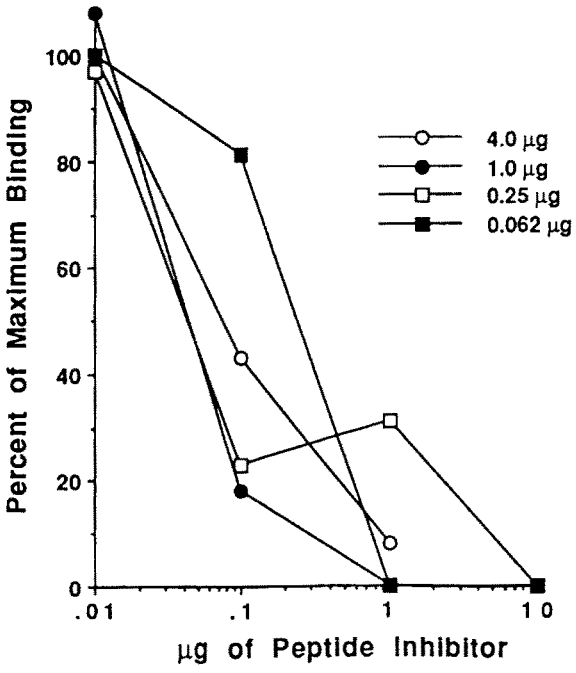

Fig. 6. Competitive interaction of unbound P240 and immobilized P240 with MAb 9B1B6 in an ELISA. Four concentrations of peptide used to coat each well are indicated on the figure

protective vaccines. The S1 has been shown to be a target for both point mutations and recombination events [37]. The apparent continuous evolution of IBV, implicated with emerging virulent strains distinct from vaccine strains, make the generation of relevant protective vaccines difficult. An ideal vaccine for a virus that is as variable as IBV would include highly conserved antigenic determinants that could contribute to the induction of responses that inhibit viral replication or the spread of virus. Antigenic determinants not associated with neutralization of virus could contribute to the control of viral infection; for example, through the induction of cellular immunity. Although epitopes that stimulate IBV neutralizing antibody appear to lie in highly variable regions, the effects of conserved antigenic regions would be universal. Computer generated comparisons of the amino acid sequences of 24 IBV strains, derived from isolations made throughout the world, identified in S1 four conserved regions separated by three variable regions, the first of which is the HVR. A region of β-turns, occupying a prominent position about 100 residues downstream of the HVR (V1) was conserved among all strains examined. This region, also conserved in amino acid sequence, was predicted to be a strongly antigenic region. Interestingly, two residues in this region were also found to be conserved in other coronavirus strains, such as MHV, bovine coronavirus, transmissible gastroenteritis virus, feline infectious peritonitis virus and human coronavirus [35]. At about position 500, a second conserved, highly antigenic determinant of 13 residues was identified in which five amino acids were common in a similar region throughout the coronavirus genus. These apparently functionally critical determinants on the protein could be useful targets for the induction of immunity that might contribute to protection.

The synthetic P240, corresponding to only 16 residues of 540 amino acids in the S1, reacted only with the one out of nine MAb examined. The P240 region was found to serve as the epitope of a well characterized MAb that had been generated against the Gray S [31]. Although, according to the present studies, the epitope was obviously within the S1 subunit, in western blot assays this MAb had been found to be conformation-independent but only reacted with the whole S and not with either subunit alone [31]. This had suggested that either the epitope lay between the two subunits and was destroyed following post-translational cleavage, or the maintenance of the intact S1 and S2 correlated with the integrity of the epitope. Another unusual property of 9B1B6 was that it had competitively inhibited the binding of most of the S-specific MAbs, where such broad competition was not common among the other MAbs [31]. The differences in competition could not be related to differences in binding constants of the MAb. It is difficult to explain the interactions of the 9B1B6 epitope with other determinants on S and the inability to recognize the S1 subunit alone except that it would appear to occupy a critical position in the S1 protein and functionally contribute to the integrity of the whole protein.

It would be of interest to determine if there is any association of this conserved region in C2 with cell attachment. Cleavage in the S could result in alterations in protein conformation that may be biologically important, for example, for viral entry into the host cell. In spite of serologic and pathogenic variations, the receptor-binding epitopes of IBV would be expected to be relatively conserved because most of the IBV strains do infect the respiratory system, as well as cultured chicken embryo kidney cells. In fact, we have identified a tissue receptor for the Gray strain from lung and kidney cells with a single molecular weight (unpubl. obs.), that could be the basis for future studies defining any direct or indirect interactions with the P240 site. Whether or not the corresponding determinant, conserved in both primary and secondary structure, is associated with cell membrane attachment, the eventual definition of the viral function that necessitates its evolutionary conservation should provide valuable insite into the overall structure and the impact of structure on S1 function.

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Authors' address: Dr. E. W. Collisson, Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77 843, U.S.A.

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