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Protein p*I* alteration related to strain variation of infectious bronchitis virus, an avian *Coronavirus*

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Summary

Viral proteins of two strains of infectious bronchitis virus (IBV), which have different tissue trophism and serology, were separated on the basis of their isoelectric points (p*I*). The viruses have four structural proteins; the protein of greatest serological importance is found at the peplomer tip. The viral structural proteins separated by isoelectric focusing were identified by comparison to SDS-PAGE separations. Three protein bands were identical in p*I* and one protein band showed a difference in p*I* between strains. When the renatured viral proteins were Western blotted and reacted with strain-specific antiserum, antigen-antibody complexing was seen only at points corresponding to the strain-specific variant bands. For IBV strain Mass-41, antigen-antibody complexing occurred at a p*I* of 6.8, and, for IBV strain Ark-99, at 7.2. No cross reaction of antisera was observed for either strain.

Since tissue affinities are a function of the viral peplomer-mediated attachment of virus to cells and are often directly related to pathogenicity, it appears that altered pathogenicity of strains of IBV may be detected by alteration of p*I* of the proteins. Classification by p*I* of proteins of at least the smaller viruses allows untypeable, highly pathogenic or persistent strains of these viruses to be characterized on the basis of variant proteins.

Coronavirus classification; Isoelectric focusing; Strain variation; Biophysical parameter

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Introduction

More than thirty IBV strains and numerous strain variants are now classified by serological criteria (Kusters et al, 1989; King and Hopkins, 1983). The varying pathology is demonstrated in animals or by embryo testing (Naqui et al., 1988). Biophysical methods such as isoelectric focusing (IEF) and high-performance liquid chromatography suggest quantitative criteria independent of conventional serology and animal testing by which virus strains can be classified. Groupings of viruses by serology do not always give clear delineations of strains and serological tests can give imprecise information with regard to minor strain variation which may result in altered pathogenicity during animal infections. The interior viral proteins tend to be evolutionarily conserved, in contrast to the outer proteins which can be highly variable (Yagyu and Ohta, 1990). The primary antigenic area of *Coronavirus* IBV, the epitope to which protective antibody is made, is located on the more distal subunit of the peplomer or spike (S-1) of the virus. This area controls cell attachment and virus neutralization (VN), determining the virus' tissue affinity and the greater part of its serological grouping (Cavanagh, 1983). Variations of these distal epitopes influence the ability of the virus to attach to specific cell receptors. Altered antigenicity as detected using serum neutralization is due to variation in the surface proteins of a virus. In the case of IBV, alteration in ability to be neutralized by antisera is due primarily to variation in the S-1 protein which forms the peplomer tip (Cavanagh, 1983). Change in the protein is caused mainly by amino acid alterations, which very often leads to an alteration of the total charge on S-1. The resulting alteration of charge can be detected by a separation of the viral proteins based on the charge on each of the constituent protein molecules. The charge of the molecules can be demonstrated by allowing the dissociated proteins to migrate in an electric field through a stabilized pH gradient until they reach their points of charge neutrality (p*I*) (Dunbar, 1989; Bond et al., 1979).

Of the many strains of IBV, two of distinctly different serotype and pathology have been chosen in order to elucidate alternative biophysical procedures with which to detect variants demonstrating altered pathology. Mass-41 strain of IBV is isolated from lung and tracheal tissue after infection of chickens (Arnold, 1984; Kawakubo et al., 1961). Ark-99 strain has been shown to have a marked preference for kidney tissue and, after initial infection of the respiratory system, can be isolated from the kidneys of infected chickens (Gelb et al., 1983; Cowen et al., 1987; Albassam et al., 1985).

The objective of this study is to show that viral strain variation can be detected by variation in serotypically important viral proteins.

Materials and Methods

Virus strains

Mass-41 was obtained from Dr. H. Van Roekel at the Massachusetts Agricultural Experiment Station and it has subsequently been passaged at least 8 times in embryonated eggs. Ark-99 was received from Dr. L. van der Heide at the University of Con-

necticut in Storrs after it was isolated from an Arkansas field infection. It has been similarly passaged three times. The two strains of virus were shown to be serologically different by King and Hopkins (1983) and Cowen and Hitchner (1975).

Virus propagation

Virus was propagated in 11-day-old embryonated eggs from White Leghorn hens and harvested for amnioallantoic fluid (AAF) after 45–48 h incubation. Virus from pooled lots of harvested AAF were concentrated by adsorption to and elution from polyethylene glycol 6000 (PEG) followed by sedimentation onto a 60% sucrose cushion at 83 100 × *g* for 90 min at 4°C. The pellet was collected from above the cushion and carefully layered onto a 20–60% sucrose gradient. This material was recentrifuged at 153 000 × *g* for 3.5 h. At the end of the isopycnic run, fractions containing visible bands or ten drops each were collected and the density determined from the refractive index.

Immuno-electron microscopy

The purified viruses were each mixed with specific antibody, stained and observed by electron microscopy.

Protein quantitation

Protein was quantitated in purified virus samples using ultraviolet absorbance scans between 200–300 nm. A micro Lowery protein assay was also done on the samples (Dunbar, 1987).

Isoelectric focusing

Focusing was done following the procedure of Dunbar (1987). Gels were formed with 15 ml of 28% Bis-acrylamide stock (28.38 g acrylamide, 1.62 g methylene bis-acrylamide, 100 ml distilled water), 12.6 g urea, 1.5 ml mercaptoethanol, 1.9 ml ampholytes, 3–10 pH range (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, U.S.A.), and made up to a volume of 30 ml with distilled water. Three hundred µl of ammonium persulfate (22.8 mg/ml) and 50 µl TEMED were added after degassing. The gel was cast to dimensions of 12 cm × 22 cm × 1 mm on Gelbond PAG film (FMC Bioproducts, Rockland, ME, U.S.A.). Electrode wicks were wet with 0.02 M arginine and 0.04 M aspartic acid, respectively. Electrodes were placed 9 cm apart. The gel was prefocused with cooling for 750 volt-hours at constant current of 8 mA. This established a pH gradient across the gel which could be measured between 4.5 at the anode and 9.6 at the cathode. After prefocusing, sample wicks were placed near the cathodal edge of the gel and loaded with 25-µl replicate samples of virus. The purified virus had been dissociated into its constituent proteins by exposure to 9 M urea, 2% NP-40, and 5% mercaptoethanol for 1 h at room temperature. Each sample applied to the gel contained approximately 40 µg of protein. IEF standards (Bio-Rad Laboratories, Rich-

mond, CA, U.S.A.) were run in parallel with virus samples, as were egg fluid samples from uninfected embryonated eggs of the same stage of development.

Gels were run for a total of 6500 volt-hours to allow the individual proteins to separate and migrate from the sample application wicks to their isoelectric points. When 6500 volt-hours was reached, the gel was removed from the flatbed apparatus and cut vertically into identical replicate thirds. One third was fixed in trichloroacetic acid to remove ampholytes. After ampholyte removal, the gel segment was stained to detect proteins using Coomassie blue R-250. Two additional replicate sections were frozen at −20°C until they were blotted.

Protein blotting

Identical replicate segments of the gel had their proteins transferred to nitrocellulose (NC) membranes. Each blotted membrane was blocked with non-specific protein (dried milk) and then allowed to react with strain-specific chicken anti-Mass IBV or anti-Ark IBV antiserum (Spafas, Inc., Storrs, CT, U.S.A.) overnight with shaking. After washing, a secondary antibody, rabbit anti-chicken horseradish peroxidase-linked antiserum (Organon Teknika-Cappel, Malvern, PA, U.S.A.) was added, followed by washing and a color developing system [HRP color development reagent (4-chloro-1-naphthol), Bio-Rad Laboratories].

Results

Virus concentration, purification and isolation

Virus was adsorbed to PEG, pelleted onto a sucrose cushion and purified by isopycnic centrifugation. Visible bands were collected for Mass-41 and Ark-99 and had respective densities of 1.20 and 1.19 g/ml.

Immuno-electron microscopy

Virus particles 130–168 nm in diameter with a regular pattern of club-shaped projections were found from the visible bands produced by isopycnic centrifugation. Viruses of each strain were observed in pelleted material and as aggregates after treatment with strain-specific antiserum.

Isoelectric focusing

The charge-based separation of the viral proteins showed two identical major bands for each strain which focused towards the anodal end of the gel (Fig. 1). By comparison to SDS-PAGE separations of the same material (data not shown), these were identified as the major proteins N and M. Towards the neutral range, minor protein bands were found between IEF standards of human carbonic anhydrase (p*I* 6.5) and whale myoglobin (p*I* 8.05). One band, positioned across from equine myoglobin at a

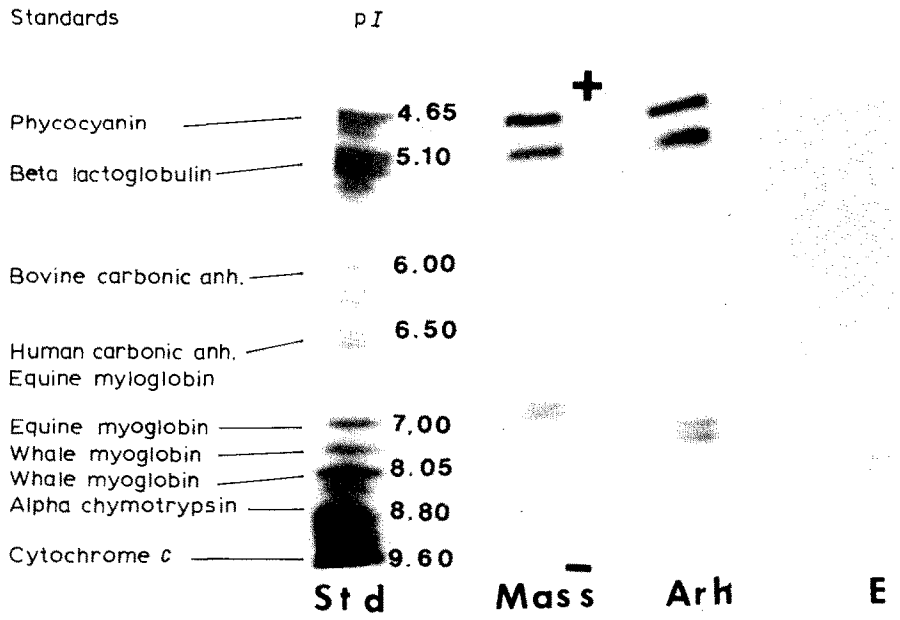

Fig. 1. Separation of constitutent proteins of two strains of infectious bronchitis virus, Mass-41 (Mass) and Ark-99 (Ark). Proteins have migrated under the influence of an electric field through a pH gradient to their points of charge neutrality (p*I*). The heavy protein bands closest to the anode (+) at the top of the Mass and Ark columns are the major structural protein M and the nucleoprotein N, each of which represent approximately 40% of the viral protein. The two minor bands in the center of the Mass and Ark columns are identified as the peplomer proteins S-1 and S-2, each of which accounts for another 8% of the total virion protein. A common band appears to focus at pH 7.00 for both strains. The serologically important band which varies between strains focuses at pH 6.8 for Mass and 7.2 for Ark. Egg fluids from sham-infected embryonated eggs of the same age are similarly separated in column E. The egg AAF proteins do not focus in common with any of the viral proteins. The standards (Std) are broad-range IEF standards.

p*I* of 7.0, was common to both strains. A variant band was found at a p*I* of 6.8 in the Mass-41 lane and in the Ark-99 lane the variant band appeared at a p*I* of 7.2, as judged by comparison to the adjacent standards.

Serological identification of the renatured protein bands

When a gel containing both strains of the viral proteins was blotted onto an NC membrane and reacted with Mass-41 strain-specific antiserum (Fig. 2), a darkly staining color reaction visualized as a doublet band developed in the center of the Mass-41 lane. No reaction with any of the Ark-99 proteins was detected with the anti-Mass antibodies. A duplicate blot of separated proteins of both viral strains was reacted with strain-specific anti-Ark (Fig. 3) and only a single band developed coordinate with the lower of the minor Ark-99 protein bands at p*I* 7.4. There was no reaction with the Mass proteins.

Fig. 2. Western blot of the IEF gel containing separated Mass and Ark proteins as shown in Fig. 1. The blot has been reacted with strain-specific anti-Mass antiserum. A specific reaction occurred only with the doublet band in the center of the Mass column which is identified as the peplomer protein S-1. There was no reaction with any of the Ark proteins. Egg fluids from embryonated eggs in column E showed no reaction with the antibody. The standards (Std) are broad-range IEF standards.

Discussion

The innermost of IBV structural proteins, the nucleocapsid or N protein, is a phosphorylated 55-kDa core polypeptide which can be shown to be evolutionary conserved (Boursnell et al., 1987; Yagyu and Ohta, 1990) and comprises 40% of the total viral protein by weight (Cavanagh, 1983). It is not glycosylated and, since it is an interior protein, protective antibody is not made to it.

The matrix protein M is a polypeptide which consists of 224–225 amino-acids and accounts for an additional approx. 40% of total viral protein mass (Cavanagh and Davis, 1988). Its relative molecular mass can vary between 23 and 36 kDa, due to heterogenous glycosylation.

The outermost structure of the virus is the peplomer or spike protein S which is produced as a precursor S-O polyprotein (Cavanagh, 1983). It is an N-linked high mannose oligomer (Niesters et al., 1987) which is cut to form two subunits: S-1 (90 kDa) and S-2 (84 kDa). The subunits are present in equimolar amounts and each subunit accounts for approx. 8% of total viral protein by weight. In the mature virion, several copies of the S-1 subunit form the tip of the peplomer and several copies of the S-2

Fig. 3. Western blot of the IEF gel containing separated Mass and Ark proteins as shown in Fig. 1. The blot has been reacted with strain-specific anti-Ark antiserum. The minor band in the center of the Ark column is identified as the peplomer protein S-1 because the specific reaction occurred with the lower of the S bands. No reaction occurred with any of the Mass proteins. Egg fluids from sham-infected embryonated eggs are similarly reacted in column E. The standards (Std) are broad-range IEF standards.

subunit together span the membrane (Kusters et al., 1989). Parts of the conformation-dependent S-1 epitopes may be clustered and linked by disulfide bonds (Niesters et al., 1986).

The S-2 has been shown to have 12 potential glycosylation sites. There are conserved epitopes and, although S-2 does not contain the dominant antigenic sites (Cavanagh et al., 1986b), binding of antibodies on S-2 epitopes may enhance peplomer conformational changes or create steric effects leading to rapid and efficient binding and neutralizing by other antibodies at S-1 epitopes (Niesters et al., 1987; Mockett et al., 1984).

S-1 is comprised of 514–519 amino-acids and is smaller than S-2 in terms of total number of amino acids. However, S-1 has a higher molecular weight due to more extensive glycosylation (Sutou et al., 1988; Cavanagh et al., 1986a). S-1 has 17 potential glycosylation sites (Sutou et al., 1988), 16 of which were shown to be conserved among 31 strains of IBV (Kusters et al., 1989). Antigenically closely related strains may have up to 97% S-1 sequence homology (Cavanagh and Davis, 1986), while distantly related strains approach 50% homology (Kusters et al., 1989). Serotype and other surface antigenic determinants are located in the N′ end of the S-1 protein (Kusters et al., 1989) and a single amino-acid change in their conformation-depen-

dent neutralization epitope(s) may create a new variant (Dea et al., 1990). A rapid rate of evolution in the S-1 protein sequence can be expected, due to the high mutation rate of the single-stranded RNA genome coupled with the selective pressure favoring the propagation of strains resistant to individual and/or flock immunity (Cavanagh and Davis, 1988). S-1 neutralization epitopes are comprised of clusters of mutations which have been shown to physically correspond to regions of mutations in the genome. There are no clearly defined hypervariable regions, but in a comparison of 31 strains of IBV, a greater number of replacements has been shown to occur in two areas (in amino acid residues 50–170 and 250–310) and a greater number of insertions and deletions can be located in a portion of the first area (in residues 120–170) (Kusters et al., 1989). Virus produced during persistent infection has been shown to have S-1 proteins antigenically distinguishable from those of the initial infecting virus (Holmes and Behnke, 1981).

The p*I*s of the S and M proteins are also affected by the kind and extent of their glycosylation. Glycosylation may affect antigenicity and tissue affinity by the carbohydrate side chains which are part of the antigenic determinant of S-1; or, the attachment of carbohydrate may affect overall folding of the S-1 protein, altering the conformation and influencing antigenicity at points far removed from the actual point of carbohydrate attachment. Viral variation may be enhanced by the microheterogenous nature of the surface protein's glycosylation. Virus released from cells in which the glycosylation has not progressed to the same level as the earlier made virions could result in 'accidentally variant' viruses which may then be able to enter a different type of cell by virtue of the altered pattern of their cell attachment sites. These variants may in turn be acted upon by different cellular proteases found in other cell types, so that the cutting of the S-O protein might be altered, thus increasing the possibility of divergent phenotypes. Mass-41 and Ark-99 strains of IBV have been shown to be genetically and serologically distinct strains by means of oligonucleotide fingerprinting (Naqui et al., 1988; Kusters et al., 1989) and extensive cross neutralization studies using VN and HI tests (King, 1988; Cowen and Hitchner, 1975). One-dimensional SDS-PAGE studies of IBV virus, which do not distinguish strains or antigenic variants, show two major proteins and two minor proteins in strains of IB virus (Cavanagh and Davis 1986). SDS-PAGE fails to distinguish between the similar sized minor protein subunits of the peplomer proteins (S-1 and S-2), nor does it detect the most frequent mutations (amino-acid substitutions or alterations of glycosylation) which do not result in substantial molecular weight differences (Lominiczi and Morser, 1988).

The anti-Mass antibody may have either reacted with both S-1 and S-2 or the S-1 band may have focused as a doublet band not detected by the Coomassie blue staining. In some focusing runs, the upper Mass band appeared as two lighter, closely-spaced bands above the lower band. During blotting, some size distortion of the gel image occurs. Spot orientation between the gel and blot was facilitated by notching the corners of the gel and blot. It was also helped by some faintly perceptible transfer of the standards to the blot. Although the difference in location of the minor band may appear slight, the detected values of S-1 and S-2 for each viral strain are supported by two independent lines of evidence: (1) the reaction of the strain-specific antiserum

TABLE 1

Mass-41 peplomer amino acid[a] charges at neutrality (pH 7.0)

	S-1	S-2
Positively charged amino acids:		
Glutamine	15	27
Asparagine	15	22
Negatively charged amino acids:		
Histidine	11	4
Lysine	17	27
Arginine	14	17
	30(−)44(+)	49(−)48(+)
Net Charge	+14	−1

[a]Amino acid sequence deduced from the genome sequence published by Sutou et al., 1988.

occurred only at those locations at which the most antigenic bands focused; (2) based upon the published sequences of Mass-41 S-1 and S-2 (Sutou et al., 1988), the charge upon the amino-acids at neutrality can be calculated and, based upon the amino-acid charges, both S-1 and S-2 protein subunits would have close to neutral p*I* with the Mass-41 S-1 subunit having a charge of +14 to the S-2's charge of −1 (Table 1). From the charges on the amino acid of S-1, it could be expected to focus as the minor reactive band closer to the anode (Fig. 1), and S-2 subunit closer to neutral, pH 7.

Although no amino acid sequencing data is available for Ark-99, a clear antigen-antibody reaction was seen at the site corresponding to the visually observed variant minor band. It is known from published sequencing studies on various other strains of IBV that the S-2 amino-acid sequences, the anchoring portion of the peplomer, are often more closely conserved from strain to strain than S-1 subunits (Niesters et al., 1986; Binns et al., 1986; Cavanagh et al., 1986a; Jordi et al., 1989). It could be expected that the S-2 subunits for Mass-41 and Ark-99 would have similar focusing points.

The four proteins' identification is strengthened by showing that the starting material is substantially purified virus. The criteria of purity have been: the comparison of published SDS-PAGE separations and their similarity to SDS-PAGE separations done in this laboratory on the same purified virus; isopycnic banding of each virus, indicating homogenous density; sedimentation rate, suggesting homogeneity of size and shape; uniform 260–280 nm ultraviolet absorption ratios, indicating relatively the same amounts of nucleic acids and proteins in each strain; the ability of strain-specific antibody to clump or agglutinate purified virus of each strain as visualized by electron microscopy. Although they do not rule out minor protein contamination, these criteria make it unlikely that bands found by IEF are other than viral structural proteins. The IEF bands produced from egg fluids (Fig. 1) did not correspond to any viral bands.

The results of these experiments show that isoelectric focusing can be used to distinguish between variant strains of infectious bronchitis virus, and that the separated

proteins, on being allowed to renature, can be verified serologically.

Isoelectric focusing is a relatively rapid phylogenetic comparison technique which can provide a quantitative basis for viral strain comparison. It might clarify viral strain identification based upon serological tests and could possibly be used to decrease animal testing for the demonstration of pathology. The use of such biophysical criteria to detect viral variant strains produced in large flocks of vaccinated chickens could allow more efficacious vaccine regimens to be suggested and pin-point the causative strains of vaccine-related disease 'breaks'.

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