Polypeptides of the Surface Projections and the Ribonucleoprotein of Avian Infectious Bronchitis Virus

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Purified avian infectious bronchitis virus was digested with bromelain (0.7 mg/ml), and the surface projections were removed. Polycracylamide gel electrophoresis of the polypeptides from these bromelain-treated particles showed that VP1, VP2, and VP5 were missing from the seven polypeptides, VP1 to VP7, that were present in untreated virus preparations. Milder bromelain treatment (0.07 mg/ml) left visible surface projections and polypeptides comprising VP1 and VP2 intact, but removed VP5. Thus, there are apparently two types of surface projections on the virus particle. The ribonucleoprotein complex was released from virus particles disrupted with 1% Nonidet P-40. The proportion of VP6 in such preparations was greatly reduced, implying that VP6 is the structural polypeptide of the ribonucleoprotein. Polypeptides VP1, VP2, VP4, and VP5 are glycosylated, but none of the polypeptides contains lipid.

Avian infectious bronchitis virus (IBV) is a member of the coronavirus group (15). These viruses develop exclusively in the cytoplasm of infected cells (10), beginning in the perinuclear region and spreading to involve the entire cytoplasm (12). IBV particles exhibit moderate pleomorphism and have peculiar club-shaped projections that are characteristically widely spaced (10). Particles of IBV equilibrate at different densities in sucrose density gradients, but all have the same morphology and contain seven polypeptides named VP1 to VP7 (11). However, no studies have yet related the individual polypeptides to the structural components of the particles. In the present paper, we report the effect of bromelain and Nonidet P-40 treatment on virus particles and identify the polypeptides that form the surface projections and the ribonucleoprotein (RNP). Our results suggest that the polypeptide structure of IBV differs from that of other coronaviruses such as transmissible gastroenteritis virus (4, 5), human coronavirus strains OC43 (7) and 229E (6), and mouse hepatitis virus (13).

MATERIALS AND METHODS

Virus growth and purification. IBV strain Connecticut (IBV 46) was used. The virus was grown in 10-day-old embryonated chicken eggs and was purified on linear 25 to 55% (wt/wt) sucrose density gradients at 4°C as previously described (11).

Electron microscopy. Virus samples were applied to grids that were washed with distilled water, negatively stained with 2% (wt/vol) potassium phosphotungstate (pH 6.5), and then examined in a Philips EM 300 electron microscope.

Bromelain treatment. Purified virus preparations were incubated for 15 min at 37°C in 50 mM diethiothreitol (pH 7.2) in Dulbecco phosphate-buffered saline A (PBSA), with bromelain at 0.7 or 0.07 mg/ml. After incubation, samples were directly layered onto linear 25 to 55% (wt/wt) sucrose density gradients in PBSA at 4°C and fractionated as previously described (11).

Nonidet P-40 treatment. Purified virus was suspended in 1% Nonidet P-40–50 mM dithiothreitol (pH 7.2) in PBSA and then immediately layered onto linear 25 to 55% (wt/wt) sucrose density gradients in PBSA at 4°C and fractionated as previously described (11).

Polycracylamide gel electrophoresis. Purified virus fractions from linear sucrose gradients were treated with 5% sodium dodecyl sulfate–2% 2-mercaptoethanol at 100°C for 1.5 min. A trace amount of bromophenol blue dye was added to the reduced polypeptides, and the polypeptides were electrophoresed through 7.5% polyacrylamide gels (11).

Polypeptide analysis. After polycracylamide gel electrophoresis, the gels were removed from their supporting tubes and stained for proteins, glycoproteins, and lipids. For protein staining, the gels were stained with 0.1% Coomassie brilliant blue in 50% methanol–7.5% acetic acid overnight and then destained over 2 to 3 days with several changes of 50% methanol–7.5% acetic acid. Glycoprotein staining was done by fixing the gels in 10% trichloroacetic acid for 2 h, washing with several changes of 3% acetic acid, and then staining with 1% periodic acid in 3% acetic acid for 1 h in the dark. The gels were then washed with 3% acetic
acid, stained with reduced acidified basic fuchsine (Schiff reagent) for 1.5 h, washed with freshly made up 0.5% sodium metabisulfite, and finally washed with several changes of 5% methanol-7.5% acetic acid. Lipid staining was done by immersing the gels in saturated oil red O in 60% ethanol for 2 h, destaining them with 50% ethanol for 2 h, and then washing them with several changes in 50% methanol-7.5% acetic acid. After staining, the gels were analyzed for material absorbing at 620 nm on a Joyce-Loebl Chromoscan densitometer.

RESULTS

Morphology and polypeptide structure of the virus particles. In a previous paper (11) we showed that IBV particles of different densities can be obtained on sucrose density gradients, although only at 1.18 g/cm³ were virus particles always seen. As these formed the majority of the virus particles, we studied them to elucidate the polypeptide composition of their surface projections and RNP. Figure 1 is an electron micrograph of IBV Connecticut showing typical spherical particles with almost complete coronas of surface projections. In no cases were virus particles observed with complete coronas of projections (11).

The polypeptides of the particles were examined by polyacrylamide gel electrophoresis (Fig. 2). Seven polypeptides, VP1 to VP7, were observed, with molecular weights of 130,000, 105,000, 97,000, 82,000, 74,000, 51,000, and 33,000, respectively (11).

Effect of bromelain and Nonidet P-40 on virus morphology. Virus particles incubated in the presence of bromelain and separated by sedimentation on sucrose density gradients are shown in Fig. 3A and B. Relatively gentle incubation conditions (0.07 mg of bromelain per ml at 37°C for 15 min) produced virus particles (Fig. 3A) that appeared essentially the same as untreated particles: the surface projections were apparently undigested by bromelain. More concentrated bromelain (0.7 mg/ml at 37°C for 15 min) produced virus particles that had lost all of their surface projections (Fig. 3B). In this case the surface projections were completely digested by bromelain (3). As shown in both Fig. 3A and B, the membranes of particles treated with bromelain appeared to be intact as the particles were not penetrated by stain. Virus particles incubated in buffer without enzyme did not lose their surface projections.

Fig. 1. Particles of IBV from sucrose density gradients. Negative staining with 2% potassium phosphotungstate, pH 6.5. Bar represents 100 nm.
Treatment of IBV with Nonidet P-40 followed by sedimentation on sucrose density gradients produced virus particles with coronas of surface projections into which the stain had penetrated, suggesting that the virus membranes had been damaged (Fig. 3C). No internal virus components could be distinguished with such treatment.

Effect of bromelain and Nonidet P-40 on the structural polypeptides. Figure 4A is an electropherogram of the polypeptides of virus particles after bromelain treatment (0.07 mg/ml at 37°C for 15 min), and a comparison with Fig. 2 shows that VP5 was missing. Treatment with bromelain at 0.7 mg/ml at 37°C for 15 min, however, produced particles that lacked VP1, VP2, and VP5 (Fig. 4B).

The polypeptide profiles showed that after treatment of IBV with Nonidet P-40, VP6 was greatly reduced (Fig. 5). Particles treated in this way were penetrated with stain and apparently lacked the internal component (Fig. 3C).

Virus glycopolypeptides. Polyacrylamide gels of IBV polypeptides were stained with Schiff reagent to determine which of the virus polypeptides were glycosylated (Fig. 6A). VP2 and VP5 always stained as glycopolypeptides, and VP1 and VP4 were stained in most preparations. Figure 6B shows a gel stained for lipid with oil red O. None of the polypeptides or glycopolypeptides stained, and hence none of them contains detectable lipid.
As shown in Fig. 6B, a component at the top of the polyacrylamide gel stained with oil red O, implying the presence of lipid. This lipid component also stained with Schiff reagent (Fig. 6A), although not with Coomassie brilliant blue (Fig. 2).

**DISCUSSION**

In this paper we have shown that purified IBV consists of spherical particles with a distinct corona of surface projections. Seven polypeptides called VP1 to VP7 have been identified (11).

Bromelain treatment (0.7 mg/ml) of the virus particles was found to remove all of the surface projections but to leave the virus particles otherwise intact. Particles stripped of projections by bromelin treatment lacked VP1, VP2, and VP5, suggesting that these are the surface projection polypeptides. However, only VP5 was removed from the virus particles treated with lower concentrations of bromelain (0.07 mg/ml), and such virus particles appeared to have normal coronas of surface projections. Similarly, treatment of virus particles with Nonidet P-40 removed VP5, but left the other surface projection polypeptides intact. In some virus preparations, VP5 was missing without any treatment, and these virus particles also appeared to have a normal corona of surface projections. We suggest that VP5 comprises a species of projection that is readily removed from the particles and that is not easily visualized by electron microscopy. Furthermore, a second type of surface projection is formed from VP1 and VP2, which are more firmly attached to the virus surface and more readily visualized by electron microscopy as typical coronavirus petal projections. Further experiments are in progress to characterize in more detail the surface projections of IBV and the polypeptides that comprise them. Preliminary results with human and mouse coronaviruses suggest that the loosely attached surface projection is a long thin component that can be visualized with difficulty by electron microscopy (M. R. Macnaughton and H. A. Davies, unpublished data).

Treatment of IBV with Nonidet P-40 causes damage to the virus membranes and apparently allows the RNP to escape (8, 9). However, we have not been able to visualize by electron microscopy any RNP associated with Nonidet P-40-treated virus particles. VP6 was greatly reduced in amount in Nonidet P-40-treated particles, and we suggest, therefore, that this polypeptide comprises the protein component of the RNP. Finally, we conclude by exclusion that VP3, VP4, and VP7 constitute the membraneous component of the virus particle.

Staining the polypeptides with Schiff reagent revealed that there are four glycopolypeptides, namely, VP1, VP2, VP4, and VP5, of which VP1, VP2, and VP5 have been shown to be surface projection polypeptides. None of the polypeptides contained lipid. A component at the top
of our polyacrylamide gels stained with both Schiff reagent and oil red O, although not with Coomassie blue. We suggest that this component is a lipopolysaccharide and may comprise virus envelope material (10). Studies are in progress to further identify this component.

In a previous paper (11) we described how IBV now appears to have only 7 polypeptides rather than up to 16 polypeptides as reported previously (1, 2). This result compares favorably with the four to seven polypeptides reported for human coronaviruses (6, 7), transmissible gastroenteritis virus (4), and mouse hepatitis virus (13). All of these reports agree that these viruses contain a polypeptide of about 50,000 daltons that is not glycosylated, and in three cases (1, 2, 13) it comprises a large proportion of the total polypeptides. Furthermore, two reports (5, 13) show that the 50,000-dalton polypeptide, corresponding to our VP6, comprises the inner core polypeptide, whereas others (1, 6, 7) show that this polypeptide is not located on the virus surface. However, there are not many similarities in size and number among the other polypeptides of these coronaviruses, although it has been shown that the polypeptides with the highest molecular weights tend to comprise the surface projection polypeptides (1, 4, 6, 7, 13). Finally, in all of the coronaviruses studied, a large proportion of the polypeptides are glycosylated (2, 5–7, 14).

It is not yet clear whether the reputed variations in the coronavirus polypeptides reflect differences among the coronaviruses. The differences in the number and sizes of the polypeptides obtained in different laboratories may be due to the use of different denaturing conditions for polypeptide analysis (11, 13). Furthermore, the molecular weights of the glycopolypeptides determined by their relative mobility in polyacrylamide gels may be inaccurate due to the presence of the carbohydrate. Work is in progress in this laboratory to investigate the structural polypeptides of a number of coronaviruses, using the same conditions of preparation and analysis to observe whether the apparent differences among the structural polypeptides of different coronaviruses are genuine.

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LITERATURE CITED
