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KEYWORDS: respiratory syncytial virus, spike glycoproteins.

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RESPIRATORY SYNCYTIAL VIRUS II. ISOLATION AND MORPHOLOGY OF THE GLYCOPROTEINS

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Abstract. Treatment of respiratory syncytial (RS) virus with Triton X-100 in solutions of low ionic strength solubilized not only glycoproteins but also some non-glycosylated proteins. Rate zonal sedimentation of the solubilized materials resulted in separation of the glycoproteins from the other components, *i.e.* one fraction predominantly composed of two glycoproteins each with molecular weight (mol. wt.) of~100,000 and 53,000 was obtained. Electron microscopic observation of this fraction revealed numerous club- or rod-shaped fine structures, suggesting that these were spikes of RS virus. After removal of Triton X-100 the structures aggregated and formed oligomers and polymers.

Key words: respiratory syncytial virus, spike glycoproteins.

The surface of RS virus is covered with spikes measuring 12 nm in length and having a club-shaped appearance (1, 2). The biological, biochemical and morphological characteristics of these spikes, however, have not been well clarified and isolation has not been reported. The spikes of influenza virus (3) and some paramyxoviruses, *e.g.* SV5 (4) and HVJ (5) are composed of glycoproteins and have biological activities such as hemagglutination, neuraminidase activity and cell fusion.

Envelope glycoproteins of RS virus were first reported by Wunner and Pringle (1976), *i.e.* one major and one minor glycoproteins each with mol. wt. of 48,000 and 42,000 (6). The major one was a sole glycoprotein (7). Levine (1977) analyzed purified RS virus in neutral sodium dodecyl sulphate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) system and resolved two major glycoproteins with mol. wts. of 79,000 and 56,000, and also one very minor glycoprotein with a mol. wt. of 22,000 (8). However, in the discontinuous SDS-8.5% PAGE system there was a shift in the migration of the glycoproteins in relation to the migration of other viral proteins (9). Thus, the system gave an approximate mol. wt. of 90,000 instead of 79,000 for the largest glycoprotein, VP1, and 49,000 instead of 56,000 for VP2, and the minor glycosylated component VP7 migrated to a portion corresponding to a size of a mol. wt. between

28,000 and 25,000. More recently, Peeples and Levine (1979) reported that two major glycoproteins removed by treatment of RS virus with trypsin were envelope proteins and treatment with Triton X-100 in the presence of increasing concentrations of NaCl solubilized not only the major glycoproteins but also non-glycosylated proteins (9).

In our previous study on analysis of purified RS virus in the SDS-10% PAGE system, only a 53,000 mol. wt. polypeptide of seven virus-specific polypeptides with mol. wts. ranging from 25,000 to 75,000 was glycosylated (10).

In this paper we report the isolation of a fraction containing glycoproteins by rate zonal sedimentation of purified RS virions solubilized with Triton X-100 and describe its morphology as seen with an electron microscope.

MATERIALS AND METHODS

Cell culture and virus preparation. Monolayer cultures of HES cells, derived from human embryonic skin and established in our laboratory, were grown in Eagle's minimum essential medium with 3% fetal calf serum and were inoculated with Long strain of RS virus as described previously (11).

Purification of RS virus. Confluent monolayers of HES cells grown in roller bottles were inoculated at a multiplicity of approximately 1 PFU per cell and incubated at 33°C. After about 36 h, the medium was harvested and clarified at 5,000×g. Polyethylene glycol in NTE buffer, which was composed of 0.15 M NaCl, 0.05 M Tris-HCl and 1 mM EDTA (pH 7.5), was added to a final concentration of 10% (w/v), and the suspension was stirred for 60 min. The precipitated virus was collected by centrifugation at 5,000×g for 20 min and suspended in 20% sucrose-NTE buffer. The virus was pelleted by centrifugation through 30% sucrose-NTE buffer at 51,000×g for 60 min and suspended again in 20% sucrose-NTE buffer. The concentrated virus thus obtained was purified by filtration through a Bio-Gel A-15m column equilibrated with 20% sucrose-NTE buffer, and by two successive sedimentations in a discontinuous sucrose gradient (35, 45 and 60%) at 165,000×g for 60 min and in a linear 30–60% sucrose gradient at 165,000×g for 180 min as described previously (10, 11).

Detergent treatment of virions. Virus from the gradients was pelleted to remove the sucrose by centrifugation through 30% sucrose-NTE buffer at 51,000×g for 60 min, and suspended in 0.01 M phosphate buffer (pH 7.2) with 10% (v/v) Triton X-100. The virus suspension was stirred for 30 min at room temperature and centrifuged at 200,000×g for 60 min. The resulting supernatants were collected and the proteins contained in these were extracted with n-butanol as described by Scheid and Choppin (1973) (12).

Isolation of glycoproteins. The proteins extracted with n-butanol were suspended in 0.01 M phosphate buffer with 1% Triton X-100 and fractionated by rate zonal sedimentation in a linear 5–25% sucrose gradient containing 1% Triton X-100 at 100,000×g for 18 h. Each fraction was collected from the top of the tube and dialyzed against 0.01 M phosphate buffer. Triton X-100 was

removed from this sample by n-butanol extraction as above. Proteins in all fractions were treated with dissociating buffer for PAGE.

SDS-PAGE. Protein samples were resuspended in 0.1 M sodium phosphate buffer (pH 7.2) containing 1% SDS, 1.7% β-mercaptoethanol and 25% glycerol, and heated for 2 min in boiling water. Electrophoresis was performed in SDS-10% polyacrylamide gels, and the resolved polypeptides were detected by staining with Coomassie brilliant blue and the carbohydrates with periodic acid-Schiff's (PAS) technique as described previously (10).

Electron microscopy. The specimens were fixed with 2.5% glutaraldehyde and negatively stained with 2% uranyl acetate. Micrographs were taken with a Hitachi HU 11 DS electron microscope.

RESULTS

Solubilization of RS virus with Triton X-100 treatment. Purified virus was solubilized with 10% Triton X-100 in 0.01 M phosphate buffer and then centrifuged at 200,000 × g for 60 min, as described under Materials and Methods. Polypeptides in the supernatant fluid as well as those in the resulting pellet were analyzed by SDS-10% PAGE (Fig. 1). In the pellet (Fig. 1b) the amount of

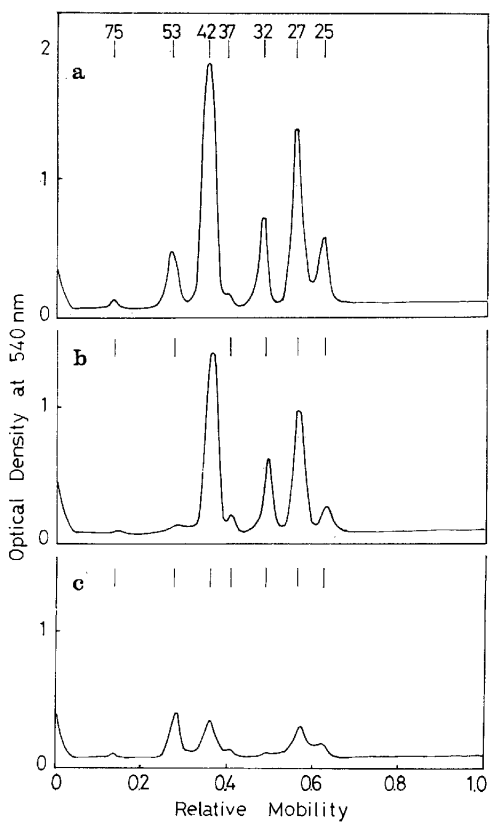

Fig. 1. Polyacrylamide gel electrophoresis of polypeptides in RS virions (a) and in the 200,000 × g pellet (b) and supernatant fluid (c) after sedimentation of solubilizing materials obtained by treatment of virions with Triton X-100. Electrophoresis of each sample was carried out on SDS-10% polyacrylamide gel as described in materials and methods, then representative gel stained with Coomassie brilliant blue was traced by a densitometer at 540 nm. The molecular weights of the polypeptides were determined by reference to the mobility of RNase, pepsin, trypsin, ovalbumin and bovine serum albumin (monomer and dimer) by the method of Weber and Osborn (13).

53,000 mol. wt. polypeptide was markedly decreased compared to that found in whole virions before the detergent-treatment (Fig. 1a), while the composition and amount of other polypeptides detected in the pellet was almost the same as those in whole virions. Proteins in the supernatant fluid (Fig. 1c) mainly comprised three polypeptides, with mol. wt. of 53,000, 42,000 and 27,000.

Separation of glycoproteins from solubilized materials of RS virus. The 200,000×g supernatant fluid of the solubilized materials obtained by treatment of RS virus with Triton X-100 was fractionated by rate zonal sedimentation and all the resulting fractions 1–10 were analyzed by SDS-10% PAGE. As seen by polypeptide banding patterns (Fig. 2) and by the densitometric pattern of the solubilized materials (Fig. 1c), the polypeptide composition of fractions 1 and 2 was

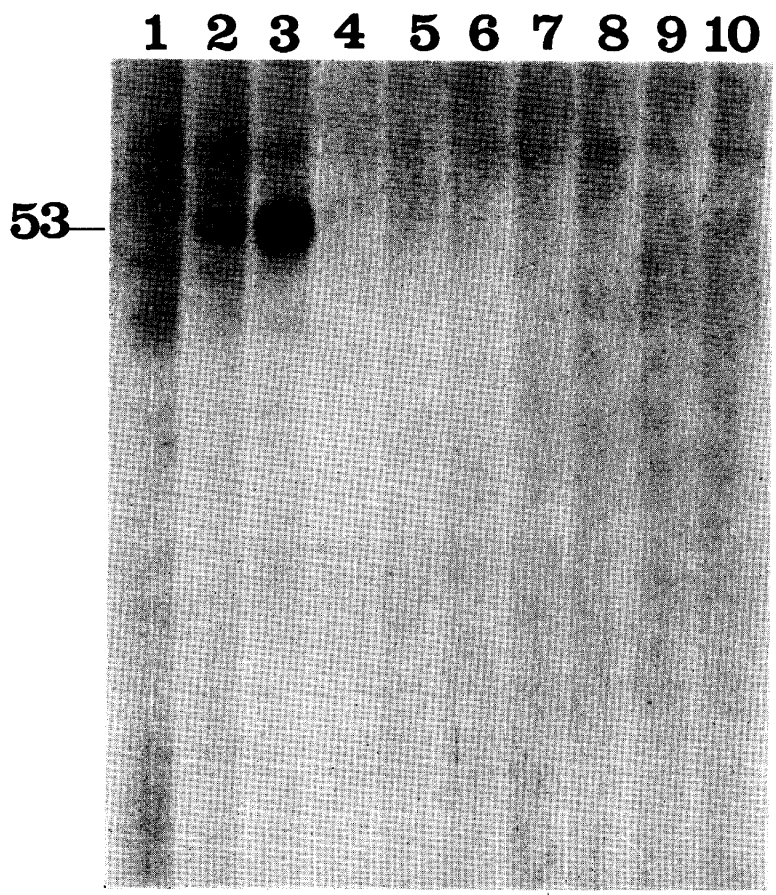

Fig. 2. Polyacrylamide gel electrophoresis of the polypeptides in all fractions obtained from rate zonal sedimentation of the 200,000×g supernatant fluid of RS virions treated with Triton X-100. The gels were stained with Coomassie brilliant blue. Mol. wt. $\times 10^3$ is shown.

similar to that of the solubilized materials with the exception that the 53,000 mol. wt. polypeptide was hardly detected in fraction 1. In fraction 3, the predominant polypeptide had a mol. wt. of 53,000. A small amount of polypeptide with a mol. wt. of 75,000 was also detected. No polypeptide was found in fraction 4. All fractions from 5 to 10 contained low levels of the 75,000 mol. wt. polypeptide.

Demonstration of two kinds of glycoproteins. Glycoproteins before and after treatment of RS virus with Triton X-100 were analyzed by SDS-5% PAGE and PAS staining. In whole virions before treatment with detergent, two peaks of PAS-positive bands were present as shown in Fig. 3b. One corresponded to the 53,000 mol. wt. polypeptide, while the other migrated more

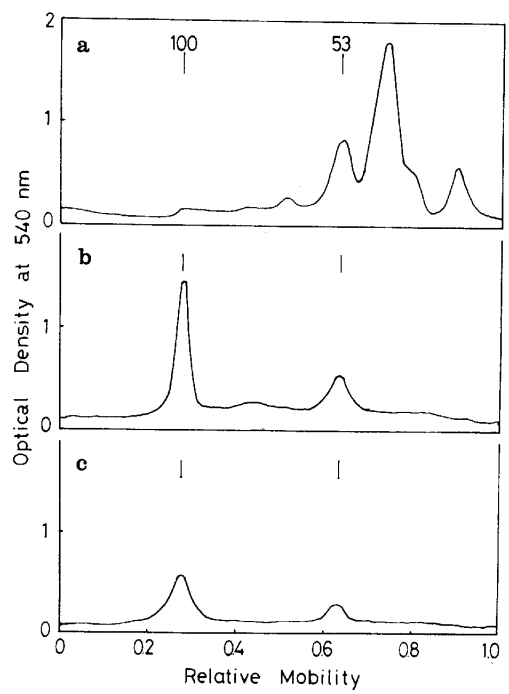

Fig. 3. Polyacrylamide gel electrophoresis of polypeptides in RS virions (a, b) and in fraction 3 (c) obtained from rate zonal sedimentation of the 200,000×g supernatant fluid of RS virions treated with Triton X-100. Gels were stained with Coomassie brilliant blue (a) and PAS technique (b, c), and were traced by densitometer at 540 nm. Mol. wt. $\times 10^3$ is shown.

slowly. The latter stained more heavily by PAS (Fig. 3b) and more lightly by Coomassie brilliant blue (Fig. 3a) than the 53,000 mol. wt. polypeptide. Its mol. wt. was estimated to be~100,000. The glycosylated polypeptide pattern of fraction 3 (Fig. 3c) was very similar to that of whole virions (Fig. 3b).

Morphology of isolated glycoproteins of RS virus in the presence of Triton X-100. Samples of fraction 3 described in the previous section were studied in the presence of the detergent using an electron microscope. As shown in Fig. 4

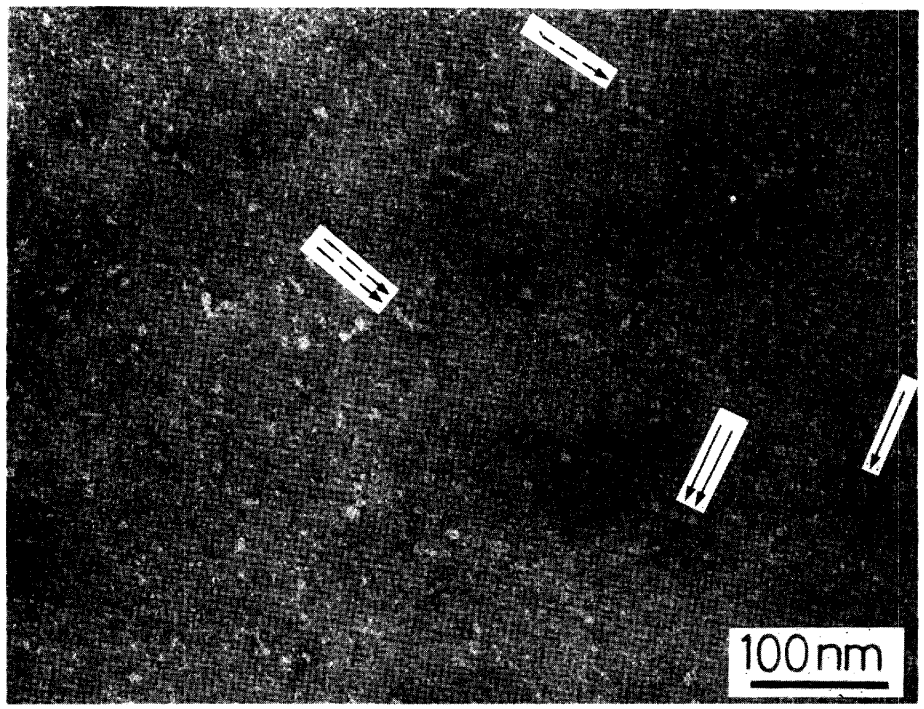

Fig. 4. Electron micrograph of glycoproteins in the presence of Triton X-100. Monomers of club-shaped ( ) and rod-shaped ( ) fine structures, and dimers composed of club-shaped ( ) and of rod-shaped ( ) ones are shown. Magnification ; ×180,000.

numerous fine structures of about 22 nm in length and with a club- or rod-shaped appearance were detected. When they showed knob-like thickness at both ends, occasionally one being a little smaller than the other, they were seen in a club-shaped or a dumbbell-shaped appearance as indicated by the single straight arrow in Fig. 4, while when knob-like thickness was not clearly seen at both ends they looked to have a rod-shaped appearance as indicated by the single broken arrow in Fig. 4. Dimers composed of two such structures were also occasionally detected (double arrow in Fig. 4). Dimers seemed to be formed by the connection of two spikes overlapping the smaller knob-like ends. The larger knob-like ends were located at the periphery of the dimers.

The morphological similarity of these fine structures to the spikes seen of the surface of virions strongly suggested that these structures were spikes isolated from the envelope of RS virus.

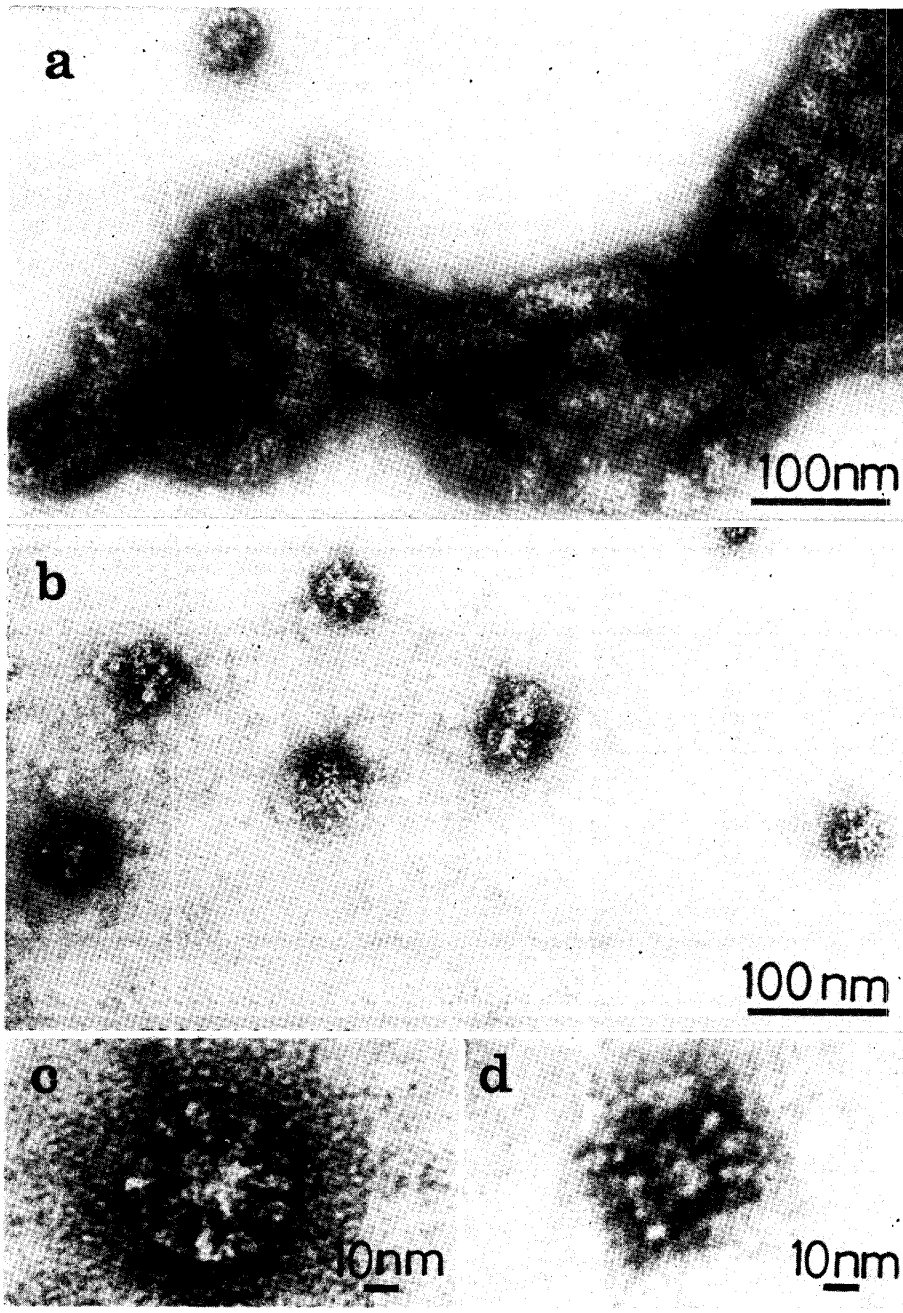

Fig. 5. Electron micrograph of glycoproteins after removal of Triton X-100. A cluster (a), polymers (b, c) and oligomers (d) composed of fine structures are observed. Magnification; a, b ×180,000, c, d ×430,000.

Morphology of isolated spikes of RS virus after removal of Triton X-100. When the detergent was removed from samples of fraction 3, spikes aggregated to form oligomers or polymers (Fig. 5b). Oligomers were composed of several dimers, which appeared to be arranged side by side at the connecting point of two spikes comprising a single dimer (Fig. 5d). Polymers were made up of more than ten spikes arranged radially or in a spherical form and had a diameter of at least 44 nm (Fig. 5c). Amorphus and enormous polymers were occasionally observed (Fig. 5a).

DISCUSSION

The solubilized fraction of enveloped RNA viruses obtained after treatment with nonionic detergent in solutions of low ionic strength is predominantly composed of glycoproteins, which are thought to be viral envelope proteins (4, 5, 12, 14–18). Our experimental results for RS virus, however, showed that detergent-solubilized viral components contained not only glycoproteins but also some non-glycosylated proteins. This suggests that RS virus is extremely fragile compared to other paramyxoviruses, a point also made by Peeples and Levine (1979) (9).

We found that the RS virion contained a large glycoprotein with a mol. wt. of ~100,000, in addition to the 53,000 mol. wt. glycoprotein previously reported (10). This large glycoprotein was more carbohydrate-rich than the 53,000 mol. wt. glycoprotein and presumably corresponds to the glycoprotein of mol. wt. 90,000, referred to by Peeples and Levine (1979), since they also reported that the glucosamine content relative to protein was much greater than that of the other major glycoprotein with mol. wt. of 49,000 (9). The difference in mol. wt. of the largest glycoprotein in their study and ours was presumably due to the different systems of SDS-PAGE used.

Wunner and Pringle (1976) were unable to separate glycoproteins from other viral proteins by centrifugation of Triton X-100 treated RS virions in a metrizamide gradient (6). Glycoproteins may have been hidden by other components contained in solubilized materials because they used partially purified virions, solubilization of RS virions in solutions of high ionic strength and no removal of non-solubilized materials by centrifugation. Recently Peeples and Levine (1979) solubilized RS virion with Triton X-100, but did not attempt separation of glycoproteins (9). We showed here that it was possible to obtain a fraction mainly composed of two glycoproteins by three successive steps, *i.e.* solubilization of purified RS virions with Triton X-100 in 0.01 M phosphate buffer, the 200,000 × g centrifugation for removal of non-solubilized materials and then rate zonal sedimentation in a linear sucrose gradient with Triton X-100 in 0.01 M phosphate buffer.

Electron microscopic observations of solubilized samples of RS virus containing two glycoproteins revealed rod- or club-shaped fine structures, suggesting that these were spikes of RS virus. It has already been shown that two glycoproteins of influenza virus (3, 21), SV5 (4) and HVJ (5) each constitute morphologically and functionally different spikes. Similarly, there is a possibility that RS virus possesses two kinds of spikes, *e.g.* club-shaped and rod-shaped ones, and the existence of two glycoproteins in the solubilized materials favours this possibility. However, we must give careful consideration to this point because such a morphological difference in spikes might merely indicate artifacts produced during electron microscopic procedures and purification.

HVJ possesses cell fusion, hemagglutination and neuraminidase activities, while RS virus has only cell fusion activity (19, 20). Whether this activity is related to any particular kind of spike needs to be investigated. The clarification of such detailed structures and of the function of spikes of RS virus is dependent on adequate isolation techniques for each glycoprotein of the virus and investigations according to this line are in progress.

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