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## Mucosal Exudation of Fibrinogen in Coronavirus-induced Common Colds

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We studied the mucosal exudation of plasma in relation to pathophysiological events during an induced common cold. Coronavirus 229E was inoculated nasally in 20 healthy volunteers under controlled conditions. Ten volunteers developed the common cold, determined by symptom scores and serology. The bulk plasma exudate was monitored, using fibrinogen (MW 340 kD) in nasal lavage fluids as an endogenous marker. Following inoculation, anterior rhinoscopy and objective registrations of nasal mucosal temperature, nasal discharge weight, and nasal blockage index by peak expiratory air flow, were followed twice daily for 6 days. Mucosal plasma exudation, as assessed by fibrinogen in lavage fluids, increased hundredfold after virus inoculation, concomitantly with the subjective symptoms and objective physiological changes. We propose that this exudation reflects the degree of subepithelial inflammation, and suggests that plasma bulk exudate, including all potent plasma protein systems may be involved in the resolution of acute viral rhinitis—common cold. *Key words: inflammation, nasal lavage, nasal mucosal temperature, peak expiratory flow, plasma exudation, respiratory tract infection, symptom scores, virus inoculation.*

### INTRODUCTION

Experimental coronavirus infections cause nasal symptoms which exhibit the general features of common colds. These include increased nasal mucosal discharge and nasal blockage along with a significant increase in nasal mucosal temperature. The changes are transient and return to pre-infection levels within a few days when the infection is aborted (1). Inoculation of coronavirus thus provides an opportunity for examining the nasal mucosal changes in the different phases of a common cold.

It is well established that proteins, such as immunoglobulins and albumin, appear at the mucosal surface during viral infections in the upper respiratory airway (2–5). However, there is still uncertainty as to whether the proteins are produced locally, or whether they are derived from plasma. The latter source has been considered of importance when the mucosa and its microcirculation have been damaged. The presence of increased amounts of the major plasma protein albumin on the mucosal surface of the airway suggests that plasma is the source of the proteins. It is not known to what extent plasma proteins larger than albumin may also traverse the mucosa along with albumin. Indeed, albumin may not always be a valid plasma tracer, since it may also be secreted by the airway mucosa (6). Although increased levels of albumin have been observed during common cold infections (1, 3–5), this information may therefore not be sufficient to establish whether or not mucosal exudation

of the plasma proteins occurs during a common cold.

The mechanisms of mucosal exudation of plasma induced by inflammation are finely regulated, and constitute an integral function of the subepithelial microcirculation and the epithelial lining (7, 8). Mucosal exudation involves a dramatic change in permeability because the large plasma macromolecules are also moved to the mucosal surface (9, 10). This process is not injurious because it leaves the ultrastructure of the mucosa intact and it does not compromise the integrity of the mucosa as an absorption barrier (11–13). The mechanism of this unidirectional outward flux of solutes involves active, mediator-induced separation of microvascular endothelial cells and the creation of increased subepithelial hydrostatic pressure as well as the suggested effects of the mechanical force on the epithelial *zonula occludens* (8).

Mucosal exudation of bulk plasma is a sensitive human airway response to inflammatory mediators (14), and a large plasma protein, such as fibrinogen, may equally, or better than albumin, reflect the mucosal exudation response in allergic inflammation of the airways (10, 15). The only known source of mucosal fibrinogen is plasma. Fibrinogen is a large molecule (MW 340 kDa) with a non-globular structure, which explains the fact that it has a high friction coefficient, 2.34. Its exudation may, therefore, represent the para-cellular movement of bulk plasma, including the circulating immunoglobulins, into the airway lumen.

In the present study we have studied the appearance of fibrinogen on the human nasal mucosa in response to nasal inoculation with coronavirus. We have thus assessed the occurrence of mucosal exudation of bulk plasma in subjects with or without symptoms of the common cold, parallel to objective measurements of nasal blockage and mucosal temperature.

## MATERIAL AND METHODS

The investigation was performed in May 1991 in accordance with the Declaration of Helsinki, after approval by the Ethics Committee at Lund University. Besides the present study other aspects of viral inflammation were studied with the same volunteers and will be reported elsewhere. All methods were chosen to avoid interference with other measurements. Volunteers were recruited by advertisements in students' newsletters at Lund University. After detailed information about the study had been given, an interview using a standardized form was carried out and supplemented with a physical examination and laboratory analyses of urine and blood. Subjects were regarded as not having allergy if they had a negative history confirmed by a negative skin-prick test (Phazett<sup>®</sup>, Pharmacia, Sweden).

Twenty healthy male volunteers 20–27 years of age (mean 24 years) were included in the study. They had no history or clinical signs of general, nasal, or allergic disease, or airway hypersensitivity, and had had no recent medication or vaccinations. The volunteers were isolated and accommodated four by four in separate two-bedroom apartments. They had strict instructions not to meet people other than their roommates at a closer range than 10 m, and not to expose themselves to potentially contagious public objects. Outdoor activities were otherwise not restricted. All meals were delivered to the apartments. Isolation was maintained for 8 days. To ensure that the volunteers were not infected at the start, they were observed during the first 2 days of isolation. During this period none of them developed symptoms or signs of a common cold. They were consequently challenged with virus by inoculation on day 0 at 9–10 p.m.

### *Virus inoculation procedure*

Human coronavirus (HCV) 229E was diluted in phosphate-buffered saline at 4°C to a concentration of 100 TCID<sub>50</sub> per ml and kept on ice until inoculated. With the volunteer in the horizontal position and the neck extended, 0.5 ml of the solution was dropped into each nasal cavity from a syringe. The volunteers were informed that, as a result of existing immunity, only few of them would become infected and develop a cold.

In order to avoid contamination during the study, the investigators in contact with the volunteers wore aprons, surgical gloves and face masks. The equipment used by the volunteers was either personal or disposable. All non-disposable equipment that came into contact with the volunteers was soaked in 70% ethanol or boiled in water for at least 2 min between applications.

### *Determination of infection*

Two methods were used to distinguish between subjects who developed a common cold and those who did not. Clinical evaluation was done by the same otolaryngologist twice daily. Each volunteer was given a daily score based on symptoms ascertained by direct questioning and by findings at physical otorhinolaryngological examinations, in a manner similar to that used in previous investigations at the Common Cold Unit, Salisbury, U.K. (16). Signs and symptoms, such as nasal discharge, nasal blockage, number of sneezes, headache, sinus pain, cervical adenitis, and hoarseness, were noted and given points (0–3) which were added to a daily score. A total score was calculated from the daily scores after inoculation. The criteria for a common cold were a total score of more than 16, and a consistent increase of the daily symptom score on the second to fifth days after inoculation. In support of the clinical evaluation, an ELISA technique was used for serological analysis of HCV 229E antibody (17). Pre-inoculation sera were compared to convalescent sera obtained 3–4 weeks after inoculation, and a fourfold increase in titer was considered a seroconversion, and an indication of an HCV 229E infection resulting from the inoculation.

### *Nasal mucosal exudation of fibrinogen*

The volunteers refrained from blowing their noses for 30 min prior to each lavage. Nasal mucosal surface liquids were collected before and 20 h after inoculation, and every following morning (at 8–9 a.m.) and evening (at 8–9 p.m.) by a nasal pool-technique (14). The nasal pool-device is a compressible plastic container equipped with a nasal adapter. The adapter was inserted into the right nostril by the subject sitting in a 60° forward flexed neck position. By compressing the container the nasal pool-fluid, 14 ml of isotonic saline, was instilled into and kept in the nasal cavity for 10 min. When the pressure was released, the fluid returned into the container and the regained volume was centrifuged ( $G = 160$  g, 10 min, 4°C). Samples for analyses were obtained from the supernatant and frozen (–20°C).

Analysis of fibrinogen (MW 340 kD) was made by a radioimmunoassay with human fibrinogen as refer-

ence (Sigma, St. Louis, MO), using rabbit anti-human fibrinogen (UCB, Brussels, Belgium) and goat anti-rabbit IgG (Draco, Lund, Sweden) for detection. The detection limit was 2 ng/ml and the intra- and inter-assay coefficients of variation were 7% and 12%, respectively (10).

#### Rhinoscopic assessment

The degrees of swelling, reddening, and rhinorrhea were estimated by anterior rhinoscopy with a four-grade scale by the same otolaryngologist twice daily. These scores were not included in the total symptom score.

#### Physical measurements

Nasal discharge was determined by weighing pre-weighed paper handkerchiefs after use. The volunteers were instructed to use only the handkerchiefs supplied, which were kept in air-tight plastic bags at all times to avoid evaporation. In the mornings the bags were collected and their post- to pre-weight difference was accredited to the previous day as the nasal discharge weight.

Nasal mucosal temperature was measured twice daily at 7 a.m. and 7 p.m. This was done by placing an electronic thermometer (ctd 85, Ellab, Rødovre, Denmark) on the mucosa of the inferior turbinate on the left side of the nose, in a manner previously described (18). Body temperature was measured sublingually by a thermometer (model 403001, Becton & Dickinson, Franklin Lakes, NJ).

Nasal peak expiratory flow (nPEF) was measured twice daily at 7 a.m. and 7 p.m. by a peak flow meter (Mini-Wright Airmed, Clement Clarke Int. Ltd., London, U.K.) in order to estimate alterations in nasal patency. The flow meter was attached to an anaesthetic mask applied over the nose and held airtight without deforming the nose. Maximal expiration was performed with the mouth closed. Oral peak expiratory flow (oPEF) was performed on the same occasions and for this measurement an appropriate mouthpiece replaced the mask. The blockage index (i.e.  $[oPEF-nPEF]/oPEF$ ) was calculated (19) and the mean value of three attempts was registered.

#### Statistics

A mean of the values obtained from the two days prior to inoculation were compared to the values from every day after inoculation by Wilcoxon's rank sum test, after an analysis of variance (ANOVA) had been found significant. Areas under the curve (AUC) after inoculation were compared between the groups by the Mann-Whitney U-test. Results are expressed as the mean  $\pm$  standard error of the mean (SEM),

except where otherwise stated.  $p$ -Values  $< 0.05$  were considered significant.

## RESULTS

Ten volunteers developed common cold as judged by the symptoms and signs. Two of them failed to show sero-conversion, but both had a progressive increase in symptom scores and high total scores (42 and 56 out of a total range in the group with colds of 21–56). Because of the convincing development of symptoms, all 10 were included in the group with the common cold. Ten individuals showed sero-conversion and 2 of these had no symptoms or signs of a common cold, and were included in the group without colds ( $n = 10$ ). Symptom scores in the group with colds peaked on day 4 (Fig. 1).

In the common cold group, the median concentration of fibrinogen was 15.8 ng/ml (interquartile range 2.77–50.3 ng/ml) before inoculation, and increased significantly on day 4 ( $p = 0.008$ ) to a peak of 1,500 ng/ml (median, 25% quartile 332, 75% quartile 1,740) on day 5 (Fig. 2). No significant changes were seen in the group without colds when comparing the values before and after inoculation. Fibrinogen concentrations after inoculation were significantly higher in the group with colds than in the group without colds ( $p = 0.006$ ).

Rhinoscopic assessment of rhinorrhea and swelling showed significantly higher scores in the common cold group as compared to the group without colds ( $p = 0.02$  and 0.04, respectively), but the scores for

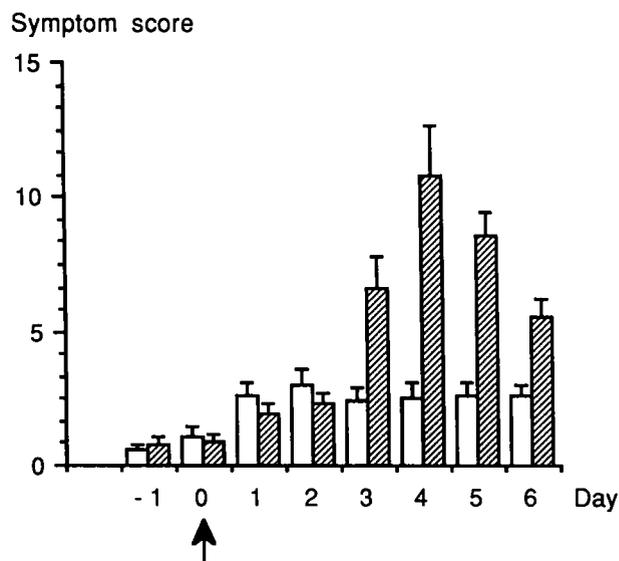


Fig. 1. Development of symptom scores during the study. The scores identified volunteers with (hatched columns,  $n = 10$ ) and without (open columns,  $n = 10$ ) common cold. Arrow indicates virus inoculation.

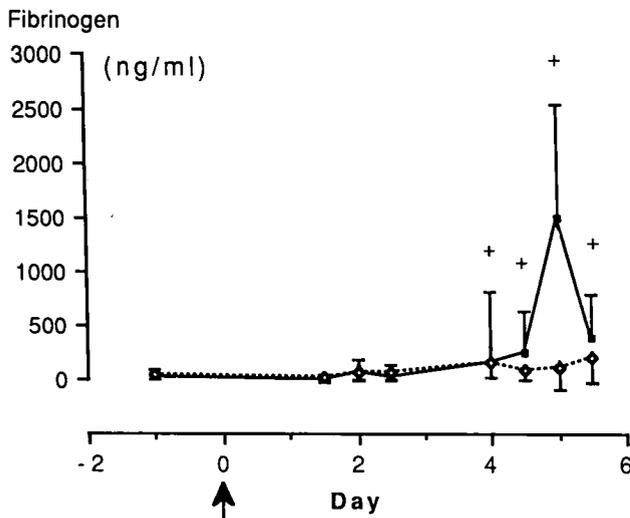


Fig. 2. Median fibrinogen concentrations in nasal lavages as an index of mucosal exudation of bulk plasma in groups with (full lines,  $n = 10$ ) and without (dotted lines,  $n = 10$ ) common cold. Only subjects with common colds exhibited a significant increase compared to control values prior to inoculation ( $+p < 0.01$ ). There was a difference between the groups ( $p = 0.006$ ). Arrow indicates virus inoculation. Error bars show quartile deviation.

redness were not significantly different between the groups. In the common cold group, scores for all rhinoscopic characteristics assessed increased significantly on day 3.

Nasal discharge weight increased significantly on day 2 after inoculation in the group with common colds ( $p = 0.02$ ), and the increase was sustained throughout the study. The weight was increased also on day 3 in the common cold group, but since the lavage protocol differed on this day, discharge weights are not reported (Fig. 3). There were significant differences between the groups after inoculation ( $p = 0.002$ ). No significant change in the weight of nasal discharge was seen in the group without colds.

Nasal mucosal temperature of all volunteers was  $30.2^\circ \pm 0.30^\circ\text{C}$  prior to inoculation. From the first day after inoculation there was a significant increase in nasal mucosal temperature in the common cold group ( $p = 0.03$ ). The highest mean value was seen on day 4 ( $35.0^\circ \pm 0.43^\circ\text{C}$ ), a  $5.1^\circ\text{C}$  increase compared to the temperature prior to inoculation ( $29.9^\circ \pm 0.60^\circ\text{C}$ ) in this group (Fig. 4a). In the group without colds, an ANOVA for repeated measurements was not significant ( $p = 0.13$ ). The difference between the groups after inoculation was significant ( $p = 0.02$ ). The sublingual temperature was  $36.3^\circ \pm 0.12^\circ\text{C}$  prior to inoculation, and no significant differences were found between the groups after inoculation. In both groups morning temperatures

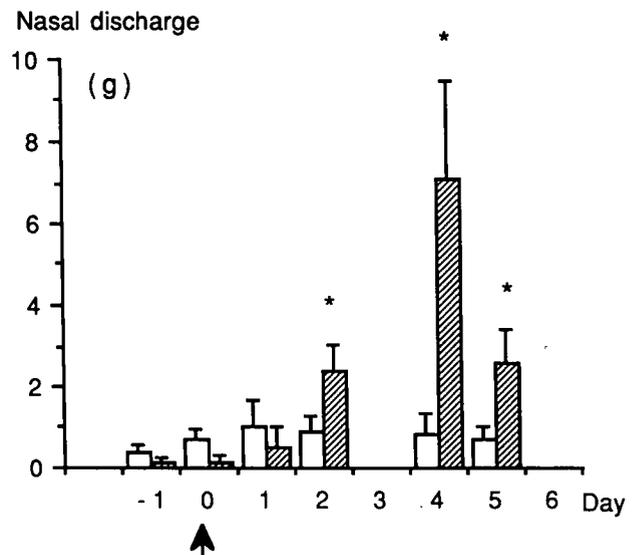


Fig. 3. Nasal discharge measured by daily weight of handkerchiefs in groups with (hatched columns,  $n = 10$ ) and without (open columns,  $n = 10$ ) common colds. Only subjects with common colds exhibited a significant increase compared to control values prior to inoculation ( $*p < 0.05$ ). There was a difference between the groups ( $p = 0.002$ ). Arrow indicates virus inoculation. Weights on day 3 are not shown.

were significantly lower than evening values. The highest single temperature recorded,  $37.8^\circ\text{C}$ , was in the group with colds.

Nasal peak expiratory flow, expressed as the blockage index, was significantly higher on the morning of day 4 in the group with common colds, as compared with pre-inoculation values ( $p = 0.03$ ) (Fig. 4b). In the group without colds, morning blockage indices were reduced as from day 2 ( $p = 0.04$ ). When comparing the groups, significantly higher blockage indices were found in the group with common colds ( $p = 0.05$ ). No difference in oral peak expiratory flow were seen between the groups.

## DISCUSSION

This study demonstrated markedly increased fibrinogen values in nasal lavage liquids during coronavirus-induced common colds. Since the only known source of this fibrinogen is the profuse subepithelial microcirculation of the nasal airways, our data suggest that coronavirus-infected subjects exude bulk plasma across the airway mucosa. The virus inoculation dose was chosen, on the basis of experience from the Common Cold Unit, Salisbury, U.K., to simulate naturally-acquired colds. The clinical infection rate (50%) corresponds well to that found in a previous investigation (1). Some individuals are expected to seroconvert without presenting symptoms of common

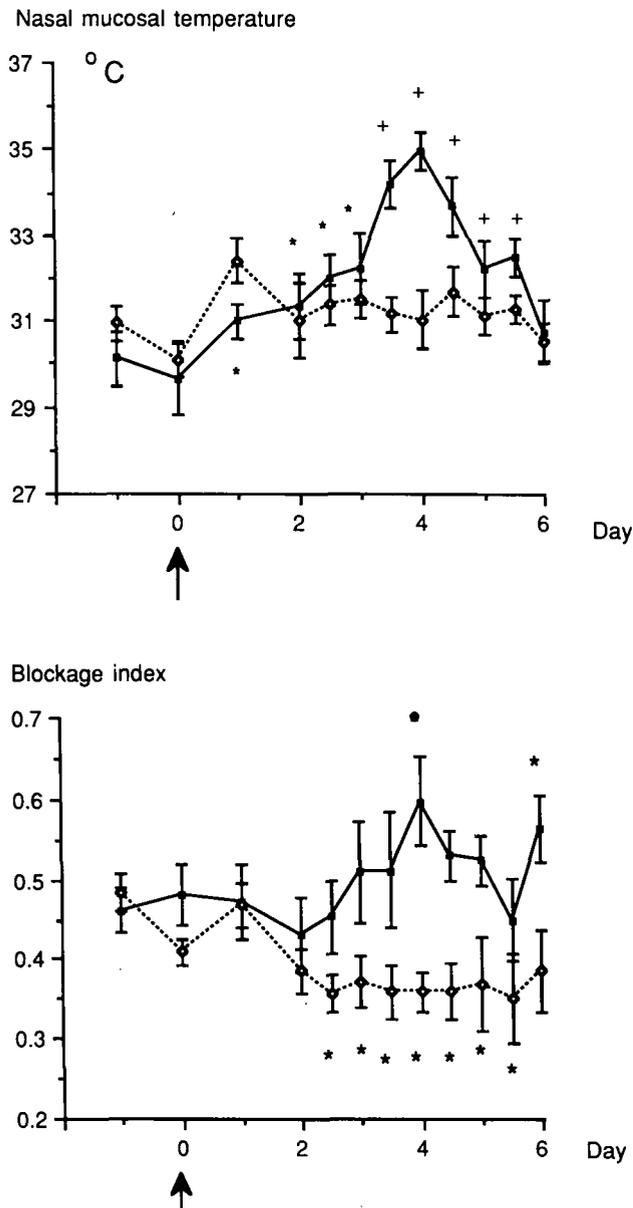


Fig. 4. Nasal mucosal temperature (a) and nasal blockage (b) indicated as blockage index calculated from nasal and oral PEF measurements in groups with (full lines,  $n = 10$ ) and without (dotted lines,  $n = 10$ ) common cold. Comparisons within the groups were made to control values prior to inoculation (\* $p < 0.05$ , + $p < 0.01$ ). There were significant differences between the groups for mucosal temperature ( $p = 0.02$ ) and blockage index ( $p = 0.05$ ). Arrows indicate virus inoculation.

cold but virtually all individuals with colds are expected to seroconvert. However, in this study the actual serological results did not fully meet the expected serological results. Still, in accordance with the purpose of the study, the group affiliation was decided from the presence or absence of common cold symptoms, and serology was not decisive. The subjective symptom scores and the objectively-measured

physical changes, including the increase in mucosal temperature, peaked 4–5 days after virus inoculation in the group with the common cold, which confirmed and extended the findings of the previous study (1).

Mucosal exudation of plasma was not increased in either group of volunteers in the non-symptomatic period of the first 2 days after inoculation in this study. Hence, an exudative response may not occur in response to the mere presence of a potentially infectious dose of coronavirus on the nasal mucosa. Mucosal exudation of bulk plasma was instead induced concomitantly with the development of symptoms of active infection.

The nasal discharge volume (weight) in infected subjects increased as early as on day 2 when the fibrinogen levels were still low in the nasal lavage liquids. Mucosal secretions may thus be induced earlier than a plasma exudation response. In contrast to the volume of the nasal discharge, the fibrinogen levels were still high on day 5. Assuming that the fibrinogen exudation would correspond to a volume of exuded undiluted plasma, this volume would be in the order of  $10 \mu\text{l}$  (calculated from a fibrinogen concentration of approximately  $1,500 \text{ ng/ml}$  in lavage and  $3 \text{ mg/ml}$  in plasma). However, it is not possible to know how much liquid was attracted and moved by the mucosal exudation process. The extravasated plasma should rapidly generate an abundance of peptide molecules. These include potent mediators, such as kinins (5, 20). The accumulation of solutes should attract fluid by osmotic forces. Hence, the volume of the plasma exudate may be far greater than  $10 \mu\text{l}$ . The discordance between the total nasal discharge volume and the mucosal exudation of fibrinogen suggests that the plasma exudate was not a major source of the nasal discharge in this study. Mucosal secretion is a non-specific response which can be produced by inflammatory agents, and by neural irritants. In contrast, plasma exudation may be induced only by factors that are inflammatory by nature (21, 22). These stimuli include mediators (histamine and leukotriene  $D_4$ , but not methacholine), allergens, occupational agents and, as in this study, infectious agents.

The present data comply with the notion that airway luminal entry of plasma exudates reflects the intensity and time-course of subepithelial inflammatory processes (10, 15). Symptoms and mucosal temperature were elevated between days 3 and 6, when the significant mucosal exudation of plasma also occurred (fibrinogen concentrations were also obtained on day 3 but are not reported here since the samples were not obtained according to the present protocol). The time-course of mucosal exudation of plasma is, moreover compatible with the possibility that plasma-

derived factors may have contributed to the resolution of the infection. The appearance of fibrinogen on the mucosal surface suggests that non-sieved plasma was being exuded. Hence, all potent protein systems of plasma, including immunoglobulins, kinin, complement, coagulation, and fibrinolysis systems, would be exuded and potentially contribute to defence reactions in the lamina propria and on the mucosal surface. Interestingly, both kinin generation (5) and complement activation (23) have been demonstrated during experimental virus infections of the upper airway. The origin of the bradykinins and complement fragments have not been fully determined. The present data suggest that exuded plasma could have been the source of these potent peptides.

Fibrinogen is not only a marker of plasma exudation. By its transformation into fibrin it also becomes an effector protein that may contribute to defence, repair and inflammation. Fibrin and fibrin-fibronectin gels may be formed in airways where the epithelial lining has been damaged. The fibrin component may, through its adhesive receptors for white cells, act as an extracellular matrix ladder for inflammatory cell traffic and also provide a matrix for re-epithelialisation and re-vascularisation of the airway mucosa (24). Fibrinolytic peptides may be generated to participate in inflammatory and defence processes. However, it has not been conclusively shown that mucosal damage occurred in this study where the infection and the symptoms lasted for only a few days. We did not examine the ultrastructure of the mucosa, but in a separate investigation we demonstrated that the present coronavirus infection was not associated with a significant increase in the mucosal absorption ability (unpublished observations). This would suggest that integrity of the epithelial barrier was not severely damaged.

In conclusion, this study has shown that by employing coronavirus inoculation in the upper airway a range of nasal symptoms and increased mucosal temperature have been seen in infected subjects. Along with these signs, mucosal exudation of fibrinogen increased about hundredfold. The fibrinogen data may reflect bulk exudation of plasma and may also reflect the degree of virus-induced subepithelial inflammation. It is suggested that fibrinogen, along with the other potent proteins systems of plasma, may contribute to the resolution of common cold infections.

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