**INTRODUCTION**

In previous papers we demonstrated that the influenza virus infection A/Aichi/2/68 (H3N2) is associated with graduated oxidative disturbances in the lung and liver as well as the blood serum as well, and cytochrome changes and decreased activity of liver cytochrome P450-dependent monooxygenases (1-3). Different conditions can favor the susceptibility of the host to virus infection, and among them cold exposure and stressors of physical, chemical and psychological origin are well-established (4-6). At the same time, cold exposure and/or immobilization stress are known as pro-ulcerogenic factors. As the lungs are generally considered the main target organ in influenza virus replication (7), and liver oxidative and metabolic disorders provoked by hepatoxic mediators (2, 6), little is known about the effects of influenza on gastric mucosal integrity and gastric damages. No data concerning gastric effects of the combined exposure to cold and/or immobilization stress and influenza infection are available.

In the present study we investigated the effects of influenza virus A/Aichi/2/68 (H3N2) on mouse gastric mucosal integrity and the possible alteration in gastric lipid peroxidation induced by either influenza virus infection alone or in combination with cold and/or immobilization stress exposure.

**MATERIALS AND METHODS**

Albino male mice, line ICR (14-16 g), were used. The animals were fed a standard diet and fasted for 24 h before the study. The mice were divided into 8 groups, as follows: i) group I (n = 10): control group (healthy animals); ii) group II (n = 10): mice subjected to immobilization stress in stereotactic frames for 4 h; iii) group III (n = 10): mice subjected to cold stress in refrigeratory camera for 4 h at 4 ºC; iv) group IV (n = 10): mice subjected to cold-restraint stress (immobilized in stereotactic frames and subjected to cold stress in refrigeratory camera for 4 h, at 4 ºC); v) group V (n = 10): mice infected with influenza virus A/Aichi/2/68 (0.5 of LD50) by intranasal inoculation; vi) group VI (n = 16): mice subjected to immobilization stress for 4 h and then infected with influenza virus A (0.5 of LD50); vii) group VII (n = 16): mice subjected to cold stress for 4 h at 4 ºC and then infected with influenza virus A (0.5 of LD50); and viii) group VIII (n = 16): mice infected with influenza virus A/Aichi/2/68 (H3N2) (0.5 of LD50) by intranasal inoculation, and then subjected to immobilization stress for 4 h.
models of oxidative stress. It was found that immobilization, cold and cold-restraint stress didn’t influence the endogenous levels of MDA and fluorescent lipofuscin-like products in the stomachs of mice that were not infected with influenza virus. Inoculation of mice with influenza virus A/Aichi/2/68(H3N2) resulted in a

\[ n = 16 \]: mice subjected to cold-restraint stress and then infected with influenza virus A (0.5 of LD50).

The animals were sacrificed by rapid decapitation and exsanguination on the 5th day after virus inoculation. The stomach was removed immediately, gently washed in ice-cold physiological salt solution, spread over the pad and observed macroscopically for appearance of mucosal lesions. The length of each lesion was measured. In case of petechia, 5 of them were considered as 1-mm lesions. The mean ulcer area (mm²) was calculated.

Gastric (mucosal) tissue was homogenized at 4 °C in 0.1 M K, Na-phosphate buffer, pH 7.4, 1:3 (w:v). Total lipids were extracted from tissue homogenates using a chloroform-methanol mixture (2:1, v:v) according to the method of Folch et al. (9). Fluorescent lipofuscin-like products were measured fluorimetrically at \( \lambda_{\text{ex}} = 360 \text{ nm} \) and \( \lambda_{\text{em}} = 420 \text{ nm} \). Endogenous free MDA was assayed by HPLC method as described in (10).

Influenza virus A/Aichi/2/68 (H3N2) was obtained from the collection of the D.I. Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow. All reagents of analytical grade were obtained from Aldrich Chem. Co., Henkel Co., Merck, Sigma Chem. Co.

Data were analyzed statistically by one-way analysis of variance (ANOVA) and expressed as mean ± SEM. A value of \( p < 0.05 \) was considered statistically significant. The statistical procedure was performed with GraphPad InStat software.

The experimental procedure was approved by the Home Office for Care and Use of Laboratory Animals and performed in a considerate and humane manner.

RESULTS

Index of ulceration

Macroscopic observation showed that immobilization, cold-restraint stress and virus infection induced gastric mucosal lesions filled with a brown-colored hematin material, varying in number and size and slightly fixed to the liable tissue (Fig. 1). It was established that the gastric mucosa lesion in the influenza virus infected mice was about 6 mm² and increased in animals subjected to combined application of influenza virus infection and cold-restraint stress (\( p < 0.001 \) vs. group I; \( p < 0.05 \) vs. group V). Combination of immobilization and virus inoculation did not influence ulceration compared with infected animals.

Levels of lipid peroxidation products

Figure 2 and Figure 3 illustrate the endogenous levels of fluorescent products and MDA in the stomach homogenates isolated from mice subjected to different

\[ \text{FIG. 1. Effect of immobilization, cold and cold-restraint stress on the index of ulceration in the stomach of mice before and after influenza virus inoculation. I: control; II: immobilization; III: cold stress; IV: cold-restraint stress; V: influenza virus infection; VI: immobilization + influenza virus infection; VII: cold stress + influenza virus infection; VIII: cold-restraint stress + influenza virus infection. } \]

\[ \text{FIG. 2. Effect of immobilization, cold and cold-restraint stress on the level of fluorescent products of lipid peroxidation in the stomach of mice before and after influenza virus inoculation. I: control; II: immobilization; III: cold stress; IV: cold-restraint stress; V: influenza virus infection; VI: immobilization + influenza virus infection; VII: cold stress + influenza virus infection; VIII: cold-restraint stress + influenza virus infection. } \]

\[ \text{FIG. 3. Effect of immobilization, cold and cold-restraint stress on the level of fluorescent lipofuscin-like products in the stomach of mice before and after influenza virus inoculation. I: control; II: immobilization; III: cold stress; IV: cold-restraint stress; V: influenza virus infection; VI: immobilization + influenza virus infection; VII: cold stress + influenza virus infection; VIII: cold-restraint stress + influenza virus infection. } \]
significant increase of both MDA and fluorescent lipofuscin-like products, by 30% and 40%, respectively, compared with animals who were not infected. Combined application of immobilization stress and influenza virus infection led to a significant increase in lipid peroxidation products compared with control animals and didn’t significantly change lipid peroxidation products compared with infected non-immobilized animals. Similar results were observed after combined application of cold stress and influenza virus infection. In this case, lipid peroxidation products were increased significantly compared with control non-infected animals. In mice subjected to cold-restraint stress and influenza virus infection the level of fluorescent products and MDA was also significantly higher compared with infected and non-stressed animals. In this case, fluorescent lipofuscin-like products increased approximately three-fold compared with control animals, and approximately two-fold compared with influenza virus inoculated and non-stressed animals; MDA increased approximately two-fold compared with control animals and approximately 1.5-fold compared with virus infected and non-stressed animals.

**DISCUSSION**

The results of the present study showed that influenza virus A/Aichi/2/68 (H3N2) challenge altered the gastric mucosal integrity and that the gastric mucosa represents a target for influenza virus infection. Mucosal damage evoked by influenza virus was more pronounced than that provoked by either cold exposition or restraint stress or their combination. Moreover, gastric damage after any of the latter pro-ulcerogenic condition(s) was aggravated by influenza virus infection. The observed alteration in gastric mucosal integrity was accompanied by significant changes in the levels of either fluorescent lipofuscin-like products of lipid peroxidation or of free MDA, strongly suggesting a relationship between lesion induction and oxidative stress development.

It is widely accepted that the pathogenesis of gastric mucosal ulceration is multifactorial and mucosal ischemia is thought to be a major causative factor implicated in cold and/or restraint stress-ulcer (11). The focal ischemic areas, which subsequently develop into erosions, and inadequate perfusion, may disturb cell metabolism with local release of tissue-damaging mediators such as oxygen-derived free radicals. A close relationship between stress-evoked gastric mucosal lesions and extensive lipid peroxidation in gastric tissue evidenced by the accumulation of MDA and depletion of glutathione levels has been already established (12-14).

Several studies have suggested that an overreaction of the host’s immune system is involved in free radical generation under influenza virus infection (6, 8, 15-17). Neutrophils and macrophages are known to produce superoxide free radicals and hydrogen peroxide, which are normally involved in the killing of microorganisms (5, 6, 8, 15, 16).

Though the effects of influenza virus infection on gastric mucosal integrity have been poorly investigated, it is known that influenza virus alters ingestive behavior, resulting in anorexia and loss of body weight (18, 19). This effect could be at least partly explained by the ability of influenza virus infection to directly damage gastric mucosa, as shown in this study, as well as to aggravate gastric injury evoked by infection predisposing factors such as cold and/or stress. The suppression of stomach antioxidant defense system before influenza virus inoculation resulted in an acceleration of oxidative stress and graduated damages. The activation of free radical processes in the stomach was followed by a compensatory enhancement in the activities of antioxidant enzymes and a decrease in the content of non-enzymic antioxidants (12, 14).

The finding that influenza virus-induced gastric mucosal injury is accompanied by extensive lipid peroxidation in gastric tissue may help the understanding of the viral pathogenesis and may provide a rational basis for the protection of target tissues against influenza toxicity through downregulation of oxidative stress development.

In conclusion, the present study provides direct experimental proof that combination of acute influenza virus infection with different models of oxidative stress, such as immobilization, cold and cold-restraint stress, is associated with marked oxidative disorders in the gastric mucosa despite the apparent absence of virus in this...
tissue. Although the gastric mucosa is not a site of virus replication, this organ has been shown to be influenced to a great extent in influenza virus infection.

REFERENCES

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