Inhalation of a steam-mixed gas containing hydrogen gas enhances the salivary titers of immunoglobulin A against pathogenic hemagglutinin antigens derived from influenza virus strains in healthy volunteers

Nobunao IKEWAKI, Tohru SONODA*, Kazuhiro AZUMA**

Abstract

In a previous study, we demonstrated a significant increase in the salivary interleukin-1β (IL-1β) levels following the administration, via inhalation, of a steam-mixed gas containing hydrogen gas (designated as XEN) in healthy volunteers. In this study, we examined the salivary titers of immunoglobulin A2 (IgA2) directed against pathogenic hemagglutinin (HA) antigens derived from influenza virus strains (hereinafter, shortened to IgA2) after XEN administration by inhalation via a nasal cannula for 15 min in healthy volunteers. The salivary titers of IgA2 directed against HA antigens were measured using an originally constructed enzyme immunoassay system. The results indicated significant increases of the salivary IgA2 directed against the HA antigens derived from the following influenza virus strains after 15 min of XEN inhalation, as compared to the titers recorded prior to the inhalation: A/California/7/2009/H1N1 ($P = 0.028$), A/Hong Kong/4801/2014/H3N2 ($P = 0.047$), vaccine-type containing HA antigens derived from influenza virus strains, A/Singapore/GP1908/2015/H1N1, A/Hong Kong/4801/2014/H3N2, B/Texas/2/2013/Victoria system, and B/Phuket/1307/2013 /Yamagata system ($P = 0.039$). Taken together, XEN rapidly enhanced the salivary titers of IgA2 directed against HA antigens derived from influenza viruses, which may imply a role in the protection against influenza virus infection by activation of humoral immunity (IgA2 action) in the whole body via the nasal mucosal immune system.

Key words : enzyme immunoassay, hemagglutinin (HA) antigen, salivary IgA2, steam-mixed gas containing hydrogen gas, XEN

Introduction

Free radicals (reactive oxygen species) are well-known to be involved in the development of various kinds of diseases, and it has been suggested that anti-oxidant therapy with molecular hydrogen (H_2) against free radicals may be useful for preventing diseases[1]. Many studies in the basic and clinical research fields have examined the usefulness of H_2 as an anti-oxidant agent, through administration of H_2-containing water, intravenous drip infusion of H_2-rich saline, or administration by inhalation of 2%-4% H_2 gas[2, 3]. In particular, the effect of H_2 gas as an anti-oxidant agent for many kinds of diseases has been examined, and H_2 gas is now acknowledged as a potentially strongly effective agent for the treatment of these diseases[4].

In a previous study, we demonstrated rapid and significant increases of the salivary interleukin-1β (IL-1β) levels in healthy volunteers following administration by inhalation of a steam-mixed gas containing hydrogen gas (designated as XEN), generated by a machine (Suisonia) by decomposition of superheated steam; our

Department of Medical Life Science, Kyushu University of Health and Welfare School of Medical Life Science, Institute of Immunology, Junsei Educational Institution, 1714-1 Yoshino-machi, Nobeoka-city, Miyazaki 882-8508, Japan
*Department of Occupational Therapy, Kyushu University of Health and Welfare School of Health Science, Institute of Immunology, Junsei Educational Institution, 1714-1 Yoshino-machi, Nobeoka-city, Miyazaki 882-8508, Japan
**Azuma Clinic of Internal Medicine, 3-8-8 Kamihirano-cho, Nichinan-city, Miyazaki 887-0022, Japan

finding strongly indicated that XEN has immunological effects[5]. However, the effects of XEN inhalation on the production levels of salivary immunoglobulin A2 (IgA2), which is well-known to be resistant to and stable against a variety of proteolytic enzymes[6], against pathogenic antigens, such as hemagglutinin (HA) antigens derived from influenza virus strains, have not yet been investigated.

The aim of the present study was to analyze the effect of XEN inhalation on the salivary IgA2 response directed against HA antigens derived from influenza virus strains in healthy volunteers, and also discuss the potential effects, at the immunological level, of XEN inhalation in the living body.

Materials and Methods

Reagents

Purified hemagglutinin (HA) antigen solutions (HA titer: 80-fold) derived from formalin-fixed influenza A virus strains, namely, A/California/7/2009/H1N1, A/Hong Kong/4801/2014/H3N2 and vaccine-type containing mixed HA antigens derived from influenza virus strains, A/Singapore/GP1908/2015/H1N1, A/Hong Kong/4801/2014/H3N2, B/Texas/2/2013/Victoria system, and B/Phuket/1307/2013 /Yamagata system (containing HA antigen 30 μg) were purchased from Denka Seiken Co., Ltd. (Niigata). Rabbit anti-influenza A and B virus HA antigen-specific polyclonal IgG antibody was purchased from Takara Bio Co., Ltd. (Tokyo). Horseradish peroxidase (HRPOD)-conjugated mouse anti-human IgA2 monoclonal antibody (mAb) and HRPOD-conjugated goat anti-rabbit IgG antibody were purchased from MBL Co., Ltd. (Nagoya).

Ethics statement

The study protocol was approved by the institutional review board (IRB) of Kyushu University of Health and Welfare (IRB number 15-058). Informed consent was obtained from each of the volunteers prior to their participation in this study.

Steam-mixed gas containing H_2 gas generator machine

A machine (Suisonia, FRJ-003) developed by Earth Engineering Co., Ltd. (Kitakyushu), that decomposes superheated steam to produce a steam-mixed gas containing H_2 gas was used in this study. Steam-mixed gas containing H_2 gas is named XEN at our laboratory[5].

Volunteers and saliva collection

Seventeen healthy adult volunteers without any abnormalities of the oral cavity (8 males, age 47.6 ± 19.9 yr.; 9 females, age 39.1 ± 8.5 yr.) were enrolled for this study. Prior to the administration of XEN by inhalation, the subjects were asked to gargle and rinse their mouth (oral cavity) 15 times with water. About 40 min later, they were seated on a chair, and XEN was administered by the inhaled route via a nasal cannula for 15 min. Thereafter, saliva samples were collected from the 17 volunteers. Briefly, saliva specimens were collected in 15-mL sterile tubes for two minutes. All the samples were then centrifuged at 3,000 rpm for 30 min, and the supernatants were harvested and stored at -80°C until assay.

Measurement of salivary IgA2 directed against HA antigens

EIA plates (Sumitomo Co., Ltd., Tokyo) were coated with hemagglutinin (HA) antigen solutions derived from influenza virus strains at x20 dilution in carbonate-bicarbonate buffer (0.01 M $NaCO_3$, 0.035 M $NaHCO_3$, pH9.6) for 24 hrs at 4°C. The wells were washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blocked with PBST containing 2% BSA (BSA-PBST) for 60 min at room temperature. Thereafter, the wells were washed three times with PBST. Confirmation of HA antigen fixation to the wells of the EIA plates was performed by a binding assay using a rabbit anti-influenza A and B virus HA antigen-specific polyclonal IgG antibody. The saliva specimens (x8 dilution with 0.1% BSA-PBST; 50 μL) were then added to each well, followed by incubation for 60 min at room temperature

with shaking. The wells were washed five times with PBST, followed by addition of HRPOD-conjugated mouse anti-human IgA2 mAb (x3,000 dilution with 0.1% BSA-PBST; 50 μL) to each well and incubation of the wells for 60 min at room temperature with shaking. The wells were then washed 10 times with PBST, followed by addition of the substrate-chromogen (TMB; Cosmo Bio Co., Ltd.; 50 μL) to each well and incubation for 20 min at room temperature with gentle shaking. The reaction was stopped by the addition of 0.5 M HCl (50 μL), and the optical density (O.D.) was measured at 450 nm using a multichannel EIA-microplate reader (TOSHO Co., Ltd.).

Statistical analysis

Data were analyzed by Wilcoxon's paired *t*-test. Differences at $P < 0.05$ were considered to be statistically significant.

Results and Discussion

In this study, we examined the salivary titers of IgA2 directed against HA antigens derived from influenza A virus strains after XEN inhalation by an originally constructed EIA system in the 17 healthy volunteers. A significant and rapid increase of the salivary titers of IgA2 directed against HA antigens derived from A/California/7/2009/H1N1 and A/Hong Kong/4801/2014/H3N2 was observed as compared to the titers measured at the baseline ($P = 0.028$ and $P = 0.047$, respectively) following XEN inhalation (Figs. 1 and 2). Furthermore, the salivary titers of IgA2 directed against mixed HA antigens (vaccine-type) derived from A/Singapore/GP1908/2015/H1N1, A/Hong Kong/4801/2014/H3N2, B/Texas/2/2013/Victoria system, and B/Phuket/1307/2013/Yamagata system also increased rapidly and significantly as compared to the titers measured at the baseline ($P = 0.039$) (Fig. 3). The mean percent increases of the salivary titers of IgA2 directed against the HA antigens after XEN inhalation were 43.4% for the HA antigen derived from A/California/7/2009/H1N1, 28.0% for the HA antigen derived from A/Hong Kong/4801/2014/H3N2, and 63.9% for the vaccine-type HA antigens derived from A/Singapore/GP1908/2015/H1N1, A/Hong Kong /4801/2014/H3N2, B/Texas/2/2013 /Victoria system, and B/Phuket/1307/2013 /Yamagata system (Fig. 4). On the other hand, no significant changes of the salivary IgA2 titers were observed in the absence of XEN inhalation (data not shown).

It is common knowledge that IgA antibodies, which occur in monomeric and dimeric forms, play a crucial role in mucosal immunity[7]. The amount of IgA, which accounts for up to 15% of the total immunoglobulins, produced in mucosal membranes is greater than that of the other types of antibodies. Two subclasses of IgA are recognized (IgA1 and IgA2); IgA1 occurs predominantly in the serum (>80%), while IgA2 is found mainly in maternal milk, tears, saliva[8] and secretions from the gastrointestinal tract, prostate and respiratory epithelium. The dimeric forms of IgA1 or IgA2 bound by a joint protein (J-chain) and secretory component (SC) are called secretory IgA1 (sIgA1) and secretory IgA2 (sIgA2), respectively. In regard to the biological and structural properties, as IgA2, as compared to IgA1, is more resistant to and stable against a variety of proteolytic enzymes produced by microbes in the living body, IgA2 plays key roles in protection against many microbes in the living body[6]. Based on the above, in order to analyze the immunological IgA responses in the saliva, we selected an originally constructed EIA system for the detection of IgA2 in the saliva in this study. We found that the salivary titers of IgA2 directed against pathogenic HA antigens derived from influenza viruses clearly increased following XEN inhalation.

The precise mechanisms underlying the increase of the salivary titers of IgA2 directed against influenza-virus-derived HA antigens after XEN inhalation remain unclear at present. However, we speculate on some possible mechanisms, as follows. XEN administered by inhalation via a nasal cannula induces activation of the nasal and oral mucosal immune systems, including in the mucosa-associated lymphoid tissues (MALTs)[9] as immune-related organizations, resulting in increase in the salivary IgA2 levels. Alternatively, the increased salivary IgA2 titers may reflect activation of the immune system in the whole living body as protection

against influenza virus infection. These findings suggest the strong potential of XEN for development as a novel type of nasal mucosal vaccine for clinical application in the future[10].

In conclusion, we report, for the first time, that inhalation of XEN, a steam-mixed gas containing H_2 gas, rapidly increased the salivary IgA2 response to influenza-virus-derived HA antigens.

Disclosure

No authors have any conflict of interest.

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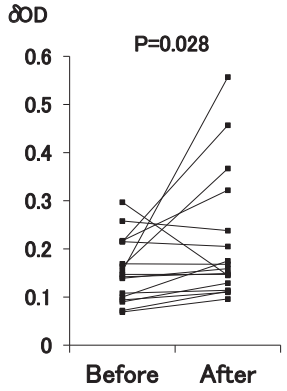

Figure 1. Detection of salivary IgA2 directed against HA antigens derived from the A/California/7/2009/H1N1 influenza virus strain.

Detection of IgA2 in salivary samples (n = 17) directed against HA antigens derived from the A/California/7/2009/H1N1 strain was performed using an originally constructed EIA system (See Materials and Methods for details). The measurements were repeated five times. P = 0.028 (before inhalation vs. after inhalation).

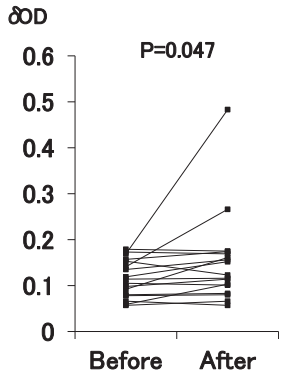

Figure 2. Detection of salivary IgA2 directed against HA antigens derived from the A/Hong Kong/4801/2014/H3N2 influenza virus strain.

Detection of IgA2 in salivary samples (n = 17) directed against HA antigens derived from the A/Hong Kong/4801/2014/H3N2 strain was performed using an originally constructed EIA system (See Materials and Methods for details). The measurements were repeated five times. P = 0.047 (before inhalation vs. after inhalation).

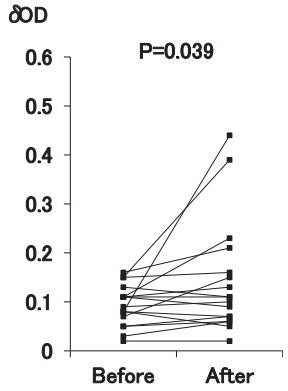

Figure 3. Detection of salivary IgA2 directed against mixed HA antigens (vaccine-type).

Detection of IgA2 in salivary samples (n = 17) directed against mixed HA antigens (vaccine-type), consisting of the A/Singapore/GP1908/2015/H1N1, A/Hong Kong/4801/2014/H3N2, B/Texas/2/2013/Victoria system, and B/Phuket/1307/2013 / Yamagata system strains, was performed using an originally constructed EIA system (See Materials and Methods for details). The measurements were repeated five times.
P = 0.039 (before inhalation vs. after inhalation).

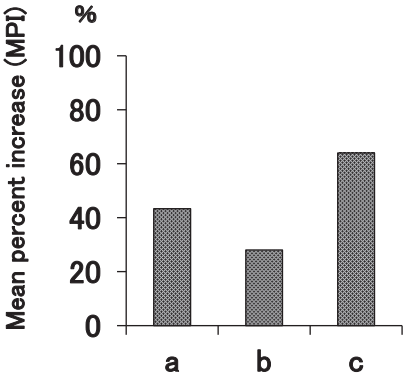

Figure 4. Mean percent increases of the salivary titers of IgA2 directed against HA antigens.

The mean percent increases (MPIs) of the salivary titers of IgA2 directed against the HA antigens after XEN inhalation as compared to those before XEN inhalation (n = 17) were calculated. The percent increases in each volunteer were calculated as follows. Percent increase (%) = {δOD value after inhalation - δOD value before inhalation)/δOD value before inhalation} x 100; then, the average values for the overall population (n = 17) were calculated. a: HA antigen derived from A/California/7/2009/H1N1; b: HA antigen derived from A/Hong Kong/4801/2014/H3N2; c: vaccine-type HA antigens derived from A/Singapore/GP1908/2015/H1N1, A/Hong Kong/4801/2014/H3N2, B/Texas/2/2013 /Victoria system, and B/Phuket/1307/2013 /Yamagata system.

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水素ガスを含む蒸気混合ガス吸入後のインフルエンザウイルス由来のhemagglutinin抗原に対する唾液中IgA抗体価の増強

池脇　信直，園田　徹*，東　和弘**

九州保健福祉大学生命医科学部生命医科学科・順正学園免疫学研究所　〒882-8508　宮崎県延岡市吉野町1714-1
*九州保健福祉大学保健科学部作業療法学科・順正学園免疫学研究所　〒882-8508　宮崎県延岡市吉野町1714-1
**東内科クリニック　〒887-0022　宮崎県日南市上平野町3-8-8

要旨

水素発生装置（スイソニア）から発生する水素ガス（濃度 0.1%～ 0.3%）を含む蒸気混合ガス XENを鼻カニューラで吸入した。吸入後、A型およびB型インフルエンザウイルス由来の hemagglutinin（HA）抗原に対する唾液中 IgA2抗体価を自主開発の酵素免疫測定法で解析した。その結果、XEN吸入 15分後、A型インフルエンザウイルス株（A/California/7/2009/H1N1と A/Hong Kong/4801/2014/H3N2）由来の HA抗原に対する唾液中 IgA2抗体価は、吸入前と比較して有意に増加した（P=0.028および P=0.047）。さらに、XEN吸入 15分後、A型と B型インフルエンザウイルス株（A/Singapore/GP1908/2015/H1N1、A/Hong Kong/4801/2014/H3N2、B/Texas/2/2013/Victoria system、B/Phuket/1307/2013 /Yamagata system）由来の HA抗原（ワクチンタイプ）に対する唾液中 IgA2抗体価も吸入前と比較して有意に増加した（P=0.039）。以上の結果から、XENには HA抗原に対する唾液中 IgA2抗体価の増強作用があることがわかった。

キーワード：酵素免疫測定法、ヘマグルチニン、唾液 IgA2、水素ガス含有蒸気混合ガス、XEN