Aging Exacerbates Restraint Stress-Induced Inhibition of Antigen-Specific Antibody Production in Mice

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ABSTRACT

Background: We have recently found that exposure to acute restraint stress suppresses antigen-specific antibody production, including IgE, in a murine model of allergic rhinitis. Although age-related alterations in immune responses are known, it remains unclear whether aging modulates the antibody production under stressful conditions. In this study, we set out to determine the effects of aging on antibody production under acute restraint stress in mice.

Methods: Both young and aged CBA/J mice were repeatedly sensitized intranasally with phospholipase A2 (PLA2) without adjuvants. Restraint stress was applied using uniform cylinders once a week for a continuous 8 h period, on 5 occasions in total. Blood samples were taken at 0, 20 and 30 days after primary sensitization, and production of PLA2-specific antibodies and levels of IL-4, IFN-γ, IL-10 and IL-1β in sera were determined by ELISA.

Results: Repeated intranasal sensitization with PLA2 induced PLA2-specific IgE, IgG1 and IgG2a production in aged mice. We found that exposure to restraint stress significantly inhibited production of PLA2-specific IgE, IgG1 and IgG2a in aged mice. In addition, antibody production under restraint stress decreased significantly in aged mice when compared with young mice. No IL-4, IFN-γ, IL-10 or IL-1β were detected in sera from non-stressed or stressed aged mice.

Conclusions: Aging exacerbates the immunosuppressive role of acute restraint stress in antigen-specific antibody production in mice.

KEY WORDS

aged mouse, immunosuppression, phospholipase A2, restraint stress, specific antibody

INTRODUCTION

It is known that aging is associated with a reduced immune function, so called immunosenescence, in both humans and animals.¹ For example, a shift in lymphocyte population from conventional T cells to NK cells and extrathymic T cells is observed in human centenarians.¹ Changes in the proportion of T cell subsets, in addition to increases in memory T cells, impairment of response to mitogens and other stimuli, and alterations in cytokine production also occur with aging.²,⁴

In terms of humoral immunity, it is known that pro-

B cells in old mice are impaired in their capacity to rearrange themselves to both D to J and V to DJ gene segments in mice.⁵ In addition, serum IgE levels and antigen-specific IgE production are known to decline with age in humans.⁶,⁷

Exposure to physical, neurological, or emotional stress can also affect both innate and acquired immune responses.⁸,¹⁰ For example, exposure to acute stress modulates antigen-specific T cell responses.¹¹ We have recently reported that inhibition of antigen-specific antibody production was confirmed using a type of restraint stress following intranasal sensitization with phospholipase A2 (PLA2) in mice.¹² How-
Method
Sensitization and restraint stress

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<td>Sensitization</td>
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<td>PLA2 10 μg</td>
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Day 0 7 14 21 28

Blood sampling

Fig. 1 Treatment schedule; Mice were intranasally sensitized to 10 μg of PLA2 in 20 μl saline. Sensitization was repeated in the same manner. Following sensitization, restraint stress was applied to mice using a single transparent cylindrical chamber and repeated once every week, for a total of 5 applications. Blood samples were taken from each tail vein at 0, 20, and 30 days after primary sensitization.

However, little is known whether aging affects stress-induced alterations in humoral immune responses.

In this study, we compared stress-induced inhibitions of antibody production between aged and young mice in an intranasal sensitization model. As physical restraint is occasionally used in geriatric care in order to prevent bed fall in hospitals, the results presented here may provide a basis for evaluating the risk of restraint stress on humoral immunity in elderly patients.

**METHODS**

**ANIMALS**

Nine-week old female, young adult mice (18–20 g) and 17-month old female, CBA/J strain mice (26–30 g) (Charles River Japan, Yokohama, Kanagawa, Japan) were used in this study. Mice were maintained in an animal house according to the guidelines of the Animal Study Committee of the Kagawa Prefectural College of Health Sciences. All animals were housed in groups of 3, each in an opaque polycarbonate mouse cage (30 × 20 × 30 cm) with access to food and water ad libitum, and were maintained on a 12-hour light-dark cycle for 2–3 weeks before the experiments began. The temperature in the animal house was maintained at 25°C.

**REAGENTS**

ELISA plates were purchased from Corning (Corning, NY, USA). Purified rat anti-mouse IgE was purchased from Biosource (Camarillo, CA, USA), extraAvidin-peroxidase conjugate, PLA2, carbonate buffer and fetal calf serum from Sigma (St. Louis, MO, USA), tetramethylbenzidine substrate from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA), phosphoric acid from Wako Pure Chemical Industries (Osaka, Japan), peroxidase-conjugated goat anti-mouse IgG1/IgG2a monoclonal antibody from Boehringer-Mannheim (Indianapolis, IN, USA) and biotin (long-arm) N-hydroxy succinimide ester from Vector Laboratories (Burlingame, CA, USA). It is known that endotoxin contamination suppresses allergen-induced immunologic responses including IgE Production on mice. Contamination of endotoxin was negligible as determined using an Endospec assay kit (Seikagaku Kogyo, Tokyo, Japan) in accordance with the manufacturer’s instructions.

**SENSITIZATION OF MICE**

Mice (n = 6–8 per group) were sensitized by nasal administration of 20 μl of saline containing 10 μg of PLA2 using a microsyringe (Hamilton, Reno, NV, USA). PLA2 was carefully given as 7–8 drops of aqueous solution into each nostril in turn. Sensitization was repeated in the same manner after 1 and 2 weeks. On day 21 and on the following 7 consecutive days, the same amount of PLA2 was given in the same manner. Blood samples were taken from the tail vein on days 0, 20, and 30 after primary sensitization (Fig. 1).

**INDUCTION FOR RESTRAINT STRESS**

Following sensitization, restraint stress was applied to mice (n = 6–8 per group) using a single transparent polymethylmethacrylate cylindrical chamber (20 mm diameter, 100 mm long) commonly used for drawing blood from mice. This chamber was placed horizontally in the mouse cage, and the mice were maintained therein for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of 5 occasions (Fig. 1). Control mice were maintained in their cages without food and water at the same time. Three separate experiments were performed to confirm reproducibility.
Restraint Stress and Immune Function in Aged Mice

**Fig. 2** Effect of restraint stress on PLA2-specific IgE (A), IgG1 (B) and IgG2a (C) production in aged and young mice. Both aged (n = 9, closed triangle) and young (n = 9, closed circle) were placed in a cylindrical chamber for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of five occasions. Control aged mice (n = 9, open triangle) were maintained in their cages without food and water at the same time. Blood samples were taken on days 0, 20 and 30 after primary sensitization, and levels of PLA2-specific antibodies were determined by ELISA. Results are expressed as mean ± SEM. Data are representative of 2 separate experiments. *P < 0.05 between stressed aged group and control aged group. †P < 0.05 between stressed aged group and stressed young group.

**PLA2-SPECIFIC IgE, IgG1, AND IgG2a IN SERUM**
Serum levels of PLA2-specific IgE, IgG1 and IgG2a were determined using ELISA. Titters for specific IgE were estimated as mean optical density (OD) at 450 nm of 1 : 4 diluted sera. Titters for specific IgG1 and IgG2a were estimated as mean OD at 450 nm of 1 : 100 diluted sera.

**TOTAL IgE, IgM, AND IgG IN SERUM**
Serum levels of total IgE in serum were measured as described previously. The detection limits of this system was 0.3 ng/ml. The levels of total IgM and total IgG were measured using ELISA Quantitation Kit (Bethyl Laboratories, Inc., Montgomery, TX, USA). The detection limits for IgM and IgG in this system were 0.4 and 0.4 ng/ml, respectively.

**CYTOKINE DETERMINATION**
Concentration of IL-4, IFN-γ, IL-10 and IL-1β in sera were measured using Opt EIA sets (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The detection limits for IL-4, IFN-γ, IL-10 and IL-1β in this system were 10, 60, 15 and 30 pg/ml, respectively.

**STATISTICAL ANALYSIS**
Data are expressed as means ± standard error of the mean (SEM) for each subject group. Statistical analysis was performed using Student’s unpaired t-test to compare titers of PLA2-specific IgE, IgG1 and IgG2a for restrained and control groups. Values of p < 0.05 were considered to indicate a statistically significant difference.

**RESULTS**

**EFFECT OF RESTRAINT STRESS ON ANTIGEN-SPECIFIC ANTIBODY PRODUCTION IN AGED MICE**
Production of PLA2-specific IgG1 was seen 20 days after the first intranasal sensitization in control aged mice, and production of PLA2-specific IgE and IgG2a, 30 days after the first sensitization. In aged mice under restraint stress, impaired production of these 3 antibodies was observed. On day 30, aged mice under stress produced significantly lower amounts of PLA2-specific IgE, IgG1 and IgG2a as compared with non-stressed aged mice (P < 0.05) (Fig. 2A, B, C).

**EFFECT OF AGING ON RESTRAINT STRESS-INDUCED INHIBITION OF ANTIGEN-SPECIFIC ANTIBODY PRODUCTION**
We then compared PLA2-specific antibody production under restraint stress between young and old mice. Young mice under stress produced PLA2-specific IgE and IgG1 20 days after the first sensitization, and produced PLA2-specific IgG2a 30 days after sensitization. The level of PLA2-specific IgE on day 20 was significantly less in aged mice under stress than young mice, and the difference could still be ob-
EFFECTS OF RESTRAINT STRESS ON SERUM LEVELS OF IL-4, IFN-γ, IL-10 AND IL-1β
Serum levels of IL-4, IFN-γ, IL-10 and IL-1β were determined in mice with and without restraint stress. None of these cytokines were detected in sera from non-stressed or stressed aged mice. In addition, these cytokines were not detected even in sera from stressed young mice.

EFFECTS OF AGING AND/or STRESS ON LEVELS OF TOTAL IgE, IgM, AND IgG IN SERA
Levels of total IgE, total IgM, and total IgG in sera did not differ between stressed aged and non-stressed aged groups. On the other hand, levels of serum total IgM and IgG but not IgE were significantly lower in the stressed young group compared with the stressed aged group throughout the experimental period (Fig. 3).

DISCUSSION
Reductions in T-cell function in aged mice have been shown to reduce IgE antibody production by impairing differentiation of IgE-containing progenitor B cells into IgE antibody-producing plasma cells. These age-associated reductions in immune function, and T-cell function in particular, are thought to affect the function of helper B cells and suppress indirect antibody production response.

In aged mice, various effects of stress in the immune system, and particularly in T cells, have been investigated in previous studies. For example, Kanno et al. reported in a study of restraint stress on mice that atrophy of the thymus and decreases in splenic T cells were observed after exposure to stress. However, young mice showed a rapid recovery of the immune function after 1 week, while the aged mice never recovered. However, little is known whether aging can affect stress-induced humoral responses despite the fact that aging and stress share similar effects on immune function.

We previously reported that the humoral immune system in young mice was suppressed by restraint stress in the early stages of antibody production following intranasal sensitization with PLA2. In this study we have further demonstrated that, although repeated intranasal sensitization with PLA2 induced PLA2-specific IgE, IgG1 and IgG2a in aged CBA/J mice, exposure to restraint stress significantly inhibited production of PLA2-specific antibodies. In addition, the present study found that aged mice underwent even more marked suppression of antibody production than young mice under restraint stress. These results suggest for the first time that aging and stress have a synergic effect on the impairment of humoral immunity, and more importantly, that aging exacerbates stress-induced inhibition of humoral re-
sponses. None or only slight differences in antibody production were found between the aged control group and young stressed group. This may be due to an aging effect, and may suggest that the impact of aging on antibody production in our model resembles that of restraint stress seen in young mice.

Restraint stress suppresses both PLA2-specific IgG1 and IgG2a production in aged mice. It is known that IgG1 and IgG2a is Th2 and Th1-type IgG isotype, respectively. Fukui et al. reported that restraint stress significantly suppressed both Th1- and Th2-type immune responses in mice. It has also been reported by Dhabhar et al. that B cells show a greater stress-induced decrease than T cells. These reports support our findings, suggesting that restraint stress suppresses both Th1- and Th2-type humoral responses in aged mice. Defective induction of functional Th2 cytokine responses has been reported in aged mice in addition to Th1 type immune response being important for the protection against intracellular pathogens such as viruses, mycobacterium and protozoan parasites. Thus susceptibility to impair Th1-type immune responses by restraint stress in elderly patients may increase the risk of suffering from infectious diseases by intracellular pathogens.

The levels of total IgE, total IgM, and total IgG in sera did not differ between stressed aged and non-stressed aged groups. This result suggests that restraint stress selectively affects antigen-specific antibody production in aged mice. Interestingly, levels of serum total IgM and IgG but not IgE were significantly lower in the stressed young group compared with the stressed aged group. This may be due to baseline differences, as serum total IgM and IgG in aged groups were higher than in young groups even before intranasal sensitization. Long-term life in the animal house under a conventional environment may increase serum total IgM and IgG levels.

Although no IL-4, IFN-γ, IL-10 or L-13 was detected in sera from non-stressed aged mice or stressed aged mice, the mechanisms involved in the suppression of antibody production in aged mice under stress have not been clearly elucidated.

Other studies have examined the application of restraint stress, and further studies are needed to clarify the direct or indirect involvement of endocrinological neuronal pathways in the initiation of allergic rhinitis. Accumulation of findings from a wide field of research focusing on the immune system and including the nervous endocrine systems is necessary.

In conclusion, we have shown that restraint stress impaired antigen-specific antibody production, especially in aged mice, and aging displays a strong impact on stress-induced inhibition of humoral immune responses. These observations may provide a basis for the management of care for elderly patients with physical restraints. In modern life, both the young and elderly are exposed to various forms of stress. Our study suggests stress as one of the mechanisms for the epidemiological finding that serum IgE levels and antigen-specific IgE production decline with age in humans.7, 8

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