Eur J Appl Physiol (2002) 87: 358–364
DOI 10.1007/s00421-002-0653-x

ORIGINAL ARTICLE

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Acute exercise induces gastrointestinal leakage of allergen in lysozyme-sensitized mice

Accepted: 17 April 2002 / Published online: 27 June 2002
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Abstract Food-dependent exercise-induced anaphylaxis (FDEIAn) is a leading cause of physical allergies. However, the mechanisms involved in the development of FDEIAn are not yet clearly understood. In this study, to investigate the leakage of allergen from the gastrointestinal tract into the circulation, lysozyme (LYZ)-sensitized B10.A mice, which have been shown to exhibit an especially high concentration of plasma antigen-specific immunoglobulin-E (IgE) by sensitization of LYZ as allergen in the white of hens' eggs, and unsensitized mice were made to run on a treadmill after oral LYZ ingestion. Resting mice as well as sensitized and unsensitized animals were used as controls. As a result of the sensitization, total IgE concentration, and levels of LYZ-specific IgE and IgG significantly increased. The running time until exhaustion decreased in the sensitized mice compared with that in the unsensitized mice. Both the sensitization and exercise affected the increase in gastrointestinal leakage of LYZ, which was estimated by immunohistochemical staining of the LYZ antibody in the liver, after oral LYZ ingestion. The exercised sensitized mice were especially closely observed. After oral LYZ ingestion, damage to the villi in the small intestine also occurred following exercise in sensitized mice. Damage to the villi was also noted to have occurred slightly in the resting sensitized mice. These results suggest that allergen leakage from the intestinal tract into the circulation was strongly induced by exercise in the LYZ-sensitized mice and that the mechanisms of FDEIAn might be related to gastrointestinal leakage of allergen due to gastrointestinal mucosal lesions.

Keywords B10.A mouse · Lysozyme · Food-dependent exercise-induced anaphylaxis · Immunoglobulin-E · Gastrointestinal erosion

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Introduction

Exercise-induced anaphylaxis (EIAn) is a well-known physical allergy (Briner and Sheffer 1992). Food-dependent EIAn (FDEIAn) is a unique form of physical allergy that has been recognized with increasing frequency (Kemp et al. 1995; Castells et al. 1999). In FDEIAn, allergic symptoms are thought to be induced by chemical mediators that can be released from activated mast cells following the binding of antigen by immunoglobulin-E (IgE) antibodies. Specific IgE antibodies against food and chemical mediators are usually detected in patients with FDEIAn, but the mechanisms involved in FDEIAn have not been well defined, because acute exercise has not in itself been found to induce production of the IgE antibody (Eliakim et al. 1997) and increase the plasma concentration of histamine (Watanabe et al. 1990). In a recent study, however, it was reported that the amount of food allergen ingested was related to the allergic reaction of FDEIAn (Hanakawa et al. 1998). Therefore, there is a need to understand better the absorption of food from the gastrointestinal tract as an associated factor of FDEIAn.

Physical stress has been shown to induce gastrointestinal mucosal lesions. In fact, some previous studies have reported that gastrointestinal bleeding and mucosal lesions (Moses 1993), and endotoxin translocation (Bosenberg et al. 1988) were induced by exercise. Thus

protein, which functions as an allergen, might also leak into the circulation, although it is still unclear as to whether gastrointestinal leakage of allergen is induced by exercise.

We have hypothesized that the food allergy reaction of FDEIAn might be accelerated by exercise, which would induce gastrointestinal mucosal lesions. In the present study, we have estimated the effect of exercise on the leakage of allergen from the gastrointestinal tract into the portal circulation in sensitized mice after oral lysozyme (LYZ) ingestion.

The mechanisms of FDEIAn still remain unclear, because a completely satisfactory mouse model of FDEIAn has not been established, although murine models have been used widely in studies of various allergy diseases (Li et al. 1999). We used LYZ-sensitized B10.A mice, which have been shown to exhibit a high level of plasma antigen-specific IgE by sensitization of LYZ as allergen in the white of hens' eggs (Anet et al. 1985), and made them run on a treadmill after oral LYZ ingestion.

Methods

Animals and sensitization

Male B10.A mice weighing 25–30 g ($n = 31$) were used in this experiment. The animals were kept on a standard diet without LYZ and had access to tap water ad libitum. The room temperature was maintained at 20 ± 2°C, and a 12/12 h (8 a.m.–8 p.m.) light/dark cycle was used. The experiment procedures employed followed the guidelines set forth in the Care and Use of Animals in the Field of Physiological Sciences approved by the Council of the Physiological Society of Japan. The sensitized mice ($n = 16$) were injected with 10 mg LYZ in 0.5 ml of an aluminum hydroxide phosphate-buffered saline solution by intraperitoneal administration with an interval of 7 days between three injections. Blood was obtained weekly from the orbital veins of both sensitized and unsensitized ($n = 15$) mice under light ether anaesthesia.

Enzyme-linked immunosorbent assay

The specific anti-LYZ IgE and IgG in mouse serum was evaluated using enzyme-linked immunosorbent assay (ELISA; Engvall and Perlmann 1971). Briefly, 100 µl of LYZ solution (10 $\mu g \cdot ml^{-1}$) was added to each well of flat-bottomed microtiter plates, and incubated overnight at 4°C. The wells were then washed with phosphate buffer saline containing 0.05% of Tween 20 (PBS-T). A 1% BSA (bovine serum albumin)/PBS-T solution was added to each well and incubated for 30 min at 37°C. Then 100 µl of each mouse serum diluted 50 times with 1% BSA/PBS-T solution was applied to each well and incubated for 3 h at 37°C. After each well was washed with PBS-T three times, 100 µl of peroxidase conjugated goat anti-mouse IgE or IgG (Nordic Immunological Laboratories, England) in 1% BSA/PBS-T solution (10^{-3}) was added to each well and incubated for 3 h at 37°C. Each well was washed with PBS-T three times, and then 100 µl of *o*-phenylenediamine in citrate-phosphate buffer (0.4 $mg \cdot ml^{-1}$) containing 0.03% H_2O_2 was added. The reaction was stopped with 50 µl of H_2SO_4 solution (2.5 $mol \cdot l^{-1}$) after 20 min at room temperature. The colour development of the well was measured photometrically at 492 nm.

The concentration of IgE in the mouse serum was analysed using an ELISA mouse IgE kit (Morinaga Institute of Biological Science, Japan) and employing a standard curve of 0.5 to 32 $ng \cdot ml^{-1}$.

Measurement of exercise performance

Before the experiment, all animals were exercised during a 3 day acclimation period. Then 2 days after the last injection of LYZ or saline, each mouse was challenged with 50 mg of LYZ in 0.5 ml of PBS ingested orally. Groups of 8 exercising mice in each of the sensitized and unsensitized groups were run on a treadmill. From an initial speed of 18 $m \cdot min^{-1}$, at a 5% gradient for 30 min, the treadmill speed was increased every 30 min by 3 $m \cdot min^{-1}$ until exhaustion. Exhaustion was defined as the point at which a mouse refused to run despite being given mild physical prodding (Woods et al. 1993). Resting mice remained in the cage for 2 h after LYZ oral ingestion and were exposed to the same handling and noise stress without actually running. The range of total duration of exercise in mice was between 41–240 min.

Immunohistochemical estimation of LYZ in liver

After exercise or rest, the mice were rapidly anaesthetized with ether. In preparation for examination using a fluorescence microscope, the liver was perfused by the portal vein with 10 ml PBS and liver specimens were fixed in O.C.T. compound (Tissue-Tek, Sakura, Japan). Then, immunohistochemical staining of the liver was performed. Next, 6 µm-thick cryostat sections were placed on acetone (4°C) for 10 min. The sections were then incubated with 10% normal rabbit serum (Nichirei, Japan) for 10 min at room temperature, followed by incubation with anti-LYZ mouse Ig antibody (BMA Biomedicals, Switzerland) at a 1:1,000 dilution overnight at 4°C. After washing with PBS-T, the sections were treated with a fluorescein isothiocyanate-labelled secondary antibody (Nichirei) for 10 min. Negative method controls were included in which the primary and/or secondary antibodies were omitted at the relevant stages. Positive control sections were of the liver of a mouse that had been injected with 10 mg LYZ in 0.5 ml of a PBS solution by the tail vein 2 h before sacrifice. For LYZ-immunohistochemical estimation, the presence of LYZ-stained liver was examined under a fluorescence microscope (BX50-LLA, Olympus, Japan) and scored on a point scale of 0–3; 0 (no stain), 1 (weak staining), 2 (moderate staining), and 3 (marked staining).

Estimation of small intestine damage

After all the animals were sacrificed, the small intestine was removed, spread open, and washed with PBS. The tissue was carefully cut into six parts and fixed with neutral-buffered 10% formalin. Each of the six parts was examined under a microscope (MZ6, Leica, Switzerland). The surface of the mucosal lesions was taken as a record of erosions. The cumulative number of all lesions in each mouse served as the measure of erosion damage; 0 (normal), 1 (one point), 2 (two points), and 3 (more than three points).

Some of the fixed tissues were embedded in paraffin and 6 µm sections were stained with haematoxylin and eosin reagent (HE staining).

The immunohistochemical estimation of liver and the count of the number of erosions in the small intestine were performed by three independent observers, each of whom were blinded to the diagnosis, and average value for each section was calculated.

Statistical analysis

Data were expressed as the means (SEM). Between-group analysis was performed using Student's two-tailed *t*-test for unpaired data. Within-group analysis was performed first with a two-way analysis of variance (ANOVA) and then, post hoc, with the Fisher PLSD test. Statistical procedures were performed using a data base and statistical package (Stat View J-4.5, Abacus Concepts, USA), in which $P < 0.05$ was considered significant.

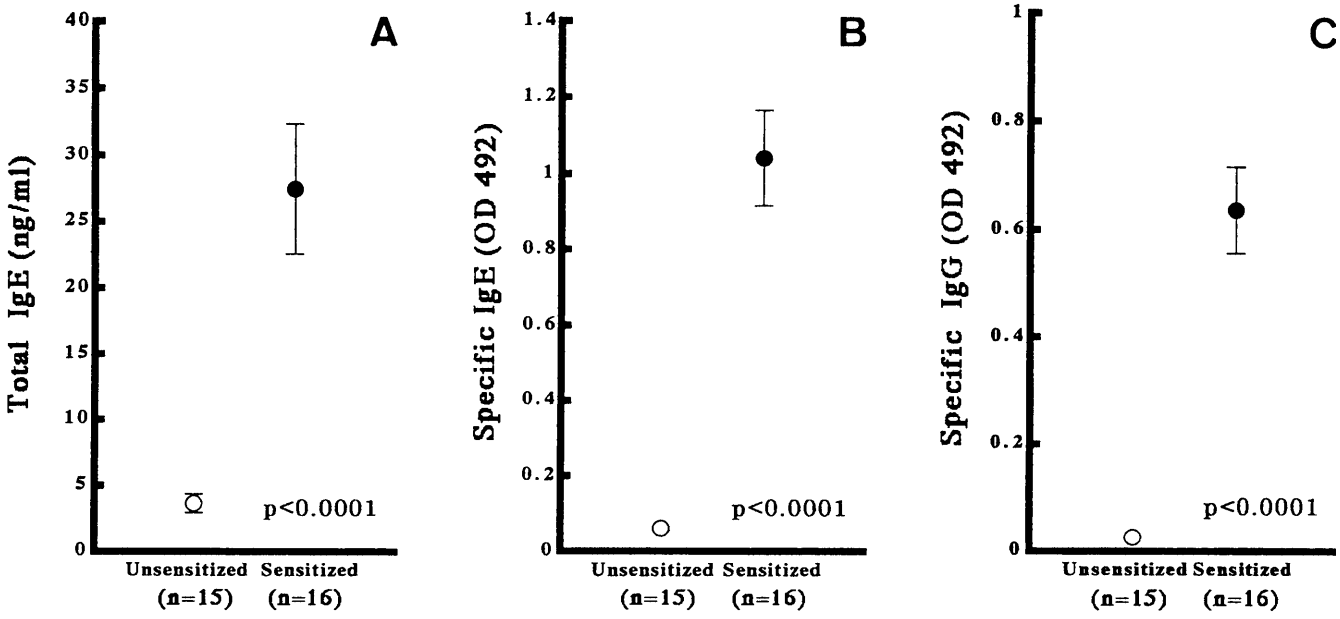

Fig. 1. Mean (SEM) effect of lysozyme (*LYZ*) sensitization on total immunoglobin-E (*IgE*) **A**, and antigen-specific IgE **B,** and IgG **C** antibodies. *OD* Optical density

Results

Since LYZ-injected mice developed significant increases in total IgE ($P < 0.0001$), and LYZ-specific IgE ($P < 0.0001$) and IgG ($P < 0.0001$) values in their serum, the mice were recognized to be sensitized with LYZ (Fig. 1).

After oral ingestion of LYZ, the sensitized and unsensitized groups ran until exhaustion. The running times to exhaustion are shown in Fig. 2. The data indicated a shorter time for the LYZ-sensitized group than for the unsensitized group [131 (16) compared to 199 (16) min, $P < 0.01$, Fig. 2].

As shown in Fig. 3 extensive LYZ positive staining was evident in the livers of the LYZ-sensitized mice. The hepatic sinusoid was stained by anti-LYZ antibody in the LYZ-sensitized mice following exercise. The two-way ANOVA test for LYZ-immunohistochemistry estimation in the liver showed statistically significant results for sensitization ($P < 0.0001$) and exercise ($P < 0.0001$). In the unsensitized group, exercise had little effect on LYZ-positive staining in liver. In the sensitized group, however, the scores for hepatic LYZ-positive staining in the exercised mice were significantly higher than those in the resting mice ($P < 0.0001$). In addition, the scores in the exercised-sensitized mice were higher than those in the exercised-unsensitized group ($P < 0.0001$, Fig. 4).

Localization of the mucosal lesions in the small intestine was not limited. As shown in Fig. 5 there was damage to the villi in the small intestine. Surface observation (Fig. 5, upper panel) revealed a mucosal lesion of the small intestine in a sensitized mouse following exercise. Furthermore, an HE-stained section showed

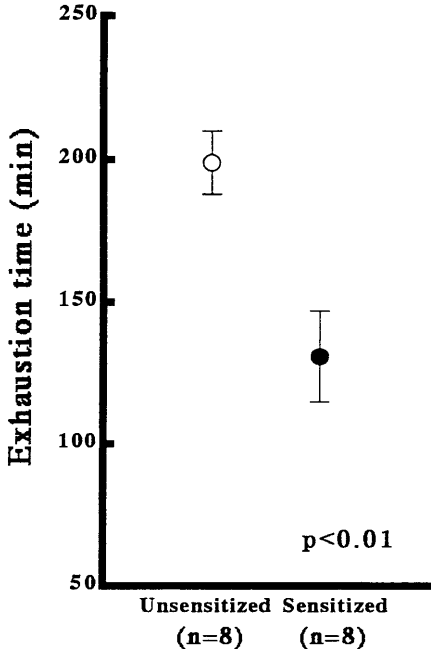

Fig. 2. Mean (SEM) effect of oral lysozyme (LYZ)-ingestion on exercise performance in LYZ-sensitized and unsensitized mice. Exercise performance was estimated from the time to run to exhaustion

damage of the surface epithelium (Fig. 5, lower). The denuded villi in one exercised sensitized mouse were equivalent to grade 4 as described by Chiu et al. (1970), who classified morphological changes in the mucosa into six grades. Regarding erosion scores, statistically significant results for sensitization ($P < 0.001$) and exercise ($P < 0.001$) were found (Fig. 6). The exercised sensitized mice had the highest erosion score. In the unsensitized group, the erosion scores were higher in the exercised mice than in the resting mice ($P < 0.01$). In addition, the

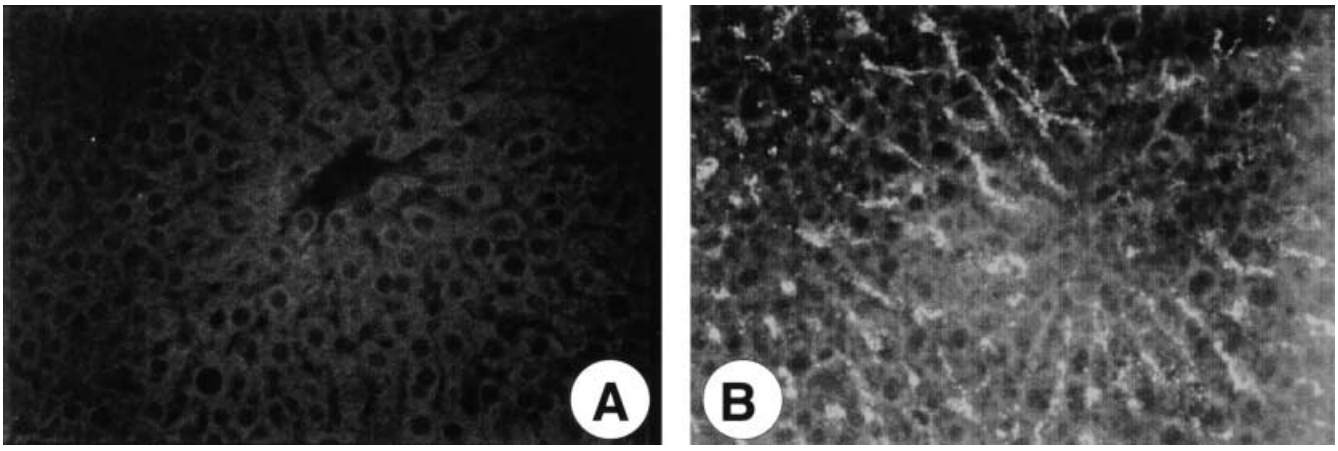

Fig. 3. Photograph of lysozyme (LYZ) positive staining in the liver of resting **A** and exercised **B** sensitized mice (×100). The hepatic sinusoid was stained using anti-LYZ antibody in the LYZ-sensitized mice following acute exercise

erosion scores in the resting sensitized mice were significantly higher than those in resting unsensitized mice ($P < 0.0001$).

Discussion

We found that acute exercise in the LYZ-sensitized B10.A mice accelerated gastrointestinal leakage of LYZ as an antigen. The hepatic sinusoid positively stained by anti-LYZ antibody showed excessive leakage of LYZ into the portal circulation after oral LYZ ingestion. There is a possibility that strenuous exercise allows antigen to pass rapidly in significant amounts into the systemic circulation. Under such circumstances, the amount of allergen might affect the mediator release from mast cells in extra-intestinal sites. Previously reported degranulation of skin mast cells in EIAn (Briner and Sheffer 1992) is suggestive of the passage of allergen into the systemic circulation. The IgE has generally been regarded as the major trigger of immediate hypersensitivity reactions (type I). Although it is clear that IgE is central to much of the allergic reaction, our finding hints at the existence of a considerable amount of leakage of allergen from the gastrointestinal tract into the systemic circulation due to exercise after sensitization, which shows a high level of serum specific IgE. Furthermore, the 34% reduction in exercise performance in the LYZ-sensitized mice after oral LYZ-ingestion could probably be explained by an FDEIAn symptom due to an immunoreaction between specific immunoglobulin and the allergen.

The LYZ-positive stained hepatic sinusoidal cells appear to be Kupffer cells, i.e. fixed hepatic macrophages. Kupffer cells represent 90% of the total body phagocyte activity and occupy a strategic position in relation to the terminal branches of the portal vein. They form the body's first line of defence, clearing the circulation of a number of antigens and other potentially immunostimulatory materials which cross to the liver from the gastrointestinal tract. However, Kupffer cells are also known to play an important role in anaphylaxis, because a Kupffer cell phagocytosis blockade has been shown to reduce greatly the mortality rates and symptoms of mouse anaphylaxis due to ovalbumin sensitization (Lazar et al. 1994). Kupffer cells activated by phagocytosis induce elevation of serotonin, an important mediator in mouse anaphylaxis, in the liver (Miller and Church 1976).

The IgE antibodies, which bind to high-affinity FcεRI receptors on mast cells, macrophages and dendritic cells, play an important role in mediating type I hypersensitivity responses. In addition, since Oettgen et al. (1994)

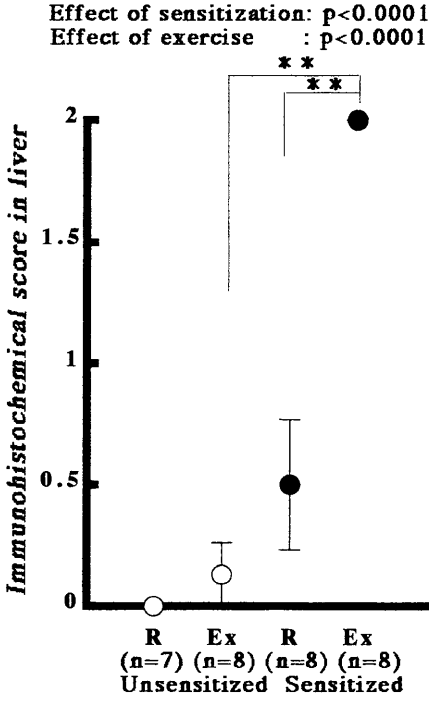

Fig. 4. Mean (SEM) effect of acute exercise on the lysozyme immunohistochemical score in the liver of the sensitized and unsensitized mice after oral ingestion. *R* Rest, *Ex* exercise. $^{**}P < 0.01$

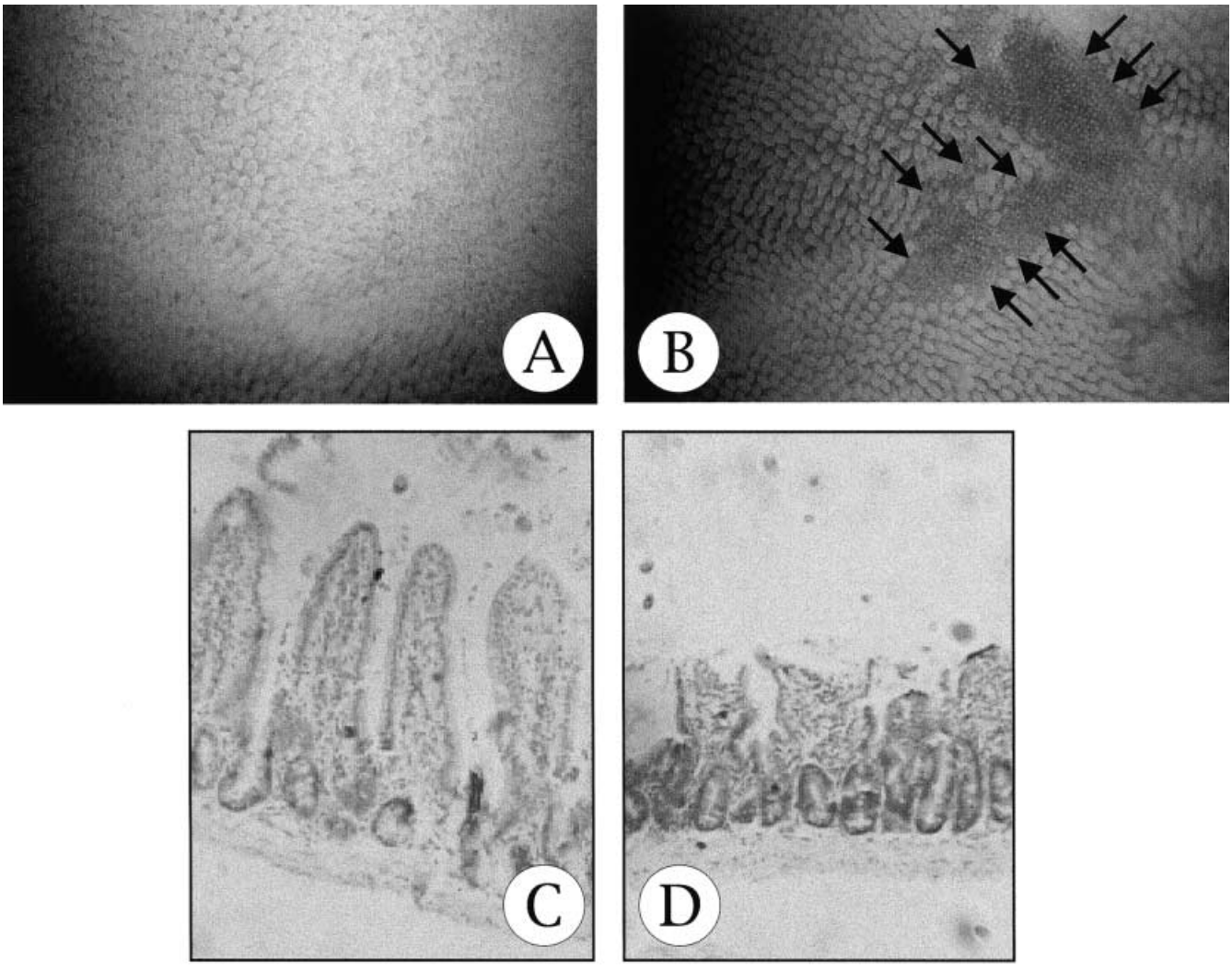

Fig. 5 A–D. Histologic alterations in damage to the villi in the small intestine in lysozyme (LYZ)-sensitized mice after acute exercise. Photographs show a surface scan (upper, ×40) and haematoxylin and eosin staining (lower, ×100) in the jejunum. **A** and **C** show the resting unsensitized mice and **B** and **D** show the exercised sensitized mice. The mucosal lesion (*surrounded by arrows*) was observed in a LYZ-sensitized mouse following acute exercise. Denuded villi, suggesting severe damage to the small intestine, can be observed in **D**

reported that non-IgE immunoglobulins mediate antigen-specific systemic anaphylactic reactions in IgE-deficient mice, IgG_1 could also mediate immediate hypersensitivity reactions in mice. In our study, LYZ-injected mice showed an increase in the LYZ-specific IgG value in serum. However, the relationship between FDEIAn symptoms and IgG remains unclear.

A second finding was that acute exercise in the LYZ-sensitized B10.A mice accelerated gastrointestinal erosion. It is generally accepted that strenuous exercise induces a great reduction in mesenteric blood flow (Bortoff 1988; Musch et al. 1987). Furthermore, intestinal ischaemia has been found to be associated with bacterial translocation and increased endotoxin absorption from the gastrointestinal tract (Tokyay et al. 1993). These reports suggest that dysfunction of the intestinal mucosal barrier occurs due to mesenteric ischaemia caused by the reduction of mesenteric blood flow during exercise. In fact, other authors have reported that exercise has induced gastrointestinal bleeding and mucosal lesions in humans (see review Moses 1993) and we have seen these in rats (Yano et al. 1999). Ahonen et al. (1972) observed ischaemic small intestinal injury after 30 min of occlusion in the dog. Another factor that has been speculated upon is exercise-induced mechanical jarring of the intestine (Brouns 1991). In a recent study, the platelet-activating factor associated with inflammatory cytokines seemed to play a role in the production of intestinal necrosis in rat anaphylaxis (Pellon et al. 1994). Although, in this study, we could not clarify the mechanism of small intestine mucosal lesions induced by acute exercise, it is evident that mucosal lesions were induced by acute exercise. Accordingly, strenuous exercise markedly accelerated intestinal damage in the sensitized mice after allergen ingestion. It seems that these mucosal lesions of the small intestine relate to the cause of gastrointestinal leakage of LYZ as an antigen,

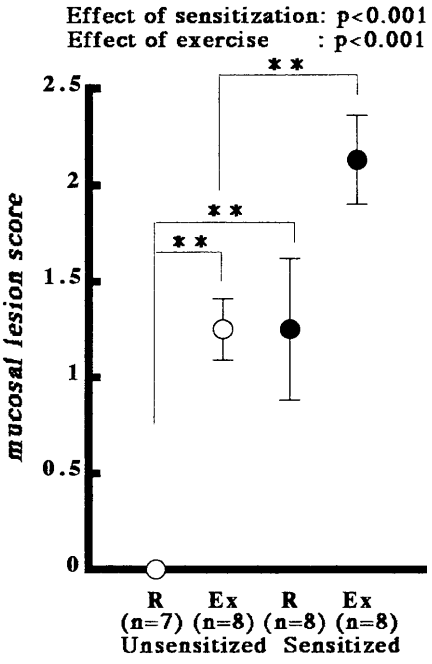

Fig. 6. Mean (SEM) effect of acute exercise on mucosal lesion scores in the small intestine of the sensitized and unsensitized mice after oral lysozyme ingestion. *R Rest*, *Ex* exercise. $^{**}P < 0.01$

because alterations caused by damage to the intestinal wall could allow the escape of LYZ from the gastrointestinal tract into the portal circulation, leading to greater exposure to Kupffer cells.

Furthermore, in this experiment, we also observed slight damage to the villi of the small intestine of the sensitized mice that were not exercised. The histologic appearance was essentially the same as that described in intestinal anaphylaxis in rats (Levine and Saltzman 1998) and mice (Li et al. 1999). Li et al. (1999) reported intestinal permeability after intragastric allergen challenge in sensitized mice.

Although various foods have been reported in association with FDEIAn, little has been published on allergen models and animal strains. In LYZ-sensitized B10.A mice, however, antigen-specific IgE antibody showed a tenfold increase following LYZ-sensitization without some adjuvant, suggesting a useful model of type I hypersensitivity. The specific IgE level was very high compared with that in previous food allergy, that is cows milk with the adjuvant of cholera toxin, in the C3H mouse model (Li et al. 1999).

In conclusion, with LYZ as the allergen, leakage from gastrointestinal tract into the circulation was strongly induced by acute exercise in LYZ-sensitized mice. This finding suggests that the mechanisms of FDEIAn might be related to the gastrointestinal leakage of allergen caused by exercise-induced gastrointestinal mucosal lesions.

Acknowledgement This study was supported by an Interdepartmental Research Fund of Kawasaki University of Medical Welfare.

References

Ahonen J, Inberg MV, Jaaskelainen AJ, Havia T, Aho AJ, Scheinin TM (1972) Effect of oxygen ventilation in mesenteric arterial occlusion in the dog. Scand J Gastroenterol 7:9–16

Anet J, Back JF, Baker RS, Barnett D, Burley RW, Howden ME (1985) Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteins. Int Arch Allergy Appl Immunol 77:364–371

Bortoff A (1988) Influence of exercise on gastrointestinal function. In: Horton ES, Terjung RL (eds) Exercise, nutrition, and energy metabolism. McMillan, New York, pp 159–171

Bosenberg AT, Brock-Utne JG, Gaffin SL, Wells MTB, Blake TW (1988) Strenuous exercise causes systemic endotoxemia. J Appl Physiol 65:106–108

Briner WW Jr, Sheffer AL (1992) Exercise-induced anaphylaxis. Med Sci Sports Exerc 24:849–850

Brouns F (1991) Etiology of gastrointestinal disturbances during endurance events. Scand J Med Sci Sports 1:66–77

Castells MC, Horan RF, Sheffer AL (1999) Exercise-induced anaphylaxis (EIA). Clin Rev Allergy Immunol 17:413–424

Chiu CJ, McArdle AH, Brown R, Scott ZHJ, Gurd FN (1970) Intestinal mucosal lesion in low flow states. Arch Surg 101:478–483

Eliakim A, Wolach B, Kodesh E, Gavrieli R, Radnay J, Ben-Tovim T, Yarom Y, Falk B (1997) Cellular and humoral immune response to exercise among gymnasts and untrained girls. Int J Sports Med 18:208–212

Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871–874

Hanakawa Y, Tohyama M, Shirakata Y, Murakami S, Hashimoto K (1998) Food-dependent exercise-induced anaphylaxis: a case related to the amount of food allergen ingested. Br J Dermatol 138:898–900

Kemp SF, Lockey RF, Wolf BL, Lieberman P (1995) Anaphylaxis a review of 266 cases. Arch Intern Med 155:1749–1754

Lazar G Jr, Lazar G, Kaszaki J, Olah J, Kiss I, Husztik E (1994) Inhibition of anaphylactic shock by gadolinium chloride-induced Kupffer cell blockade. Agents Actions 41:C97-C98

Levine S, Saltzman A (1998) Distributions of small intestinal lesions in anaphylaxis of rats. Int Arch Allergy Immunol 115:312–315

Li X, Schofield BH, Huang C, Kleiner GI, Sampson HA (1999) A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 103:206–214

Miller P, Curch MK (1976) Sequential release of histamine and 5-hydroxytryptamine during pinnal anaphylaxis in mouse. Int Arch Allergy Appl Immunol 52:53–58

Moses FM (1993) Gastrointestinal bleeding and the athlete. Am J Gastroenterol 88:1157–1159

Musch IT, Friedman DB, Pitetti KH, Haidet GC, Stray-Gundersen J, Mitchell JH, Ordway GA (1987) Regional distribution of blood flow of dogs during graded dynamic exercise. J Appl Physiol 63:2269–2277

Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P (1994) Active anaphylaxis in IgE-deficient mice. Nature 370:367–370

Pellon MI, Steil AA, Furio V, Crespo MS (1994) Study of the effector mechanism involved in the production of haemorrhagic necrosis of the small intestine in rat passive anaphylaxis. Br J Pharmacol 112:1101–1108

Tokyay R, Zeigler ST, Traber DL, Stothert JC Jr, Loick HM, Heggers JP, Herndon DN (1993) Postburn gastrointestinal vasoconstriction increases bacterial and endotoxin translocation. J Appl Physiol 74:1521–1527

Watanabe T, Sakamoto Y, Tomonaga H, Inuyama M, Sasayama H, Hara K (1990) A case of food-dependent exrcise-induced anaphylaxis (in Japanese with English abstract). Arerugi 39:1529–1540

Woods JA, Davis JM, Mayer EP, Ghaffar A, Pate RR (1993) Exercise increases inflammatory macrophage antitumor cytotoxicity. J Appl Physiol 75:879–886

Yano H, Kinoshita S, Yano L (1999) Role of Kupffer cells in acute exercise-induced intestinal mucosal lesions in rats. In: Wisse E, Knook DL, Zanger R de, Fraser R (eds) Cells of the hepatic sinusoid, vol 7, Kupffer Cell Foundation, Leiden, pp 247–248