β_2 -Agonist Clenbuterol Suppresses Bacterial Phagocytosis of Splenic Macrophages Expressing High Levels of Macrophage Receptor with Collagenous Structure

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Splenic marginal zone macrophages expressing macrophage receptor with collagenous structure (MARCO) contribute to the clearance of blood-borne pathogens. We determined a splenic adherent cell fraction abundantly containing cells expressing a higher level of MARCO by flow cytometry, and examined the effects of daily administration of an anabolic dose of β_2 -agonist clenbuterol on the phagocytic capacity of the cells in mice. After 6 weeks of clenbuterol (1.0 mg/kg body weight/d) or vehicle administration to the mice, splenic adherent cells were isolated. These cells were separated into three cell-size subpopulations. Among them, the small-cell subpopulation contained abundantly the cells with markedly higher levels of MARCO and exhibited more intense phagocytic capacity against *Escherichia coli*, as compared with the other subpopulations. The phagocytic capacity of the small cells was significantly reduced after clenbuterol administration. These results suggest that the utilization of clenbuterol as doping drug impairs bacterial clearance in the spleen.

Key words β_2 -agonist; doping; splenic macrophage; phagocytosis; macrophage receptor with collagenous structure

Macrophage phagocytosis plays a key role in the first line of the innate immune response against microorganisms.¹⁾ Macrophages are distributed in every tissue of the body and acquire specialized physiological roles according to their anatomical locations.²⁾ Among these locations is the spleen, which is composed of a branched splenic artery that eventually ends in venous sinuses.³⁾ Although the terminal arterioles from the central arteriole run through the lymphoid compartment white pulp, most of the arterial blood ends in the marginal zone of the spleen, located at an anatomical border between the white pulp and the more innate scavenging compartment of the red pulp.^{3–5)} Therefore, the splenic marginal zone is a strategically important site for the removal of microorganisms in the blood.³⁾

The splenic marginal zone contains highly phagocytic macrophages, such as marginal zone macrophages, that are a minor subset of splenic macrophages constitutively expressing macrophage receptor with collagenous structure (MARCO).^{3,6–8)} The expression of MARCO is restricted to marginal zone macrophages and macrophages residing in the peritoneal cavity and medullary cord of the lymph nodes.⁶⁾ It is known that MARCO binds to Gram-negative and Gram-positive bacteria,⁶⁾ and its constitutive expression facilitates efficient clearance of *Streptococcus pneumoniae* in the spleen.^{9–12)}

Macrophages express β -adrenergic receptors (β -ARs) on the cell surface,¹³⁾ and their cellular functions are negatively regulated by sympathetic nervous activity.^{14,15)} The down-regulation of β_2 -AR after exercise training has been shown to increase the lipopolysaccharide-induced inflammatory responses of resident peritoneal macrophages in mice.^{16–18)} Furthermore,

the results of several *in vitro* studies have implied that macrophage phagocytic capacity against bacterial pathogens is suppressed by sympathetic overactivation.^{19–23)}

β_2 -Agonist clenbuterol is commonly used as a nonsteroidal anabolic drug for sports doping. According to the World Anti-Doping Agency documents, clenbuterol was the seventh most commonly used anabolic agent in 2009, accounting for 2.0% of all cases.²⁴⁾ It is of critical importance to elucidate how clenbuterol affect the bacterial phagocytosis of specialized subsets of splenic macrophages, because the cells contribute to the clearance of blood-borne pathogens.^{6,9–12)} The elucidation of these issues will provide further scientific evidence to demonstrate the side effects of clenbuterol on host defense systems. Therefore, we determined a splenic adherent cell fraction abundantly containing cells expressing a higher level of MARCO by flow cytometry, and examined the effects of daily administration of an anabolic dose of clenbuterol on the phagocytic capacity of the cells in mice.

MATERIALS AND METHODS

Animal Care Five-week-old male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and pre-fed for 5 d to allow adaptation to their new environment. The mice were housed individually in stainless mesh cages at 23–25°C and 50–60% humidity with a fixed light/dark cycle (light at 09:00–21:00 h and dark at 21:00–09:00 h).^{25,26)} Animal food (CE-2 cubic type, CLEA Japan) and once-boiled tap water were available *ad libitum*.^{25,26)} After the adaptation period, the mice were divided into clenbuterol-administered (CLE) and control (CON) groups. All the experimental and animal care procedures were approved by the Committee on Animal Care and Use at Waseda University (No. 2011-A003) and in ac-

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cordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan, based on the Declaration of Helsinki, 1964.

Administration of Clenbuterol Clenbuterol hydrochloride (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in 0.9% NaCl as a vehicle to make the clenbuterol solution at a concentration of 0.02%.²⁵⁾ The solution (1.0 mg/kg body weight/d)^{25,27,28)} was administered by intraperitoneal injection to the CLE group mice for 6 weeks. In contrast, an equivalent volume of the 0.9% NaCl vehicle was administered to the CON group mice in the same manner.

Preparation of Splenic Adherent Cells After 24 h from the final clenbuterol/vehicle administration, the mice were anesthetized using isoflurane (Mylan, Osaka, Japan), and their spleens were immediately isolated. The spleens were minced using a 22-gauge needle (Terumo, Tokyo, Japan) in phosphate-buffered saline (PBS; pH 7.4) and then filtrated with a cell strainer (70 μ m; Becton, Dickinson and Company [BD], Franklin Lakes, NJ, U.S.A.). After centrifuging at 1000 rpm for 3 min at 4°C, red blood cells were selectively lysed by incubating for 5 min at room temperature, with a hemolytic buffer containing 0.83% NH_4Cl and 0.17 mol/L Tris-HCl (pH 7.65) at a 9:1 volume ratio. After centrifugation, the cells were washed twice with PBS and then resuspended with RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (BioWest, Nuaille, France), 100 units/mL penicillin, and 100 mg/mL streptomycin (Nakalai Tesque, Kyoto, Japan).²⁶⁾ The cells were then seeded into plastic culture dishes. After culturing for 2 h in a CO_2 incubator with 5% CO_2 , non-adherent cells were discarded, and the adherent cells, including the splenic macrophages, were washed three times with PBS and collected by scraping with a silicone rubber sheet.

Flow-Cytometry Analysis of Cell Surface MARCO Expression and *Escherichia coli* Phagocytosis The splenic adherent cells were counted using a disposable one-cell counter (Wakenyaku, Kyoto, Japan), and then 5×10^5 cells were resuspended in PBS. The cells were Fc blocked with 2 μ g of mouse immunoglobulin G (IgG) (BD) for 15 min at room temperature. After blocking, the cells were stained with monoclonal anti-mouse MARCO-phycoerythrin (PE) (R&D Systems, Minneapolis, MN, U.S.A.) or rat IgG₁ isotype control conjugated with PE (R&D Systems) for 30 min at room temperature in the dark. The cells were washed twice with PBS and then resuspended in 500 mL of PBS. After filtration with a cell strainer (40 μ m; BD), the samples were analyzed using a FACSCalibur flow cytometer (BD) with the FL2 (PE) detector. For bacterial phagocytic analysis, splenic adherent cells were incubated with *E. coli* transformed with pRSET-EmGFP vector (Invitrogen, Carlsbad, CA, U.S.A.) at the multiplicity of infection (MOI) of 30:1 for 30 min in the CO_2 incubator. After washing twice with PBS, the samples were then filtrated with a cell strainer (40 μ m; BD) and analyzed using FACSCalibur with the FL1 (GFP) detector.

In Vitro Treatment of Splenic Adherent Cells with β_2 -Agonists After removal of non-adherent splenic cells, the splenic adherent cells were cultured with the complete medium, including 100 μ M clenbuterol hydrochloride (Sigma-Aldrich), 100 μ M (–)-isoproterenol (+)-bitartrate salt (Sigma-Aldrich) or vehicle (H_2O) for 3 h, and then the cells were collected by scraping with a silicone rubber sheet, and 5×10^5

Fig. 1. Fractionation of Splenic Adherent Cells Based on Cell Size and Intracellular Complexity

(A) Forward light scatter (FSC) and side light scatter (SSC) plots of splenic adherent cells. (B) The ratios of the large, medium, and small splenic adherent cells. The values are shown as mean $\pm$ S.E.M. ($n=9$). ** $p<0.01$.

cells were subjected for bacterial phagocytic analysis as described above.

Statistical Analysis The experimental data were presented as mean $\pm$ S.E.M. Equivalence of data or group means was tested by one-way analysis of variance (ANOVA). *Post hoc* comparisons of significant differences among three data sets were performed by the Bonferroni test. Differences were considered statistically significant at $p<0.05$.

RESULTS

Splenic Adherent Cell Subpopulations We first fractionated splenic adherent cells based on relative cell size, using forward light scatter, and intracellular complexity, using side light scatter. As shown in Fig. 1A, splenic adherent cells were separated into three subpopulations according to size. When these subpopulations were individually gated and each dot count was analyzed, the counts of the large-, medium-, and small-cell subpopulations accounted for $57.6 \pm 2.6\%$, $34.1 \pm 2.6\%$, and $5.7 \pm 0.8\%$ of the total splenic adherent cells, respectively (Fig. 1B).

Cell Surface MARCO Expression Levels and Phagocytic Capacity of the Splenic Adherent Cell Subpopulations The histograms of the MARCO-stained and isotype control-stained cells for the large-, medium-, and small-cell subpopulations indicated that the fluorescence intensity of the MARCO-stained cells was higher than that of the isotype control-stained cells for all three subpopulations (Fig. 2A1). However, the expression level of MARCO was dramatically higher for the small cells than for the large- and medium-cell subpopulations (Fig. 2A2). The histograms for the *E. coli*-phagocytized cells in the large-, medium-, and small-cell subpopulations also indicated that the *E. coli*-phagocytized cells were appeared in all three subpopulations (Fig. 2B1), although the phagocytic capacity of the small splenic adherent cells was markedly higher than the large- and medium-cell subpopulations (Fig. 2B2).

Effects of Clenbuterol Administration on the Phagocytic Capacity and Cell Surface MARCO Expression Levels of the Splenic Adherent Cells We next examined the effects of clenbuterol administration on the phagocytic capacity

Fig. 2. Flow Cytometry Analysis of the Cell Surface Expression Levels of MARCO (A) and *E. coli* Phagocytosis (B) for the Large-, Medium-, and Small-Splenic Adherent Cell Subpopulations

(A1) The histograms of the anti-MARCO- and isotype control-stained cells for each size group, and (A2) the mean fluorescence intensity (MFI) of the cells for each size group. The values in the parentheses are the relative ratios of the anti-MARCO-stained cells to the isotype control-stained cells. (B1) The histograms of *E. coli*-phagocytized and *E. coli*-free cells for the large-, medium-, and small-cell groups, and (B2) the MFI of the cells for each size group. The values in the parentheses are the relative ratios of the *E. coli*-phagocytized cells to the *E. coli*-free cells. The values are shown as mean $\pm$ S.E.M. ($n=9$). *** $p<0.001$.

against *E. coli* for each splenic adherent cell subpopulation. The phagocytic capacities of the large- and medium-cell subpopulations were not affected by the daily administration of an anabolic dose of clenbuterol for 6 weeks (Fig. 3A). However, the capacity of the small cells abundantly containing the cells expressing a higher level of MARCO was significantly reduced after the experimental period (Fig. 3A). On the other

hand, the expression levels of MARCO on all three subpopulations of splenic adherent cells were not changed after the experimental period (Fig. 3B).

In Vitro Effects of β_2 -Agonists on the Bacterial Phagocytosis of the Splenic Adherent Cells To examine the direct action of β_2 -agonists against the bacterial phagocytosis of the splenic adherent cells, the cells isolated were directly cultured

with medium including clenbuterol and isoproterenol for 3 h. As shown in Fig. 4, the cell ratio of *E. coli*-phagocytized cells in the large-cell subpopulation was significantly decreased by both β_2 -agonists, although the phagocytic capacities of all three subpopulations were not affected (data not shown). By contrast, the cell ratio of *E. coli*-phagocytized cells in the medium-cell subpopulation was significantly decreased by clenbuterol but not by isoproterenol (Fig. 4).

DISCUSSION

Under pathogen-free conditions,²⁹⁾ the expression of MARCO is restricted to marginal zone macrophages in the spleen and macrophages in the peritoneal cavity and the medullary cord of lymph nodes.⁶⁾ Marginal zone macrophages are a small subset of splenic macrophages and have been shown to interact with blood-borne antigens.⁹⁾ We found that adherent

cells in the spleen can be clearly separated into three subpopulations according to their size. Of these groups, the small-cell subpopulation accounted for only a minor fraction of the total cells but then exhibited markedly higher levels of MARCO on their cell surfaces compared with the other size groups.

MARCO is a class A macrophage scavenger receptor that is shown to bind to gram-negative bacteria such as *E. coli* and Gram-positive bacteria such as *Staphylococcus aureus*.⁶⁾ The constitutive expression of MARCO on the cell surface of marginal zone macrophages facilitates efficient clearance of *Streptococcus pneumoniae* in the spleen.^{9–12)} In addition, marginal zone macrophages display greater phagocytic capability than other subsets of splenic macrophages, including marginal metallophilic macrophages.^{4,30)} In the present study, we demonstrated that the small-cell subpopulation of splenic adherent cells exhibits greater phagocytic capacity against *E. coli* than the medium- and large-cell subpopulations. These results suggest that marginal zone macrophages are included in this small cell group.

There have been a number of *in vitro* studies investigating the effects of adrenergic receptor stimulation on phagocytic capacity using various subsets of active state macrophages. Among these studies, Abrass *et al.*¹³⁾ initially reported that phagocytosis of aggregated γ -globulin by Bacille de Calmette et Guérin (BCG) vaccine-activated macrophages from rat peritoneal cavity was suppressed by treatment with the moderately selective β_2 -agonist metaproterenol. In another study on macrophages isolated from a murine cutaneous injury site, the phagocytic capacity against *E. coli* was reduced by physiologic and pharmacologic concentrations of noradrenalin; however, the capacity of macrophages from the spleen was not affected by these stimuli.²²⁾ In contrast, several studies have implied that the phagocytic activity of splenic macrophages is reduced by sympathetic overactivation.^{21–23)}

However, it still remains unclear how adrenergic receptor stimulations affect the phagocytic capacity of certain subsets of splenic macrophages, mainly because of the technical difficulties in isolating the scarce and fragile cells for functional analysis *in vitro*.^{31,32)} Our study demonstrates that the phagocytic capacity against *E. coli* of the small splenic adherent cells, abundantly containing the cells expressing a higher level of MARCO, is significantly reduced after daily administration of an anabolic dose of clenbuterol for 6 weeks. However, the capacity of most splenic adherent cells, including other types of splenic macrophages, was not affected by the

Fig. 3. Effects of Clenbuterol Administration on the *E. coli* Phagocytosis (A) and the Cell Surface Expression Levels of MARCO (B) for the Large-, Medium-, and Small-Splenic Adherent Cell Subpopulations

(A) The relative mean fluorescence intensity (MFI) is the relative ratio of the MFI of the *E. coli*-phagocytized cells to that of the *E. coli*-free cells. The value in the parenthesis is the relative ratio of the CLE value to the CON value. (B) The relative MFI is the relative ratio of the MFI of the anti-MARCO-stained cells to that of isotype control-stained cells. The values are shown as mean $\pm$ S.E.M. ($n=9$). ** $p<0.01$.

Fig. 4. *In Vitro* Effects of Clenbuterol and Isoproterenol Treatments on the Cell Ratios of *E. coli*-Phagocytized Cells for the Large-, Medium-, and Small-Splenic Adherent Cell Subpopulations

The splenic adherent cells were treated with vehicle (control: CON), 100 μ M clenbuterol (CLE) or 100 μ M isoproterenol (ISO) for 3 h. The cell ratio of *E. coli*-phagocytized cells is presented as the percentage in each cell subpopulation. The values are shown as mean $\pm$ S.E.M. ($n=7$). * $p<0.05$ and ** $p<0.01$.

administration of clenbuterol. These results suggest that the reaction to β_2 -adrenergic stimulation differs between various subsets of splenic macrophages. Furthermore, it is conceivable that the susceptibility against desensitizing effects of chronic administration is different among these cells.

The expression levels of MARCO on the cell surface of splenic adherent cells were not affected by clenbuterol administration for 6 weeks. In addition, the mRNA expression levels of macrophage scavenger receptor 1 (MSR1), which is abundantly expressed on every macrophage lineage, were not affected by the stimuli in splenic adherent cells (data not shown). These results suggest that the reduction in phagocytic capacity of the cells after chronic administration of clenbuterol was caused by factors other than the expression levels of such macrophage scavenger receptors. For instance, Gosain *et al.*²²⁾ demonstrated that noradrenalin-induced suppression of the macrophage phagocytosis is mediated by the α and β receptors-cAMP-protein kinase A (PKA) signaling cascade. Recently, it was found that morphine-induced reduction of Fc γ receptor-mediated bacterial phagocytosis may be due to inhibition of Rac1-GTPase activity caused by elevation of the intracellular cAMP levels and the resulting actin polymerization attenuation and reduction in membrane ruffling.³³⁾ Moreover, another study³⁴⁾ found that *Bacillus anthracis* edema toxin weakened macrophage phagocytosis through the activation of cAMP-PKA signaling, resulting to a decrease in cell spreading and F-actin content. These findings indicate that clenbuterol affects cellular membrane trafficking via β_2 -adrenergic receptor signaling cascade.

On the other hand, our *in vitro* experiment demonstrated that the treatments of the splenic adherent cells with β_2 -agonists, such as clenbuterol and isoproterenol, resulted in the decrease of the cell ratio of *E. coli*-phagocytized cells in the large-cell subpopulation. In addition, the effect was also observed in the medium-cell subpopulation in the case with clenbuterol but not with isoproterenol. However, the reduction of the phagocytic capacity of the small-cell subpopulation observed in the *in vivo* experiment was not induced by the direct stimulation with both β_2 -agonists. These results indicate at least that the reduction of the bacterial phagocytic capacity by daily administration of clenbuterol for 6 weeks was indirect effects of β_2 -adrenoceptor, although the experimental conditions were fundamentally different between the *in vivo* chronic administration and *in vitro* acute treatment.

Chronic administration of an anabolic dose of clenbuterol to animals was generally maintained for 4–6 weeks.^{35–39)} Among these durations, we selected the most prolonged period, and demonstrated that such stimuli suppresses the phagocytic activity of splenic adherent cells expressing high levels of MARCO. It is necessary to elucidate the mechanisms of β_2 -agonist action by examining in various periods, including the acute experiment.

From our findings, it can be concluded that chronic administration of an anabolic dose of clenbuterol impaired phagocytic capacity of splenic macrophages expressing a higher level of MARCO, and this may lead to a reduction in blood-borne pathogen clearance.

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