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Molecules Associated with Antigen-Presenting Activity
on Bronchoalveolar Lavage Cells in Rats

(オゾン曝露はラット肺胞洗浄液中の細胞の抗原提
示に関わる細胞表面分子の発現を増加させる)

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Ozone Exposure Enhances Expression of Cell-Surface Molecules
Associated with Antigen-Presenting Activity
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Abstract

In this study, we investigated the effects of ozone (O₃) exposure on expression of cell-surface molecules associated with antigen presentation and on accessory activity of bronchoalveolar lavage cells (BAL cells). Rats were exposed to 1 ppm O₃ for 3 days. Expression of cell-surface molecules was measured by flow cytometry. Accessory activity of BAL cells was assessed by the allogeneic mixed lymphocyte reaction (MLR) and specific antigen-presenting activity. O₃ exposure increased the expression of Ia, B7.1, B7.2, and CD11b/c on BAL cells. Morphological and immunological studies showed the Ia-positive cells to have monocyte-like features. Peripheral blood monocytes expressed Ia, B7.1, B7.2, and CD11b/c. The Ia expression on the monocytes was further increased by treatment of them with BAL fluid from O₃-exposed rats (O₃-BALF). Resident alveolar macrophages, however, did not express Ia antigen, and the Ia expression was not increased by O₃-BALF. Neutrophils, which also infiltrated in response to O₃ exposure, did not express Ia, B7.1, and B7.2. Therefore, infiltrating monocytes may have caused the increased expression of Ia and B7 molecules on BAL cells exposed to O₃. The accessory activity of BAL cells in terms of MLR and specific antigen-presenting activity was also enhanced by O₃ exposure. The present study suggests that monocytes infiltrating in response to O₃ exposure caused enhancements of the expression of Ia and costimulatory molecules on and the accessory activity of BAL cells.

Key Words: ozone, bronchoalveolar lavage cells, accessory activity, Ia antigen, B7 molecules, CD11b/c, and monocytes

Abbreviations: AEC, alveolar epithelial cells; Air-BALF, BAL fluid from air-exposed rats; AM, alveolar macrophages; BAL cells, bronchoalveolar lavage cells; BALF, BAL fluid; BrdU, 5 bromo-2'-deoxyuridine; BSA, bovine serum albumin; FBS, fetal bovine serum; LNC, lymph node cells; MLR, mixed lymphocyte reaction; O₃, ozone; O₃-BALF, BALF from O₃-exposed rats; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; R10, RPMI 1640 medium containing 10% heat-inactivated FBS, 100 mg/ml penicillin, and 100 U/ml streptomycin

INTRODUCTION

Recently, allergic asthma and rhinitis appear to be increasing in many countries, and previous reports have shown that these allergic disorders are associated with air pollutants (Leikauf *et al.*, 1995; Koren, 1995). Epidemiological studies have also shown a relationship between O₃ and the prevalence of asthma (Balmes, 1993). Accordingly, O₃ may induce an attack of airway allergic disorders or aggravation of symptoms. Airway hyperresponsiveness and enhancement of IgE production are features of airway allergic disorders. The mechanism of airway hyperresponsiveness induced by O₃ exposure has been analyzed from various points of view (Kobayashi, 1996). Exposure to O₃ enhances the activity of IgE-containing cells in the lungs (Gershwin *et al.*, 1981). There are many factors that contribute to IgE production, such as antigen-presenting activity, differentiation and proliferation of type II helper T-cells and B-cells, cytokines, and chemokines. It is thus necessary to examine the effect of O₃ exposure on these factors in order to clarify the mechanisms of enhancement of IgE production.

Antigen-laden bronchoalveolar lavage (BAL) cells may present the antigen to helper T-cells in the lungs and lymph nodes after migration. Therefore, an increase in the antigen-presenting activity of BAL cells would play an important role in the recruitment of eosinophils and/or enhancement of IgE production. To our knowledge, however, there are no reports that show the effect of O₃ on the antigen-presenting activity of BAL cells. It has been reported that monoclonal antibodies against MHC class II (Ia) antigen can suppress the T-cell proliferation elicited by MLR (Gotze *et al.*, 1975) and specific antigen presentation (Niederhuber *et al.*, 1977). Therefore, the expression of Ia is essential to antigen presentation. Resident alveolar macrophages (AM) function poorly as antigen-presenting cells. It was reported, however, that Ia expression in BAL cells was increased by exposure to silica, asbestos, and tobacco smoke (Struhar *et al.*, 1989; Hartmann *et al.*, 1984; Higashimoto *et al.*, 1994). Monoclonal antibodies against costimulatory molecules such as B7.1, B7.2, CD11b or CD11c could also suppress the T-cell proliferation caused by MLR (Lanier *et al.*, 1995; Van-Gool *et al.*, 1994; Landry *et al.*, 1990; Xu *et al.*, 1992) or by specific antigen presentation (Lenschow *et al.*, 1993; Freeman *et al.*, 1993; Meunier *et al.*, 1994). These data suggest that Ia antigen and costimulatory

molecules are necessary for MLR as they are for antigen presentation. Therefore, the accessory function in the MLR can equally estimate that in antigen-presentation. Exposure to O₃ increases the markers of allergic lung disease such as IgE-containing cells (Gershwin *et al.*, 1981) or eosinophils (Bassett *et al.*, 2000) in the lungs. O₃-induced stimuli in the lungs may also increase the expression of cell-surface molecules associated with antigen-presenting activity in BAL cells. Then the BAL cells expressing these cell-surface molecules may transit to the lymph nodes and present the antigen to T-cells. As there are no reports that show the effect of O₃ on the expression of Ia and costimulatory molecules on BAL cells, it is thus important to elucidate whether O₃ affects the expression of these cell-surface molecules on the BAL cells.

Alveolar macrophages are accounted for primarily by the recruitment of circulating blood monocytes (Struhar *et al.*, 1990; Tryka *et al.*, 1984). The number of BAL cells increases in response to a variety of inflammatory stimuli, and these infiltrating cells express Ia antigen to a greater degree than resident AM (Bowden and Adamson, 1980; Blusse *et al.*, 1983). Therefore, O₃ exposure may increase the expression of cell-surface molecules associated with antigen-presenting activity on BAL cells by affecting the infiltrating monocytes.

In this study, we investigated 1) the effect of O₃ exposure on the expression on BAL cells of cell-surface molecules associated with antigen presentation (Ia antigen, B7.1, B7.2, and CD11b/c), 2) whether infiltration of monocytes may reflect the O₃-induced changes in the expression of BAL cell-surface molecules, and 3) the effect of O₃ exposure on the accessory activity of BAL cells.

METHODS

Animals. Specific pathogen-free, male Wistar rats (4 weeks old) and Fisher rats (4 weeks old) were obtained from Japan Charles River Inc. (Yokohama, Japan) and were used at 8-10 weeks of age (weighing 310-380g and 200-250g, respectively). The animals were given sterile distilled water and CE-2 food (CLEA JAPAN Inc., Tokyo, Japan). Wistar rats were used in all experiments except for the preparation of allogeneic lymphocytes in the MLR.

Exposure to O₃. Wistar rats were placed in stainless-steel cages and exposed to 1±0.1 ppm O₃ or filtered air for 3 d (24 h/d) in two identical chambers (volume 1.16 m³) made of stainless-steel and glass. Food and water was provided *ad libitum* to the animals during the exposure period. The conditions in the chambers were as follow: temperature, 25±1°C; humidity, 55±10%; pressure, -5 mm H₂O relative to atmospheric pressure; total air flow rate, 90-100 m³/h. O₃ was generated by a silent discharge apparatus (ML9811, 22245 Network Place, Chicago, IL). The concentration of O₃ was continuously monitored with a chemiluminescence-based O₃ analyzer (Model 8410, Monitor Lab. Inc., U.S.). Rats were exposed to O₃ or filtered air for 3 d in all experiments except the time course study.

Preparation of BAL cells. Air- or O₃-exposed Wistar rats were anesthetized with sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan) given intraperitoneally (50 mg/kg), and exsanguinated from the abdominal aorta. An incision was made between the cartilaginous rings of the trachea, and a cannula was inserted into the trachea and secured with a suture. Lungs and trachea were lavaged twice with 10 ml of 37°C Dulbecco's calcium and magnesium-free, phosphate-buffered saline (PBS [-]; Dainippon Pharmaceutical Co., Osaka, Japan). BAL cells were collected by centrifugation at 400 g for 10 min at 4°C and resuspended in R10, which is RPMI 1640 medium (Dainippon Pharmaceutical Co., Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (FBS; Dainippon Pharmaceutical Co., Osaka, Japan), 100 µg/ml penicillin, and 100 U/ml streptomycin (Sigma, St. Louis, MO). The numbers of viable cells were determined by the trypan blue (Gibco Laboratories, Grand Island, NY) exclusion method.

Preparation of neutrophils. Neutrophils that had infiltrated the BAL cell population were isolated from O₃-exposed rats by Percoll (Sigma, St. Louis, MO) discontinuous gradient centrifugation. Neutrophils were derived from a pool of three animals. Percoll was diluted from 100% stock with PBS [-] containing 10% FBS to strengths of 30, 40, 50, 60, 70, and 80%. Five-step discontinuous gradients (40-80%) were prepared (3 ml of each gradient). BAL cells in each animal were suspended in 2 ml of 30% Percoll, layered onto 40% Percoll layer, and then centrifuged at 400 g for 20 min at 20°C. Neutrophils (> 80% pure) were recovered

from the 70-80% Percoll fraction, washed three times with PBS [-], and resuspended in R10.

Preparation of monocytes. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood from normal Wistar rats by Ficoll Paque (Amersham Pharmacia Biotech, Sweden) gradient centrifugation, and monocytes were purified from PBMC by adherence to plastic dishes. Monocytes were derived from a pool of three animals. Heparinized blood was diluted 1:2 in PBS [-]. The diluted blood (30-35 ml) was layered over 15 ml of Ficoll-Paque, and centrifuged at 400 g for 20 min at 20°C. The PBMC at the interface were recovered and washed three times with PBS [-]. Then, the PBMC ($< 5 \times 10^7$) were resuspended in 10 ml of R10. Before the incubation, plastic culture dishes (100 mm diameter) were treated with FBS at 4°C, overnight. PBMC were incubated in the plastic culture dishes for 1 h at 37°C in a 5% CO₂/95% air atmosphere. Nonadherent cells were removed by gently flushing the dishes three times with R10. Adherent cells were incubated with 10 ml of 0.5% EDTA in PBS [-] containing 5% FBS for 30 min at 4°C. After incubation, the liberated monocytes were collected and resuspended in R10. The numbers of viable cells were determined by the trypan blue exclusion method.

Preparation of lymph node cells (LNC). LNC for use in the allogenic MLR experiments were derived from a pool of superficial, facial, and posterior mediastinal lymph nodes from normal Fisher rats (two rats). The lymph nodes were pushed through a sterile stainless wire mesh (200 mesh), and the resulting cells were suspended in 37°C PBS [-]. Cells were collected by centrifugation at 400 g for 10 min at 20°C and resuspended in R10. The numbers of viable cells were determined by the trypan blue exclusion method.

Preparation of ovalbumin (OVA)-sensitized T-cells. OVA-sensitized T-cells for use in the experiments on the antigen-presenting activity of BAL cells were derived from a pool of superficial, facial, and posterior mediastinal lymph nodes from OVA-sensitized Wistar rats (two rats). Rats were immunized with 100 µg OVA (Seikagaku, Co, Tokyo, Japan) and 3 mg Al(OH)₃ in 500 µl of saline. Lymph nodes were harvested at day 14, and LNC were prepared as described above. LNC (1×10^8) in 1 ml of R10 were loaded onto a nylon fiber column (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the column was

incubated for 45 min at 37°C in a 5% CO₂ / 95% air atmosphere. After incubation, the column was washed with R10, and the fraction of non-adherent cells (T-cells) was collected. These T-cells were collected by centrifugation at 400 g for 10 min at 20°C and resuspended in R10. The numbers of viable cells were determined by the trypan blue exclusion method.

Diff-Quik stain of BAL cells. BAL cells were centrifuged onto glass slides for 5 min at 500 rpm. The slides were allowed to air dry for 5 min. Differential cell counts were determined by use of Diff-Quik (International Reagents, Kobe, Japan) stain according to the manufacturer's instructions. Briefly, the slides were dipped in fix reagent and two dye reagents for 3 times severally. The slides were washed with distilled water and allowed to air dry.

Immunohistochemical staining of Ia on BAL cells. BAL cells were centrifuged onto glass slides for 5 min at 500 rpm. The slides were allowed to air dry for 5 min. The slides were into cold acetone (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and cells were fixed for 5 min. The slides were allowed to air dry and rinsed with PBS [-]. Endogenous peroxidase activity was blocked by incubation for 30 min with methanol containing 0.3% H₂O₂ and slides were rinsed with PBS [-] for 5 min. The cells were then treated with 5 µg/ml monoclonal mouse anti-rat Ia IgG1 (Harlan Sera-Lab, Loughborough, UK) in staining buffer, which is PBS [-] containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) and 10% FBS for 30 min. The slides were rinsed with PBS [-] for 5 min. The cells were then treated with biotinylated anti-mouse IgG (Amersham, UK) diluted 1:100 in staining buffer for 30 min, and the slides were rinsed with PBS [-] for 5 min. The cells were then treated with streptavidin-horseradish peroxidase conjugate (Amersham, UK) diluted 1:100 in staining buffer for 30 min, and the slides were rinsed with PBS [-] for 5 min. Thereafter, the cells were treated with 3,3'-diaminobenzidine tetrahydrochloride (2.8 mM) and 0.05% H₂O₂ in PBS [-] as a substrate of the peroxidase for 10 min. The slides were rinsed with PBS [-] for 5 min. Differential cell counts were determined by use of Diff-Quik stain.

FACS analysis. For FACS analysis, the following monoclonal antibodies were used:

FITC-labeled Ox6 (anti-rat Ia), PE-labeled 3H5 (anti-rat B7.1), PE-labeled 24F (anti-rat B7.2) and PE-labeled Ox42 (anti-rat CD11b/c) (Pharmingen, San Diego, CA). Cells (1×10^6) were resuspended in 100 μ l PBS [-] with 0.3% BSA and 0.05% sodium azide (Wako Pure Chemical Industries, Osaka, Japan) and incubated with 1 μ g FITC-Ox6/PE-3H5, FITC-Ox6/PE-24F or FITC-Ox6/PE-Ox42 for 30 min on ice. After incubation, the cells were washed, and the fluorescence was measured by a FACScan flow cytometer (Becton Dickinson, Tokyo, Japan). Fluorescence data were expressed as the percentage of positive cells.

Preparation of BALF. Air- or O₃-exposed Wistar rats were anesthetized with sodium pentobarbital, and a cannula was inserted into the trachea and secured with a suture as described in preparation of BAL cells. Lungs and trachea were lavaged with 5 ml/300 g body wt of 37°C RPMI 1640 medium. The lavage was done with the same RPMI 1640 medium repeatedly by slowly instilling and withdrawing the instillation 10 times. The recovered BALF was centrifuged at 400 g for 10 min at 4°C, and the cells were removed. The acellular lavage fluid was then sterilized by filtration through a MILLEX-HA (0.45 μ m: Millipore, Bedford, MA). Ten percent heat-inactivated FBS, 100 μ g/ml penicillin, and 100 U/ml streptomycin were added to each filtrate of the BALF from Wistar rats exposed to either air or O₃ for 3 d, and these final solutions were designated as Air- or O₃-BALF. There was no difference in the pH of the BALF between the O₃- and air-exposed rats.

Culture of AM and monocytes with BALF. AM and peripheral blood monocytes were prepared from normal Wistar rats. Cells of each type (5×10^5) were cultured with 200 μ l of R10, Air-BALF or O₃-BALF in 96-well flat-bottom plates (Nunc, Denmark) in triplicate for 2 d at 37°C in a 5% CO₂ / 95% air atmosphere. After incubation, Ia expression on AM or monocytes was measured by FACScan flow cytometry. Fluorescence data were expressed as the percentage of Ia positive cells.

MLR. LNC as responder cells were prepared from Fisher rats and BAL cells as stimulator cells were prepared from air- or O₃-exposed Wistar rats. BAL cells were treated with 50 μ g/ml mitomycin C (Kyowa, Tokyo, Japan) for 30 min in water bath at 37°C. Then,

the cells were washed three times with R10 and resuspended in R10. LNC (4×10^5) were cocultured or not with BAL cells (3×10^3 - 2×10^5) in 96-well flat-bottom plates. The MLR was performed in 200 μ l of R10 in triplicate for 4 d at 37°C in a 5% CO₂ / 95% air atmosphere. LNC proliferation was measured after a 4-d culture period.

OVA-specific AP activity of BAL cells. BAL cells were treated with mitomycin C as described above. OVA-sensitized T-cells (4×10^5) were cocultured or not with BAL cells (1.2×10^4 - 4.8×10^4) in the presence of OVA (40 μ g) in 96-well flat-bottom plates. These cell cultures were performed in 200 μ l of R10 in triplicate for 4 d at 37°C in a 5% CO₂ / 95% air atmosphere. T-cell proliferation was measured after a 4-d culture period.

Measurement of cell proliferation. Cell proliferation was measured with a Cell-Proliferation-ELISA Kit (Boehringer Mannheim, Mannheim, Germany). This technique is based on the incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. BrdU incorporated into DNA is measured by a sandwich-type enzyme immunoassay using monoclonal anti-BrdU antibody. This technique is at least as sensitive as the traditional counting of [³H]-thymidine (Porstmann *et al.*, 1985). Cell proliferation was measured by adding 20 μ l of 100 μ M BrdU to each well 18 h before the measurement. After incubation, the plate was centrifuged at 350 x g for 10 min, the culture medium was removed. Then the cells were fixed and DNA denatured by adding FixDenat (200 μ l/well) and incubating the plate for 30 min at room temperature. FixDenat solution was decanted from wells and residual solution was removed thoroughly by tapping the plate on clean paper toweling, anti-BrdU-peroxidase (100 μ l/well) added, and the plate incubated for 90 min at room temperature. Excess antibody conjugate was removed by decantation and wells were rinsed three times with washing solution (PBS) (200 μ l/well). Washing solution was decanted from wells and residual solution was removed thoroughly by tapping the plate on clean paper toweling, substrate (tetramethylbenzidine) (100 μ l/well) was added, and the plate was incubated for 30 min at room temperature. Substrate reaction was stopped by adding 1M sulfuric acid (25 μ l/well) and mix. Absorbance of the samples were measured in an ELISA reader (ImmunoReader NJ-2000, Inter Med, Tokyo, Japan) at the

wavelength of 450 nm (reference wavelength: 620 nm).

Statistical analysis. Data were represented as the mean \pm SEM of three animals from one experiment representative of three experiments. For statistical analysis, the unpaired Student's two-tailed *t* test was used. A *p* value of < 0.05 was considered to indicate a significant difference between the two groups.

RESULTS

Changes in the differential BAL cell counts and the percentage of Ia-positive cells by O₃ exposure

Figure 1(a) shows the changes in the numbers of the different types of BAL cells during a 3-d exposure to 1 ppm O₃. The total number of cells decreased until 12 h, then recovered and significantly increased on day 3. The number of AM and monocytes decreased until 12 h and then tended to recover. The number of neutrophils increased time dependently through day 2 of exposure. The numbers of eosinophils and lymphocytes only slightly increased. Figure 1(b) shows the changes in the percentage of Ia-positive cells during the 3-d exposure to 1ppm O₃. BAL cells from control rats did not express Ia antigen. The percentage of Ia-positive BAL cells increased in a time-dependent manner during O₃ exposure.

Effect of O₃ on the expression of Ia antigen and B7 costimulatory molecules on BAL cells

Figures 2(a) and 3(a) show typical examples of the effects of O₃ on the expression of Ia, B7.1 and B7.2 on BAL cells in dot plots. Figures 2(b) and 3(b) show the percentage of expression of Ia, B7.1, and B7.2 on BAL cells. Most of the BAL cells from air-exposed rats did not express these cell-surface molecules. The expression of Ia, B7.1, and B7.2 molecules on BAL cells was increased by O₃ exposure. The number of double-positive cells also increased in the BAL cells from O₃-exposed rats.

Effect of O₃ on the expression of Ia antigen and CD11b/c on BAL cells

Figure 4(a) and 4(b) show that the expression of Ia antigen and CD11b/c on BAL cells was increased by O₃ exposure. These figures show that double-positive cells also increased in number in the BAL cell population from O₃-exposed rats. Infiltration of cells expressing Ia

and costimulatory molecules and increases in the expression induced by the changes in the microenvironment of the lungs may have caused the increases in the expression of these molecules on BAL cells. Thus, these possibilities were examined next.

Morphology of Ia-positive BAL cells increased by O₃ exposure

Figure 5 shows photomicrographs of BAL cell population. Figures 5(a) and 5(b) are only Diff-Quik-stained BAL cells. O₃ exposure increased the number of neutrophils or monocyte-like cells. Figures 5(c) and 5(d) are BAL cells immunostained with Ox6 antibody. Normal BAL cells were Ia negative, but in the BAL cells from O₃-exposed rats, Ia-positive cells had increased in number. The Ia-positive cells were small-sized and looked like monocytes morphologically.

Expression of cell-surface molecules of infiltrating neutrophils in the BAL cell population

Figure 6 shows that neutrophils, that had infiltrated the lungs during O₃ exposure did not express Ia, B7.1 or B7.2 molecules. However, approximately 90% of the neutrophils expressed CD11b/c.

Expression of Ia antigen and costimulatory molecules on peripheral blood monocytes

The expression of these molecules on peripheral blood monocytes before the cells had infiltrated the alveoli was examined. Both monocytes and AM were obtained from normal rats, and the expression on the monocytes was compared with that on the AM. Figure 7(a) and 7(b) show that monocytes highly expressed Ia, B7.1, B7.2, and CD11b/c molecules in comparison with resident AM. These figures also show that double-positive monocytes were observed.

Effect of O₃-BALF on the expression of Ia antigen on AM and monocytes

Figure 8(a) shows that the Ia expression on the resident AM was very minimal and was not affected by culture with either Air-BALF or O₃-BALF for 2 d. However, the Ia expression on the peripheral blood monocytes, which was found on approximately 20% of the cells, was increased by culture with O₃-BALF but not Air-BALF (Fig. 8(b)).

Accessory activity of BAL cells

Figure 9(a) shows that proliferation of LNC was increased by the addition of BAL cells from O₃-exposed rats. Figure 9(a) also shows that BAL cells from air-exposed rats (>99%

AM) tended to suppress the proliferation of LNC, in a BAL cell number-dependent manner. The accessory activity of BAL cells in the MLR was thus enhanced by O₃ exposure. The capacity of BAL cells to serve as antigen-presenting cells for OVA-sensitized T-cells was also examined. Figure 9(b) shows that OVA-specific proliferation of T-cells was stimulated by BAL cells. BAL cells from O₃-exposed rats, compared with those from air-exposed rats, caused increased proliferation of T-cells. The OVA-specific antigen-presenting activity of BAL cells was thus also enhanced by O₃ exposure.

DISCUSSION

Figure 1(a) showed that the total number of BAL cells decreased until 12 h. This decrease in the recovery of BAL cells may have been caused by adherence of cells to the alveolar epithelium. It is well known that recovery of BAL cells is decreased by short-term exposure to O₃ (Dowell *et al.*, 1970). Figures 1(b) showed that most resident AM (BAL cells from air-exposed rats) did not express Ia antigen or costimulatory molecules, B7.1, B7.2, and CD11b/c. It has been reported that resident AM function poorly as antigen-presenting cells and rather suppress T-cell activation (Koike *et al.*, 1998; Koike *et al.*, 1999; Strickland *et al.*, 1993). In contrast, Fig.1(b) showed that expression of Ia antigen on BAL cells was increased by exposure to O₃ for 3 d, time dependently. Previous reports have shown that the expression of Ia antigen on BAL cells is increased by exposure to silica, asbestos, and tobacco smoke (Struhar *et al.*, 1989; Hartmann *et al.*, 1984; Higashimoto *et al.*, 1994). Figures 2-4 showed that the expression of B7.1, B7.2, and CD11b/c was also increased by O₃ exposure. It has been reported that BAL cells in lung inflammation caused by asthma showed increased expression of B7.1 and B7.2 (Burastero *et al.*, 1999; Agea *et al.*, 1998). Therefore, O₃-induced changes in the microenvironment of the lungs caused the increase in expression of Ia antigen and costimulatory molecules on BAL cells. Furthermore, double-positive cells, which expressed Ia/B7.1, Ia/B7.2, and Ia/CD11b/c, were increased in number by the O₃ exposure (Figs. 2-4). This paper is the first report that O₃ exposure enhanced the number of BAL cell double positive for Ia antigen and costimulatory molecules associated with antigen presentation. This

accumulation of the cells could contribute to antigen presentation and subsequent antibody production.

There are many factors that induce expression of Ia antigen and costimulatory molecules on BAL cells during O₃ exposure. Firstly, O₃-induced changes in the microenvironment of the lungs may affect the expression of these cell-surface molecules on resident AM. It has been reported that IFN- γ induced Ia expression on AM (Struhar *et al.*, 1989). TH1 cytokines, such as IFN- γ and IL-15, induced expression of both B7.1 and B7.2 (Agostini *et al.*, 1999), and IL-16 induced only B7.1 expression on AM (Hermann *et al.*, 1999). TH2 cytokine such as IL-4, and GM-CSF, induced only B7.2 expression on AM (Agea *et al.*, 1998). GM-CSF also induced expression of CD11b and CD11c on AM (Rivier *et al.*, 1994; Eischen *et al.*, 1992). Figure 8(a) showed that expression of Ia antigen on the very few positive resident AM was not increased by treatment with either Air-BALF or O₃-BALF. These results suggest that O₃-induced changes in the microenvironment of the lungs may not affect the expression on resident AM of cell-surface molecules associated with antigen-presentation. Alveolar epithelial cells (AEC) desquamated by O₃ exposure might affect the enhancement of Ia, B7.1, and B7.2 expression on BAL cells. It has been reported that Ia antigen, B7.1, and B7.2 are aberrantly expressed on AEC in idiopathic pulmonary fibrosis (Kaneko *et al.*, 2000), and Ia antigen on AEC is also increased by exposure to silica (Struhar *et al.*, 1989). Thus, O₃ might enhance the expression of these cell-surface molecules on AEC. There are no reports, however, that show O₃ enhancement of the expression of these cell-surface molecules on AEC as far as we know. After 3 d of O₃ exposure, the percentage of AEC in BAL cells as measured by keratin staining was about 5% (data not shown). In contrast, the percentage of Ia-positive cells was about 35%. These data suggest that AEC desquamated by O₃ exposure can hardly account for the increased number of Ia-positive BAL cells.

Secondly, infiltration of cells expressing Ia antigen and costimulatory molecules may cause the increase in the number of BAL cells expressing these cell-surface molecules. It has been reported that small-sized cells (Mochitate and Miura, 1989) and CD11b-positive cells (Bhalla, 1996) in the BAL cell population are increased by O₃ exposure. Actually, Fig. 5(b) showed

that small-sized cells, which had monocyte-like feature and neutrophils, were increased by O₃ exposure. Most of the infiltrating neutrophils expressed CD11b/c, but did not express Ia antigen, B7.1, or B7.2 (Fig. 6). Accordingly, infiltrating neutrophils did not contribute to the increase in the number of BAL cells expressing Ia antigen, B7.1, and B7.2 by O₃ exposure. In contrast, peripheral blood monocytes generally expressed Ia antigen, B7.1, B7.2, and CD11b/c (Figs. 7). Figure 5(d) also demonstrated that the Ia-positive cells were monocyte-like cells. These results suggest that monocytes infiltrating into the alveoli would cause the O₃-induced enhancement of the number of BAL cells expressing cell-surface molecules associated with antigen presentation.

There are many cytokines that induce the expression of these cell-surface molecules on monocytes. IFN- γ , GM-CSF, and TNF- α are known to enhance the expression of Ia antigen on monocytes (Chang and Lee, 1986; Alvaro *et al.*, 1989). A TH1 cytokine, IFN- γ , enhanced the expression of both B7.1 and B7.2; and two TH2 cytokines, IL-4 and IL-10, up-regulated B7.1 and down-regulated B7.2 expression (Debs *et al.*, 1988; Creery *et al.*, 1996). Figure 8(b) showed that expression of Ia antigen on peripheral blood monocytes was increased by treatment with O₃-BALF but not Air-BALF. Accordingly, O₃-induced changes in the lung microenvironment may have enhanced the expression of cell-surface molecules associated with antigen-presentation on monocytes that had infiltrated the lungs.

As for the effect of O₃ exposure on the functions of BAL cells, Fig. 9(a) showed that the accessory activity of BAL cells in the MLR was enhanced by O₃ exposure. Furthermore, O₃ exposure also enhanced OVA-specific antigen-presenting activity of BAL cells for sensitized T cells (Fig. 9(b)). These enhancements of the accessory activity of BAL cells in terms of MLR and OVA-specific antigen-presentation were associated with the O₃-induced increase in the number of Ia antigen and costimulatory molecules on the BAL cells. Such enhancements could also be associated with O₃-induced inhibition of the immunosuppressive activity of BAL cells. Our previous reports showed, however, that O₃ inhibited the suppressive activity of BAL cells toward T-cell function through changing the microenvironment of the lungs. These studies also showed that O₃ did not change the immunosuppressive activity of the BAL cells

themselves (Koike *et al.*, 1998, 1999). Accordingly, the enhanced accessory activity of the BAL cells may not be attributed to the immunosuppressive activity of the BAL cells.

In summary, the number of BAL cells expressing cell-surface molecules associated with antigen presentation was increased by O₃ exposure. Morphological and immunological studies suggested that the Ia-positive BAL cells were infiltrating monocytes. The accessory activity of BAL cells in MLR and OVA-specific antigen-presentation was also enhanced by O₃ exposure. These enhancements of accessory activity may be caused by the increase in the number of BAL cells expressing Ia antigen, B7.1, B7.2, and CD11b/c. Furthermore, the enhancement of these cell-surface molecules on BAL cells may be associated with the infiltration of monocytes expressing these molecules and/or the increase in their expression on infiltrating monocytes caused by O₃-induced changes in the microenvironment of the lungs. On the basis of the present results, we need to conduct further research on the effect of exposure to the ambient level of O₃ on the expression of cell-surface molecules associated with antigen-presenting activity of BAL cells.

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Figure Legends

Fig.1. (a) Differential BAL cell counts. The changes in the number of AM and monocytes, neutrophils, eosinophils, and lymphocytes during 0-3-d exposure to 1 ppm O₃ were examined. BAL cells were isolated from filtered air- or 1 ppm O₃-exposed rats. Differential cell counts was determined by Diff-Quik staining after cytopsin preparation. Solid, shaded, hatched, dotted, and open bars represent total BAL cells, AM and monocytes, neutrophils, eosinophils, and lymphocytes, respectively. (b) Changes in the percentage of Ia-positive BAL cells during a 3-d exposure to 1ppm O₃. BAL cells were isolated from filtered air- or 1 ppm O₃-exposed rats. The cells were treated with mouse anti-rat Ia monoclonal antibody and stained by the indirect avidin-biotin immunoperoxidase method. Values are means ± SEM of three animals. (* p<0.05, ** p<0.01 compared with BAL cells from air-exposed rats).

Fig.2. Effect of O₃ exposure on the expression of Ia and B7.1 on BAL cells. BAL cells (10⁶ cells) were incubated with FITC-Ox6 and PE-3H5 antibodies and analyzed by FACS. (a) Examples of typical Ia and B7.1 expression on BAL cells in dot plots. (b) Percentages of each cell type in the BAL cells. Open and solid bars represent BAL cells from rats exposed to air and 1 ppm O₃, respectively, for 3 d. Data represent the mean ± SEM of three animals from one experiment representative of three experiments. (* p<0.05, ** p<0.01 compared with BAL cells from air-exposed rats).

Fig.3. Effect of O₃ exposure on the expression of Ia and B7.2 on BAL cells. BAL cells (10⁶ cells) were incubated with FITC-Ox6 and PE-24F antibodies and analyzed by FACS. (a) Examples of typical Ia and B7.2 expression of BAL cells in dot plots. (b) Percentages of each cell population in BAL cells. Open and solid bar represent BAL cells from rats exposed to air and 1 ppm O₃, respectively, for 3 d. Data represent the mean ± SEM of three animals from one experiment representative of three experiments. (* p<0.05, ** p<0.01 compared with BAL cells from air-exposed rats).

Fig.4. Effect of O₃ exposure on the expression of Ia and CD11b/c on BAL cells. The cells (10⁶ cells) were incubated with FITC-Ox6 and PE-Ox42 antibodies and analyzed by FACS. (a) Examples of typical Ia and CD11b/c expression of BAL cells in dot plots. (b) Percentages of each cell type in the BAL cells. Open and solid bar represent BAL cells from rats exposed to air and 1 ppm O₃, respectively, for 3 d. Data represent the mean ± SEM of three animals from one experiment representative of three experiments. (* p<0.05, ** p<0.01 compared with BAL cells from air-exposed rats).

Fig.5. Morphological study of Ia-positive BAL cells. Light micrographs of BAL cells isolated from rats exposed to filtered air (a, c) or 1 ppm O₃ for 3 d (b, d). The cells were stained with Diff-Quik stain with (c, d) or without (a, b) immunostaining for Ia antigen. Arrows in Fig.5(b) show monocyte-like cells. Arrows in Fig.5(d) show an Ia-positive cell (brown). Magnification x 300

Fig.6. Expression of cell-surface molecules on neutrophils that infiltrated in the lungs of O₃-exposed rats. Neutrophils that had infiltrated the lungs were isolated by Percoll discontinuous gradient centrifugation. The neutrophils (10⁶ cells) were incubated with FITC-Ox6 and PE-3H5, PE-24F or PE-Ox42 antibodies and analyzed by FACS. Data represent those of three animals in one of four similar experiments.

Fig.7. Expression of Ia and B7.1, B7.2 or CD11b/c on monocytes. Peripheral blood monocytes were prepared from normal rats, and the cells (10⁶ cells) were incubated with FITC-Ox6 and PE-3H5, PE-24F or PE-Ox42 antibodies and analyzed by FACS. (a) Examples of typical Ia and B7.1, Ia and B7.2, and Ia and CD11b/c expression on monocytes in dot plots. (b) Percentages of each cell population. Open and solid bar represent resident AM and monocytes, respectively. Data represent the mean ± SEM of three animals from one experiment representative of three experiments. (* p<0.05, ** p<0.01 compared with resident AM).

Fig.8. Effect of O₃-BALF on the expression of Ia antigen on AM and monocytes. Resident AM and peripheral blood monocytes were prepared from normal rats. Cells of each type (5×10^5) were cultured with R10, Air-BALF or O₃-BALF for 2 d. Then, these cultured cells were incubated with FITC-Ox6 antibody and analyzed by FACS. Fluorescence data are expressed as the percentage of Ia-positive cells in AM (a) and monocytes (b). Data represent those of one of three similar experiments.

Fig. 9. Accessory activity of BAL cells. BAL cells were prepared from rats exposed to 1 ppm O₃ or filtered air for 3 d. (a) MLR. LNC (2×10^6 /ml) were cultured in R10 (200 μ l/well) in the presence of graded numbers (0.75 to 50% of the LNC number) of BAL cells from rats exposed to air (open bar) or O₃ (solid bar). Cells were cultured for 4 d, and BrdU was added 18 h before measuring cell proliferation. Data represent the mean \pm SEM of three animals from one experiment representative of three experiments. (* $p < 0.05$, ** $p < 0.01$ compared with BAL cells from air-exposed rats, † $p < 0.05$, †† $p < 0.01$ compared with LNC alone). (b) OVA-specific antigen presenting activity. OVA-sensitized T-cells (4×10^5) were cultured in R10 (200 μ l/well), in the presence of OVA (200 μ g/ml) and graded numbers (3.125 to 12.5% of the T-cell number) of BAL cells from rats exposed to air (open bar) or O₃ (solid bar). Cells were cultured for 4 d, and BrdU was added 18 h before measuring cell proliferation. Data represent the mean \pm SEM of three animals from one experiment representative of three experiments. (* $p < 0.05$ compared with BAL cells from air-exposed rats, † $p < 0.05$, †† $p < 0.01$ compared with T-cells with OVA).

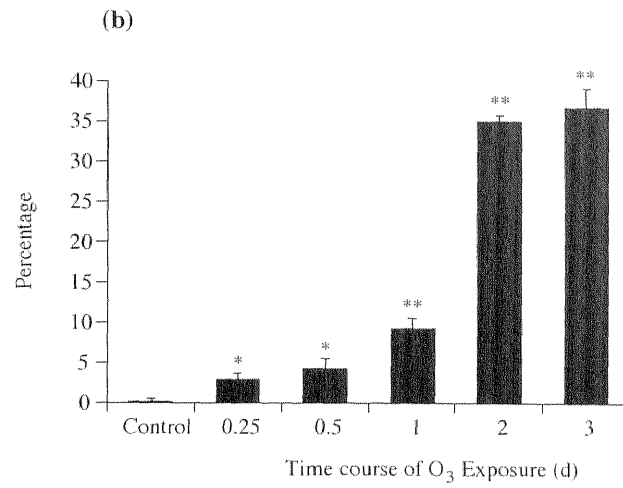
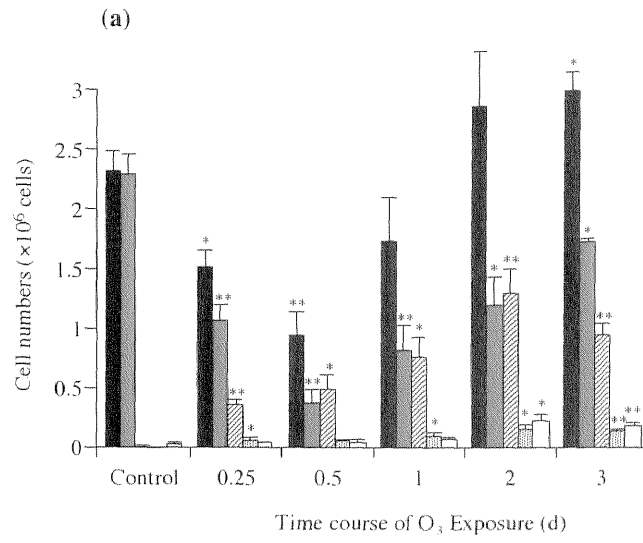


Fig. 1

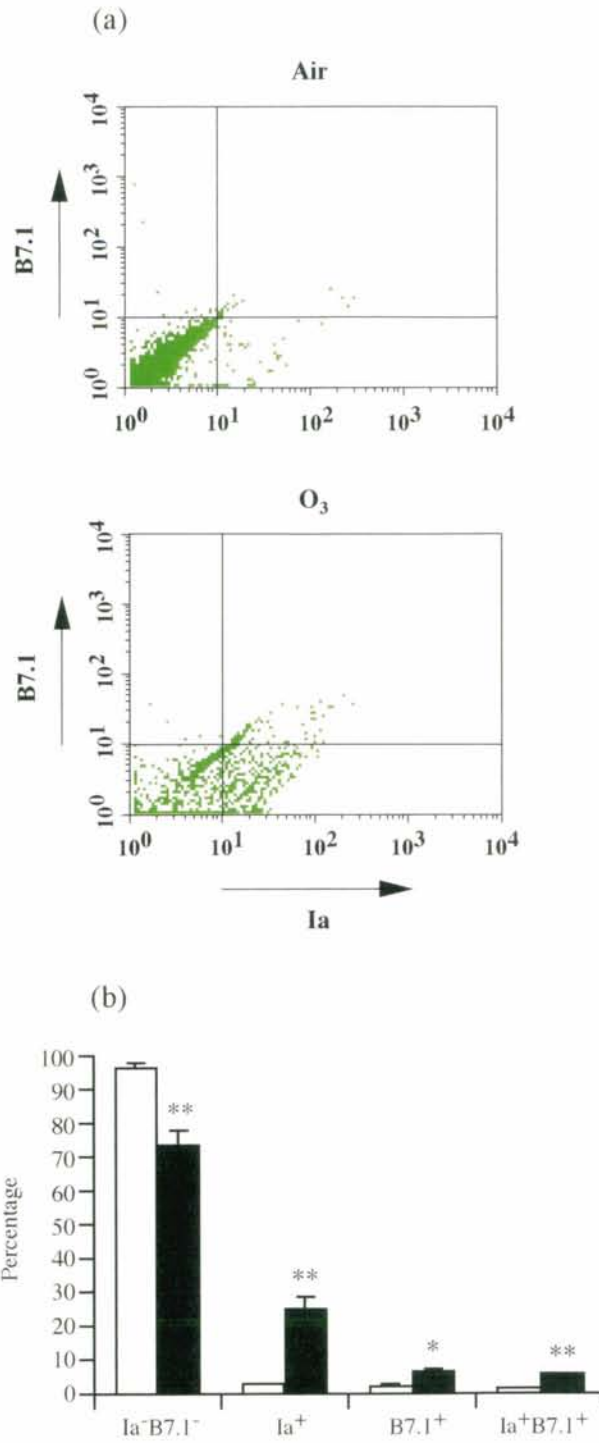


Fig. 2

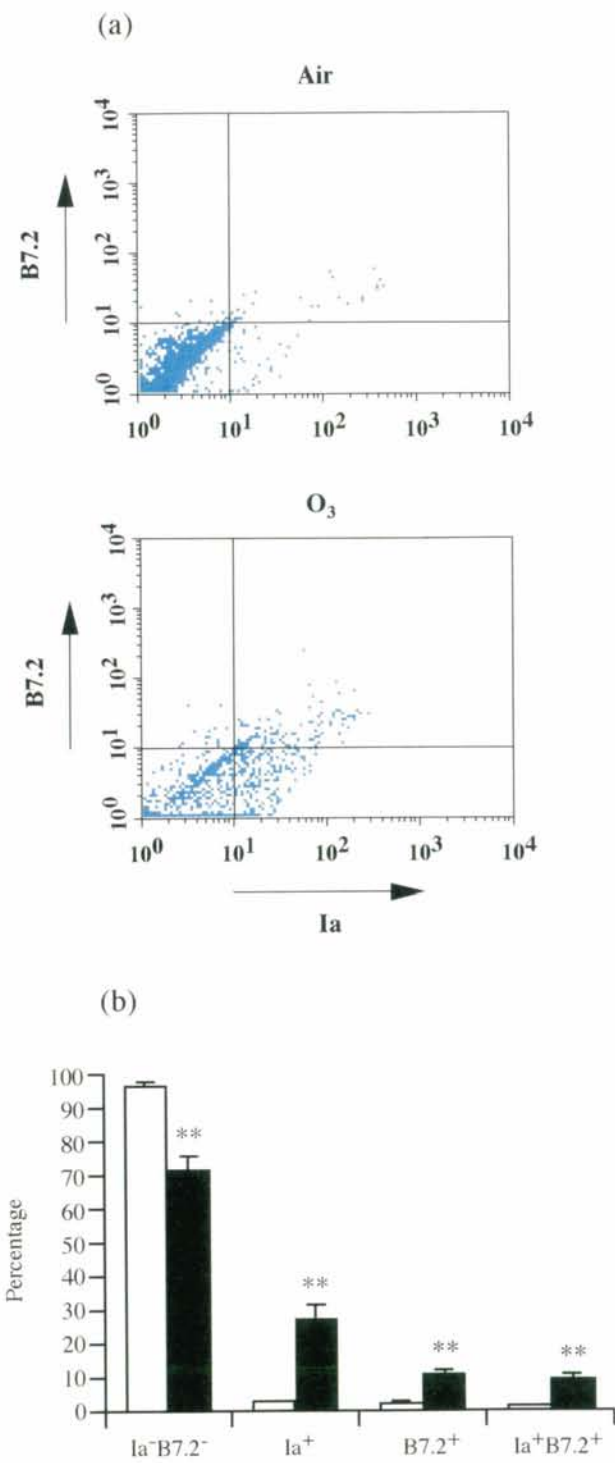


Fig. 3

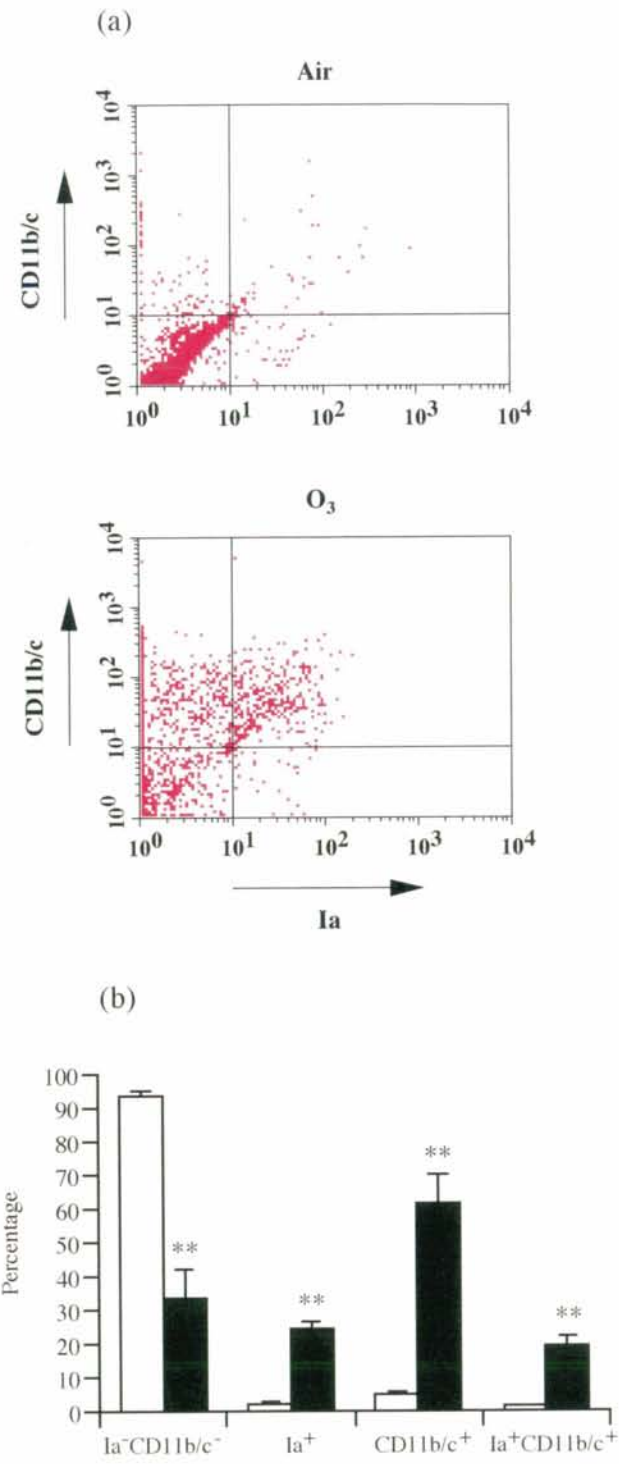


Fig. 4

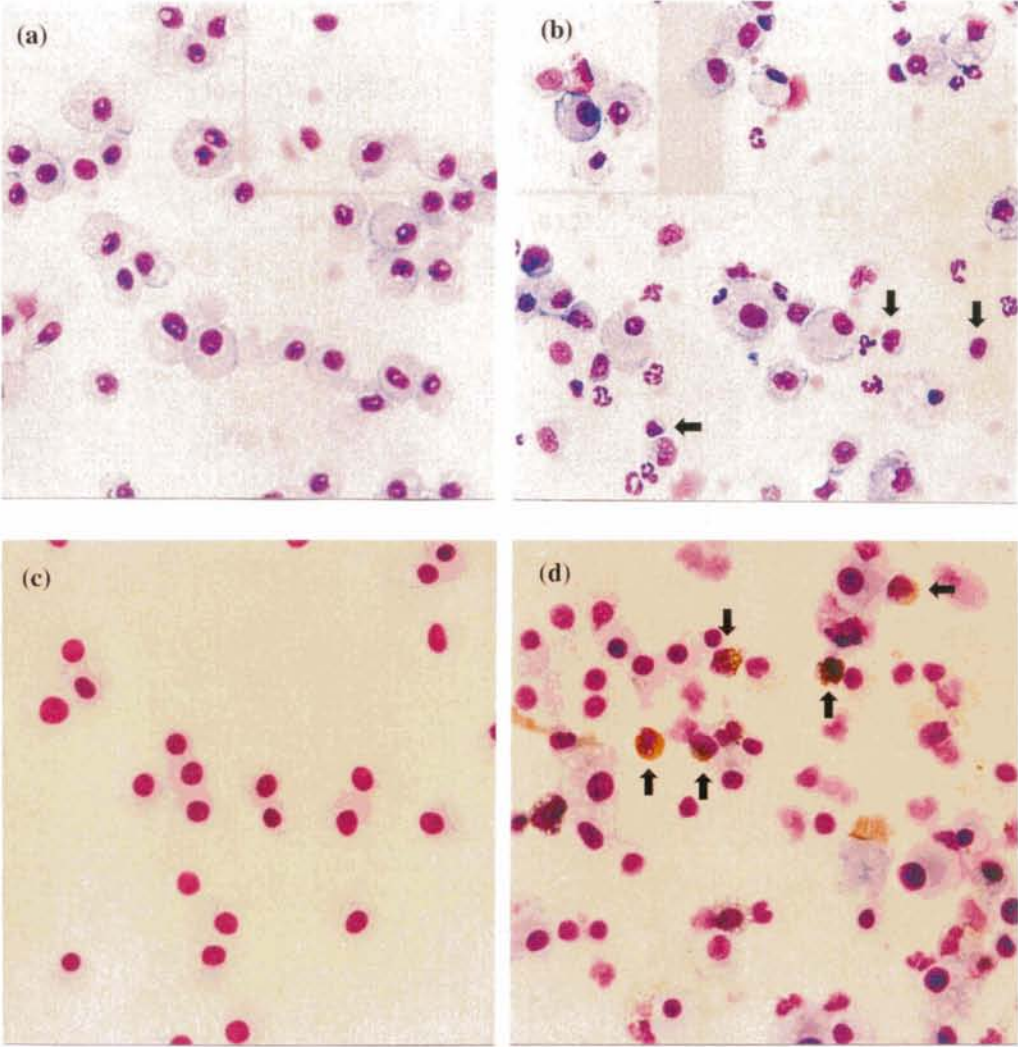


Fig. 5

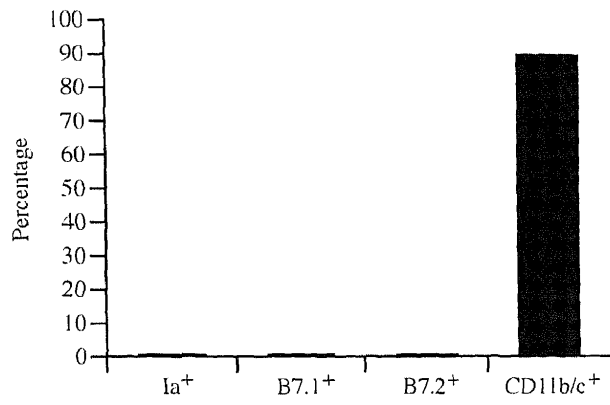


Fig. 6

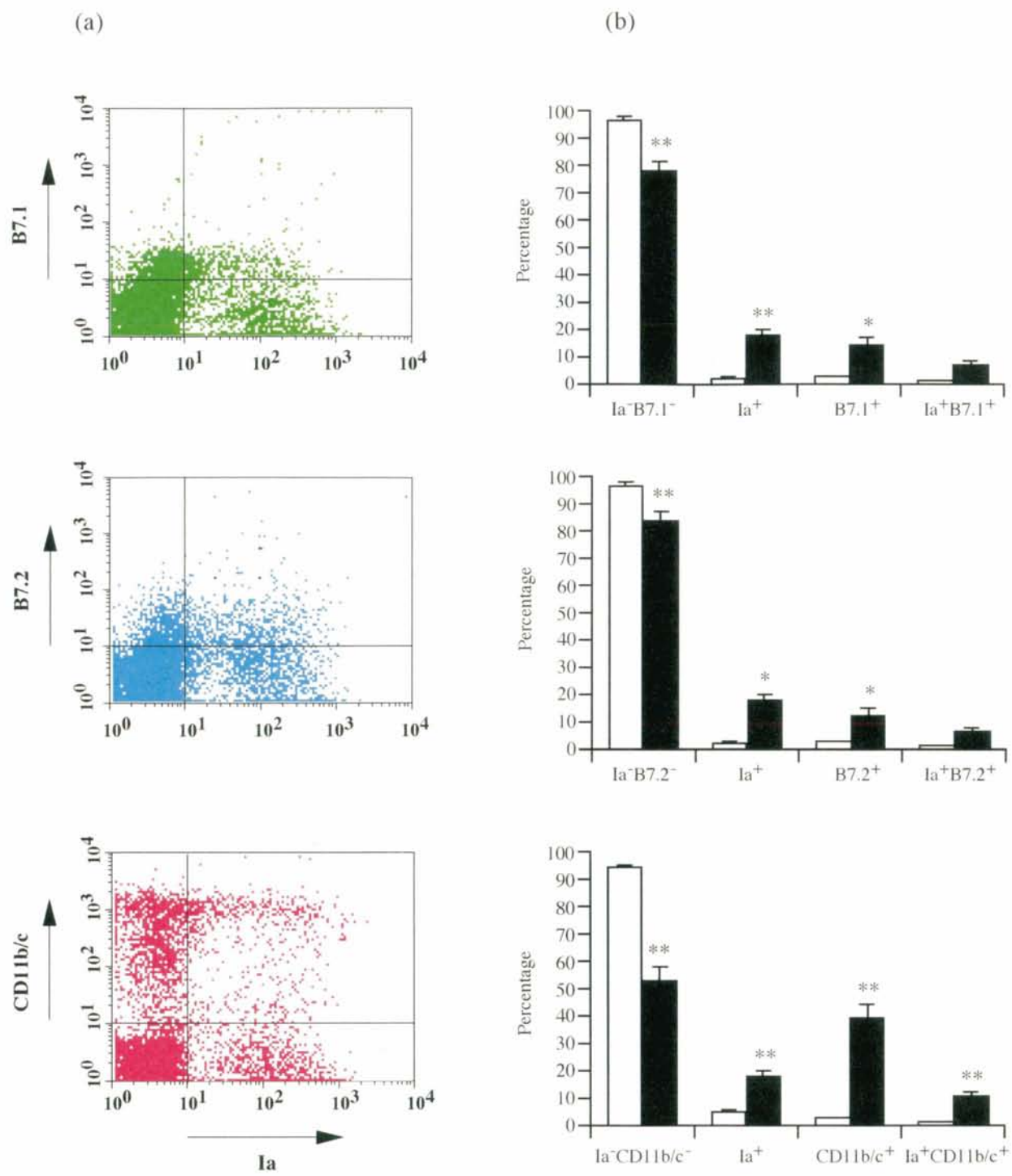


Fig. 7

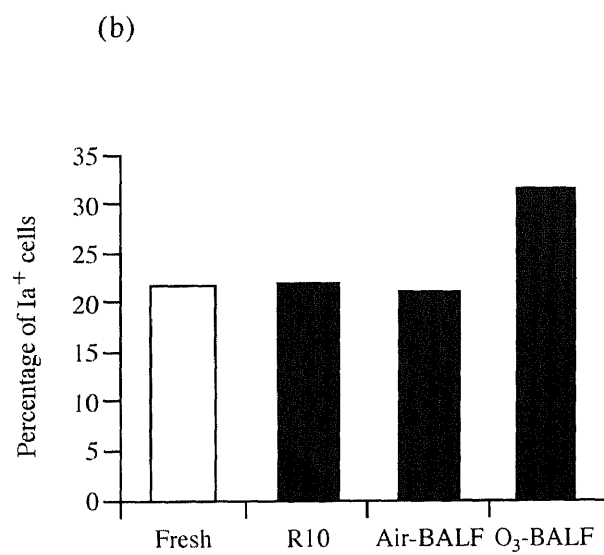
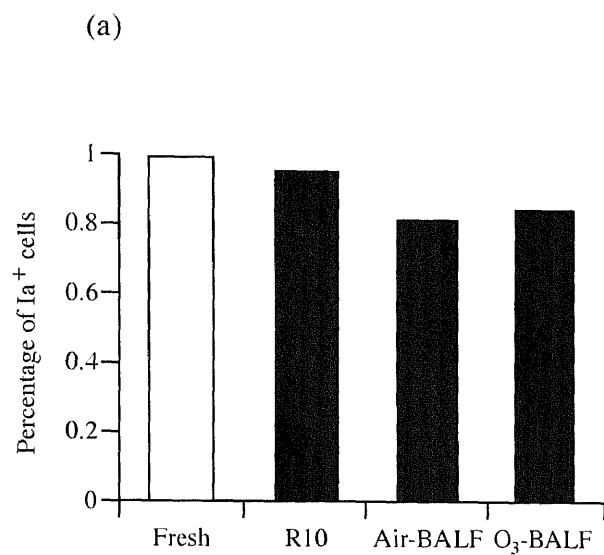


Fig. 8

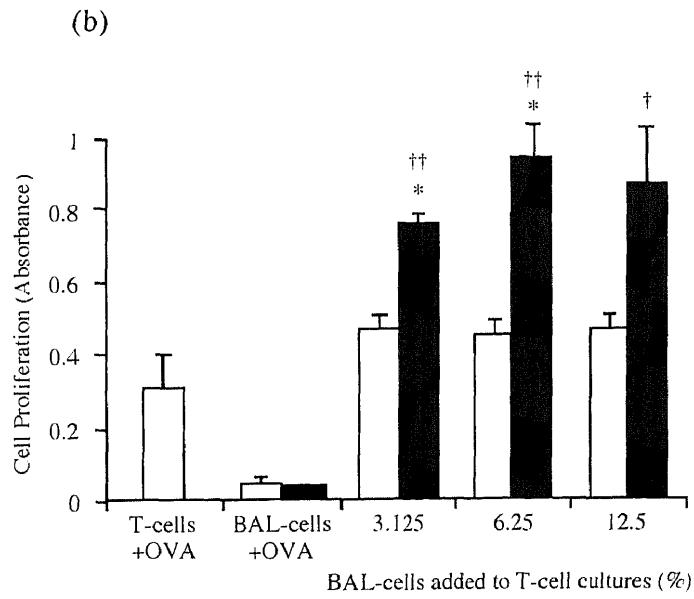
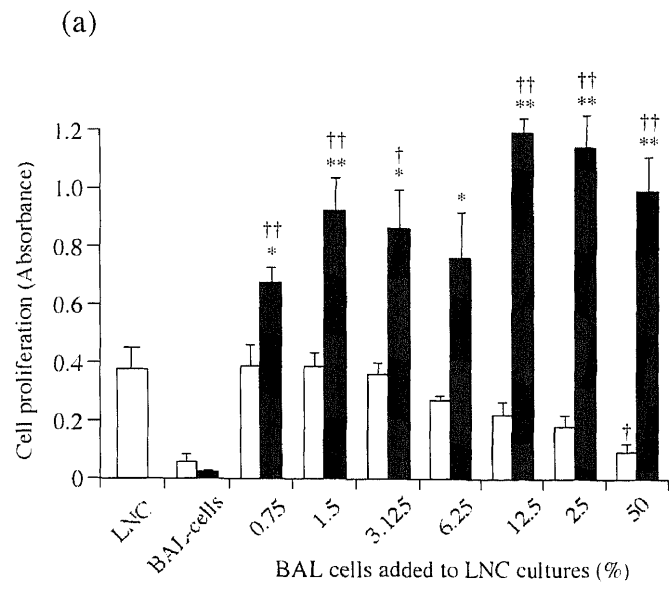


Fig. 9