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ORIGINAL ARTICLE

Yukiko Sumi · Hiroshi Nagura · Makoto Takeuchi
Masasumi Miyakawa

Granulomatous lesions in the lung induced by inhalation of mold spores

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Abstract The health hazards associated with grain dust exposure have been recognized as a cause of lung diseases. In the present study, we used germ-free rats exposed to *Aspergillus versicolor* to elucidate the mechanism for the lung damage induced by grain dust exposure. One month after exposure to the mold, remarkable proliferation of bronchus-associated lymphoid tissues with germinal centres was induced by aspiration of mold spores. After 1 month, alveolar macrophages increased, becoming foamy macrophages by ingestion and digestion of mold spores. They expressed interleukin (IL)-1, Ia antigens and intercellular adhesion molecule-1 intensely and occasionally bound lymphocytes. Numerous lymphocytes infiltrated the granulomatous lesions which consisted of accumulated foamy macrophages and some T lymphocytes which carried IL-2 receptor. Granulomatous lesions were identified in the entire lung, especially around bronchioles. They extended from alveolar ducts to alveolar spaces for 6 months after exposure to the mold. The macrophage appears to be a key effector cell in granulomatous reactions to inhaled molds.

Key words Lung granuloma
Bronchus-associated lymphoid tissue · Mold inhalation
Aspergillus versicolor · Germ-free rat

Introduction

Health hazards associated with grain dust exposure have been recognized and include Farmer's lung. There are large numbers of relevant case reports and series of hypersensitivity pneumonitis, invasive aspergillosis and granulomatous lesions associated with grain dust exposure in immunocompetent hosts [3, 5, 17, 35].

Although grain dust contains a variety of organic materials which have varied biological activity, the main antigens responsible for the lung damage have been shown to be molds, particularly *Aspergillus* species, and hypersensitivity reactions to antigens are important in pathogenesis [25, 28, 33, 39]. However, there are significant discrepancies in patho-histological findings and their interpretation in previous animal studies. This may be due to differences in the antigen, the animal species used, and duration and concentration antigen exposure [1, 8, 31, 32].

Aspergillus versicolor (*A. versicolor*) is quite common in the human environment and as a contaminant in various grains, such as wheat, rice and bean [6]. However, as far as we are aware, there is no previous report on the occurrence of lung lesions in experimental animals induced by exposure to the mold. We have speculated that co-existence of other microbes in the respiratory tract modulates the intrinsic character of inhaled molds and/or the host reaction to them through the mucosal immune system in the lung. The present study was undertaken to examine the response of the lung to the inhalation of this mold in the absence of other microbes, using germ-free rats.

Y. Sumi (✉) · M. Takeuchi · M. Miyakawa
Laboratory of Germfree Life,
Research Institute for Disease Mechanism and Control,
Nagoya University School of Medicine,
65 Tsuruma-cho, Showa-ku,
Nagoya 466, Japan

H. Nagura
Department of Pathology,
Tohoku University School of Medicine,
Sendai, Japan

Materials and methods

Germ-free Wistar rats were produced by our own technique [19], and have been maintained for more than 50 generations in plastic isolators in the author's laboratory. The diet was prepared in our laboratory; its composition has been reported previously [19], and the germ-free status was checked as described by Wagner [34].

A rubber capped test tube within which *A. versicolor* was cultured on Sabouraud dextrose agar was introduced into an autoclave attached to the germ-free isolator, and its surface was sterilized under peracetic acid spray. The tube was then transferred into the germ-free isolator, and the pellet diet therein was contaminated with *A. versicolor* alone. As time passed after the monoassociation, the mold not only grew on the diet but also was found everywhere in the isolator. The colonies were observed on the inner surface of the plastic film of the isolator. Five months later, float-

ing conidiospores (6±4/ml air) were recognized at the outlet within the isolator.

Twenty germ-free male rats of 30 days of age were transferred into the moldy isolator, and maintained under these condition as experimental rats, while 10 germ-free rats as untreated control were maintained in the germ-free condition. Five rats were sacrificed by ether anaesthesia at 1, 2, 3, and 6 months after exposure to the mold, with 2 control germ-free rats.

Tissue specimens were taken from whole lobes of the lungs. They were fixed in 10% formalin immediately after the sacrifice, and embedded in paraffin. Sections were stained with haematoxylin and eosin for histological examination, and chromic acid-Schiff for the mold. Cryostat sections of formalin-fixed frozen tissues were stained with Sudan III for neutral fat.

For immunohistochemical examination, lung tissues were fixed in periodate-lysine-4% paraformaldehyde [16] for 6 h at 4° C, washed in increasing concentrations of sucrose in phosphate-buffered saline, and finally placed in 20% sucrose. The fixed specimens were embedded in OCT compound (Miles Scientific, Naperville, Ill.), frozen in dry-ice/ethanol, and sectioned at 6 μm thickness on a cryostat microtome. Sections were placed on albumin-coated slides and dried in air for 30 min.

Monoclonal antibodies (mAb), W3/25 (CD4), OX-8 (CD8), OX-19 (CD5), OX-39 [interleukin (IL)-2 receptor] and OX-6 [major histocompatibility complex (MHC) class II-Ia] were purchased from Sera Lab (Cosmo Bio, Tokyo), ED-1 (monocytes, macrophages, dendritic cells) and R73 ($\alpha\beta$ T cell receptor) were from Serotec (Cosmo), and mAb to intercellular adhesion molecule (ICAM)-1 (CD54) were from Seikagaku Kogyo, Tokyo. Polyclonal Ab to rat IgM, IgG and IgA were purchased from Cappel (Cosmo), and human IL-1 from Genzyme (Cosmo). Anti-human joining (J) chain was kindly donated by Prof. K. Kobayashi (Hokkaido University). Antisera to human IL-1 and J chain had been previously proved to react with relevant rat antigen [15]. Antibodies and their optimum dilution used in the present study are listed in Table 1.

Antigens in the tissue sections were localized by the immunoperoxidase method as described previously [23]. The monoclonal antibodies were stained by the avidin-biotin-complex variant method, using a biotin-streptavidin amplified system purchased from Bio Genex (Seikagaku Kogyo). The others were stained by the indirect enzyme-labelled antibody method.

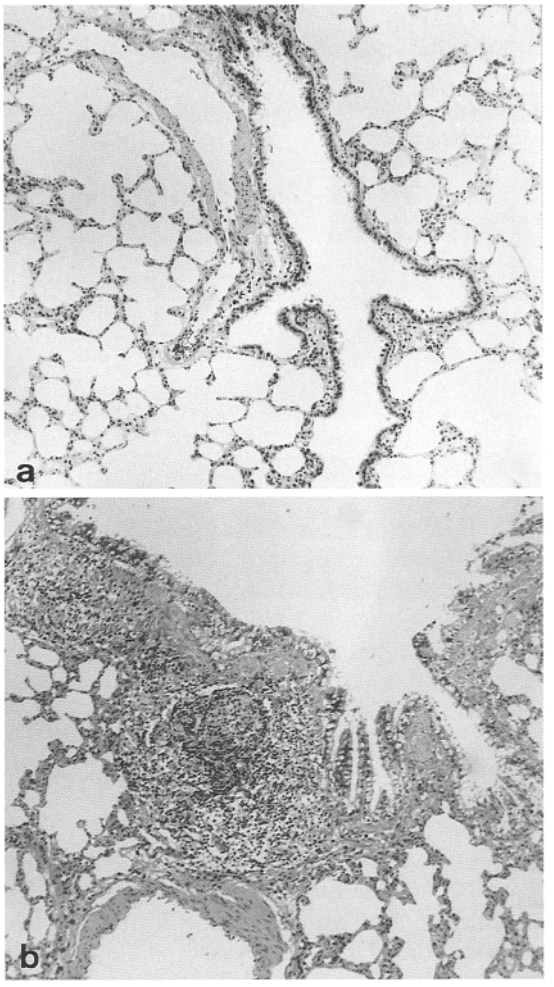

Fig. 1a, b Control germ-free rat, 7 months of age. Neither inflammatory reaction nor germinal centre development in bronchus-associated lymphoid tissue (BALT) is identified, (×200)

Results

In control germ-free rats, there were no inflammatory reactions and oedematous changes, nor germinal centre de-

Table 1 Antibodies used in the study (*IL* interleukin, *MHC* major histocompatibility molecule, *ICAM* intercellular adhesion molecule, *J* joining)

Antibodies	CD	Specificities	Dilution
W3/25	CD4	Helper/inducer T, macrophages	1:1500
OX-8	CD8	Suppressor/cytotoxic T	1:1500
OX-19	CD5	Pan T	1:1500
OX-39	CD25	IL-2 receptor	1:1500
OX-6		MHC class II (Ia)	1:2000
ED-1		Monocytes, macrophages, dendritic cells	1:1500
R73		$\alpha\beta$ T cell receptor	1:1500
ICAM-1	CD54	ICAM-1	1:1500
Anti-rat IgA		Rat IgA (α chains)	1:400
Anti-rat IgG		Rat IgG (γ chains)	1:400
Anti-rat IgM		Rat IgM (μ chains)	1:400
Anti-human IL-1		Reacts with rat IL-1	1:400
Anti-human J chain		Reacts with rat J chain	1:200

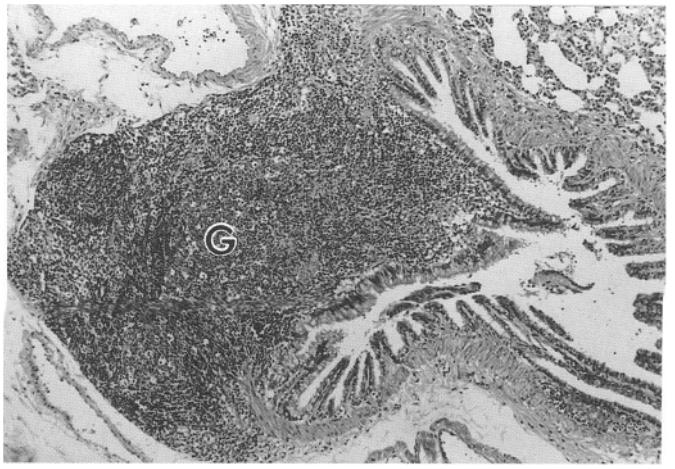

Fig. 2 BALT with a germinal centre (*G*) in the germ-free rat, 1 month after exposure to *A. versicolor*, (×130)

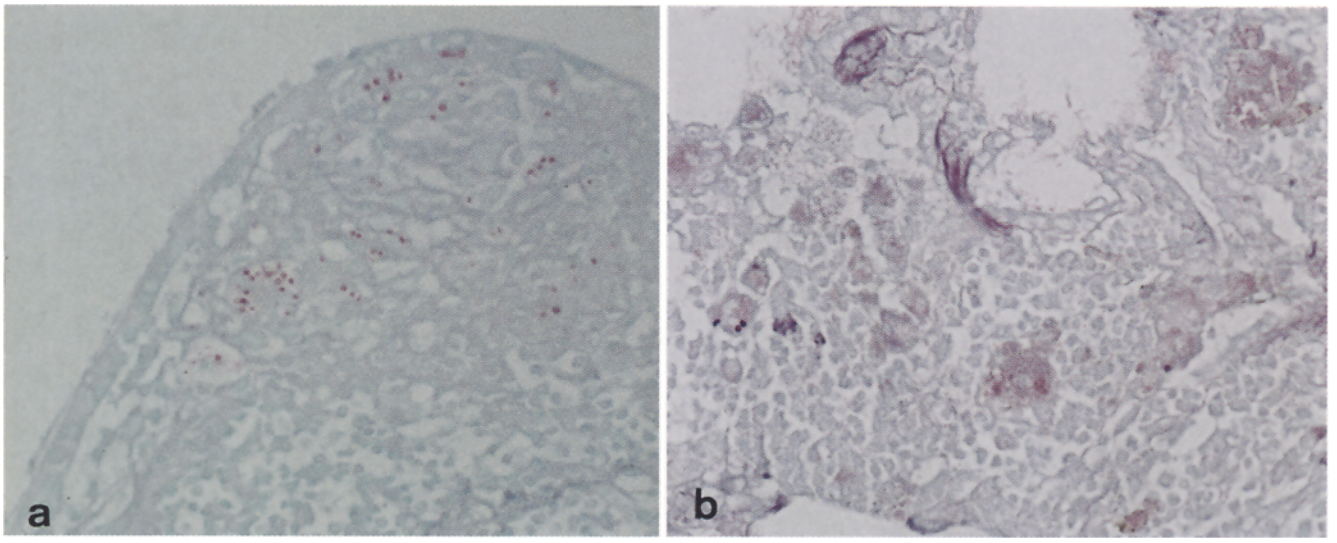

Fig. 3 **a** Mold spores ingested by macrophages in BALT, 1 month after exposure to the mold. **b** Mold spores are ingested and digested by macrophages. The cytoplasm of these foamy macrophages is stained diffusely in pink, 6 months after exposure to the mold, (chromic acid-Schiff stain, ×700)

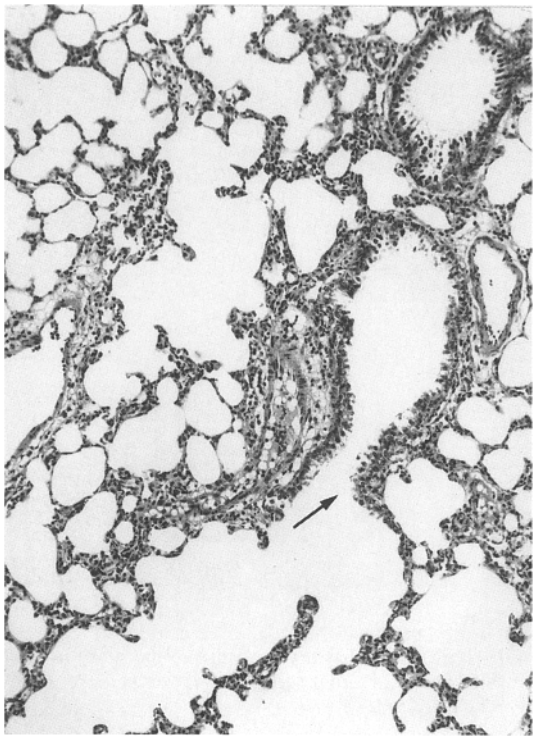

Fig. 4 One month after exposure to the mold, the alveolar structure is well preserved, and mononuclear cells are present in the alveolar wall and around the alveolar duct (*arrow*), (×250)

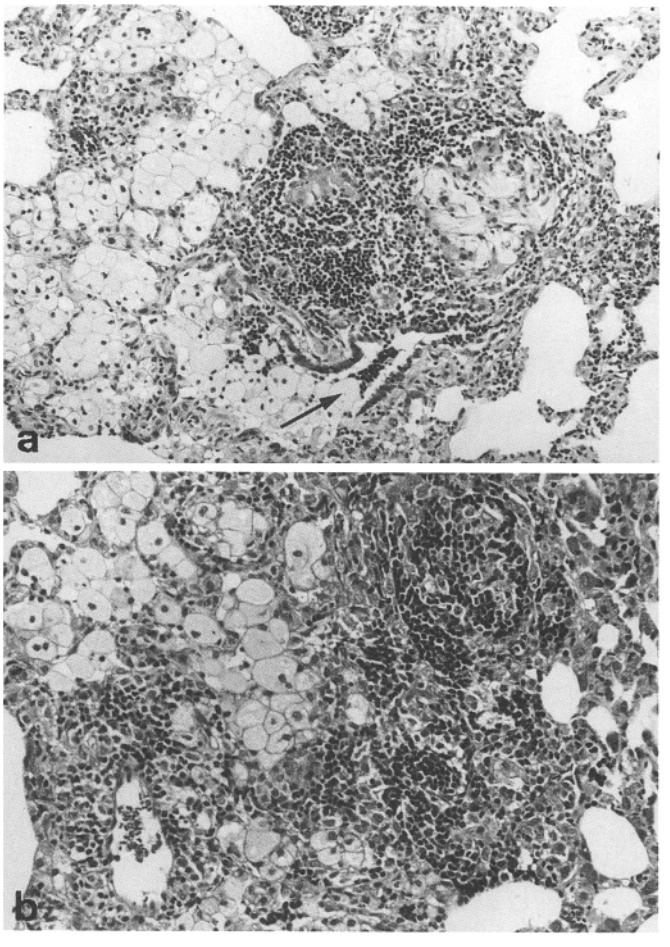

Fig. 5 **a** Granuloma composed of foamy macrophages and lymphocytes around the bronchiole, 6 months after exposure to the mold, (×260). **b** Another granulomatous lesion, 3 months after exposure to the mold in higher magnification, (×400)

velopment in the bronchus-associated lymphoid tissue (BALT) (Fig. 1).

One month after exposure to the mold, remarkable proliferation of BALT with germinal centres formation was observed (Fig. 2). Chromic acid-Schiff staining revealed that macrophages ingesting mold spores migrated into BALT through overlying lymphoepithelium (Fig. 3a). Macrophages increased moderately in perivascular areas adjacent to bronchioles and alveolar walls (Fig. 4). A few lymphocytes also infiltrated but granulocytes were

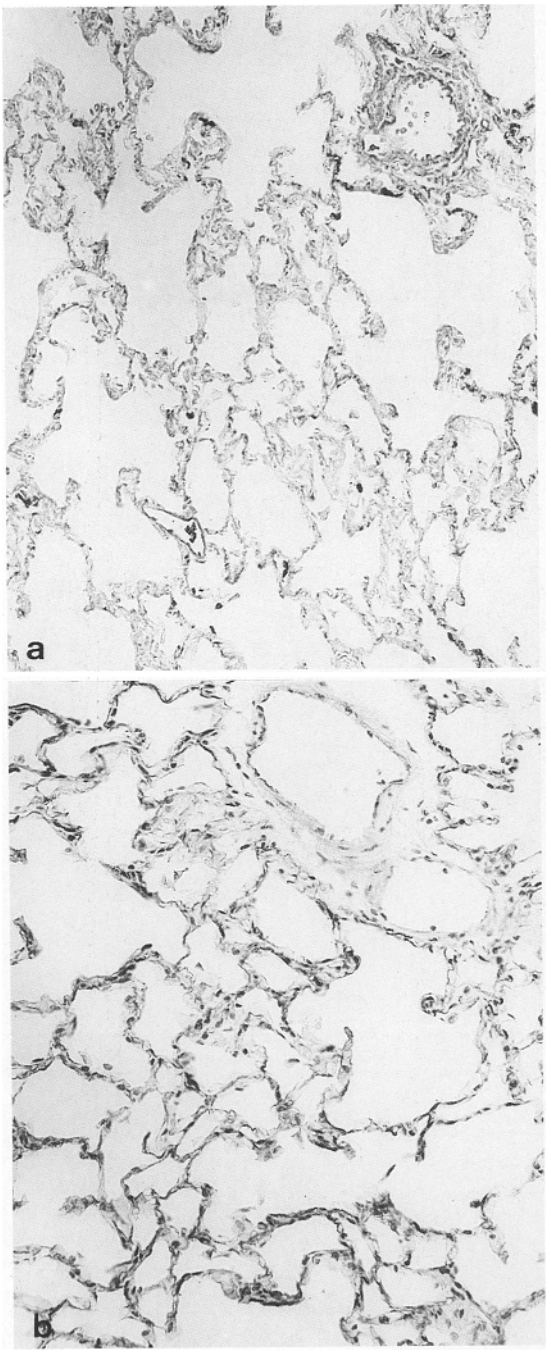

Fig. 6 Immunohistochemical localization of interleukin (IL)-1 (**a**), and intercellular adhesion molecule (ICAM)-1 (**b**) in the control germ-free rat, 7 months of age. Blood vessels in alveolar septa are positive for IL-1 and ICAM-1, but alveolar macrophages are negative for both antigens, (×500)

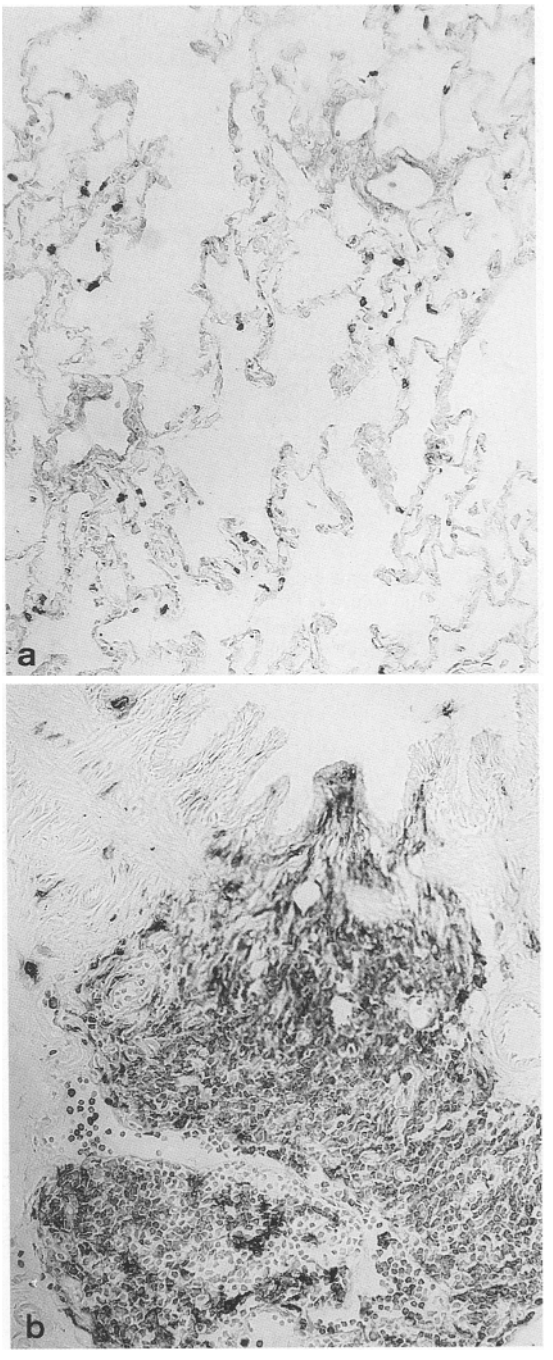

Fig. 7a, b Immunohistochemical localization of IL-1$^+$ and Ia$^+$ cells in the lung 1 month after exposure to the mold. **a** IL-1$^+$ cells are infiltrated in the alveolar septum. **b** Ia$^+$ cells increase in BALT, (×500)

scarce in this lesion. The alveolar structure was well preserved, except for oedematous changes in the perivascular area.

Two months later, numerous lymphoid follicules appeared in the more distal peribronchiolar area. The lumen of bronchioles was narrowed by the protrusion of the mucous membrane into the lumen with conspicuous mononuclear cell infiltration.

Alveolar macrophages increased in the inflamed lesion and vacuolated to become foamy macrophages. There was massive accumulation of these macrophages in distal air spaces and alveolar septa. Mold spores were ingested by these macrophages whose cytoplasm was stained diffusely in pink by chromic acid-Schiff, suggesting that spores had been digested (Fig. 3b). In addition, the cytoplasm was filled with numerous neutral fat

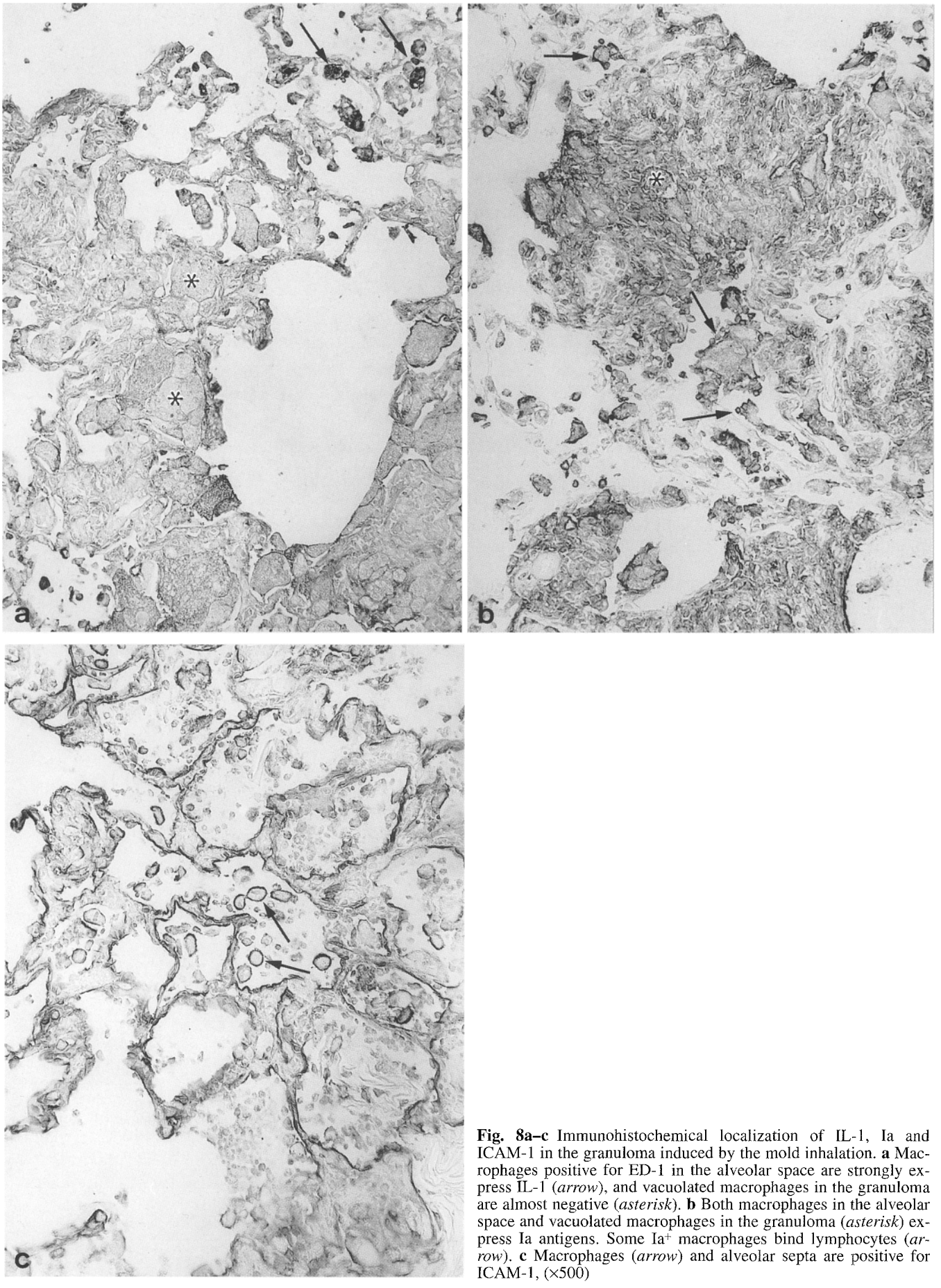

Fig. 8a–c Immunohistochemical localization of IL-1, Ia and ICAM-1 in the granuloma induced by the mold inhalation. **a** Macrophages positive for ED-1 in the alveolar space are strongly express IL-1 (*arrow*), and vacuolated macrophages in the granuloma are almost negative (*asterisk*). **b** Both macrophages in the alveolar space and vacuolated macrophages in the granuloma (*asterisk*) express Ia antigens. Some Ia^+ macrophages bind lymphocytes (*arrow*). **c** Macrophages (*arrow*) and alveolar septa are positive for ICAM-1, (×500)

Table 2 Changes in macrophage population in the lung after mold inhalation

	Ia	IL-1	ICAM-1	
Macrophages[a] in alveolar spaces				
Germ-free	–	–	–	Few not vacuolated
One month after mold exposure	±[c]	±[c]	–	Few not vacuolated
Two months after mold exposure	+	+ or –[b]	+	Many vacuolated
Macrophages[a] in granuloma	+	+ to –[b]	±	Vacuolated

[a] ED-1 positive
[b] Accumulated macrophages in alveolar spaces and within the granulomas
[c] Weakly positive

droplets positive for Sudan III. Numerous lymphocytes infiltrated around this granulomatous lesion, consisting of foamy macrophages (Fig. 5). Such lesions appeared in the entire lung, especially around bronchioles, and extended from alveolar ducts to alveolar spaces until 6 months after exposure to the mold. There was no evidence of lung fibrosis.

Hyphal form of *A. versicolor* was not found in or out of the granuloma.

In germ-free rats, alveolar macrophages stained for ED-1 were few in number and small in size, and almost negative for IL-1, Ia, and ICAM-1 (Fig. 6). A very few immunoglobulin-positive cells were recognized in the peribronchiolar area.

One month after exposure to the mold, IL-1$^+$ monocytes and granulocytes were infiltrated in the alveolar septum and space. Class II MHC$^+$ (Ia$^+$) cells increased in BALT, but slightly in perivascular areas and alveolar septa (Fig. 7). Macrophages found free in alveolar spaces expressed Ia antigens weakly.

Two months later, macrophages stained by mAb ED-1 increased in number and vacuolated. They were strongly positive for IL-1 in their cytoplasm and plasma membrane, although, among these large vacuolated macrophages, aggregated ones in alveolar spaces and in the granulomas were IL-1 negative. They expressed Ia antigens and ICAM-1 intensely, and occasionally bound lymphocytes which were Ia$^+$ or Ia$^-$, W3/25$^+$ or OX-8$^+$ (Fig. 8). These macrophage-lymphocyte clusters were seen throughout the lung. The changes in macrophage population in the lung after mold inhalation are summarized in Table 2. IgM-, IgG-, and IgA-positive cells increased in BALT, peribronchiolar areas and alveolar septa. The surface of bronchioles and blood vessels were strongly stained with anti-IgG and anti-IgA antibodies. The granulomas were composed of Ia$^+$ macrophages, and T and B lymphocytes. T lymphocytes included both CD4 (W3/25$^+$) and CD8 (OX-8$^+$) subsets, but W3/25$^+$ lymphocytes were predominant. Moreover, the majority of T lymphocytes beared $\alpha\beta$ receptor, and some of them ex-

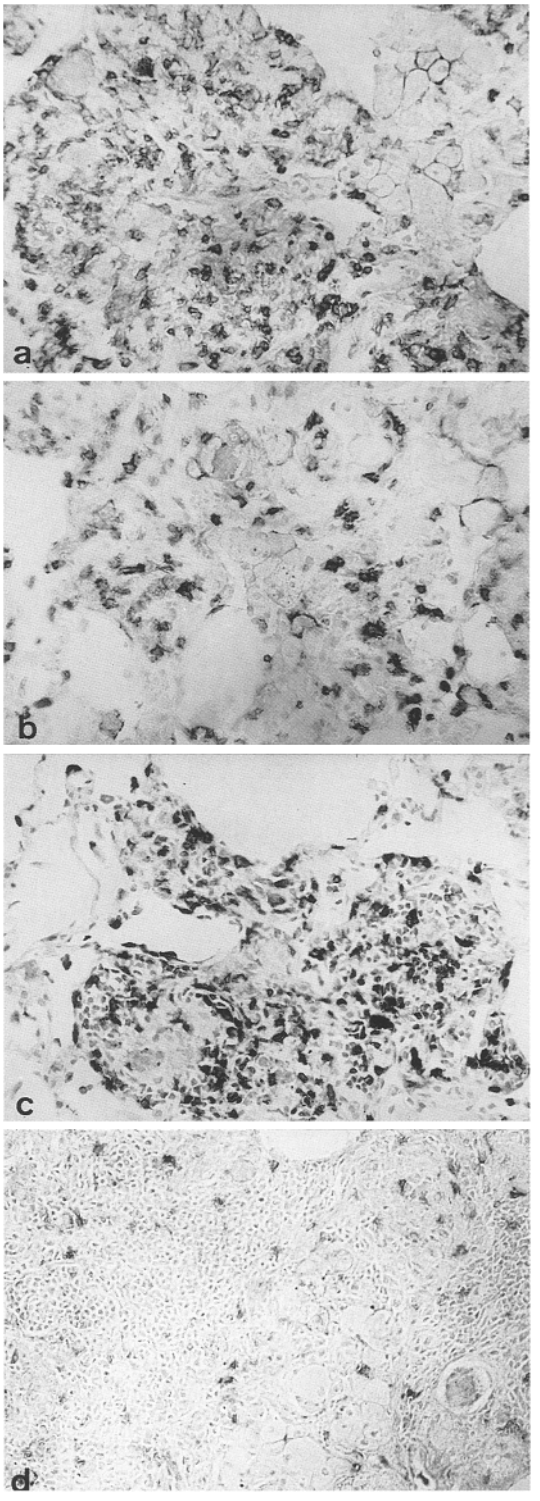

Fig. 9a–d Immunohistochemical localization of W3/25$^+$ (**a**), OX-8$^+$ (**b**), $\alpha\beta$ T cell receptor$^+$ (**c**), and IL-2 receptor$^+$ (**d**) cells in the granuloma. T lymphocytes include both CD4 (W3/25$^+$) and CD8 (OX-8$^+$) subsets. The majority of them bear $\alpha\beta$ receptor and some of them express IL-2 receptor, (×510)

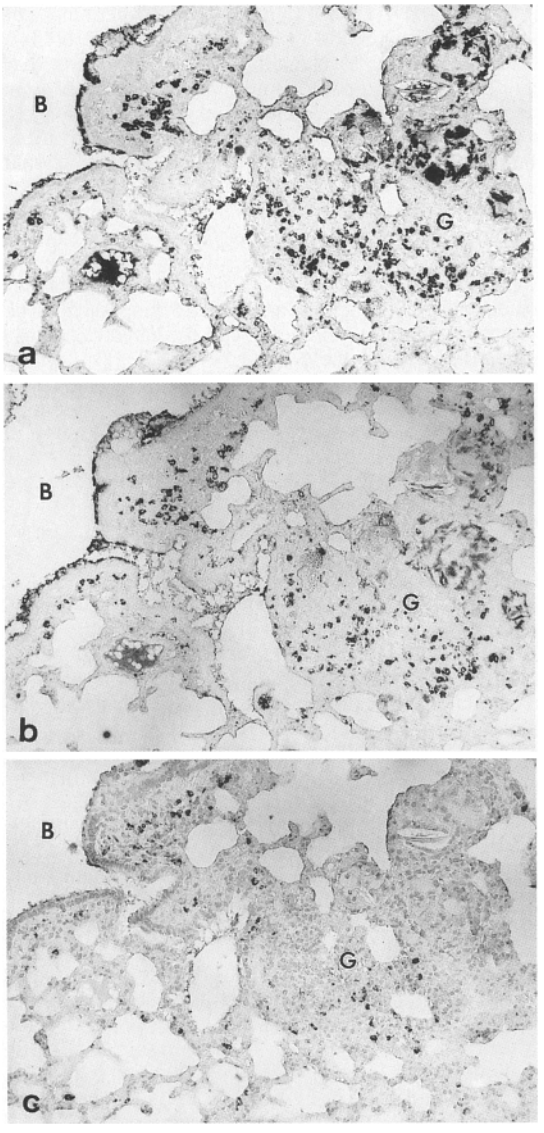

Fig. 10a–c Immunohistochemical localization of IgG$^+$ (**a**), IgA$^+$ (**b**), and joining (J) chain$^+$ (**c**) cells in the granuloma (*G*) around the bronchiole (*B*). Large numbers of IgG$^+$, IgA$^+$ and J chain$^+$ cells are scattered in the granuloma. The surface of bronchiole is strongly stained with anti-IgG and anti-IgA antibodies, (×260)

pressed IL-2 receptors (Fig. 9). Large numbers of IgM$^+$, IgG$^+$, IgA$^+$, and J chain$^+$ cells were scattered in the granulomas (Fig. 10).

Discussion

In the early stage of mold exposure, BALT hyperplasia and development of the germinal centres were characteristic findings. Macrophages phagocytizing mold spores were recognized in BALT, suggesting that mold spores entered into the BALT through the covering lymphoepithelium. Specialized membrane (M) cells within the lymphoepithelial layer are capable of sampling various antigens as for example, the M cells of Peyer's patches [24]. Using inhaled horseradish peroxidase and bacillus Calmette-Guérin, Rácz et al. reported that the lymphoepithelium over BALT represented a major route of antigen entry to the lung [26, 27]. Uptake of the mold by M cells might be the first line of immune reaction in the lung, followed by the enlargement of the BALT and appearance of macrophages ingesting mold spores. Two months after exposure, large vacuolated alveolar macrophages had increased, and macrophage-lymphocyte clusters and granulomas were formed.

It has been generally accepted that alveolar macrophages suppress blastogenic responses of lymphocytes to mitogens under a normal steady-state condition [2, 10, 11, 12, 13]. However, alveolar macrophages, if activated by some exogenous agents, have the potential to induce lymphocyte responses [4, 9, 14, 36]. In our present study, alveolar macrophages in control germ-free rats were devoid of secretion of IL-1 and expression of Ia antigens and ICAM-1. After mold inhalation, they secreted IL-1 and expressed Ia antigens and ICAM-1 intensely during the phagocytosis and processing of the mold spores. Occasionally these macrophages made clusters with many lymphocytes.

A number of investigations have suggested that proliferation of resting lymphocytes requires at least two extracellular signals [37]. The first signal is provided by binding of the T cell receptor to antigen associated with Ia molecules on the surface of accessory cells [30] or by cross-linking the receptor with mitogenic lectin or anti-receptor antibody [7, 29, 38]. The second signal is generated through the binding of the cytokine, IL-1 to its receptor on T lymphocytes [18, 20, 38]. Since large numbers of IgA plasma cells probably containing J chain and IgG plasma cells were recognized all over of the lung in our study, in addition to granuloma formation, it was postulated that B cells were also activated and maturated, and both mucosal and systemic immune responses were augmented [21, 22]. Thus, the macrophage appears to be a key effector cell in these granulomatous reactions to inhaled molds, and inhalation of the mold in the high concentration in environment may induce the lung damage even in the absence of microbial infection. The other components of grain dust, the influence of microbial infections, and the effect of immunosuppressants including steroid hormones on the pulmonary response are worthy of study.

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