Comparative ultrastructure and immunolabeling of MHC-II antigens of alveolar macrophages obtained from patients with paracoccidioidomycosis and other lung diseases

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Abstract: Samples of alveolar macrophages (AM) obtained by bronchoalveolar lavage from patients with either paracoccidioidomycosis, silicosis, sarcoidosis, or allergic alveolitis were investigated by electron microscopy and immunocytochemistry to compare cellular ultrastructure and expression of MHC-II antigens in the AM cell surface. All samples of AM obtained from patients with these pathologies showed heterogeneous structural features. Although, this morphological diversity is also present in AM of healthy donors, our observations seem to indicate that in the diseases studied this morphofunctional diversity is associated with additional ultrastructural characteristics inherent to each disease. In paracoccidioidomycosis the proportion of vacuolated macrophages is significantly lower than in other diseases; this might indicate that in paracoccidioidomycosis the proportion of activated AM is smaller. We observed significant differences in the expression of MHC-II antigens. Silicosis, sarcoidosis, and allergic alveolitis do not differ significantly in the quantity of immunolabeled AM or in the distribution of the label. The percentage of AM from paracoccidioidomycosis that exhibit the MHC-II molecule is very low with poor immunolabeling. In this disease the low expression of the MHC-II molecule could be related to a decrease of the antigen presenting function by AM. ***J. Leukoc. Biol.*** **57: 101-109; 1995.**

Key Words: *alveolitis • bronchoalveolar lavage • electron microscopy • sarcoidosis • silicosis*

INTRODUCTION

The macrophages, an accessory cellular type of the immune system, express a variety of molecules in their surface whose action is directed to the appropriate presentation of processed antigens in an immunogenic form to T cells [1-4]. These include antigens that belong to the major histocompatibility complex Class II (MHC-II) molecules: HLA-DR in humans and H-2 in mice. The expression of MHC-II molecules in the surface of mononuclear phagocytes is related directly to the clonal recognition of the antigens and to regulatory mechanisms of immune response caused by infection with microorganisms and other pathologic agents [5-8]. The expression of these molecules on macrophages can be induced and modified by infections or other stimuli [9-14].

Alveolar macrophages (AM) are important in pathological and physiological processes that occur in the lungs [9, 15, 16]. In addition to changes in expression of MHC-II molecules, AM also display morphological changes related to these processes [13, 17-20]. A specific digestive deficiency against *Paracoccidioides brasiliensis* has been observed in phagocytes of patients with paracoccidioidomycosis [21, 22]. In addition, the characterization of leukocyte immunophenotypes by means of light microscopy of cells obtained by bronchoalveolar lavage (BAL) from patients with paracoccidiodomycosis, sarcoidosis, and silicosis has shown differential expression of MHC-II molecules [14]. Our goal was to detect MHC-II molecules on the surface of AM by immunolabeling techniques and to study ultrastructural features of BAL macrophages obtained from patients with paracoccidioidomycosis, sarcoidosis, silicosis and allergic alveolitis.

MATERIALS AND METHODS

Antibodies and immunolabels

As primary antibody we used a monoclonal antibody that recognizes MHC-II antigens, (B33.1.1, HLA-DR, kindly donated by Dr. Giorgio Trinchieri (Wistar Institute of Pathology, Philadelphia). The primary antibody was diluted 1:40 with fresh 0.01 M phosphate-buffered saline (PBS), 0.14 M sodium chloride, pH 7.2. The diluted antibodies were divided into 0.5 ml aliquots and stored at −20°C until use.

Goat antimouse antibodies (GAM; Amersham Intl., Amersham, UK) coupled to colloidal gold, 10 nm mean particle diameter (GAM-G10), were obtained as follows: 2.5 ml of 1% trihydrated tetrachloroauric acid stock solution (Riedel deHaën, Seelze, Hannover, Germany), in double glass distilled water was reduced to colloidal gold particles of 10 nm average diameter, with 247 ml of a 0.061% solution

Abbreviations: AM, alveolar macrophages; BAL, broncho alveolar lavage; B-GAM, biotinylated GAM; BHI, brain-heart infusion liquid medium; BSA, bovine serum albumin; FCS, fetal calf serum; GAM, goat antimouse antibodies; GAM-G10, GAM coupled to colloidal gold; HBSS, Hanks' balanced salt solution; HHG, HBSS-heparin-gentamicin; HHGFCS, HHG+20% FCS; MHC-II, major histocompatibility complex Class II; PBS, phosphate-buffered saline; PBS-BSA, PBS+1% BSA; St-G10, streptavidine-colloidal gold.

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of dihydrated trisodic citrate in boiling distilled water, according to the procedure modified by De Mey [23]. The complex GAM-G10 was maintained without dilution in an amber-colored flask at 4°C until use.

A solution of biotinylated GAM (B-GAM) (Amersham Intl.) was diluted 1:100 in PBS, divided into aliquots of 0.3 ml, and kept at −20°C until use. Streptavidine-colloidal gold, 10 nm mean particle diameter (St-G10) (Sigma Chemical Co., St. Louis, MO), was diluted 1:4 in PBS and kept in aliquots of 0.5 ml at 4°C until use.

Fungi

Viable yeast phase *P. brasiliensis,* isolate 6688 (48092, American Type Culture Collection, Rockville, MD) was grown in brain-heart infusion liquid medium (BHI) and harvested at the end of the log phase. Single-cell suspensions were obtained by mild sonication [24] and the fungi were processed to study their interaction with the pulmonary macrophages, according to the method for neutrophils described by Goihman-Yahr et al. [21]. A final concentration of 2×10^6 organisms per ml was used in all experiments.

Patients and cell sampling

Patients were studied at the Instituto Nacional de Tuberculosis, Caracas. Bronchoscopy was medically indicated in all cases and procedures were approved by the Ethics Commission of the Department of Tuberculosis and Lung diseases of the Ministry of Health of Venezuela. Individuals had either paracoccidioidomycosis (n = 10), silicosis (n = 15), sarcoidosis (n = 5), or alveolitis (n = 5). Criteria of classification

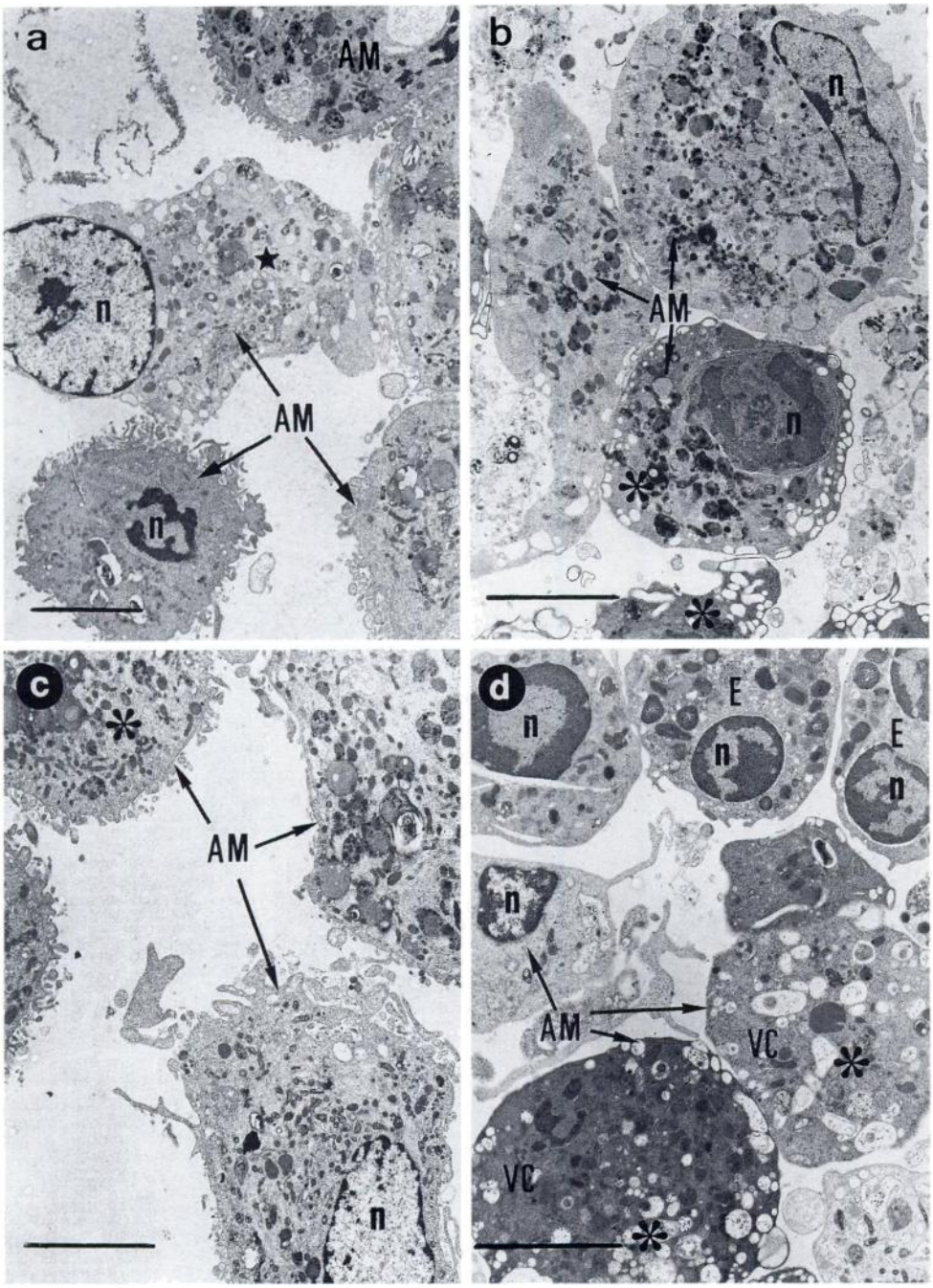

Fig. 1. Diversity of cellular structure of AM obtained from paracoccidioidomycosis (a), silicosis (b), sarcoidosis (c), and alveolitis (d). Clear cell (★) is observed in (a). Electron-dense cells (*) can be seen in (b), (c), and (d). Multivesiculated cells are present in (a), (b), and (c), and multivacuolated cells (VC) only are observed in (d). Microvilli and nuclear shapes also are very heterogeneous in all samples. N, nucleus; AM, alveolar macrophages; VC, vacuolated cell; E, eosinophil. Bars = 5 μm.

were those of Franco et al. [25], Dauber [26], Rossman and Dauber [27], and Shellito [28], respectively. Most cases were of chronic (adult) paracoccidioidomycosis, chronic or accelerated silicosis, stage II sarcoidosis, and allergic alveolitis. For quantitative determinations fewer patients were analyzed (see Results section). Most patients were moderate or severe smokers. Normal individuals were not included because of ethical reasons. In contrast to the practice in many countries, the Ethics Commission of the Instituto Nacional de Tuberculosis did not approve bronchoscopy and BAL of normal volunteers. In addition, volunteer recruiting is not commonly approved in Venezuelan medical practice.

BAL was done by the method of Goihman-Yahr, as outlined in Tapia et al. [14]. Warm sterile heparinized Hanks' balanced salt solution (HBSS) was used as the lavage fluid and samples were maintained in a cold medium composed of 4% fetal calf serum (FCS; GIBCO, Grand Island, New York), gentamicin, and Medium 199 (GIBCO) until final processing. The cellular suspensions were washed five times in HBSS-heparin-gentamicin (HHG) and 20% FCS (HHGFCS) [29]. Aliquots of the samples were adjusted to 1×10^6 cells per ml in HHG, cytocentrifuged, and stained with Wright-Giemsa and cell types counted. Mean percentage of macrophages exceeded 80% in all groups except for alveolitis, where it was 65%. A viability test using erythrosin B [30] was routinely performed. The BAL cell suspension was standardized to 1×10^7 cells per ml in HHGFCS and divided into three fractions that were processed as follows: (1) Fraction A, at time 0, constituted by 0.25 ml BAL cell suspension and 0.75 ml HHGFCS medium, processed immediately for immunolabeling; (2) Fraction B with 0.25 ml BAL cell suspension, 0.5 ml HHGFCS medium, and 0.25 ml autologous serum, incubated 2.5 h at 37°C; and (3) Fraction C with 0.25 ml BAL cell suspension, 0.25 ml HHGFCS medium, 0.25 ml ABO compatible serum, and 0.25 ml isolate 6688, incubated 2.5 h at 37°C. After incubation all samples were processed for the immunolabeling procedure.

Immunolabeling procedure

Incubated BAL cell suspensions were centrifuged at 1500*g* for 10 min at room temperature, the supernatant was discarded and the cellular sediment prefixed in 0.5 ml 2.5% glutaraldehyde (Ladd Research Industries Inc., Burlington, VT) in PBS 0.1 M, pH 7.2, 30 min at room temperature. The prefixed sediments were washed three times in 5 ml PBS with 1% bovine serum albumin (BSA) (Sigma) and incubated overnight at 4°C, in 100 μl anti-MHC-II diluted 1:40 in PBS. The cells were washed extensively in PBS-BSA and incubated in 100 μl B-GAM, diluted 1:100 in PBS, for 60 min at room temperature. Immediately thereafter, the cells were again washed three times in PBS-BSA and incubated in 50 μl St-G10, diluted 1:4 in PBS, for 60 min at room temperature [31].

Some BAL cell sediments were immunolabeled with GAM-G10: cells previously incubated with anti-MHC-II and washed in PBS-BSA were incubated overnight at 4°C with 50 μl GAM-G10, diluted 1:4 in PBS.

In all cases, cell sediments incubated directly with B-GAM and St-G10 only or with GAM-G10 only were included as controls. We also included controls with St-G10 diluted adequately in PBS. All immunolabeled samples of BAL were washed and processed for electron microscopy.

Electron microscopy

Samples from all patients were kept separate. All immunolabeled cell sediments and their respective controls were postfixed in 0.3 ml 1% osmium tetroxide (Ladd) in distilled water for 1 h at room temperature. Samples were then centrifuged at 1500*g* and supernatant was extracted with a micropipet without disturbing the sediment; cells were washed three times in distilled water. The cell sediments were carefully detached from the bottom of centrifuge tubes with fine wood applicators, placed in small vials and dehydrated in increasing concentrations of ethanol and propylene oxide.

The samples were impregnated and polymerized in LX-112 epoxy resin (Ladd). Block sections of 0.2–0.5 μm of all samples were obtained, mounted on glass slides, and stained with toluidine blue for cell type evaluation. Blocks of all samples were sectioned at 40–90 nm with a Porter-Blum MT-2B ultramicrotome. Sections were taken at different levels in the sediment inclusions of all blocks. Sections were mounted on 200 mesh copper grids and counterstained with uranyl acetate and lead citrate. The mounted sections were observed and photographed with a transmission electron microscope (Hitachi H; 500 to 75 KV). Quantitative evaluations of cell types were also carried out on paper prints at ×3750 and ×4500 magnification. Only cells with a nuclear profile were counted. A cell was judged as labeled when at least 10 individual particles or patches of two or more colloidal gold particles were present throughout the external cellular membrane.

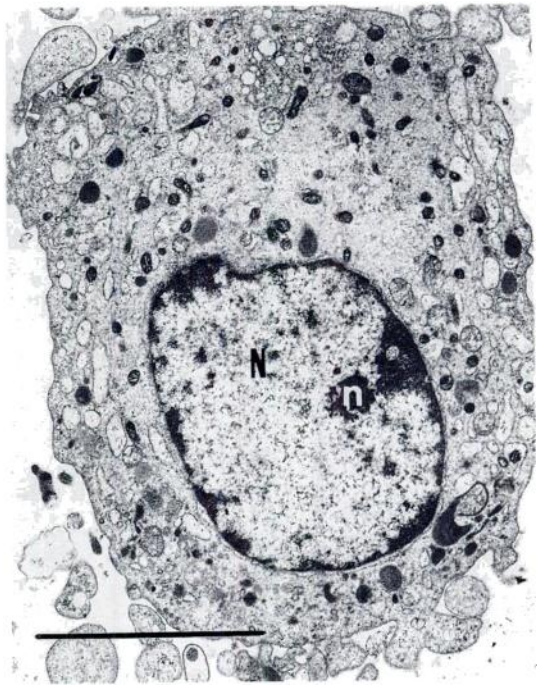

Fig. 2. Section of AM obtained from alveolitis. The cell shows a clear cytoplasm with small and electron dense mitochondria. Vesicles and granules can be seen dispersed in the cytoplasm. Numerous vacuoles are disposed in the periphery of the cell. The microvilli in the plasma membrane are discrete. N, nucleus; n, nucleolus; Bar = 5 μm.

Statistical analysis

Statistical information is expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for evaluation of the significance level ($P < 0.01$) among groups. All statistical tests were performed with GraphPAD InStat software V.: 1.14 (GraphPAD Software Inc., San Diego, CA).

RESULTS

All samples from patients with paracoccidioidomycosis, silicosis, sarcoidosis, or allergic alveolitis showed heterogeneous structural features. This heterogeneity was reflected in the diversity and density of cellular granules, electron density and vacuolization of the cytoplasmic matrix, nuclear contour, and pseudopodia of the cell membrane. **Figure 1** shows details of this diversity of the cellular structure of AM.

In all samples, at least three characteristic cell types may be distinguished: (1) Cells exhibiting a large cytoplasm of low electron density (**Figs. 2** and 1a), vacuolated, with few cytoplasmic inclusions of variable aspect. The nucleus is generally large and rounded with scarce heterochromatin peripherally distributed around the nuclear membrane and evident nucleolus. The plasma membrane is smooth and with few pseudopods and microvilli. (2) Macrophage subpopulation represented by cells with a cytoplasmic matrix of intermediate density (**Figs. 3b,** 1a, 1b, and 1c) with abundant, small mitochondria, variable quantity of electron dense granules, and vesicles with very dense inclusions. These cells can present some vacuoles, generally near the plasma membrane. The nucleus is lengthened, of spindle-shaped form, with irregularities in the nuclear membrane, and with scanty heterochromatin disposed toward the nuclear periphery. The nucleolus is not always evident. The plasma membrane presents pseudopods or microvilli intercalated with rounded and smooth areas of cell surface. (3) The last cellular group is constituted by cells of very electron-dense cytoplasmic matrix (**Figs. 4,** 1b, 1c, and 1d). The granules and cytoplasmic vesicles are abundant and electron-dense, with inclusions variable in content and density. The mitochondria are

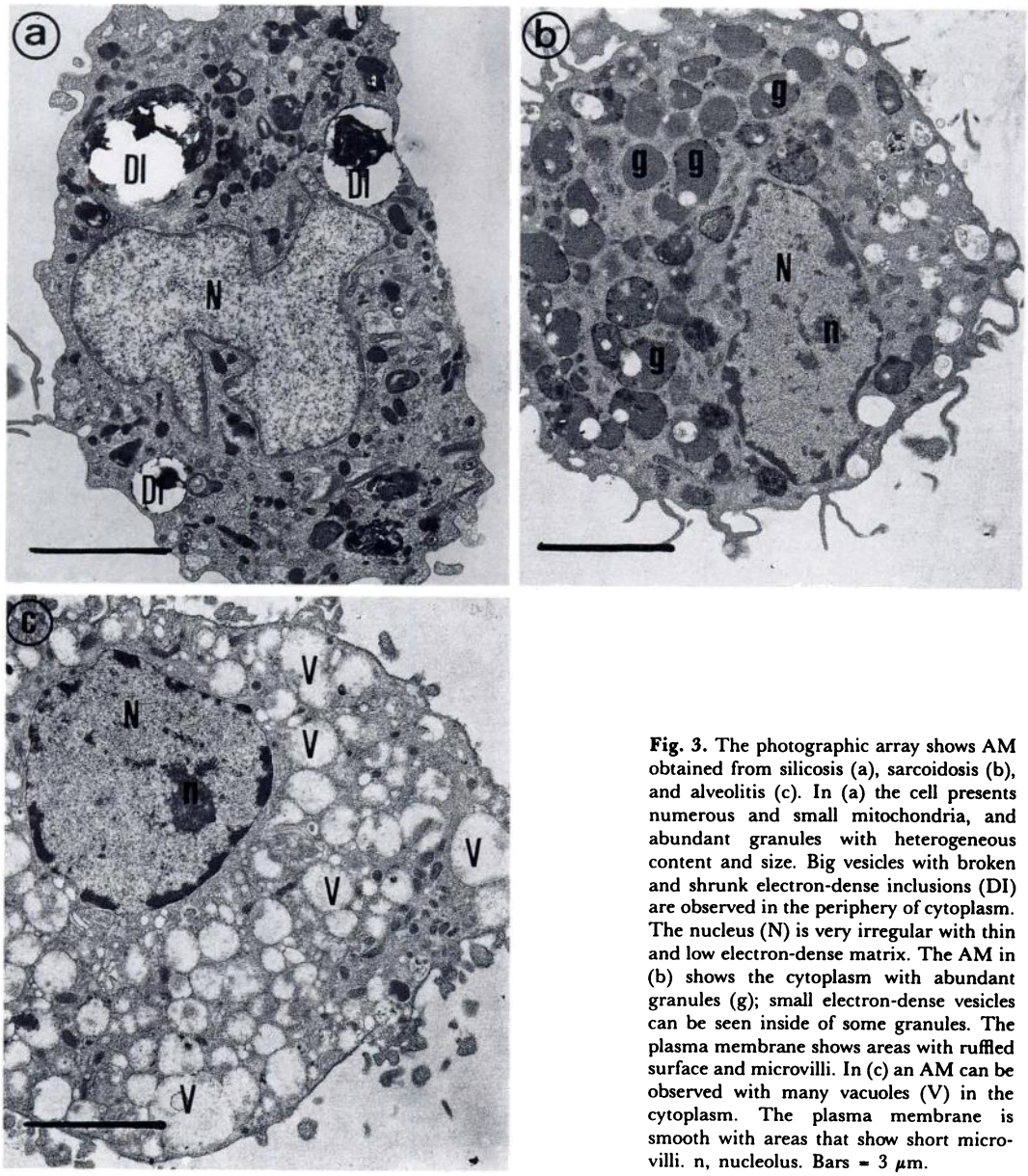

Fig. 3. The photographic array shows AM obtained from silicosis (a), sarcoidosis (b), and alveolitis (c). In (a) the cell presents numerous and small mitochondria, and abundant granules with heterogeneous content and size. Big vesicles with broken and shrunk electron-dense inclusions (DI) are observed in the periphery of cytoplasm. The nucleus (N) is very irregular with thin and low electron-dense matrix. The AM in (b) shows the cytoplasm with abundant granules (g); small electron-dense vesicles can be seen inside of some granules. The plasma membrane shows areas with ruffled surface and microvilli. In (c) an AM can be observed with many vacuoles (V) in the cytoplasm. The plasma membrane is smooth with areas that show short microvilli. n, nucleolus. Bars = 3 μm.

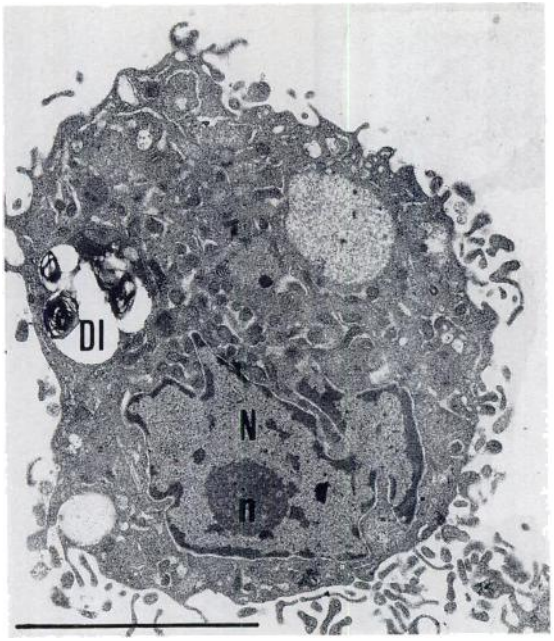

Fig. 4. Section of AM from paracoccidioidomycosis showing electron-dense cytoplasm and very ruffled plasma membrane. The nucleus (N) has an irregular contour with evident nucleolus (n). Electron dense inclusion (DI) near plasma membrane can be seen. The mitochondria are abundant, small, and with very electron-dense matrix. Bar = 5 μm.

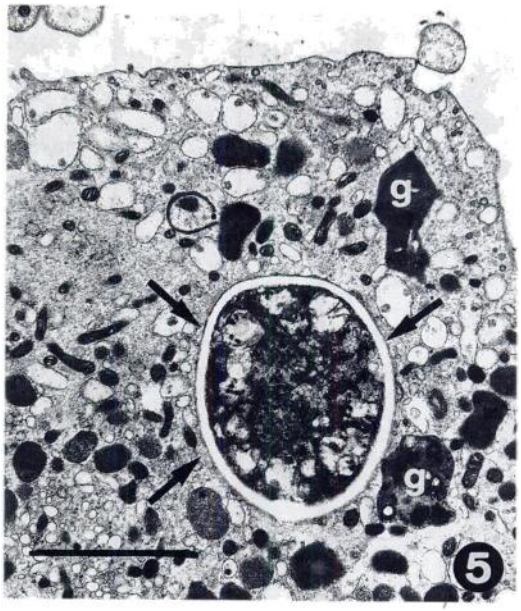

Fig. 5. Phagocytosed fungi (arrows) can be observed in a section of AM cytoplasm from paracoccidioidomycosis. Granules: g; Bar = 3 μm.

small and abundant with very dense matrix. The nucleus presents very irregular contours without or with heterochromatin disposed in patches. These cells present plasma membrane highly ruffled with a large quantity of pseudopods and microvilli distributed over all the cellular surface (Figs. 4 and 1c) or smooth surface with vacuolated cytoplasm near the plasma membrane (Figs. 1b and 1d).

The samples of paracoccidioidomycosis, sarcoidosis, silicosis, and alveolitis have a high proportion of cells with a large number of vacuoles and cytoplasmic matrix of very low electron density, (See **Table 1**). The percentages of vacuolated and low electron-dense cells are comparable in silicosis, sarcoidosis, and alveolitis. Samples from paracoccidioidomycosis have a significantly lower percentage of such cells ($P < 0.01$) than those from silicosis and alveolitis.

AM obtained from patients with silicosis (Fig. 3a), show very electron-dense cytoplasmic inclusions of great size that are not embedded adequately in the epoxy resins. They detach from the sections and leave empty spaces in the cells. In the samples of sarcoidosis (Fig. 3b), the cells have more discrete and peripherally located vacuoles. In addition, allergic alveolitis shows many vacuolated macrophages with a large number of vacuoles distributed frequently in all cytoplasmic matrix (Fig. 3c). The proportion of vacuolated cells was always higher in allergic alveolitis than in the other diseases. Differences, however, did not reach statistical significance.

All samples incubated in vitro with *P. brasiliensis* showed phagocytosed fungi (**Fig. 5**). There were no noteworthy structural differences between macrophages that ingested *P. brasiliensis* and those that had not done so. Immunolabeling studies showed differences of proportion, distribution, and density of MHC-II molecules on the surface of the AM between paracoccidioidomycosis and other diseases analyzed.

TABLE 1. Alveolar Macrophage Subpopulations

Lung disease	% vacuolated and low electron-dense cells	% intermediate and electron-dense cells
Paracoccidioidomycosis $n = 6$	14.66 ± 0.84*	78.66 ± 1.97
Silicosis $n = 8$	20.75 ± 1.35	70.50 ± 1.76
Sarcoidosis $n = 4$	19.00 ± 1.29	75.50 ± 2.98
Alveolitis $n = 4$	23.00 ± 1.91	70.50 ± 1.89

Percentage (mean ± SEM) of AM with the observed ultrastructural characteristics in the studied pulmonary diseases. The number of evaluated cells per sample was 50. Samples from paracoccidioidomycosis (*) have a significantly lower percentage of vacuolated and low electron-dense cells ($P < 0.01$, determined by ANOVA) than those from silicosis and alveolitis.

TABLE 2. Immunolabeled Alveolar Macrophages

Lung disease	% immunolabeled cells
Paracoccidioidomycosis $n = 4$	2.50 ± 0.50*
Silicosis $n = 4$	9.00 ± 1.29
Sarcoidosis $n = 4$	8.00 ± 0.81
Alveolitis $n = 4$	10.50 ± 0.95

Percentage (mean ± SEM) of AM in the studied pulmonary diseases with immunolabeling for MHC-II antigen. The number of evaluated cells per sample was 50. There are significant differences ($P < 0.01$ as determined by ANOVA) between samples from paracoccidioidomycosis (*) and samples from the other diseases.

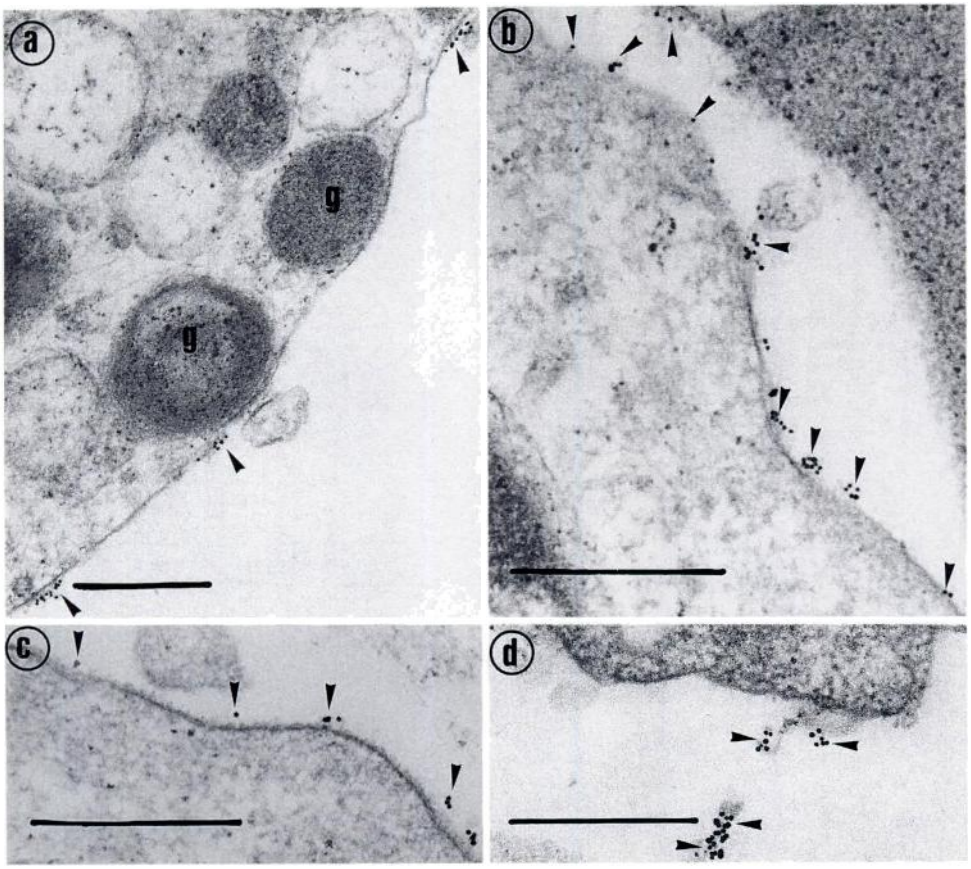

Fig. 6. Distribution of immunogold staining for MHC-II molecules in the surface of AM obtained from alveolitis. (a) and (b) Colloidal gold particles (arrow heads) are distributed in patches containing 4–12 particles in smooth areas of cellular surface. (c) Areas labeled with individual gold particles (arrow heads) or in series of 2 or 3 particles. (d) Strong immunolabeling (arrow heads) is associated with low electron density amorphous material, attached to external face of the macrophage cell membrane. Bars = 0.5 μm.

Table 2 shows that in alveolitis, sarcoidosis, and silicosis percentages of immunolabeled cells were significantly higher ($P < 0.01$) than in paracoccidioidomycosis. In addition to proportion, the distribution of MHC-II immunolabeling is worth describing. As shown in **Figure 6,** in the case of alveolitis most cells have colloidal gold particles distributed in patches containing six to 12 particles (Figs. 6a and 6b). They are present, discontinuously, in the areas of cellular surface that lack pseudopods. There are also areas labeled with individual gold particles in short intervals or in series of two or three particles (Fig. 6c). In some AM there was intense immunolabeling associated with an amorphous clot-like material attached to the cell surface (Fig. 6d). In sarcoidosis (**Fig. 7**), the patches of colloidal gold particles are scarcer and more widely spaced. Immunostaining associated with digitiform areas on the cell surface is often observed (Fig. 7b). In silicosis, immunolabeling for MHC-II molecules on the surface of AM is similar to the other cases described (**Fig. 8a**). However, it is possible to observe colloidal gold particles associated with the pseudopods of the plasma membrane (Figs. 8b and c). Some AM obtained from silicosis show a marked immunolabeling on the plasma membrane and even in the cytoplasm near the cell surface (Fig. 8d).

In paracoccidiodomycosis, few AM express MHC-II molecules on their surface (Table 2). The gold particles on the surface are very scarce, isolated, and dispersed (**Fig. 9**). At times they are associated with an amorphous and clotty material adhered to the plasma membrane (Fig. 9a). Fungi in the process of being ingested by AM may show some gold particles attached to the cell wall in the areas not folded by the pseudopods (**Fig. 10**).

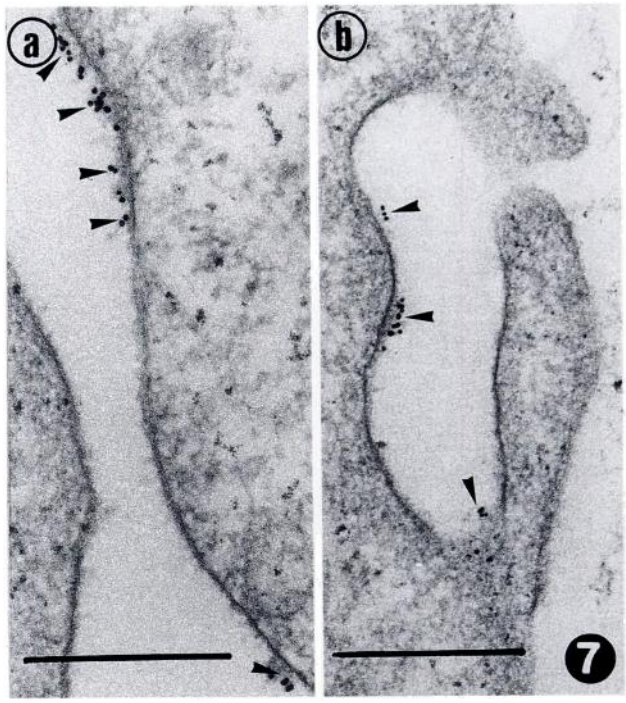

Fig. 7. Immunolabel for MHC-II molecules in the cell surface of AM obtained from sarcoidosis. (a) Colloidal gold particles (arrow heads) are distributed in patches on the surface of the plasma membrane similar to alveolitis but the patches are more widely spaced and scarcer. Bar = 0.3 μm. (b) Immunogold staining (arrow heads) associated with digitiform areas of cell surface of the AM. Bar = 0.5 μm.

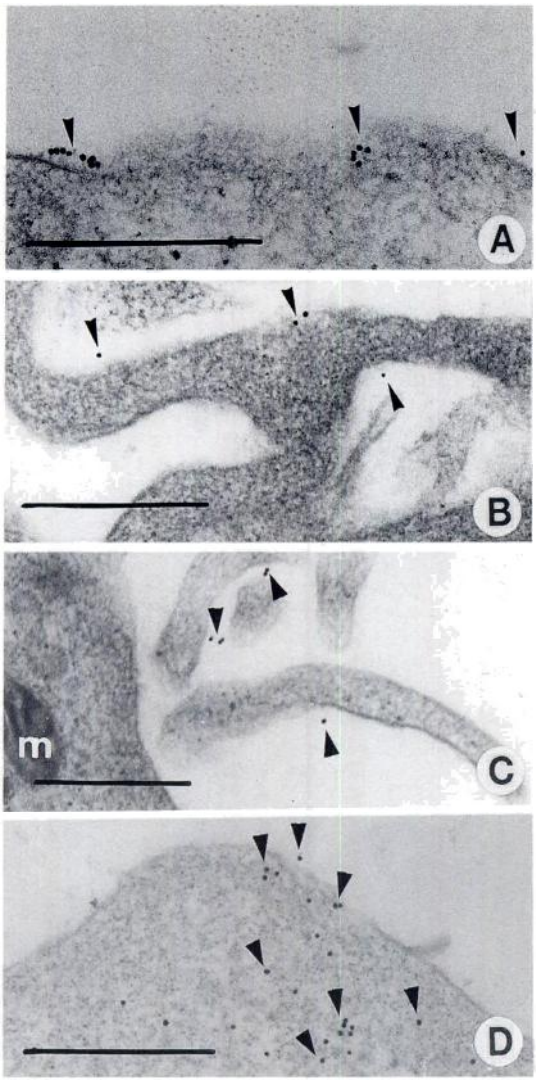

Fig. 8. Immunogold staining of MHC-II molecules in cell surface and outermost cytoplasm of AM from silicosis. (a) Colloidal gold particles (arrow heads) are distributed in patches attached to external face of plasma membrane. Bar = 0.3 μm. (b) and (c) Gold particles (arrow heads) associated with microvilli on cell surface. Bar = 0.5 μm. (d) When plasma membrane shows ruptures or disruptions, immunogold particles (arrows) are seen on plasma membrane and internalized in cytoplasm near cell surface. Bar = 0.5 μm. m, mitochondria.

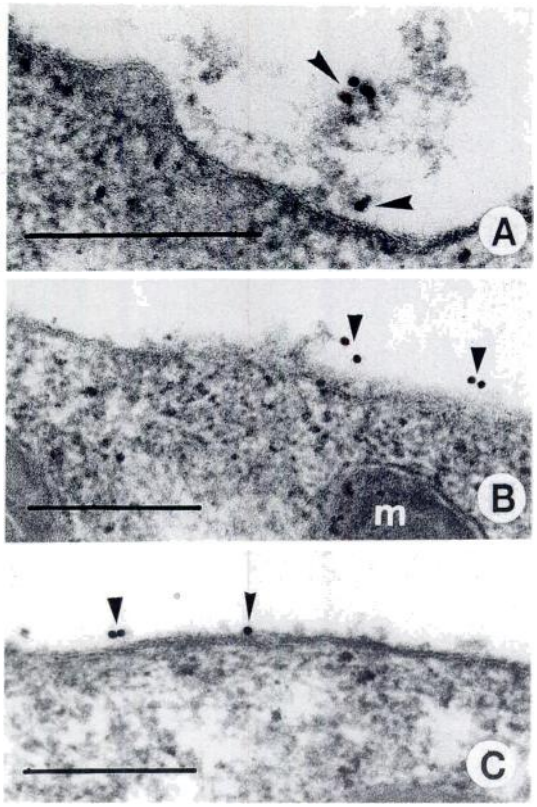

Fig. 9. Immunogold staining of MHC-II molecules on the plasma membrane of AM from paracoccidiodomycosis. (a) Immunogold particles (arrow heads) associated to low electron density amorphous material attached to external face of the plasma membrane. (b) and (c) Isolated and dispersed immunogold particles (arrow heads) can be seen on the plasma membrane of some macrophages. m, mitochondria. Bars = 0.3 μm.

It should be pointed out that neither ultrastructural nor immunolabeling differences were observed when the cells were incubated in vitro for 2.5 h at 37°C in nutrient medium with or without *P. brasiliensis* (fractions B and C).

DISCUSSION

Ultrastructural and functional observations of AM obtained from BAL of healthy donors and experimental animals show that these cells present morphological variations associated with maturity, cell activation, physiological processes, and cell microenvironment [13, 17–20]. This heterogeneity seemed more patent in the AM of primates than of other animals [32]. AM may be separated physically and characterized by their morphofunctional features. Our observations seem to indicate that in the diseases studied this diversity (morphological and functional) persists but is associated with additional ultrastructural characteristics inherent to each disease. We found a higher proportion of vacuolated and low electron density macrophages than the values reported by Nakstad et al. [18] in smokers and nonsmoker healthy individuals. In our opinion, this difference is not explained by variations in methodology, but by the disease states themselves. The increase in proportion of vacuolated macrophages could indicate a more active maturation and degradation of AM in the diseases studied. It is noteworthy that in paracoccidioidomycosis, the proportion of vacuolated macrophages is significantly lower than in other diseases studied. This might indicate that in paracoccidioidomycosis

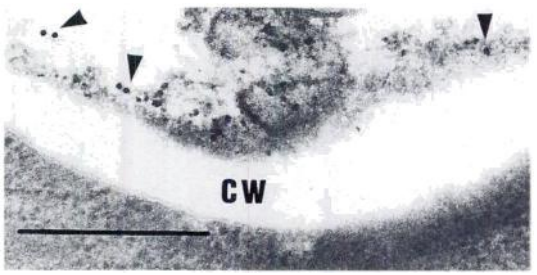

Fig. 10. The micrograph shows the cell wall (cw) of a fungus in the process of being ingested by an AM. In the areas not folded by the pseudopods immunogold staining (arrow heads) can be seen. Bars = 0.5 μm.

the proportion of activated AM is smaller and their processing and defense functions towards *P. brasiliensis* are impaired. A deficiency of digestive properties of phagocytes vis à vis *P. brasiliensis* in paracoccidioidomycosis was shown by Goihman-Yahr et al. [16, 22]. In addition, the relationship between AM subpopulations and immunological activation has been established in animal species [33-35].

The values obtained in the expression of MHC-II molecules in AM are very variable and seem to depend on the species studied and on methodologies used [10, 12-14, 36]. In our study, the proportion of macrophages expressing the MHC-II antigens is low (2-11%); however, it is noteworthy that the expression of this molecule is related to the population heterogeneity in AM [8].

In addition to the ultrastructural changes observed in the AM of the studied samples, we observed significant differences in the expression of MHC-II antigens. The percentage of AM from paracoccidioidomycosis that exhibit the MHC-II molecule is very low with poor immunolabeling. The function of AM as antigen presenting cells has been clearly established in other species [2, 10, 11].

Silicosis, sarcoidosis, and alveolitis do not differ significantly in the quantity of immunolabeled AM or in the distribution of the label. The distribution of label (and hence antigen) in patches and the frequency of the intervals of immunolabeling on the surface of AM in these diseases could mean strong macrophage activation that may be causing the stimulus of the cellular immune response. In sarcoidosis and hypersensivity pneumonitis it has been shown that the increase of surface receptors and HLA-DR (Ia) antigens is related to the increase in macrophage activation and cellular immune response [14, 37-40]. In contrast, in paracoccidioidomycosis the low expression of the MHC-II molecule could be related to a decrease of the antigen presenting function by AM; this might cause a poor stimulation of the T lymphocyte population in the respiratory tract and elsewhere, resulting in a defective cellular immune response against *P. brasiliensis*.

Similar assays, with other surface antigens and receptors in AM should be done to achieve a better understanding of the role of alveolar mononuclear phagocytes in pulmonary diseases.

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