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Suppression of Superoxide-Generating Ability during Differentiation of Monocytes to Dendritic Cells

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Human peripheral monocytes cultured with GM-CSF and IL-4 differentiated to dendritic cells (DCs) and with GM-CSF alone to macrophages. Superoxide-generating ability in such DCs was found to be suppressed whereas that in macrophages remained constant. To examine the reason for the suppression in DCs, we evaluated by immunoblotting the levels of essential components of the superoxide generating system in the cells during the differentiation. In contrast to the levels of cytosolic 47- and 65-kDa components and Rac-p21, which remained constant throughout cultivation, those of the large and the small subunits of cytochrome b_{558} were found to decrease quickly by day 2 during cultivation of monocytes with GM-CSF and IL-4. DCs obtained after 7 days of cultivation had lost the large subunit almost completely and most of the small subunit. A cell surface epitope of the cytochrome detected by a monoclonal antibody also decreased during the differentiation. On the other hand, these components, including both subunits of cytochrome b_{558}, were maintained in the cells during differentiation of monocytes to macrophages. These results indicate that the decreased levels of cytochrome b_{558}, especially that of the large subunit, is responsible for the low level of superoxide-generating ability of DCs and that the suppression is caused by IL-4.

Key words: dendritic cells, GM-CSF, IL-4, monocytes, superoxide-generating ability.

Dendritic cells (DCs) are identified as potent antigen-presenting cells like macrophages and B lymphocytes (*1-4*). DCs had been considered to be derived from a common progenitor cell for these cells, granulocytes and monocytes in bone marrow and peripheral blood (*5-8*). There is a limited number of mature DCs and progenitor cells in bone marrow and in blood (*9*). Recently, blood monocyte pool was shown to mature into cytologically and functionally characteristic DCs (*10-12*). The cooperative effect of GM-CSF and IL-4 on the development of DCs from blood mononuclear cells was reported (*13*). Akagawa and Takasuka found that when highly-purified monocytes from peripheral blood were cultured with combination of GM-CSF and IL-4, the cells became DCs that exhibited a typical dendritic morphology and characteristic surface antigens. With GM-CSF alone, the monocytes matured to macrophages (*14, 15*).

Results of our preliminary experiments showed that GM-CSF/IL-4-induced DCs exhibited no superoxide generation upon stimulation with PMA. This is in contrast with other potent antigen-presenting cells such as macrophages and B lymphocytes, which generate superoxide anion upon cross-linking of surface antigens (*16-18*). We are interested in the alteration of levels of superoxide-generating components during differentiation of monocytes to DCs. The superoxide-generating system in monocytes, like that in neutrophils and B lymphocytes, is an enzyme system that includes cytochrome b_{558}, a heterodimer compound of the large and small subunits, and two cytosolic components with molecular masses of 47 and 65 kDa. A defect in any of these components is known to cause chronic granulomatous disease (*19*). A small GTP-binding protein, Rac-p21, is also involved in the system.

To examine the reason for the loss of superoxide-generating ability in DCs, we studied levels of each protein component necessary for superoxide generation during differentiation of monocytes to DCs using the antibodies against these components (*20*). We show in this paper that monocytes lose the subunits of the cytochrome rapidly in the early days of cultivation with GM-CSF and IL-4.

Abbreviations: BPB, bromophenol blue; DCs, dendritic cells; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; MEM, minimum essential medium; PBS, phosphate-buffered saline; PI, propidium iodide; PMA, phorbolmyristate acetate; PVDF, polyvinylidene difluoride; SOD, superoxide dismutase; TBS, Tris-buffered saline; TCA, trichloroacetic acid; MLR, mixed lymphocyte reaction.

MATERIALS AND METHODS

Reagents—Bovine serum albumin (BSA), phorbolmyristate acetate (PMA), and trypsin were purchased from Gibco, superoxide dismutase (SOD) and propidium iodide (PI) from Sigma (St. Louis, MO), sodium lauryl sulfate (SDS) from Nacalai Tesque (Kyoto), RPMI1640 and minimum essential medium (MEM) from Nissui (Tokyo). Human rIL-4 and rGM-CSF were kindly provided by Professor K. Arai of this Institute.

Antibodies—The following peptide-directed polyclonal antibodies were used to identify and quantify each component of the superoxide-generating system: anti-L_C, anti-

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S_C, anti-47_N, and anti-65_N, respectively, against a synthetic peptide corresponding to residues 550-569 of the large subunit of cytochrome b_{558}, to residues 175-194 of the small subunit of the cytochrome, to residues 81-101 of the cytosolic 47-kDa protein, and to residues 23-42 of the cytosolic 65-kDa protein (*20, 21*). The monoclonal antibody 7D5 against cytochrome b_{558} was conjugated with fluorescein isothiocyanate for cytochemical analysis of the cytochrome (*22*). FITC-conjugated isotypic control mouse IgG1 was purchased from DAKO A/S (Glostrup). Rabbit polyclonal antibody against Rac2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation and Cultivation of Monocytes—Human peripheral blood monocytes were obtained from mononuclear cell fraction isolated from buffy coat by density gradient centrifugation on Ficoll-Conray solution and purified by two-step adherent processes. Briefly, washed mononuclear cells were suspended in RPMI1640 medium supplemented with penicillin (10 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), and 10% FBS. The cell suspension was placed in 100-mm glass dishes and incubated at 37°C for 15 min under humidified air containing 5% CO_2. Non-adherent cells were removed by three times washing with phosphate-buffered saline (PBS). The adherent cells were released from the plates by vigorous pipetting with PBS and resuspended in RPMI1640 medium. The procedure was repeated using MSP-plates (Japan Immuno-research Laboratories, Saitama) instead of glass plates. Finally the adherent cells on the MSP-plate were detached, collected, and suspended in RPMI1640 medium at a concentration of 5×10^5/ml. Six-well tissue culture plates (Sumilon, Tokyo) were used for the experiments and 1×10^6 cells in 2 ml were seeded in each well. The cells were allowed to adhere at 37°C for 15 min and the medium was changed for one supplemented with 8 ng/ml IL-4 and 1 ng/ml GM-CSF or 1 ng/ml GM-CSF alone. After 0, 1, 2, and 7 days, the cells were harvested with 0.25% trypsin in PBS. The monocyte-derived DCs were non-adherent and recovered after 7 days of culture.

Cytochemistry—Purity of monocytes was determined by peroxidase staining and flow cytometry. In the case of peroxidase staining, the cells were spun down onto glass slides using cytospin II (Shandon, Pittsburgh, PA) at $50\times g$ for 3 min. After treating the slides with 0.5% $CuSO_4$ for 30 s, the air-dried cells were incubated for 2 min at room temperature with one drop of Benzidine-H_2O_2 solution containing 0.1% benzidine, and two drops of 3% H_2O_2 in 100 ml of solution. The cells were stained with 1% safranin for 2 min, washed and observed under a microscope. Some dark blue particles can be observed within monocytes but not in lymphocytes. Monocyte purity was determined by counting over 200 cells in various fields. Monocyte purity was also determined using a FACScan (Becton Dickinson, San Jose, CA) by setting the gate in forward scatter and side scatter (FSC/SSC) dot blot. The results obtained by these methods agreed well with each other. Viability of the cells was determined by trypan blue exclusion method and over 90% of the cells excluded the dye during cultivation.

Assay of Superoxide Generation—To measure superoxide anion, we used the lucigenin-dependent SOD-inhibitable chemiluminescence method and the SOD-inhibitable cytochrome *c* reduction method. Varying numbers of monocytes or monocyte-derived DCs suspended in MEM containing 20 mM Hepes (pH = 7.3) were incubated for 5 min at 37°C. To the suspension, 50 μM lucigenin was added and incubated for further 2 min. The reaction was started by the addition of 200 ng/ml PMA. Chemiluminescent response was measured using a Berthold LB9505 luminometer (Wildbad). The peak count was used as an indicator of superoxide generation.

The superoxide production of monocytes, macrophages and dendritic cells in tissue culture plates was assayed by the SOD-inhibitable cytochrome *c* reduction assay. The cells cultured in 24-well tissue culture plates (3×10^5 cells/ml/well) were washed gently twice with warm HBSS and incubated further at 37°C for 5 min in the presence of 100 μM ferricytochrome *c*. The reaction was started with 200 ng/ml PMA. Control wells contained 500 U SOD. Aliquots of 80 μl of the reaction mixture were poured into 400 ml of ice cold HBSS every 10 min. The maximum rate of superoxide generation was quantified as that of the cytochrome reduction measured at 550 nm.

Immunoblotting Analysis—Cells were treated with 10% trichloroacetic acid (TCA, v/v) and the precipitated proteins were disrupted by sonication in the presence of 9 M urea, 2% Triton X-100, and 5% 2-mercaptoethanol. The lysate was mixed with 0.5 M Tris-HCl (pH 6.8) containing 3% (w/v) SDS, 8% glycerol, and Bromophenol blue (BPB). The samples were subjected to SDS-PAGE according to Laemmli (*23*). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) using a semi-dry blotting apparatus (Model BE-300, Bio-Craft, Tokyo). The membranes were incubated with polyclonal antibodies that recognized each component of the superoxide-generating system at 4°C for 12 h. After blocking the nonspecific binding with 20 mg/ml BSA at room temperature for 1 h, the membrane was allowed to react with alkaline phosphatase-conjugated goat anti-rabbit IgG at 4°C for 12 h. Color development was performed using 5-bromo-4-chloro-3-indolylphosphate and Nitroblue tetrazolium. The stained bands were quantified on a Bio Image Analyser (Millipore, Ann Arbor, MI) by comparing the integral densitometry (*24*).

FACS Analysis of Cytochrome b_{558} on Cell Surface—Fresh monocytes or monocyte-derived DCs (5×10^5) were incubated with mouse IgG1 (25 μg/ml) for 20 min at room temperature to block Fc receptors. The cells were labeled at room temperature for 1 h with FITC-conjugated monoclonal antibody 7D5 against cytochrome b_{558} or IgG1 (isotypic control) in 0.2 ml TBS containing 2% BSA, 10 mM NaN_3, and 0.5 mM EGTA. The stained cells were analyzed on a FACScan. Gates on monocytes and dendritic cells were set in the forward and side scatter (FSC/SSC) dot plots. PI was used to stain the dead cells. Only the living cells were evaluated for fluorescence emission. Using the FACScan software, mean fluorescence intensity (MFI) was evaluated for comparing difference in fluorescence intensity.

RESULTS

DCs are distinguished from monocytes as large, irregularly shaped cells with multiple dendrite-like cellular projections. When monocytes isolated from human peripheral blood and adhered on a plastic bottle (Fig. 1a) were cultured with GM-CSF and IL-4 for a week, almost all cells became DCs, which were non-adherent and exhibited typical veils

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and dendrites (Fig. 1b). The cells were negative in non-specific esterase activity, non-phagocytic, and expressed highly CD1, CD11c, and MHCII, whereas expression of CD14 and CD71 was suppressed. The cells exhibited high MLR stimulatory capacity. When monocytes were incubated with GM-CSF alone, they differentiated into macrophages (Fig. 1c). Without the growth factor, few monocytes could survive.

As shown in Fig. 2, the monocyte-derived DCs exhibited little superoxide-generating ability as evaluated by the lucigenin-dependent chemiluminescence method, while freshly isolated monocytes exhibited high superoxide-generating potential in a cell number-dependent manner. The results suggest alteration of superoxide-generating ability of the cells during differentiation of monocytes to DCs. The cells incubated with GM-CSF and IL-4 lost most of superoxide-generating ability by day 2 and DCs obtained after 7 days of culture did not generate superoxide anion upon stimulation (Fig. 3A). This is in contrast with the cells incubated with GM-CSF alone, which maintained high superoxide-generating ability (Fig. 3B).

To ascertain the reason for the decreased superoxide-generating ability of the cells during differentiation of monocytes to DCs, we analyzed the levels of four components essential for the generation by immunoblotting using peptide-directed antibodies. These are the large and small subunits of cytochrome b_{558}, 47- and 65-kDa cytosolic components. As shown in Fig. 4A, apparent decrease in the cytochrome components were seen in contrast to the cytosolic 47- and 65-kDa components. An equivalent level of Rac-p21 protein, a fifth component of the superoxide-generating system, was found in monocytes and in DCs (data not shown).

The immunoblot bands were quantified using Milligen Bio Image analyzer as described in "MATERIALS AND METHODS" and integrated optical densities were plotted against the time of cultivation. As shown in Fig. 4B, the levels of cytochrome components decreased rapidly by day 2 in the cells cultured with GM-CSF and IL-4, which differentiate to DCs, and the level of the large subunit of the

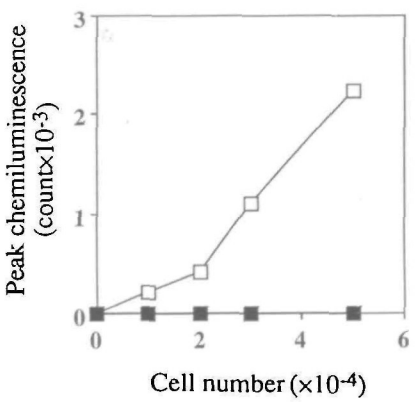

Fig. 2 **Chemiluminescence response of monocytes (□) and monocyte-derived dendritic cells (■).** SOD-inhibitable chemiluminescence was measured in response to PMA using lucigenin as the chemiluminescence enhancer. The peak value of count per minute was used as the index of superoxide production

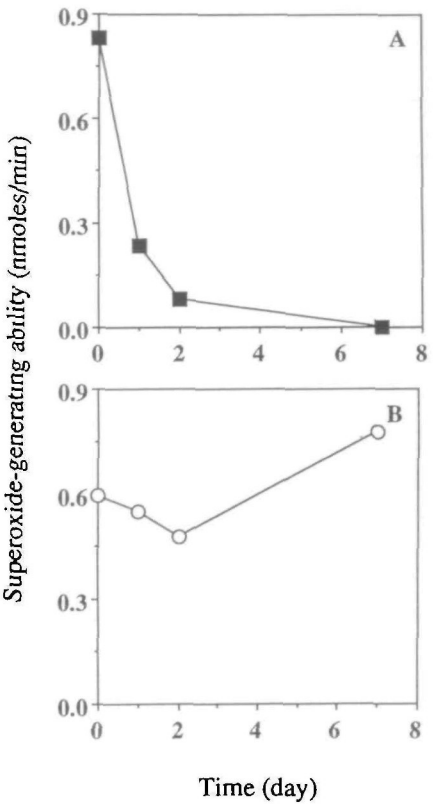

Fig. 3 **Change of the superoxide-generating potential during cultivation of monocytes.** The superoxide-generating potential of monocytes differentiated to dendritic cells (A) and macrophages (B) was assayed on day 0, 1, 2, and 7 by the cytochrome *c* reduction method. PMA was used as a stimulus. The maximum velocities of superoxide production are plotted against culture periods.

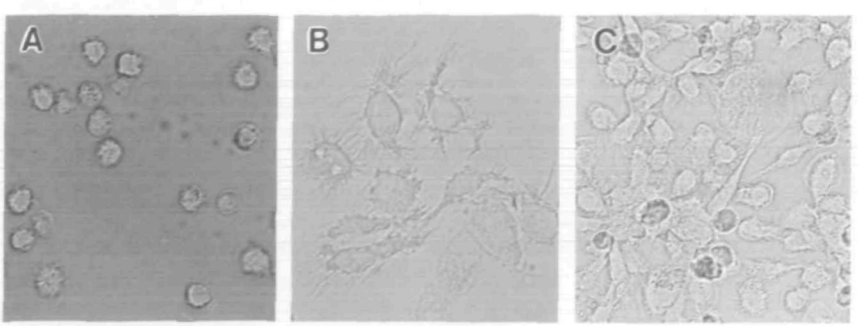

Fig 1 **Morphology of blood monocytes (A) and monocyte-derived dendritic cells (B) and macrophages (C).** Human peripheral monocytes differentiated into dendritic cells in the presence of GM-CSF plus IL-4, and macrophages in the presence of GM-CSF alone after 1 week of culture. Despite the firm adherence of the monocytes the derived dendritic cells were non-adherent and showed the typical morphology of peripheral dendritic cells: dendrites, veils, and processes. In contrast, the derived macrophages maintain the adherence and spread. The cells were photographed under an inverted microscope (Olympus, Tokyo) at magnification ×400.

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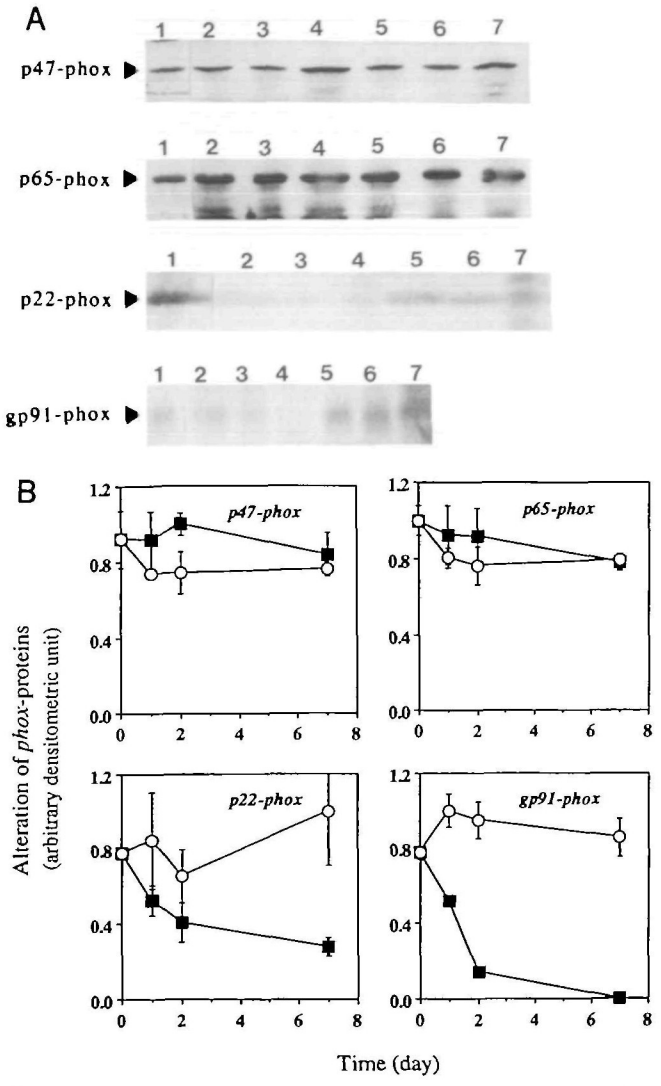

Fig. 4. **Levels of the components of superoxide-generating system during cultivation of monocytes.** (A) Representative immunoblotting data are shown of the large (gp91-*phox*) and small (p22-*phox*) subunits of cytochrome b_{558} and cytosolic 47-(p47-*phox*) and 65-kDa (p65-*phox*) proteins in fresh monocytes (lane 1), dendritic cells (lane 2, 3, 4), and macrophages (lane 5, 6, 7). Results of day 0, 1, 2, and 7 are shown. (B) Changes in components of the superoxide-generating system during cultivation. Immunoblot bands shown in (A) and in three other experiments were quantified using a Bio Image analyzer. The mean integral densities in DCs (■) and macrophages (○) are shown in the figure. For the small subunit of the cytochrome, the same results were obtained with anti-S_N antibody, a polyclonal antibody raised against a synthetic peptide corresponding to residues 1-25 of the protein.

cytochrome decreased further during prolonged cultivation. No essential change was found in the levels of cytosolic 47- and 65-kDa components in these cells. In contrast, the levels of all four proteins were maintained throughout the cultivation in the cells cultured with GM-CSF alone, which differentiate to macrophages.

We also examined flow-cytometrically the alteration of surface cytochrome b_{558} during differentiation of monocytes to DCs using monoclonal antibody 7D5, which is reported to stain the small subunit of the cytochrome (*22*). As shown in Fig. 5, the epitope was significantly reduced in DCs ob-

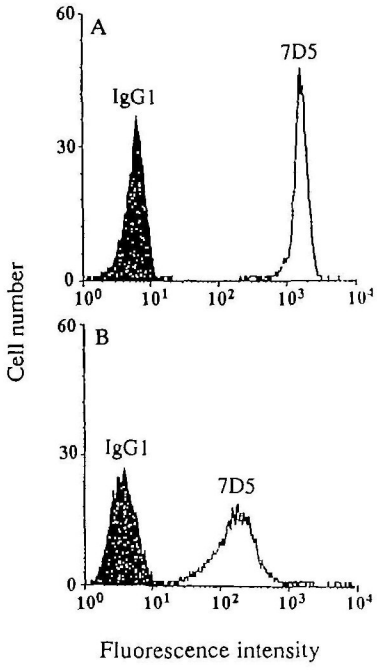

Fig. 5. **Cell surface cytochrome b_{558} on monocytes and on monocyte-derived dendritic cells.** Flow-cytometric analyses of cytochrome b_{558} on the membrane of fresh monocytes (A) and monocyte-derived dendritic cells (B) were performed using monoclonal antibody 7D5 and mouse IgG1 as the isotypic control.

tained after 7 days of cultivation, in good agreement with the immunoblotting results.

DISCUSSION

In this paper we confirmed and extended observations that purified monocytes derived from human peripheral blood differentiated to cells that exhibited various characters of DCs when they were cultured with GM-CSF and IL-4. GM-CSF supports the growth of myeloid cells including DCs in bone marrow (*9*) and in peripheral blood (*25*). It was reported that the CSF promotes the survival of human peripheral blood DCs and induces further differentiation of DCs to mobile, reversibly adherent cells with long-branched projections (*25*). Without GM-CSF, few monocytes can survive to differentiate to macrophages, and the majority of the cells die due to apoptosis (*14, 26-29*). On the other hand, the B cell growth factor IL-4 was shown to enhance cell surface expression of class II MHC antigen and CR3 but to down-regulate monocyte functions such as production of inflammatory cytokines (*29*). IL-4, therefore, is considered as an important negative regulator of macrophage inflammatory activities. As shown in this paper, monocytes cultured with GM-CSF alone differentiated to macrophages. For the development of DCs, the cooperative effect of GM-CSF and IL-4 was essential.

We showed that DCs derived from peripheral monocytes did not exhibit superoxide-generating ability on stimulation. Like neutrophils and eosinophils, monocytes and macrophages have a special superoxide-generating system on their cell surface that generates the superoxide anion upon stimulation (*22*). The anion is released from the cells and converted to other, more toxic active oxygen species

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(*30*), which may be used for killing microorganisms. Recent observation disclosed, however, that exactly the same superoxide-generating system is present in mature B lymphocytes (*16-18*), though the amount of superoxide produced by the cells was much lower than that produced by neutrophils. Since the system in peripheral and tonsil B lymphocytes was activated upon cross-linking surface antigens such as sIgM or sIgD (*16, 31, 32*), it was assumed that superoxide generation by these cells was concerned with antigen presentation or a similar function of the cells. Although DCs are known as potent antigen-presenting cells and distributed widely in tissues, we could not detect superoxide-generating ability in DCs derived from monocytes (in this paper) and tissue DCs (unpublished observation). The hypothesis, therefore, seems not to be applicable to DCs.

Monocytes cultured with GM-CSF and IL-4 lost their superoxide-generating ability rapidly in the early stages of cultivation. Among the components essential for superoxide generation, levels of the large and small subunits of cytochrome b_{558}, a unique electron transport component in the superoxide-generating system, were found to be decreased rapidly by day 2 of the cultivation, and the results correlated well with the decreased superoxide-generating ability of the cells. Regulating cytosolic 47- and 65-kDa components remained constant throughout the cultivation. It is likely, therefore, that the loss of cytochrome in DCs was responsible for their loss of superoxide-generating ability.

Superoxide-generating ability of monocytes/macrophages is known to be suppressed during cultivation without addition of cytokines (*28*). Under these conditions, the cells are known to undergo apoptosis (*29*). It has also been shown that IL-4 down-regulated various macrophage functions including superoxide-generating ability (*33, 34*) and that the suppression of this ability in porcine alveolar macrophages was due to a reduction in the level of mRNA for the large subunit of cytochrome b_{558} (*35*). Monocytes/macrophages treated with IL-4 also undergo apoptosis (*29*). Therefore, the possibility remained that the suppression of superoxide-generating ability in monocytes/macrophages during cultivation was related to some event occurring during apoptosis of the cells. The present results eliminated this possibility.

The antibodies used for the immunoblotting in the present study recognize either the carboxyl terminus of the large subunit or the carboxyl or amino terminus of the small subunit. Since all these termini face the cytoplasm and are sensitive to proteinase digestion (*21, 22*), they seem to have decomposed during differentiation of monocytes to DCs by proteolysis. These termini were shown to be essential for the assembly of a functionally active superoxide-generating complex in the cytoplasmic membrane (*22*). Although small amounts of the small subunit of the cytochrome and its surface epitope remained in the cells after carboxyl terminus of the large subunit had almost completely disappeared, the remaining portion of the cytochrome may have lost its function when the carboxyl terminus of the large subunit was decomposed. Further studies are needed to clarify these points.

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