Chlamydia pneumoniae Infection Induces Differentiation of Monocytes into Macrophages

Hiroyuki Yamaguchi, Shusaku Haranaga, Ray Widen, Herman Friedman, and Yoshimasa Yamamoto*

Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, Florida 33612

Received 7 September 2001/Returned for modification 29 October 2001/Accepted 30 January 2002

Migration and differentiation of monocytes to the intima of blood vessels may be a crucial first step in the development of atherosclerosis associated with Chlamydia (Chlamydomphila) pneumoniae. However, the involvement of C. pneumoniae infection in such steps is not clear. In the present study, therefore, the differentiation-inducing activity of C. pneumoniae to monocytes was examined. Human THP-1 monocytic cell line cells were infected with C. pneumoniae, and the differentiation of monocytes to macrophages was assessed by cell morphology, phagocytic activity, and expression of a cell surface adhesion molecule. The monocytes infected with viable bacteria markedly differentiated into macrophages associated with diffused cell morphology, increased uptake of polystyrene beads and increased ICAM-1 (intercellular adhesion molecule 1) expression on the cell surfaces. Heat-killed bacteria did not induce any morphological changes or increase of phagocytosis, but they did induce an increase of cell surface ICAM-1 expressions in THP-1 monocytic cells. The antibiotic minocycline treatment of infected cells resulted in marked inhibition of the cell differentiation as well as C. pneumoniae growth in the cells, but not ICAM-1 expression. In addition, the experiments with human peripheral blood monocytes infected with C. pneumoniae also showed the differentiation of macrophages assessed by morphological change and phagocytic activity. These results indicate that C. pneumoniae infection may directly induce the differentiation of monocytes to macrophages. However, antigenic stimulation of monocytes with bacteria may not be sufficient for a full macrophage differentiation.

It is widely recognized that atherosclerosis is a chronic inflammatory disease and that the formation of the atherosclerotic plaque, which is known to be caused by excessive inflammatory fibroproliferation with the damage of endothelial and smooth muscle cells, is critical in coronary artery disease (24). Vasoregulatory molecules, growth factors, and cytokines following host immunomodulation have been implicated in this process (26). Current studies indicate that Chlamydia (Chlamydomphila) pneumoniae, an obligate intracellular bacterium associated with respiratory tract infection, may be involved in the process of atherosclerosis (5, 25).

C. pneumoniae preferentially infects respiratory tract epithelial cells as well as macrophages associated with the immune defense system, and this is related to the possible mechanism by which this bacterium colonizes the human host. Moreover, in recent years, findings that not only bacterial DNA and antigens but also viable bacteria are detected in the atherosclerosis lesions have been reported (3, 4, 7, 18). This raises an important question of how C. pneumoniae organisms reach the site of the intima, which is the major site of atherogenesis, from respiratory tracts, the gate for this pathogen. A previous report showed that in experimental infection of mice with C. pneumoniae the bacteria were spread via peripheral blood mononuclear cells, and the authors speculated that the responsive cell vehicle may be monocytes (19). However, the mechanisms of monocyte infection with C. pneumoniae and migration of infected cells to the intima are still unclear. In this regard, our present study found important evidence regarding macrophage differentiation caused by C. pneumoniae infection. That is, when monocytes were infected with C. pneumoniae, marked differentiation of monocytes to macrophages was observed. Furthermore, it was also evident that the differentiation associated with the macrophage function was caused by infection, not by stimulation with a bacterial antigen. Since macrophage differentiation allows cells to adhere to tissues, such as endothelial cells, and the cells eventually migrate into the intima, the finding obtained in this study may provide an infected cell recruitment mechanism for C. pneumoniae.

MATERIALS AND METHODS

Bacteria. C. pneumoniae strain AR39 was obtained from the American Type Culture Collection, Manassas, Va. The bacteria were propagated in the HEp-2 cell culture system according to the methods described previously (23). In brief, the infected cells were harvested on day 3 and disrupted by freezing-thawing and ultrasonication (Sonic Dismembrator 60; Fisher Scientific, Pittsburgh, Pa.). After centrifugation at 500 × g for 30 min to remove cell debris, bacteria were concentrated by a high-speed centrifugation at 30,000 × g for 30 min. The bacterial pellets were resuspended in sucrose-phosphate-glutamic acid buffer (0.2 M sucrose, 3.8 mM KH₂PO₄, 6.7 mM Na₂HPO₄, 5 mM L-glutamic acid [pH 7.4]) and then stored at −80°C until used. The organisms resuspended in RPMI 1640 medium were used for experiments. The bacterial suspensions were confirmed to be mycoplasm-free by PCR, as previously reported (20). The control inocula were prepared according to the same procedure with uninected HEp-2 cells. The number of infectious C. pneumoniae cells was determined as inclusion forming units by counting chlamydial inclusions formed in HEp-2 cells with fluorescein isothiocyanate (FITC)-conjugated monoclonal antichlamydia antibody specific to chlamydia lipopolysaccharide (LPS) (Research Diagnostics, Flanders, N.J.) (9, 23). Heat-killed bacteria were prepared by heating at 70°C for 45 min as previously reported (10). The viability of the heat-killed bacteria determined by specific inclusion formation showed that no viable bacteria remained.

Human peripheral blood monocytes. Human peripheral blood monocytes were isolated from buffy coats provided by the Florida Blood Services, St. Petersburg, by density gradient centrifugation with Histopaque-1077 (Sigma Chemical, St. Louis, Mo.). The resulting peripheral blood mononuclear cells were washed three times with Hanks’ balanced salt solution (HBSS) and suspended in

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., Tampa, FL 33612. Phone: (813) 974-2332. Fax: (813) 974-4151. E-mail: yyamamot@hsc.usf.edu.
C. pneumoniae and Monocyte Differentiation

C. pneumoniae infection. The human monocyte cell line THP-1 was obtained from the American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics at 37°C in 5% CO2. The infection of THP-1 and human peripheral monocytes with C. pneumoniae was performed as follows. The cells—at a concentration of 10⁶ cells/well (96-well plate) (for cell counting and assay for phagocytic activity), 2 × 10⁵ cells/well (24-well plate) with or without glass coverslips (12 mm in diameter) (for flow cytometry analysis, morphology observation and assay for infectious rate), or 10⁶ cells/well (six-transwell plates with polyester membranes; pore size, 0.4 μm; Costar, Corning, Inc., Corning, N.Y.) with coverslips into both chambers—were infected with bacteria without any centrifugation and incubated for up to 7 days at 37°C in 5% CO2. The infection ratio was 10 bacteria per cell. In some experiments, the cells were also incubated with heat-killed bacteria (10 bacteria per cell) as well as uninfected HEP-2 extracts. As a positive control for cell differentiation, the cells were treated with a 10⁻⁷ M concentration of phorbol myristate acetate (PMA) (Sigma) (22).

Cell count. At 1, 3, 5, or 7 days after the incubation, the culture plates were centrifuged at 210 × g for 10 min. After carefully removing culture supernatants, the cells were detached using the cell dissociation solution. The resulting cell suspensions were then used for viable cell counts by the trypan blue dye exclusion method.

Assessment of morphology. The cells on a coverslip were fixed in methanol, stained with a Giemsa stain solution (Harleco Inc., Gibbstown, N.J.), and examined with a microscope (BH-2; Olympus Co., Tokyo, Japan). The percentage of the differentiated cells which showed amoeboid or diffused morphology for each experiment was measured under a microscope by counting at least 500 cells in four random fields of each specimen.

Determination of infection. The infected cells were fixed in methanol and stained with FITC-conjugated anti-C. pneumoniae major outer membrane protein monoclonal antibody (Accurate Chemical and Scientific, Westbury, N.Y.) followed by staining with goat F(ab')₂ anti-mouse immunoglobulin G (IgG) FITC-labeled antibody (Southern Biotechnology, Birmingham, Ala.). The number of chlamydia inclusion-positive cells was observed for six randomly selected fields containing more than 500 cells for each specimen by fluorescence microscopy.

Phagocytosis assay. Phagocytosis activity of cells for carboxylate-modified fluorescence-labeled polystyrene beads (average diameter 0.5 μm; excitation wavelength, 575 nm; emission wavelength, 610 nm; Sigma) was performed as follows. The cells cultured infected or not infected with C. pneumoniae were incubated with 0.025% (vol/vol) beads for 24 h. After washing by centrifugation with HBSS, the cells were lysed in 0.2% saponin (Sigma), and the relative fluorescence of cell lysates was determined by a fluorescence microplate reader (Molecular Devices, Sunnyvale, Calif.). As a standard, a series of diluted fluorescence-labeled beads were spiked into the cell lysates.

ICAM-1 expression assay. The expression of the cell surface marker intercellular adhesion molecule 1 (ICAM-1) was examined by flow cytometry with R-phycocerythrin-conjugated anti-ICAM-1 (EB5451) monoclonal antibody (Bio Source International, Camarillo, Calif.) or mouse IgG1 isotype control. The cells at 3 days after infection were detached from the wells using the cell dissociation solution and were treated with antibody or control IgG for 1 h at 4°C. After washing with HBSS, the cells were fixed in 1% paraformaldehyde and then analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, Calif.). The change in mean channel fluorescence was calculated as follows: Δ mean channel fluorescence of R-phycocerythrin-positive cells – (mean channel fluorescence of ICAM-1-positive cells) (mean channel fluorescence of IgG1 isotype control-positive cells).

Antibiotic treatment. The cells infected with C. pneumoniae were treated with the antibiotic minocycline (Sigma) at a concentration of 0.6 μg/ml, which is 10 times the MIC (0.06 μg/ml) (8), for up to 7 days. The cells were then analyzed for infection, morphology, phagocytic activity, and ICAM-1 expression.

Statistical analysis. Statistical analysis was performed with the unpaired Student t test.

RESULTS

Effect of C. pneumoniae infection on THP-1 cell growth. THP-1 monocytes are continuous-growth cell line cells, and when the terminal differentiation of the cells to macrophages induced by a differentiation-inducing agent, such as PMA, occurs, cell proliferation ceases. Therefore, in order to determine the possible differentiation-inducing activity of C. pneumoniae infection, the effect of infection on THP-1 cell proliferation was determined initially. Culture of THP-1 cells up to 7 days, with or without viable or heat-killed C. pneumoniae, was performed, and the number of THP-1 cells was counted during the incubation. The PMA-stimulated cells were used as a reference for cell differentiation. As shown in Fig. 1, cells infected with viable C. pneumoniae did not proliferate during the experiments, as seen for PMA-treated cells. In contrast, heat-killed bacteria permitted some proliferation of the THP-1 cells, but this was limited.

Change of cell morphology by infection. Since the macrophage-differentiated cells are readily distinguished from monocytic cells by cell morphology, the morphology of C. pneumoniae-infected THP-1 cells was assessed by microscopy after Giemsa staining. Figure 2 shows representative Giemsa-stained microscopic images of THP-1 cells at 3 days after incubation with or without bacteria or PMA. The control cultures (Fig. 2A and E) without any stimulation or infection maintained the round shape of the cells. In contrast, the morphology of the cells infected with viable bacteria (Fig. 2B and F) as well as the cells stimulated with PMA (Fig. 2D and H) showed an obvious change. That is, the cells showed a diffused and enlarged shape with many vacuoles. Heat-killed bacteria (Fig. 2C and G) induced minimal morphological changes com-
pared with control cells. These data clearly indicate that the morphological changes were induced by infection, not by bacterial antigen stimulation.

The cell differentiation of monocytic cells to macrophages may be induced by several mechanisms, including an involvement of soluble factors produced by monocytes as well as bacteria. In order to determine such a possibility in the differentiation induced by *C. pneumoniae*, transwell plates with a membrane were utilized. Both chambers of the transwell plates were cultured with monocytic cells. The cells in the upper chamber were treated with viable bacteria, but the lower chamber contained only cells without bacteria. The percentage of diffused cells determined by microscopy of each chamber was measured up to 7 days after incubation. The results are shown in Fig. 3. The PMA-treated reference cells showed a high percentage of differentiated cells for both chambers. In contrast, the cells treated with viable bacteria showed change to a diffused morphology in only the upper chamber, not the lower chamber, which did not receive bacteria at the beginning of the culture. Even though the cells were cultured for as long as 7 days, no diffused cells were observed in the lower chamber for the viable bacterium-infected group. The percentage of morphological changes for the viable bacterium-infected cells was minimal at 1 day after incubation, but at 3 days after incubation the changes markedly increased, similar to those seen in the PMA-treated control group. These data indicate that the cell differentiation induced by *C. pneumoniae* infection does not appear to be mediated by soluble factors for monocytes induced by infection and bacterial secretions.

**Differentiation of infected cells.** To determine whether morphologically changed cells are infected with *C. pneumoniae*, the cells infected with bacteria were stained with FITC-conjugated antichlamydia antibody to measure chlamydia inclusions in a cell. At 3 days after incubation with viable bacteria, approximately 50% of cells showed obvious chlamydia inclusions demonstrated by specific staining with antichlamydia monoclonal antibody and FITC-conjugated secondary antibody (Fig. 4). Most chlamydia inclusion-positive cells were diffused cells, which were easily distinguished from the compact-round shape of noninfected control cells. Cells from other groups, such as noninfected and PMA-treated groups, did not show any chlamydia inclusions. Heat-killed-bacterium-treated cells showed a few chlamydia inclusion-positive cells which may have been due to cluster formation of bacteria in cells. Thus, the results indicate that *C. pneumoniae*-infected cells become differentiated cells.
Phagocytic activity of differentiated cells. The differentiation of monocytes to macrophages should be associated with macrophage functions, such as increased phagocytic ability. Therefore, whether morphologically differentiated cells induced by \textit{C. pneumoniae} infection were associated with an increased phagocytic ability was determined using fluorescence-labeled polystyrene beads. At 3 days after incubation of THP-1 cells with viable bacteria, there was a markedly increased phagocytic activity against beads, followed by a decrease of the activity due to the burden of infection (Fig. 5). In contrast, nontreated control cells and heat-killed-bacterium-treated cells showed minimal phagocytic activity, even though activity was measured as long as 7 days after cultivation. The PMA-treated cells showed remarkable phagocytic activity, indicating that the experimental system was appropriate.

**ICAM-1 expression.** The degree of cell surface adhesion molecule expression is an index of the differentiation of monocytes to macrophages (13). Therefore, the increase of a cell surface adhesion molecule, such as ICAM-1, by infection as well as stimulation with heat-killed bacteria in THP-1 cells was assessed by flow cytometry assay. At 3 days after incubation, with or without bacteria or PMA, the ICAM-1 expression was
significantly increased in both viable and heat-killed-bacterium-treated groups as well as the PMA-treated control group (Fig. 6). However, it is notable that the expression levels of ICAM-1 in viable and heat-killed-bacterium-treated cells were the same. These results indicate that the expression of ICAM-1 was induced not only by infection but also by stimulation with C. pneumoniae antigen.

**Effect of antibiotic on the differentiation induced by infection.** Since the findings indicate the possibility that the differentiation of monocytes to macrophages may be caused by the direct infection with C. pneumoniae, the likelihood that antibiotics can prevent the differentiation of monocytes was assessed. Minocycline, known to be active against C. pneumoniae in cells (6, 16, 20), was utilized in this study. As shown in Table 1, the treatment of C. pneumoniae-infected THP-1 cells with minocycline (0.6 μg/ml; 10 times of MIC) markedly reduced the infection as determined by detection of chlamydia inclusions. The differentiation of monocytic cells to macrophages assessed by morphological determination and phagocytic activity, but not ICAM-1 expression, was significantly reduced by the treatment with minocycline. The induction of ICAM-1 expression on the surfaces of cells induced by infection was not affected by the antibiotic treatment. The lower concentration of minocycline, such as 0.06 μg/ml (MIC of minocycline for C. pneumoniae), did not cause any effect on the induction of the differentiation (data not shown). Nevertheless, the results clearly support the argument that the differentiation of monocytes to macrophages is induced by C. pneumoniae infection, but the induction of adhesion molecule ICAM-1 expression of the cells may not be caused by infection.

**Differentiation of human peripheral blood monocytes.** In order to determine the possible differentiation of primary blood monocytes by infection as observed in established THP-1 monocytic cells, human peripheral blood monocytes were infected with C. pneumoniae and the differentiation of monocytes was assessed. Figure 7A and B show representative Giemsa stainings of human peripheral blood monocytes at 3 days after infection with or without viable bacteria. The control cultures (Fig. 7A) without infection maintained the round shape of the cells. In contrast, cells infected with viable bacteria (Fig. 7B) showed an obvious change in cell morphology, which was a diffused and enlarged shape with many vacuoles. Heat-killed bacteria induced only minimal morphological changes compared with control cells (data not shown). The phagocytic activity of human peripheral blood monocytes infected with viable bacteria was markedly increased for all three donor monocytes tested (Fig. 7C). In contrast, heat-killed bacteria induced a moderate phagocytic activity, which was dependent on the donors. The control noninfected monocytes did not show any significant increase of phagocytic activity during the culture.

**DISCUSSION**

THP-1, a well-characterized human monocytic leukemic cell line, has been used extensively in studies of macrophage differentiation (1, 2). For example, it was demonstrated that the PMA-induced differentiation of THP-1 monocytic cells is associated with alterations in cell morphology, cell adherence, phagocytosis, cell growth, and expression of several genes (1). Therefore, using THP-1 cells for analysis of macrophage dif-

---

**TABLE 1. Effect of minocycline on the differentiation of THP-1 cells induced by C. pneumoniae infection**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Day(s) after infection</th>
<th>Treatment with minocycline</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Infectious rate (%)a</td>
<td>1</td>
<td>0.5 ± 0.4</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>49.5 ± 13.3</td>
<td>4.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>48.6 ± 4.8</td>
<td>8.7 ± 5.9</td>
</tr>
<tr>
<td>Differentiated cells (%)b</td>
<td>1</td>
<td>12.1 ± 2.1</td>
<td>3.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>85.5 ± 3.2</td>
<td>18.3 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>82.9 ± 1.5</td>
<td>35.6 ± 17.5</td>
</tr>
<tr>
<td>Phagocytic activityc</td>
<td>1</td>
<td>2,124.6 ± 78.2</td>
<td>102.3 ± 20.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3,188.3 ± 145.2</td>
<td>623.6 ± 132.4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>926.3 ± 23.5</td>
<td>370.3 ± 60.9</td>
</tr>
<tr>
<td>ICAM-1 expressiond</td>
<td>3</td>
<td>795.3 ± 39.6</td>
<td>672.2 ± 46.1</td>
</tr>
</tbody>
</table>

---

a. Concentration of minocycline was 0.6 μg/ml. Data are means ± standard deviation for three experiments except for the ICAM-1 assay (two experiments).
b. Percentage of chlamydia inclusion-positive cells.
c. Percentage of morphologically differentiated cells (diffused and enlarged).
d. Relative fluorescence units.
e. Change in mean channel fluorescence of ICAM-1.
f. p, significant difference between treated with and without minocycline. NS, not significant.
differentiation-inducing activity of C. pneumoniae may be appropriate due to the well-characterized nature of THP-1 cells. The proliferation of THP-1 cells infected with C. pneumoniae as well as stimulated with PMA was significantly inhibited in comparison with control nontreated cells. However, such inhibition of cell proliferation was not due to the cell death determined by the trypan blue dye exclusion method (data not shown). Since PMA is known to modulate cell cycle such as diffused and enlarged cell shape, may indicate a differentiation of the cells to macrophages after infection with C. pneumoniae. Particularly, it is notable that many vacuoles in C. pneumoniae-infected as well as PMA-stimulated cells (Fig. 2) were filled with lipids, as demonstrated by oil red staining (data not shown). Since the formation of macrophage foam cells in the intima is the hallmark of early lesions in atherosclerosis, the presence of lipid-containing vacuoles in C. pneumoniae-infected cells observed in this study indicates a possible contribution to the formation of foam cells. In this regard, it has already been demonstrated that C. pneumoniae infection causes in vitro foam cell formation (15).

The morphological changes indicating the differentiation of THP-1 cells and human peripheral blood monocytes were observed in the viable-bacterium-treated cells, not in the heat-killed-bacterium-treated cells. The UV-irradiated C. pneumoniae also did not show any morphological change under the same experimental conditions (data not shown). These results clearly indicate that the differentiation of THP-1 cells as well as human peripheral blood monocytes may be induced by infection, not by antigen stimulation. Furthermore, the detection of chlamydia inclusions in the differentiated cells strengthened such a possible mechanism for direct infection-induced differentiation. In addition, the studies using the transwell plates showed that the involvement of soluble factors produced by THP-1 cells and/or bacteria may be less likely to have a role in the differentiation, even though the experimental conditions were limited and the results were not sufficient to completely rule out a possible involvement of soluble factors. In other words, the differentiation may be caused by direct infection of cells with C. pneumoniae, because the cells in the lower chamber isolated from the infected and differentiated cells in the upper chamber by a membrane were not differentiated.

The demonstration of increased phagocytic ability of the morphologically differentiated THP-1 cells and human peripheral blood monocytes by C. pneumoniae infection using polystyrene beads shows that the differentiation is associated with the functional activity of macrophages. The phagocytic activity of human peripheral blood monocytes treated with heat-killed bacteria was also higher than that of control cells, although the phagocytic activity was lower than that of cells infected with viable bacteria. In the case of THP-1 cells, however, heat-killed bacteria did not induce any significant increase of phagocytic activity. The difference between the responses of primary cells and cell line cells to heat-killed bacteria is not clear. The
induction of the adhesion molecule ICAM-1 in C. pneumoniae-infected cells indicates that the differentiation was also related to the level of surface antigen expression, which is important in the migration of macrophages. However, heat-killed bacteria also induced an increase of ICAM-1 expression in the cells, even though the cells showed minimal differentiation in terms of morphology as well as at functional levels. In this regard, it has been reported that bacterial LPS up-regulates the ICAM-1 expression of THP-1 cells and enhances monocyte differentiation (11, 14). Therefore, the results of increased ICAM-1 induction as well as moderate increase of phagocytic activity in peripheral blood monocytes by heat-killed bacteria may be due to bacterial components such as C. pneumoniae LPS. Because heat-killed bacteria did not induce any morphological change in THP-1 cells but induced a weak functional differentiation in peripheral blood monocytes, the stimulation caused by bacterial antigen may not be sufficient to induce a full macrophage differentiation.

The findings of morphological changes, increased phagocytic ability, and ICAM-1 induction by C. pneumoniae infection in monocyte cells strongly indicate the possibility that the differentiation of monocytes to macrophages is caused by direct infection. The use of the antibiotic minocycline for prevention of the macrophage differentiation caused by C. pneumoniae infection, therefore, appears appropriate to determine whether such a pathophysiologically important event can be prevented. The experiments conducted in this study indicate that the use of an appropriate antibiotic, such as minocycline, which is a potent antibiotic against C. pneumoniae in cells (6, 16, 20), can control the differentiation induced by C. pneumoniae infection. However, a relatively higher concentration of antibiotic, such as 10 times the MIC, was required. Although tetracycline and macrolides have been widely used for treatment of chlamydial infections (21), the effectiveness of these antibiotics against C. pneumoniae infection in monocytes/macrophages is not well understood. In this regard, a current report revealed the important clinical issue that C. pneumoniae infection in circulating monocytes resists antibiotic treatment with azithromycin and rifampin (12). Therefore, the in vitro model of C. pneumoniae infection and macrophage differentiation established in this study may be valuable for analyzing the effect of antibiotic on not only microbiological but also pathophysiological issues of infection.

In conclusion, it was evident in this study using THP-1 monocyte cell lines as well as human peripheral blood monocytes that differentiation of monocytes to macrophages can be induced by C. pneumoniae infection. However, the macrophage differentiation may be prevented by treatment with antibiotics at a relatively higher concentration.

REFERENCES


Editor: B. B. Finlay