Identification and Purification of Arginine Deiminase That Originated from *Mycoplasma arginini*

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A lymphocyte blastogenesis inhibitory factor, (LBIF), was purified from the culture supernatant of human histiocytic lymphoma U937 by fast protein liquid chromatography. In this study, we demonstrated, first, that LBIF originated from a mycoplasma, *Mycoplasma arginini*, infecting U937 cells, and second, that LBIF bore the arginine deiminase activity. The implication of in vivo immunosuppression induced by arginine-utilizing mycoplasma species is discussed.

Mycoplasma and viral infections often radically change host cell metabolism in vitro (2, 4, 5). Because of the seriousness of the resultant diseases, more progress has been made in the study of viruses than mycoplasmas. Mycoplasmas are the smallest of procaryotic organisms. Mycoplasmas or their products have been shown not only to inhibit the stimulation of lymphocytes by allogeneic cells or mitogens but also to serve as mitogens for T and B lymphocytes (4).

Historically, Copperman and Morton first described (in 1966) the inhibition of mitosis in lymphocyte cultures by *Mycoplasma hominis* (5). In 1968, Barile and Levineih reported that the inhibitory effect of *M. hominis* on phytohemagglutinin-mediated lymphocyte stimulation was due to depletion of arginine from the growth medium (2). In 1963, Schimke and Barile had shown that arginine is a major energy source for nonfermentative mycoplasma species which convert prodigious amounts of arginine to citrulline and ornithine via the arginine dihydrolase pathway (12). For almost 3 decades since then, it has been believed that the mycoplasma suppressive effect is mainly due to the depletion of arginine from the nutritional source for cells.

Recently, we identified a lymphocyte blastogenesis inhibitory factor (LBIF) in the culture supernatant of human histiocytic lymphoma U937 (16, 17). The factor was purified by fast protein liquid chromatography (FPLC). LBIF is a single polypeptide chain, as estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). LBIF shows a pl of approximately 4.5 upon chromatofocusing. Partial amino acid sequencing analysis shows that LBIF is a novel immunoregulatory factor. The cell growth inhibitory activity of LBIF was characterized by the growth of lectin-stimulated T lymphocytes (17) as well as various tumor cell lines (15). In this study, we demonstrate that LBIF originates from a mycoplasma, *M. arginini*, and bears the arginine deiminase activity.

**MATERIALS AND METHODS**

**Cell lines.** Human histiocytic lymphoma U937 was obtained from H. Fujiwara, Habikino Hospital, Osaka, Japan, and coded as U937-F or from the American Type Culture Collection, Rockville, Md., and coded as U937-ATCC. A human melanoma cell line, A375, was provided by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. U937-F is a producer of LBIF and has been shown to be infected with a mycoplasma, *M. arginini*, which was determined by metabolism inhibition tests (10), as described below.

**Purification of LBIF.** LBIF was purified from crude supernatant of a human histiocytic lymphoma cell line, U937-F, as described previously (16, 17). Briefly, cells were cultured at 1 × 10^6 cells per ml in serum-free RPMI 1640 medium. The serum-free supernatant was concentrated by an ultrafiltration membrane module, AIL-1010 (Asahi Chemical Industry Co., Ltd., Tokyo, Japan). The crude concentration was fractionated on a DEAE-SPW TSK gel (21.5 mm by 15 cm) (Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris hydrochloride (pH 7.7). The separation was done by a linear gradient from 0 to 0.5 M NaCl. LBIF activity was tested as described below. The active fractions were subsequently fractionated on a Mono P chromatofocusing column (HR 5/20; Pharmacia, Inc.). A fraction of LBIF was further resolved on a hydroxyapatite column (21 by 100 mm; TAPS-05210; Tosen, Tokyo, Japan). The purity of the LBIF preparation was assessed by SDS-PAGE and reversed-phase high-performance liquid chromatography of an RP-304 column (Bio-Rad Laboratories, Inc.), as described previously. The homogeneity of LBIF used in this study is indicated by the results shown in Fig. 2. One unit of LBIF was defined as the amount of LBIF sample required to induce a half-maximum response in the LBIF assay, as previously described. Approximately 10 ng of LBIF sample corresponds to 1 U.

**LBIF assay.** The LBIF assay was originally performed by using interleukin-1 (IL-1)-stimulated murine thymocytes as described previously (16, 17). Our experience showed that a human melanoma cell line A375 could be used as an indicator cell line in this LBIF bioassay. The sensitivity to detect LBIF is the same as that of the original LBIF assay. Furthermore, the LBIF assay using A375 cells is simple and much easier to handle than the original assay. In this study, the LBIF assay was done by using A375 cells, as described previously (14). Briefly, A375 cells were cultured at 5 × 10^5 cells per 200 μl per well in a flat-bottomed 96-well culture plate (Falcon 3072) in the presence or absence of LBIF. After 3 days, cells were pulsed with 18.5 kBq of [3H]
thymidine (625 Bq/mm; TRK-61; Amersham, United Kingdom) per well for the last 4 h of the culture and collected by a cell harvester (Labo Science Co., Ltd.). When the cells were harvested, culture supernatants were removed by a cell harvester and 50 μl of 0.25% trypsin-0.02% EDTA (GIBCO) was added to each well. After the cells were detached, they were harvested on the same filter by the harvester. \(^{3}H\) thymidine incorporation was measured by a liquid scintillation counter (Beckman LS3801). The viable cell number was counted directly by a trypan blue dye exclusion test (see Fig. 4).

**Detection of mycoplasmas.** Mycoplasmas were detected by two standard methods. One detection assay was carried out by using the Mycoplasma Tissue Culture Detection System (GEN-PROBE, San Diego, Calif.) according to the instructions of the manufacturer. Briefly, cell-free supernatant (1.5 ml) was centrifuged at 15,000 \(\times g\) for 10 min. The mycoplasma pellets were hybridized with the \(^{3}H\)-labeled probe at 72 ± 2°C for 2 h. After the addition of the separation suspension (hydroxyapatite), the hydroxyapatite was sedimented by centrifugation at 500 \(\times g\) for 1 min and washed twice with a wash solution, and the radioactivity of the \(^{3}H\)-labeled probe hybridized with mycoplasma DNA was counted with a liquid scintillation counter. The second method was that of Russell et al. (11) with some modification. Briefly, mycoplasma-free murine 3T6 cells \((5 \times 10^{5})\) mixed with U937-F cells \((0.5 \times 10^{5})\) were cultured in a Lab-Tek 4808 chamber (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). 3T6 cells were proven to be mycoplasma-free by using the Mycoplasma Tissue Culture Detection System. After 4 days, monolayers of cells on cover slips were washed with phosphate-buffered saline and fixed twice with Carnoire solution for 5 min. The fixed cells were stained with Hoechst 33258 solution for 15 min. The preparation was washed with distilled water three times, air dried, and mounted and examined by a fluorescence microscope equipped with a UG1 filter (excitation frequency, about 365 nm) and a L420 cut filter (emission, about 450 nm).

**Isolation and identification of mycoplasmas.** Isolation of mycoplasmas from cell cultures was performed by the methods of Barile and McGarrity (3) with modifications. Briefly, PPLO broth (Difco Laboratories) supplemented with horse serum (20%) and fresh yeast extract (10%) was inoculated with cell culture fluid, making serial 10-fold dilutions up to 10\(^{-4}\), and incubated at 37°C for 2 weeks. At 7 and 14 days of incubation, 0.01 ml of culture fluid from each dilution was subcultured onto an agar medium solidified with 1.2% Noble agar (Difco Laboratories) in duplicate. One of the duplicates was incubated aerobically, and the other was incubated anaerobically at 37°C for 2 weeks. A single colony was transferred to an agar plate. This was repeated three times to obtain pure cultures.

Identification of the isolates was carried out by metabolism inhibition tests by the method of Purcell et al. (10). Rabbit antisera against *M. buccale* CH20247, *M. fermentans* PG18, *M. hominis* PG21, *M. lipophilum* MaBy, *M. orale* CH19299, and *M. salivarium* PG20 and mule antiserum against *M. arginini* G230 were employed. Each rabbit antiserum was prepared by the method of Senterfitt (14). Mule antiserum and mycoplasma strains used to prepare rabbit antisera were kindly supplied by M. F. Barile, National Institutes of Health, Bethesda, Md.

**Clearance of mycoplasma.** Mycoplasma-infected U937-F cells \((1 \times 10^{5})\) were cleared of mycoplasma infection by subcutaneous transplantation to an immunosuppressed new-born hamster less than 24 h old which had been pretreated with anti-hamster hamster cytome, as described previously (7, 18). After 3 weeks, U937 cells were recovered from animals, separated from dead cells by Ficoll-Hypaque separation solution, and cultured in vitro for 2 weeks. The U937 cells were determined to be mycoplasma-free by the mycoplasma detection protocols described above. The isozyme patterns (glucose-6-phosphate dehydrogenase, lactate dehydrogenase, and purine nucleotide phosphorylase) were also determined by using the Authentic Kit (Corning Glass Works, Corning, N.Y.) in order to confirm that the recovered cell line was U937.

**Preparation of anti-LBIF antiserum.** BALB/c mice were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Mice were immunized intraperitoneally (i.p.) with 0.2 ml of Freund incomplete adjuvant containing 20 μg of purified LBIF and 100 μg of muramylidipetide every 3 weeks. After four or five immunizations, polyclonal anti-LBIF serum was obtained.

**Assay for enzymatic activity.** L-Arginine (GIBCO) (1 mM) was incubated with various concentrations of LBIF in 5 mM phosphate buffer, pH 6.5, at 37°C for 1 h (9). After heating the samples for 5 min, the reaction mixture was subsequently analyzed by the concentration of citrulline and ornithine by an amino acid analyzer (Hitachi-835; Hitachi, Japan) according to standard procedure.

**RESULTS**

**Dose-response curve of FPLC-purified LBIF.** A375 cells were cultured in the presence of various concentrations of purified LBIF. After 3 days, cell proliferation was monitored by visual examination and by determining the level of incorporation of \(^{3}H\)thymidine for the final 4 h (Fig. 1). The SDS-PAGE pattern of LBIF (U937 cell culture derived) employed throughout this study is seen in Fig. 2. It should be noted that LBIF abruptly inhibited the growth of A37 cells at a very narrow concentration range (approximately 1.5 to 2.5 U/ml) (Fig. 1). This switchlike, all-or-none characteristic
of LBIF was also present in the original LBIF assay system, in which murine thymocytes were stimulated with IL-1 in the presence or absence of LBIF. This inhibition was due to cytostatic but not cytotoxic effects of LBIF (15, 15a, 17).

**LBIF produced by mycoplasma-infected cells.** Although the molecular characterization of LBIF has been carried out, we have not extensively examined possible origins of the LBIF producers such as the U937 cells or infectious organisms (mycoplasmas or viruses). The U937-F cells employed in this study were previously reported to be mycoplasma-free, as judged by the absence of [3H]thymidine incorporation in the culture supernatant and negative staining with ethidium bromide-acridine orange (6). However, we reexamined in this study the U937-F cell by using two highly sensitive mycoplasma detection procedures which have been recently developed. The results were that U937-F was clearly mycoplasma positive. Therefore, in order to determine which organism produces LBIF, we first attempted to clear mycoplasmas from U937-F cells and examine the LBIF production of mycoplasma-free U937-F cells. The clearance of mycoplasmas was done according to the in vivo passage procedure described previously (7). Cells (1 x 10^6/ml) were cultured for 2 days. Then, LBIF and mycoplasma were examined in the supernatant and U937 cells, respectively. LBIF production was not detected in mycoplasma-cleared or mycoplasma-free U937 cells (Table 1). However, when mycoplasma-free U937 cells were infected with mycoplasma contained in the supernatant of U937 cells, these cells again produced LBIF in the culture supernatant. The time course of LBIF production and mycoplasma growth were also examined in the culture of U937-F cells, showing that LBIF production paralleled mycoplasma growth (data not shown). We also investigated the possibility that reinfected U937 cells might produce a suppressor factor distinct from LBIF produced by U937-F cells. To do this, the culture supernatant of reinfected U937 cells was collected and fractionated by sequential FPLC by using DEAE-5PW, Mono P, and hydroxypapatite columns. The yield and effluent pattern of the suppressor factor at each step of the chromatography and the pI value and molecular weight estimated by SDS-PAGE were the same as those of LBIF. Furthermore, peptide mapping analysis and partial amino acid sequencing analysis confirmed that reinfected cells produced LBIF (unpublished data).

These results suggested two possibilities, one being that mycoplasma infection stimulated U937 cells to produce LBIF and the other being that mycoplasma produced LBIF.

**M. arginini produces LBIF.** To examine these possibilities, isolation and serological identification of mycoplasma were carried out. As a result, arginine-catabolizing mycoplasmas were isolated in high titers (10^7 CFU/ml of cell culture fluid). The isolate was cloned three times on agar medium. Four strains were obtained by single colony cloning and subjected to a metabolism inhibition test. Arginine catabolism of all four strains was inhibited by the antiserum against *M. arginini* G230 at the dilutions of 1:1,280 or 1:2,560 but not by other antiserum diluted 1:40. Each antiserum used here showed metabolism-inhibiting antibody titers of 1:640 or higher with homologous organisms and did not cross-react with heterologous organisms at the dilution of 1:40. A representative strain of the isolates identified as *M. arginini* was inoculated into broth containing arginine and processed as follows. The mycoplasma-free supernatant was obtained after 5 days of incubation by centrifugation at 15,000 x g for 10 min and filtered through a membrane module (molecular exclusion limit, 6,000 daltons; AIL-1010 which cut off molecules over M_weights, 6,000). The supernatant was dialyzed with 20 mM Tris hydrochloride buffer (pH 7.8) and fractionated on a

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**TABLE 1. Mycoplasma-infected cells produce LBIF**

<table>
<thead>
<tr>
<th>Cells and condition</th>
<th>Production of LBIF (U/ml)</th>
<th>Detection of mycoplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937-F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycoplasma infected</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>Mycoplasma cleared^d</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Mycoplasma reinfected^d</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>U937-ATCC</td>
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<td></td>
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<tr>
<td>Mycoplasma free</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Mycoplasma infected</td>
<td>4</td>
<td>++</td>
</tr>
</tbody>
</table>

^a Cells (1 x 10^6/ml) were cultured for 2 days. The cell-free supernatant was concentrated 10-fold by ultrafiltration, and the cell growth inhibitory activity was assayed by the LBIF assay, as described in Materials and Methods.

^b Test was performed by staining cells with Hoechst 33258 as described previously (11).

^c Cells were prepared by the in vivo passage method with hamsters, as described previously (7).

^d Mycoplasma-cleared cells were cultured in medium containing 30% culture supernatant of mycoplasma-infected U937-F cells for 7 days.
DEAE-5PW TSK gel (7.5 mm by 7.5 cm; Tosoh) under a linear gradient from 0 to 0.5 M NaCl and subsequently on a G3000 SWXL TSK gel (7.8 by 300 mm; Tosoh). LBIF activity was assayed on each fraction. A single peak of fractions with LBIF activity was detected in materials derived from mycoplasma culture. This elution pattern was identical to those of authentic LBIF on DEAE-5PW and TSK G3000 SWXL gels (Fig. 3). These results suggested that M. arginini might produce LBIF.

Neutralization of mycoplasma-derived inhibitory factor with anti-LBIF antiserum. By using partially purified materials (Fig. 3) derived from mycoplasma culture, we determined whether mycoplasma inhibitory activity could be neutralized by anti-LBIF antiserum prepared by hyperimmunization with the purified LBIF. Anti-LBIF antiserum was added at various concentrations of LBIF in the LBIF assay system. Anti-LBIF antiserum almost completely neutralized the inhibitory activity of mycoplasma materials, whereas control serum did not (Fig. 4A). The same results were obtained in the case of authentic LBIF (Fig. 4B). Thus, considering these results collectively, it was concluded that LBIF was of M. arginini origin.

LBIF has arginine deiminase activity. It is believed that the mycoplasma suppressive effect is mainly due to the depletion of arginine from the nutritional source for cells, and arginine deiminase may be a candidate for a mycoplasmal inhibitory factor.

Therefore, we first determined whether the addition of excess amounts of L-arginine could reverse the inhibiting activity of LBIF. Figure 5 clearly shows that LBIF activity was effectively blocked by the addition of L-arginine in dose-response fashion while not being affected by the addition of L-lysine, indicating that LBIF might be arginine deiminase or arginase. To answer this question, the product resulting from the incubation of the mixture of LBIF and L-arginine in vitro was determined (9). In the case of arginine deiminase, the end products are citrulline and NH₃, whereas arginase produces ornithine and urea. Table 2 clearly shows that LBIF produces citrulline but not ornithine, demonstrating that LBIF is arginine deiminase.

DISCUSSION

In previous studies, we established the molecular nature of LBIF, which was purified from the culture supernatant of human histiocytic lymphoma U937. We have demonstrated here that LBIF originates from M. arginini-infected U937 cells. A number of studies have suggested that soluble mycoplasma products may cause significant alteration of lymphoid cell functions (2, 4, 5). However, the molecular characterization of these factors has remained preliminary. The in vitro culture of mycoplasmas requires PPLO medium containing 20% horse serum, 10% yeast extract, and a number of other supplements including glucose, amino ac-
FIG. 4. Neutralization of mycoplasma-derived inhibitory factor with anti-LBIF antiserum. (A) *M. arginini*-derived materials which were purified on DEAE-5PW and G3000 SWXL TSK gels, as shown in Fig. 3. (B) U937-derived LBIF which were purified as described in Materials and Methods. A375 cells (5 × 10^4) were cultured with various concentrations of *M. arginini*-derived materials (nondiluted material) or U937-derived LBIF (nondiluted material) and LBIF at 16.9 U/mL in the presence of 21-fold-diluted murine anti-LBIF antiserum (○) or control serum (preimmune serum) (□) for 3 days, as described in Materials and Methods. The viable cell number was counted directly by a trypan blue dye exclusion test. Each dot represents the mean value for 12 wells. A375 cell number (percent) = ([cell number of experimental group - 5,000]/[cell number of control group without LBIF] - 5,000)] × 100. The cell number of control group was 1.9 × 10^4 per well. n (a multiplier of dilution rate) is shown on the x axis.

FIG. 5. Blocking of LBIF activity by the addition of L-arginine. The LBIF sample shown in Fig. 2 was used. A375 cells (5 × 10^4) were cultured with (○) or without (□) LBIF (5 U/mL) in the presence of various doses of L-arginine. After 3 days, cell proliferation was monitored by observing the level of [3H]thymidine pulse-labeling for the final 4 h. The effect of L-lysine was also examined, and no influence was confirmed up to 7 mM.

example of how mammalian cells can be used as the best carrier of a nutritional matrix for the growth of mycoplasmas. Mycoplasma-infected cells can often be cultured in a serum-free medium. Accordingly, we were easily able to purify the factor, LBIF, to homogeneity by FPLC from the serum-free supernatant of mammalian cells. This allowed us to analyze the amino acid sequence of LBIF and to prepare the antiserum specific to LBIF.

It has been demonstrated that an immunosuppressive substance derived from mycoplasmas may be the arginine deiminase which was suggested by a few classical studies published about 3 decades ago (2, 4, 5). It was clearly demonstrated (Fig. 4 and Table 2) that LBIF bore the arginine deiminase activity.

In previous studies, we observed that LBIF is biologically very specific and its effects are exerted in a selective manner in vitro. LBIF does not inhibit IL-2 production or p55 IL-2 receptor expression (17). Moreover, although LBIF drastically inhibits the constitutive growth of various tumor cell lines in vitro, there are several cell lines whose cell growth is not influenced by LBIF (15). If these results are attributable to the simple nutritional depletion of L-arginine by arginine deiminase, L-arginine must play an important role in regulating cell growth and differentiation. In this regard, it has been reported that arginine deprivation inhibits the replication of tumor cells, parasites, and viruses and suppresses cellular immune functions in vitro (13).

Hibbs et al. (8) showed that the activated macrophage cytotoxic effector system is associated with L-arginine deiminase activity and that the imino nitrogen removed from the guanido group of L-arginine by the deiminase reaction subsequently undergoes oxidation to nitrite. Thus, it has been suggested that arginine deiminase might induce multiple metabolic changes in mammalian cells. Furthermore, Albina et al. (1) recently demonstrated that the culturing of resident macrophages in L-arginine-free medium results in a generalized increase in activation-associated functions, not only morphologically pronounced spreading and elongation but also augmented tumoricidal activity, superoxide production, phagocytosis, and protein synthesis.

A number of studies have suggested that arginine-utilizing mycoplasma species can exert an immunosuppressive effect in vivo (4). However, it is difficult to envisage how organisms could deplete a host of arginine. Thus, the in vivo significance of mycoplasma inhibition of lymphoid cells in vitro appears to be difficult to evaluate. However, examining these results collectively, it is conceivable that macrophages may be involved in an immunosuppressive effect in an L-arginine-deficient microenvironment in vivo. The purified LBIF may be useful to explain parts of disease pathogenesis caused by mycoplasma infection in vivo.

### Table 2. Arginine deiminase activity of LBIF

<table>
<thead>
<tr>
<th>LBIF (U/mL)</th>
<th>L-Arginine (mM)</th>
<th>Conc (µM) of product:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L-Citrulline</td>
<td>L-Ornithine</td>
</tr>
<tr>
<td>0</td>
<td>1.08</td>
<td>ND</td>
<td>2.66</td>
</tr>
<tr>
<td>0.1</td>
<td>1.08</td>
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<td>2.60</td>
</tr>
<tr>
<td>0.3</td>
<td>1.10</td>
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<td>1.67</td>
<td>70.5</td>
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</tr>
<tr>
<td>10</td>
<td>0.95</td>
<td>234.0</td>
<td>2.86</td>
</tr>
<tr>
<td>50</td>
<td>0.59</td>
<td>623.0</td>
<td>2.86</td>
</tr>
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</table>

* L-Arginine (1 mM) was incubated with various concentrations of LBIF in 5 mM phosphate buffer, pH 6.5, at 37°C for 1 h. The reaction mixture was analyzed by the Hitachi-835 amino acid analyzer.
ACKNOWLEDGMENTS

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LITERATURE CITED