ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1991, p. 2526–2530
0066-4804/91/122526-05$02.00/0

Vol. 35, No. 12

Susceptibility of *Legionella pneumophila* Grown Extracellularly and in Human Monocytes to Indole-3-Propionic Acid

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Received 22 March 1991/Accepted 19 September 1991

Indole-3-propionic acid (IPA), a phytohormone derivative, is a potent inhibitor of growth of *Legionella pneumophila* cultivated extracellularly in a chemically defined hypotonic medium and intracellularly in human monocytes. The inhibitory activity turns into bactericidal activity with increasing concentrations. The susceptibility of the microorganism to IPA was more evident in "fast-growing" cultures (under conditions of vigorous shaking) than in static cultures growing under an atmosphere of 5% CO_2–95% air, which resulted in a decreased growth rate. The MIC, after incubation with the drug for 48 h and as determined by counting of the CFU, was 1.58 μM for fast-growing cultures and 2.64 μM for those grown under static conditions. The MBCs were 5.28 and 26.43 μM, respectively. Tryptophan (Trp) at 150 μM prevented the inhibition caused by 2.64 μM IPA, increased the MIC about 3-fold, and increased the MBC by 10-fold. The effect of Trp was less remarkable in "slow-growing" cultures. The susceptibility of *L. pneumophila* proliferating in human monocytes was markedly lower than that when it was cultivated extracellularly in the chemically defined hypotonic medium. The MIC after incubation for 48 h was 5.28 μM, and a decrease in viable count was achieved with 105.70 μM. The lower susceptibility was apparently due (at least partially) to the presence of Trp (24.50 μM) in the RPMI 1640 medium that was used for the monocyte cultures. The effect of IPA was time dependent, and prolonged exposure enhanced the bactericidal activity and turned the inhibitory dose into a bactericidal dose. The present data demonstrate that IPA is a potent anti-*L. pneumophila* factor, although it has a markedly lower activity against bacteria growing intracellularly compared with its activity against extracellularly proliferating microorganisms.

Legionella pneumophila, a microorganism known to cause the Legionnaires' disease, is a facultative intracellular pathogen (8) that is capable of proliferating in human monocytes, pulmonary alveolar macrophages, protozoa, and a variety of tissue culture cells (6, 8, 9). After invasion of the phagocytic cell by attachment to complement receptors and coiling phagocytosis, the bacteria grow within the phagocyte and eventually cause its disruption (7, 8, 11).

L. pneumophila is a nutritionally fastidious microorganism that requires amino acids as a source of energy and carbon (3, 17), and its ability to grow in chemically defined media has been investigated by others (1, 3, 12, 14, 15, 17, 19) as well as in our laboratory (4). By studying the effects of various amino acids, their precursors, and the derivatives on the growth of *L. pneumophila*, it was found that indole-3-acetic acid, which is an auxin (phytohormone), and another indole derivative, indole-3-propionic acid (IPA), are potent inhibitors of growth of this microorganism (5). As assayed by turbidity measurements, IPA was found to be an about 20-fold more effective inhibitor than indole-3-acetic acid (5). We therefore focused our studies on further investigation of the susceptibility of *L. pneumophila* to IPA using viable count methods, namely, determination of CFU, which made it possible to distinguish between the inhibitory (bacteriostatic) and the bactericidal activities of the compound.

Since in vitro susceptibility data are not always reliable in predicting the efficacy of an antimicrobial agent, particularly for *L. pneumophila*, we followed the IPA effect on bacteria proliferating in human monocytes. Such systems were previously used by others for the evaluation of erythromycin, rifampin (9, 18), various quinolones, and other compounds as effective anti-*L. pneumophila* factors (13, 18).

The structural resemblance of IPA to tryptophan (Trp) or to the precursors of its biosynthetic pathway (10, 20) prompted us to further investigate the effect of this amino acid on the anti-*L. pneumophila* activity of IPA.

MATERIALS AND METHODS

Materials. IPA, L-tryptophan, and the other amino acids used in this study were from Sigma Chemical Company, St. Louis, Mo.

Bacteria and growth conditions. *L. pneumophila* serogroup 1 Philadelphia 1 was obtained from Herve Bercovier, Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem. The microorganism was grown in a chemically defined buffered hypotonic medium supplemented with minerals and amino acids (CDHM), to which the essential L-cysteine (200 μg/ml) was added aseptically (5). The cultures were incubated for 24 to 48 h at 37°C with vigorous shaking on a rotatory shaker (Gyrotory shaker, model G2; New Brunswick Scientific Co., Inc., Edison, N.J.). For susceptibility experiments, logarithmically growing bacteria (overnight culture) were inoculated into tubes containing various concentrations of IPA in 5 ml of CDHM. After incubation at 37°C with shaking, proliferation of the bacteria was determined turbidimetrically by using a Coleman spectrophotometer (model 6/20A, Junior 11A; Perkin-Elmer Coleman Instruments Division, Oak Brook, Ill.) at 550 nm and by counting the colonies growing on buffered charcoal yeast extract (BCYE) agar (2) contain-

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ing 50 mM potassium phosphate buffer instead of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer and supplemented with 0.1% α-ketoglutarate and 0.02% L-cysteine. The bacteria were incubated for 3 to 4 days at 37°C.

Isolation and cultivation of human blood monocytes. Monocytes were prepared from the blood of a healthy donor with no history of legionellosis by a modification of the method described by Horwitz and Silverstein (8). Blood was collected in an anticoagulant (heparin) and diluted 1:1 in phosphate-buffered saline (PBS), and 35 ml was layered over 14 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). After centrifugation at 400 × *g* for 30 min, layers containing the mononuclear cells were aspirated, washed twice with PBS, and resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 2% fetal bovine serum. The cells were counted in a hemacytometer and adjusted to about 2×10^6 cells per ml. The viability was more than 99%, as assessed by trypan blue exclusion. A total of 2 ml of the cell suspension was placed into plastic petri dishes (35- by 10-mm tissue culture dishes; A/S; Nunc, Roskilde, Denmark), and the dishes were incubated for 2 h at 37°C in a humidified 5% CO_2 incubator. The nonadherent cells were removed by two washes with the medium described above, and then 2 ml of the medium with 15% fetal bovine serum was added to the monocyte layer. The cells were incubated for 6 days and then washed twice with RPMI 1640 medium without antibiotics, and dishes containing the monocytes in 2 ml of medium were used for infection with *L. pneumophila*.

Infection of the monocytes. *L. pneumophila* (5×10^6 in 0.05 ml) at the logarithmic stage of growth (20-h culture grown in CDHM with aeration) was added to dishes containing 10^6 monocytes in 2 ml of RPMI 1640 medium. The cultures were rotated on a Gyrotory shaker (100 rpm) for 1 h at 37°C; thereafter, IPA (in H_2O) was added and incubation was continued for 96 h under static conditions in a humidified 5% CO_2 incubator. The dishes were examined microscopically each day, and the monocytes were roughly quantitated to detect lysis and/or detachment of cells from the monolayers. The proliferation of the bacteria in monocytes was determined by the following procedure. The medium was aspirated, and the cell layer was washed twice with 2 ml of PBS and was subsequently incubated with 2 ml of double-distilled water for 40 min at 37°C with shaking on a Gyrotory shaker (150 rpm). This procedure caused complete lysis of the monocytes (as observed microscopically) without affecting the bacteria. After appropriate dilutions in H_2O, the contents of the dishes were assayed for CFU. The viability of the extracellular bacteria (nonphagocytosed) present in the RPMI 1640 medium was assessed in 0.1-ml samples taken at various time intervals, diluted in H_2O, and plated onto BCYE agar.

To eliminate the possibility of a drug carryover effect on subcultures of bacteria grown in the presence of high concentrations of IPA (105.70 and 211.40 μM), the infected monocytes were lysed and transferred from the dishes into plastic tubes and then pelleted by centrifugation at 12,000 × *g*. After one wash with 2 ml of water, the pellet was resuspended in the same volume of water and samples were plated onto BCYE agar. The same washing procedure was used to count the microorganisms in the RPMI 1640 medium. Since the viable count of the washed bacteria treated with the highest drug concentrations used did not significantly differ from that obtained by direct plating, this step was omitted when lower concentrations of IPA were used.

Determination of the rate of extracellular growth of *L. pneumophila* cultivated in static cultures under an atmosphere of 5% CO_2. The bacteria were grown in tubes containing 5 ml of CDHM with the indicated additions and under static conditions in a humidified 5% CO_2 incubator. At various time intervals, samples were taken, diluted, and plated onto BCYE agar as described above.

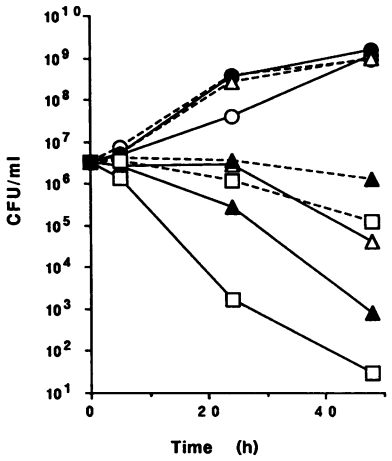

FIG. 1. Effect of IPA on growth of *L. pneumophila* in aerated cultures in the presence and absence of Trp. The bacteria were grown at 37°C with vigorous shaking in CDHM supplemented with the indicated additions. For determination of the CFU per milliliter, samples were taken at various time intervals, diluted in H_2O, and plated onto BCYE agar. The colonies were counted after incubation for 3 to 4 days at 37°C. There was no evidence of any antimicrobial carryover, because the number of CFU decreased linearly with dilution of the IPA-containing cultures. The lowest detectable number of viable bacteria permitted by the assay was 20/ml. The following concentrations of IPA (in micromolar) were used: none (●), 0.53 (○), 2.64 (△), 5.28 (▲), 26.43 (□). Dotted lines are for cultures supplemented with Trp (150 μM) and with the various concentrations of IPA given above. The inoculum size was the same (as determined turbidmetrically) in each experiment. Points represent the mean value of three experiments in duplicate, and standard errors are within the symbols.

RESULTS

Effect of IPA on growth of *L. pneumophila* in aerated cultures in the presence and absence of Trp. *L. pneumophila* cultivated under conditions of vigorous shaking, a "fast-growing" culture, was highly susceptible to IPA, and increasing concentrations of the compound caused an increased rate of cell death (Fig. 1). IPA (0.53 μM) caused a slight inhibition of growth during incubation for 24 h, but upon incubation for 48 h, the number of CFU was the same as that in controls incubated without IPA. The MIC, defined as the minimum concentration of drug that inhibited growth, appeared to be 2.64 μM. However, upon prolonged incubation (48 h), this concentration caused a marked decrease in the number of CFU, and the MIC at that time was 1.58 μM (Table 1).

The MBC, which was defined as the lowest concentration required to achieve a 99.9% decrease in the initial CFU, was 5.28 μM IPA, which, upon incubation with the bacteria for 48 h, resulted in a ≥3-log-unit cell killing (Fig. 1; Table 1). After the initial 5 h of incubation, a decrease in the viable count was found only with 26.43 μM IPA. Thus, the effect of IPA appears to be exposure time dependent.

The presence of Trp (150 μM) in the growth medium

TABLE 1. MIC and MBC of IPA for *L. pneumophila* in fast- and slow-growing cultures in the presence and absence of Trp[a]

Culture	MIC (μM)		MBC (μM)	
	−	+	−	+
Fast-growing	1.58	5.28	5.28	52.85
Slow-growing	2.64	5.28	26.43	105.70

[a] For fast-growing cultures, the bacteria were cultivated with vigorous shaking (150 rpm) at 37°C for 48 h in CDHM with (+) or without (−) Trp (150 μM) and with various concentrations of IPA. The slow-growing cultures were incubated for 48 h under static conditions in a humidified 5% CO_2 incubator. The data represent the means of three experiments done in duplicate.

completely prevented the inhibition caused by 0.53 and 2.64 μM IPA in a Trp-less medium and turned the bactericidal concentrations of 5.28 and 26.43 μM into bacteriostatic and slightly bactericidal, respectively.

Inhibition of growth of *L. pneumophila* within human monocytes by IPA. As depicted in Fig. 2, the susceptibility to IPA of *L. pneumophila* proliferating in human monocytes was markedly lower than that when it was growing in CDHM. A decrease of about 1 log unit in the number of CFU was obtained with 105.70 μM IPA after incubation for 72 h, whereas the MIC was 5.28 μM. The proliferation of bacteria in the presence of 1.06 μM was the same as that in controls incubated without IPA (data not shown). The extracellular number of bacteria in the RPMI 1640 medium after incubation for 48 h increased by about 1 log unit compared with the initial CFU per milliliter and decreased with increasing IPA concentrations (Table 2). IPA per se did not affect the viability of the monocytes, as assessed by trypan blue exclusion by cells preincubated with 105.70 μM IPA for 96 h.

Effect of IPA on *L. pneumophila* grown under static conditions in a humidified 5% CO_2 incubator. To examine the effect of conditions of growth on the susceptibility to IPA, *L. pneumophila* was cultivated in static cultures in a humidified 5% CO_2 incubator in CDHM with and without Trp and with various concentrations of IPA. The growth conditions were thus partially similar to those applied when the bacteria were cultivated in the monocytes. The data presented in Fig. 3 demonstrate that the growth rate under these conditions was markedly lower than that in aerated cultures, and after incubation for 24 h, almost no increase in the CFU could be observed. The presence of Trp did not significantly improve growth, although it decreased the susceptibility of the organism to IPA (Fig. 3; Table 1). The MIC of IPA in a "slow-growing" culture was higher than that for fast-growing bacteria, and the effect of Trp was less remarkable under the latter conditions as well (Fig. 3; Table 1). A small decrease in viable count was achieved with 5.28 μM IPA, whereas increasing concentrations up to 105.70 and 52.85 μM were bactericidal and reduced the number of CFU by almost 6 log units as the result of incubation with the drug for 48 and 72 h, respectively (Fig. 3). The MBC of IPA for slow-growing cultures was 5-fold higher than that for fast-growing microorganisms, and the addition of Trp increased the MBC by 4- and 10-fold, respectively (Table 1).

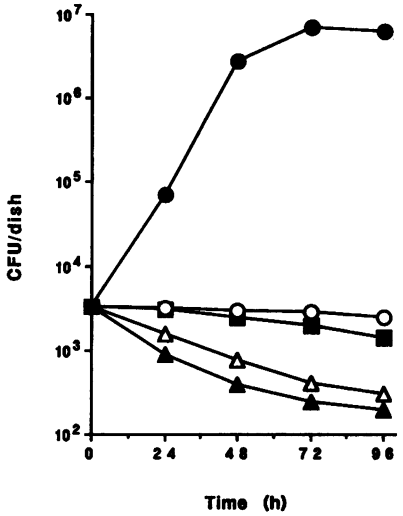

FIG. 2. Inhibition by IPA of *L. pneumophila* cultivated in human monocytes. The cells infected with *L. pneumophila* (see text) were incubated in a humidified incubator under an atmosphere of 5% CO_2. IPA was added 1 h after infection. Viable cell counts were determined at the indicated time intervals after RPMI 1640 medium was removed and washing and lysis of the monocytes (see text). The bacteria, at appropriate dilutions in H_2O, were plated onto BCYE agar. The following concentrations of IPA (in micromolar) were used: none (●), 5.28 (○), 26.43 (■), 105.70 (△), 211.40 (▲). Each point represents the mean of three experiments in duplicate, and standard errors are within the symbols.

DISCUSSION

IPA is a potent and specific inhibitor of growth of *L. pneumophila*, a facultatively intracellular pathogen. It has been previously shown in this laboratory that IPA at a concentration of 26.40 μM selectively inhibits the growth of several *L. pneumophila* strains, whereas other bacterial genera, like *Escherichia coli*, *Mycobacterium smegmatis*,

TABLE 2. Effect of IPA on extracellular *L. pneumophila* incubated in RPMI 1640 medium in cultures with monocytes

Concn IPA (μM)	CFU/ml at the following times (h)[a]:				
	0	24	48	72	96
None	$(2.15 \pm 0.09) \times 10^6$	$(4.61 \pm 0.21) \times 10^6$	$(1.84 \pm 0.10) \times 10^7$	$(9.51 \pm 0.54) \times 10^6$	$(6.10 \pm 0.30) \times 10^6$
5.28		$(2.50 \pm 0.14) \times 10^6$	$(9.92 \pm 0.59) \times 10^5$	$(5.23 \pm 0.32) \times 10^5$	$(1.90 \pm 0.13) \times 10^5$
24.43		$(8.82 \pm 0.69) \times 10^4$	$(2.15 \pm 0.19) \times 10^4$	$(7.93 \pm 0.51) \times 10^3$	$(4.90 \pm 0.41) \times 10^3$
105.70		$(9.04 \pm 0.55) \times 10^3$	$(4.91 \pm 0.42) \times 10^3$	$(1.01 \pm 0.09) \times 10^3$	$(3.85 \pm 0.36) \times 10^2$
211.40		$(4.80 \pm 0.40) \times 10^3$	$(1.63 \pm 0.15) \times 10^3$	$(3.10 \pm 0.45) \times 10^2$	$(1.25 \pm 0.14) \times 10^2$

[a] *L. pneumophila* (5×10^6) at the logarithmic stage of growth was added to plastic dishes (35 by 10 mm) containing adherent monocytes in 2 ml of RPMI 1640 medium. The cultures were incubated for 1 h at 37°C on a Gyrotory shaker prior to the addition of the indicated concentrations of IPA and, subsequently, under static conditions in a humidified 5% CO_2 incubator (for further experimental details, see text). The viability of the extracellular bacteria in the RPMI 1640 medium was assessed in 0.1-ml samples taken at the indicated time intervals, diluted in H_2O, and plated onto BCYE agar. Values are means ± standard errors of three experiments in duplicate.

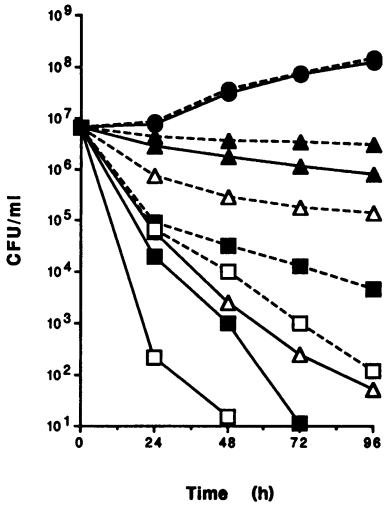

FIG. 3. Effect of IPA on *L. pneumophila* growing in static cultures under an atmosphere of 5% CO_2. The bacteria were cultivated in CDHM with the additions described in the legend to Fig. 1. The cultures were incubated under static conditions in a humidified 5% CO_2 incubator at 37°C. Other experimental procedures were as described in the legend to Fig. 1. The following concentrations of IPA (in micromolar) were used: none (●), 5.28 (▲), 26.43 (△), 52.85 (■), 105.70 (□). Dotted lines are for cultures supplemented with Trp. The inoculum size was the same in each experiment. Each point represents the mean of three experiments in duplicate, and standard errors are within the symbols.

Proteus morganii, *Salmonella typhi*, *Shigella flexneri*, *Pseudomonas aeruginosa*, and yeasts like *Candida albicans* and *Saccharomyces cerevisiae* were not affected (5). Further studies have shown that other strains of *L. pneumophila*, such as serogroup 1 AAM-4, AAM-17, and AAM-26; serogroup 6 AAM-26; and recently obtained serogroup 1 Philadelphia 1 BC 1636 (all from the Centers for Disease Control, Atlanta, Ga.), exhibited susceptibility to IPA at the same range of concentrations and were responsive to Trp, as was the strain used in the experiments described here (unpublished data).

The inhibitory activity of IPA changes into cidal activity with elevated concentrations and prolonged times of exposure. However, a decrease in the growth rate caused by incubation for 24 h with a low concentration of IPA (0.53 μM) was reversible, and a normal bacterial yield was achieved upon incubation for 48 h (Fig. 1). A concentration of 2.64 μM was the MIC for the bacteria incubated for 24 h, but it resulted in a 2-log-unit decrease in viable cells at 48 h of incubation. After a relatively short time of exposure to the drug (5 h), a decrease in CFU was obtained only in the presence of a relatively high IPA concentration in the medium (26.43 μM), whereas the MBC at 48 h of incubation was 5.28 μM (Fig. 1), which indicates that the effect of IPA is exposure time dependent.

The physiological state of the microorganism obtained at favorable or nonfavorable conditions of growth markedly affected the susceptibility to IPA (Table 1). Since aeration was reported to increase the growth rate of legionellae (16), we examined the effect of IPA on aerated cultures (fast-growing) and on microorganisms grown under static conditions under an atmosphere of 5% CO_2, which supported a markedly lower growth rate (Fig. 3). A concentration of 1.58 μM IPA was the MIC for *L. pneumophila* incubated for 48 h in aerated cultures, whereas 2.64 μM was the MIC for bacteria at a low growth rate. The bactericidal potency was markedly more affected by the physiological conditions, and the MBC for aerated cultures was fivefold lower than that for bacteria cultivated under static conditions (Fig. 1 and 3; Table 1). The possibility of a better contact between IPA and bacterial cells in cultures incubated under shaking conditions should be considered as well.

Since IPA is structurally related to Trp, a biologically essential metabolite, it is plausible to assume that the compound blocks the biosynthesis of this amino acid and/or its physiologically important metabolic reactions. Trp synthase from bacteria, and particularly that from *E. coli*, has been extensively studied and has been found to catalyze the three following reactions (10, 20): (i) indole-3-glycerol phosphate ⇆ indole + D-glyceraldehyde-3-phosphate; (ii) indole + L-serine $\xrightarrow{\text{pyridoxal phosphate}}$ L-tryptophan + H_2O; (iii) indole-3-glycerol phosphate + L-serine $\xrightarrow{\text{pyridoxal phosphate}}$ L-tryptophan + D-glyceraldehyde-3-phosphate + H_2O. Regarding the chemical relationship, IPA may possibly inhibit at least one of these biosynthetic reactions. It was thus reasonable to study the effect of Trp on the susceptibility to IPA, particularly bearing in mind that *L. pneumophila* is prototropic for this amino acid.

Trp at a concentration of 150 μM in the growth medium prevented the inhibition caused by 2.64 μM IPA and increased the MIC by more than 3-fold and the MBC by 10-fold (Fig. 1; Table 1). The effect of Trp was less prominent in slow-growing cultures (Fig. 3; Table 1), which further emphasizes the importance of the physiological conditions in estimating the susceptibility of *L. pneumophila* to IPA as well as the ability of Trp to overcome or moderate susceptibility.

In vitro susceptibility data do not always correlate with the in vivo efficacy of an antimicrobial factor, particularly in the case of an intracellular pathogen like *L. pneumophila* (9). Therefore, only drugs which can penetrate the phagocytic cells can attack the microorganisms in their intracellular niche. We thus found it important to examine the effect of IPA on bacteria proliferating in human monocytes. The data depicted in Fig. 2 demonstrate that the susceptibility to IPA of *L. pneumophila* growing in monocytes was markedly lower than that of the microorganism cultivated extracellularly. The MIC upon incubation for 48 h was 3.3-fold higher than that for the extracellularly growing bacteria and corresponds, rather, to the MIC for legionellae growing in the presence of Trp (Fig. 2; Table 1). Incubation with IPA at 105.70 μM for 48 h caused a decrease in the CFU by 76% compared with that of the initial viable count. This low susceptibility may have been at least partially due to the presence of Trp (24.50 μM) in the RPMI 1640 medium. Other factors, like limited accumulation of IPA in the monocytes, should be considered as well.

In preliminary experiments with white mice, we found that IPA possesses a relatively low toxicity; concentrations up to 0.5 g/kg of body weight introduced intraperitoneally did not appear to be lethal. Although the therapeutic potential of IPA seems to be debatable, considering, at least, the presence of Trp in blood, the drug might possibly be used for disinfecting water.

In conclusion, the susceptibility of *L. pneumophila* to IPA drastically decreases with the addition of Trp to the growth medium, which indicates that IPA inhibits a biosynthetic pathway of this amino acid or its metabolism or the latter prevents the uptake and intracellular accumulation of IPA.

Further studies are in progress to elucidate the mechanism of the anti-*L. pneumophila* activity of IPA.

ACKNOWLEDGMENT

The research was supported in part by the George M. Jaffin Fund, New York, N.Y.

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