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Direct Activation of Human Monocyte-Derived Macrophages by a Bacterial Glycoprotein Extract Inhibits the Intracellular Multiplication of Virulent *Legionella pneumophila* Serogroup 1

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Intracellular multiplication of virulent *Legionella pneumophila* serogroup 1 was inhibited by human monocyte-derived macrophages activated by a glycoprotein extract of *Klebsiella pneumoniae*, RU 41.740. Macrophage cultures were infected with *L. pneumophila* in the presence of immune antibodies on day 7 of culture. Extracellular bacteria were removed an hour after infection, and various concentrations of RU 41.740 or an antibiotic, erythromycin, were added. Intracellular multiplication in the presence of RU 41.740 was significantly slowed down compared with that of cultures without RU 41.740. The reduction was, however, significantly less than that effected by erythromycin, which was used as a positive control for inhibition of intracellular multiplication. Cultures incubated with RU 41.740 before infection also demonstrated a significant reduction in the intracellular multiplication of *L. pneumophila*. In addition, RU 41.740 increased superoxide anion production from human monocytes in suspension in the presence of *L. pneumophila*. These results show that direct nonspecific activation of macrophages by a bacterial glycoprotein inhibits the intracellular multiplication of *L. pneumophila* and may suggest a role for activated macrophages in host defense against intracellular pathogens.

Legionella pneumophila is a facultative, intracellular gram-negative bacillus. Upon ingestion by cells of the monocyte-macrophage series, it resists destruction and, in fact, multiplies within these cells (6, 7, 26, 48). Indirect activation of human monocytes in vitro by concanavalin A-stimulated lymphocyte supernatant has been shown to inhibit the intracellular multiplication of virulent *L. pneumophila* (27). In this study, we evaluated the possibility of directly activating human monocyte-derived macrophages (MDMs) to restrict the intracellular multiplication of virulent *L. pneumophila* serogroup 1. The activation of the phagocytic cells was achieved with a bimolecular glycoprotein compound, RU 41.740, extracted from *Klebsiella pneumoniae* K201 (18–20, 46). Activated macrophages are known to inhibit the growth of intracellular pathogens by both oxygen-independent and oxygen-dependent mechanisms, which involve the release of toxic oxygen radicals (5, 34, 42, 43). MDMs activated by recombinant interferon gamma (8; J. L. Vildé, P. Rajagopalan, J. J. Pocidalo, and M. Brandely, Prog. Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1071, 1986) also inhibit the intracellular growth of *L. pneumophila*. In these reports and in an earlier report by Horwitz and Silverstein (27), the cells were activated with products derived from T lymphocytes. In this study, the macrophages were activated nonspecifically and directly by a bacterial glycoprotein extract. Many bacterial products containing lipopolysaccharides have been shown to be potent activators of macrophages (3). The *K. pneumoniae* extract RU 41.740 has been shown to modulate the immune response (21, 39, 50, 51) and hence was used in this study to directly activate the cultured macrophages.

In the model used in this study, we demonstrate that (i) monocytes preincubated with a bacterial glycoprotein exhibit metabolic oxidative burst in a chemiluminescence (CL) assay and (ii) direct nonspecific activation of MDMs by this glycoprotein slows the intracellular multiplication of virulent *L. pneumophila*.

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MATERIALS AND METHODS

Bacteria. *L. pneumophila* serogroup 1 (strain Paris CB 81-13) was isolated from the lung of a patient who died from Legionnaires disease during a nosocomial outbreak in Paris, France. Crude lung homogenate was plated on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYE) and antibiotics. Plates were incubated at 35°C with 2.5% CO_2 and 95% humidity. A single colony was taken after 72 h and plated onto BCYE plates, and bacteria were grown at 35°C to mid-logarithmic phase. The bacteria were harvested in sterile distilled water and tested for viability and the absence of contaminating bacteria. The concentration of the bacteria was then adjusted to 10^9 bacteria per ml, divided into samples, and stored at −70°C until use. The virulence of this strain, passaged twice in BCYE agar, was assessed by intraperitoneal infection of guinea pigs as described recently (14).

Immune serum. Immune serum was obtained from a patient recently recovered from Legionnaires disease, who was no longer under medication. The anti-*L. pneumophila* antibody titer as measured by indirect immunofluorescence was 1/512. Immediately after collection, the serum was filtered (Millipore Corp., Bedford, Mass.) and stored in samples at −70°C until use.

Normal serum. Sera from five healthy donors free of anti-*L. pneumophila* antibodies were pooled, filtered, and stored in portions at −20°C until use.

RU 41.740. Radiosterilized lyophilized RU 41.740 (2 mg)

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was reconstituted in sterile, deionized double-distilled water, divided into samples, and stored at −70°C till use. The product was provided by Laboratoire Cassenne, Osny, France.

MDMs. Mononuclear cells were separated from blood with Ficoll-Hypaque gradient (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and monocytes were isolated by adherence to plastic substrates. Briefly, 20 to 30 ml of heparinized venous blood was obtained from healthy donors seronegative for *L. pneumophila* antibodies. Whole blood was diluted (1:2) with the tissue culture medium RPMI 1640 (GIBCO, Paisley, Scotland), and three parts of the diluted blood were carefully layered over two parts of the gradient and centrifuged at 400 × *g* for 30 min at room temperature. The mononuclear cell band was carefully aspirated and suspended in three times the original volume with RPMI 1640 and centrifuged at 400 × *g* for 15 min. The pelleted cells were washed twice and suspended in RPMI 1640 (supplemented with 2 mM L-glutamine and 25 mM hydroxyethylpiperazine ethanesulfonic acid buffer; GIBCO), and the concentration was adjusted to 6×10^6 cells per ml. The percent esterase-positive cells was determined by differential staining with May-Grunwald-Giemsa alpha naphthyl butyrate esterase (47), and the concentration was adjusted to contain 10^6 monocytes per ml. A 1-ml suspension containing approximately 10^6 monocytes was layered onto sterile Lab-Tek plastic tissue culture wells (Miles Laboratories, Inc., Elkhart, Ind.) with 10% pooled normal serum and left for adherence for 1.5 h at 37°C with 5% CO_2. The nonadherent cells were removed after 1.5 h, and the adhering cells were washed gently with the tissue culture medium twice more to remove any remaining nonadherent cells. This adherent monolayer was fed with fresh RPMI 1640 and 10% normal serum and incubated at 37°C with 5% CO_2. The medium was changed on alternate days. A homogeneous layer of well-spread macrophages was obtained on day 7 of culture.

Macrophage toxicity. The effect of various concentrations of RU 41.740 on the viability of MDMs was assessed by the lactic dehydrogenase (LDH)-release assay (31) by using a commercially available LDH kit (optimized UV Merkotest; E. Merck AG, Darmstadt, Federal Republic of Germany) and a trypan blue dye exclusion test.

Infection of the macrophage monolayers. On day 7, the monolayer of MDMs was infected with *L. pneumophila* and 10% fresh immune serum in RPMI 1640 (bacteria-cell ratio, 10:1). At 1 h, in one well the supernatant containing the extracellular bacteria was removed and the intracellular *L. pneumophila* were enumerated after hypotonic lysis with sterile distilled water. In the rest of the wells, the supernatant was removed and the monolayers were rinsed vigorously with sterile phosphate-buffered saline (pH 7.2; 0.05 M) to remove any noninternalized bacteria and fed with fresh medium containing 10% normal serum, with (1.5 to 200 μg/ml) or without RU 41.740. The cultures were reincubated at 37°C with 5% CO_2 for 24 h.

Erythromycin, which is the reference drug for the treatment of Legionnaires disease (16, 40) and which is known to inhibit the intracellular multiplication of virulent *L. pneumophila* within human monocytes (28, 49), was used as a positive control for inhibition of multiplication. Therefore, in certain cultures 0.1 to 1.0 μg of erythromycin lactobionate (Abbott Laboratories, Paris, France) per ml was added instead of RU 41.740, and the experiment was done as stated above.

Preincubation of the MDMs with RU 41.740. In certain experiments, on day 4 of culture MDM monolayers were preincubated with 50 to 200 μg of RU 41.740 for 72 h (until day 7 of culture). Cultures were then vigorously rinsed with RPMI 1640 and infected with *L. pneumophila* (as described above). Control cultures consisted of monolayers not treated with RU 41.740.

Controls. The control cultures consisted of (i) only the medium and the bacteria to ensure that no multiplication occurred in the absence of macrophages and (ii) 1.5 to 200 μg of RU 41.740 per ml and bacteria only to verify that no reduction of *L. pneumophila* by RU 41.740 alone resulted in decreased multiplication.

Enumeration of CFU. CFU were enumerated at 1 h (as stated in the infection of macrophage monolayer section) and at 24 h. At 24 h, the supernatant was aspirated onto a sterile tube and the intracellular bacteria were released by hypotonic lysis with 1 ml of sterile distilled water and pooled onto the supernatant. Serial dilutions of the suspensions were plated onto BCYE plates and incubated at 35°C with 2.5% CO_2 and 95% humidity. CFU were enumerated after 5 days and represented as $\log_{10}$ value.

Expression of results. Results were expressed as (i) the $\log_{10}$ ratio of CFU of *L. pneumophila* at 24 h with RU 41.740/CFU of *L. pneumophila* at 1 h without RU 41.740 (the intracellular bacteria at 1 h are considered the inoculum, because *L. pneumophila* is an intracellular bacterium and the legionellae at 24 h are thus representative of intracellular multiplication; a $\log_{10}$ value more than 0 indicates no reduction of multiplication) and (ii) the $\log_{10}$ ratio of CFU of *L. pneumophila* at 24 h with RU 41.740/CFU of *L. pneumophila* at 24 h without RU 41.740 (a $\log_{10}$ value less than 0 indicates reduction of multiplication). Results were expressed as mean $\log_{10}$ ratio ± standard deviation.

Assay for activation of monocytes with the bacterial glycoprotein. CL was measured as described by Allen and Loose (1) with a Packard Picolite 6500 luminometer (Packard Instrument Co., Inc., Rockville, Md.). It records the light emission by phagocytic cells stimulated during oxidative metabolism. This light, emitted as photons of free oxygen radicals, is amplified by luminol, a cyclic hydrazide compound (11). For the CL assay, each reaction vial contained 10^6 monocytes in suspension (as stated above); 10% (vol/vol) immune serum; and 50, 100, or 200 μg of RU 41.740 per ml. The cell suspension was incubated with *L. pneumophila* (bacteria-cell ratio, 10:1) at 37°C for 30 min in the luminometer for dark adaptation. Controls consisted of (i) cells alone, (ii) cells plus RU 41.740, and (iii) cells plus bacteria. Background counts were recorded for 10 min, CL was initiated by the addition of 100 μl of luminol (final concentration, 10^{-5} M; Sigma Chemical Co., St. Louis, Mo.) and CL was recorded every minute until a peak and a decreasing slope were obtained. Each vial was run in duplicate.

Statistical test. A one-way analysis of variance was used to test the significance. The level of significance was considered to be $P < 0.05$.

RESULTS

Macrophage toxicity. Viability of the MDMs assessed by trypan blue dye exclusion test was always greater than 95%; the LDH release by the macrophages treated with RU 41.740 and those not treated was comparable ($P > 0.05$; Table 1), thus demonstrating no toxic effect of RU 41.740 at the concentrations used.

Multiplication of *L. pneumophila* within MDMs. The $\log_{10}$ number of *L. pneumophila* CFU within MDMs at 24 h postinfection (7.07 ± 0.4 [standard deviation]; 13 experi-

TABLE 1. Cytoplasmic LDH release assay of MDMs treated with RU 41.740

Concn of RU 41.740 (μg/ml)	LDH (IU/liter)/10^6 cells[a]
0	42.5 ± 8.66[b]
50	40.8 ± 9.96
100	45.8 ± 7.93
200	40.0 ± 7.38

[a] Mean LDH released by mocrophages preincubated for 72 h with RU 41.740 ± standard deviation of three experiments. The total LDH content, determined by lysis of the macrophages with a nonionic surface detergent, Nonidet P-40 (Sigma), was 182.25 ± 13.57 (n = 3).

[b] None of the values was significantly different in the cultures whether or not RU 41.740 was present.

ments) was approximately 150 times greater than the intracellular inoculum at 1 h postinfection (5.11 ± 0.5 [standard deviation]; 11 experiments) ($P < 0.001$).

Controls without MDMs. In the presence of various concentrations of RU 41.740 alone, neither reduction nor multiplication of *L. pneumophila* was observed and the CFU were comparable to the CFU of *L. pneumophila* at 24 h without RU 41.740 (Table 2).

Multiplication of *L. pneumophila* within MDMs in the presence of RU 41.740. Comparison of the intracellular multiplication at 24 h with various concentrations of RU 41.740 to that of the intracellular inoculum at 60 min is represented as a ratio in Fig. 1. Compared with the intracellular inoculum, 0.1 to 1.0 μg of erythromycin per ml, the positive control completely inhibited the intracellular multiplication of legionellae. Although there was inhibition of multiplication with 3.1 to 200 μg of RU 41.740 per ml ($P <$ 0.01 to 10^{-5}), it was significantly less compared with erythromycin. The level of reduction in the intracellular multiplication of legionellae for concentrations ranging from 3.1 to 200 μg of RU 41.740 per ml did not show any dose-dependent effect.

Comparison of the intracellular multiplication at 24 h with RU 41.740 to that without RU 41.740 is represented as a ratio in Fig. 2. In the presence of concentrations of RU 41.740 ranging from 3.1 to 200 μg/ml, there was a significant reduction of the multiplication ($\log_{10}$ ratio below 0) compared with 1.5 μg of RU 41.740 per ml ($P < 0.03$ to 0.001). The level of inhibition was not dose dependent. As observed with ratio 1, the reduction of the intracellular multiplication was significantly less ($P < 0.01$) compared with that of erythromycin.

Multiplication of *L. pneumophila* within MDMs preincubated for 72 h with RU 41.740. In the presence of macrophages preincubated with RU 41.740, at 24 h the $\log_{10}$ of *L. pneumophila* CFU within MDMs was approximately 10 times less ($P < 0.001$) compared with cultures without RU 41.740 (Fig. 3). The intracellular inoculum at 1 h within RU 41.740-preincubated macrophages (mean $\log_{10}$ ± standard deviation value, 4.93 ± 0.03, 4.99 ± 0.05, and 5.07 ± 0.06 for 50, 100, and 200 μg of RU 41.740 per ml, respectively) was similar to that of nonpreincubated macrophages (mean $\log_{10}$ ± standard deviation value, 4.88 ± 0.32).

Monocytes activated with RU 41.740 trigger an oxidative metabolic burst. With RU 41.740 and *L. pneumophila*-incubated monocytes, the metabolic burst was significantly more than either with the cells plus bacteria or with cells plus

TABLE 2. Effect of RU 41.740 on *L. pneumophila* after 24 h of incubation in the absence of MDMs

Concn of RU 41.740 (μg/ml)	$\log_{10}$ CFU (no. of expts)[a]
0	7.00 ± 0.2 (6)
1.5	6.96 ± 0.1 (3)
3.1	7.02 ± 0.1 (3)
6.2	6.96 ± 0.0 (3)
12.5	7.06 ± 0.1 (3)
25.0	7.10 ± 0.1 (4)
50.0	6.93 ± 0.1 (3)
100.0	6.94 ± 0.1 (6)
200.0	6.94 ± 0.0 (9)

[a] Mean $\log_{10}$ CFU ± standard deviation. None of the values was significantly different in cultures whether or not RU 41.740 was present.

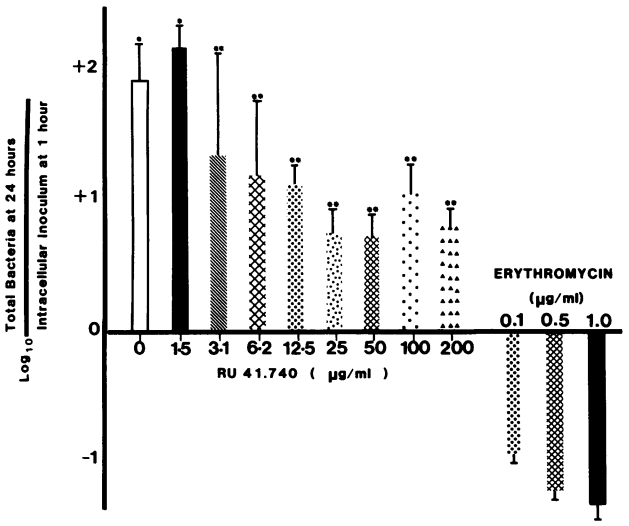

FIG. 1. Comparison of the $\log_{10}$ ratio of total bacteria at 24 h with or without RU 41.740 to the intracellular inoculum at 1 h. Each bar represents the mean ± standard deviation of 3 to 11 experiments. A $\log_{10}$ value above 0 indicates multiplication and a value below 0 indicates reduction of intracellular multiplication of *L. pneumophila*. $P < 0.01$ to 10^{-5} for * versus **. The effect of erythromycin represents a positive control for the inhibition of the intracellular multiplication.

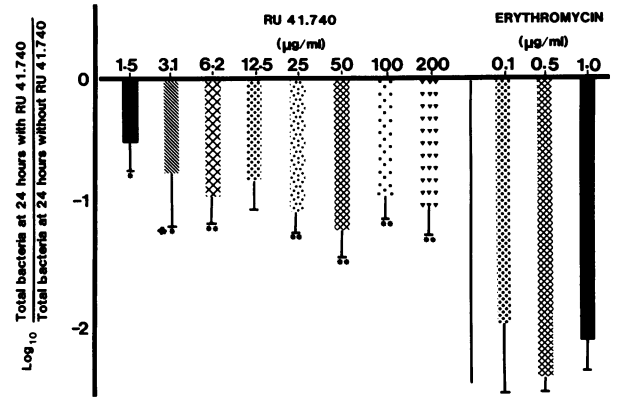

FIG. 2. Comparison of the $\log_{10}$ ratio of total bacteria at 24 h with RU 41.740 to total bacteria at 24 h without RU 41.740. Each bar represents the mean ± standard deviation of three to nine experiments. A $\log_{10}$ value below 0 indicates reduction of intracellular multiplication of *L. pneumophila*. $P < 0.03$ to 0.001 for * versus **. Compared with erythromycin, the reduction of the intracellular multiplication of *L. pneumophila* with RU 41.740 was significantly less ($P < 0.01$).

TABLE 3. CL of human monocytes with virulent *L. pneumophila* serogroup 1 and RU 41.740

RU 41.740 concn (μg/ml) and combination	Mean cpm ± SD[a]		*P*
	Peak	Cumulative	
0 cells alone[b]	36,357 ± 3,276	32,420 ± 5,981	
			>0.05
0 cells + bacteria[c]	35,718 ± 3,844	30,638 ± 7,915	
50 + cells[d]	37,426 ± 4,337	34,201 ± 4,240	
			>0.05
50 + cells bacteria[e]	46,477 ± 2,166	39,527 ± 6,055	
100 + cells[f]	53,934 ± 5,265	49,104 ± 7,340	
			<0.02
100 + cells + bacteria[g]	66,614 ± 2,206	57,011 ± 9,656	
200 + cells[h]	57,359 ± 1,271	54,881 ± 4,889	
			<0.001
200 + cells + bacteria[i]	84,791 ± 7,214	75,060 ± 13,022	

[a] Mean counts per minute ± standard deviation of three experiments. Cumulative counts per minute were taken for comparing the significance.
[b] $P > 0.05$ for *b* versus *d*; $P < 0.001$ for *b* versus *f* and *h*. $P < 0.001$ for *d* versus *f* and *h*; $P > 0.05$ for *f* versus *h*. $P < 0.001$ for *c* versus *e*, *g*, and *i*; for *e* versus *g* and *i*; and for *g* versus *i*.

RU 41.740 ($P < 0.05$). The metabolic burst was dose dependent. Results are summarized in Table 3.

DISCUSSION

L. pneumophila is a facultative intracellular bacterium multiplying within monocytes and macrophages of susceptible hosts (6, 7, 26, 48). Upon ingestion, it multiplies within a specialized phagosome-lysosomal vacuole (22, 24). Destruction of the bacteria is avoided by the inhibition of the fusion of the phagosome-lysosome (25) and nonacidification of the internal milieu of the vacuole (23). Furthermore, the virulent bacteria, upon interaction with the phagocytic cells, inhibit the production of toxic oxygen-free radicals (J. L. Vildé, P. Rajagopalan, and E. Dournon, Prog. Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 65, 1983; P. Rajagopalan and E. Dournon, 4th Int. Symp. Bioluminescence Chemiluminescence, abstr. no. C 21, September 1986) to which it is otherwise susceptible (35, 36). The bacteria are thus endowed with a host of antibacterial mechanisms which contribute to their intracellular survival.

Horwitz and Silverstein (27) have shown that indirect activation of human monocytes in vitro with concanavalin A-stimulated lymphocyte supernatant can overcome the intracellular multiplication of virulent *L. pneumophila*. Gibson et al. (17) have provided the first evidence in vivo that activation of the nonspecific efferent arm (macrophage) of the immune response with mycobacteria can protect guinea pigs against a lethal *L. pneumophila* lung infection. It is fairly well known that macrophage activation by numerous microorganisms (37) and acidic polymers protects animals against infections and tumors (2). Activated mononuclear phagocytes (12, 38, 45), particularly animal macrophages, have been reported to kill or inhibit the multiplication of *Leishmania enrietti* (9), *Toxoplasma gondii* (4), *Trypanosoma cruzi* (44), *Rickettsia tsutsugamushi* (41), and the bacterial pathogens *Listeria monocytogenes* (13, 15, 32) and *Mycobacterium tuberculosis* (10).

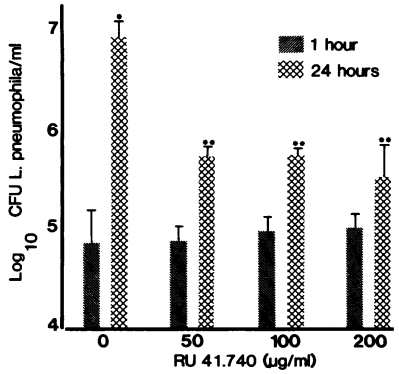

FIG. 3. Preincubation of 4-day-old monocyte-macrophage culture with 0, 50, 100, or 200 μg of RU 41.740 per ml for 72 h. On day 7, RU 41.740 was rinsed off and the monolayers were infected with *L. pneumophila* as stated in the Materials and Methods section. Each bar represents the mean $\log_{10}$ CFU per ml ± standard deviation (four experiments for control cultures and three experiments for RU 41.740). $P < 0.001$ for * versus ** at 24 h. The number of bacteria internalized at 1 h did not differ significantly whether or not the cultures were preincubated with RU 41.740.

The overall data of this study showed that in vitro the intracellular multiplication of *L. pneumophila* was significantly reduced in the presence of a bacterial glycoprotein (Fig. 2) but was not fully abolished as can be achieved with antibiotics (Fig. 1). The reduction in the intracellular multiplication of legionellae was observed with activation done before or after infection. This effect was observed for concentrations as low as 6.2 μg/ml but was not significantly modified by increasing the concentrations up to 200 μg/ml.

The inhibition of the intracellular multiplication of *L. pneumophila*, in this model, was most probably due to the activation of macrophages for three reasons. (i) RU 41.740 alone, in the absence of macrophages, did not result in the reduction of the CFU of *L. pneumophila* (see above). (ii) RU 41.740 did not affect either the viability or the number of adhered macrophages. Since legionellae need macrophages for proliferation, reduction in the quantity of the cells would result in a limited rate of multiplication; this was verified by the normal yield and shape of the adhered macrophages, absence of the vital dye (trypan blue) uptake, and nonleakage of cytoplasmic LDH. (iii) In the absence of RU 41.740, the bacteria multiplied within cultured macrophages and did not trigger the metabolic burst, as observed in the CL assay.

That nonspecific activation of MDMs results in reduced multiplication of legionellae has been demonstrated by Horwitz and Silverstein (27). In their experiments, the monocytes were indirectly activated by supernatants of concanavalin A-stimulated lymphocytes. Such monocytes, when challenged with virulent *L. pneumophila*, ingested fewer bacteria and slowed the multiplication of those internalized bacteria. Decreased phagocytosis, resulting in a

limited multiplication, may be one of the mechanisms by which activated phagocytes restrict intracellular growth of the pathogens (2, 27, 37). In our experiments, however, the MDMs, whether stimulated before or after infection, phagocytosed similar numbers of bacteria.

Thus, both pre- and postincubation of MDMs with RU 41.740 inhibited the multiplication of virulent *L. pneumophila*. Neither the exact mechanism nor the constituent of RU 41.740 responsible for the activation of the macrophages is known. One of the characteristics of activated macrophages is an enhanced generation of oxygen-derived products such as O_2^- and H_2O_2, which can kill intracellular pathogens (5, 29, 30, 33, 34, 42, 43). Since in acellular models these toxic oxygen radicals have been shown to be deleterious to *L. pneumophila* (35, 36), one can speculate that RU 41.740-activated MDMs which produce free radicals, as evidenced by the CL assay, may inhibit the intracellular multiplication of virulent *L. pneumophila*.

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