Interaction of *Aeromonas* spp. human isolates with murine macrophages

Sylwia Krzymińska, Adam Kaznowski, Marta Puk

Department of Microbiology, Faculty of Biology, A. Mickiewicz University, Poznań, Umultowska, Poland

──────────── SUMMARY ────────────

This study aimed to evaluate phagocytic and bactericidal activity of macrophages originated from murine J774 macrophage cell line against 26 strains of *Aeromonas* caviae, *Aeromonas veronii* biotype sobria and *Aeromonas hydrophila* isolated from human fecal specimens. The analysis of phagocytic index (PI) indicated these strains were phagocytosed in low numbers, below 1.1×10^{-3} for 65% strains of *A. veronii* biotype sobria and *A. hydrophila*. The highest PI value (0.019) was noted in murine macrophages infected with 19% of *A. caviae* isolates. Analysis of the number of viable bacteria in phagocytes revealed that 31% of *Aeromonas* spp. strains could replicate for an initial 3 h after gentamicin treatment. Opsonisation of the bacteria with non-immune serum did not affect phagocytic activity and bacterial killing. Stimulation of macrophages with gamma-interferon (IFN-γ) at a concentration of 10 IU/ml caused the highest increase in phagocytic activity but had no effect on the rate of bacteria elimination by murine macrophages. *Aeromonas* spp. isolates showed an invasive ability toward non-phagocytic epithelial cells. These findings indicate that *Aeromonas* spp. strains have evolved mechanisms to avoid phagocytosis, suggesting that murine macrophages, even activated by IFN-γ, have a limited ability to eliminate these bacteria, which may invade epithelial cells and spread in the illeum.

KEY WORDS: *Aeromonas* spp., Phagocytosis, Macrophages, Invasion epithelial cells

INTRODUCTION

The genus *Aeromonas* comprises Gram-negative rods and currently includes at least 21 species differentiated on the basis of DNA-DNA hybridization (Figueras, 2005; Harf-Monteil *et al.*, 2004; Miñana-Galbis *et al.*, 2007). These bacteria are widespread in natural habitats like soil and aquatic environments, and are frequently isolated from raw and processed food (Hänninen *et al.*, 1997; Martins *et al.*, 2002). Members of the genus *Aeromonas* are pathogens of human and animals. There have been a number of epidemiological studies indicating *Aeromonas* species as a cause

of diarrheal disease in children, elderly people and immunocompromised patients (Figueras, 2005; von Gravaenitz, 2007). An increasing number of reports have implicated *Aeromonas* sp. strains as the etiologic agent in acute diarrhea. *Aeromonas* sp.-associated diarrhea ranges from a mild self-limiting to a more severe, invasive *Shigella*-like dysenteric form (Figueras, 2005; von Gravaenitz, 2007). There have been case reports of complications of intestinal infections including chronic colitis post *Aeromonas* sp. gastrointestinal infection, and association with pancreatitis and small bowel obstruction (Figueras, 2005). The most prevalent species isolated from fecal specimens are *Aeromonas caviae* (75%), followed by *Aeromonas veronii* biotype sobria and *Aeromonas hydrophila* (Joseph, 1996; von Gravaenitz, 2007). The possible mechanisms of the pathogenesis of gastroenteritis are complex and multifactorial with the involvement of a number of putative virulence factors whose role in dis-

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Corresponding author
Adam Kaznowski
Department of Microbiology
A. Mickiewicz University
61-614 Poznań, ul. Umultowska 89, Poland
E-mail: akazn@amu.edu.pl

ease development is still not clear. After ingestion and adhesion to the epithelial cells of the intestine *Aeromonas* spp. strains produce many potential virulence factors, including exoenzymes, cytotoxic and cytotonic enterotoxins (Chopra and Houston, 1999; Krzymińska *et al.*, 2003; Laohachai *et al.*, 2003). Some strains are also able to invade epithelial cells (Shaw *et al.*, 1995; Thornley *et al.*, 1997; Krzymińska *et al.*, 2003). Phagocytes, either resident in tissues or circulating in blood, contribute to the primary line of innate defence against bacterial pathogens by providing their removal and destruction at the level of the epithelial barrier (Philpott *et al.*, 2001). Some bacterial enteropathogens have developed strategies to escape the attack of phagocytes by avoiding phagocytosis, surviving inside or killing the phagocytes. These bacteria also enter non-phagocytic cells, such as epithelial cells (Sansonetti, 2001).

Understanding how the host immune system responds to *Aeromonas* spp. is especially important because the majority of acquired infections occur in very young children or immunocompromised patients. Therefore the present study was designed to evaluate the role of phagocyte-mediated recognition and killing in primary host defence against these isolates. For this aim we used the well established macrophage-like mouse cell line J774, which has features similar to those of normal macrophages and has frequently been used in functional studies on phagocytosis (Ralph *et al.*, 1981). Moreover, we estimated phagocytic activity of macrophages after stimulation with gamma interferon (IFN-γ) and examined the invasion of epithelial cells by *Aeromonas* spp. strains.

MATERIALS AND METHODS

Bacterial strains

Twenty six *Aeromonas* spp. strains: sixteen of *A. caviae* (MPU A375, MPU A376 377, 378, 379, 380, 383, 384, 385, 386, 388, 390, 393, 545 549, 550) two of *A. hydrophila* (MPU A541, 542) and eight of *A. veronii* biotype sobria (MPU A382, 387, 389, 391, 392, 551, 552, 554) used in this study were isolated from fecal specimens of patients suffering from gastroenteritis. The examined isolates were identified on the basis of their phenotypic prop-

erties, DNA-DNA hybridization (Szczuka and Kaznowski, 2004) and by the 16S rDNA RFLP method described previously by Figueras *et al.*, (2000). As a control, an invasive strain of *Yersinia enterocolitica* O:8/1B (pYV⁺) and non-pathogenic strain of *E. coli* K12C600 were included. The isolates were maintained at -75°C in brain heart infusion broth (BHI, Difco) containing 50% (vol/vol) glycerol.

Opsonization of bacteria

For opsonization, 0.5 ml of bacterial suspension were incubated in 5% fresh non-immune human serum for 45 min at 37°C. Then the supernatant was discarded and the pellet resuspended and the bacteria centrifuged at 1000×g for 30 min, washed twice with 0.5 ml of phosphate-buffered saline (PBS, Biomed) and resuspended in 0.5 ml PBS (Persson *et al.*, 2001).

Cell culture

Murine J774 macrophage cell line derived from BALB/c reticulum cell sarcoma was maintained in growth medium (GM), containing RPMI 1640 (Biomed, Poland) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), gentamicin (5 μg/ml) and 2 mM L-glutamine. Human epidermal carcinoma cells of larynx (HEp-2) cells were grown in Eagle minimal essential medium (MEM, Sigma) with 5% fetal calf serum containing 2 mM glutamine, penicillin (50 U/ml), streptomycin (100 μg/ml) and nystatin (1 mg/ml). The cells were incubated at 37°C in an atmosphere with 5% CO_2 (Musso *et al.*, 2001).

Activation of murine J774 macrophage cell line with IFN-γ

In these experiments murine J774 macrophage cell line was pretreated with RPMI containing recombinant mouse IFN-γ (Bender MedSystems) at concentrations of 10 U/ml, 100 U/ml and 500 U/ml for 24 h prior to infection (Eze *et al.*, 2000, Musso *et al.*, 2001).

Phagocytosis and bactericidal assays

The phagocytosis assay was performed according to Musso *et al.* (2001) with minor modifications. Bacterial strains were cultured on BHI with 1.5% agar, harvested and resuspended in PBS to 1 McFarland scale and diluted 1:100 in GM to a number 1.4-1.6×10⁷/ ml. The aliquots were dilut-

ed and viable bacteria quantified. Murine J774 macrophage cells were seeded into 96-well, flat bottom microtiter plates (Nunc) in number 2×10^5 cells per well. The cells were grown in GM with gentamicin at 37°C for 24 h. Then the medium was replaced with 0.1 ml of fresh GM without gentamicin, incubated for a further 24 h and infected with 0.1 ml of opsonized or non-opsonized bacterial culture at a multiplicity of infection (MOI) 1:10 (2×10^5 cells incubated with approximately 2×10^6 bacteria) (Eze *et al.*, 2000). To perform phagocytosis assay, the cell cultures were incubated with bacteria for 60 minutes then gentamicin (100 µg/ml) was added to kill extracellular bacteria. After a two-hour incubation the samples were washed twice with PBS and 0.1 ml of 1% (vol/vol) Triton X-100 solution was added to each well. The number of intracellular viable *Aeromonas* spp. strains was determined in duplicate by quantitatively plating the lysates onto BHI with 1.5% agar. The degree of phagocytosis expressed as phagocytic index (PI) was calculated by the formula based on the number of bacteria inside macrophages (CFU per well) per number of phagocytosing macrophages (Jensch-Junior *et al.*, 2006). To assess bactericidal assay, murine J774 macrophage cells were exposed to bacteria for 60 minutes, and the number of CFU determined at 1, 3, 24 and 48 h after gentamicin treatment. Intracellular killing, expressed as percentage killing of bacteria was assayed in activated with 10 IU/ml INF-γ and non-activated murine J774 macrophage cells. Each experiment was performed in triplicate.

Statistical analysis

All results are expressed as means and standard deviation of PI of two separate experiments. One-way analysis of variance (ANOVA) and comparison of mean values using Tukey's HSD test at significance level P<0.05 were performed using Statistica PL software (StatSoft Poland Inc., USA).

Invasion of human epithelial cells

Invasion assay was performed as described previously by Krzymińska *et al.* (2003). The number of bacteria recovered after a three-hour infection and gentamicin treatment was compared to the initial bacterial inoculum to determine the invasion index (Shaw *et al.*, 1995). It was expressed as the percentage of bacteria recovered from the lysed monolayer which were compared with the original inoculum (1×10^5 CFU/ml). Assays were performed in duplicate in two separate experiments for each isolate. Strains which indicated an index higher than 0.2% were classified as invasive according to the criteria of Shaw *et al.* (1995).

RESULTS

Phagocytic activity of murine J774 macrophage cell line against *Aeromonas* spp.

Murine J774 macrophage cell line revealed a large variation in phagocytic activity of *Aeromonas* spp. cells following a one-hour incubation with bacteria. The phagoytic index varied significantly between J774 cells infected by different strains. The statistical analysis of the phagocytic index (PI) showed six groups (Table 1). The maximum PI value (PI=0.019) was observed in macrophages infected with *A. caviae* MPU A545 followed by strains MPU A376 (PI=0.017) and MPU A549 (PI=0.012). Murine J774 macrophage cells revealed significantly the lowest efficiency of phagocytosis for seven *A. caviae* strains (MPU A377, MPU A378, MPU A383, MPU A384 MPU A385, MPU A388 and MPU A393), one *A. hydrophila* MPU A541 and all *A. veronii* biotype sobria iso-

TABLE 1 - *Phagocytic activity (PI) of murine J774 macrophage cells infected with Aeromonas spp. Strains.*

Group	Number of strains	PI range ($\times 10^{-4}$)
a[1]	2	192 (20) - 177 (10)[2]
b	1	121(15)
c	2	73 (8) - 70 (9)
d	3	42 (6) - 32 (5)
e	4	32 (5) - 12 (2)
f	14	11 (3) - 0.13 (0.03)

[1]Means in the group did not differ significantly at P<0.05 according Tukey's HSD test; [2]Means of number of intracellular viable bacteria in J774 cells per number of murine J774 macrophage cells phagocytosing (2×10^5) and (standard deviation) of two separate experiments performed in triplicate.

lates. The mean number of phagocytosed bacterial cells for these strains ranged between 213 (PI=1.1×10^{-3}) and 2.7 (PI=1.35×10^{-5}) per 2×10^5 murine J774 macrophage cells. Strain of *E. coli* K12C600, non-pathogenic control was phagocytosed in a low number 13 cells (PI=6.5×10^{-5}). An invasive strain of *Y. enterocolitica* O:8/1B was recovered in murine J774 macrophage cells in a significantly greater number than *E. coli* and *Aeromonas* spp. strains with mean CFU counts exceeded 29×10^4 bacterial cells (PI=1.46).

Bactericidal activity of non-activated murine J774 macrophage cell line

The killing of *Aeromonas* spp. strains by murine J774 macrophage cell line was measured by viable count technique. The results showed that the cell line revealed statistically significant variation in bactericidal activity against the isolates (Table 2). The number of viable bacteria inside non-activated cells infected with *A. caviae* MPU A545 increased approximately 2-fold at 1 h after gentamicin treatment. The increase in the number of bacterial cells were also obtained from murine J774 macrophage cells infected with three strains of *A. caviae* (MPU A375, MPU A379, MPU A386) and two isolates of *A. veronii* biotype sobria (MPU A382 and MPU A554). After a three-hour incubation CFU counts significantly increased for seven *A. caviae* strains (MPU A375 MPU A379, MPU

A380, MPU A388, MPU A393, MPU A549, MPU A550) and one isolate of *A. veronii* biotype sobria MPU A552. A gradual decreasing in number of bacteria inside J774 cells was noted for 73% isolates at 24 h postinfection. The percentage killing ranged between 76% (MPU A545) and 100% (MPU A375, MPU A377, MPU A379, MPU A384). Effective bactericidal activity of murine J774 macrophage cells was observed against 65% of strains after 48 h of incubation. Four *A. caviae* (25%), one *A. hydrophila* and three *A. veronii* biotype sobria isolates (40%) caused destruction of the monolayer after 24 h.

Killing of *E. coli* K12C600 non-pathogenic control reached 31% after 1 h of incubation and increased to 100% at 3 h postinfection. Invasive strains of *Y. enterocolitica* O:8/1B revealed the ability to replicate and persist inside J774 cells. CFU inside murine J774 macrophage cell line reached 5.3×10^5 and 1×10^7 at 1 h and 3 h of incubation respectively. Destruction of the monolayer was observed after 24 h and 48 h.

Effect of stimulation of murine J774 macrophage cells with IFN-γ

Murine J774 macrophage cells were pre-stimulated with different concentrations (10 U/ml, 100 U/ml and 500 U/ml) of recombinant IFN-γ prior to the experiment. Intracellular bacteria were measured after serial dilutions and plating on

TABLE 2 - *The maximum and minimal Phagocytic Index (PI) of murine J774 macrophage cells infected with Aeromonas spp. and intracellular killing.*

Strain No.	PI (SD)[a] ($\times 10^{-4}$)	Percentage killing after incubation for:			
		1 h	3 h	24 h	48 h
A. caviae					
MPU A545	192 (20) a[b]	*[c]	*	76	DM[d]
MPU A377	13.5 (5.1)^f	100	100	100	100
A. hydrophila					
MPU A541	4.6 (0.8) f	75	86	90	100
A. veronii biotype sobria					
MPU A387	2.7 (0.1) f	*	36	DM	DM
MPU A551	0.8 (0.2) f	25	6	80	100
E. coli					
K12C600	0.65 (0.2) f	30	100	100	100

[a]Means of number of intracellular viable bacteria in murine J774 macrophage cells per number of cells phagocytosing (2×10^5) and (standard deviation) of two separate experiments performed in triplicate; [b]Within each column, means designated by the same letter (a or f) did not differ significantly at P<0.05 according to Tukey's HSD test; [c](*) Multiplication of bacteria inside murine J774 macrophage cells; [d]DM Destruction of J774 cellular monolayer.
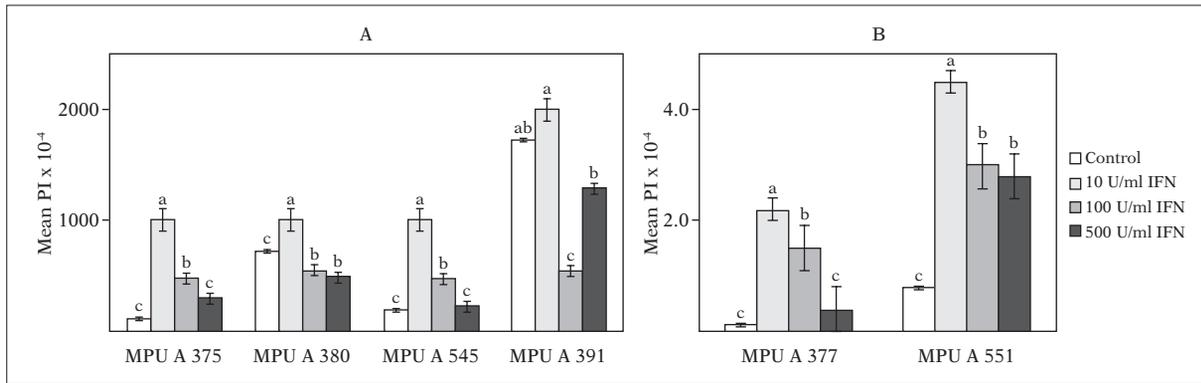
FIGURE 1 - *Effect of IFN-γ stimulation (10 U/ml, 100 U/ml and 500 U/ml) on phagocytic index (PI) of murine J774 macrophage cells infected with Aeromonas spp. strains. (A) The highest PI in J774 cells infected with A. caviae MPU A375, MPU A380, MPU A545 and A. veronii biotype sobria MPU A391 respectively. (B) The lowest phagocytic activity of J774 cells infected with A. caviae MPU A377 and A. veronii biotype sobria MPU A551. Control C, phagocytic activity in non-stimulated murine J774 macrophage cells. Results were expressed as the mean ± standard deviation (error bars) of two separate experiments performed in triplicate. Values followed by different letters (a, b or c) are significantly different (p<0.001) according to Tukey's HSD test.*

BHI with 1.5% agar. There were significant (P<0.05) differences between INF-γ concentration and phagocytic activity of murine J774 macrophage cells (Figure 1). The highest phagocytosis was observed in cells stimulated with INF-γ at a concentration of 10 U/ml, which caused the maximal 20-fold increase in the effectiveness of phagocytosis for *A. caviae* MPU A377 in comparison with non-activated control (Figure 1A). The enhancement of phagocytosis occurred in murine J774 macrophage cells infected with *A. veronii* biotype sobria MPU A551 (6-fold), MPU A391 (5-fold) and MPU A387 (4.5-fold). In contrast, IFN-γ at concentration 100 U/ml and 500 U/ml did not enhance phagocytic activity.

The effect of IFN-γ at concentration 10 IU on bactericidal activity of murine J774 macrophage cells against *Aeromonas* spp. is shown in Table 3. There were no statistically significant differences between the percentage killing of *Aeromonas* spp. strains inside treated and untreated cells.

Invasion of epithelial cells
Seven *Aeromonas* spp. isolates (70%) revealed an invasion ability to HEp-2 cells, the invasion index ranging from 0.22% to 1.22% (Table 4). Six of eight *A. veronii* biotype sobria isolates (75%) and one *A. hydrophila* strain were invasive to epithelial cells. One *A. hydrophila* strain caused destruction of HEp-2 cells monolayer making the

TABLE 3 - *Effect of IFN-γ (10 IU) stimulation on bactericidal activity of murine J774 macrophage cells.*

Strain No.	Percentage killing after incubation for:			
	1 h	*3 h*	*24 h*	*48 h*
A. caviae				
MPU A545	*a	19	DM[b]	DM
MPU A377	18	97	100	100
A. hydrophila				
MPU A541	11	29	DM	DM
A. veronii biotype sobria				
MPU A387	24	10	100	100
MPU A551	*	28	100	100

[a](*) Multiplication of bacteria inside macrophages; b(DM) Destruction of J774 cell monolayer.

TABLE 4 - *Invasive ability of A. hydrophila and A. veronii biotype sobria isolates.*

Type of isolate	No of strains with invasive ability	
	A. hydrophila (n=2)[a]	*A. veronii biotype sobria (n=8)*
Invasive[b]	1	6
Noninvasive[c]	1	2

[a]Total number of examined strains; [b]Invasion index >0.2; [c]Invasion index <0.2.

analysis of invasion activity impossible. The invasion indexes of *E. coli* negative control and Y. *enterocolitica* O:8/1B positive control were 0.01% and 9.8% respectively. Invasion of epithelial cells by *A. caviae s*trains was reported in earlier studies (Krzymińska *et al.,* 2003).

DISCUSSION

One of the first steps in a primary host response during a bacterial infection is the recognition and ingestion of the pathogen by professional phagocytes, such as macrophages and neutrophils, which express specialized receptors that promote phagocytosis (Sansonetti, 2001; Greenberg and Grinstein, 2002). These receptors, like the mannose receptor (MR), transmembrane Toll-like receptors (TLR), cytosolic Nod-like receptor (NLR) or opsonin-dependent receptors: Fcγ receptors (FcγR) and complement receptors (CR1, CR3 and CR4) are involved in the detection and recognition of certain bacterial surface structures on the exterior of the bacteria such as LPS or flagellin, called pathogen-associated molecular patterns (Underhill and Ozinsky, 2002). Phagocytes in the innate immune response to the early phase of *Aeromonas* sp. infection may be critical step in its pathogenesis. So far there are no data on the role of macrophages, the most efficient phagocytic cells in host protection against strains isolated from human specimens.

In the present study we analysed the phagocytic and bactericidal activity of murine J774 macrophage cell line infected with *Aeromonas* spp. isolates associated with human diarrhoea. Moreover, we focused on explaining the role of complement and IFN-γ in phagocytosis and phagocytic killing. Our study revealed that a low number of *Aeromonas* spp cells were phagocy-

tosed after a one-hour incubation with murine J774 macrophage cells. The maximal phagocytic index for 19% of *A. caviae* strains did not exceed 0.19% of total bacterial inoculum. Strains of *A. veronii* and *A. hydrophila* were taken up by phagocytes with lower efficiency, below 0.03% of total bacterial inoculum although the phagocytic index was higher than that against non-pathogenic control of *E. coli* K12C600. The results suggest the hypothesis that *Aeromonas* spp. strains revealed resistance to phagocytosis.

Many enteropathogens are able to avoid host recognition or diminish the immune activation through elaborated interactions with the host (Underhill and Ozinsky, 2002, Backert and König, 2005). Protein toxins are one group of bacterial factors which play a major role in these processes. One of the most potent virulence factors of *Aeromonas* spp. is cytotoxic enterotoxin, which in our laboratory was isolated from *A. hydrophila* isolates MPU A541 and MPU A 542 and *A. veronii* biotype sobria strains MPU A 551, MPU A552 and characterized (Krzymińska *et al.* 2006). These toxins revealed cytotoxic, cytotonic and hemolytic activities. In our previous research we have noted that *A. caviae* strains produced both cytotoxic and cytotonic toxins (Krzymińska *et al.,* 2003). Galindo *et al.* (2006) stated that cytolytic and cytotonic enterotoxins isolated from *A. hydrophila* generated arachidonic acid metabolites with synthesis of prostaglandin E_2 and subsequent activation of adenylate cyclase and production of cAMP which precedes the formation of proinflammatory cytokines. By accumulating cAMP in the host, these toxins may diminish the activity of phagocytes (Backert and König, 2005). Different species of bacteria and the differences in the techniques used to quantify the bacterial uptake make it difficult to compare the data with our current findings. The resistance to phagocy-

tosis has been previously described for *H. pylori* isolates (Ramaro and Meyer, 2001, Baldari *et al.*, 2005), *Pseudomonas aeruginosa* and enteropathogenic *E. coli* strains (Celli and Finlay, 2002). On the contrary the highest efficiency of phagocytosis we observed in murine macrophages infected with an invasive strain of *Y. enterocolitica* O:8/1B (pYV$^+$). A similar observation was made by Koning-Ward *et al.* (1998) who suggested that these bacteria actively invaded phagocytes because the plasmid-containing strain expressed invasin, which binds to β_1 integrins on the macrophage surface and stimulates them to phagocytosis.

Our analysis of the number of viable bacteria in murine J774 macrophage cell line revealed that 31% of *Aeromonas* spp. strains could replicate in phagocytes for an initial 3 h after gentamicin treatment. These results suggested that *Aeromonas* spp. isolates evolved effective mechanisms for avoiding microbicidal activity within phagocytes at the initial stage of infection. Previously, Leung *et al.* (2000) also demonstrated the ability of *A. hydrophila* strains to effectively multiply within fish phagocytes. In the present study we observed that opsonisation of the bacteria with non-immune serum had no effect on the uptake and phagocytic killing (data not shown). Galindo *et al.* (2006) suggested that in resistance to non-immune serum may be involved *Aeromonas* sp. lipopolisaccharide (LPS), bacterial cell wall component. We observed statistically significant differences in phagocytic activity of macrophages activated with different concentrations of IFN-γ. Stimulation of J774 cells with IFN-γ at a concentration of 10 U/ml resulted in the highest enhancement of phagocytosis, suggesting that this cytokine may contribute to the development of cell-mediated immunity against *Aeromonas* sp. strains. However, the highest efficiency of phagocytosis after stimulation with IFN-γ (10 U/ml) did not exceed 4300 cells, to make 0.21% of total inoculum. Evena 20-fold increase in the phagocytosis of *A. caviae* MPU A377 strain caused elimination only of 4×10^{-4}% total bacterial inoculum. Stimulation of phagocytes with IFN-γ had no effect on bactericidal activity compared with non-stimulated cells, suggesting that IFN-γ did not affect the rate of bacterial elimination.

Several virulent bacteria developed a series of strategies to avoid phagocytosis that contribute to an essential part of their pathogenic ability. Some strains are not really phagocytosed by professional phagocytes because they invade other cells (Sansonetti 2001). In the study 10 of 18 *Aeromonas* spp strains (55%) invaded HEp-2 cells. The most invasive were *A. veroni*i biotype sobria isolates (53%) with the highest invasion index (0.22-1.22) but significantly lower than that of *Y. enterocolitica* positive control (9.8). Previously, investigations by Thornley *et al.* (1997) reported that *A. veronii* biotype sobria were the most invasive species within genus *Aeromonas*. Shaw *et al.* (1995) reported that 9% of *A. caviae* isolates were invasive of epithelial cells whereas our earlier study (Krzymińska *et al.,* 2003) showed that 4 out of 20 *A. caviae* strains were invasive of HEp-2 cells.

Our findings suggest that *Aeromonas* spp. strains have developed strategies to avoid phagocytosis by murine macrophages. These phagocytes activated with IFN-γ in the presence of non-immune serum phagocytosed of *Aeromonas* spp. strains in low numbers leading to the conclusion that one of the major defence mechanisms of host nonspecific immunity was not sufficient to overcome *Aeromonas* sp.-mediated infections. These bacteria may avoid phagocytes by penetrating epithelial cells leading to dissemination in the ileum.

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