SHORT COMMUNICATION

Phagocytosis of *Campylobacter jejuni* and *C. coli* by Peritoneal Macrophages

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Guinea-pig resident peritoneal macrophages had no activity against freshly isolated *Campylobacter jejuni*, whilst *C. coli* was phagocytosed and killed. The number of bacteria killed by macrophages always exceeded the number of those ingested, suggesting an extracellular mechanism of killing.

INTRODUCTION

Thermophilic *Campylobacter* have recently been recognized as the leading bacterial cause of acute human gastroenteritis in many countries (Blaser *et al.*, 1983; Feldman & Pearson, 1982), but the mechanism of pathogenesis is poorly understood. A cholera-like enterotoxin has been isolated from *Campylobacter jejuni* and its biological and immunological properties have been described (Ruis Palacios *et al.*, 1983; Klipstein & Engert, 1984; Olsvik *et al.*, 1984). However, the presence of blood and leucocytes in the stools of infected persons and the presence of bacteraemia associated with the infection indicate that *C. jejuni* and *C. coli* can be invasive (Blaser & Reller, 1981; Duffy *et al.*, 1980; Lastovica & Penner, 1983; Newell, 1984), although the latter species is less frequently isolated in man and is of lower virulence in experimental animals (Karmali & Skirrow, 1984; Kakoyannis *et al.*, 1985).

The purpose of this study was to examine the role of phagocytosis in the control of *Campylobacter* infection, by investigating the interactions between guinea-pig macrophage populations and suspensions of *C. jejuni* and *C. coli* isolated from patients with acute diarrhoea.

METHODS

Organisms and media. The *C. jejuni* and *C. coli* strains were faecal isolates from persons with secretory-type diarrhoea and were identified by the biochemical tests described by Morris & Patton (1985). All strains belonged to biotype 1, and were stored at -70°C , until required, in small portions in Brucella broth (Difco) containing 15% (v/v) glycerol. Working stocks of strains were obtained by culturing a frozen sample into the liquid medium described by Mehlman & Romero (1982), at 42°C in catalyst-free anaerobic jars (GasPack anaerobic system; BBL). Thus, each strain underwent a single subculture. When necessary for viable counting, Columbia agar plates (Oxoid) supplemented with ferrous bisulphite pyruvate (FBP) growth supplement (Oxoid) were used.

Macrophages. Peritoneal macrophages were harvested from outbred guinea-pigs by washing the peritoneal cavity with 30–50 ml Krebs Ringer phosphate buffer (KRP), pH 7.4, containing 0.5 mM-CaCl₂, 5 mM-glucose and 10% (v/v) citric acid/citrate dextrose (ACD). Macrophages were purified by centrifugation of the peritoneal washings on a Ficoll-paque (1.077 = d₂₀; Pharmacia) gradient at 800 g for 15 min at 18°C , according to the method of Boyum (1968). The band-forming cells were withdrawn, washed twice in KRP, resuspended in the same medium and counted electronically (Coulter counter ZBI). The cells were adjusted to a concentration of $6\text{--}10 \times 10^6$ macrophages ml⁻¹, and were 90% viable, as judged by the erythrosin B exclusion test (Sigma). Macrophages accounted for 90% of the total cells, the remainder being lymphocytes, as judged by Wright and Giesma stained specimens prepared by cytocentrifugation (Cytospin 2, Shandon) (Soderberg & Solotorovsky, 1979).

Macrophage-bacteria incubation. Macrophages [$3-5 \times 10^6$ (ml KRP) $^{-1}$] and *Campylobacter* strains in ratios 1:1 to 400:1 bacteria to macrophages were incubated for 10-30 min at 37 °C with the addition of fresh or heat-inactivated (56 °C for 30 min) guinea-pig serum to 10% (v/v) concentration, the final volume being 1 ml. Fresh pooled guinea-pig sera were absorbed twice with 5% (v/v) suspensions of 10^{10} campylobacters ml $^{-1}$ at 4 °C for 15 min, freed of campylobacters by centrifugation and stored in small portions at -70 °C until required. Samples of the mixture were withdrawn at various times and were processed for the quantitative measure of either phagocytosis or killing.

Phagocytic assay. Phagocytosis was quantified on Wright and Giemsa stained specimens prepared by cytocentrifugation of the bacteria-macrophage mixture diluted tenfold with KRP containing 1% (w/v) BSA. The centrifugation was done at 75 g, which allows free bacteria to remain in suspension. Bacteria which appeared to be clearly engulfed in a phagocytic vacuole were considered as phagocytosed and, those adhering to cells but not included in a vacuole were considered as trapped. The sum of phagocytosed and trapped bacteria is referred to as 'macrophage-associated bacteria'. At least 300 macrophages were scored for each sample.

Killing assay. The survival of bacteria was evaluated by plating the specimens in triplicate after appropriate tenfold dilutions and treatment with 1% (w/v) saponin. Viable counting of bacteria was done on c.f.u. developed after 48 h incubation at 42 °C under microaerophilic conditions. Similar samples taken from mixtures of fresh or heat-inactivated serum mixed with campylobacters or from campylobacters alone served as controls, and these gave 100% survival, with no differences between fresh and heat-inactivated serum. The levels of significance were calculated by the F test and regression analysis (Lison, 1961).

RESULTS AND DISCUSSION

C. jejuni and *C. coli* were phagocytosed to different extents by guinea-pig peritoneal resident macrophages. *C. jejuni* was neither phagocytosed nor trapped at any of the bacteria-macrophage ratios tested, while *C. coli* was taken up by macrophages (Table 1). Phagocytosis of *C. coli* (Table 1) seemed to increase as the bacteria-macrophage ratio was increased, until a maximum value was reached, as can be observed in other *in vitro* models of phagocytosis (Van Furth *et al.*, 1978). The different behaviour of macrophages toward the two *Campylobacter* species suggested different interactions between the outer surfaces of *C. coli* and *C. jejuni* and the macrophage membrane; this could possibly be due to the existence of an antiphagocytic capsular-like material in *C. jejuni*, similar to that of *C. fetus* (McCoy *et al.*, 1975). The failure of macrophages to kill *C. jejuni* and their ability to kill *C. coli* is in keeping with this suggestion (Table 1). It is noteworthy, however, that in all experiments the number of killed *C. coli* greatly exceeded the number of macrophage-associated micro-organisms. In fact after 30 min contact between bacteria and macrophages, the number of *C. coli* associated with macrophages, i.e. the sum of endocytosed and trapped bacteria, could be increased by increasing the ratio of bacteria to macrophages in the incubation mixture (Fig. 1, lower line). The upper line in Fig. 1 represents the number of killed *C. coli* with respect to the ratio of bacteria to macrophages: increase in this ratio resulted in greater numbers of campylobacters killed. Dead bacteria exceeded associated bacteria by about 20- to 100-fold, suggesting that neither phagocytosis nor trapping could account for the number of killed micro-organisms. In addition, on the basis of microscopic counts, it seems that adhesion but not internalization is required for macrophages to kill bacteria. Our specimens were prepared after dilution and cytocentrifugation of the sample; most of the attached bacteria detach from macrophages during these procedures. Thus, only firmly attached bacteria, which were likely to be engaged in the phagocytic process, were stained and counted as trapped bacteria. The results suggest that there was a fraction of the detached and/or free micro-organisms that underwent killing without being counted as adherent to or trapped by macrophages, and were therefore killed outside of the phagosome. Phagocytosis itself seems, therefore, not to be the only mechanism involved in the killing of *C. coli*, and macrophages may be able to exert their bactericidal action via an extracellular mechanism. Such behaviour has been described for neutrophil-mediated killing of *Escherichia coli* (Weiss *et al.*, 1985). Further work is in progress in our laboratories to investigate the mechanism by which macrophages may exert this effect on *C. coli*.

In conclusion, our experimental model reveals that the two species of *Campylobacter* studied differed in their interactions with macrophages. *C. coli* was phagocytosed and killed, whereas *C. jejuni* scarcely interacted with guinea-pig peritoneal macrophages during 30 min contact. The

Fig. 1. Interactions between peritoneal guinea-pig macrophages and *C. coli* after 30 min incubation. ○, Bacteria associated with macrophages (bacteria engulfed plus bacteria trapped; $r = 0.88$, $P < 0.01$); ●, bacteria killed by macrophages ($r = 0.93$, $P < 0.001$). Each point represents the mean of two determinations.

Table 1. Phagocytosis and killing of *C. coli* and *C. jejuni* by guinea-pig macrophages

	Initial bacteria/macrophage ratio	Macrophages (%) with associated bacteria*		No. of bacteria per 100 macrophages				Surviving bacteria (%)†	
				Phagocytosed		Trapped			
		10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min
<i>C. coli</i>	10	4.0	4.0	2.0	2.5	3.0	2.5	46.0	50.0
	50	43.0	48.5	19.0	31.0	55.0	57.0	80.0	80.0
	150	30.0	65.0	8.0	30.0	100.0	115.0	ND	46.0
	200	40.0	37.0	17.0	19.0	81.0	172.0	ND	80.0
<i>C. jejuni</i>	10	4.3	6.0	2.5	5.0	3.0	5.0	93.0	93.5
	150	9.0	6.5	3.0	3.0	2.5	5.0	99.0	98.5
	400	ND	9.0	ND	3.0	ND	8.5	ND	98.0

ND, Not done.

* Percentage of macrophages showing phagocytosed and/or trapped bacteria.

† Percentage of surviving bacteria (c.f.u.) with respect to the total starting number.

failure of macrophages to ingest and/or kill *C. jejuni* may play a role in the determination of its virulence, as suggested by Kiehlbauch *et al.* (1985). Furthermore, our data are consistent with the existence of an extracellular killing mechanism, at least against *C. coli*.

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