COMPLEMENT MEDIATED LYSIS, ADHESION, INVASION, AND MULTIPLICATION WITHIN HeLa CELLS AND HUMAN MONOCYTE MACROPHAGES OF SUSCEPTIBLE AND MULTIPLE DRUG-RESISTANT *S. typhi*

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ABSTRACT

Twenty strains each of susceptible and multiple drug resistant strains of *S. typhi* were compared for susceptibility to complement lysis and cellular invasion. Drug resistant strains showed increased resistance to complement killing. Antibody increased the efficiency of complement killing of *S. typhi*. Susceptible and drug resistant strains showed almost the same rates of adherence and invasion of HeLa cells and human monocyte cell lines. However, the resistant strains showed a relatively higher rate of intracellular multiplication after 6 hours within human mononuclear macrophages. Decreased killing by complement and the relatively higher multiplication within mononuclear macrophages of the drug resistant strains may be the reason for their increased virulence.

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KEY WORDS : Complement; Drug resistance; HeLa cells; Human monocyte macrophages; Plasmid; *S. typhi*.

Introduction

The resurgence of typhoid fever in Asia and the Far East has been marked by emergence of multiple drug resistant strains (MDRST) [1]. MDRST cause a fulminant illness with a higher incidence of organomegaly and hyperpyrexia, greater duration of hospitalization and longer time for defervescence after appropriate antimicrobial therapy [2]. This study was carried out to compare, in vitro, the interaction of antibiotic susceptible *S. typhi* (SST) and MDRST with complement and cultured human cell lines so as to provide a pointer to increased virulence of MDRST.

Material and Methods

S. typhi isolated from blood cultures of patients suffering from typhoid fever were identified conventionally [3]. Disc susceptibility testing was done by the Stokes method [4]. MDRST showed resistance to at least four antibiotics i.e. sulphamethoxazole, trimethoprim, ampicillin and chloramphenicol. Twenty isolates each of SST and MDRST were used in this study.

Late log phase organisms used were incubated without shaking for 6 hours in 20 per cent CO₂. Complement and monocytes were obtained from donors who were serologically negative for HIV, HBsAg, and syphilis and had no history of typhoid fever or recent history of typhoid vaccination (TO titre < 20). Serum was inactivated by incubating at 56°C for 30 minutes. Inactivated and pooled sera of patients recovering from typhoid fever (Widal titres > 640) were used as a source for antibody. Bacterial killing by the alternate complement pathway was assayed as previously described [5,6]. Complement lysis by the classical and alternate pathways were compared. Bacterial concentration was adjusted to approximately 10⁵ bacteria per mL. Ten µL of the high titre sera were added to 100 µL of the bacterial suspension (no evidence of agglutination was seen on microscopy) and incubated with complement for 30 minutes. Ten µL were plated and mean of bacterial CFU's after overnight incubation was counted. The difference in the CFU's surviving incubation in heat-inactivated serum versus normal serum augmented by antibody was expressed as kill rates. Killing of

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SST (and MDRST) by alternate and direct complement pathways was compared using the paired 't' test. Killing of MDRST and SST by the direct complement pathway (and alternate pathway) was compared by the unpaired 't' test.

HeLa cell monolayers in a 96-well flat bottom tissue culture plate were used for the epithelial cell invasion assay. Human monocyte-derived macrophage monolayers were also obtained, as previously described, in 96-well plates [7]. Bacteria grown in Luria Bertanni broth modified by addition of 1 mM Ca^{++} [8] were re-suspended to a concentration of 10^8 . Ten μL was inoculated into each well. To study adherence after thirty minutes of contact monolayers were washed thrice with phosphate buffer saline (PBS) and lysed with 0.2 mL of 1 per cent Triton X-100 by incubating at 37°C for 5 minutes. Ten μL were spread on agar plates and CFU's counted to give adherence levels. To study adhesion, invasion, and intracellular multiplication monolayers were incubated in fresh MEM with 10 micrograms of gentamicin per mL and bacterial CFU's at 2, 6, 12 and 24 hours counted as for adherence assays. Mean of CFU's were compared by the unpaired 't' test.

Plasmids were extracted by a modification of the alkaline lysis method of Birnboim and Doly [9] and molecular weight determined by the method of Meyers et al [10]. The molecular weight standards used in the study was *E. coli* V 517 [11].

Results

The 20 MDRST showed the presence of a single large plasmid with molecular weight ranging from 90 to 110 megadaltons. The 20 SST did not show the presence of any plasmid DNA. Log kill rates of MDRST and SST by the alternate pathway showed marginal differences in kill rates which was accentuated at a concentration of about 10^4 bacteria per 100 μL . The results are shown in Fig 1. Assays of complement lysis by the direct and indirect pathways was done at this concentration. The mean, standard deviation and 't' test values of the CFU's grown after overnight incubation are shown in Table 1. MDRST appear to be more resistant to complement mediated killing than SST. Antibody increases the efficiency of complement mediated bacterial lysis. The results are represented graphically in Fig 2.

Fig. 1: This fig shows the lysis of SST and MDRST by human complement over a wide range of bacterial concentrations. The X axis denotes a serial 1 to 10 dilution of bacteria. The results are log values of kill rates (i.e. kill rates = CFU's after incubation in inactivated sera - CFU's after 1 hour incubation in pooled complement containing sera).

Fig. 2: This fig shows the lysis of SST and MDRST by human complement at a concentration of 10^5 bacterial/mL and the effect of antibody on bacterial killing. The results are expressed in % kill rates in the graph. (i.e. % kill rates = kill rate/CFU's after incubation in inactivated sera % 100).

TABLE 1
Killing of *S. typhi* by serum

	Serum kill rate	Serum + Ab kill rate	t value (paired 't' test)
MDRST (Mean)	35.40	42.75	6.44
SD	9.53	8.09	
SST (Mean)	41.90	49.50	6.48
SD	10.81	10.30	

't' value (unpaired 't' test) 1.97 2.25

Table 1 shows kill rate, i.e., the difference in cfu count between serum, serum with antibody and inactivated serum per 10 μL of serum overnight incubation.

The mean, standard deviation and 't' test values for infection of HeLa and human macrophage cell lines are shown in Table 2. There was no statistically discernible difference in the rates of adhesion, invasion and intracellular multiplication of MDRST and SST within HeLa cells. MDRST

however were more successful in surviving within HeLa cell lines. There was no statistically discernible difference in the rates of adhesion and invasion. However, intracellular multiplication of MDRST was more pronounced than SST within human monocyte macrophages after 6 hours (Ta-

Fig. 3 : HeLa cell adhesion, invasion and intracellular survival by SST and MDRST. Shows Log of CFU's per well on the Y axis. The X axis denotes time.

Fig. 4 : Human monocyte macrophage monolayer adhesion, invasion and intracellular survival by SST and MDRST. Shows log of CFU's per well on the Y axis. The X axis denotes time.

TABLE 2

HeLa cell adhesion, invasion, intracellular multiplication

	0 hrs	30 mins	2 hrs	6 hrs	12 hrs	24 hrs
SST						
Mean	107.6	32.4	31.15	30.35	23	17.35
SD	24.79	7.64	4.91	6.6	5.6	2.85
MDRST						
Mean	103.1	33.85	33.4	33.25	33.95	35
SD	20.54	7.51	6.89	6.45	6.73	6.24
't' value	0.6	0.6	1.16	1.37	5.45	11.2

Table 2 shows that MDRST show improved survival with HeLa cells as compared to SST after 12 hours. The value of 't' from table for 1% level of significance for 38 d. f. is 2.71.

TABLE 3

Macrophage adhesion, invasion, intracellular multiplication

	0 hrs	30 mins	2 hrs	6 hrs	12 hrs	24 hrs
SST						
Mean	111.4	31.15	29.15	30.45	31.65	33.2
SD	17.6	8.76	7.21	7.02	6.04	7.38
MDRST						
Mean	111.95	34.05	31.2	34.9	41.65	56.2
	15.8	7.04	7.74	7.65	9.12	14.64
't' value	0.1	1.12	0.84	1.86	3.98	6.11

The survival and multiplication of MDRST within macrophages shows a 't' value of 1.86, 3.98 and 6.11 respectively at 6, 12 and 24 hours. The values are more than the 't' values from the table for 10%, 1% and 1% level of significance for 38 d. f. respectively.

ble 3). The interaction of HeLa cells and human monocyte macrophages with SST and MDRST are shown graphically in Fig 3 and 4 respectively.

Discussion

Typhoid fever is an unusual form of septicemia with a very low concentration of *S. typhi* cells in the blood, (less than 15 bacteria per mL of blood) [12]. The role of complement in neisserial and meningococcal septicemias have been studied [6,13]. Outer membrane proteins (OMP) are the initial site of complement activation and help Gram negative bacteria resist the bactericidal action of complement. Studies on *S. typhimurium* have shown the importance of *rck*, *rsk* and *traT* genes carried on the virulence plasmid in altering the expression of OMP and their effect on complement killing [14-16]. The number and length of lipopolysaccharide (LPS) side chains are important determinants in bacterial virulence. By activating complement at a distance from the cell surface LPS precludes insertion of the membrane attack complex in the outer membrane [13]. The rate of activation of the alternate complement pathway by the salmonella 'O' polysaccharide has been a property inversely proportional to virulence in mice [17]. Phagocytosis of salmonellae by macrophage has been shown, in vitro, to be dependent on activation of a serum factor belonging to the alternate pathway by the LPS [15]. Thus salmonellae which activate complement poorly escape phagocytosis and cause disease.

This study is unique in that it is the first to report results of complement mediated lysis of *S. typhi* by both the classical and alternate pathways. MDRST show an increased ability to withstand complement lysis compared to SST both by the alternate and classical pathways over a wide range of bacterial concentrations. Antibody enhances bacterial lysis by complement both in SST and MDRST (SST more than MDRST).

Many studies on epithelial cell lines and mononuclear cell adhesion and invasion by salmonellae have been reported [19-22]. However only a few have studied invasion and adherence by *S. typhi* [7,8]. None of the reports compared invasiveness between the SST and MDRST. This study is unique in this aspect and has been done to correlate the clinical evidence of increased severity of

illness and organomegaly in MDRST. It suggests that epithelial cell adhesion and invasion by *S. typhi* is not affected by the presence of the multiple drug resistance plasmid. This is in concordance with various other studies in salmonellae [21,22]. Gulig, in an article reviewing salmonella virulence plasmids, suggested a similarity in the common virulence region of salmonella virulence plasmids [23]. There is a significant increase in the number of bacterial CFU's within macrophages after 6 hours. This is probably responsible for the increased incidence of organomegaly and hyperpyrexia in MDRST infections as compared to SST.

Reitmeyer et al have shown that salmonella cytotoxin is a component of the OMP [24]. The OMP is intimately involved in macrophage pathogen interactions and it is probable that the megaplasmid carried by MDRST probably codes for or alters the OMP's. This may explain decreased effectiveness of complement in killing MDRST and improved intracellular multiplication within macrophages thus enhancing virulence.

Further studies on OMP expression, plasmid sequencing, protein homology studies and mutational analysis could offer an insight into the molecular strategies employed by bacteria in promoting invasion and evading host defence mechanisms.

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