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Distinct Adherence Mechanisms in *Salmonella Typhimurium* Strains 1826 ATCC 14028 to Macrophages

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Abstract. The adherence of *Salmonella typhimurium* (*ST*) strains 1826 and ATCC 14028 were assessed using a visual binding assay. *ST* 14028 exhibited better adherence to Møs than *ST* 1826. Adherence of *ST* 14028 exceeded 80% in the third hour when incubated with Møs which was significantly higher compared to *ST* 1826. Cytochalasin-B at 50 µg/ml was used to prevent phagocytosis and uptake of microorganism without affecting the Mø viability. This was essential for the binding visual assay used in this study. There was a distinct variation between the two strains in the adherence process to the mouse macrophages (Mø) which indicates that the two strains used different mechanisms to infect Møs.

Keywords: Adherence, *Salmonella typhimurium*, macrophage.

1. Introduction

Salmonellosis continues to be the leading bacterial disease around the world [1]. About 25-30% of salmonellosis is due to *Salmonella typhimurium* (*ST*) [2] which is also a prominent cause of salmonellosis in animals. With exception of *S. typhi*, animals are the main reservoirs of *Salmonella*. The infection occurs as enteric fever, gastroenteritis, empyema, bone and joint infection or a combination of these complications as a result of ingestion of contaminated water and food of animal origin [3]. Only strains that cause enteric fever penetrate the intestinal epithelial cells and infect a target cell, the macrophage, and are subsequently transported to spleen and liver [4].

It is known that specific antibody (Ab) directed at exposed structures of the microbial surface enhance phagocytosis and uptake of microorganisms via FC (fragment of constant) receptors by opsonisation [3]. Deposition of activated complement components on salmonellae surface also enhances phagocytosis [5]. Under some conditions, *Salmonella* sp is able to bind directly to host Møs in the absence of either antibody or complement [3]. This direct association is an important requirement for intracellular pathogens, such as *Salmonella* to allow them to adhere, invade and eventually survive intracellularly in Møs. However, the infection process may differ among the *ST* strains.

In this study, the mouse model was used to monitor adherence of *ST* 1826 and ATCC 14028 strains to the Møs, to evaluate the efficiency of the adherence in different media, and to determine the viability of Møs during the adherence assay. *ST* 1826 was isolated from human urinary tract, while *ST* ATCC 14028 was isolated from cow liver dying with septicaemia.

2. Materials and Methods

2.1. Preparation of Bacteria

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Salmonella typhimurium ATCC 1826 and 14028 were chosen for their ability to adhere to mouse peritoneal Mø following the method described by Al-Bhary and Pistole [3]. Bacteria were grown to late logarithmic growth phase in LB broth (Becton and Dickinson, USA), incubated at 37℃. Bacteria were centrifuged at 10000 x g for 1 h, washed twice in phosphate-buffered saline (PBS), pH 7.0. The cells were then resuspended in PBS, and adjusted to a concentration of 2 x $l0^9$ cells/ml in RPM1 1640 medium (Life technologies, USA).

2.2. Preparation of Macrophages

Eight to twelve weeks old Male Balb/c mice were used in this study for source of Mø. Mice were injected intraperitoneally with 2 ml of aged Brewer thioglycollate broth (Becton and Dickinson, USA). Mice were sacrificed by CO_2 inhalation after 72 h. Peritoneal Møs were collected in 10 ml of cold Dulbecco's PBS (DPBS), centrifuged at 100 x g for 10 min and washed 3-4 times in 2 ml of cold RPM1 medium. The cells were then counted using haemocytometer and the concentration adjusted for visual binding assay [3].

2.3. Visual Binding Assay

Four types of diluents were tested to determine the optimal conditions for assessing the adherence of *ST* to Møs. These were DPBS, RPMI 1640, RPMI supplemented with essential amino acids, but without glutamine.

ST adherence was determined by direct microscopic examination [3]. Each binding assay contained approximately 3 x $l0^5$ Mø and 3 x 10^8 bacteria. The contents were incubated at 37 ^oC for 1, 3, 6 and 9 h in the presence of 5% CO_2 to allow adherence of to the Mø. Adherent *ST* was determined after each incubation period. Mø to which 5 or more bacteria attached were considered positive.

2.4. Determination of Cell Viability

Approximately 1x10^6 Mø/ml suspended in RPMI and were incubated as explained above. At time zero and every two hours up to ten hours thereafter, 0.1 ml of the sample was removed and diluted 1:10 in trypan blue using dye exclusion assay [6].

2.5. Effect of Cytochalasin-B

Three hundred microliters of cell suspension containing 1x10^6 Mø/ml were prepared as above and incubated for 1 h. Varying concentration (10, 50, 150 and 200 µg/ml) of cytochalasin-B were used and the mixture was incubated for 30 min. 3 x 10^8 bacteria of were added to each assay system. The reaction mixture was incubated for 3 h and the adherent microbes were counted as above [3].

2.6. Statistical Analysis

Data were analysed statistically, using analysis of variance Scheffe's test of significance.

3. Results

3.1. Adherence of ST Strain 1826 to Peritoneal Møs

Adherence of ST strain 1826 to macrophages was determined by binding visual assay and was tested in four different growth media. The media used were DPBS, RPMI 199 without glutamine, RPMI 199 with glutamine, and RPMI 199 containing all essential amino acids (Fig. 1). The experiments were followed within 9 h period in 3 h intervals, the first, the third, the sixth and the ninth hour. Only Møs that bound to 5 bacterial cells or more were considered as positive. Counting total number of bound Møs to bacteria was repeated three times counting 500 total cells of bound and unbound Møs. The average of these counts was calculated and the percentage of bound from unbound Møs was determined. Adherence of ST 1826 bound poorly up to the sixth hour in all tested media. Maximum adherence was observed at the ninth hour. At this hour, more than 80% of Møs that bound to bacteria were from the RPMI 199 medium that has no amino acid supplement. However, viability of Møs that were grown in RPMI 199 medium dropped from 95% at the beginning of the experiment to 81% in the tenth hour of the experiment (Fig. 2).

3.2. Adherence of ST Strain 14826 to Peritoneal Møs

Because of poor adherence of *ST* strain 1826 to Møs, another bacterial strain of *ST* strain 14826 was tested in all four culture media during 9 hours period (Fig. 3). After the first hour of incubation adherence of 5 bacteria or more to Møs was less than 20% in DPBS medium, and continued to increase gradually up to 84% in the ninth hour. Interestingly, number of adhered Møs to bacteria in almost all RPMI media increased up to 60% during the first hour of incubation. At the third hour of incubation, the adhered Møs to bacteria were above 80% and were maintained at this range until the ninth hour. The comparative adherence assay of the two bacterial strains to Møs in RPMI 199 is illustrated in Fig. 4. As a result, of poor binding of *ST* strain 1826, *ST*, strain14826 was used for the rest of the experiments. Since RPMI 199 without amino acids was better than DPBS medium and showed no significant difference in adherence process of Møs when compared with supplemented RPMI 199 with amino acids, it was used for all of the preceding experiments. Also, because of the maximum adherence was obtained at the third hour of the assay, all of the preceding assays were conducted at the third hour of the experiments.

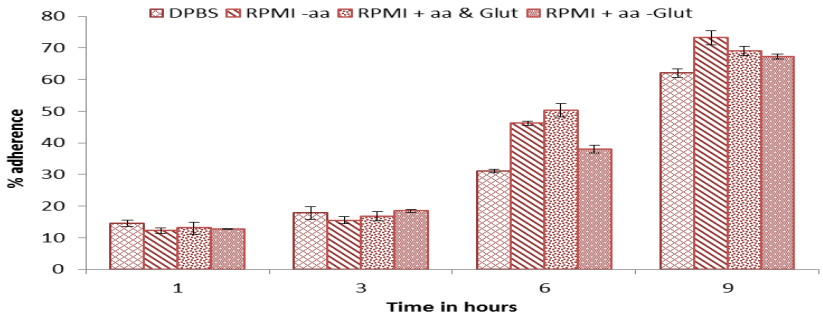

Fig. 1: Adherence of *S.T* 1826 to mouse peritoneal Møs in four media during 1, 3, 6 and 9 hr of incubation.

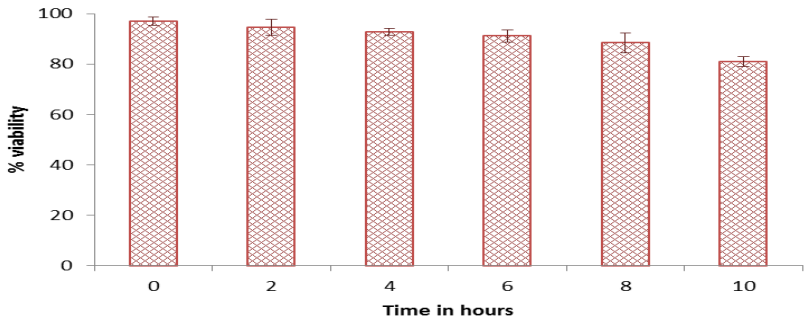

Fig. 2: Viability of Møs grown in RPMI medium without essential amino acids between 0 to 10 hours.

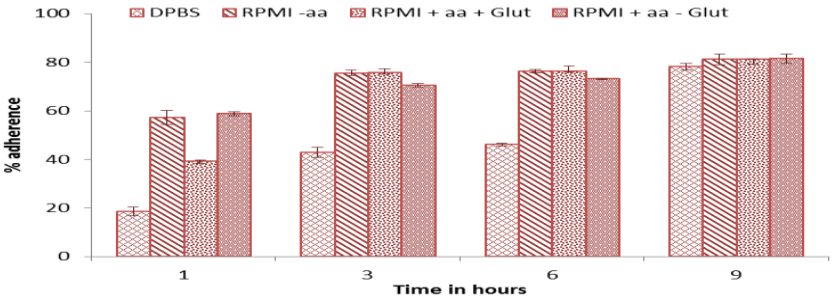

Fig. 3: Adherence of *S.T* 14028 to mouse peritoneal Møs in four media during 1, 3, 6 and 9 hr of incubation.

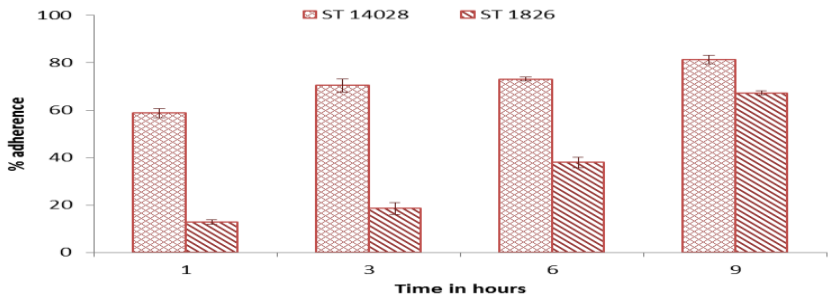

Fig. 4: Comapartive adherence of the two *ST* strains to mouse peritoneal Møs in four media during 1, 3, 6 and 9 hr of incubation.

3.3. Effect of Cytochalasin-B in the Adherence of Bacteria to Macrophages

Because of the difficulty in counting phagocytized bacteria, cytochalasin-B was used to inhibit phagocytosis of bacteria by Møs and thus minimizing false results. Adherence of bacteria in the absence of cytochalasin-B was as low as 70% (Fig. 5). At concentrations of 10 µg/ml, cytochalasin-B has no significant effect. However, counting bound bacteria to Møs was amplified to 94% when cytochalasin-B concentration was elevated to 50 µg/ml, about 10% increase. Increasing cytochalasin-B beyond this concentration causes a decline in adherence which indicates toxicity to Møs. Viability of Møs was conducted in the presence of cytochalasin-B at concentrations 0, 10, 50 100, 150 and 200 µg/ml by dye exclusion assay (Fig. 5). There were no vital changes in the uptake of the dye by Møs between 0-50 µg/ml. However, Mø uptake to dye started to increase with the increase of cytochalasin-B concentration indicating the death of Møs in the medium. Because of these two factors, inhibition of phagocytosis and prevention of Mø death by cytochalasin-B, all of the preceding experiments for the visual binding assays, the Møs were pre-treated with 50 µg/ml cytohalasin-B in a combination with the experimental components.

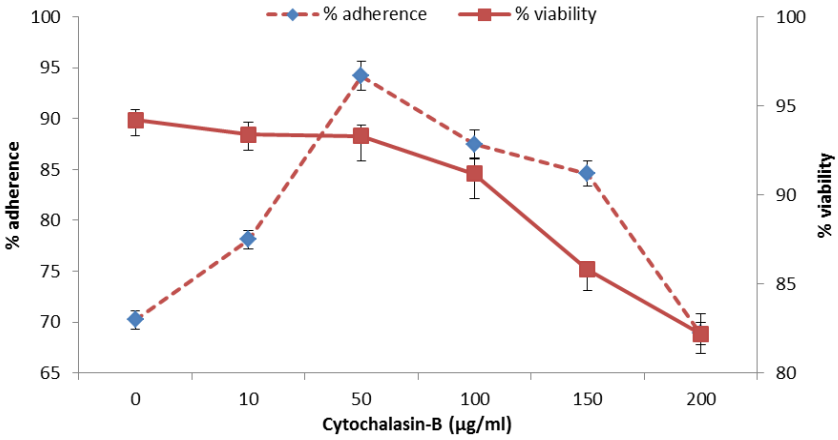

Fig. 5: Effect of cytochalasin-B on the adherence of *ST* 14028 to mouse peritoneal Møs. Viability of Møs at different concentrations of cytochalasin-B

4. Discussion

Due to phagocytosis during the course of incubation, many bacteria were observed to be internalized by Møs which consequently gives false results. This is because of the difficulty to visualize engulfed bacteria. Cytochalasin-B was used to prevent phagocytosis process and therefore to be able to visualize all bound bacteria on Mø surface. Unbound Møs to bacteria increased with the elevation of cytochalasin-B concentrations probably because of cytotoxicity of this component. Although this component inhibits phagocytosis, Sveum *et al*., 1985 [7] stated that it does not inhibit the adherence. Cell viability dropped sharply with the increase of cytochalasin-B concentrations.

Adherence of *ST* 1826 to Møs was initially tested in DPBS medium. The results were not encouraging because of the weak adherence that was achieved especially during the first hours of the assay. However, significant improvement was observed after 6^{th}-9^{th} hour of the assay. Substitution of DPBS medium to

RPMI 199 medium, even when supplemented with amino acids, did not enhance adherence of *ST* 1826 which is similar to the adherence assay in DPBS between the 6^{th}-9^{th} hour. Due to the declining in viability of Møs at the 9^{th} hr of *ST* 1826 adherence, the assay was discontinued. This suggests that *ST* 1826 uses a different mechanism of infection by preventing its adherence to Møs and subsequent invasion and intracellular survival. Also, this indicates that *ST* 1826 is probably not a facultative intracellular pathogen.

Adherence of *ST* 14028 to Mø reached to 60% at the first hour when the assay was conducted in RPMI 199 medium with or without amino acid supplements. Adherence of this strain at the third hour was the highest, more than 80%, and continued at this range up to the 9^{th}. The results of this study are similar to those reported by [3]. However, adherence of this strain in DPBS was similar to *ST* 1826. The difference of adherence of the two strains was clearly demonstrated in this study. It was concluded that that DPBS is not a proper medium for the adherence assays. Data obtained from this investigation indicates that the prime target for *ST* 14028 infection are macrophages. The process of infection therefore differs from that of *ST* 1826 suggesting that *ST* 14028 is a facultative intracellular pathogen.

In this investigation, it was revealed that although both strains belong to *S. typhimurium* the two strains use different mechanisms of infection which probably determines their tissue specificity. The mechanism of *ST* 14028 has been well documented. It will be interesting to determine the components used by *ST* 1826 which disable the adherence process of Mø and subsequently prevents its engulfment.

5. References

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