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DETECTION OF *SALMONELLA TYPHI* ANTIGENS ON THE SURFACE OF MOUSE PERITONEAL MACROPHAGES USING FLUORESCENT TAGGED ANTIBODIES

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ABSTRACT

To see the possibilities of detecting *Salmonella typhi* antigens on Macrophage surface, mouse peritoneal macrophages were infected with *S. typhi in vitro* and the macrophages were examined for morphological changes and expression of *S. typhi* surface antigens on the macrophage surfaces, using FITC tagged specific antibodies. The study showed positive results as fluorescence could be observed on the macrophage surfaces, however, the intensity of fluorescence was low.

Keywords: *S. typhi* antigen, mouse peritoneal macrophages, phagocytosis, FITC tagged antibodies.

INTRODUCTION

Globally Typhoid fever continues to be a public health problem. It is an infectious disease and is a major cause of morbidity and mortality in developing countries. Only humans are affected and most often the acquisition of *S. typhi* occurs via ingestion of food and water contaminated with excreta from carriers of bacterium (Hornick *et al*., 1970). *Salmonella* species efficiently invade the gut mucosa and servive as intracellular pathogens of macrophages (Finlay and Falkow, 1989). It is the ability to survive intracellularly that limits the effectiveness of antibody and neutrophill mediated damage and that allows the pathogens to disseminate systemically from mucosal sites. Typhoid fever is predominately a disease of school age children and young adults (Balraj *et al*., 1992)

World Health Organization (2000) estimates the annual global incidence of typhoid fever at 0.3% corresponding to about 16 million cases of which approx. 600,000 ends in death. In some developing countries of Asia and Africa the annual incidence may reach 1% with fatality rates as high as 10%. About 70% of fatalities from typhoid fever occur in Asia. Laboratory diagnosis is made by blood, bone marrow or stool cultures and with the Widal test (demonstration of *salmonella* antibodies against O-somatic and H-flagellar antigens). The Widal test is time consuming and most of the times when diagnosis is reached, it is too late to start an antibiotic regimen. Isolation of *S. typhi* is the definitive diagnosis but the bacterium is rarely isolated.

The present study was designed to see the possibilities of detecting *S. typhi* antigens on mouse macrophages by artificially infecting these with *S. typhi*. The study showed some promising initial results and the experiments need to be repeated with human circulating macrophages and then in macrophages of typhoid patients using some more sensitive tools to reach to any final conclusion about the utility of this approach.

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MATERIALS AND METHODS

Test bacterial strains

Five strains of *Salmonella typhi*, all from invasive sites, received from NSEC (National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India) were used as test strains. The strains were revived on nutrient agar and identified as *S. typhi* on the basis of biochemical and serological characterization using standard methods.

Preparation of Fluorescent tagged antibodies

Antibodies raised against *S. typhi* "O" somatic antigen and "H" flagellar antigen were concentrated using salting out principle (Wolfson *et al*., 1948). The concentrated antibodies were then conjugated with iso-thiocyanate dye using conjugate buffer (0.5 M carbonate /bicarbonate, pH 9.5) (Johnson, 1963). The unreacted (free) dye was removed by gel filtration using a sephadex column 20 cm long and 3cm diameter. The quality of the fluorescent dye tagged antibodies was checked to ensure that the specific antibodies are tagged which react specifically with its inducer antigens. For this antigen smear was prepared on a glass slide and stained with tagged antibodies and examined under fluorescent microscope.

Collection of mouse peritoneal macrophages (Ray and Dittel, 2010)

A group of 10-13 healthy Swiss albino mice were scarified by deep ether anesthesia. Peritoneal cavity was exposed by cutting the skin of the abdominal wall as shown in figure 1. 10 ml of chilled RPMI-1640 was

Fig. 1. The peritoneal cavity of Swiss albino mouse injected with chilled RPMI.

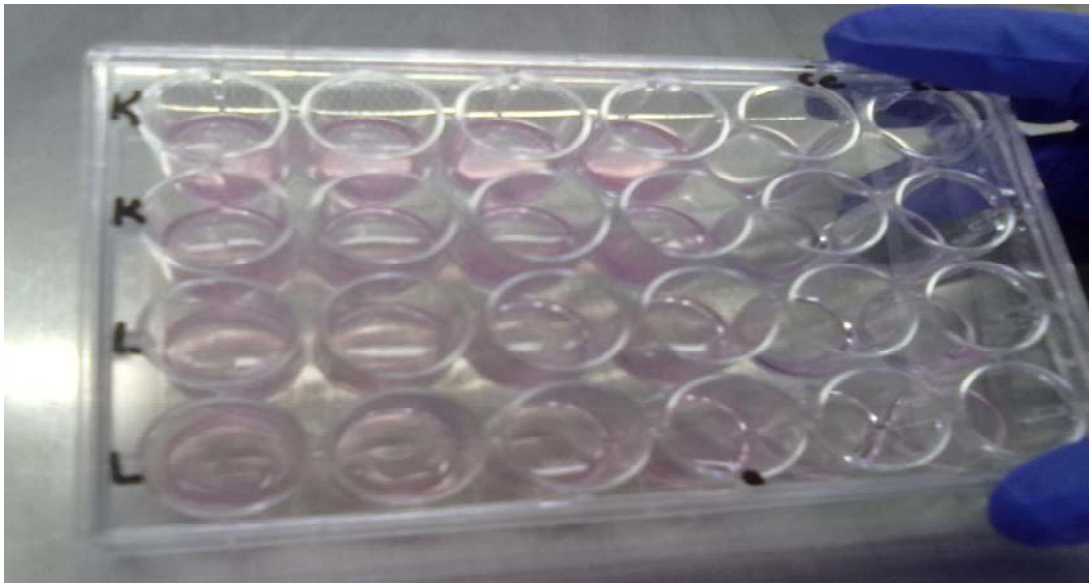

Fig. 2. The cell 24- well castor containing mouse peritoneal macrophages infected with *S. typhi.*

injected into the peritoneal cavity of each mouse. The mice were shaken vigorously to release peritoneal macrophages. The injected fluid was aspirated and collected in sterile 50 ml conical tubes.

The harvest was centrifuged at 3,000 rpm for 3 minutes at 4ºC and sediment was washed twice with RPMI. The cells thus collected were counted for validity as evaluated by trypan blue exclusion and suitably diluted in RPMI 1640 with 10% FCS to give count $1x10^6$ viable cell per ml.

1ml of cell suspension was inoculated in each well of 24 well cell castors with sterile 13mm round cover slip in each well. The cells were incubated at 37ºC for 4 hours in CO_2. After 4 hrs, cell castors were examined under the inverted microscope for the attachment of cell. The cover slips were washed with plain RPMI-1640 (without FCS) to remove non –adherent cells. Adherent macrophages on cover slips were then used for further experiment.

Infection of macrophages with S. typhi

Live and killed suspension of S. typhi was prepared. The prepared suspension of live and killed *S. typhi* were added in the wells containing adhered macrophages as shown in figure 2.

Coverslips were removed after an interval of 2, 4, 6 and 8 hours of infection with antigens and examined for

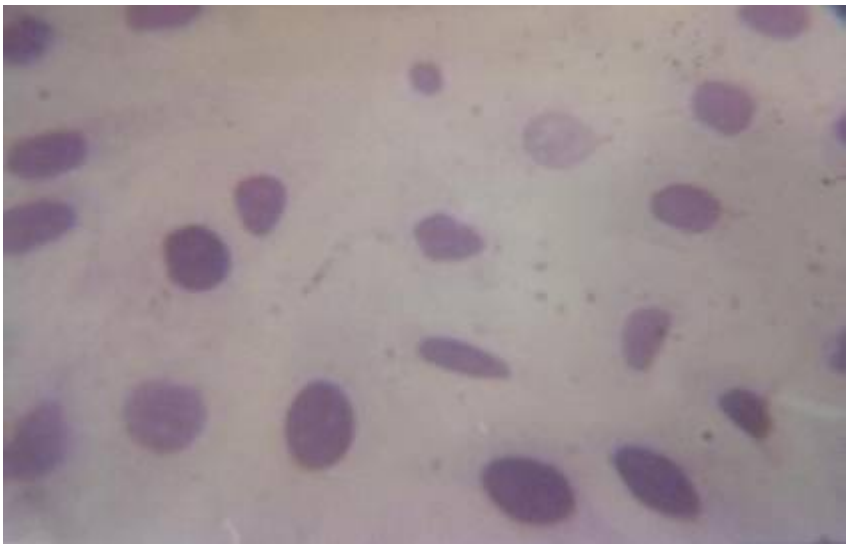

Fig. 3. Bacteria-macophage interaction after 2 hr of incubation.

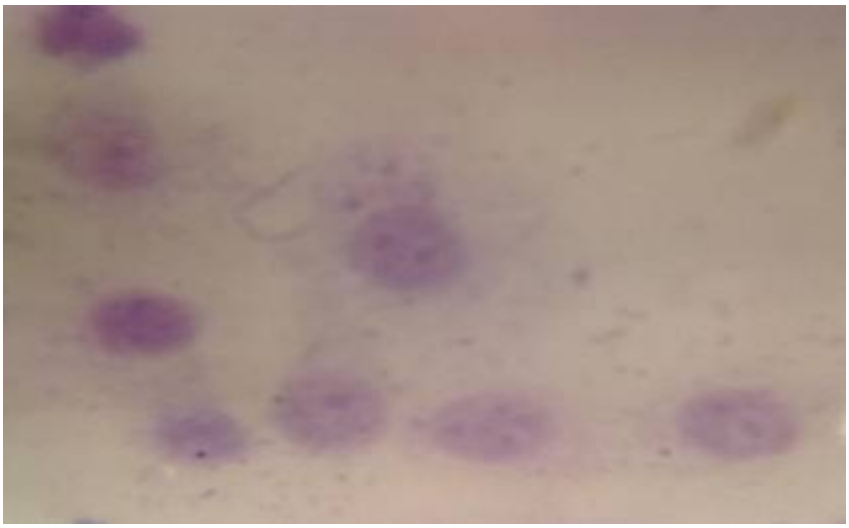

Fig. 4. Bacteria-macophage interaction after 4hr of incubation.

phagocytosis and detection of antigens on the macrophage surface.

Study of phagocytosis by macrophages (Jenkin and Benacerraf, 1960)

The cover slips were washed three times with PBS, then fixed with acetone for 30 seconds and were stained with methylene blue. Again washing with PBS was given. Coverslips were examined under inverted microscope.

Staining of activated macrophages with FITC labeled antibodies

The cover slips from the cell caster were removed after 2h, 4h, 6h, & 8h of infection and were washed 3-4 times in PBS. To check the expression of *S. typhi* O- antigen on the surface of the macrophages, the slides were stained with FITC-labeled antibodies specific to the O-antigen and observed under fluorescent microscope (10X).

RESULTS AND DISCUSSION

Bacteria –Macrophages interaction - phagocytosis

From the results it was observed that the macrophages interacted with bacteria, 2 hours after infection showing attachment and intracellular bacteria with increase in size as in figure 3. After 4 hours incubation majority of cells showed attachment as well as intracellular bacteria with enlargement in size and reversal of cytoplasm to nucleus ratio as shown in figure 4.

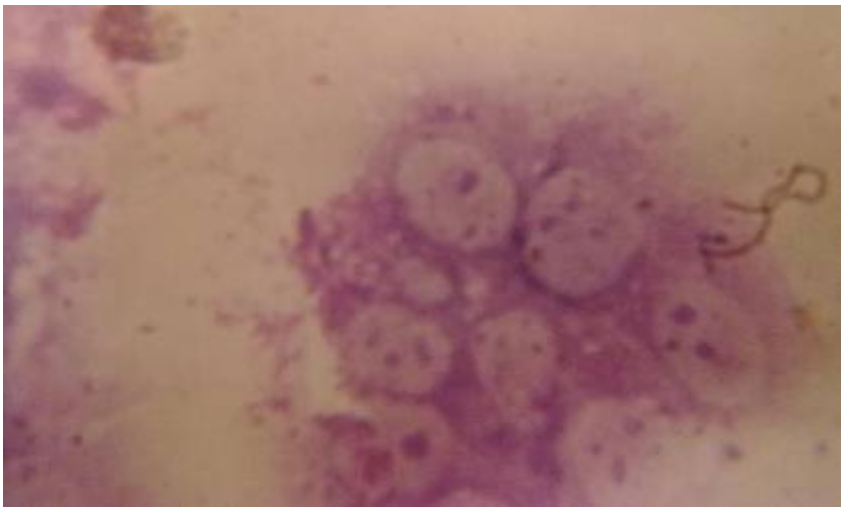

Fig. 5. Bacteria-macophage interaction after 6 hr of incubation.

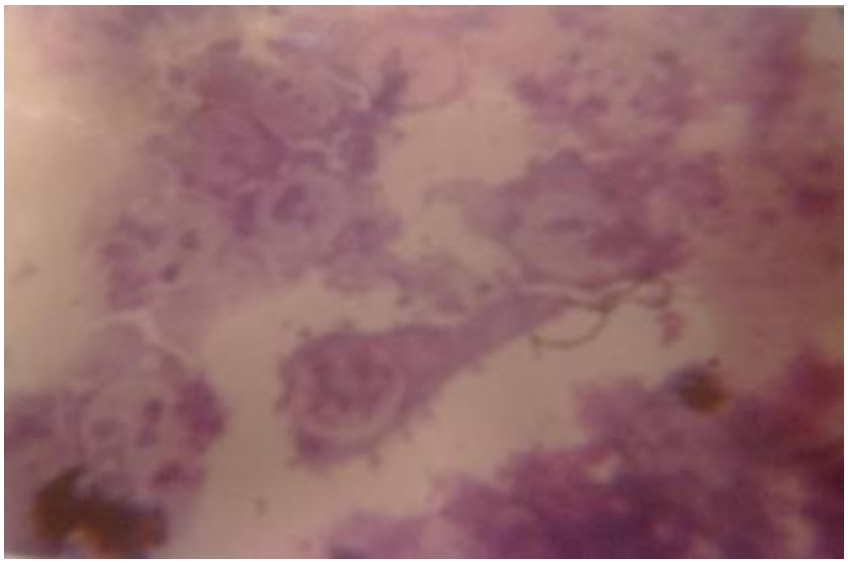

Fig. 6. Bacteria-macophage interaction after 8 hr of incubation.

After 6 hours of incubation, the activation of macrophages continued and showed a further increase in size of a cell. Granules appeared in cytoplasm and vacoulation started. At this stage bacteria were clearly visible intra-cellularly figure 5. After 8 hour few of cell ruptured while majority of cells were intact. The cells were still sticking to coverslip and enlargement was significantin as shown in figure 6.

Detection of S. typhi antigens on macrophages by staining of infected macrophages with FITC labeled antibodies.

When viewed under fluorescent microscope the infected macrophages showed flouresence on their surfaces indicating presence of *S. typhi* antigens. However, the intensity of the fluorescence was low. The results of the staining of the macrophages with fluorescent labeled antibodies have been presented in the figure 7.

Salmonellosis is recognized to be a global problem. Human salmonellosis accounts for 60-70% of all reported cases of food borne diseases (WHO, 1988). Laboratory diagnosis of the infection is made by detection of antibodies against *S. typhi* somatic O- antigen and flagellar H-antigen in the serum of the infected individual using Widal test. Early diagnosis of the disease is very important since it results in early containment of the infection and hence rapid management and treatment of the cases.

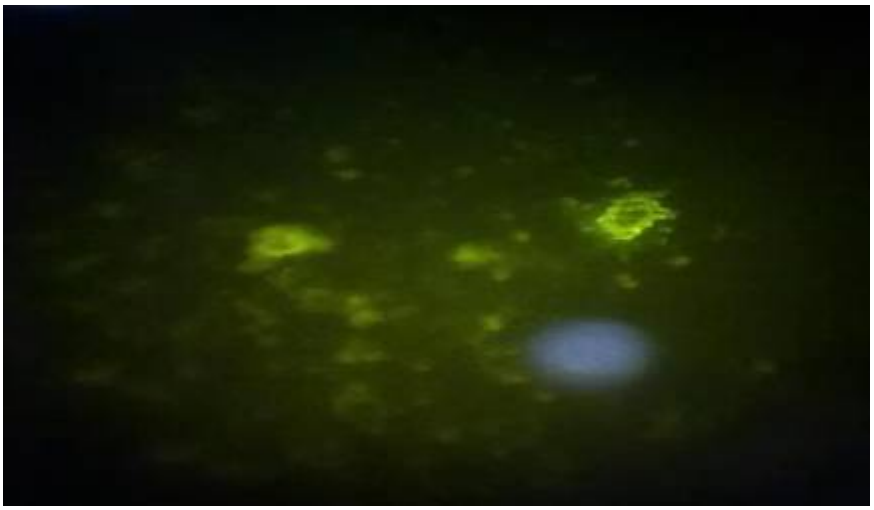

Fig. 7. Macrophages showing fluorescence under fluorescent Microscope.

As macrophages and other antigen presenting cells do carry antigenic moieties of the infecting organisms to release antibodies by the antibody producing cells hence in this study an attempt was made to see the possibility of detecting O antigens of S. typhi on mouse peritoneal macrophages and getting initial, positive results to continue the experiments with human circulating (blood) macrophages and to see the feasibility of this approach as a tool for early detection of typhoid cases. In this study, we studied the interaction between the *S. typhi* and the mouse peritoneal macrophages in-vitro. It was observed that after infection with *S. typhi*, the macrophages showed clear signs of activation like increase in size, vacoulation in the cytoplasm, irregular margins. These change were evident after 2h of infection and progressed in the same manner as is evident from the observation made after 4,6 to 8 hrs that the granulation occurred in the cytoplasm, the cell size increased considerably, the bacterial uptake by the macrophages was found to increase significantly and a few cells got ruptured while the majority of the cells were still intact. The next objective of the study was to detect the presence of the expressed O-antigens on the surface of the macrophages. For that we produced antisera against the O-antigen in rabbits. The antibodies so obtained were then labeled with FITC to stain the macrophage cells for demonstration of the surface O-antigen. To check the efficacy of the labeled antibodies we first stained the pure suspension of *S. typhi* and observed under the fluorescent microscope. The efficacy of the preparation was verified as the bacterial cells were showing fluorescence under the fluorescent microscope. FITC-labeled antibody treatment of macrophages showed fluorescence under the fluorescent microscope. The intensity of fluorescence was very small which can be due to the low quantity of expressed O-antigens on the macrophages or low sensitivity of the method used. Though fluorescent labeled antibodies treatment of the infected macrophages showed low flourscent intensity but the methylene blue stained smears revealed clear evidence of macrophage activation after infection with the *S. typhi* strains. Hence some more refined/sensitive techniques (like electron microscopy) may give better results to show the presence of antigenic moieties on the macrophage surface. Thus this approach has more scope for further studies.

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