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FUTA Journal of Research in Sciences

ISSN: 2315-8239

FUTA Journal of Research in Sciences, 2013 (1): 87-93

INFLUENCE OF MACROPHAGES IN CONFERRING IMMUNITY AGAINST *Escherichia coli* O157:H7 INFECTION IN ALBINO RATS

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ABSTRACT

The influence of macrophages in conferring immunity against *Escherichia coli* O157:H7 infection in albino rats was studied by monitoring the number of organisms that were trapped in the liver, spleen, and ileum of the rats that were administered carrageenan, which has a selective cytopathic effect on macrophages. At different intervals, starting from 30 minutes to 168 hours, the selected organs were dissected out and the number of trapped *E. coli* O157:H7 was counted using standard microbiological techniques. The number of trapped *E. coli* O157:H7 in the spleen, liver and ileum of carrageenan treated rats (5.66, 5.58 and 5.50 $\log_{10}$ cfu/ml respectively) was observed to be significantly greater ($P \leq 0.05$) than the number that was trapped in the spleen, liver and ileum of those that were not administered with carrageenan prior to infection (5.26, 5.28 and 5.37 $\log_{10}$ cfu/ml respectively) at the end of 168 hours. The highest number of *E. coli* O157:H7 (5.77 $\log_{10}$ cfu/ml) was however recovered from the spleen of carrageenan treated rats after 96 hours of infection. From this investigation, the administration of carrageenan resulted in high recovery of *E. coli* O157:H7 from the organs of infected rats. Thus, macrophages played a significant role in fighting against this organism in the host's immune system.

Keywords: Macrophages, Immunity, *E. coli* O157:H7, Liver, Spleen, Ileum, Cytopathy

INTRODUCTION

Immunity is the ability of the host system to be able to recognise different arrays of foreign substances (antigens) in the body and react against them. The response of a host to any particular pathogen may involve a complex set of responses; the humoral and/or cellular arms of the specific (adaptive) immune response and the non-specific (innate) responses (Janeway *et al*., 2005).

Macrophages are important in the regulation of immune responses. They are often referred to as scavengers or antigen-presenting cells (APC) because they pick up and ingest foreign materials, and present these antigens to other cells of the immune system such as T cells and B cells (Janeway *et al*., 2001). On the other hand, macrophages after ingestion of the antigens can destroy them through the action of enzymes such as toxic peroxidases present in their cytoplasm, they are known to digest more than 100 bacterial cells before they die (Ryan and Ray, 2004).

Escherichia coli O157:H7 colonizes the gastrointestinal tract and causes a condition known as hemorrhagic colitis (HC) or bloody diarrhoea (Karch *et al*., 2005). This group of *E. coli* is solely defined by its capacity to produce Shiga toxin type 1 (Stx1), Shiga toxin type 2 (Stx2), or both toxins which has been linked to their virulence (Manning and Motiwala, 2008).

Transmission of the organism has been linked with the consumption of raw or undercooked foods of bovine origin, vegetables, apple cider, cantaloupe, mayonnaise-containing salad dressing and cabbage (Blackburn and McCarthy,

2000). Others are person to person transmission, drinking of contaminated water and contact with faeces of bovine or human origin (Treor, 2008).

Carrageenan is a sulphated polysaccharide isolated from marine algae. It has cytopathic effect on macrophages thereby surpressing immune response mediated by these cells both *in vivo* and *in vitro* (Thomson and Fowler, 1981). This study was embarked on to know whether carrageenan sensitive macrophages play any role in mediating immunity against infection caused by *E. coli* O157:H7 in rats.

MATERIALS AND METHODS

Experimental animals

Seven to eight weeks old, adult male albino rats, with average weight of 65g were used for this experiment. They were housed in cages with adequate ventilation and fed with clean water and growers mash which was made up of 88% dry matter, 15% crude protein, 6% crude fibre, 1.2% calcium, 0.40% phosphorus and energy kcal/kg 2650, a product of Jesme Feed Depot, Akure, Nigeria. They were fed once daily for 2 weeks during which period they were observed for any sign of illness.

The organism used

The *Escherichia coli* O157:H7 (single plasmid, molecular weight 24,980bp) used was collected on an agar slant from the Nigerian Institute of Medical Research (NIMR) in Yaba, Lagos, and transported to the laboratory.

Preparation of cells of *Escherichia coli* O157:H7

This was done according to the method of Olorunfemi and Adebolu (2012). The organism was transferred from an agar slant into 10ml nutrient broth using a sterile inoculating loop, mixed well and incubated at 37^0C for 24 hours. After incubation, the content was centrifuged using MSE Minor 35 model, at a speed of 3000 revolution per minute (rpm) for 10 minutes to harvest the cells. The supernatant was discarded and the harvested cells were washed with sterile distilled water and re-centrifuged. The cells were then re-suspended in 10ml sterile distilled water which served as the stock cell solution for serial dilution. The number of cells per ml was then determined using pour plate techniques.

Determination of Infective dose of *E. coli* O157:H7

This was carried out according to the method of Olorunfemi and Adebolu (2012). Sterile test tubes were arranged serially, 9ml sterile distilled water was dispensed into 6 set of test tubes, and 1ml of the stock (cell) solution was introduced into the first tube making 1:10 dilution. This procedure was repeated for the remaining 5 tubes. One ml of each dilution was pour plated on Sorbitol MacConkey agar. The plates were incubated at 37^0C for 18 - 24 hours. Visible colonies were counted and estimated according to the dilution factor. The rats, 4 groups of 3 each were challenged orogastrically with 1ml of the different corresponding dilutions. They were observed for 1 week for any clinical symptom of infection (diarrhoea). The dose that was able to produce clinical effect such as unformed stool on the animal was calculated and used as the infectivity dose of the organism.

Effect of Carrageenan treatment on the growth of *Escherichia coli* O157:H7 in selected organs of albino rats.

The rats were injected intraperitoneally with carrageenan according to the method of Tatsukawa *et al*. (1979). After 24 hours, they were orogastrically dosed with the infectious dose of the test organism, according to the method of Onifade and Audu (1995) with slight modification. At 30 minutes, 1 hour, 6, 24, 48, 72, 96, 120, 144, and 168 hours, the liver, spleen, and ileum were dissected out after anesthetizing the animals with cottonwool soaked in chloroform in a metallic container for one minute. The liver and spleen were homogenized separately in sterile mortar and pestle, while the ileum (10cm in length) was washed in a sterile bijou bottle containing 10ml of sterile distilled water. One ml of the homogenized liver, spleen, and washings of the ileum were serially diluted and pour plated using Sorbitol MacConkey Agar. The plates were incubated at 37^0C for 18 - 24 hours. The number of viable bacteria were counted and expressed as $\log_{10}$ (bacterial count). For the control experiment, rats that were not pretreated with carrageenan were dosed with the infectious dose of *E. coli* O157:H7 and the liver, spleen, and ileum were dissected out and analysed as described for the carrageenan treated rats.

Statistical analysis

Statistical analysis of data was carried out using analysis of variance (ANOVA) and Duncan's New Multiple Range Test for the estimation of means. The 't' value was tested at 95% confidence interval.

RESULTS

Estimated infective dose (ID) of *Escherichia coli* O157:H7 in albino rats

The estimated infective dose was observed to be 2.7 x 10^2cfu/ml (Table 1). Stool inconsistency was used as a sign of diarrhoeic infection in the rats. The stool of infected rats was observed to be sausage shaped, unusually long, and blotted compared with the stool of healthy rats which was solid and of short rod shape. This was observed between 72 to 120 hours after the infection.

Time Course Growth of *Escherichia coli* O157:H7 in the Liver, Spleen, and Ileum of rats treated with Carrageenan.

In the carrageenan treated rats, there was an increase in the number of bacterial count in the organs examined at 1 hour, followed by a reduction at the 6th hour. However, the bacterial count later increased at 24 hours. The highest bacterial growth was recorded in these organs at 96 hours, followed by a gradual decrease till 168 hours. The results in figures 1, 2 and 3 showed the growth of *E. coli* O157:H7 in the liver, spleen, and ileum of the infected rats as compared with the control rats respectively. The same pattern was observed in the control rats, except that maximum bacterial growth was seen in the spleen at 72 hours instead of 96 hours in the carrageenan treated rats.

The percentage recovery and mean value of $\log_{10}$ bacterial count (cfu/ml) of *E. coli* O157:H7 that was recovered from the carrageenan treated rats was greater than that of the non treated rats throughout the experiment. The load (expressed as $\log_{10}$cfu/ml x10^3) and percentage of *E. coli* O157:H7 that was recovered from the liver (4.64, 1.72%), spleen (4.86, 1.80%) and ileum (4.69, 1.73%) of carrageenan treated rats was higher than that of the liver (4.28, 1.59%), spleen (4.00, 1.48%) and ileum (4.48, 1.66%) of those that were not administered with carrageenan after 30 minutes of infection.

However, after 1 hour of infection, the bacterial count rose to 4.85, 5.12 and 5.22, in the liver (1.80%), spleen (1.90%) and ileum (1.93%) of carrageenan treated rats respectively, and 4.69, 4.57 and 4.73 in the liver (1.74%), spleen (1.69%) and ileum (1.75%) of the control rats respectively. At 6 hours of infection, there was a reduction in the number of the trapped *E. coli* O157:H7 in the treated and non treated rats, as 1.75%, 1.84% and 1.74% was recovered from the liver (4.73), spleen (4.97) and ileum (4.70) of the treated rats respectively, while 1.63%, 1.50% and 1.74% was recovered from the liver (4.40), spleen (4.04) and ileum (4.69) of the non treated rats respectively. At 24 hours of infection, the bacterial count increased significantly ($P \leq 0.05$) to 5.30, 5.37 and 5.41 in the liver (1.96%), spleen (1.99%) and ileum (2.00%) of carrageenan treated rats respectively, and 5.11, 5.04 and 5.18 in the liver (1.89%), spleen (1.87%) and ileum (1.92%) of the control rats respectively. This increased till 96 hours of infection.

At 96 hours where maximum growth of *E. coli* O157:H7 was observed in the treated rats, the percentage of trapped *E. coli* O157:H7 increased to 2.12%, 2.14% and 2.12% in the liver (5.72), spleen (5.77) and ileum (5.71) of the treated rats respectively, while 2.03%, 1.97% and 2.04% was recovered from the liver (5.48), spleen (5.33) and ileum (5.51) of the non-treated rats respectively. However, maximum bacterial growth was observed in the spleen of control rats at 72 hours (1.98%, 5.35). The bacterial count decreased gradually till the last hour in the treated rats, but rose again in the control rat at 144 hours before it finally reduced to 2.07%, 2.10% and 2.04% in the liver (5.58), spleen (5.66) and ileum (5.50) of the treated rats respectively and 1.96%, 1.95% and 1.99% in the liver (5.28), spleen (5.26) and ileum (5.37) of non-treated rats respectively 168 hours.

Table 1: Physical parameters of rats and their stool after infecting with different population of *E. coli* O157:H7 in order to determine the infectious dose.

Treatment	Symptoms (stool parameters)						
(cfu/ml)	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs	168 hrs
$2.7x10^2$	FS,A,E	FS,A,E	US,W,LA	US,W,LA	US,W,LA	FS,A,E	FS,A,E
$2.3x10^2$	FS,A,E	FS,A,E	US,A,E	US,A,E	FS,A,E	FS,A,E	FS,A,E
$1.2x10^2$	FS,A,E	FS,A,E	FS,A,E	FS,A,E	FS,A,E	FS,A,E	FS,A,E

Key: FS- formed stool, US- unformed stool, A- active, W- weak, LA- loss of appetite, E- eaten well

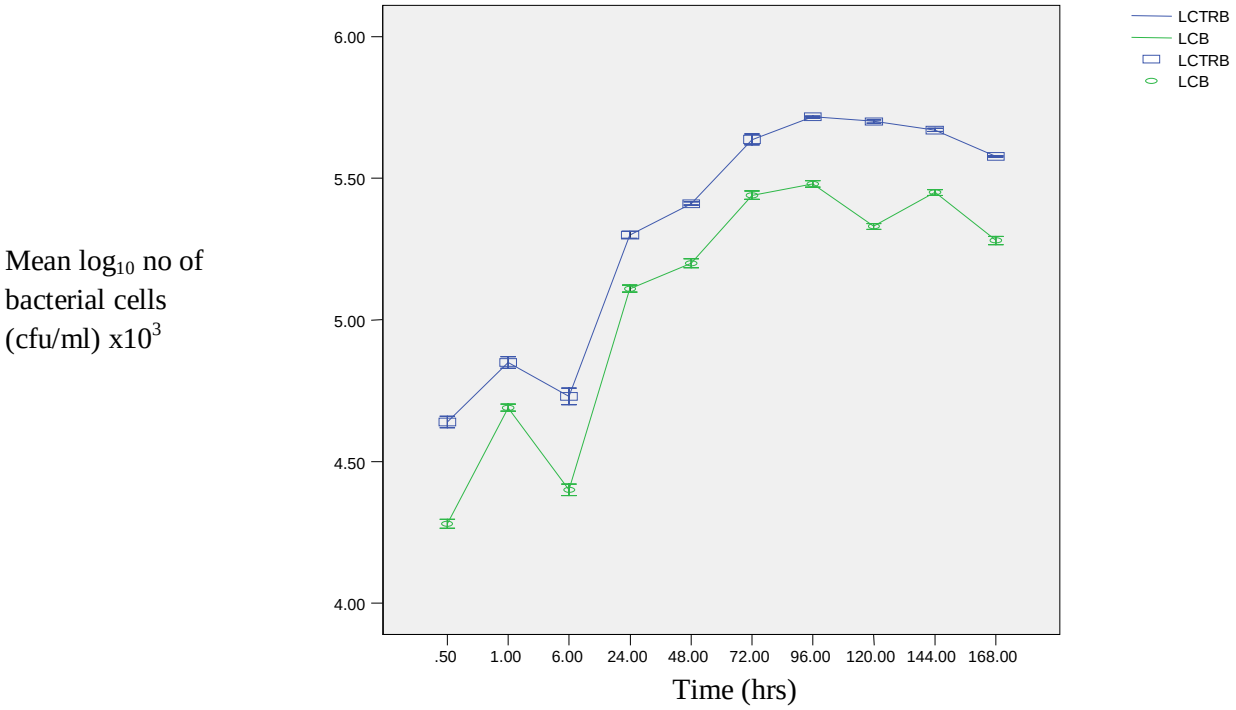

Figure 1: Time course growth of *E. coli* O157:H7 in the Liver of Carrageenan treated rats after challenge with the infective dose of the organism as compared with the control rats
Keys: LCTRB – Liver of Carrageenan treated rats, LCB – Liver of control rats

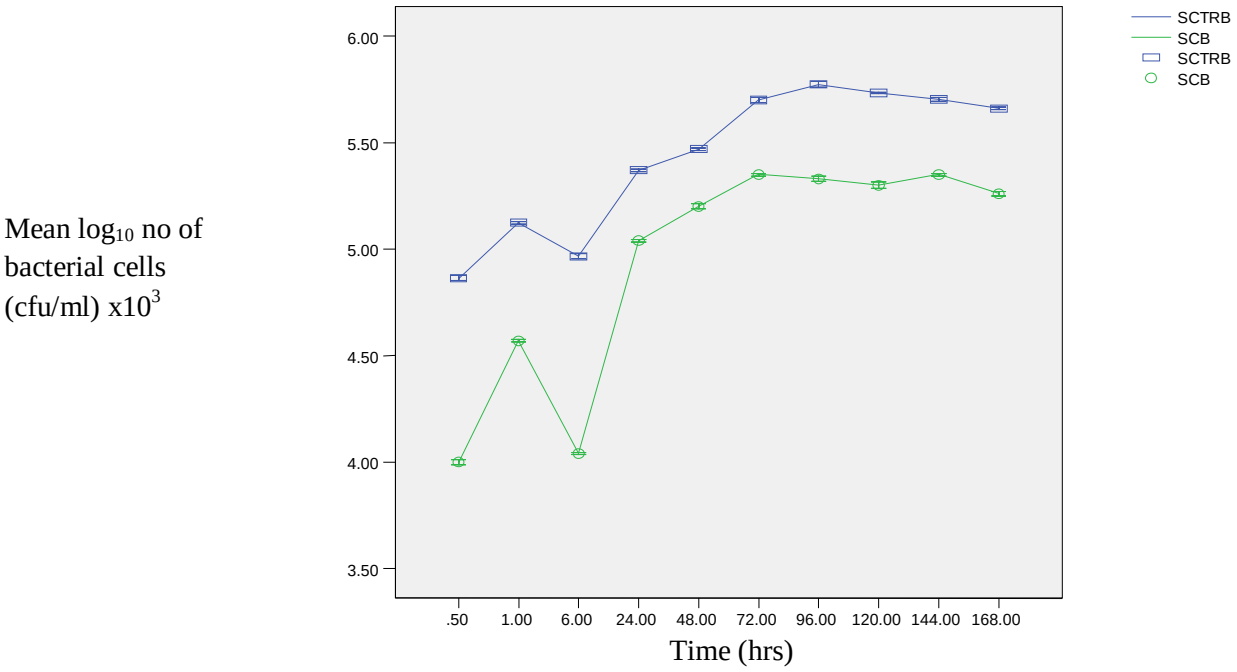

Figure 2: Time course growth of *E. coli* O157:H7 in the Spleen of Carrageenan treated rats after challenge with the infective dose of the organism as compared with the control rats
Keys: SCTRB – Spleen of Carrageenan treated rats, SCB – Spleen of control rats

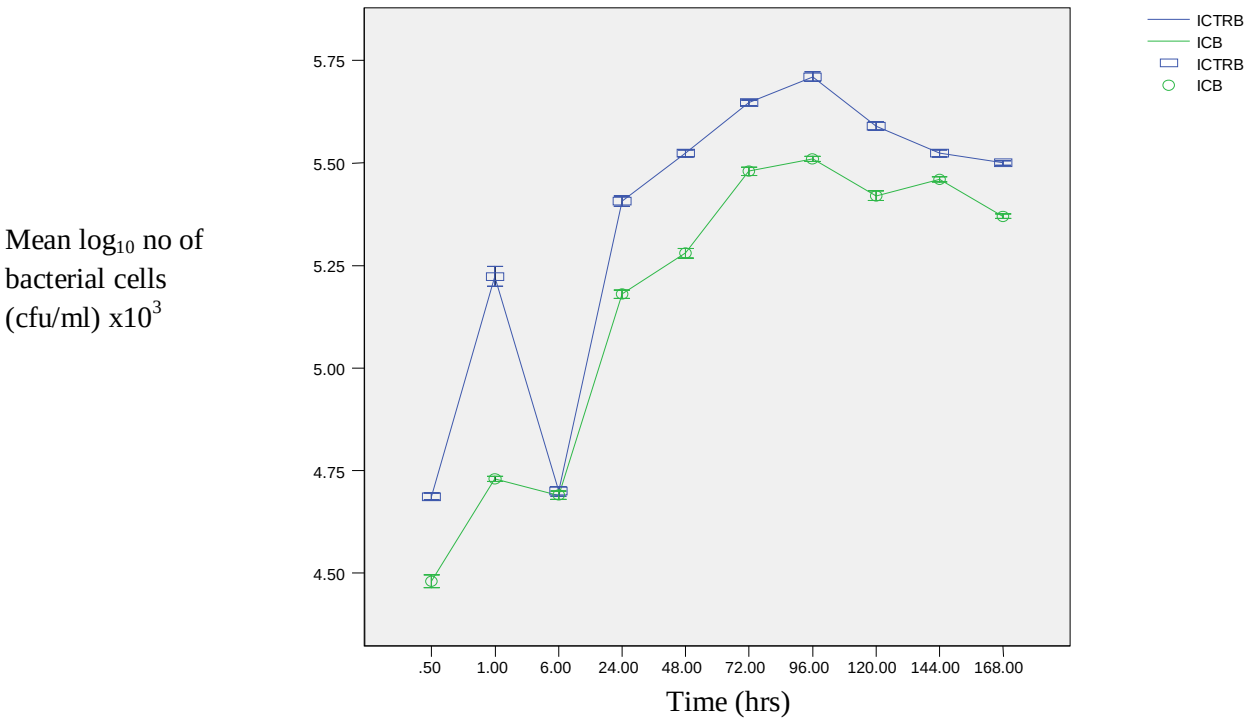

Figure 3: Time course growth of *E. coli* O157:H7 in the Ileum of Carrageenan treated rats after challenge with the infective dose of the organism as compared with the control rats
Keys: ICTRB – Ileum of Carrageenan treated rats, ICB – Ileum of control rats

DISCUSSION

In the carrageenan treated rats, destruction of the macrophages by carrageenan caused an increase in the number of *E. coli* O157:H7 in the organs examined. This shows that macrophages play significant role in fighting against this organism in the host's immune system. This agrees with the report of Tatsukawa *et al*. (1979) in their study on the different contributions of polymorphonuclear cells and macrophages to protection against *Listeria monocytogenes* and *Pseudomonas aeruginosa*, in which the destruction of macrophages led to a progressive increase in the number of the organisms that were trapped in the liver and spleen. Onifade and Audu (1995) also had similar observation when an investigation was carried out on the effect of carrageenan treatment on the immune response of mice against *Escherichia coli*.

The recovery of bacteria however, was greater in the spleen of carrageenan treated rats than the other organs examined. This might be due to the fact that the percentage of macrophages present in the spleen was higher than the other organs examined and when these were destroyed by the administered carrageenan, they were not available to phagocytose and destroy the invading bacteria. This is an important consideration in the interpretation of the effects of carrageenan *in vivo* and precludes its use as a clinical immune suppressant (Thomson and Fowler, 1981).

The ileum recorded the highest bacterial count in the control experiment which could be attributed to the fact that this part of the gastrointestinal tract is the principal site of localization of the organism (Treor, 2008). Moreover, only few immune cell types are found there. For example, the only immunoglobulin that is present is IgA, phagocytes play significant role when the organisms enter the lymphatic channels.

The decrease in bacterial count observed in the liver and spleen at 6 hours might be due to the collaborative activity of the cells of the immune system including the macrophages in fighting against the bacteria. This assertion is as a result of the fact that despite the destruction of macrophages by carrageenan, there was still a decrease in the count of *E. coli* O157:H7 in the carrageenan treated as well as those that were not pretreated with carrageenan by 6 hours of infection although the ones not pretreated with carrageenan recorded a lower count ($P \leq 0.05$). However in the ileum, there was no significant difference ($P \geq 0.05$) in the bacterial counts of both the pretreated and the control rats showing that macrophages play no role in the ileum at 6 hours. The sharp increase in bacterial growth at 24 hours could be attributed to the high virulence of the organism. Those organisms that were able to escape the initial trapping and killing by the phagocytes began to proliferate. Among these virulence factors are (a) periplasmic catalase, which is encoded on the pO157 plasmid and (b) shiga-like toxins (Stxs) functionally identical to toxins produced by virulent *Shigella* species. According to Robinson *et al.* (2006), Stxs can promote colonization by *Escherichia coli* O157:H7 and the toxin play a role in gastrointestinal colonization.

More *E. coli* O157:H7 was recovered from the carrageenan treated rats than the control rats in the selected organs, particularly the spleen and this shows that macrophages play a significant role in conferring protection against the infection. The immune response against the infection caused by *E. coli* O157:H7 therefore requires the activity of macrophages.

ACKNOWLEDGEMENT

The Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, is highly appreciated for the provision of *Escherichia coli* O157:H7 used.

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