ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Effect of non porine (49 K.D) outer membrane proteins of *Salmonella typhimurium* against Salmonellosis in mice leukocytes and phagocytosis

Noor A. ALridha Kazim
Department of Biotechnology. College of Science. Al-Nahrain University.

Manuscript Info

Manuscript History:

Received: 21 October 2014
Final Accepted: 22 November 2014
Published Online: December 2014

Key words:

****Corresponding Author***

Noor A. ALridha Kazim

Abstract

The research aimed to investigate the protective effect of non porins outer membrane proteins (OMPs) of *Salmonella typhimurium* against salmonellosis. The outer membrane non porin proteins (OMPs) of this bacterium was extracted and analyzed by Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The desired proteins Purification by ion exchange chromatography on DEAE- cellulose and gel filtration chromatography on Sephacryl S- 200 was carried out to obtain non porin proteins with Molecular weight 49 kDa. To evaluate the immunological parameters (total and absolute count of leucocytes, Phagocytic index) .For this purpose 15 albino mice divided into three groups before subjection to various treatments. The results of immunological parameters were as follows: Group III showed a significant increase in total count of leucocytes, neutrophils, monocyte and lymphocyte. No significant differences were detected in the total counts of. Group III showed a significant increase in phagocytic index and total immunoglobulin level.

Introduction

The vaccine targets bacteria called *Salmonella typhimurium*, organisms which cause food poisoning in humans called salmonellosis. The bacteria are found in the intestinal tracts of animals and humans and are easily spread. Thousands of people are hospitalized in the US each year with salmonella infection which can be fatal. About half of these cases are attributed to infected chicken eggs, *S. typhimurium* bacteria shed in feces of infected animals or people and cause diarrheal illness, which can be quite severe in the young, elderly and individuals with weakened immune systems [17] .

Different antigens such as O, H, Vi polysaccharide, heat shock proteins and whole cell parental vaccines have been exploited for their protective role against typhoid, The outer membrane proteins have been recently expected to become an effective tool for conferring protection against typhoid that could be used in vaccine formulations [4 , 20] .For importance mentioned above the aims of study were extraction and purification of proteins. outer membrane protein from *Salmonella typhimurium*.

Materials and Methods

Isolation of *S. typhimurium* and extraction of outer membrane protein

S. typhimurium previously isolated from stool sample of infant suffering from diahrria. The extraction of outer membrane proteins from *S. typhimurium* outer surface was carried out according to [15, 8]

Procedure

- Bacterial isolate was grown on brain heart infusion agar at 37˚C over night.
- Bacterial cells were harvested and washed three times with Tris-Hcl buffer (0.01M at pH 7.8) and centrifuged at 3500 rpm for 10 min.
- Ten grams of wet weight of bacterial cells were suspended in 100 ml of 2% triton and left over night, and

then cells were centrifuged at 4750 rpm for 10 min, then 100 ml of (2%) triton x-100-EDTA (0.01M) was added to pellet and left in an incubator at 37˚C for 10 min.

- Twenty mg of lysozyme was added to the mixture and left in incubator for 1 hr, and then the mixture was centrifuged at 5000 rpm for 20min. pellet was washed with 100 ml of Tris (0.01M) –EDTA (0.01M) pH7.8 buffer and centrifuged at 4000rpm for 20 min.
- Pellet was washed with 100 ml of (triton x-100 –Mgcl2) pH 7.8, and centrifuged at 6000 rpm for 10 min.
- Finally pellet was washed with 100 ml Tris-Hcl buffer (1M) at pH 8.8 and centrifuged at 5000 rpm for 15 min and the pellet was collected for further study.

Protein estimation and detection

Protein concentration in pellets was determined according to Lowery method using bovine serum albumin as standard protein solution for the preparation of a standard curve [10]**.**

Protein purification

- DEAE-Cellulose preparation

A DEAE-Cellulose column was prepared according to [19] . Then beads were left to settle down and were washed several times with D.W until getting clear appearance. The suspension was filtered throughout Whattman No.1 using Buchner funnel under discharging.

The resin was resuspended in 0.25 M sodium chloride and sodium hydroxide solution. The suspension then was filtered again as mentioned above and washed several times with 0.25 M hydrochloric acid solution and next by distilled water before it was equilibrated with 0.01M phosphate buffer pH 7 after preparation, an outer membrane proteins was applied to ion exchange chromatography column packed with DEAE-Cellulose equilibrated previously with 0.01 M phosphate buffer pH 7. Then column was washed with an equal volume of the same buffer, while attached proteins were stepwise eluted with gradual concentrations of sodium chloride:(0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 0.8, 0.9,1M respectively).

Flow rate throughout the column was 3ml/fraction and the absorbance of each fraction was measured at 280 nm using UV-VIS spectrophotometer. Each peak of elution fraction was collected and checked by SDS-PAGE for outer membrane presence.

- **Preparation of Sephacryl S-200**

Sephacryl S-200 was prepared as recommended by Pharmacia Fine Chemicals Company. A quantity of Sephacryl S-200 was suspended in 0.1 M phosphate buffer (pH 7), degassed, and packed in a glass column (1.5x63 cm), and equilibrated with 0.1M phosphate buffer (pH7).

A volume of concentrated outer membrane proteins obtained after the ion exchange purification step (fractions number 55-73) was applied onto Sephacryl S-200 column in dimensions of 1.5cm x 63cm, equilibrated previously with 0.01M phosphate buffer pH7. Elution was achieved at a flow rate of 3 ml/ fraction using the same buffer for equilibration. Absorbance of each fraction was measured at 280nm. Then each peak was collected and checked by SDS-PAGE for outer membrane presence.

Experimental design

Twenty Albino male mice were used with (8-10) weeks, and the weight of each was (28-30) grams. They were divided into 4 groups (each group contain 5 mice); and kept in a separate plastic cage, the animals were hosted at (20- 25) ˚C. [18]:

Group- I-: Mice injected with 0.25ml of normal saline.

Group- II-: mice infected with 100µl ($1x10^7$ cfu/ml) *S. typhimurium*.

Group- III-: mice subcutaneously injected with 0.25ml of outer membrane proteins and 0.25ml of complete Freund´ s adjuvant and then orally infected with (100µl) of *S. typhimurium.*

Immunological parameters

Total leukocyte count

Blood samples were collected after two weeks of infection by heart puncture using a disposable insulin syringe (1 ml) precoated with heparin. The method of [3] was followed, in which, an aliquot of 0.02 ml blood was mixed with 0.38 ml of leukocyte diluents in a test tube, and left at room temperature for 5 min. A drop of the mixture was applied To the surface of Neuberger chamber under the cover slip, and the chamber was left for 3 minutes to settle the cells.

The leucocytes were counted in 4 large squares (each with 16 small squares), and the total count of leucocytes was obtained using the following equation: Total Count (cell/cu.mm. Blood) =
Average number of chamber W.B.C x dilution (20) / volume (0.4).

Differential count of leucocytes

One drop of blood was smeared on a clean slide using another slide and left to dry at room temperature. The smear was stained with Leishman stain for 5 minutes and buffered for 10 minutes, and then washed with tap water. The slide was air-dried, and then examined under oil immersion lens (100X) [3]. At least 100 leucocytes were examined, and the percentage of each type was recorded, while the total count of each type was obtained using the following equation:

$$\text{Total Count (cell/cu.mm. Blood)} = \left(\frac{\text{Percentage of Cells x Total Count}}{100} \right)$$

Phagocytic index

The evaluation of phagocytosis was carried out on phagocytes obtained from the peritoneum of mice. The procedure of [14] was followed with some modifications.

Results and discussion

Detection of outer membrane protein purity

Analysis of protein profile of OMPs by SDS_PAGE gave two bands 55kDa and 49kDa after purification by using ion exchange chromatography on DEAE-cellulose compared with the marker proteins (figure 3-3). The purity of OMPs were detected by SDS-PAGE showed that one band of OMPs with molecular weight 49 kDa purification by using gel filtration on Sephacryl S-200 as indicate in (figure 3-4). This results agreed with Samuelson et al. (2002) who reported that non porin OMPs of *S.typhimurium* with different molecular masses (15, 33, 37 and 49) kDa.

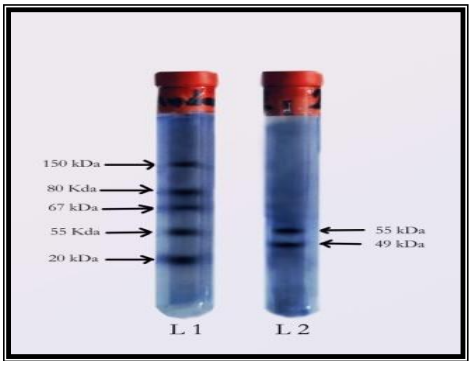

Figure (1): Polyacrylamide gel electrophoresis of purified outer membrane protein of *S. typhimurium* after ion exchange on DEAE-cellulose: Lane (1): protein markers, Lane (2): partial Purified OMPs.

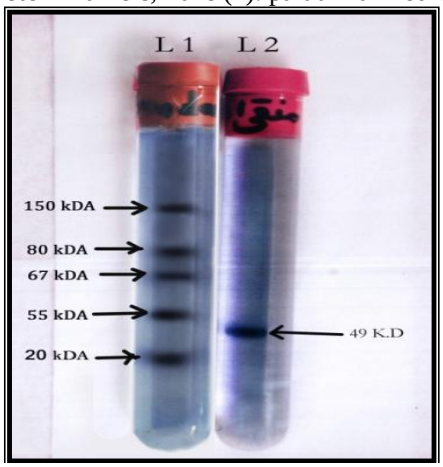

Figure (2): Polyacrylamide gel electrophoresis of purified outer membrane protein of *S. typhimurium* after gel filtration on Sephacryl S-200: Lane (1): protein markers, Lane (2): Purified 49 k.d, OMP .

Table (1): shows the total and differential leukocytes in mice group after injected with 49 k.d OMP of *S. typhimurium*

Mice group	**Total leukocyte count (M*+SE) (cells /cu.mm. blood**	**Neutrophil count (M*+SE) (cells /cu.mm. blood)**	**Lymphocyte count (M*+SE) (cells /cu.mm. blood)**	**Monocytes count (M*+SE) (cells /cu.mm. blood)**
Group-I- (negative control)	b 5660+321.46	d 1988+211.24	d 2370+203.6	c 248+84.51
Group -II- (positive control)	b 305+64.34	b 2965+284.47	c 309+218.65	c 4750+266.7
Group-III (mice subcutaneously injected outer membrane proteins orally infected with (100µl) of S. typhimurium.	a 372+64.50	a 3174+229.43	a 2554 +320	a 6200+410.08

* Different letters: Significant difference ($P \leq 0.05$) between means of column.

Phagocytic index

Table (2): Phagocytosis index in mice treated with 49 kDa non porin outer membrane proteins and infected with *S. typhimurium*.

Mice groupe	Phagocytosis (%) (M*±SE) **(cells /cu.mm. blood)**
Group-I- (negative control)	C 29.600±3.507
Group -II- (positive control)	b 40.600±3.647
Group-IIII-(mice treated with outer membrane protein and infected with *S. typhimurium*).	a 50.600 ±5.459

* Different letters: Significant difference ($P \leq 0.05$) between means of column.

Discussion

Mice infected with *S. typhimurium* exhibited a significant increase in total count of leucocytes, lymphocyte , neutrophils and monocytes ; no significantly increase in eosinophilis and basophiles counts. These results come in accordance with [2 , 5]who mentioned that the total white blood cell is low and anemia may be involved in typhoid

patients and the significant leucopenia commonly observed in typhoid fever is attributed to invasion of haemopoietic organs such as bone marrow, lymph nodes and spleen by Salmonella.

Mice infected with *S. typhimurium* exhibited a significant increase in phagocytosis activity. This result come in accordance with results of [12] who reported that the normal and Salmonella-infected mice increased their phagocytic capacity when exposed to a high bacterial load.

It has been recognized that macrophages play an important role in the recovery from infection by Salmonella; this function is enhanced by T-lymphocytes in association with the development of immunity [7].

Moreover, *Salmonella* induces cytokine production including IL-1, IL-6, IL-8, and tumor necrosis factor (TNF). LPS activates macrophages to enhanced phagocytosis and cytotoxicity. Macrophages are stimulated to produce and release lysosomal enzymes, IL-1 and tumor necrosis factor, as well as other cytokines and mediators [13]

Mice treated with OMPs of *S. typhimurium* and infected with *S. typhimurium* shows significant increase in total leukocyte counts, lymphocyte count and in neutrophil. These results come in accordance with [1] who observed that the OMPs are good immunogens in the induction of protective immunity against the *Salmonella* infections. This protective effect could be attributed to OMPs ability to activate dendritic cells (DCs) and enhance Th1 polarization. It is known that DCs can capture degraded bacteria or protein of bacteria and present their antigens on major histocompatibility complex (MHC) class molecules to T cells. As a result, an adaptive immune response that specifically targets bacteria-derived antigens is initiated.

Conclusions and Recommendations

Conclusions

.

- Outer membrane protein with molecular weight 49kDa were extracted and purified .
- Effect of *Salmonella typhimurium* (49kDa) OMP on leucocytes and phagocytosis.

Recommendations:

- Studying the effect of *Salmonella typhimurium* non porin proteins with different molecular weight against salmonellosis.

References

1- Armando isibasi ; vianney ortiz, and jesus kumate . (1988). Protection against Salmonella typhi Infection in Mice after Immunization with Outer Membrane Proteins Isolated from Salmonella typhi 9, 12, d, Vi . Infection and immunity, 56, 11:2953-2959.

2- Cheesbrough, M. (1999) .District laboratory practical manual in tropical countries. J innate immunol. 11: 23-33.

3- Haen, P. J. (1995). Principles of Hematology. Edited by L. H. Young and W.B. Publisher, London. 310 – 325.

4- Hamid, N.;. and Jain, S. (2008). Characterization of an Outer Membrane Protein of *Salmonella enterica Serovar typhimurium* That Confers Protection against Typhoid. clinical and vaccine immunology, 15(9): 1461–1471.

5-Hoffbrand, A.; Mitchell, L. and Edward, G D. (1996) .Postgraduate hematology (4th Ed) Oxford University Press Inc, New York. 219-222.

6- Holt, J.G.; krieg, N.R. and Williams, S.T. (1994). Brgey´s Manual of determinative bacteriology. (9th Ed). Williams and Wilkins.USA.

7-Kita, E.; Emoto, M. and Yasui, K. (1986). Cellular aspects of the longer-lasting immunity against mouse typhoid infection afforded by the live-cell and ribossomal vaccines. Immunology 57:431-435.

8-Kuusi, N.; Nurminen, M. and Sarvas, M. (1981). Immunochemical characterization of major outer membrane components from *Salmonella typhimurium*. Infect. Immun. 33:750-757.

9-Laemmli, M. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680–685.

10-Lowery, C.H.; Rosebrough, N. and Farr, A. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 93: 266-275.

11-MacFarlane, A. (1999). In vitro blockage of nitric oxide with aminoguanidine inhibits immune suppression induced by an attenuated strain of *Salmonella typhimurium*, potentiates *Salmonella* infection, and inhibits macrophage and polymorphonuclear leukocyte influx into the spleen. Infect. Immun. 67:891–898.

12-Maria, I.; Muniz, J. and Aluízio, P. (1997). Factors influencing phagocytosis of Salmonella typhimurium by macrophages in murine schistosomiasis. Revista da Sociedade Brasileira de Medicina Tropical 30(2):101-106, mar-abr, 1997.

13-Mastroeni, P., Harrison, J. A. and Chabalgoity, J. A. (1996). Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. Infect. Immun. 64, 189–196.

14-Metcalf, J. A.; Gallin, J. I. and Nauseef, W. (1986). Laboratory Manual of Neutrophil Function. Raven Press, New York. U.S.A.

15-Nurminen, M. (1976). A mild procedure to isolate the 34K, 35K, and 36K porins of the outer membrane of *Salmonella typhimurium*. Fem. Microbiol. Lett. 3:331-334.

16-Samuelson, p.; Gunneriusson, E and Stahl, S. (2002). Display of proteins on bacteria. j Biotech 96 , 129-154.

17-The center for security and public health. (2006). Salmonellosis. Fact sheet.

18- Udhayakumart, V. and Muthukkaruppan, V. (1987). Protective immunity induced by outer membrane proteins of *Salmonella typhimurium* in mice. Infection and immunity, 55(3):816-821.

19-Whitaker, J. R. and Bernard, R. A. (1972). Experiments for: An Introduction for Enzymology, the Whiber Press.

20 -World Health Organization. (2007). Background Paper on Vaccination against Typhoid Fever using New Generation Vaccines