A COMPARATIVE STUDY ON THE SPECIFIC MUCOSAL AND SYSTEMIC IMMUNE RESPONSES TO TWO SHIGELLA SPECIES IN RABBITS

A thesis

Submitted to the Council of College of Science

University of Babylon

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy of Science in Biology / Microbiology

By

Adnan Kareem Abid Al-Salamy

Shaaban – 1426 *September 2005*

بسم الله الرحمن الرحيم

قالوا سبحانك لا عِلم لنا إلا ما علمتنا إنك أنت

العِليم الحِكيمE

صدق الله العلي العظيم

سورة البقرة

(الآية 32)

Dedication

To my Mother……

To the memory of my Father…..

To my Brothers, my Sister and my Wife

With my endless love

Adnan

AcknowledgementS

I would like greatly to thank Prof.Dr. Ibrahim M.S. Shnawa for his research planning and advising.

Thanks to the University of Babylon, College of Science, Department of Biology and University of Kufa, College of Medicine, Department of Microbiology for providing the necessary facilities during this study.

I would like to extend special thanks to Dr.Ali Muhsin Naahma, Dr. Salman Azeez, Dr.Ahmed Mousa, Dr. Sabah, Dr.Abdullah-Al-Hatamee and Dr. Arkan Kareem-Al-Salamy, for their kind advice and help.

My great thanks go to the postgraduate students (Hind, Gameel, Seham, Ali). for their assistance and encouragement. Thanks also to Miss Suaad for typing this thesis.

Certification

I certify that this thesis was prepared under my supervision at the Department of Biology, College of Science, University of Babylon as a partial requirements for the degree of Doctor Philosophy in Biology (Microbiology) and this work has never been published anywhere.

Signature

Prof. Dr. Ibrahim M.S. Shnawa

College of Science, Dep. Of Biology

Babylon University

Date : / /

In view of the available recommendations, we forward this thesis for debate by the examining committee

Signature

Prof. Ali S.M.Al-araji

Head of Dept. of Biology

College of Science

Babylon University

Date: / /

List of Contents

List of Tables

List of Figures

Summary

The present work was aimed for investigating *Shigella flexneri, Shigella dysenteriae* specific antitoxic immunity at both humoral and cellular immune response as well as mucosal and systemic arms of the lapin immune system (Oral priming) in which exotoxin, endotoxin as well as *Shigella* protein were prepared and assayed. Haemaglutation was used to detect humoral arm while NBT, E-rosette, LIF and skin DTH test were the means to investigate for the cellular arm of the immune response.

The safe immunogenic doses of *Shigella flexneri* LPS, *Shigella dysenteriae* LPS were (1.5,2.5 mg/ Kg) whereas(0.5, 1 mg/Kg) were safe and immunogenic dose for *S. dysenteriae* exotoxin .The immunization was carried out using eighty five mature male rabbits (about 2kg of weight). These rabbits were divided into ten groups, from one to group seven, each group consist of ten replicates from eight to group ten, each groups consist of five replicates.

LPS of *S.flexneri* and *S.dysenteriae* dose(1.5,2.5 mg/ Kg) were able to stimulate humoral and cellular immune response at both systemic and mucosal levels, but the mean values of mucosal system, specially at appendix(mucosal samples included appendix and duodenum samples)were statistically high significant where the mean values of spesfic antibody titers were 48, 48, 41.6, 44.8for *S.flexneri* LPS and *S.dysenteriae* LPS dose (1.5,2.5 mg/ Kg), respectively compared with the mean values of systemic humoral immune response for *S.flexneri* LPS dose (1.5,2.5 mg/ Kg) were 304, 320and 336, 304 for *S.dysenteriae*. Similarly when the rabbits immunized with exotoxin dose (0.5,1mg/Kg) revealed mean values higher at mucosal system, especially at appendix and they was 44.8, 48 but the mean values at systemic level were lower than that of mucosal 256, 288. Nitrobluetetrazolium reduction test for

neutrophil phagocyte shows high mucosal neutrophil phagocytic activity in all endotoxin doses (1.5,2.5mg/Kg) of both *S.flexneri* LPS and *S.dysenteriae* LPS, particularly at appendix and it was noted that the mean values of *S. flexneri* NBT were 45,47.2 for appendix at dose(1.5, 2.5mg /Kg LPS), respectively .For *S.dysenteriae* the mean values of NBT were 46.3, 49 for cells of appendix for the used doses, respectively . The results of NBT at mucosal system of rabbits immunized with both doses (0.5,1mg/Kg exotoxin) were also increased, but lower than endotoxin ,especially at duodenum level and their mean values were 29.4, 31.2 at duodenum for dose (0.5,1mg/Kg), respectively .

Systemic increase in NBT activity was also observed but was lower than mucosal, especially after exotoxin immunization and these were 35, 38.4 for *S.flexneri* LPS, 37.4, 38.7 for *S.dysenteriae* LPS dose (1.5,2.5mg /Kg) and 26.4, 27.5 in *S.dysenteriae* dose (0.5,1mg /Kg exotoxin) for each one of the doses, respectively.

Highly significant results were observed for LIF at mucosal level and the mean values were higher than systemic particularley for both LPS doses of both organisms particularly for those cells of the appendix. Nevertheless, lower findings were noticed in both doses (0.5,1mg/Kg exotoxins) of *S.dysenteriae* .

Systemic LIF was showing lower mean values when exotoxin was used as a sensitizer compared with LPS of *S.flexneri* and *S.dysenteriae* at both doses (0.5, 1mg /Kg) where the mean values was 57,54.8 for each doses, respectively.

The mucosal lymphocytes E-rosette formation was higher as 47, 49, 48, 48.3 at appendix than of systemic which were 37.4, 39.9, 39.4, 40.6 for *S.flexneri* and *S. dysenteriae* dose (1.5,2.5mg /Kg LPS), respectively. Exotoxin dose (0.5,1mg/Kg) was also causing increase in mean values of E-rosette but less than that of mucosal E-rosette when the rabbits were

immunized with LPS with the above two doses. However, their mean values were 40,44.4 at appendix for (0.5,1mg/Kg), respectively .Systemic E-rosette of exotoxin was also less than that of mucosal and the mean values were 30.2, 30.4 at dose (0.5, 1 mg/Kg), respectively.

Skin delayed type hypersensitivity of tuberculin type was noted using *Shigella* exotoxin protein dose (1mg/Kg LPS) of *S.flexneri* dose (2.5mg/Kg) and LPS of *S.dysenteriae* dose (2.5mg/Kg) showed positive reaction within the specified period of time which prove again the involvement of T cell (cell mediated immunity) in the immune response to exotoxin and endotoxin of the organism *Shigella*.

List of Abbreviation

Abbreviation	Meaning
AIDS	Acquired immunodeficiency syndrome
APCs	Antigen presenting cells
BALT	Bronchus- associated lymphoid tissue.
BEM	Basal eagle medium
BHI	Brain heart infusion.
CMI	Cell mediated immunity
CMIS	Common mucosal immune response system.
CMTs	Common mucosal immune system
CPU	Colony forming unite
CTL	Cytotoxic T-lymphocyte
CTL	Cytotoxic T-lymphocytes
DCS	Dendritic cells
D-IgA	Dimeric IgA.
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EAC	Erythrocyte- antibody –complement-rosette
EIEC	Entero invasive EScherishia coli.
ELISA	Enzyme linked immunosorbent assay
E-rosette	Erythrocyte- rosette
FAE	Follice- associated epithelium
GALT	Gut- associated lymphoid tissues.
H_2O_2	Hydrogen peroxide
HIR	Humoral immune response.
HIV	Human immunodeficiency virus
ICDDR,B	International center for Diarrheal Diseases Research ,Bangladesh.

I-EC	Intestinal epithelial cell
IEL	Intra epithelial lymphocytes
IFN	Interferon-gamma
IgA	Class A immunoglobulin
IgM	Class M immunoglobulin
IgS	Immunoglobulins.
IL-1	Interleukin -1 ,4,6,10
IPA	Invasive plasmid antigen.
LIF	Leukocyte migration inhibitory factor
LN	Lymph nodes
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharide
M	Mean
M.W.	Molecular weight
MAbS	Mucosal antibodies
MALT	Mucosa associated lymphoid tissue.
MHC	Major histocompatibility complex.
NADP	Nicotinamide adenine dinucleotide phosphate-oxidase
NBT	Nitroblue tetrazolium reduction test
NK cells	Natural killer cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylenglycol.
PF	Permeability altering factor
PHA	Passivehaemagglutination test
PIgR	Polymeric Ig receptor
PMNs	Polymorph nuclear leukocytes
PP	Peyer's patches

PRR	Pattern recognition receptor
RBCs	Red blood corpuscles
ROI	Reactive oxygen intermediates
SA	Standard deviation
SC	Secretory component.
$SHET_1$	Shigella enterotoxin 1
$SHET_2$	Shigella enterotoxin 2
S-IgA	Secretory immunoglobulin A
S-IgM	Secretory immunoglobulin M
SRBCs	Sheep red blood corpuscles
TCR	T cell receptor
TH_1	T-helper 1
TLRs	Toll-like receptor
TNF	Tumor necrosis factor.
TNF-α	Tumor necrosis factor- alpha
TNF-β	Tumor necrosis factor- beta

Figure (11): Photomicrograph of positive skin test (tuberculin type).

Figure (6): Photomicrograph of exotoxin bioassay (mice leg paralysis) .

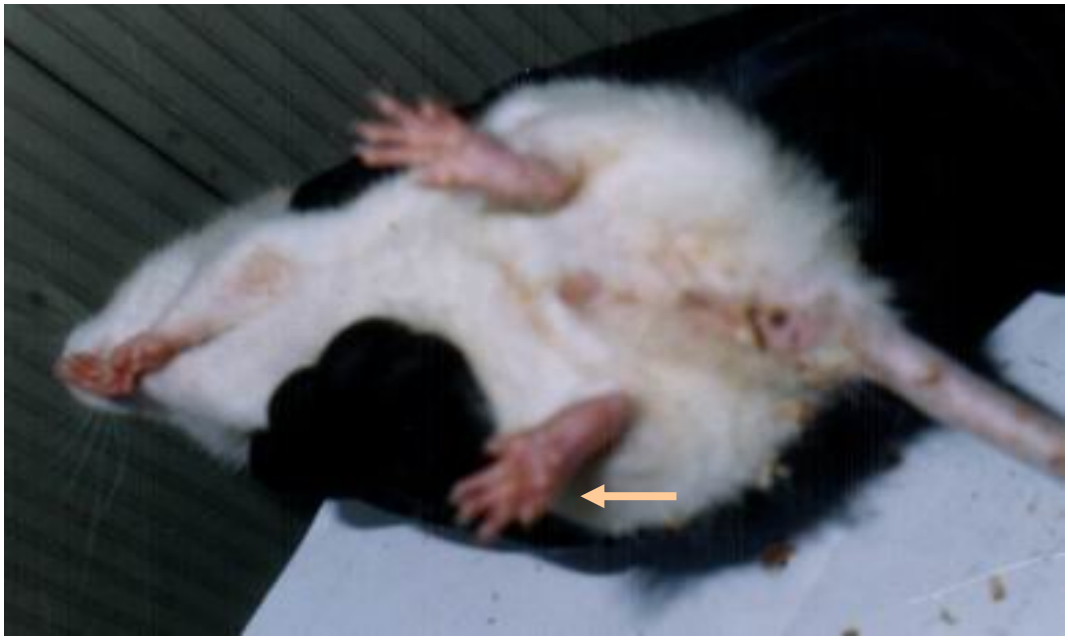

Figure (8): Photomicrograph of endotoxin bioassay (foot pad reaction in rate).

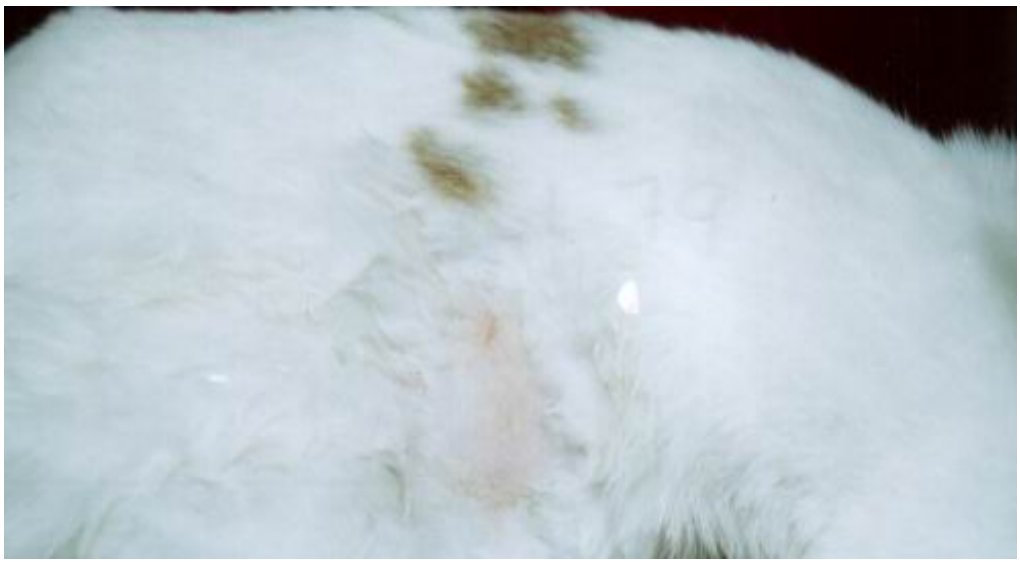

Figure (7): **Photomicrograph of exotoxin bioassay (PF negative**).

Figure (9-A): Photomicrograph of a positive NBT – neutrophil (⟶)(1000X)

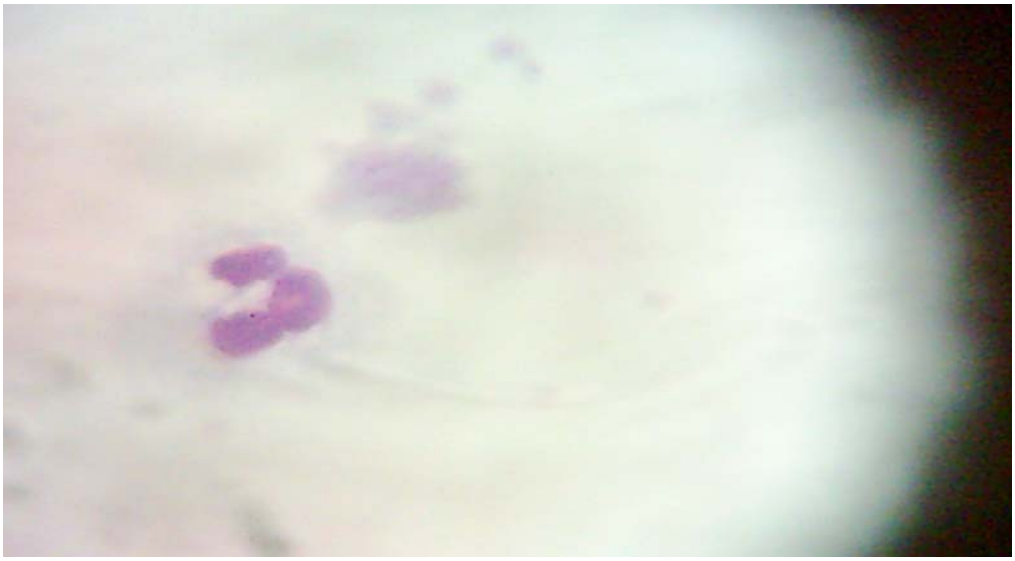

Figure (9-B): Photomicrograph of a negative NBT – neutrophil (⟶)(1000X)

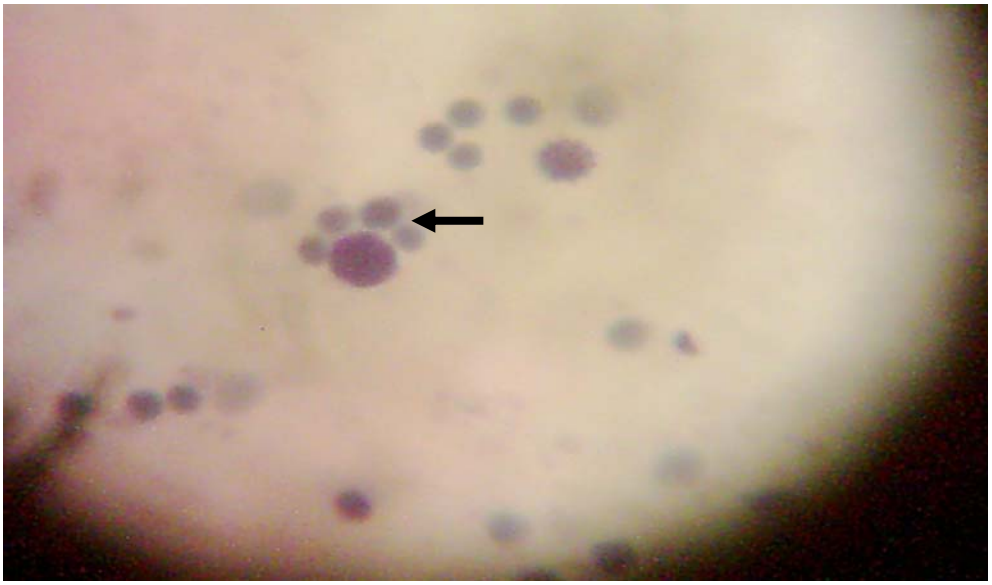

Figure (10-A): **Photomicrograph of an E-rosette positive T-lymphocyte (→) (1000X)**.

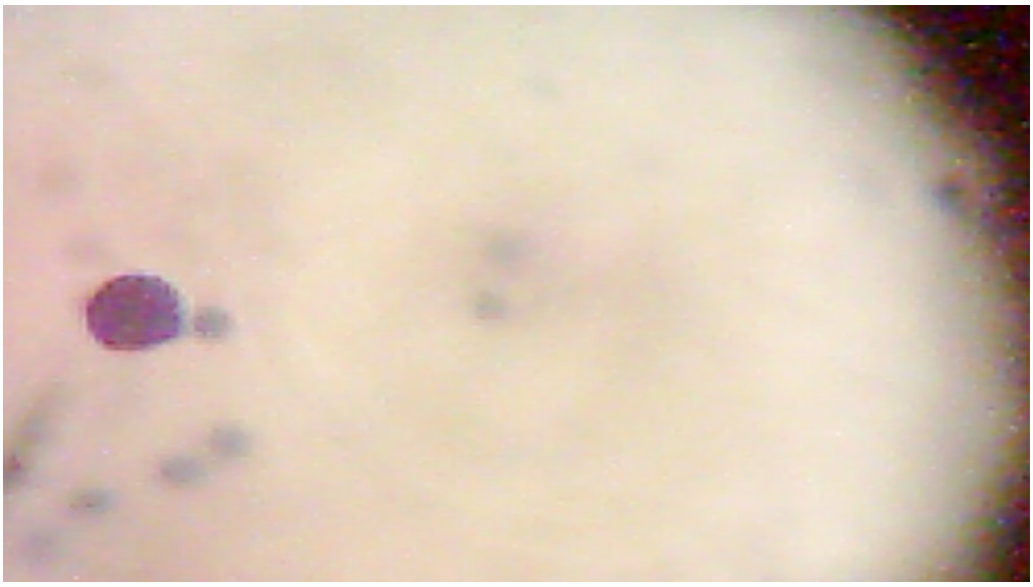

Figure (10-B): **Photomicrograph of an E-rosette an negative T-lymphocyte (→) (1000X)**.

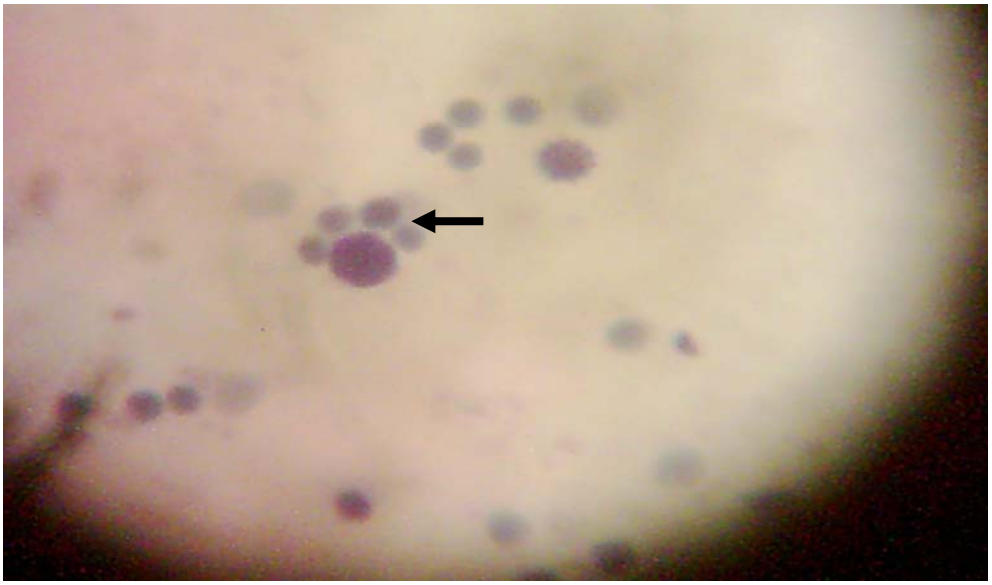

Figure (10-A): **Photomicrograph of an E-rosette positive T-lymphocyte (→) (1000X)**.

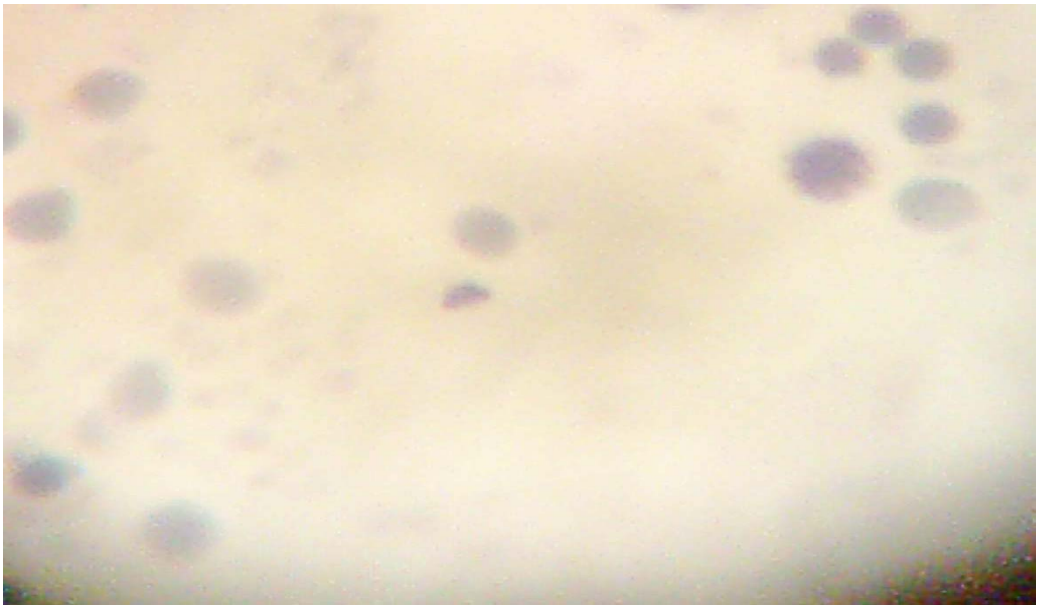

Figure (10-B): **Photomicrograph of an E-rosette an negative T-lymphocyte (→) (1000X)**.

Figure (9-A): Photomicrograph of a positive NBT – neutrophil (⟶)(1000X)

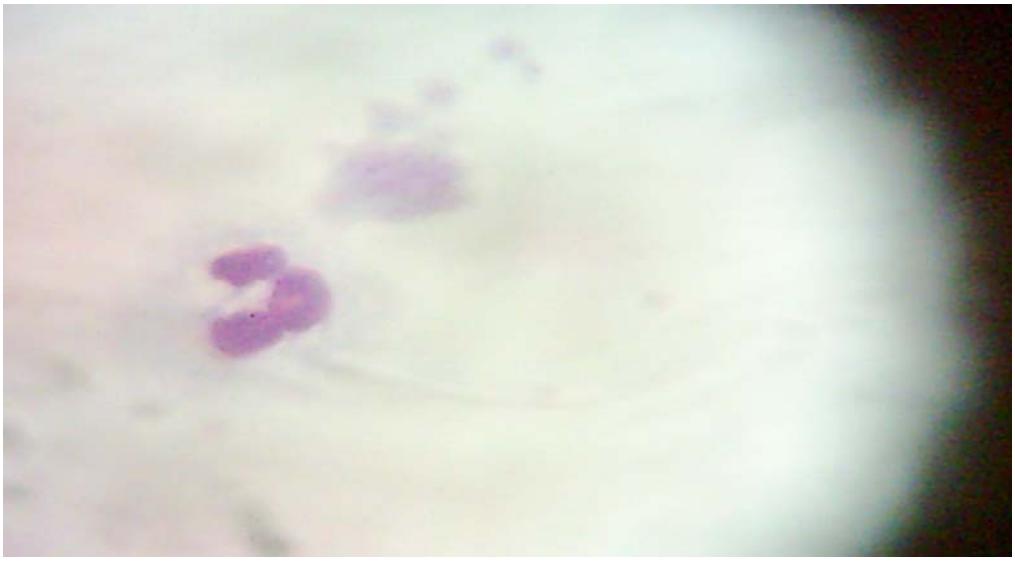

Figure (9-B): Photomicrograph of a negative NBT – neutrophil (⟶)(1000X)

Chapter One
Introduction

1.1 Overview

Shigella are gram-negative,non sporulating , facultative anaerobic rods like ,, non lactose ferment ,non motile ,non H_2S producing ,some of them ferment of mannitol, belong to the Enterobacteriaceae family .They cause Shigellosis, or bacillary dysentery , an invasive infection of the human colon that affects a spectrum of clinical presentation, from shortlasting watery diarrhea to acute inflammatory bowel disease; the classic expression of bacillary dysentery is characterized by the traid of fever, intestinal cramps, and bloody diarrhea with mucopurulent feces (Hale,1998).

The bacterium belongs to the genus *Shigella*, which comprises four different species *S. flexneri* (6 serotypes) and *S.sonnei* (1 serotype) accounting for the endemic disease, the former being prevalent in the developing world ,the latter in the industrialized world .*S.dysenteriae* (16 serotypes) includes serotype 1 ,the "Shiga bacillus" which accounts for deadly epidemics in the poorest countries, largely due to its capacity to produce Shiga toxin ,a potent cytotoxin .*S.boydii* (8 serotypes) remains restricted to the Indian subcontinent .In terms of public health (Kotoloff *et al.,* 1999), Shigellosis shows three major characteristics :1) it is mostly a pediatric disease , > 60% of the cases occurring in children between the age of 1 and 5 years; 2) it is a third world disease, with ~ 150 million cases occurring every year, compared with 1.5 million cases in industrialized countries; and 3)it is also a deadly disease with 1~ 1 million deaths every year, again infecting mostly infants and young children .Lack of hygiene is the major, if not exclusive ,contributing factor, the disease being transmitted by person- to- person contact or contaminated food .

In addition to poverty being the primary factor favouring occurrence of Shigellosis, other disease specific parameters aggravate the public

health burden of Shigellosis. They are essentially four, all of which deserve increased research attention :1) extension of antibiotic multiresistance in both endemic and epidemic areas; 2) very low infectivity ,10-100 microorganisms administered orally being able to cause the disease in adult volunteers;3) severity of acute complication ,particularly in infants and malnourished children, with complex and yet often unexplained pathogeneses, some of them being lethal, as is the case for acute hypoglycemia, seizures, toxic mega colon, pseudoleukemoid reaction and hemolytic–uremic syndrome, intestinal perforations ,peritonitis, and Gram negative septicemia; and 4) the recently recognized importance of delayed complications, characterized by a prolonged state of malnutrition whose pathogenesis is still unclear and indeed poses another challenge (Sansonetti , 2001).

1.2 Historic Perspective

Dysentery is an ancient scourge of humans living under conditions allowing faecal –oral transmission .This infectious inflammatory bowel disease is caused by bacteria that invade the mucosa of large intestine .In the latter portion of the nineteenth century; *Entamoeba histolytica* (a parasitic amoeba) was identified as an aetiological agent of dysentery. By the turn of the century ,however, bacillus dysenteriae was also recognized as a distinct agent of bacterial (bacillary) dysentery .Over the subsequent decades, three additional species of dysentery bacilli were identified by systemic epidemiological ,physiological and serological investigation of outbreaks .As a result, the 1950 congress of the international association of the Microbiologist *Shigella* commission adopted the generic name of *Shigella*, in the honour of Shiga, the Japanese bacteriologist who first described dysentery bacilli in 1898.The commission also designated

species subgroups A (*S.dysenteriae*), B (*S.flexneri*) C(*S. boydii*) and D (*S. sonnei*) (Enterobacteriaceae sub- committee Reportes 1954 ,Bensted 1956). By the mid-1960s the virulence mechanism of *Shigella* species was characterized as "enteroinvasive" .This discovery was predicated on bacterial invasion of cultured mammalian cells, invasion of intestinal epithelium of compromised guinea pigs (LaBrec *et al.,* 1964) ,invasion of rabbit ileal 1oops (Voino- Yasenetsky and Khavkin, 1964) and invasion of the colonic epithelium of Rhesus monkeys (Takeuchi et al.,1968) .In the 1970s it became apparent that some strains which are biochemically and serologically classified as *Escherichia coli* also express the enteroinvasive phenotype (enteroinvasive *Escherichia coli* or EIEC strains) (DuPont *et al.,* 1971).

In the 1980s, *Shigella*- EIEC virulence plasmids were discovered and investigation of the genetic basis of pathogenesis at the molecular level was begun. In the past decade, the interaction between mammalian cells and enteroinvasive pathogens has been explored and the roles of host cell inflammatory elements and cytoskeleton components in the initiation and propagation of *Shigella* infection have been investigated (Hale, 1998).

1.3 Infection sources and interrupted transmission of disease

Infections are transmitted via the faecal –oral route and are usually the result of direct person-to-person contact or the consumption of contaminated water or food (DuPont *et al.,* 1989). Transmission of shigellosis is readily interrupted by public health measures that preclude contact with contaminated faeces. When clean water and adequate sanitary facilities are available, safe disposal of excreta, protection of food from unwashed hands and extermination of flies are obvious prophylactic measures against single source outbreaks. In endemic areas, however, effective prophylaxis requiring combination of infrastructural

and social interventions (Henry, 1991).Unfortunately, national strategies for the improvement of environmental service (e.g .safe water and sewage disposal) in developing countries are frequently inadequate or short- live. On the other hand, educational services encouraging conscious avoidance of faecal contamination, breast feeding and nutritional support for children with dysentery or per health measures reduce the incidence of bacillary dysentery (Rohde, 1984).

1.4 Clinical manifestation of the disease

Dysentery, the definitive clinical manifestation of Shigellosis, is defined as frequent passage of bloody stools with mucus and abdominal pain. Constitutional symptoms such as rectal tenesmus, fever, mild tenderness over the left colon upon palpation and presence of faecal leucocytes are also suggestive of shigellosis (Mathan and Mathan, 1991a).Nevertheless ,the specificity of this combination of symptoms is limited .In a survey of hospital admissions at the international center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), for example, only 10% of the *S. sonnei* patients excreted bloody stools whereas 83% and 55% of patients with *S. dysenteriae* 1 and *S.flexneri,* respectively ,passed gross faecal blood .In contrast, *S. sonnei* infection were associated with watery diarrhea in approximately 75% of patients but *S. flexneri* and *S. dysenteriae* typ 1 were associated with diarrhea in only 33% and 22% of patients, respectively (Stoll *et al.,* 1982) .

Shigellosis is usually a self-limiting disease, but retrospective analysis of ICDDR, B hospital admission records revealed fatal infections in 7% of patients (Bennish *et al.,* 1990). Analysis of these data indicated that age (<1 year), altered consciousness (lethargy), abnormally low serum protein level and thrombocytopenia (<1 X 10^5 platelet mm^{-3}) are lethal risk factors. Severe shigellosis (duration of dysentery > 10 days) is often

associated with stunted growth in children of the developing world (Henry, 1991) .Haemolytic- uraemic syndrome (HUS) can be a sequela of infection with S. dysenteriae serotype 1 that characteristically express the Shiga cytotoxin (Lopez *et al.,* 1989). Reactive arthritis (Reiter syndrome) is a rare sequela of *S.flexneri* infection. The HLA –B27 histo compatibility antigen phenotype is a strong associating and predisposing factor for the latter complication (Bunning *et al*., 1988). *Shigella* bacteremia in HIV- positive patients is being an increasing common complication requiring aggressive antibiotic therapy and prophylaxis (Nelson *et al.,* 1992; Huebner *et al.,* 1993). Shigellaemia occurs in this setting even in the absence of diarrhea or positive stool culture.

1.5 Pathogenesis

In humans, the major pathologic finding is in the colon, where *Shigella* invades the mucosa and results in an inflammatory colitis of varying severity (Islam *et al.,* 1994). Lesions are more common and profound in the distal colon and become progressively less severe in the transverse colon and ascending colon (Speelman *et al.,* 1984).Histologic finding include mucosal edema and haemorrhage, crypt hyperplasia, goblet- cell discharge, a marked inflammatory infiltrate in the lamina propria , epithellial cell damage and death, superficial ulcerations, and an inflammatory exudates in the lumen and the stool.

The invasive process is quite complex, requires the contribution of multiple genetic loci in the organism, both chromosomal and plasmid are essential for virulence (Parsot, 1994). The nature of the initial contact between the non motile organism and the target host cell is not understood; however contact of the pathogen with the surface of the colonic cell triggers ingestion within membrane-bounded vesicles as the organism induces the polymerization of acting fibrils in a process

analogous to that phagocytosis (Goldbery and Sansonetti, 1993 Chen *et el*., 1996);this process is followed by rapid lysis of the phagocytic vacuole with release of the invading organism into the cytoplasm, where it is able to multiply. Actin polymerization then occurs at one pole of the organism, resulting in a propulsive force dubbed the "actin motor", which drives the bacteria to the plasma membrane of the host cell, using energy derived from hydrolysis of adenosine triphosphate resulting from a bacterial adenosine triphosphatase (Zychlinsky *et al.,* 1994). This force is enough to propel the bacterium, still within the host cell and bounded by the host cell membrane, to protrude into adjacent cells (Bernardini *et al.,* 1989); subsequent fusion of the host cell membranes results in transfer of *Shigella* organisms from one cell to the next without ever exiting the intra cellular milieu (Allaoui *et al.,* 1992).In this manner , foci of infection develop, leading to local cell injury , death and sloughing and the production of the characteristic –ulceration of the intestinal mucosa associated with dysentery.

Various data suggest the importance of the induced inflammatory response early in the course of mucosal infection to pathogenesis .In vitro studies in cultured human intestinal cell monolayer , addition of bacteria to the apical surface of the cells and of human neutrophils to the basal surface results in considerably increased transfer of bacteria across the monolayer ,occurring primarily at intracellular tight junctions (Perdomo *et al.,* 1994) .Invasion of *Shigella* in vivo in the ligated rabbit ileal loop model also supports this role of host cell neutophilic response because the number of invading bacteria increase dramatically when neutrophilic infiltration occurs. Introduction of antibody to neutrophil surface antigen, CD18, can reduce the extent of the neutrophilic response (Perdomo *et al.,* 1994). Perfusion of animals with the cytokine antagonist interleukin-1 receptor antagonist before infection of ligated ileal loops with *S.flexneri*

significantly reduces bacterial invasion, the extent of mucosal inflammation and tissue destruction (Sansonetti *et al.,* 1995).

The cellular inflammatory response is accompanied by the production of inflammatory cytokines (such as IL-1), which are detectable in both serum and stool (Raqib *et al.,* 1995).An increase in all cytokine –producing cells in rectal mucosa has been demonstrated in humans with dysentery (Raqib *et al.,* 1995).

In addition to effecting invasion S.dysenteriae 1 produces the cytotoxine protein Shiga toxin (Fontaine *et al.,* 1988). .Shiga toxin binds to host cells expressing the blood group active glycolipid Gb3 (globotriaosylceramide),specially to its terminal galactose–α 1-4 galactose disaccharide (Jacewicz *et al.,* 1986).The effect of Shiga toxin on tissue culture and rabbit intestinal cell that express this determinant because it are able to bind toxin .When toxin is translocated to the cytoplasm by a receptor mediated endocytosis, the RNA glycohydrolase enzymatic activity of the A subunit cleaves a specific residue in the 28s ribosomal RNA of the 60s ribosomal subunit, irreversibly inhabiting protein synthesis and leading to cell death (Acheson and Keusch,1995).Biological effects are determined in large part by selectivity in toxin binding .In the rabbit small bowel, for example, only villous cells express the principal glycolipid toxin receptor (Gb3),bind toxin ,and are inhibited in their protein synthesis (Kandel *et al.,* 1989) .These findings are associated with depressed sodium absorption ,which is a major function of the villus cell.

It is likely that Shiga toxin directly contributes to the pathogenesis of hemolytic-uremic syndrome associated with *S.dysenteriae* type 1 infection, because the same complication occurs after infection with Shiga toxin –producing serotypes of E. coli, such as O157:H7, but not with non-toxin producing serotypes (Hofmann, 1993). It is postulated to

be related to the ability of Shiga toxin to bind to and damage the endothelial cells.

Metabolic response generally associated with inflammatory bowel diseases, such as muscle catabolic and protein –losing enteropathy, may be manifestations of the release of metabolically active cytokines such as in interleukin -6 and tumor necrosis factor or other similar mediator peptides in the inflammation bowel as characteristic of the infection (De Silva *et al.,* 1993).

1.6 Diagnosis of *Shigella*

Patients with diarrhea and fever ,or with dysentery (bloody ,mucoid stools), are suspected of having shigellosis .However , the differential diagnosis should include Entroinvasive *Escherichia coli* (EIEC),*Campylobacter species ,Salmonella enteridis ,Yersinia entcrocolitica and Entamoeba histolytica* .Blood in the stools of patients with bacillary dysentery is bright red whereas that of patients infected with *E.histolytica* is usually dark brown. Microscopic examination of stool smears from patients with amoebiasis reveals erythrophagocytic trophozoites with few PMNs. By contrast, shigellosis is characterized by sheets of PMNs with more than 50 faecal leukocytes per high power microscope field (Speelman *et al.,* 1987). A parameter of 10 leukocytes per field can help to differentiate *Shigella* infection from EIEC, Campylobacter, *Salmonella, Enterotoxigenic E.coli* and rotavirus (Echeverria *et al.,* 1991).

In addition, clinical diagnosis of bacillary dysentery should be confirmed by isolation and identification of *Shigella* from faeces. During the acute phase of the disease, positive culture is readily obtained from blood-tinged plugs of mucus in freshly passed stool specimens or from rectal swabs .*Shigella* can actually be isolated with greater frequency

from rectal swabs than from stool specimens (Adkins and Santingo.1987).Stool specimens should be processed immediately, but swabs should be held overnight at 4C˚if they are suspended in phosphate glycerol saline holding solution (Wells and Morris, 1981). Further, polymerase chain reaction (PCR) using *virf* plasmid gene primers is more sensitive than standard culture techniques for the identification of *Shigella* species if the stools are pre incubated for 4h in brain –heart infusion (BHI) broth. These techniques are rapid (less than 8h) and relatively simple for a well equipped laboratory with experienced technical personnel .PCR is particularly useful for the detection of *Shigella* species in stools of patients after treatment with bactericidal antibiotics such as ampicillin or ciprofloxacin (Sethabutr *et al.*, 1993).

1.7 Aim of the study

Vast published works are available about the serology and bacteriology of *Shigella*. Meanwhile, there is fragmentary published information elsewhere in the world on some aspects of immunology of Shigellosis in man and laboratory animals .Thus, the present work is focused on developing laboratory animals model for the immunology of *Shigella flexneri* and *Shigella dysenteriae* exotoxin and endotoxin specific immune response at both mucosal and systemic levels. To verify this aim the steps mentioned below are followed:

1-Isolatoin of *Shigella flexneri, Shigella dysenteriae* from clinical specimens and confirming diagnosis.

2-Prepare partially purified exotoxin.

3- Prepare, partially purified endotoxin.

4-Bioassay the partially purified exotoxin.

5- Bioassay the partially purified endotoxin .

6-Immunization of rabbits with two concentrations of each of exotoxin and endotoxin separately.

7-Studying mucosal and serum antibody responses, phagocytosis by Nitroblue tetrazolium reduction test(NBT)

8-Studying mucosal and peripheral blood non- specific cellular responses.

9-Studying skin test and Leukocyte migration inhibitory factor(LIF)of rabbits immunized with exotoxin and endotoxin.

10-Studying the T cell count via E-rosette testing.

11- Calculate the ratios of systemic to mucosal immune responses in *Shigella* primed rabbits.

12-Correlate the immune feature for *Shigella flexneri serotype 2, Shigella dysenteriae*s *serotype1* .

Chapter Two
Literature Review

2.1 An Overview to Systemic Immune Response

Blood cells are red corpuscles and white cells. White cells are: neutrophils, basophils, eosinophils, monocyte and lymphocytes. Lymphocytes precursors are originated from fetal yolk sac, and then transfer to fetal liver, then fatal bone marrow. From bone marrow, pro lymphocytes are matured to B lymphocytes. (In birds they develop in the bursa of Fabricius) or migrated to thymus then differentiated through positive and negative selection by the action of thymic hormones and thymic factors to mature T lymphocyte (Roitt *et al.,* 2001).

B cells rearrange their immunoglobulin genes and express a unique receptor for antigen on their cell surface. At this point, they migrate to a secondary lymphoid organ-for example spleen and may be activated by an encounter with antigen to become antibody –secreting plasma cells (Abbas *et al.,* 1997).

T cells are lymphocytes that require maturation in the thymus and form many subclasses with specific functions. They are the source of cell-mediated immunity (Roitt *et al.,* 2001).T cell is of subsets as helper, suppress or, delayed hypersensitivity and killer cells(Parslow *et al.,* 2001).Helper T cell activity ,in addition to stimulating B cells to produce antibodies, promotes the development of delayed hypersensitivity and servers in the defense against intracellular agents, including intracellular bacteria .Cytotoxic T cell activity is aimed mainly at the destruction of cells in tissue grafts ,tumor cells , or cells infected by some viruses .Thus, T cells are mainly utilized to activate B cell response and to cope with intracellular pathogen (Stites *et al.,* 1994).

On the other hand ,the two major categories of cells participating in phagocytosis process are the polymorph nuclear phagocytes (granulocytes) and the mononuclear phagocytes (Monocytes and macrophages), both originating in the bone marrow but belonging to

different cell lineages .These cells act as non specific cells defending against invading microorganism (Van Furth ,1970).

2.2 An Overview to Mucosal Immune System

2.2.1 Basic Structure.

The mucosal immune system can be morphologically and functionally subdivided into two major parts (1) organized lymphoid tissues consisting of the mucosal follicles (also celled gut- associated lymphoid tissues) [GALT] and bronchus- associated lymphoid tissues [BALT] and (2) a diffuse lymphoid tissue consisting of the widely distributed cells located in the mucosal lamina propria. The former (or organized) tissues are "afferent" lymphoid area, where antigens enter the system and induce immune responses ,and the latter or diffuse tissues are " efferent" lymphoid areas, where antigens interact with differentiated cells and brings the secretion of antibodies by B cells or induce cytotoxic reactions by T cells (Stitis *et al.*, 1994) .

GALT and other mucosa- associated lymphoid tissue (MALT) structures are covered by a characteristic follicle –associated epithelium (FAE) ,which contain membranous (M) cells ,these cells specialized thin epithelial cells are particularly effective in uptake of live and dead antigens from the gut lumen, especially when particulate in nature, many enteropathogenic infectious bacterial and viral use M cells as portals of entry (Hathaway and Kraehenbuhl,2000).GALT structures resemble lymph nodes with B-cells follicles, intervening T-cell areas, and a variety of antigen-presenting cell (APC) subset ,but there are different lymphatic supplying antigen for immunological stimulation. Therefore, the exogenous stimuli must come directly from the gut lumen, probably in the main via the M cells (Brandtzaeg *et al.,* 1999).

Mucosal surface contain also numerous lymphoid cells, as well as cells of non- specific immune response (granulocytes, macrophages) disseminated along the gut. Some lymphocytes are inter spread among epithelial cells and are called intraepithelial lymphocytes (IEL) .The majority of these cells are T- lymphocytes (80- 90%).Others are located in the connective tissue of the lamina propria, and called lamina propria lymphocytes (LPL) .In contrast to the epithelial compartment, lamina propria lymphocytes include 40 to 90% of T. cells and thus also substantial population of B cells. These cells (LPL and IEL) are repress and the diffused lymphoid tissues of the mucosal immune system. (Stitis *et al.,* 1994; Abreu-Martin and Targan ,1996).

2.2.2 Inductive and Effector Sites of the Mucosal Immune System

The mucosal immune system consists of two functionally distinct types of tissue: 1) inductive sites, where naïve B and T cells are clonally selected and expanded upon Ag contact; and 2) effector sites, where activated B and T cells were migrated and homed (relocated) after Ag-priming in inductive sites to express their effectors functions. This concept is best established for the components of the intestinal mucosal immune system. They are organized in the so-called gut –associated lymphoid tissue (GALT) ,and include peyer's patches (PP), mesenteric lymph nodes (LN) , Appendix, and dispersed lymphoid cells in the epithelial layer and in the gut lamina propria (Zuercher *et al.,* 2002) .PP are inductive sites and have been described as the major location for Ag-specific B cell activation and isotype switching to IgA and generation of IgA^+ memory B cells as well as for the induction of Ag-specific CTL (Brantdzaeg *et al.,* 1999) .The lamina propria and epithelial compartment constitute effector sites ,where antigen interact with differentiated cells (Brantdzaege *et al.,* 1996).

2.2.3 Mucosal Humoral Antibody

Mucosal surfaces are protected specifically by secretory immunoglobululin A (SIgA) and SIgM generated through external translocation of locally produced dimeric IgA and pentameric IgM. Their active transport is mediated by the epithelial polymeric Ig receptor (pIgR), also called the Transmembrane secretory component (Johansen *et al.,* 1999). It is believed that secretory (SIgA) and to lesser extent, SIgM antibodies enhance the epithelial barrier function by a mechanism termed immune exclusion (Stokes *et al.,* 1975). These antibodies ,which may act both within secretory epithelia and at mucosal surface (Mazanec *et al.,* 1992; Mazanec *et al.,* 1993 ; Brandtzaeg *et al.,* 1999), are generated by a unique cooperation between two distinct cell types (Brandtzaeg and Prgdz, 1984 ;Brandtzaeg ,1985) (1) J chain- expressing plasma cells that produce polymeric (P) IgA (mainly dimmers as a result from combination two monomeric IgA via J chain) or pentameric IgM .

The J chain is a short 1500 Dalton chain which links the two monomers via disulphide bonds at the foot of two Y- shaped molecules like IgA; the J-chain is a plasma cell product, and J-chain synthesis is largely distributed in the sites from which d. IgA is derived , adjacent to mucosa (French, 1988) (2) secretory epithelial cells that express the PIgR, also known as the Tran membrane secretory component (SC) .This receptor mediates active transport of PIgA and pentameric IgM to exocrine secretions (Brandtzaeg *et al.,* 1998; Mostov ,1994). Cleavage of unoccupied and ligand –complexed PIgR ,respectively releases free SC and SIgA or SIgM into the lumen .Secretory IgA (S-IgA) is a combination of dimeric-IgA (d-IgA) covalently linked to a secretory component (SC) ,the total molecular weight being approximately 395000 Daltons. Sc is notably the product of plasma cells but of epithelial cells,

SC is intimately linked with transport of d-IgA through the epithelial cells to reach the mucosal surface (Lindh ,1975).

Secretory IgA produced by lamina propria plasma cells are transported by enterocytes into the bowel lumen .Unlike other immunoglobulin classes, they do not activate complement or inflammatory responses, which makes them ideal for protecting mucosal surface .Their major function seems to protect digestive epithelium by cross-linking microorganisms or macromolecules, thus facilitating their elimination by peristalitism or mucociliari movements ,and preventing their contact with the surface of epithelial cells (Corthesy and Kraehenbuhl ,1999).

SIgA prevents the attachment of microorganism to the epithelium, indirectly by limiting their diffusion across the mucus layer when coated with IgAs, or directly blocking the microbial sites that mediate attachments. IgA can also neutralize microorganisms that are internalized by epithelial cells by mucosal tissues (Gebbers and Laissue, 1989; Corthesy and Kraehenbuhl, 1999). Secretory IgA may adhere selectively to M cells in the lumen of the gut and present them to underlying lymphoid follicles (Neutra, 1999);they represent a key effectors of correctly regulated intestinal immune response.

Elaboration of IgA is strongly T cell dependent, as is shown not only by in vitro analysis of lymphocytes behavior, but by the IgA production deficiency in some clinical T-cell deficiencies in man. It is now widely accepted that the generation of antibody is closely modulatal by T cell, both helper and suppressor T cell being involved (Elson *et al.,* 1979; Kudsk, 2002).

2.2.4 Mucosal Cellular Immunity

Beside the humoral immune mechanisms, mucosal immune system contains also cellular immune mechanisms such as $CD8^+$ T cell mediated

immunity and $CD4^+$ cytokine production ,and forms the early line of defense. Besides supporting humoral immunity, $CD4^+$ T- helper cells function in CMI as producer of cytokines, which mediate delayed type hypersensitivity and support cytotoxinc T- lymphocyte (CTLS) which as such are critical components of the CMI response to intracellular pathogens. Both antigen specific and non- specific (Natural Killer cells, antibody dependent cytotoxicity) mucosal cytotoxic cell types can control growth of intracellular pathogens by two distinct mechanisms; first they can respond to the infection by secreting a number of cytokines such as macrophage inflammatory protein-1 a 8 (Yang *et al.,* 1997; Fehniger *et al.,* 1998). These soluble factors inhibit growth of intracellular pathogens such as virus without destroying the host cell. Second, cytotoxic cells can effectively and efficiently recognize and lyses infected cells and prevent multiplication of virus.

Cytotoxic T- lymphocytes play an important role in the elimination of cells infected with various intracellular pathogens by recognizing specific antigen / MHC complex pathogens and help to terminal infections compartmentalization of pathogen specific CTL responses has been reported and located at the site of initial infection ,for example, CTL preferentially compartmentalization in mucosa associated lymphoreticular tissue (Frederik *et al.,* 2000).

On the other hand, the ratio between $CD4^+$ and $CD8^+$ cells in mucosal system is similar to that other peripheral T cell population .In addition,B cell aggregate together with T cells in the M cell pockets ,which thus represent the firs contact site between immune cells and luminal antigens (Yamanaka *et al.,* 2001; Brandtzaeg,2001).

The B cells may perform important antigen-presenting functions, perhaps promoting antibody diversification and immunological memory or contributing to tolerance .Other types of professional APCS,

macrophages and dendritic cells (DCS), are located below FAE and between the follicles (Brandtzaeg *et al.,* 1999).

2.3 An Overview of Immunity to *Shigella*

Natural *Shigella* infection induces a mucosal and systemic immune response to *Shigella* antigens **.**Passive hemoagglutination and enzyme-linked immunosorbent assay (ELISA) has been employed for detection of serum antibodies of various immunoglobulin classes developed against *Shigella* serogroup specific lipopolysaccharide (LPS) (Cohen *et al.,* 1988 ;Cohen *et al.,* 1989).It has been reported that the rise in serum immunoglobulin A (IgA) to *Shigella* LPS indicates recent infection with the homologue organism while IgG specific antibodies are markers of more distant exposure to *Shigella* strains (Cohen *et al.,* 1989) .A strong correlation between preexisting IgG anti- LPS serum antibodies and acquired natural immunity against Shigellosis was demonstrated previously (Cohen *et al.,* 1990).In the immunogenicity studies that have been performed on candidate *Shigella* vaccines ,a significant rise in serum antibodies to homologous LPS was used as evidence for stimulation of immunocompetent cells (Taylor *et al.,* 1993;Cohen *et al.,* 1994)

A few studies have shown that antibodies to various bacterial antigens can be detected after natural infection of the urinary tract or other mucosal sites (Alemohammad *et al.,* 1993; Fliedner *et al.,* 1986; Shotrliffe *et al.,* 1989), and that at least part of these antibodies is of the mucosal origin (Kruze *et al.,* 1989; Prentice *et al.,* 1991).

Disease due to *Shigella* involves bacterial invasion of clonic enterocytes and uptake by M cells (Mathan and Mathan 1991, a) with induction of an intense acute inflammatory response (Raqib *et al.,* 1995). Within minutes of uptake into mammalian cells,*Shigella* lyse the

phagocytic vacuoles and are thereby release into the cell cytoplasm, where they divide and use actin-based motility to move and spread directly into adjacent cells (Bernardini *et al.,* 1989 ; Prevost *et al.,* 1992).In this way ,once within an intestinal epithelial cell ,*Shigella* is able to spread from one infected cell into adjacent cells without reentering the extra cellular environment. *Shigella* is also taken up by phagocytic cells, including macrophages and polymorphonuclear leukocytes (Zychinsky *et al.,* 1996).

As described above, *Shigella* resides within the cytoplasm of host cells and is thereby protected from opsonizing antibodies and immune cell surface Igs but is susceptible to cytotoxic T-cell activity and professional phagocytic cell-mediated killing. This intracellular localization and the mechanism of *Shigella* cell-to cell spread are strikingly similar to those of the Gram-positive pathogen *Listeria monocytogenes*. In this model, CD^{+8} T. Lymphocytes are the predominant protective cell type (Mielke *et al.,* 1989;Harty and Bevan 1995), although CD^{+4} T- lympocytes also provide protective effects (Bishop and Hinrcihs- 1987; Kaufmann *et al.,* 1987; Magee and Wing 1988).

2.4 Characteristics of *Shigella* Antigens and Immunogens

2.4.1 Exotoxin (Shiga toxin)

S.dysenteriae type 1 was first shown in 1903 to produce a lethal toxin that became known as Shiga neurotoxin because it resulted first in limb paralysis in animals .Since then ,this toxin has been found to cause fluid accumulation in rabbit gut and to be cytotoxic for cells in culture (Vicari *et al.,* 1960 ;Keusch *et al.,* 1985). Shiga toxin is the prototype of a family of enzymatically and structurally similar to Shiga-like toxins produced by other members of this genus and by certain *E.coli* serotype (O'Brien *et al.,* 1984,a).On the basis of these common features, the toxin

nomenclature has changed (Stx ,Stx-1,Stx-2) .Although the *E.coli* produced Shiga toxins, Stx-1, and Stx-2 ,are encoded by genes present in transforming phage, the Shiga toxin (Stx) gene is uniquely present on the chromosome of *S.dysenteriae* type 1 ,near the *pyrf* locus (Strockbine *et al.,* 1988).

The toxin is composed of two different peptide subunits ,an enzymatically active 32-kDa subunit A and a complex of five identical 7.8KDa subunits B responsible for binding to the host cell receptor (Donohue-Rolf *et al.,* 1989), a glycolipid containing a terminal disaccharide composed of galactose- linked α1⟶4 to galactose (Jacewicz *et al*.,1986).The subunit A is an N-glycosidase identical in action to the plant toxin ricin ,which cleaves a specific adenine base in the 28s ribosomal RNA of the 60s ribosomal subunit and permanently inactivates the ribosome in protein synthesis (Endo *et al.,* 1988).The active site of the A subunit has not been fully characterized; however, glutamic acid 167 appears to be a critical residue ,and even a conservative change to aspartic acid reduces the A subunit enzymic activity by more than 1000 fold .(Hovde *et al.,* 1988).The X-ray crystallographic structure of the B subunit of *E.coli* stx-1 ,which is identical to Shiga toxin itself, has been solved (Stein *et al.,* 1992).The three-dimensional view suggests that a conserved carbohydrate binding site is formed by β-sheet interactive between adjacent B monomers ,which are arranged in a pentameric structure, producing five potential binding sites per holo-B subunit. Site directed mutagenesis of a separate residues at positions 16 and 17, which are within the potential binding cleft noted in the crystal structure (Stein *et al.,* 1992), alters the toxin's binding capability (Jackson *et al.,* 1990).

The structural genes for the A and B subunits formed an operon that is organized in tandem translated in-frame ,with the operon reading frame for Stx AS to StxB and separated from one another by 12 nucleotides

(Strockbine *et al.,* 1988), Shiga toxin production is regulated by iron concentration through the *fur* locus (Calder wood *et al.,* 1987), an iron-dependent negative regulator of a number of iron regulated genes , including those controlling the synthesis of iron-binding siderophores and inner and outer membrane proteins involved in the uptake of the iron-siderophore complex. The *fur* gene also appears to control the *E.coli* Stx-1 genes but not the Stx-2 gene (Weinstein *et al.,* 1988).A binding site for the *fur* gene product has been found 5 to StxA in *S. dysenteriae* type 1 and in a region of dyad symmetry within the Stx promoter (Delorenzo *et al.,* 1987). When iron is present in sufficient quantities ,it complexes with the *fur* protein ,enabling *fur* to bind to sites near or within the promoters of iron-regulated genes, which serve to down-regulate these genes at the transcription level.

Two new *Shigella* toxins have been reported ,called ShET1 and ShET2 (for *Shigella* enterotoxin)because they increase net electrolyte transport by rabbit small bowel tissue in vitro and cause net fluid secretion in vivo in ligated rabbit ileal loops (Nataro *et al.,* 1993; Fasano *et al.,* 1995) .These toxins are much less active on a weight basis then Shiga toxin, which causes the same physiological response by intestinal tissue .Humans infected with *Shigella* develop neutralizing antibody to ShET1 and ShET2, indicating that they are produced in vivo. The former is encoded by a chromosomal gene, whereas the latter is controlled by an iron-regulated plasmid gene and is homologous with a previously described enteroinvasive *E.coli* enterotoxin (Fasono *et al.,* 1990) Although the role of the ShET toxins in disease pathogenesis remains uncertain, the potential importance of ShET1 is limited by the observation that the gene is found predominantly in *S.flexneri* type 2 and not other *S. flexneri* serotypes or *Shigella* species (Noriega *et al.,* 1995).

Shiga toxin B subunit induce peptide-into the MHC class 1 pathway and induced peptide-specific CTL in mice (Lee *et al.,* 1998; Haicheur *et al.,* 2000).

2.4.2 Lipopolysaccharide (LPS)

Bacterial endotoxins were first described at the end of the 19th century after the recognition that, in addition to then known secreted toxin (exotoxins), certain types of bacteria also produce biologically active, heat-stable molecules associated with the bacterial cell wall itself (Rietschel *et al.,* 1996).Endotoxins are now known to be a component of the outer membrane of all Gram-negative bacteria (Glauser *et al.,* 1991; Remick. 1995; Rietschel *et al.,* 1996).

Endotoxin is a complex glycolipid composed of a biologically active lipid (lipid A) linked to polysaccharide region (Glauser *et al.,* 1991; Rietschel *et al.,* 1996; Ulevitch and Tobioas, 1995). The basic endotoxin molecular structure consists of two distinct regions: a hydrophilic carbohydrate (polysaccharide) portion which includes an O-specific side chain and an inner and outer core region, and the hydrophobic toxic lipid A component (Glauser *et al.,* 1991; Rietschel *et al.,* 1996).Although the general structure is highly conserved among Gram negative bacteria, there is considerable structural variability at the O- specific chain between species.

Since the O-specific chain is enzymatically constructed by the sequential addition of oligosaccharides, the endotoxin of a given bacterium at a given point in time is a heterogeneous mixture of molecules with short, intermediate, and long O-specific chains.

The lipid A component of endotoxin is highly conserved from one Gram-negative bacterial family to another and gives the endotoxin molecule its toxicity (Rietschel *et al.,* 1996), whereas a component of a

viable microorganism or when shed from the cell wall. The most powerful evidence implicating lipid A as the biologically -active portion of endotoxin involves studies using synthetic molecules. These show that lipid A, independent from all carbohydrate constituents, is as toxic as its naturally occurring endotoxin counterpart (Ulevitch and Tobias, 1995). The actual endotoxic activity of LPS is believed to be dependent upon the specific conformation of the lipid A portion of the molecule. At high concentration this conformation appears to be a three- dimensional non lamellar structure. It is believed that this conformation enables endotoxin to maximally interact with specific humoral and cellular factors, triggering the inflammatory cascade (Ulevitch and Tobias, 1995).

The O- specific polysaccharide (O-SP) domain of lipopolysaccharide is both an essential virulence factor and protective antigen of *Shigella* (Robbins and Schneerson, 1992). Convalescence from shigellosis confesr LPS specific immunity ,although incomplete and of limited duration (Bing-nan et al.,2000;Herrington *et al.,* 1990 ;Kotloff *et al.,* 1999;Robbins *et al.,* 1992). There is correlation between the level of IgG LPS antibodies and resistance to shigellosis (Cohen *et al.,* 1988 ;Cohen *et al.,* 1991).

O-antigens of *Shigella* serologic cross-reactivity with certain *E.coli* , for example *S.dysenteriae* type 1 has a unique repeating unit (3α-D-GlcNACl- 3α-L-R hal 3- α-L Rha 1 2- α-D –Gal 1), which is shared only with *E.coli* O1 and O120 and not with other species of *Shigella*. The somatic antigens of two other group A serotypes, *S.dysenteriae* types 2 and 3 are also known to be identical to the O antigen of *E.coli* O112 and O124, respectively ,but distinct from that of *S.dysenteriae* type 1 (Carlin *et al.,* 1984).

All species *S.flexneri* serotypes except *S. flexneri* type 6 have a common repeating unit of four sugar (2 α –L- Rha 1 2- α- L Rha 1 3 α-

L Rha 1 3-B-D-GlCNaC),which constitute serological specificity,the determinant group,the immunodominant epitopes (Carlin *et al.*, 1984).The more frequently found 1a-5b seroreactivities of *S.flexneri* result from the attachment of α- D- glucopyronosyl and O-acetyl group to various sites on the basic repeat unit. These sorts of antigen are relatively common among enterobacteriaceae; as a result the are many cross- reaction between *S. flexneri* and *E.coli*.

The rest of the LPS molecules is similar among all gram negative organism (Anderson *et al.,* 1989).The 2-Keto -3 deoxyoctonate inner core region of the *Shigella* LPS resembles that of Salmonella or *E.coli*, whereas the short outer core region is markedly similar in all *Shigella* species .There is no evidence of significant variation in lipid A structure or biologic activity from *Shigella* to *E.coli* (Carlin *et al.,* 1984).

Lipopolysaccharide exerts its highly complex array of pathophysiologic effects by interacting in the host with a panoply of naturally- occurring cellular and humoral elements (Glauser *et al.,* 1991; Bone *et al.,* 1992; Remick ,1995 ;Rietschel *et al.,* 1996).

Endotoxin interacts with virtually all components of the cellular immune system .It is taken by neutrophils ,leading to cell activation and the subsequent enhancement of the phagocytic ability of these cells. Further it may be activate neutrophils to express cell adhesion molecules with mediate sorts of bindings like neutrophil- to –neutrophil ,neutrophil-to vascular endothelial cell and neutrophil-to-tissue binding , causing local inflammation and vascular leakage (Glauser *et al.,* 1991; Rietschel *et al.,* 1996).

LPS also appear to affect various populations of lymphocytes, stimulating B-cell proliferation and antibody production ,activating T-cell to secrete cytokines and down –regulating T-suppressor cells (Rietschel *et al.,* 1996).The most widely studied and probably the most significant

cellular effects of endotoxin involve the interaction with cells of the monocyte /macrophage lineage (Glauser *et al.*, 1991 ; Rietschel *et al.*, 1996; Remick, 1995) ,which express a membrane receptor known as CD14 (Remick ,1995). Circulating LPS is bound by a glycoprotein serum factor, LBP, which facilitates binding of LPS to its principal cellular receptor, the CD14 molecule (Ulevitch and Tobias, 1995). The importance of this interaction is demonstriated by experiments in which preventing LPS-LBP binding to monocytes blocks the activity of endotoxin (Remick *et al.,* 1995).Binding of LPS –LBP to CD14 induces monocytes to produce and secrete a myriod of pro and antinflammatory cytokines ,including interleukins (IL-1 IL-6 ,IL-8, IL-10),macrophage migration inhibitor factor and tumor necrosis factor (TNF)(Rietschel *et al.,* 1996; Remick *et al.,* 1995; Ulevitch and Tobias ,1995).

LPS can stimulate B-cell proliferation to production antibodies (Rietschel *et al.,* 1996), and IgM antibody is the early immunoglobulin produced in response to LPS special to lipid A (Caceres and Mata,1974); meantime LPS can activate the complement system through two separated pathways bacteria and bacteria cell wall components complex with antibodies activating the classical (antibody dependent) complement pathway intended to kill the bacteria ,while the bacteria and endotoxin directly activate the alternative (non-antibody)pathway (Glauser *et al.,* 1991).The resulting complement cascade induced by LPS produces, among other mediators, the anaphylotoxin C3a and C5a ,which contribute to vasodilatation, increased vascular permeability ,and circulatory collapse. In addition, complement components induce adhesion and activation platelets and neutrophils, stimulating the secondary events of platelet aggregation, releasc of lysozymal enzymes and arachidonic acid metabolites (Rietschel *et al.,* 1996).

LPS induced the activation of susceptible cell such as neutrophils, leading to the release of prostaglandins, leukotrienes, and other agents with vasoactive and pro inflammatory effects.(Colmam,1989).

One of the factors that can effect chemical characters and biologic activities of LPS is the method of extraction in addition to the type of strain and their growth (Leive and Morris, 1972), and LPS characters are different in accordance with the nature of the extraction method (Morrison and Leive ,1975).

Many methods have been developed for the extraction of endotoxic lipopolysaccharides from Gram-negative bacteria (Luderitz *et al.,* 1966) such a procedure includes extraction with trichloro acetic acid at 4C° (Boivin and Mesrobeanu, 1933), but LPS extracted by this procedure forms complex with other bacterial components such as proteins and phospholipids, whereas these materials are removed by phenol extraction, extraction with aqueous ether at 6-12C° (Ribir *et al.,* 1961), extraction with water at 80C° (Roberts ,1966), and extraction with aqueous phenol (Westphal *et al.,* 1952; Westphal and Jan ,1965).Of these methods, extraction with phenol –water is widely used because it is feasible applicable to many different groups of bacteria and because it is one of the few methods by which also lipopolysaccharides from rough mutant bacteria can be extracted .In this method ,dried bacteria are treated in mixture of phenol and water separated into a phenol phase containing mainly proteins, and a water phase containing lipopolysaccharide .

2.4.3 Invasion Plasmid Antigens (IPA)

The invasion processes of host cells by the member species of *Shigella* are rather complex and multifactorial events in which many different bacterial proteins are involved .Many of the key virulence proteins genes

of *Shigella* are located on a 140 MDa plasmid and are conserved in all *Shigella spp*. These antigens (IpaA 70KDa), IpaB (62KDa), IpaC (43KDa) ,and IpaD(38KDa)), are essential virulence factors (Buysse *et al.,* 1987; Menard *et al*.,1996).Previously it has been shown that IpaB ,IpaC and IpaD ,along with LPS are major antigens recognized by *Shigella* infected individuals (Oaks *et al.,* 1986 ;Li *et al.,* 1993; VanNhieu *et al.,* 1999; Samandari *et al.,* 2000).

2.5 The Immune Status of Shigellosis

Shigellosis is an important etiologic agent of diarrheal disease and dysentery all over the world, causing an estimated annual death of 1.3 million, particularly among infants and young children (Kotloff *et al.,* 1999).

Information on the human immune response to *Shigella* infection is limited primarily to humoral immunity, and attempts to correlate specific humoral immunity and protection have been inconsistent. In humans, systemic and mucosal antibodies are directed primarily to *Shigella* LPS, with a lesser response against the invasion plasmid antigens (Ipa) A, B, C, and D (Black *et al.,* 1987; Li *et al.,* 1994).

Epidemiological studies have found that disease due to endemic species of *Shigella* occurs primarily in children while disease due to epidemic species is equally prevalent in all age groups, which suggests that the protection following natural infection can occur (Taylor *et al.,* 1989).

Partial *Shigclla* species- specific and serotype- specific protection have been described following natural acquired *Shigella* infection in humans (Formal *et al.,* 1991).

The peripheral immune response of patients infected with *Shigella dysenteriae* 1, and *Shigella flexneri* 2 was studied against various

lipopolysaccharide and invasion plasmid-coded antigen (Ipa-S). The patient reacts with significant titer increase in the immunoglobulin A and G classes against the lipopolyasccharide and invasive plasmid antigen (Cam *et al.,*1993).Furthermore, infection with *Shigella* causes an increase in secretory IgA in intestinal secretion and an increase in the number of IgA antibody –producing cells in intestinal mucosa (Orr *et at*., 1992).

Anti *Shigella* antibodies are bactericidal in complement fixation or opzonization assay in vitro (Reed and Albright.,1974; Reed.,1975) .Antibody response to *Shigella* is an important component of adaptive immunity and antibody can protect against disease .Furthermore, since serotype specificity of *Shigella* is defined by the composition of the oligosaccharide repeating unit of the 0-antigen of the bacterial lipopolysaccharide (Lindberg *et al.,* 1991), the serotype specificity of the response in certain studies indicates than an antibody response to bacterial LPS is an important component of protection (Way *et al.,* 1999) .The significance of antibody response to antigens other than LPS (e.g Ipa BCD, ICsA or ShETs) is not known (Nataro *et al.,* 1995).

Knowledge of mucosal immune response especially that in the human gut is limited due to obvious limitations in investigation. By humanitarian legalization acts, the priming of local antibody responses following mucosal infection or oral vaccination have been studied by enumeration of specific circulating antibody –secreting cells(ASC) using an enzyme –linked immunospot assay (Sedgwick and Hoit., 1983). The *Shigella* specific circulating ASC have essentially been evaluated after oral immunization with live attenuated vaccines (Li *et al.,* 1994; Karnell and Li., 1995) to assess their ability to induce mucosal immune response. Only two reports have studied , by measurement of circulating ASC directed against LPS homologous to that of the infective strain ,the mucosal response induced by natural infection in the area of endemicity

(Orr *et al.,*1992; Raqib *et al.*,1993) .IgA response is mainly directed against serotype specific detriment (Rasolofo- Razanaparany *et al.*,2001). Human infected with *Shigella* produces antibody predominantly to IpaB and IpaC and at lower frequency to IpaA, Ipa D and VirG protein (Oaks *et al.,* 1986).

Since *Shigella* is an intracellular pathogen it has been hypothesized that cell-mediated immunity (CMI) may be essential for the immune defense against shigellosis. Evidences are accumulating to support this theory. In humam- derived peripheral blood NK cells were shown to kill *Shigella* Hela cells(Klimpel *et al.,* 1986) .In another study, Bangladeshi patients with acute Shigellosis ,elevated levels of IFN - γ were detected in rectal biopsies and serum as well as stool, which contained particularly high levels (Raqib *et al.,* 1995; Raqib *et al.,* 1995). Subsequently, the same investigators found an up-regulation of IFN- γ production and expression of the IFN- γ receptor in the epithelial lining of rectal biopsies from patients convalescing from *S.dyenteriae* 1 infection (Raqib *et al.,* 1996). *Shigella* infection has also been found to elicit the appearance of activated T cells in circulation during the course of human disease (Hartman *et al.,* 1991) .Despite these observations suggesting an important role for CMI , prospective data are not yet available in humans to correlate the generation of CMI responses with protection against *Shigella* (Samanderi *et al.,* 2000).

An essential requisite for an induction of a potent immune response by an immunogen, the antigen presenting cells (APC)). These cells (APC) constitute major targets for intracellular bacteria such as *Shigella*(Germain, 1995). Moreover, infection of APC with most intra cellular bacteria results in the activation of these cells and subsequent induction of a robust immune response (Kaufmann *et al.,* 1999).

Consequently , in the case of many intracellular bacteria, ,infection induces an immune response , without the need for addional adjuvant .However, the simple induction of an immune response is not the end of the game; the quality of the immune response is equally decisive .To fight against infections with intracellular bacteria must induce a T-helper 1 (Th1) immune response (Kaufmann *et al.,* 1999) .The Th1 response is characterized by IFN-γ secreting CD^{+4} cells and the generation of cytotoxic T .lymphocytes (CTL) (Abbas *et al.,* 1996). Both ,IFN- and CTL play a pivotal role in fighting infection with intracellular pathogens .IFN- γ activates professional phagocytes like macrophages to kill the engulfed pathogens, and CTL kill cells that fail to control their predators.

Indeed, the induction of a sustained Th1 response is the hallmark of infection with intracellular bacteria (Kaufmann *et al.,* 1999). Although unique microbial factors are involved in the generation of the primary cytokine milieu and APC activation after infection ,pattern recognition receptor (PRR) on APC and other cells of the immune system turns out to be of central importance (Medzhitov and Janeway, 2000). Recent focus increasingly turned towards a subfamily of PRR, the toll-like receptor (TLR), amenders of this family recognize different bacterial agents, including lipopolysaccharide (Qureshi *et al.,* 1999). Recognition of bacterial agents by TLR results in signaling via the IL-1 receptor pathway and finally the activation of the cells (Muzio and Mantovani., 2000).

2.6 *Shigella* Vaccination

The supposition that a virulent mutant strain can be employed as live, attenuated ,oral vaccines have been a guiding principle of *Shigella* vaccine development for four decades .In the 1960s,there were spontaneously an rising,a virulent mutants which were successfully tested in field trials as oral vaccines (Mel *et al.,* 1971; Meitert and Pencu.,

1984). More recent genetic analysis of these mutants indicates that they are non-invasive due to deletions in the virulence plasmid (Mallett and VanDeverg., 1993).The major disadvantage of these non-invasive candidates is the impracticality of immunization regimens consisting of 5 oral doses, escalating to 10^{11} colony forming unit/ml(CfU) .In addition ,vomiting or diarrhea occurs in some vaccines following ingestion of large numbers of a virulent *Shigella* (Mel *et al.,* 1971) partial elucidation of the genetic basis of *Shigella* virulence in the 1980s and 1990s allowed the construction of live, attenuated vaccine candidates that retain enteroinvasive phenotype. Since these vaccines express the IpaBC invasion, they actively colonize lymphoid follicles in the large intestine of primates ,delivering bacteria to the underlying antigen processing cells .Examples of attenuated, enteroinvasive *S.flexneri* vaccine candidates include (1) The icsA mutant that are unable to spread within the absorptive epithelium (Sansonetti *et al.,* 1991;Noriega *et al.,* 1994).(2)The auxotrophic aromutants are attenuated mutants obtained by a blockage in biosynthesis of aromatic metabolites such as the chorismic acid precursor of P-aminobenzoic acid (Karnel *et al.,* 1993) and (3) an *E.coli* K-12 hybrid, aroD mutant is expressing *S.flexneri* somatic antigen and the plasmid-encoded *Shigella* vaccine phenotype (Kotloff *et al.,* 1995,b).Thus for, clinical trials of these enteroinvasive vaccine candidates have been characterized by a narrow window delimiting the immunizing doses from the overtly reactogenic intestinal challenge doses (Hale ,1995).

The release of inflammatory cytokines (like IL-1) during the early stages of *Shigella* infection (Sansonetti *et al.,* 1995) is probably necessary for an optimal mucosal immune response and these cytokines also constitute the mediators for vaccine reactions such as headache ,fever and diarrhea .Nevertheless ,there was an ongoing research for defining

the enteroinvasive mutants possible to eventually succeed in establishing the optimal balance between immunogenicity and reactogenicity in live *Shigella* vaccines .

Serotype specific immunity suggests that O-specific polysaccharide (O-SP) subunit vaccines could protect against shigellosis (Robbins *et al.,* 1992).Parenteral injection of subunit vaccines consisting of acid-hydrolysed ,detoxified O-sp, covalently conjugated to a protein carrier such as *pseudomonas aeroginosa* exoprotein A(rEPA), evokes only mild local reactions and elicits significant rises in serum antibody (IgG, IgM , IgA) (Taylor *et al.,* 1993). A *P. Shigelloides* O-SP-rEPA conjugate was evaluated as a vaccine against *S.sonnei* infection in a recent field trial conducted by the Israel Defense Force Medical Corps and the significant protection against diarrhea was demonstrated (Hale, 1995).

Alternative sub cellular delivery vehicles for O-SP include parenteral ribosomal vaccines (Levenson *et al.,* 1991) and oral or intranasal proteosome –LPS vaccines (Orr *et al.,* 1993; Mallett *et al.,* 1995).Like the chemically haptenated protein polysaccharide conjugates ,these nucleic acid and /or protein carriers elicit T cell dependent immune response against O-SP. The safety of subunit or sub cellular O-Sp vaccines facilitates volunteer studies and these products may represent the best hope for the immunoprophylaxis of shigellosis in the near feature (Hale, 1998).

2.7 Laboratory Animal Models for *Shigella* Immunity

In animals model, mice or guinea pigs immunized intranasal or orally with virulence antigens of *Shigella*, including IpaB, IpaC and IpaD (invasion plasmid antigen)and LPS without any additional adjuvant ,mounted a significant immunoglobulin G (IgG) and IgA antibody response against the *Shigella* virulence antigens and LPS. The virulence –specific response was very similar to that noted in primates infected with

Shigella, Guinea pigs (Keratoconjunctivitis model) or mice lung model that are immunized intranasally on 0.4 and 28 and challenged 3 weeks latter with virulent *Shigella* were protected from disease for both animal models.(Turbyfill *et al.,* 2000).

Guinea pigs were immunized with two *S. flexneri* serotypes 2a and 3a (which together bear all of the major antigenic group factors of this group) produced serum and mucosal antibodies cross- reacted with all the *S.flexneri* serotypes test (except of *S. flexneri* serotype 6) ,(Noriega *et al.*, 1999). In mice, secretory IgA provided via "back pack" hybridoma tumor or mixed with bacterial inoculum is able to protect against the disease caused by *Shigella* of the homologous serotype (Phalipon *et al.*, 1995), Although mice lack IgA (IgA mice) have no increase in susceptibility to challenge after vaccination (Way *et al.*, 1999).The ability of vaccination to confer protection to IgA –mice suggests that IgA is not essential for protection and that non-IgA isotypes are likely to be important in this process.

In a mouse pulmonary model of *Shigella* infection, IFN-γ was produced by naive and at even higher level, by immune mice in response to *Shigella* infection (VanDeVerg *et al*., 1995).Using mice deficient in IFN-γ and beige mice (a mouse strain deficient in NK cell activity), one group concluded that NK cell –mediated IFN-γ is essential for resistance following primary *Shigella* infection (Way *et al.*, 1998).

In mice , adaptive immunity to *Shigella* is mediated by T lymphocytes. Primary T lymphocytes harvested from *Shigella* vaccinated mice or T lymphocyte clones that recognize *Shigella* peptide epitopes are able to confer protection to naive mice (Bishop and Hinrichs. 1987; Kaufmann *et al.,* 1988; Harty *et al.,* 1992).In this model CD^{+8}T lymphocytes are predominant protective cell type (Mielke *et al.,* 1988; Mielke *et al.,* 1989), although CD^{+4}T lymphocytes also provide protective effect

(Kaufmann *et al*.,1987;Magee and Wing 1988).CD^{+8} and CD^{+4}T lymphocytes confer protection via different mechanisms ,the former via perforin- dependent, gamma interferon (IFN-γ) independent mechanism (Kagi *et al.,* 1994) and the latter via an IFN-γ mediated mechanism (Harty *et al.,* 1992).

Vaccination with *S.flexneri* serotype 2a confer protection to mice that lack T lymphocyte or gamma interferon (IFN- γ), specific depletion of T lymphocyte does not alter the protection, and adaptive transfer of splenocytes from vaccinated mice does not confer protection to naïve mice .In contrast, vaccination conferred no protection to mice that lack B lymphocytes and adaptive transfer of immune sera conferred partial protection to naive mice. These data demonstrate that in the mouse broncho pulmonary model, adaptive immunity to *S.flexneri* 2a is an antibody –mediated, B-lymphocyte –dependent process and can be generated in the absence of T lymphocytes or IFN- γ (Way *et al.,* 1999).

In rabbit model, an intravenous injection of live culture of *S.dysenteriae* into rabbit produces enteritis and paralysis (Buxton and Fraser,1977).While,pure *S.dysenteriae* exotoxin preparation was enterotoxic in rabbit ligated ileal loop technique (Gyles *et al*., 1986) .*S.flexneri* with an additional chromosomal genes that encoded for their O-antigens can penetrate rabbit intestinal epithelium (Gyles *et al*., 1986).In a rabbit intestinal model of *Shigella* M cells have a role in antigen uptake and ulcer formation through the destruction of APC(Wassef *et al*., 1989).

Thus from the aforementioned base line information on the immunology of Shigellosis it appears that no previous studies gave the major and delineate it from the minor immune features of an exotoxin and endotoxin mediated immunity in rabbits.

Chapter Three
Materials and Methods

3.1 Solutions and buffers

3.1.A General solution

3.1.A.1 Cleaning solution

It was prepared by dissolving 63 gm of potassium dichromate in 35 ml distilled water with the aid of heating the volume was carefully made up to 1000 ml with concentrated H_2SO_4 , it was used for cleaning the glass ware (Mackie and McCartney., 1996).

3.1.A.2 Normal saline

It was prepared in concentration 0.85% by dissolving 0.85 gm from sodium chloride (NaCl; BDH Company ;U.K ,M.W;58.44) in small amount of distilled water ;the volume was completed to 100 ml and autoclaved at 121℃ , 15 pound/inch^2, for 15 min. This solution was used in titration of antibodies in systemic and mucosal with causative agent antigens of LPS and exotoxin (Garvey *et al.*,1977).

3.1.A.3 Phosphate buffered saline (PBS) pH7.2

This buffer was prepared by dissolving one buffered disc in 100 ml distilled water and sterilized by autoclaving (121˚C,15 pound / inch^2 ,for 15 min.) in accordance with the instructions of manufacturer (BDH).

3.1.A.4 Protein estimation solution

These solutions were prepared according to Lowry *et al.*(1951) as follows:-

Solution A

$CuSO_4$	0.5 gm
Potassium sodium tartarate	1 gm
Distilled water	100 ml

Solution B

NaOH	2 gm
Na_2CO_3	10 gm
Distilled water	500 ml

Solution C

Solution A	1 ml
Solution B	50 ml

Solution D

Folin reagent	5 ml
Distilled water	5 ml

3.1.A.5 Giemsa's stain solution

It was prepared according to Mackie and McCartney (1996) as follows:

Solution A:

Prepared by adding 2 gm of Giemsa's stain powder (BDH, Company) to 100 ml of equal volume of methanol (Absolute) and glycerol. The mixture was stirred vigorously at 37C° for 3 days and stored as stock solution .

Solution B:

Prepared by mixing 1 ml of each of stock solution A and 4 ml of Sornes buffer ,and filtrated with filter paper (Whatman No.1) and stored as work solution .This stain was used to staining lymphocyte and neutrophil cells.

3.1.B Immunology

3.1.1 Formaldehyde saline

It was prepared by mixing 3.75 ml formaldehyde (40% concentration; H –CHO, BDH Company; M.W= 303) with 20 ml normal saline .The

volume then was completed to 300 ml with normal saline (0.85%).The final formaldehyde concentration would be 0.5% .This solution was used to solvent immunoglubulins from appendix and duodenum mucosa (Lehmann *et al*., 1968).

3.1.2 Buffer A

This was prepared by dissolving 3.72 gm KCL ,2 gm $MgCl_2$,and 2.42 gm Tris- hydrochloride into 1000 ml distilled water, the pH was adjusted to 7.4 ,then autoclaved at 121C˚ for 15 min., and stored at 4 C˚ until use. This buffer was used to prepare the *S. dysenteriae* sonic lysate exotoxin (SLE) (O'Brien and Laveck., 1982,a).

3.1.3 Tris buffer

This buffer was prepared by dissolving 12 gm of tris $(NH_2C\ CH_2OH)_3$; BDH Company; U.K ; M.W 12.4) in a small amount of distilled water ; the volume was then completed to 1000 ml and the pH adjusted to 7.0 by using HCL (0.1 normal) .It was used in preparation of polyethlenglycol (Johnston and Thorpe ; 1982).

3.1.4 Sornes buffer

Sornes buffer was prepared by dissolving 7.08 gm of Na_2HPO_4 and 6.7 gm of KH_2PO_4 in 1000 ml distilled water .The pH adjusted to 7.2 by using HCL (0.1 normal) .It was used in washing films that have been stained with Giemsa's stain.

3.1.5 Tannic acid solution

The solution was prepared by dissolving 0.5 gm tannic acid powder ($C_{76}\ H_{52}O_{46}$; BDH Company; M.W= 1701.22) in 20 ml distilled water.

The volume was then completed to 100ml with distilled water to obtain the final tannic acid concentration of 0.5% .This solution was used to tan RBCs i.e to mope remove the Ags found on sheep erythrocytes surface (Garvey *et al.,* 1977).

3.1.6 Alsever's solution

Alsever's solution is an isotonic anticoagulant blood preserving solution that permits the storage of whole blood at refrigeration temperature for about 10 days. This solution was prepared by dissolving 24.6 gm glucose (BDH Company); 9.6 gm tri-sodium citrate (BDH company) and 5.04 gm sodium chloride (BDH Company) in 1200 ml distilled water ; the pH was then adjusted to 6.1 with 15% citric acid; the solution then was filtered through 0.45µm pores diameter membrane filter using micro filtration unit (Garvey *et al*.,1977).

3-1.7 Polyethylenglycol solution

It was prepared by dissolving 6 gm from polyetheneglycol ($HO(C_2H_4O)_n$ H; BDH Company; M.W= 6000- 7500) in small amount of tris buffer .It was completed to 100 ml .The final concentration was 6% ; the pH was adjusted to 7.3 .It was used in separation of secretory immunoglobulins. (Johnstone and Thorpe; 1982).

3.1.8 Ficoll- paque solution (Lymphoprep)

Sterile solution of ficoll- paque produced by (Nycomed-Norway) was used for in vitro lymphocytes isolation .

3.1.9 Nitrobluetetrazolium solution

It was prepared by dissolving 0.1 gm of stain powder in 2 ml methanol alcohol, and then adding 50 ml phosphate buffer saline .Kept at 4℃ away of light and used in NBT test (Metcalf *et al.,* 1986).

3.2 Media

3.2.A Bacteriology

Standard derived culture media from different sources and locally prepared media were used for reviving and biomass preparation of toxin production were mentioned in the table (1) as the following:-

Table (1) : Culture media

Medium	Class	Uses	Company	Preparation condition
Brain- heart infusion	Cultivation medium	Cultivation *Shigella spp*. for prepared LPS	Biolife	According to the manufacturers instructions
S.S agar	Selective medium	Isolation and identification *Shigella*	Oxoid	According to the manufacturers instructions
Master agarA A	Solidifying medium	Cultivation and isolation *Shigella*	Difico	According to the manufacturers instructions
blood agar	Enriched medium	Cultivation *Shigella* for prepare exotoxin	Biolife	According to the manufacturers instructions
Syncase broth	Cultivate media	Maintenance *Shigella spp*.	Prepared media	10 gm casamino acid,1.17gm NH_4Cl, 5 gm KH_2PO_4, 5 gm $Na_2H\ PO_4$ and 1 ml trace salt + (5%$MgSO_4$, 0.5% $MnCl_2$ in 0.001N H_2SO_4)dissolved in 1000 ml D.W ,autoclaved and then cooling at 56C˚ .0.2% glucose, 0.002% nicotinic acid and 0.004% L-tryptophan were added after filtrated.
Maintenance	Maintenance media		Prepared media	Nutrient broth+5% glycerol

3.2.B Immunology

3.2.1 Basal eagle medium (BEM)

It was prepared following Sigma-Company instruction and being summarized as one gram of eagle medium and dissolved in sterile distilled water (100 ml).This solution was membrane filtered through 0.45 µm sterile Millipore filter by syringe filter device .The solution preserved at sterile plastic universal in refrigerator at 4C.˚This BEM was used as cell nutritive solution helpful for measuring LIF of leukocyte in blood and mucosal samples.

3.3 Bacteriologic procedure eligible for immunogen preparation

3.3.1 Organism

Clinical isolates of *Shigella* was recovered from shigellosis patients *S dysenteriae* serotype 1 and *S. flexneri* serotype 2 were identified by classical biochemical test .(Figure 1).

Growth onto MacConkey agar Or S-S-agar

↓

Lactose fermentation

↓

Urease test

↓

H_2S production

↓

Shigella spp, *Providential spp*, *Salmonlla spp*

Using identification keys (MacFaddin, 2000)

↓

Shigella species

Figure (1):Flow chart Identification keys for *Shigella*

3.3.2 Confirmative identification

In addition to classical biochemical test, EPI 20 E miniaturized test was used to confirm the diagnosis of this bacteria and then was sent to Central Health Laboratory ,Baghdad for serotyping by using slide agglutination with type specific antisera .

3.4 Immunologic procedures

3.4.1 Antigen and immunogen preparation as well as bioassay

a- Exotoxin preparation

Shigella dysenteriae was grown in iron-depleted syncase broth and incubated for 48hr with shaking (260 oscillation/ min) at 37C˚ .The sample size was distributed as one liter in two liter flask size. The bacteria were harvested by centrifugation at 10,000 rpm at 4C˚ for 20 min. and washed twice in 0.85% NaCl .The organisms were then resuspended in 20 ml of buffer (0.05 m KCL, 0.01 M Mg CL_2, and 0.02 M tris at pH 7.4) and disrupted by 3min of intermittence (155 on,105 off) sonic oscillation sow(sonifer cell disrupter model 150W).The sonic extract were clarified by centrifugation at 12,000 rpm at 4C ˚ for 2hr. Discard pellet and the supernatant were filtered through 0.45µm pores diameter membrane filter and saved for biological assay and immunization programme (O'Brien and Laveck,1982,a; O'Brien and Laveck,1982,b).

-Endotoxin preparation

b-1 Preparation of bacterial dry weight

Th isolated *S. dysenteriae* or *S .flexneri* were grown in 40 ml brain heart infusion for 18 hr at 37C˚. Two liters of brain –heart infusion were prepared and distributed as one liter in two liter flask each one of liter flask was inoculated with 20 ml of grown bacteria and then incubated at

37˚ C for 24hr in shaking water bath (260 rpm).The bacteria were harvested by centrifugation at 9000 rpm at 4C˚ for 15 min. and then they were washed with distilled water ,ethyl alcohol ,acetone and twice with ether, respectively. The pellets of bacteria were dried in vacuo or in incubator for 48hr at37 ˚C .

The method of LPS preparation is devised by Westphals, *et al*., (1952). Bacteria (10 gm of dry weight bacteria) were suspended in 160 ml of distilled water and mixed with 265 ml of 75% phenol (prepared by adding 65 ml of water to 200 gm of Liquid phenol).The mixture left at 35C˚ for 30 min with occasional shaking ,then centrifuged at 3000 to 4000 rpm. The upper aqueous phase should be collected .The remaining material can be washed with water, centrifuged, and the upper layer combined with the first supernatant .This fluid was dialyzed for 2 or 3 days against tap water and 1 day against distilled water, then concentrated to a 40-50 ml volume .LPS was precipitated from dialyzed with six volumes of acetone mixed with a small volume of saturated alcohol in sodium acetate solution. The sediment was collected by centrifugation, washed with alcohol and acetone, and dried in vacuo or in incubator.(Figure 2).

C- Toxin dose determination

Serial concentration of both exotoxin and endotoxin of *S .dysenteriae* serotype 1 and *S.flexneri* serotype 2 which ranges from 1-10 mg/ Kg .For each one three rabbits were used to avoid the individual variations in the immune reactions .The immunization protocols were the same for exotoxin and endotoxin as in the following.

1-Sex: all rabbits used in this study were male.

2-Weight : The weight of rabbits was about 2 kg.

3-Adaptation: All rabbits were adapted to two weeks before immunization with exotoxin or endotoxin.

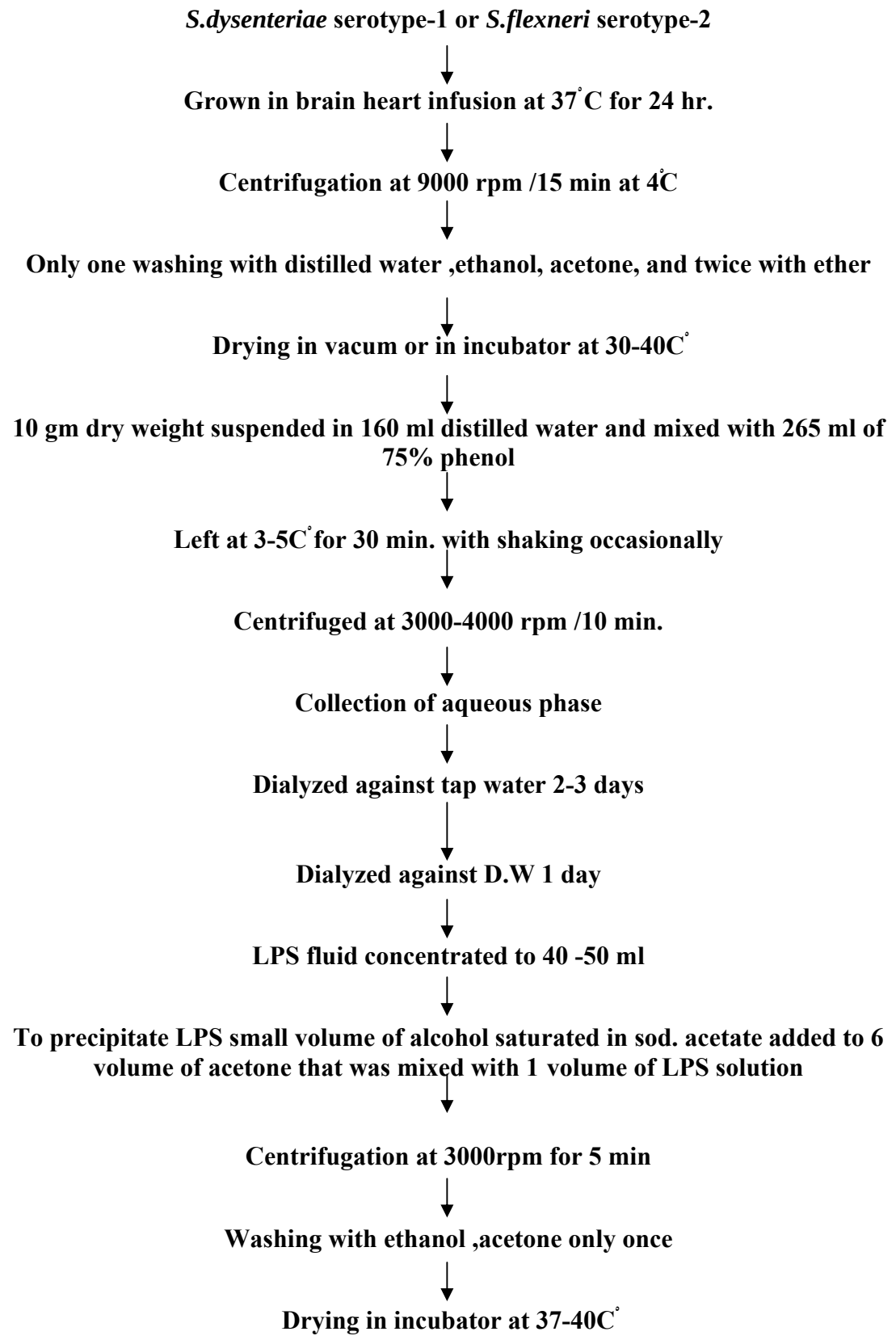

Figure (2) : Flow chart LPS preparation according to Westphals(1952)

4-All rabbits were immunized orally with exotoxin and endotoxin.

The immune reaction for toxin was bioassayed by PF, paralysis and specific antibody reaction for exotoxin while rat foot pad reaction as well as specific antibody reaction were used to detect of LPS immunogenicity. From these approaches, it was found that 1, 2 mg /5ml were the safe and immunogenic dose for exotoxin whereas 3, 5mg /5ml were the safe and immunogenic dose for LPS (Table 2).

D- Exotoxin bioassay

D.1 Neurotoxin activity

White mice 20 gm were I.P injected with 0.2 ml crude exotoxin. Flaccid paralysis indicates neurotoxin activity of this toxin (Mata *et al*., 1970).

D.2 Permeability altering factor (PF)

Rabbit skin injected intradermaly with 0.2ml crude exotoxin. Indurations of 8 mm diameter or more at 24 hr. define as positive response (Duhamel., *et al*., 1970).

E- Bioassay of endotoxin

E-1 Pyrogenicity

An amount of 0.1 gm of LPS was suspended in 10 ml normal saline, from this suspension 0.3 ml was injected within foot pad of white rat. The rise in temperature was recorded as positive results (Kumazowa *et al*., 1988).

E.2 Mitogenic activity of LPS

Mitogenic activity was assessed by injection 0.3 ml of LPS that was prepared by dissolving 0.1gm of LPS in 10 ml normal saline in foot pad

of white rats. Thickness, redness and edema were measured .(Kumazawa *et al*.,1988).

F- Protein determination

Protein concentration was determined by method of Lowry *et al*., (1951) with bovine serum albumin as a standard figure (3).

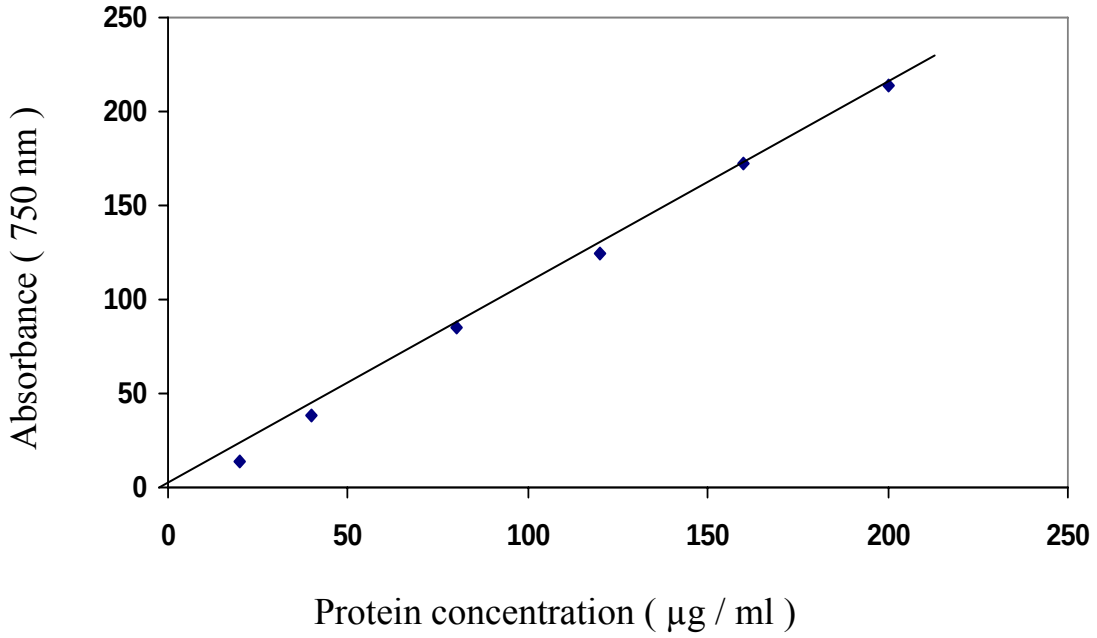

Figure (3): Standard curve protein determination

3.4.2 Rabbit groups:

Eighty five (85) rabbits were left for two weeks for adapted conditions and kept at libitum conditions of food and drinks and divided into ten groups . The groups are IIX,IX and X, each of which contains five replicates that are used two detect skin test .(Table 2)

3.4.3 Immunization Protocols

Eighty five (85) mature male rabbits were the laboratory animals. Each rabbit was about 2kg of weight. The animals were immunized orally

with LPS of *S.dysenteriae* dose 3,5mg /5ml and LPS of *S.flexneri* dose 1.5, 2.5 mg/ Kg as well as exotoxin of *S.dysenteriae* dose 0.5, 1mg /Kg in 5 ml normal saline orally for ten replicate of rabbits . LPS of *S.dysenteriae* dose 5mg /5ml and LPS of *S.flexneri* dose 5mg/5ml as well as exotoxin of *S.dysenteriae* dose 2mg/5ml were administrated orally to five replicates that used to detect skin test. All these doses were administrated in four doses, between each dose five days. (Table 3).

Table (2) : Rabbit groups and doses.

Parameters	Groups	Dose	No.
Rabbits immunized with *S.dysenteriae* Exo. 1 mg	I	0.5 mg/Kg	10
Rabbits immunized with *S.dysenteriae* Exo. 2 mg	II	1 mg/Kg	10
Rabbits immunized with *S.dysenteriae* LPS 3 mg	III	1.5 mg/Kg	10
Rabbits immunized with *S.dysenteriae* LPS 5mg	IV	2.5 mg/Kg	10
Rabbits immunized with *S.flexneri* LPS 3 mg	V	1.5 mg/Kg	10
Rabbits immunized with *S.flexneri* LPS 5 mg	VI	2.5 mg/Kg	10
Non immunized Rabbits (Control)	VII	-	10
*Immunized *S.dysenteriae* ***exo. of 2 mg	IIX	1 mg/Kg	5
*Immunized with LPS of *S.dysenteriae* 5 mg	IX	2.5 mg/Kg	5
*Immunized with **LPS of *S.flexneri* 5 mg	X	2.5 mg/Kg	5

*For skin DTH tests.

** Lipopolysaccharide

*** Exotoxin

3.4.4 Blood sampling

A total of 85 blood samples were collected from 60 immunized rabbits and 10 non- immunized rabbits as a control group .From each rabbit, a blood sample of 3-5 ml was drawn aseptically by heart puncture in a disposable syringe .Half of the blood samples were left at room temperature till being clotted, and then were centrifuged at 3000 rpm for 5 min. The sera were aspirated from the whole blood , then divided into 0.5 ml small test tubes, and stored at 20C° till testing time .Each tube was used once to avoid repeated freezing and thawing.

The second half of blood samples were tubed in EDTA tube as anticoagulant .The samples were processed for LIF and for separation T-lymphocyte to detect E-rosette formation as in Figure (4).

Table (3) :Immunization protocols of rabbits with *S. dysenteriae* LPS, exotoxin and *S.flexneri* LPS antigens that were dissolved in 5ml normal saline and given orally.

Time	*S.flexneri LPS*		*S.dysenteriae LPS*		*S.dysenteriae exotoxin.*		*Control*	*S.dysenteriae exotoxin*	*S.dysenteriae LPS*	*S.flexneri LPS*
	G. I	**G.II**	**G.III**	**G.IV**	**G.V**	**G.VI**	**G.VII**	**G.IIX**	**G.IX**	**G.X**
1st and 2nd week	Adaptation									
1st 5 days	1.5 mg LPS	2.5 mg LPS	1.5 mg LPS	2.5 mg LPS	0.5 mg exotoxin	1 mg exotoxin	5 ml N.S	1 mg exotoxin	2.5 mg LPS	2.5 mg LPS
2nd 5.days	1.5 mg LPS	2.5 mg LPS	1.5 mg LPS	2.5 mg LPS	0.5 mg exotoxin	1 mg exotoxin	5 ml N.S	1 mg exotoxin	2.5 mg LPS	2.5 mg LPS
3rd 5days	1.5 mg LPS	2.5 mg LPS	1.5 mg LPS	2.5 mg LPS	0.5 mg exotoxin	1 mg exotoxin	5 ml N.S	1 mg exotoxin	2.5 mg LPS	2.5 mg LPS
4th 5days	1.5 mg LPS	2.5 mg LPS	1.5 mg LPS	2.5 mg LPS	0.5 mg exotoxin	1 mg exotoxin	5 ml N.S	1 mg exotoxin	2.5 mg LPS	2.5 mg LPS

LPS =Lipopolysaccharide , N.S =Normal saline , G. = group

3.4.5 Entero mucosal sampling (Appendix and duodenum)

Entero mucosal sampling was collected from all immunized rabbits according to (Shnawa and Thwaini ,2002) as follows .

1-From each rabbit, about 10-12cm from appendix and duodenum were collected in an aseptically method.

2-The appendix and duodenum were opened by using sterile and clean scissor. The digested material was removed from appendix and duodenum by washing them with normal saline .The mucosa and sub mucosa were scrapped by sterile surgical scalpel and then they were placed in another sterile petridishe containing 10 ml of normal saline.

3-By sterile Pasteur pipette the suspension was transfered to sterile plastic test tube.

4-The suspension was centrifuged at 4000 rpm for 20 min. Supernatant was transferred to another sterile test tube and then used for separating of secretory immunoglobulin.

5-The precipitate was washed three times with normal saline and preserved in at ratio 1:1 in Alsever's solution for studying MIF ,E-rosette, and NBT test.

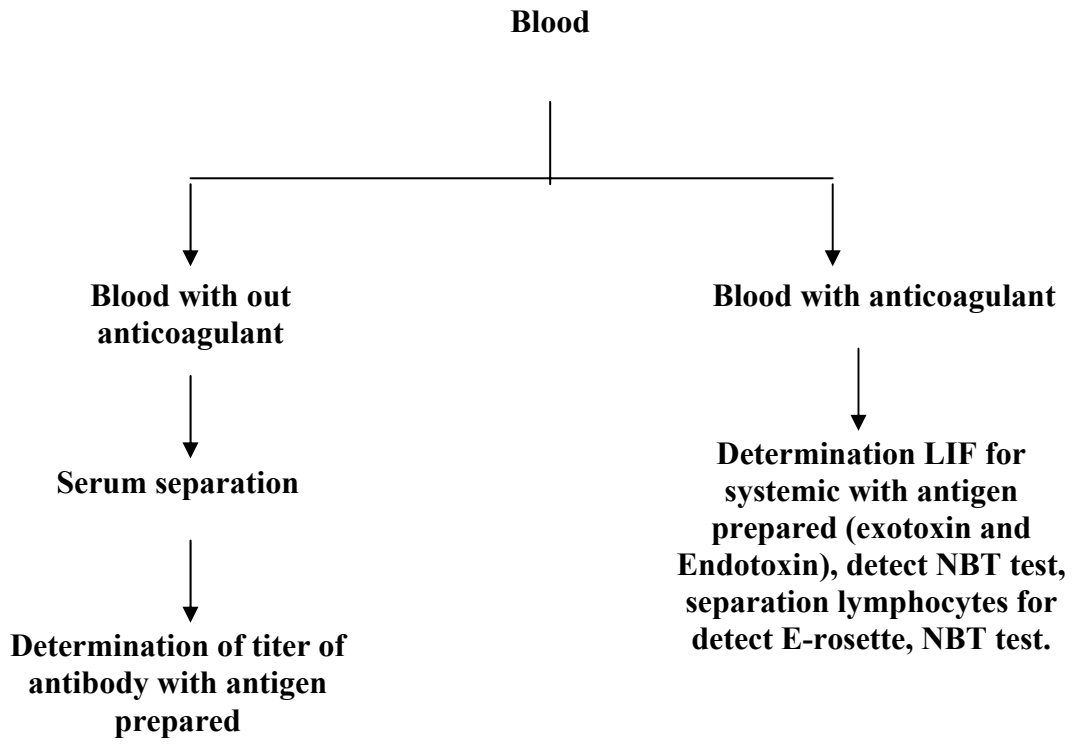

Figure (4): Flow chart for the investigation systemic immune response to rabbit that immunized with *S. dysenteriae* and *S. flexneri* (Systemic sample blood).

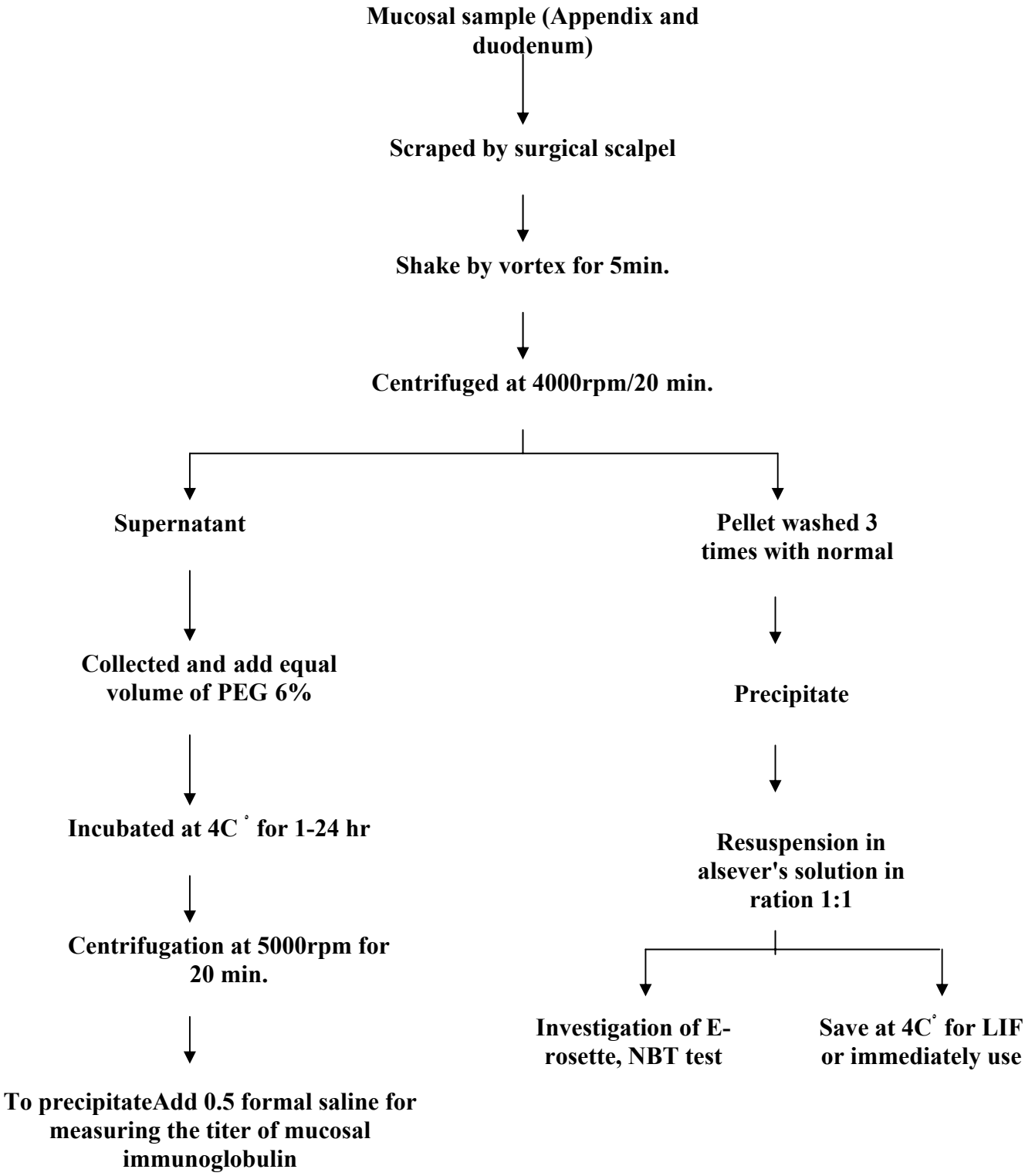

Figure (5): Flow chart for investigation mucosal immune response to rabbits that was immunized with *S. dysenteria*e and *S. flexneri* (Mucosal Appendix and duodenum).

3.5 Specific antibody separation

a) Separation of serum immunoglobulin

1-To 1 ml of serum, equal volume of polyethylenglycol was added .The mixture was left in refrigerator for 30 min. at 4˚C .

2-Centrifuged at 4000 rpm for 20 min.

3-Supernatant was discarded and the precipitate was taken.

4- Formal saline(5ml) and 5ml of PEG was added, mixed well and left at room temperature for 10-15 min.

5-Centrifuged at 3500rpm for 20min.

6-Discard the supernatant and the pellet was Dissolve in 0.25 ml formaldehyde saline (Garvey *et al*., 1977).

.

3.5(b) Separation of mucosal secretory immunoglobulins from mucosa rabbits by polyethylenglycol (PEG)

1-Equal volume of PEG was added to supernatant that was prepared in section (3.4.5) and it was left in refrigerator at 4C˚ from 1-24 hr. for precipitate of the secretory immunoglobulin.

2-Centrifuged at 4000 rpm for 20min., the supernatant was discarded .To the sediment, 1ml of formaldehyde saline was added to dissolve secretory immunoglobulins precipitated in the bottom test tube.

3-The sediment was stored in refrigerator at 4C˚till use time (Shnawa and Thwaini ,2002) .

3.6 Procedure of Lymphocytes Separation

1-Lymphocytes were separated from heparinized heart blood samples of rabbits by layering the blood and diluted with RPMI in ratio 1:1 over 3 ml of ficoll-paque into sterile centrifuge tube.

2-Centrifuged at 2000-3000 rpm for 30 min.

3-Four layers appeared including diluted plasma ,lymphocytes, ficoll-paque and the final layer which consists of red blood cells RBCs granulocytes and platelets. The cell band (lymphocytes) was transferred into sterile test tube that containing PBS, washed three times and centrifuged at 1000-2000 rpm for 10 min.

4-The pellet after the last wash was transferred into sterile test tube containing 1 ml PBS.

5-The lymphocytes (0.5ml) that was prepared above mixed gently with 0.5 ml sheep RBCs and then incubated at 4˚C for 1 hr.

6-The smear was prepared by placing 20 µl of suspension in section (5), left to dry and fixed with methanol for 5 min.

7-The smear was stained by Giemsa's stain for 3 min., washed by Sornes buffer, left to dry,and examined under oil immersion. (Lefkovits, 1997).

3.7 Humoral immune function test

3.7.1 Standard tube agglutination test

a) Standard tube agglutination method of mucosal using modified (Garvey *et al.*, 1977) and being stated as fallows

1-Set up 11 tubes in a test tube rack.

2- Add 0.25 ml of diluents (physiological saline) to each tube.

3-Add 0.25ml of mucosal immunoglobulin to tube 1. Mix and transfer 0.25 ml to tube 2 .Continue mixing and transferring, and discarding 0.25 ml from tube 10 .Tube 11 will then serve as an antigen control.

4-Add 0.25 ml of antigen suspension to each tube .The dilutions thus obtained were 1:1 ,1:2 , 1:4 , 1:8, 1:16 ,1:32 , 1:64, 1:128 ,1:256 , and 1:512 accordingly .

5- The rack was shacked , and incubated at 37C˚ for 24 hr., after the tubes were covered with parafilm. After wards, the results were read.

6-The appearance of clumps in the bottom of the tube with irregular edges and clear upper layer of the supernatant indicated positive results .If negative results were obtained ,further 24 hr. incubation was carried out to achieve a stable end point.

b) Standard tube agglutination method of systemic (Garvey *et al*., 1977).

1-Set out 11 sterile test tube in a test tube rack.

2-Place 0.9 ml of diluents (physiological saline) in tube 1 and 0.5 ml of diluents (physiological saline) in tubes 2 through 11.

3-Add 0.1 ml of serum immunoglobulin to tube 1 ,mix and transfer 0.5 ml to tube 2 ,mix and transfer 0.5 ml tube 3, and so on, through tube 10. Tube 11will serve as a control .By this method; dilutions will be 1:10 to 1: 2560.

4-Add 0.5 ml of antigen suspension to each tube.

5-The tube was then covered with parafilm and incubates at 37C˚ for 24 hours. Afterwards, the results were read.

6- The results were scored as highest dilution that gives positive agglutination results(Both for mucosal and systemic)

3.7.2 Coating tanned sheep red cells

According to the modified method of Garvey *et al*., (1977) passive haemgglutination test(HA) was used to determine antibody titer in serum and mucosal rabbits that was immunized with exotoxin .The procedure was carried out as follows:

1-Sheep erythrocyte suspension was placed in sterile test tube.

2-Alsever's solution was added to sheep erythrocyte suspension as anti coagulant in percentage 1:1, mixed gently and preserve at 4˚C .

3-3ml of sheep erythrocytes was transferred into sterile centrifuged tube and then centrifuged at 2500/5 min. The supernatant was discarded and the sediment was used.

4- Normal saline(10ml) was added to the sediment and centrifuged at 2500 rpm for 5 min. The supernatant was discarded and the sediment was used.

5-Sediment erythrocyte was resuspension in 10 ml of normal saline and then mixed well by sterile Pasteur pipette.

6-3ml of suspension in section (5) was added to 3 ml of tannic acid (0.5%) and mixed well by sterile Pasteur pipette, and then the test tube was placed in water bath at 37˚C for 10 min.

7-Centrifuged at 2500 rpm for 5min.The supernatant was discarded and the sediment was used.

8-3ml of normal saline were added to sheep erythrocytes sediment and well mixed by sterile Pasteur pipette .To this suspension 3ml of antigen(exotoxin)were added to coating all erythrocytes by this antigen. The suspension was left at room temperature for 10 min.

9-Centrifugation at 2500 rpm for 5min .The supernatant was discarded and the sediment was used .To the sediment 5ml of normal saline was added for washing the cells and then centrifuged at 2500 rpm for 5min; the supernatant was discarded and the sediment was used.

10- To the erythrocytes sediment was coated with antigen, 4 ml of normal saline were added for use in titration of mucosal and systemic antibodies.

a) Passive haemagglutination test of mucosal antibody

1-50 µl normal saline was dispensed in each well of the antibodies round buttom micro titration plate wells (12 wells).

2-50 µl of mucosal antibody suspension was added to the first well and mixed .Then serial dilutions were prepared by transferring 50 µl from the first well to the second one, mixed well and from it 50 µl was transferred to the third well and so on. From well 11 50µl was discarded .Serial dilutions thus were obtained from the first well till the eleventh on 1:2, 1:4 ,1:8,1:16, 1:32, 1:64 ,1:128, 1:256, 1:512 ,1:1044 and 1:1048 accordingly.

3-To the twelfth well, 50µl of tanned sheep erythrocytes coated with the exotoxin was added (the last well represented the negative control well which was lacking the presence of mucosal specific antibody.

4-The micro titration plates was shaken gently for about two min. and then incubated at 37˚C for 45 min.

5) Appearance of haemagglutination clumps that were diffused from the center of the well as carpet this reaction between antigens –antibody was recorded as positive results. The appearance of thick ring from the cell in the center of well was recorded as negative results.HA titer represented the last dilution that gave a positive reaction.

b) Passive haemagglutination test of systemic antibodies.

1-50µl of diluents (Physiological saline) was placed in each well of the micro titration plate well (12 wells).

2-0.1ml of rabbit's serum was added to 0.9 ml normal saline in a sterile test tube and mixed well.

3-50µl of diluted rabbit's serum (1:10) was added to the first well and mixed .Then serial dilutions were prepared by transferring 50µl from the first well to the second one, mixed well and from it 50µl was transferred to the third well and so on. From the eleventh well 50µl was discarded .Serial dilutions thus were obtained from the first well

to the eleventh one are :1:20, 1:40 ,1:80 ,1:160 ,1:320,1:640 ,1:1280 ,1:2560 ,1:5120 ,1:10240 and 1:20480 accordingly.

4-To the twelfth well, 50µl of tanned sheep erythrocytes coated with exotoxin was added (the last well represented the negative control well which was lacking the presence of rabbit's serum specific antibody .

5-The micro titration plates were shaken gently for about two minutes and then incubated at 37˚C for 45 min. Then the presence or absence of haemoagglutination was recorded as in (HA section a).HA titer represented the last dilution that gave marked matt formation within 45 min. at 37˚C or overnight at 4˚C with moistened covered plastic. Half matt half bottom indicates partial, and button formation indicates negative results.

3.8 Cellular immune function test

3.8.1.1 Preparation of the leukocytes sensitizers

The preparation of exotoxin by cultivating *S.dysenteriae* in syncase broth for 48hr with shaking and liquid media was centrifuged at 10,000 rpm for 20 min. at 4C˚.The sediment resuspended in 20 ml of buffer pH 7.2 and disrupted by 3 min. of intermittent sonic 0.5 oscillations at 50 W. The sonic extract was clarified by centrifugation at 12,000rpm, for 2hr. at 4C˚. The supernatant was filtered and distributed in sterile bottle and then preserved in refrigerator until it was used as sensitizer (O'Brein and Ladeck, 1982,a).

LPS was prepared as sensitizer by dissolving 1.5, 5mg LPS in 5ml normal saline and then used in the same dose that was administrated to rabbits in section(3.4.1,a) .

3.8.1.2 Leukocyte migration inhibition factor (LIF)of mucosal leukocytes

This test was done for measuring the factor that inhibited the migration of macrophage in case of mucosal system of rabbits immunized with endotoxin and exotoxin antigens .The procedure was carried out as follows:

1- Agar- A medium was prepared in sterile plastic plate (2% agar), and two wells were done with 2cm in diameter on plate of agar.

2-From cell of appendix or duodenum in step (3-4-5) was preserved in alsever's solution, capillary tube was filled and put in the well after centrifugation by haematocrit for 10 min.

3- In each well 0.1 ml of eagle Basal medium was added and one of the wells was considered as control.

4-Addition of 0.1 of exotoxin or suspension LPS that was prepared in step (2.7, 2.8) for one well and the other well was left as control.

5-The plate was incubated at 37˚C in jar in a humid environment for 24 hr, and then inhibition was measured.

6-Measuring the factor of inhibition of macrophage was done by oculometor.

3.8.1.3 Leukocyte migration inhibition factor(LIF) of peripheral blood.

Measurement of migration inhibition factorwas done by:-

1-Preparation of agar-A medium in sterile plastic plates and two wells were made as in (section 3.8.1.2).

2-Capillary tube containing systemic blood from rabbits have been immunized with exotoxin or endotoxin was put in each well after centrifugation by haematocrit for 10 min.

3-Eagle basal medium (0.1 ml) was put in each well; one of these wells was considered as control.

4- 0.1 ml of antigen (exotoxin fluid or LPS suspension)of *S. dysenteriae* or *S. flexneri* was added in one well.

5-Incubate at 37C° for 24 hr in humid environment.

6-Mcasurement of MIF by oculometor, same steps were used for control and normal saline was added instead of exotoxin or LPS suspension as mentioned by Soberg (1969).

MIF was measured as follows:

$$*LIF\ Persent = \frac{\text{Distance with sensitizer}}{\text{Distance without sensitizer}} \times 100$$

*Inhibition of 30% or more is significant.

3.8.2.1 Separation 0f mucosal lymphocytes

1-Lymphocytes were separated from mucosal samples (appendix and duodenum) prepared in section (3-4-5) by layering the mucosal suspension, which was diluted with RPMI in ration 1:1 over 3 ml of ficoll-paque into sterile centrifuge tube.

2-Centrifuged at 2000-3000 rpm for 30 min.

3-Three layers appeared including lymphocytes, ficoll-paque and final layer which consist of sediment of mucosa. The cell band (Lymphocytes) was transferred into sterile tube containing PBS, washed three times and centrifuged at 1000-2000 rpm for 10 min.

4-The pellets after the wash were transferred into sterile test tubes containing 1 ml PBS.

5-0.5 ml of lymphocytes was mixed gently with 0.5 ml of sheep RBCs and incubated at 4˚C for 1 hr.

6-Smear was made by putting 20µl suspension in section (5) on the slide, left to dry, and then the smear is fixed with methanol for 5min.

7-The smear was covered by Giemsa's stain for 2-5 min. and washed by Sornes buffer. Left to dry and then examined under oil immersion and lymphocytes that from E-rosette were counted.

3.8.2.2 Separation of peripheral blood lymphocytes.

1-Lymphocytes were separated from heparinized heart blood samples of rabbits by layering the blood, diluted with RPMI in ratio 1:1 over 3 ml of ficoll-paque into sterile centrifuge tube.

2-Centrifuged at 2000 -3000 rpm for 30 min.

3-Four layers appeared including diluted plasma, lymphocytes, ficoll-paque and the final layer which consisted of red blood cells ((RBCs), granulocytes, and platelets. The cells bands (lymphocytes) was transferred into sterile test tube containing PBS, washed three times and centrifuged at 1000-2000 rpm for 10 min.

4-The pellets after the last wash were transferred into sterile tube containing 1 ml PBS.

5-The lymphocytes (0.5ml) were prepared above mixed gently with 0.5 ml sheep RBCs and then incubated at 4C˚for 1 hr.

6-A smear was prepared by placing 20 µl of suspension in section (5), left to dry and then fixed with methanol for 5 min.

7-The smear was stained by Giemsa's stain for 3 min., washed by Sornes buffer, left to dry and examined under oil immersion and T cells that form E- rosette were counted (Lefkovits. 1997).

3.8.3 Nitrobluetetrazolium reduction test (NBT)

0.5 ml blood or mucosal suspension that were prepared in section (3-4-4 or 3-4-5), mixed gently with equal amount of NBT solution in siliconized tube, then incubated with humidity source at 37˚C for 30 min.. At the end of this period, the blood or mucosal suspension / NBT mixture was again mixed and a thin film was made. The film was dried in air and stained with Giemsa's stain .The slide examined under the microscope with oil lens immersion and 100 neutrophils was counted. Only those with a large black deposit (Formozan stippling) were classified as NBT positive and the percentage of these cells was recorded.This test is used for detect phagocytic activity of phagocytic cells. (Park *et al*., 1968)

3.8.4 Shigella toxin allergen (STA)

It was prepared in a concentration 2mg as in (Obrien and Laveck,1982,a) and separation of protein by PEG was done as in Johnstone and Thorpe (1982) as in the following steps:

1-2ml from exotoxin concentration 2mg was added to equal volume of PEG (6%).

2-Left at 4C˚for 1 hr. and then centrifuged at 4000 rpm for 20min.

3-The sediment resuspended in 2ml PBS and from it 0.1ml of *Shigella* protein was injected intradermally (ID) into rabbits.

3.8. 5 Skin delayed type hypersensitivity (DTH)

This was done for Shigella exotoxin primed rabbit as in Burrell (1979)

3.9 Test battery

Exotoxin was assayed by skin permeability, paralytic effect, and specific antibody reaction .Endotoxin was assayed by foot pad reaction,

temperature rose and specific antibody reaction in rats that was injected with endotoxin. Humoral immune responses were assayed by agglutination and passive haemagglutination. Cellular immune responses were assayed by NBT, LIF, E- rosette formation and skin delayed type hypersensitivity reactions test.

3.10 Score

Antibody titers scored as the reciprocal of the highest dilution that gave clear positive results .The phagocytic activity of neutrophils by NBT were scored as formazan stippling in the neurophils cytoplasm. Cell count was assayed through the formation of E-rosette, leukocyte inhibitory factor that was scored as percent migration.

3.11 Statistics

The range and mean were calculated based upon (Dawd and Al-Yas ,1991).

3.12 Normal values

Clinical assessment needs normal function levels .Below such normal function (Normal value NV) levels known as subnormal value (SV) and above the (NV) is termed as abnormal values (AV). For this experimental immunologic approach, normal values were affixed.

Chapter Four
Results

4.1 Control (Unimmunized rabbits).

Shigella Specific antibody was of nil titer in control animals NBT, LIF and E-rosette normal values .

4.2 Toxin Bioassay

4.2.1 Exotoxin:

Five replicates of the rabbits and five mice were used for bioassay of exotoxin; the five mice were injected intraperitonial with 0.2 ml exotoxin, paralysis in rear legs of these mice was observed while the paralysis is not observed in mice were injected intraperitonial with 0.2ml normal saline (control group). In the five rabbits skin permeability altering factor (PF) was also attempted as another bioassay parameter for exotoxin (Table 4, figures: 6-A,6-B,7-A,7-B) .

4.2.2 Endotoxin

The bioassay of LPS was investigated by using five rats. These rats were injected in foot pad with 0.3 ml crude LPS. The test parameters were thickness; Temperature, color and edema were used for assessing LPS. The difference in thickness of tested rats was between 3.7-4.5mm compared with control rats that was between 1-1.6mm, color changed from pale color into red (erythema)in all tested rats and was associated with edema .Temperature rose to more than 1C° in injected rats and its ranged between 38-39C° (Table 5, figure 8).

Table (4): Bioassay of Exotoxin in mice (paralysis) and rabbit (PF induration).

Seq. of mice inj. I.P with 0.2 ml Exotoxin	Test	Control normal saline inj.	Seq.of rabbit inj. parenteral with 0.2 ml Exotoxin	Test
	paralysis	Paralysis		PF indurations
1	With paralysis	Without paralysis	**1**	8.9 mm
2	With paralysis	Without paralysis	**2**	9 mm
3	With paralysis	Without paralysis	**3**	9 mm
4	With paralysis	Without paralysis	**4**	9 mm
5	With paralysis	Without paralysis	**5**	10 mm

Table (5): Bioassay of LPS in rats(skin thickness,temp.,color, and edema).

Seq. of rate inj. In foot pad with 0.3 ml LPS	Bioassay of LPS							
	Test character				Control character			
	Thickness	Temp.	Color	Edema	Thickness	Temp.	Color	Edema
1	3.7 mm	38 ℃	redness	With edema	1 mm	37 ℃	pale	Without edema
2	3.9 mm	38 ℃	redness	With edema	1 mm	37 ℃	pale	Without edema
3	4 mm	39 ℃	redness	With edema	1.4 mm	37 ℃	pale	Without edema
4	4 mm	39 ℃	redness	With edema	1.5 mm	37 ℃	pale	Without edema
5	4.5 mm	39 ℃	redness	With edema	1.6 mm	37 ℃	pale	Without edema

PF= Permeability factor
Seq. = Sequence
Inj = injected

Figure (6-A): Exotoxin bioassay (mice leg with paralysis)

Figure (6-B): Exotoxin bioassay (mice leg without paralysis)

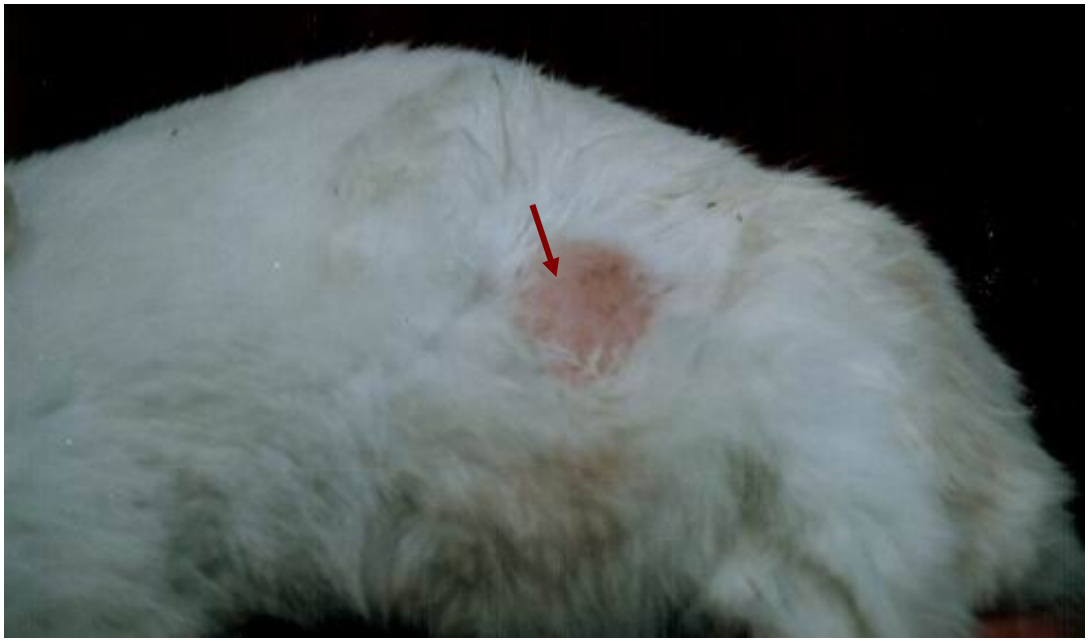

Figure (7-A): **Exotoxin bioassay in rabbit (PF positive**).

Figure (7-B): **Exotoxin bioassay in rabbit (PF negative**).

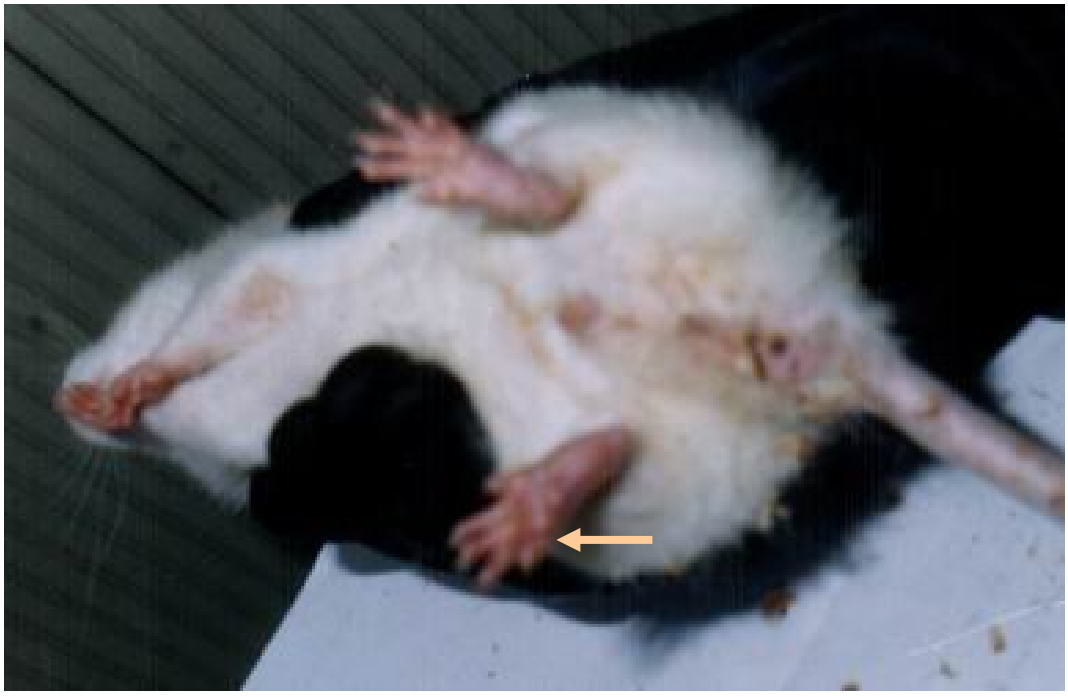

Figure (8): Endotoxin bioassay (foot pad reaction in rat).

4.3 *Shigella* specific humoral antibody response in rabbits

The mean values of serum *S. flexneri* specific antibody in LPS primed rabbits were 304, 320 at systemic level and 48, 48 at the mucosal level of the appendix and 40, 41.6 at the mucosa of the duodenum for the two doses (1.5, 2.5 mg /Kg LPS) while the mean values of specific antibody titer in rabbits immunized with *S .dysenteriae* LPS were 336, 304 at systemic and 41.6 , 44.8 at mucosal appendix and 32, 38.4 at mucosal duodenum for dose (1.5, 2.5 mg /Kg LPS), accordingly.

Serum and mucosal specific antibody titer of rabbits that were immunized with *S. dysenteriae* exotoxin dose (0.5, 1 mg/Kg) were determined by using passive haemagglutination test (HA) ,and their mean values were 256, 288 at systemic level for dose (0.5, 1 mg/Kg), accordingly whereas the mean values were 44.8 ,48 at of the appendix and 27.2 ,30.4 at mucosal level of the duodenum for dose(0.5, 1 mg/Kg), respectively .(Table 6)

Apparently the titer of the mucosa antibody in the appendix of rabbit primed with LPS showed higher than of those mucosal duodenum and systemic antibody titer for both endotoxin and exotoxin doses; on the other hand, systemic antibody titer of rabbits immunized with exotoxin was lower than of those primed with LPS.

4.4 Immune function test

4.4.1 Nitrobluetetrazolium reduction test (NBT)

Nitrobluetetrazolium reduction test was used for neutrophil phagocyte assessment and the results were showed high mucosal neutrophil phagocytic activity in all endotoxin dose (1.5, 2.5 mg /Kg) of both *S. flexneri* LPS and *S.dysenteriae* LPS especially of the appendix. From the present data (Table 7) it was noted that the mean values of *S. flexneri* NBT were 45 ,47.2 for appendix and 39 , 41.1 for duodenum at dose (1.5, 2.5 mg / Kg LPS), respectively . In *S. dysenteriae* mean values of

Table (6):Humoral systemic and mucosal bacterial specific antibody titer mean%.

Microorganism Antigen	Systemic	Mucosal	
		Appendix*	Duodenum
S. flexneri **LPS 3 mg**	304	48	40
S. flexneri **LPS 5 mg**	320	48	41.6
S. dysenteriae **LPS 3 mg**	336	41.6	32
S. dysenteriae **LPS 5 mg**	304	44.8	38.4
S. dysenteriae **Exotoxin 1 mg**	256	44.8	27.2
S. dysenteriae **Exotoxin 2 mg**	288	48	30.4

***** Election of these two parts , because of high peyer's patches in appendix and low duodenum . appendix act as inductive sites and duodenum by far is the effector sites .

NBT at both doses (1.5, 2.5 mg /Kg LPS) were 46.3 ,49 at appendix and 42 ,44.4 at duodenum ,respectively.

Neutrophil phagocytic activity at mucosal system of rabbits immunized with both doses (0.5, 1 mg /Kg exotoxin) also increased, but lower than endotoxin particularly at duodenum and the mean value was 32.3, 36.4 at appendix and 29.4 ,31.2 at duodenum, respectively .

Systemic increase in NBT activity was also observed but was lower than mucosal activity especially, when exotoxin was used . The mean values were 35, 38.4 for *S. flexneri* dose (1.5, 2.5 mg/Kg LPS) while they were 37.4 ,38.7 in *S. dysenteriae* for that dose and 26.4 ,27.5 in *S. dysenteriae* dose(0.5, 1 mg /Kg exotoxin) for each one of the doses, respectively (Table 7).

Table (7): Mean % of Nitrobluetetrazolium reduction test for neutrophil phagocytes in rabbit immunized with *S.flexneri* and *S.dysenteriae* antigens at different doses

Microorganism antigen	Systemic	Mucosal	
		Appendix	Duodenum
S.flexneri **LPS 1.5 mg/Kg**	35	45	39
S.flexneri **LPS 2.5 mg/Kg**	38.4	47.2	41.1
S.dysenteriae **LPS 1.5 mg/Kg**	37.4	46.3	42
S.dysenteriae **LPS 2.5 mg/Kg**	38.7	49	44.4
S.dysenteriae **Exotoxin 0.5 mg/Kg**	26.4	32.3	29.4
S.dysenteriae **Exotoxin 1 mg/Kg**	27.5	36.4	31.2
NBT control	17.2	17.2	17.2

4.4.2 Leukocyte migration inhibitory factor (LIF) test

The assessment of mucosal LIF in the sense of migration percent was showing highly significant LIF than systemic LIF for both *S. flexneri* and *S .dysenteriae* dose (1.5, 2.5 mg/Kg LPS) and less than in *S. dysenteriae* dose (0.5, 1 mg /Kg exotoxin) .The mean value of the appendix mucosa was higher than those of duodenal mucosa and systemic for all endotoxin and exotoxin doses; the mean values of LIF were 35.4, 33.5 at mucosal appendix and 40.3 ,39 at duodeual mucosa for *S. flexeneri* dose (1.5, 2.5 mg/Kg LPS) while the mean values were 34.1, 32.4 at the mucosa of the appendix and 38.5, 39.9 at the duodenal mucosa for *S.dysenteriae* dose (1.5, 2.5mg /Kg LPS) , respectively.

Mucosal LIF mean values were 42.1, 39.6 at appendix and 51.1, 49.5 at duodenum when exotoxin doses were (0.5, 1 mg /Kg) of *S. dysenteriae* used as a sensitizer, accordingly.

Systemic LIF was showing lower mean values when the exotoxin was used as a sensitizer at both doses (0.5, 1 mg /Kg) and the mean values were 48, 45 in *S. flexneri* and 46 ,42.3 in *S. dysenteriae* at dose (1.5, 2.5 mg/Kg) of LPS, accordingly .For exotoxin dose ,the mean values were57 for dose(0.5 mg/Kg) ,54.8 for dose (1 mg /Kg) (Table 8).

Table (8): Mean % of LIF test for rabbit immunized with *S.flexneri* and *S.dysenteriae* antigens at different doses.

Microorganism antigen	Systemic	Mucosal	
		Appendix	Duodenum
S .flexneri **LPS 1.5 mg/Kg**	48	35.4	40.3
S. flexneri **LPS 2.5 mg/Kg**	45	33	39
S. dysenteriae **LPS 1.5 mg/Kg**	46	34.1	38.5
S. dysenteriae **LPS 2.5 mg/Kg**	42.3	32.4	39.9
S.dysenteriae **Exotoxin 0.5 mg/Kg**	57.6	42.1	51.1
S. dysenteriae **Exotoxin 1 mg/Kg**	54.8	39	49.5
LIF control	90.3	90.3	90.3

Figure (9-A): Positive NBT – neutrophil (Giemsa stain , 1000X).

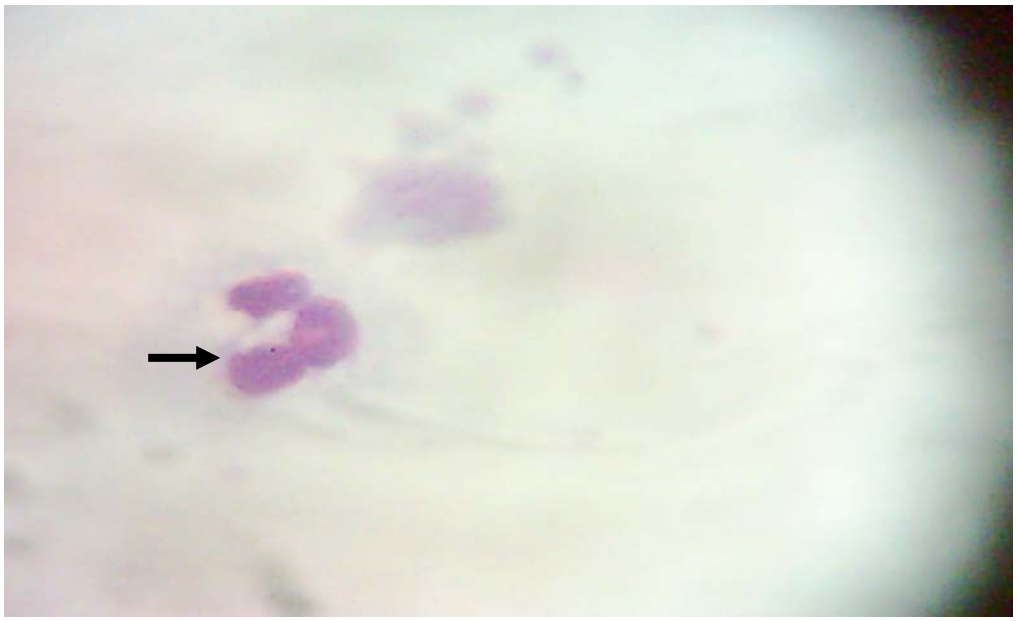

Figure (9-B): Negative NBT – neutrophil (Giemsa stain , 1000X).

4.4.3 Erythrocyte-rosette formation test (E-rosette test)

The mucosal lymphocytes E-rosette formation was higher as;47,49 at appendix and 40.2 ,42 at duodenum than of systemic which were 37.4, 39.9 for *S. flexneri* the doses(1.5 ,2.5mg /Kg LPS), respectively, also mucosal lymphocytes E-rosette formation was higher 48, 48.3 at appendix and 38, 40.4 at duodenum than that of systemic 36.4,40.6 for *S.dysenteriae* doses (1.5, 2.5mg /Kg LPS),respectively .

The mucosal lymphocytes E-rosette formation was also increased when the rabbits were immunized with two doses of exotoxin (0.5,1 mg /Kg)but less than of mucosal E-rosette when the rabbits were immunized with LPS as above ,the mean values were 40,44.4 at appendix and 32, 35.3 at duodenum for *S. dysenteriae* doses(0.5,1mg/Kg exotoxin), respectively .

The systemic lymphocytes E-rosette formation of exotoxin primed rabbits with two doses (0.5, 1mg/Kg) were also less than that of mucosal and the mean values were 30.2, 30.4 at dose (0.5,1mg/Kg), respectively.

Table (9) Mean% of E- rosette test for rabbit immunized with *S.flexneri* and *S.dysenteriae* antigens at different doses.

Microorganism antigen	Systemic	Mucosal	
		Appendix	Duodenum
S.flexneri LPS1.5 mg/Kg	37.4	47	40.2
S..flexneri LPS2.5 mg/Kg	39.9	49	42
S.dysenteriae LPS1.5 mg/Kg	36.4	48	38
*S.dysanteriae*LPS2.5 mg/Kg	40.6	48.3	40.4
S.dysenteriae Exotoxin 0.5 mg/Kg	30.2	40	32
S.dysenteriae Exotoxin 1 mg/Kg	30.4	44.1	35.3
E- rosette control	19.9	19.9	19.9

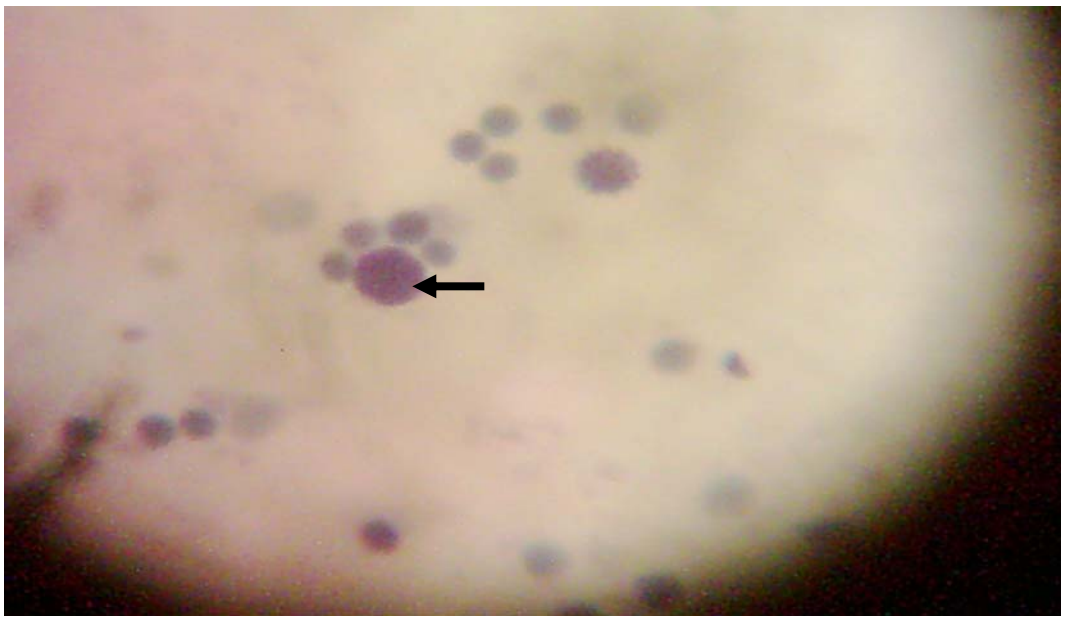

Figure (10-A): E-rosette positive T-lymphocyte (1000X).

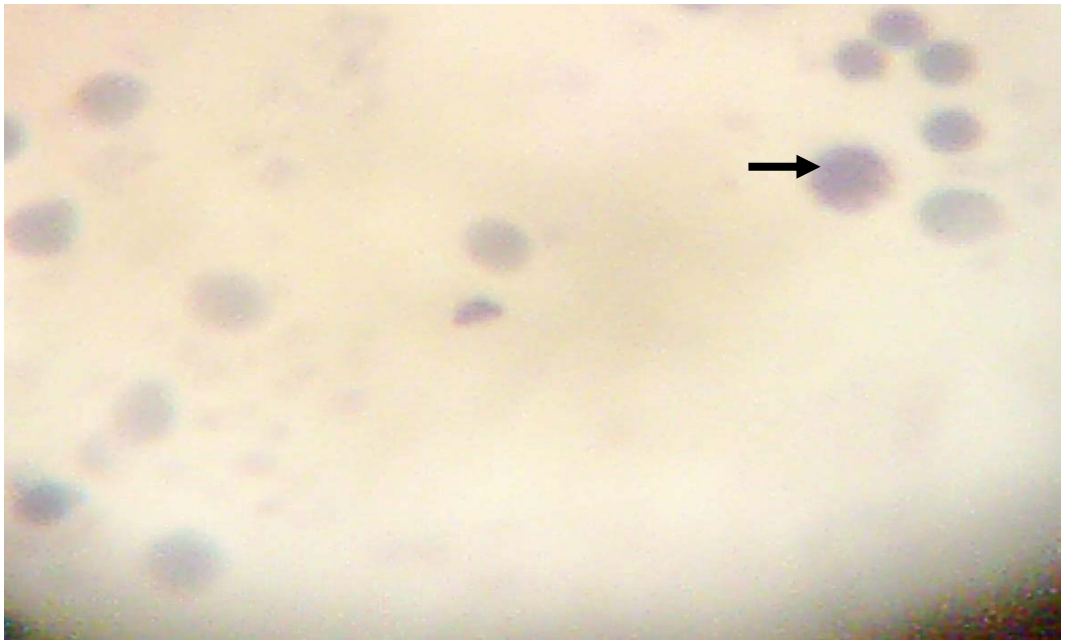

Figure (10-B): E-rosette negative T-lymphocyte (1000X).

4.4.4 Skin delayed type hypersensitivity.

Skin test of the exotoxin of primed rabbits with *Shigella* protein was of DTH tuberculin types. Likewise, for those rabbits primed with LPS of *S.flxneri* and *S.dysenteriae* .They showed erythem, induration and then necrosis. The induration diameter ranged from 10-12 mm in those primed with exotoxin and 15-20mm in those primed with LPS of both species .LIF of those exotoxin primed rabbits were with near boarder line significant of LIF 57-68% while those primed with LPS ranged from 31-46% (Table 10).

4.5 Systemic and mucosal immune response to *Shigella* in rabbits

4.5.1 Systemic immune response to *S .flexneri* LPS

The systemic humoral immune response was determined by specific antibody titer, that ranged between 160-640 with mean value of 304 for dose (1.5/Kg LPS) and between 160-640 with mean value of 320 for dose (2.5mg /Kg LPS).

The systemic neutrophile NBT phagocytosis test was between 25-45 with mean value 35 at dose (1.5mg/Kg LPS), and it ranges between 29-45 with mean value38.4 at dose (2.5mg /Kg LPS).

The systemic LIF of *S.flexneri* at both doses (1.5, 2.5mg/ Kg LPS) ranged between 39-57 with mean value 48.1 and between34-57 with mean value 45.2 for each dose, respectively.

E-rosette formation of systemic *S.flexneri* LPS primed rabbit was performed at both doses as above and it ranges between 27-49 with mean value 37.4 and between 30-51 with mean value 39.9 for each dose, respectively (Table 11).

Table (10): Skin delayed type hypersensitivity (DTH) test and LIF test of rabbits immunized with *Shigella* species .

Sequence rabbits	*S.dysenteriae* exotoxin 2mg		Sequence rabbits	*S.dysenteriae* LPS 5mg		Sequence rabbits	*S.flexneri* LPS 5mg	
	Skin test	LIF %		Skin test	LIF %		Skin test	LIF %
1	E.I.N.* within 24-48hrs 10mm	57	**1**	E.I.N. within 24-48hrs 15mm	34	**1**	E.I.N. within 24-48hrs 16mm	31
2	E.I.N. within 24-48 hrs 10mm	60	**2**	E.I.N. within 24-48 hrs 16mm	36	**2**	E.I.N. within 24-48hrs 17mm	35
3	E.I.N. within 24-48hrs 12mm	64	**3**	E.I.N. within 24-48hrs 17mm	40	**3**	E.I.N. within 24-48hrs 18mm	38
4	E.I.N. within 24-48hrs 12mm	66	**4**	E.I.N. within 24-48hrs 17mm	43	**4**	E.I.N. within 24-48hrs 18mm	41
5	E.I.N. within 24-48hrs 12mm	68	**5**	E.I.N. within 24-48hrs 18mm	48	**5**	E.I.N. within 24-48hrs 20mm	46

*E= Erythema , I= Induration , N=Necrosis , mm= Indurations diameters in mm

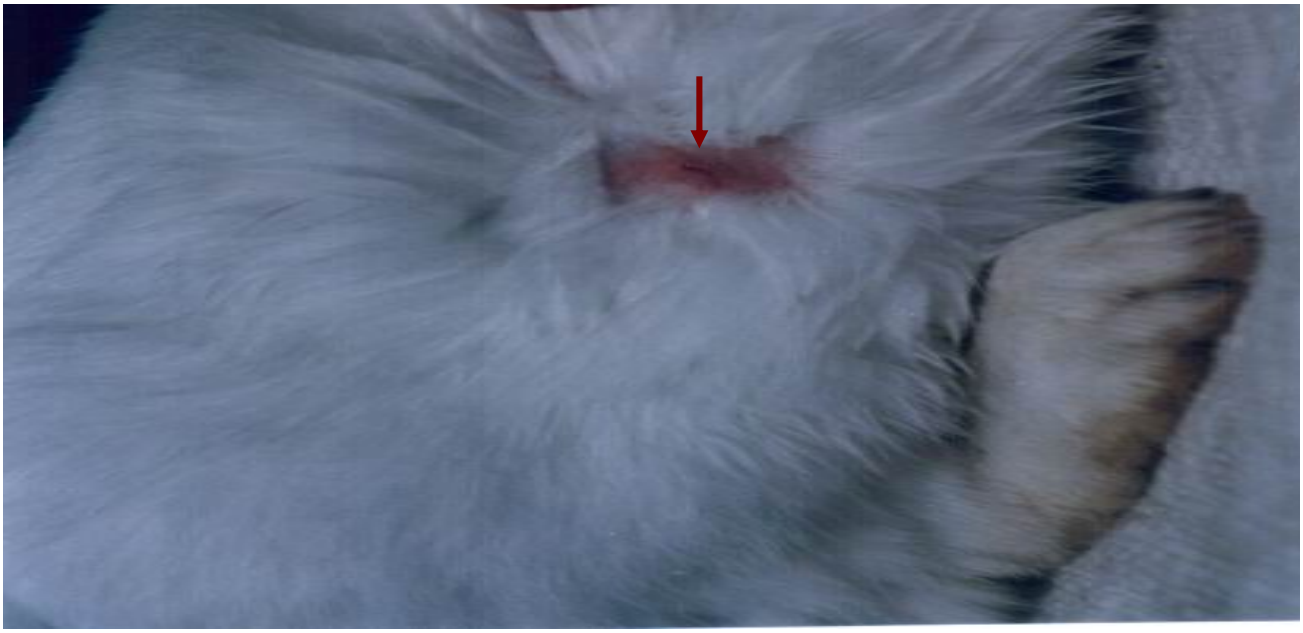

Figure (11): Positive skin test in rabbit (tuberculin type).

Table (11) :Systemic humorul (titer)and cellular(LIF, NBT,E-rosette) immune response to *Shigella flexneri* LPS in rabbit

Parameters	Specific immunopriming of rabbits with LPS 1.5 / Kg				Specific immunopriming of rabbits with LPS 2.5 / Kg			
	range	median	mean	SD ±	range	median	mean	SD ±
Humoral titer	160-640	320	304	140	160-640	320	320	130
Cellular NBT	25-45	35.5	35	6.9	29-45	40	38.4	5.5
Cellular LIF	39-57	48.5	48.1	6.2	34-57	45	45.2	7.6
Cellular E-rosette	27-49	37.5	37.4	6.9	30-51	40	39.9	7

SD= Standard deviation

4-5.2 Mucosal immune response to *S.flexneri* LPS

As in *S. dysenteriae* LPS was administrated in two doses (1.5,2.5mg /Kg). Ten rabbits were used for each concentration .The mucosal specific antibody titer was evaluated by using standardized tube agglutination test at both appendix and duodenum; at appendix the antibody titer ranges between32-64 with mean value48 for dose (LPS 1.5mg/Kg), meantime the

duodenal ranges between 16-64 with mean value 40 at the same dose, but at dose (LPS 2.5mg/Kg) the range was between 32-64 with mean value 48for dose (LPS 1.5mg/Kg), while the duodenal ranges between 16-64 with mean value 40 at the same dose, but at dose (LPS 2.5mg/Kg) the range was between 32-64 with mean value 48 and between 32-64 with mean value 41.6 for appendix and duodenum, respectively.

Non specific mucosal cellular immune response was investigated by neutrophil NBT test and its range was between 34-54 with mean value 45 at appendix and between30-48 with mean value of 39 at duodenum for dose (1.5mg/Kg LPS) whereas it ranged between 38-58 with mean value 47.2 at appendix and between 31-48 with mean value 41.1 at duodenum for dose (2.5mg /Kg LPS).

The mucosal LIF of appendix in case of (1.5mg/ Kg endotoxin) dose ranged between 26-42 with mean value of 35.4 and between 33-49 with mean value 40.3 at duodenum while the range of specific cellular LIF at dose (2.5mg/Kg LPS) between 23-40 with mean value of 33.5 and between 30-50 with mean value 39 for appendix and duodenum, accordingly. The lymphocytes E-rosette formation range was between 38-53 with mean value 47 for appendix and it ranges between 31-52 with mean value of 40.2 for duodenum at dose (1.5 mg/Kg LPS) while, the range of E-rosette as specific cellular immune response was as 34-51 with mean value of 49 and between 30-48 with mean value of 42 for (2.5mg /Kg LPS) at appendix and duodenum, respectively (Table 12).

Table (12) :Mucosal humoral (Titer) and cellular (LIF,NBT,E-rosette) immune response to *Shigella flexneri* LPS in rabbit.

Parameters	Specific immunopriming of rabbits with LPS 1.5 / Kg				Specific immunopriming of rabbits with LPS 2.5 / Kg			
	range	**median**	**mean**	**SD ±**	**range**	**median**	**mean**	**SD ±**
Humoral titer appendix	32-64	48	48	16.8	32-64	48	48	16.8
Humoral titer duodenum	16-64	32	40	17.2	32-64	32	41	17
Cellular appendix NBT	34-54	46.5	45	6.8	38-58	47.5	47.2	7.3
Cellular appendix LIF	26-42	36.5	35.4	5.3	25-40	34.5	33.5	5.6
Cellular appendix E-rosette	38-53	48.5	47	5.5	34-51	47.5	49	7.2
Cellular duodenum NBT	30-48	38.5	39	6	31-48	41	41.1	5.7
Cellular duodenum LIF	33-49	39.5	40.3	4.6	30-50	38.5	39	7
Cellular duodenum E-rosette	31-52	39	40.2	7.3	30-48	40	42	6.7

Table (13): Systemic and mucosal mean% of the immune response parameters (Titer ,LIF ,NBT, E-rosette) to *Shigella flexneri* Endotoxin

Parameters	Systemic mean %		mucosal			
			Appendix		Duodenum	
	LPS					
	1.5 mg/Kg	2.5 mg/Kg	1.5 mg/Kg	2.5 mg/Kg	1.5 mg/Kg	2.5 mg/Kg
LPS Humoral mean % titer	304	320	48	48	40	41.6
LPS Cellular NBT mean %	35	38.4	45	47.2	39	41.1
LPS Cellular LIF mean %	48	45	35.4	33.5	40.3	39
LPS Cellular E-rosette mean %	37.4	39.9	47	49	40.3	42

4.5.3 Systemic immune response to *S. dysenteriae* LPS

At systemic immune response, the specific antibody titers was also estimated by using standard tube agglutination test, and it ranges were between 160-640 with mean value 336 and between 160-640 with mean value 304 for doses (1.5, 2.5mg /kg)*S.dysenteriae* LPS.(Table 14).

Non specific cellular immune response of *S. dysenteriae* LPS was investigated by NBT test and the range for it at dose (1.5mg/Kg LPS)was between 31-44 with mean value 37.4 whereas it range was between 30-48with mean value 38.7 at dose (2.5ml /Kg LPS), respectively.

LIF for systemic was performed as specific cellular parameters and LIF at dose 1.5 mg/ Kg was showing range between 39-53 with mean value 46 but at dose 2.5mg /Kg LPS was showing range between 34-52 with mean value 42.3 .

E-rosette formation was appearing to range between 31-49 with mean value 36.4 at dose (1.5 mg/Kg LPS) whereas was appearing range between 33-48 with mean value 40.6 at dose(2.5mg/Kg LPS) of *S. dysenteriae* .

Table (14): Systemic humoral (titer) and cellular (LIF,NBT,E-rosette) immune response to *Shigella dysenteriae* LPS in rabbit

Parameters	Specific immunopriming of rabbits with LPS 1.5 mg/Kg				Specific immunopriming of rabbits with LPS 2.5 mg/Kg			
	range	median	mean	SD ±	range	median	mean	SD ±
Humoral titer	160-640	320	336	176	160-640	320	304	140
Cellular NBT	31-44	37.5	37.4	4.2	30-38	37.5	38.7	6.3
Cellular LIF	39-53	46.5	46	5.1	34-52	41.5	42.3	6.2
Cellular E- rosette	31-49	38	36.4	6.7	33-38	41.5	40.6	5.5
NBT control	15-20	16.5	17.2		15-20	16.5	17.2	
LIF control	88-93	90	90.3		88-93	90	90.3	
E- rosette control	17-22	20.5	19.9		17-22	20.5	19.9	

4.5.4 Mucosal immune response to *S.dysenteriae* LPS

The LPS of *S. dysenteriae* was used in two doses (1.5,2.5mg/ Kg).(Table 15).For each concentration ten rabbits were used .The specific mucosal humoral immune response was assessed by the determination of antibody titers by using standardized tube agglutination test. The titer ranges were between 32-64 with mean value 41.6 at appendix and between 32-32 with mean value 32 at duodenum for(1.5mg /Kg LPS dose), while for (2.5mg /Kg LPS dose) ,the range was 32-64 with mean value44.8 and as32-64 with mean value 38.4 at appendix and duodenum, respectively.

Non specific cellular immune response of the rabbits primed with *S. dysenteriae* LPS performed at both appendix and duodenum with two doses as above by NBT test used to detect phagocytic activity, however ,at appendix the range was between 40-53 with mean value 46.3 for LPS of dose (1.5mg/ Kg,),and at the same dose the range of neutrophil NBT of duodenum was between 34-49 with mean value 42,but at dose (2.5ml/Kg LPS) NBT range was as 40-57 with mean value 48.6 and as 35-52 with mean value 44.4 for appendix and duodenum ,respectively.

A specific cellular immune response of the rabbits to this bacterial toxin was evaluated by LIF and E-rosette. The LIF range at appendix for dose (1.5mg /Kg LPS) was between 30-40 with mean value 34.1 and duodenal LIF at this dose was between 31-48.5 with mean value 38.5 with the range of appendix LIF between 27-37 with mean value 32.4 and for duodenum, the range was between 33-49 with mean value 39.9 at (2.5mg/ Kg LPS).

E- rosette formation value ranged, determined and found to be between 37-55 with mean value 48 at appendix for dose 1.5mg /Kg LPS as well as duodenum as 32-45 with mean value as 38 .While, the E- rosette formation ranged between 42-56 with mean value 48.3 and between 33-48 with mean value 40.4 for dose (2.5mg /Kg LPS) at appendix and duodenum, accordingly .

Table (15) :Mucosal humoral (titer) and cellular (LIF,NBT,E-rosette) immune response to *Shigella dysenteriae* LPS in rabbit .

Parameters	Specific immunopriming of rabbits with LPS 1.5 mg/Kg				Specific immunopriming of rabbits with LPS 2.5 mg/Kg			
	range	median	mean	SD ±	range	median	mean	SD ±
Humoral titer appendix	32-64	32	41.6	13.5	32-64	32	44.8	16.5
Humoral titer duodenum	32	32	32	0	32-64	32	38.4	15.8
Cellular appendix NBT	40-53	45	46.3	4.3	40-57	48.5	48.6	5.3
Cellular appendix LIF	30-40	34	34.1	3.4	27-37	33	32.4	3.4
Cellular appendix E-rosette	37-55	48	48	5.2	42-56	48	48.3	5.4
Cellular duodenum NBT	34-49	43	42	5	35-52	45.5	44.4	5.7
Cellular duodenum LIF	31-48	37	38.5	5.8	33-49	38.5	39.9	5.6
Cellular duodenum E-rosette	32-45	36.5	48	4.3	33-48	40.5	40.4	4.7
NBT control	15-20	16.5	17.2		15-20	16.5	17.2	
LIF control	88-93	90	90.3		88-93	90	90.3	
E- rosette control	17-22	20.5	19.9		17-22	20.5	19.9	

4.5.5 Systemic immune response to *S.dysenteriae* exotoxin

For systemic humoral immune response the ranges of specific antibody titer were 160-320 with mean value 256 for (0.5mg /Kg exotoxin) dose and 160-640 with mean value 288 for (1mg/Kg exotoxin) dose.(Table 16)

The range of systemic neutrophils NBT test as non specific cellular immune response was between 22-32 with mean value 26.4 and 22-36 with mean value 27.5 for the doses (0.5, 1mg /Kg), respectively.

At the both doses of exotoxin (0.5, 1mg /Kg), LIF ranges of systemic immune response were between 48-67 with mean value 57.6 and 47-66 with mean value 54.8 for each doses, accordingly.

E-rosette formation range at systemic immune response was between 23-37 with mean value 30.2 for (0.5mg/ Kg exotoxin) dose and it ranges as 24-38 with mean value of 30.4 at the dose of (1mg /Kg).

Table (16) :Systemic humoral (HA) and cellular (LIF,NBT,E-rosette) immune response to *Shigella dysenteriae* exotoxin in rabbit.

Parameters	Specific immunopriming of rabbits with exotoxin 0.5mg/Kg				Specific immunopriming of rabbits with exotoxin 1mg/Kg			
	range	median	mean	SD ±	range	median	mean	SD ±
Humoral HA titer	160-320	320	256	82.6	160-640	320	288	147
Cellular NBT	22-32	26.5	26.4	3.6	22-36	31	27.5	5.1
Cellular LIF	48-67	57	57.6	7.3	47-66	53	54.8	6.4
Cellular E- rosette	23-37	31	30.2	4.6	24-38	29	30.4	4.8
NBT control	15-20	16.5	17.2		15-20	16.5	17.2	
LIF control	88-93	90	90.3		88-93	90	90.3	
E- rosette control	17-22	20.5	19.9		17-22	20.5	19.9	

4.5.6 Mucosal immune response to *S.dysenteriae* exotoxins

The exotoxin was attempted in two doses (0.5,1mg /Kg) .For each one concentration, ten rabbits were used using standardized immunization protocols. The mucosal humoral immune response as probed by HA and the specific antibody titers ranged between 32-64 with mean value44.8 at appendix and it ranges between 16-32 with mean value 27.2 at duodenum for (0.5 mg/Kg exotoxin) dose whereas they ranged between 32-64 with mean value 48 for appendix and between 16-32 with mean value 30.4 for duodenum at a dose (1 mg/Kg exotoxin).

At the cellular level different parameters were considered including neutrophil phagocytic activity (NBT), LIF and E-rosette formation by T-lymphocytes.

Non specific cellular immune response was studied represented by NBT test at both of appendix and duodenum with (0.5,1 mg/Kg exotoxin) for each one. At appendix (0.5 mg /Kg exotoxin) dose, it gives the values that ranges between 26-38 with mean value 32.2 and for duodenum, the range was between 23-37 with mean value 29.4, but for (1mg/Kg exotoxin) dose, NBT range was between 30-45 with mean value 36.4 for appendix and 25-40 with mean value 31.2 for duodenum. (Table 17)

Specific cellular immune response was studied and the mucosal LIF of appendix in case of (0.5 mg /Kg exotoxin) dose was ranged between 34-52 with mean value 42.1, while for the duodenum (0.5mg/Kg exotoxin),the LIF values ranged between 41-60 with mean value 51.1, meantime the range of (1 mg/ Kg exotoxin) dose was between 32-49 at appendix and between 37-59 at duodenum with mean value 39.6 and 49.5 for appendix and duodenum, accordingly.

E-rosette formations were also investigated as specific cellular immune response and they ranged at the appendix (0.5 mg/Kg exotoxin) dose was between 29-48 with mean value 40.In the same above mentioned dose of

exotoxin ,duodenal E-rosette formation ranged between 27-39 with mean value 32 .The range of appendix and duodenal E-rosette formation for the dose of (1mg/Kg exotoxin) were between 35-53 and 28-41 with mean value 44.1 and 35.3, respectively .

Table (17) :Mucosal humoral (HA) and cellular (LIF,NBT,E-rosette) immune response to *Shigella dysenteriae* exotoxin in rabbit.

Parameters	Specific immunopriming of rabbits with exotoxin 0.5mg/Kg				Specific immunopriming of rabbits with exotoxin 1mg/Kg			
	range	median	mean	SD ±	range	median	mean	SD ±
Humoral HA titer appendix	32-64	32	44.8	16.5	32-64	48	48	16.8
Humoral HA titer duodenum	16-32	32	27.2	8.4	16-32	32	30.4	6.9
Cellular appendix NBT	26-38	32.5	32.3	4.1	30-45	36.5	36.4	5.3
Cellular appendix LIF	34-52	41	42.1	6.4	32-49	39	39.6	5.8
Cellular appendix E-rosette	29-48	37	40	7.1	35-53	45	44.1	5.7
Cellular duodenum NBT	23-37	29	29.9	4.6	25-40	30.5	31.2	5.1
Cellular duodenum LIF	41-60	51	51.1	7.2	37-59	49.5	49.5	6.2
Cellular duodenum E-rosette	27-39	32.5	32	4.3	28-41	36	35.3	4.1
NBT control	15-20	16.5	17.2		15-20	16.5	17.2	
LIF control	88-93	90	90.3		88-93	90	90.3	
E- rosette control	17-22	20.5	19.9		17-22	20.5	19.9	

Table (18): Systemic and mucosal humoral (HA) and cellular (LIF, NBT, E-rosette) mean % to *Shigella. dysenteriae* exotoxin and LPS.

Parameters	Systemic Exotoxin		Mucosal Appendix Exotoxin		Mucosal Duodenum Exotoxin		Parameters	Systemic LPS		Mucosal Appendix LPS		Mucosal Duodenum LPS	
	0.5 mg	1 mg	0.5 mg	1 mg	0.5 mg	1 mg		1.5 mg	2.5 mg	1.5 mg	2.5 mg	1.5 mg	2.5 mg
Exotoxin humoral HA titer mean %	256	288	44.8	48	27.2	30.4	Endotoxin humoral titer mean %	336	304	41.6	44.8	32	38.8
Exotoxin cellular NBT mean %	576	548	32.3	36.4	29.4	31.2	Endotoxin cellular NBT mean %	46	433	46.3	49	42	44.4
Exotoxin cellular LIF mean %	26.4	27.5	42.1	39.6	51.1	49.5	Endotoxin cellular LIF mean %	37.4	38.7	34.1	32.4	38.5	39.9
Exotoxin cellular E-rosette mean %	30.2	30.4	40	44.1	32	35.3	Endotoxin cellular E-rosette mean %	36.4	40.6	48	48.3	38	40.4

4.5.7 Systemic versus mucosal immune responses to Shigella toxins(immunogen).

The ratio of systemic to mucosal responses is of dynamic state ,it varies in various immunological changes ,such as immune responses to immunogen and ad quant effect.(Table 19) .

Table (19) : The ratio of systemic to mucosal immune responses to *Shigella* toxins.

Ration *S /**M	*S.dysenteriae* exotoxin				*S.dysenteriae LPS*				*S.flexneri LPS*			
	Systemic		**Systemic**		**Systemic**		**Systemic**		**Systemic**		**Systemic**	
	Mucosal Appendix		**Mucosal Duodenum**		**Mucosal Appendix**		**Mucosal Duodenum**		**Mucosal Appendix**		**Mucosal Duodenum**	
	0.5 mg	**1 mg**	**0.5 mg**	**1 mg**	**1.5 mg**	**2.5 mg**	**1.5 mg**	**2.5 mg**	**1.5 mg**	**2.5 mg**	**1.5 mg**	**2.5 mg**
***Shigella* Specific Ab. Titer**	5.7/1	6/1	9.4/1	9.4/1	8/1	6.7/1	10.5/1	7.91/1	6.3/1	6.6/1	7.6/1	7.6/1
NBT	0.81/1	0.7/1	0.89/1	0.88/1	0.8/1	0.79/1	0.89/1	0.87/1	0.7/1	0.81/1	0.89/1	0.93/1
LIF	1.36/1	1.38/1	1.12/1	1.1/1	1.34/1	1.3/1	1.19/1	1/1	1.37/1	1.36/1	1.2/1	1.15/1
E-rosette	0.75/1	0.69/1	0.94/1	0.86/1	0.75/1	0.84/1	0.95/1	1/1	0.79/1	0.81/1	0.93/1	0.95/1

Ab=Antibody
S= Systemic
M=Mucosal

Chapter Five
Discussion

5.1 Systemic and mucosal humoral immune response to *Shigella* in rabbits

5.1.1 Systemic humoral immune response to *S.flexneri* LPS

Table (11) showed that LPS of *S.flexneri* at dose 1.5,2.5 mg/Kg able to stimulate specific systemic humoral immune response, but statistically non-significant ($p > 0.05$) and the results were lower than that of mucosal humoral immune response of rabbit immunized with this toxin at the same doses which is consistent with(Van-Deverg *et al.*,1992; Van-Deverg *et al.,* 1995) who mentioned that mice infected with *S.flexneri* LPS developed significantly elevated level of serum immunoglobulin .In guinea pigs infected with *S.flexneri* LPS revealed an increase in serum immunoglobulin titers (Noriega *et al.,* 1999).

In humans infection with *S.flexneri* revealed the production of serum immunoglobulin IgG, which is specific for some bacterial virulence protein and for LPS, the major bacterial surface component (Sansonetti *et al.,* 1996). In addition, several studies reported that the serum anti-LPS IgA mediated antibody response is the major protective response against homologous reinfection with *S.flexneri* (Sansonetti *et al.,* 1996 ;Cohen *et al.,* 1997).

On the other hand, Karnell *et al.,* 1993 who reported that serum immunoglobulin against LPS of *S.flexneri* was lesser than that of secretory immunoglobulin IgA that was in agreement with the result above. This may interpret that LPS of *S.flexneri* causes an increase in mucosal humoral immune response as compared with systemic humoral immune response because of its direct contact of LPS (given orally) with mucosal follicles involved in the preferential synthesis of IgA. However, cell derived from mucosal lymphoid aggregates, but not those from the spleen, have the capacity to induce secretory IgM (SIgM)- positive B cells to undergo isotype switching to SIgA-positive B cells. Likewise,

there is evidence that certain T cells secret IL-5, which, along with other lymphokines such as IL-6 has preferential effect on IgA B cell differentiation. Either these type of T cells having effect on IgA B cell differentiation may be more abundant in mucosal areas than elsewhere and may act in concert with switch cells to lead to preferential IgA B cell maturation in the mucosal follicles (Sites *et al.,* 1994).

5.1.2 Mucosal humoral immune response to *S. flexneri* LPS

The results expressed in table (12) revealed that LPS of *S.flexneri* is able to stimulate the mucosal humoral immune response for rabbits immunized with 1.5,2.5mg/Kg LPS and their mean titer values were significant($P<0.05$)increased, particularly at appendix for both doses.(Karnell *et al.,*1993)reported that LPS of *S.flexneri* cause significant increase in specific secretory IgA in monkeys after four doses with LPS that given orally. In human (Salmatova *et al.,*1993)reported that IgA antibodies were found to be active mainly against common determinant of *S.flexneri* LPS in secretion .In other studies (Islam *et al.,* 1997; Herias *et al.,*1997) reported that infection with *S.flexneri* cause significant increase in mucosal humoral immune response. Secretory IgA (SIgA) is the most characteristic component of productive immunity; this is particularly true for the gastero intestinal tract, where the large number of Ig- producing immunocytes in mucosal effector site such as lamina propria or appendix of the gut, which contain high number of plasma cells, committed to the secretory of IgA antibody (Brandtzage *et al.,* 1999).

5.1.3 Systemic humoral immune response to *S.dysenteriae* LPS

Likewise, LPS of S.dysenteriae at both doses 1.5, 2.5 mg /Kg cause increase in systemic humoral immune response, generally, the mean titer

values of systemic humoral immune response were less than mucosal humoral immune response of *S.dysenteriae* LPS at the same doses (Table 14) .The ability of *S.dysenteriae* to stimulate systemic humoral immune response was in agreement with that reported by (Cohen *et al.*,1988; Azim *et al.,* 1996)

The results above may depend on the structure of systemic and mucosal compartment where the last system contains numerous number of plasma cells in mucosa –associated lymphoid tissues that produce SIgA. These cells mainly reticulate within the mucosal lymphoid system. Thus, lymphoid cells stimulate in Peyer's patches pass via regional lymph nodes to the blood stream and then home back into the intestinal lamina propria. Specific recirculation is made possible because the lymphoid cells expressing homing molecules attach to adhesion molecules specifically expressed on endothelial cell adhesions of the mucosal postcapillary venles ,but these are absent from lymph node .Thus ,antigen stimulation at one mucosal area elicits an antibody response, largely restricted to the MALT (Roitt *et al.,* 2001).

On the other hand, there is a group of Th2 cytokines shown to be an essential signaling factor in the triggering of $SIgA^+$ B cells to become IgA –secreting plasma cells, such as ,IL-5 ,IL-6 and / or IL -10 were detected in vitro after $SIgA^+$ B cells had differentiated into plasma cells, resulting in IgA synthesis (Beagley *et al.,* 1988 ; Lebman and Coffman.,1988).In addition , IL-15, Like the above mentioned IgA –enhancing Th2-cytokines, affected IgA B cell development in mucosal but not in systemic compartments (Hiroi *et al.,* 2000).

B-1 cells could be a major source of common mucosal immune system (CMIS) –independent IgA plasma cells by suggesting that the IL-15 and IL-15 resignaling cascade is an essential element for differentiation of

CMIS- independent $SIgA^+$ B-1 cells into IgA plasma cells (Hirio *et al.*, 1995).

5.1.4 Mucosal humoral immune response to *S.dysenteriae* LPS

Table (15) showed that LPS of *S.dysenteriae* is able to stimulate mucosal humoral immune response at doses of 3, 5 mg and lead to increase in mean titer values, especially at appendix (Levenson and Egorova,1990) .It has been reported that LPS of S.dysenteriae was inducing highly active mucosal O-antibody response and protection against Shigella infection in guinea pigs and monkeys. In mice, *S.dysenteriae* LPS cause increase in secretory IgA level (Stein *et al.*, 1992). Several essential virulence factors of *Shigella dysenteriae* including LPS elicit substantial antibody responses in secretion after infection in this bacterium in humans (Oberhelman *et al.,*1991).On the other hand; there is substantial evidence which indicates that LPS is able to activate humoral immune response at mucosal levels (Remick *et al.*, 1995).

The increase in specific mucosal humoral immune response at mucosal system, especially at appendix may be interpretation on the basis that oral administrations of antigen best stimulation of gut IgA immunity (Lycke, 1998).Furthermore, the follicle-associated epithelium in rabbit appendix has a highly enhanced capacity to transmit antigen from the gut lumen into the tissue where it can activate a mucosal immune response (Schaffner *et al.,* 1974). Furthermore, the rabbit appendix considered in the same category as the Peyer's patches (Mage, 1998); those acting as mucosal effector tissue are enriched in plasma cell that produced secretory IgA. (Hiroi *et al.,* 2000) .Secretory IgA are the predominant type of immunoglobulin secreted by the B lymphocytes of the gut (70-90%) of all immunoglobulin present in normal intestine mucosa (Lamm,

1997).In contrast to IgA in serum, secrtory IgA are present in dimeric or polymeric form and are thus resistant to intraluminal proteolysis (Bouvet and Fischetti, 1999). Moreover B-1 cells (mucosal B cells can be classified into B-1 cells and conventional B(B-2) cells based on the expression of B220 ,IgM,IgD ,CD5 ,and Mac-1 and based on surface CD5 expression ,the B-1 cell divided into a $CD^{+}5$ B-1 a cell and a $CD^{-}5$-B-1b) constituted a major fraction of B cells in mucosal effectors tissue including intestinal Peyer's patches .Among enriched B-1 cells ,the B-1b cell fraction showed a particularly strong expression of surface (s) IgA (Hiroi *et al.,* 1999). In addition, it has been suggested that B-1 cells are a major supplier for IgA plasma cells in mucosal effectors tissue (Kroese *et al.,* 1989; Solvason *et al.,* 1991 Kroces *et al.,*1993).Furthermore, a selected cytokine produced mainly by Th2-type T cells such as IL-5 has been shown to tightly regulate the differentiation of mucosal B-1 cells into IgA antibody-producing cells (McGhee *et al.,* 1989).Furthermore, IL-15 that can be produced from intestinal epithelial cells in mice , rats and human when infected with *S.dysenteriae* have important role in B-proliferation and immunoglobulin synthesis in mucosal effector tissues that increase production secretory IgA in this tissue (Armitage *et al.,* 1995;Reinecker *et al.,*1996; Inagaki *et al.,* 1997;Grieble *et al.,*1999).

On the other hand, the significance in mean titer value at mucosal levels ,may depend on that bulk of the body's lymphoid tissue is found associated with the mucosal system ,especially the gut- associated lymphoid tissues (GALT) , as this is a major pathway of entry for external antigens (Roitt *et al.,* 2001) .These lymphoid tissues such as Peyer's patches or rabbit appendix represent large lymphoid aggregates in the intestinal wall and contain many follicles packed with proliferating B cells, progenitors of the plasma cells that manufacture antibody during immune response .

Isotype switching is an important event in humoral immunity and this is the phase of B cell development that is most commonly associated with the Peyer's patches that represent in the same category as the rabbit appendix. Commitment of B cells to the IgA lineage by switching to the production of the heavy chain is an important aspect of Peyer's patches function .This is largely a gut centric model in which the B cells that are activated in the rabbit appendix will emigrate via lymphatic so that these can disseminate along the length of the gut where they become IgA B cells (McIntgre *et al.,* 1999).Likewise ,there are a number of species in which Peyer's patches like (PP) structure are important for B cell ontogeny and repertoire expansion .In rabbits , pp-like structures are sites of diversification of V_H genes by hyper mutation and mechanisms that resemble gene conversion (Mage ,1998). In recent studies it was found that the excision of pp-like structures from neonatal rabbits (including appendix and succubus rotundas) causes a considerable reduction at the level of somatic hypermutation in the Ig genes (Vajdy *et al.,* 1998).

(Waksman and Ozer ,1976)described the pp-like structures as factories for the production of lymphocytes .In sheep it has been estimated that almost three billion B cells are produced each hour in pp- like structures (Reynolds ,1986).

5.1.5 Systemic humoral immune response *to S.dysenteriae* exotoxin

Table (16) showed that exotoxin of *S.dysenteriae* at systemic levels is able to stimulate systemic specific humoral immune response for both doses 0.5,1 mg/Kg given orally but this response was statistically less than that of exotoxin at mucosal system (p>0.05).This result is in agreement with those reported by (Levine *et al.,* 1992 ;Azim *et al.,* 1999) who mention that *Shigella*-toxin (exotoxin)antibody titers were

determined in the serum of children infected with *S.dysenteriae* 1. (Oberhelman *et al.,*1991; Cam *et al.,*1993) reported that humoral immune response in systemic compartments has been well documented after infection with *S.dysenteriae* exotoxin.

5.1.6 Mucosal humoral immune response to *S.dysentriae* exotoxin

Table (17) showed that exotoxin of *S.dysenteriae* at doses 0.5,1 mg/Kg stimulates mucosal humoral immunce response ,especially at appendix , but lesser than that of rabbits immunized with *S.dysenteriae* LPS doses 3 , 5 mg at mucosal level .These results are in agreement with those reported by (Yamada *et al.,* 1994; Azim *et al.,* 1995) in which they mention that antibody titer to exotoxin increases in secretion but less than that of LPS of patient with shigellosis.Other reported (Islam *et al.,* 1997) indicate that infection with *S.dysenteriae* causes an increase in mucosal humoral immune response to *S.dysenteriae* exotoxin (Sts). Furthermore, (MeIver *et a.,*1975; Keusch *et al*., 1995) reported that human infected with *S.dysenteriae* developed antibody to exotoxin at mucosal levels.

The decrease in mucosal humoral mean titer of rabbits immunized with exotoxin doses 1,2 mg as compared with those of rabbits immunized with *S.dysenteriae* LPS may depend on inhibition of antibody secreting cells by Shiga toxin (Sts) as has been shown to inhibit stem cells producing IgG and IgA secreting cells (Cohen *et al.,* 1990) .Likewise, the decrease in mucosal humoral immune response to exotoxin may be related to the ability of Shiga toxin (exotoxin) to bind to and damage endothelial cells in intestine in addition to inhibit of protein synthesis (Kandel *et al.,* 1989). In the same time, LPS represent the major *Shigella* somatic antigen elicits multiple host response, including activation of cells of the innate immune system (B cell mitogen rich) (Bohuslav *et al.,* 1998).

5.2 Cellular immune function test

5.2.1 Nitrobluetetrazolium reduction test (NBT)

This test was used to measuring neutrophil phagocytic activity as non-specific cellular immunity .The NBT test is used to detect the presence of reactive oxygen species generated as a result of the phagocytic process. Phagocytic cells are known to generate reactive oxygen species such as O_2^- and H_2O_2 when exposed to a variety of agent interacting with the cell membrane .This reaction –known as the oxidative burst that involves one electron reduction of oxygen to O_2^- catalysed by an NADPH or NADH oxidase utilizing NADPH or NADH as electron donors. A portion of the O_2^- is converted to H_2O_2 via spontaneous or enzyme-facilitated dismutation (Klepanoff, 1991; Chohan *et al.,* 2001).

$$NADPH + O_2 \rightleftarrows NADP + 2O_2^- + \text{(superoxide anion)}.$$

NBT is a soluble, yellow- coloured which acts as an electron acceptor and it is used to detect indirectly the production of superoxide by stimulated PMNs, NBT is converted to a dark- blue formazan as outline in the following equation:

$$NBT + O^-_2 \longrightarrow \text{formazan stippling} + O_2$$

The dark- blue formazan stippling can be seen microscopically therefore, providing an assay method to screen neutrophils for the capacity to undergo oxidative metabolism (Metacalf, 1986).

In the present study, NBT test was used to detect phagocytic activity of neutrophils in rabbits immunized with LPS of *S.flexneri* and *S.dysenteriae* for doses 3, 5 mg and *S.dysenteriae* exotoxin at mucosal and systemic levels- Both .LPS *S. flexneri* and *S.dysenteriae* lead to increase in phagocytic activity at mucosal and systemic levels, but the mean values of mucosal system particularly at appendix were statistically significant ($p<0.05$) than of systemic for both bacteria (Table 7, 13, 15). Similar results were showed when the rabbits were immunized with

S.dysenteriae exotoxin for both doses 0.5,1mg/Kg, but the ability of exotoxin to stimulate phagocytic cells seem to be lesser than that of LPS (table 7, 18).

NBT test results above are in accordance with that reported by (Adam *et al.*, 1995) who mention that invasion of epithelial cells by *Shigella* is an essential process of bacterium- induced phagocytosis. (Mathan and Mathan, 1991, b) reported that interaction with *Shigella* was associated with streaming of polymorphnuclear leukocytes (PMNs) into the intestinal lumen. (Sansonetti *et al.,* 1999; Buchrieser *et al.,* 2000) showed that *Shigella* pathogencity island (a 214-kb virulence plasmid contains most of the genes required to express the keys of the invasive phenotype) is necessary and sufficient to cause entry into epithelial cells via macropinocytosis, macrophage apoptotic death and activation of polymorphnuclear cells.

Similarly, (Anand *et al.,* 1986) mention that infection with *Shigella* causes infiltration of PMNs cells into the lamina propria. On the other hand, the ability of *Shigella* LPS to activation of neutrophils is in agreement with those reported by (Rietschel *et al.,* 1996) who showed that LPS interacts with virtually all components of the cellular immune system; it is taken up by neutrophils, leading to cell activation and the subsequent of the phagocytic ability of these cells. (Parrillo, 1993; Hansbrough *et al.,* 1990) also mention that LPS induced activation of susceptible cells, such as neutrophils, lead to the release of prostoglandins,leukotrienes and other agents.

Likewise, LPS directly activate the alternative pathway ,the resulting complement cascade induces by LPS produced among other mediators , the anophylotoxins C_3a and C_5a which contributed to activation neutrophils (Glauser *et al.,* 1991). Furthermore, induces the phagocytic

process may occurs by the *Shigella* protein such as exotoxin (Menard *et al.,* 1993).

The increase in NBT test mean values at mucosal system, particularly ay appendix as compared with systemic NBT mean values when the rabbits immunized with LPS or exotoxin ,may be interpret on the basis that mucosal surface contains numerous nonspecific cellular immune response (granulocytes, Macrophages) cells widely distributed in the lamina propria; these cells are high stimulated especially by LPS (Granucei and Riceiardi –Castagnoli ,2003).In addition ,the mucosal immune system of the gastro intestinal tract represents one of the largest immunologic compartments in the body and it contains abundant lymphoid cells (i.e ,B and T lymphocytes) and myeloid cells (i.e. Macrophages ,neutrophils) that can be activated by LPS of *Shigella* (Blumberg *et al.,* 1999). However, there are several evidence that LPS stimulates reaction such as the release of cytokines (IL-1 , IL-6, IFN- γ) from endothelial cells ,monocytes and colonic epithelial cells ;these cells produce a large array of cytokines particularly at mucosal surface because these cells are abundant in the gut mucosal associated tissues (Remick *et al.,* 1995 ; Philpott *et al.,* 2000) .Furthermore IL-18 ,which is a potent interferon (IFN- γ) inducer, allows the innate immune system to establish proper conditions for eradication of the *Shigella* inoculum, since IFN- γ is essential for the killing of *Shigella* (Way *et al.,* 1998) Although, IFN- γ can be produced from mucosal cells after activation by LPS and then IFN- γ stimulate neutrophils cells. (Raqib *et al.,* 1995; Kisick *et al.,* 2002).

In additions, epithelial cells infected with Shigella secrete large amounts of IL-8, a potent proinflammatory cytokine and chemoattractant for neutrophils (Arondel *et al.,* 1999; Jung *et al.,* 1995). At the same time, neutrophils engulf and kill *Shigella* .Even though the bacteria do not

survive within these cells (Perdomo *et al.,* 1994) In other words; control of infection with *Shigella* is mediated primarily via IL-18, IFN-γ and activation of phagocytes by LPS. Furthermore, killing of phagocytized *Shigella* not by oxidative process, but by other antimicrobial activity such as lyzozyme, myeloperoxidase, lactoferrin, elastase, cathepsin cathilicidin and other cationic protein (Klebanoff ,1970 ; Roitt *et al.,* 2001).

5.2.2 Leukocyte migration- inhibitor factor (LIF) test

There are soluble products of activated lymphocytes (Sometime called lymphocytes activation products) produced by exposure of sensitized lymphocytes to the sensitizing antigen. They are believed to serve three main functions (1) recruitment of uncommitted lymphocytes, (2) retention of such cells and phagocytes as the inflammatory site and (3) activation of the retained cells so that they can take part in the inflammatory response. These products can be thought of as chemical messengers that allow communication between the cells and also as agents that amplify the response. They act on polymorphs, lymphocytes, macrophages and also on other non lymphoid cells (Hahn *et al.,* 1976).

The best known of these non-antibody factors released by sensitized lymphocytes on contact with antigen has the property of preventing the in vitro leukocytes migration on a glass surface used for the detection of cell- mediated immune reactions in clinics (Silobreic *et al.,* 1975). About thirteen in vitro activates have been identified as lymphokine –mediated, but the best characterized lymphokine is LIF (Bernhagen *et al.,* 1998). LIF is firstly described as a factor produced by T- lymphocyte that was associated with the migration of macrophages during delayed –type hypersensitivity response (Juttner and Bernhagen, 1998).

The data in Table (8) indicate that LPS of *S.flexneri* for both doses induce specific cellular immunity at mucosal level especially at appendix. Also LPS of this bacteria elicited the ability to induce specific cellular immunity at systemic level, but mean values of LIF were statistically (P>0.05) less than that of mucosal mean values as in table (11). Although, LPS of *S. dyseneiriae* was able to induce cellular immunity at mucosal levels where the mean values were statistically higher significant(P<0.05) particularly at appendix as compared with control. In the same time ,LPS of *S.dysenteriae* at both doses 1.5,2.5 mg/Kg induce cellular immunity at systemic levels but elicits mean vales of LIF lesser statistically (P>0.05) than that of mean values of mucosal system (Table 14,15).

On the other hand, exotoxin of *S.dysenteriae* at doses 0.5,1 mg/Kg leads to induce specific cellular immunity at mucosal levels and systemic levels , at mucosal system mean values of LIF which were statistically higher significant (P<0.05) also at appendix but less than that of LPS for *S. flexneri*, *S.dysenteriae* (Table 16,17) . Likewise, exotoxin induces systemic specific cellular immunity with less mean values of LPS as compared with those of LIF at mucosal system (Table 18).

The significant decrease to more than 30% inhibition in index mean values of LIF at mucosal system may refer to that mucosal system containing higher number of activated T cells especially in inductive sites, in which cells directly contact with sensitizer when given orally (Stites *et al.,* 1994).However ,decrease in LIF mean values index with the rabbits immunized with LPS of *Shigella* as compared with exotoxin may depend on high ability of LPS to induction T cell to secrete cytokines including LIF (Reitschel *et al.,* 1996), and that is in agreement with

(Soborg,1986)who mentions that LIF depends on sensitivity of cells and antigen concentration.

5.2.3 E-rosette test (Activated)

Identification of T lymphocytes by their ability to form rosettes with sheep erythrocytes (erythrocyte rosette- forming cell or E-RFC) has been used to study cellular immunity and it was used as marker for T lymphocytes of humans and most mammals (Stiehm *et al.*,1979). However, E- RFCs appear initially in the thymus at about the 11 the week of gestation and increase to a maximal level in the thymus by 16 to 18 weeks (Wybran *et al.,* 1971) .E- rosette formation relies on the fact that some lymphocytes population have receptors for sheep erythrocytes. Human T cells and most mammals have receptor for sheep erythrocytes (E); these are CD_2 molecules. In addition to CD_2 (Bernard *et al.,* 1988) reported that another T-cell surface molecules (32KDa, 20KDa and E_2) may be involved in spontaneous rosette formation with sheep RBCs. Although, this test is different from EAC- rosette test, which is used as a tool for the quantitative assay of B-lymphocytes where EAC test needs for interaction of antibodies (A), complement (C), sheep erythrocytes (E) and specific legends on the surface of B- lymphocytes (Moretta *et al.,* 1977 ; Roitt *et al.,* 2001, Kotton. 2002).

The results (Table 9,11,12,13) showed that LPS at doses 3,5 mg of *S.flexneri* cause significant increase in mean values of E- rosette test in rabbits immunized with this toxin at mucosal level, as compared with control ($P<0.05$).Likewise, LPS of this bacteria lead to the increase in mean values of E-rosette test at the systemic level for the same doses(Table 9,11) .However, the increase in mean values at system was less than of mucosal system. Similar results were observed as above when the rabbits were immunized with *S.dysenteriae* LPS at the same doses

(Table 9, 14, 15) .On the other hand, exotoxin of *S.dysenteriae* for doses 0.5, 1 mg/Kg was able to increase the ability of T- lymphocytes to forming E-rosette at systemic (Table 9, 16) and mucosal levels (Table 17), but the mean values of E-rosette test for mucosal system, especially at appendix were higher than that of systemic. Moreover, present results were elicited that mean values of E- rosette test for rabbits immunized with LPS of *S.flexneri* and *S.dysenteriae* were higher significant ($P<0.05$) than that of rabbits immunized with exotoxin (Table 18).

The ability of LPS in the present study to activation T- lymphocytes, particularly at mucosal system (appendix) in accordance with those reported by (Flad *et al.,* 1993; Mayeux ,1997) who mention that LPS induction of the massive host response early infection and then, LPS is sensed via specific pattern recognition receptor (PRR) on APC and other cells of the innate immune system (Medzhitov and Janeway, 2000), notably CD_{14} and toll-like receptor (TLR_4) ,a receptor family recognizes different bacterial agents, including LPS (Poltorak *et al.,* 1998;Qureshi *et al.,* 1999).As a result, a variety of proinfamammatory cytokines (including INF-α ,IL-1 , IL-6 ,IL-12 and IL-18) and chemokines are produced; this cytokine milieu, especially IL-12 and IL-18 ,promotes Th_1 polarization of the adaptive immune response (Trinchieri, 1995; Munder *et al.,* 1998;).

Similar results were reported by (Reitschel *et al.,* 1996) who mention that LPS induce activation of T-lymphocytes. However, another study (Islam *et al.,* 1995) reported that infection with *S. dysenteriae* 1 or *S.flexneri* induces cellular activation T cells and leads to significant increase in the $CD_{45}RO^+$ cells in both CD_4^+ and CD_8^+ T cells and found evidence for sequential T cell activation, as shown by increased proportion of CD_{25} and CD_4^+ cells, HLA-DR and CD_{38} on CD_8^+ cells, and CD_{54} on CD_4^+ and CD_8^+ cells.

The mucosal surface has numerous lymphoid cells disseminated a long the gut. Some lymphocytes are interspersed among epithelial cells and called intraepithelial lymphocytes (IEL). The majority of these cells are T-lymphocytes (80-90%).Others are located in the connective tissue of the lamina propria, and they are called lamina properia lymphocytes (LPL).In contrast to the epithelial compartment, lamina propria lymphocytes are predominantly activated T cells (Abren-Martin and Targan 1996; Roitt *et al.,* 2001). Furthermore intestinal epithelial cells (i-EC) can produce IL-15 especially ,when infected with *Shigella* ,in which the host secreted interleukin has been shown to control the development of the CD_4^+ $CD_{8\alpha\alpha}$ intestinal intraepithelial lymphocytes (i-IEL) , a fraction of T- cells considered to be extra- thymically developed mucosal T cells (Burton *et al.,* 1994 ; Yamada *et al.,* 1997;Ohteki *et al.,* 1998;). Such extrathymic developed T cells may interpret the increase in the E-rosette formation at mucosal levels .On the other hand, (Haicheur *et al.,* 2003) report that exotoxin of *S.dysenteriae* elicits cytotoxic T cell response associated with a Th_1 –dominant polarization in mice.

Activation of professional APC at the early stage of infection leads to the presentation of epitopes derived from *Shigella* proteins by MHC class I molecules (Lee *et al.,* 1998; Haicheur *et al.,* 2000). The critical T cells for clearing *Shigella* infection are CD_8^+ T cells (Kaufmann *et al.,* 1979), also INF-α produced by CD_8^+ T cells plays an important role in protection from infection with *Shigella* (Way *et al.,* 1998). Beside CD_8^+ T cells, Shigella also induces CD_4^+ T cells (Kaufmann *et al.,* 1982) .

The major task of a next generation vaccine is the efficient establishment of a memory pool of T cells and induction of a Th_1 type response, intra cellular bacteria fulfill these demands effectively .The first step is the early generation of a cytokine milieu promoting the Th_1

immune response .As described previously, *Shigella* infection causes early secretion of IFN-γ, IL-12. IFN-γ counteracts the production of IL-4 while IL-12 promote activation of CD_4^+ T cells of the Th_1 phenotype. In addition, several bacterial factors (LPS) full activate professional APC through binding to TLR(Kaisho and Akira ., 2001) and up regulating costimulatory molecules like B7-1 , B7-2, VLA-4 ,ICAM-1, CD_4 and also MHC class 1 and MHC II molecules (Reissousa *et al.,* 1999).

5.3 Immune features of *Shigella*

Shigella gain entry to human being and laboratory animal via oral route, the *Shigella* population may undergo population reduction due to gastric acidity (Hornick *et al.,* 1970). The remaining *Shigellia* population load may colonize colonic of the gut ,the invasion plasmid antigen make *Shigella* able to cross the epithelial barrier in selected areas corresponding to M cells of the follicle- associated epithelium (FAE);the inductive site for local mucosal immune system (Sansonetti and Phalipon, 1999). This together with inflammatory response (induced by virulence-associated proteins IpaB, IpaC, and IpaD) are achieved by cellular components of the intestinal barriers (Buchrieser *et al.,* 2000).

The *Shigella* immunodominant epitopese especially those concerning exotoxin and endotoxin can be direct B cell activator leading to specific antibody secretion on T helper 2(Th_2)dependent leading to specific antibody formation with the help of some cytokines like IL-2 or it can be Th_1 activator with or without cytotoxic T cell activation leading to the production of LIF cytokines like IL-15 ,IL-8 and delayed type hyper sensitivity associated cytokine and mediating tuberculin type delayed type hypersensitivity (Zubbler ,1998; Hyde ,2000).

The basic major immune features of *Shigella* immunoprimed rabbits as compared between human being and rabbits from literature and this experimental studies (Acheson and Keusch, 1995).

Literature	Present study
* *Shigella* use intra cellular characters	*Specific anti LPS antibody systemic
**Shigella* are both invasive (Invasion plasmid antigens) and toxigenic (exotoxin and endotoxin)	*Specific antitoxin antibody local systemic
*Disease manifestation are based mainly upon toxin related effect	*Raise up of NBT neutrophil phagocytosis
*Stimulation of local gut immunity may be the key to the immune protection	*Significant LIF at mucosal and peripheral blood
*Antitoxin may prevent disease or a mediorate its course	* Raise up of E- rosette forming cells
*Secretory IgA as well as IgG may be critical in preventing attachment to epithelial cells of the potential pathogen.	*Skin delayed hypersensitivity tuberculin type, using *Shigella* protein preparation (Table 10).
**Shigella* SIgA or SIgG protease trying cleavage such antibody allowing infection.	*The more the concentration the more the effect within the limit of tolerance
*Humoral immune response is type specific Elevated cytokine level has been detected in the stool and plasma of infected patients	
*Effective *Shigella* vaccine should be invasive.	

Conclusions

1-Rabbit immune system was found responding to and usable model for the study of *Shigella* exotoxin and endotoxin immunity using oral priming protocols.

2-*Shigella* toxins primed rabbits showed that mucosal non specific cellular (NBT) and T cell E-rosetting were higher than those of peripheral blood, whereas, systemic humoral immune responses were higher than those of mucosal antibody responses.

3-Rabbit immune system responds more to *Shigella* LPS immunogen than to *Shigella* exotoxin immunogen preparations.

4-Rabbit T cell can form E-rosetting just like that of human being since both bear CD_2 markers.

5- A protein fraction obtainable by PEG (MW 6000) 6% solution from an exotoxin preparation of *Shigella* acts as protein preparation derived (PPD) like allergen at concentration 2mg /5ml can be designated as" *Shigella"* usable for DTH skin test were developed.

6-*Shigella* exotoxin primed rabbits showed mucosal and systemic *Shigella* specific haemagglutinins as well as elevated neutrophil NBT phagocytosis, elevated T cell E-rosetting and border line LIF as well as low limits of tuberculin type skin DTH test.

7-*Shigella* endotoxin primed rabbits showed mucosal and systemic specific antibody reaction as well as elevated neutrophil NBT phagocytosis, elevated T cell E-rosetting and significant LIF as well as high positive tuberculin type skin DTH test.

8-*S.flexneri* and *S.dysenteriae* toxins are basically of similar immunogenic potentials but with minor difference.

Recommendations

1-Performing further investigation for the *Shigella* mucosal rather than systemic vaccination and protective immunity through challenging studies.

2-Checking the roles of cytokines such as IL-15, IL-8 and IFN-γ as a activator in cellular immune response to *Shigella*.

References

Abbas, A.K.; Lichtman, A.H.; and pober, J.S.(1997): Cellular and molecular immunology, 3rd ed. Saunder.

Abbas, A.K.; Murphy, K.M.; and Sher, A.(1996): Functional diversity of helper T lymphocytes. Nature. 383: 787-793.

Abreu-Martin, M.T.; and Targan, S.R. (1996): Regulation of immune responses of the intestinal mucosal. Critic. Rev. Immunol .16: 227-309.

Acheson, D.W.K .and Keusch, G.T.(1995): *Shigella* and enteroinvasive *Escherichia coli*. In blaser, M.J.; Smith, P.D.; and Ravdin, J.I.(eds): Infections of the Gastrointestinal Tract. New York, Raven Press, 1995, PP 763-784.

Adam, T.; Arpin, M.; Prevost, M.C. ; Gounon, P.; and Sansonetti, P.J. (1995): Cytoskeletal rearrange ments and the functional role of T-Plastin during entery of *Shigella flexneri* into. Hela Cell.J cell Biol. 129: 367-381.

Adkins, H.J.; and Santingo, L.T. (1987): Increased recovery of enteric pathogens by use of both stool and rectal swab specimens. J. Clin. Microbiol. 25: 158-159.

Alemohammade, M.M.; Foley, T.J.; and Cohen, H. (1993): Detection of immunoglobulin G antibodies to *Helicobacter pylori* in urine by enzyme immunoassay mothod. J. Clin. Microbiol. 31:2174-2177.

Allaoui, A.; Sansonetti, P.J; and Parsot, C.(1992): Mxij, a lipoprotein involved in secretion of *Shigella* Ipa invasions, is homologous to Ysecj a secretion factor of the *Yersina* Yop proteins J Bacteriol. 174: 7661- 7669.

Anand, B.S.; Malhora, V.; Bhattacharya, S.K.; Datta, P.; Sen, D.; Bhattacharya, M.K.; Mukherjee, P.P., and Pal, S.C.(1986):Rectal histology in a cute bacillary dysentery. Gastroenterology. 90: 654-660.

Anderson, P.W., Pichichero, M.E.; Stein, E.C.; Porcalli, S.; Betts, R.F.; Connuck, D.M.; Lorones, D.; Insel, R.A.; Zahradnik, J.M.; and Eby, R.(1989): Effect of oligosaccharide chain length, exposed terminal group, and hapten loading on the antibody response of human adults and infants to vaccine consisting *Haemophilus influenzae* type capsular antigen unterminally coupled to the *Diphtheria* protein CRM197. J Immunol 142: 2464-2468.

Armitage, R.J.; Macduff, B.M.; Eisenman,J.; Paxton, R.; and Grabstein, K.H.(1995): IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation.J.Immunol. 154: 483-487.

Arondel, J.; Singer, M; Matsukawa, A.; Zychlinsky, A.; and Sansonetti, p.J.(1999): Increased IL-1 and imbalance between L-1 and IL-1 receptor antagonist during acute inflammation in experimental Shigellosis .Infect Immun 67: 6056-6066.

Azim, T.; Halder, R.C.; Sarker, M.S.; Ahmed, S., Hamadani, J.Chowdhury, A.; Qadri, F.; Salam, M.A. Sack, R.B.; and Albert, M.J.(1995): Cytokines in the stools of children with complication Shigellosis :Clin. Diagn. Lab. Immunol. 2: 492-494.

Azim, T.; Rashid, A.; Qadri, F.; Sarker, M.S.; Hamadani, J.; Salam, M.A.; Wahed, M. A.; and Al-bert, M.J.(1999): Antibodies to Shiga toxin in the serum of children with *Shigella*-associated haemolytic uremic syndrome. J Med Microbiol. 48: 14-16.

Beagley, K.W.; Eldridge, J.H.; Kiyono, H.; Everson, M.P.; Koopman, W.J.; Honjo, T.; and McGhee,J. R.(1988): Recombination IL-5 induces high rate of IgA synthesis in cycling IgA- positive Peyer's patch B cells .J. Immunol. 141:2035.

Bennish, M.L.; Salam, M.A.; Wahed, M.A.(1990): Therapy for Shigellosis : II. Randomized, double- blind comparison of ciprofloxacin and ampicillin. J Infect..Dis. 162:711-716.

Bensted, H.J.(1956): Dysentery bacilli- a brief historical review. Can .J Microbiol, 2: 163-174.

Bernardini, M. L.; Mounier, J.; D'Hauteville, H.; Coquis-Rondon, M.; and Sansonetti, P.J. (1989): Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and inter cellular spread through interaction with F-actin.Proc. Natl. Acad. Sci. USA 86: 3867-3871.

Bernard,A.C.; Lawlor,;G.J.; Neumann,C.G.;and Chandra R.K.(1988):Decread rosette-forming lymphocytes in malnutrition and intrauterine growth retardation.J.Pediatr.84-717-725.

Bernhagen,j.; Bacher, M.; Calandra ,T.; Metz, C.N.; Doty, S.B.; Donnelly, T., and Bucala, R.(1998): An essential role for macrophage migration inhibitory factor in the tuberculin delayed type hypersensitivity reaction .J Exp.Med.83: 277-282.

Bing-nan. F.; Zhang, G.R.; Yang, H.C.; Zhao, D.B.; and Liu, M.X.(2000): Epidemiological analysis of the duration of intestinal immunity after natural infection with *Shigella* .Chin. Med.J.123:1145-1150.

Bishop, D. K.; and Hinrichs, D.J. (1987): Adaptive transfer of immunity to *Listeria monocytogenes.* The influence of in-vitro stimulation on lymphocyte subset requirements .J Immunol. 139: 2005-2009.

Black, R.E.; Levine ,M.M.; Clements, M.L.; Losonsky, G.; Herrington, D.; Berman, S.; and Formal, S.B.(1987): Prevention of Shigellosis by a *salmonella typhi- Shigella sonnei* bivalent vaccine .J Infect. Dis. 155: 1260.

Blumberg, R.S.; Sanbermann, L.J.; and Strober, W.(1999): Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. Current opinions in immunology, 11: 648-656.

Bohuslav, J.; Kravchenko, V.V.; Parry, G.C.N.; Erlich, J.H.; Nigelmackman, S. G.; and Ulevitch R.J.(1998): Regulation of an essential innate immune response by the PSO subunit of NF-KB .J.Clin. Invest. 102: 1645-1652.

Boivin, A. and Mesrobeanu, L. (1933): Contribution a letude composition chimique of bacteria .Substance azotes phosphorase "acido-soluble" .C.R. séance Soc. Biol. Fil. 112: 76.

Bone, R.; Balk, R.; Cerra, F.; Dellinger, P.; Fein, A.; Knaus, W.; Schein, R.; and Sibbald, W. (1992): Deffinitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis .Chest. 1644-1656.

Bouvet, J.P. and Fischetti,V.A.(1999): Diversity of antibody mediated immunity at the mucosal barriers. Infection Immun. 97: 3687-3691.

Brandtzaeg , P.; and Prgdz, H. (1984): Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglubulins. Nature. 311: 71-73.

Brandtzaeg, P. (1985): Role of J Chain and secretory component inreseptor mediated glandular and hepatic transport of immunoglubulins in man. Scand .J. Immunol. 22:111-146.

Brandtzaeg, P. (2001): Nature and function of gastrointestinal antigen-presenting cells. Allergy 56:16-20.

Brandtzaeg, P., Farstad, I.N., Johansen, F, e., Morton, H.C., Norderhang, I.N., Yamanaka, T. (1999): The –cell system of human mucosae and exocrine gland .Immunal .Rev.20:161-166

Brandtzaeg, P.; Baekkevold, E.S.; and Farstad, I.N. (1999): Regional specialization in mucosal immune system. Immunol. Today 20: 141-151.

Brandtzaeg, P.; Johansen F.E; Krajci, P.; and Natvig, I. (1998): Human secretory component (the polymeric immunoglobulin receptor) .In

Roill D.P., ed. Encyclopedia of Immunologgy . 2nd ed London. Academic press, 2152-2158.

Brandtzaeg, P.; Johnsen, F.L.; and Farstad ,IU.N.(1996): immune function and immunopathology of the mucosal of the upper respiratory pathways. Acta .Otolaryngol. 116: 149-159.

Buchries, C.; Glaser, P.; Rusniok ,C.; Nedjari, H.; Dhauteville,H.; Kunst, F.; Sansonetti, P.J.; and Parsot, C.(2000): The virulence plasmid pWRloo and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri* .Mol .Microbiol 38: 1-14.

Bunning, V.K.; Raybourne, R.B.; and Archer, D.L.(1988): Foodborne enterobacterial pathogens and rheumatoid disease .J Appl. Bacteriol. Symposium supplement: 87C.1075.

Burrel, R.(1979): Experimental immunology, 5th ed, PP: 23-24. Burgess publishing Company, Minnesota.

Burton, J. D.; Bamford, R.N.; Peters, C., Grant, A.J.; Kurys, G.; Foldman, C.K.; Brennan, J.; Roessler, E.; and Waldmann, T.A.(1994): A lymphokine, Provisionally designated interleukin T and Produced by a human. Pro.Natl. Acad. Sci USA 91: 4935.

Buxton, A. and Fraser, G.(1977): Animal Microbiology vol.1 Blackwell Scientific publication, Oxford.117-120.

Buysse, J.M.; Stover, C.K.; Oaks, E.V.; Venkatesan, M.; and Kopecko, D.J. (1987): Molecular cloning of invasion plasmid antigen (Ipa) genes from *Shigella flexneri* : Analysis of Ipa gene product and gentic mapping. J Bacteriol. 169: 2561-25-69.

Caceres, A.; and Mata, L.J.(1974): serologic response of patients with Shiga dysentery. J.Infect. Dis. 129: 439-443.

Calderwood, S.B.; and Mekalanos, J.J. (1987): Iron regulation of Shiga – like toxin expression is *Escherichia coli* is mediated by the *fur* locus. J Bactriol 169: 4759-4764.

Cam, P.D.; Pal, T.; and Lindberg, A.A.(1993): Immune response against lipopolysaccharide and invasion plasmid coded antigens of *Shigella* in Vietnamese and Swedish dysenteric patients .J. Clin. Microbiol. 31: 454-457.

Carlin, N.I.; Lindberg, A.A.; Rock, K.; and Bundle D.R. (1984): The *Shigella flexneri* O-antigenic polysaccharide chain. Nature of the biological repeating unit. Eur.J. Biochem. 139: 189-194.

Chen, Y.Smith, M. R.; Thirumalai, K.; and Zychlinsky, A. (1996): A bacterial invasion induces macrophage apoptosis by binding directly to ICE. EMBO J.15: 3853-3860.

Chohan, N.D.; Hughes, A.A.; and Johnson, P.J.(2001): Clinical laboratory test : values and implications .3rd ed. Pensylvania: Springhouse;*P*385.

Cohen, A.; Mardried- Marina, V.; Estrov, Z.; Freedman, M.H.; Ling wood, C.A.; and Dosch. H.M. (1990) : Expression of glycolipid receptors to Shiga-like toxin on human B lymphocytes: a mechanism for the failure of long-lived antibody response to dysentery disease .Int .Immunol 2: 1-8.

Cohen, D.; Ashkenazi, S.; Green, M.; Yavzori, M.; Orr, N.; and Wiener ,M.(1994): Safty and immunogenicity of the oral *E-coli* K12-*S. flexneri* 2a vaccine (Ecsf 2a-2)among Israeli solders. Vaccine 12: 1436-1442.

Cohen, D.; Ashkenazi, S.; Green, M.S.; Gdalevich, M.; Robin, G.; Slepon, R.; Yavzpori, M.; Orr, N.; block, C.; Ashkenazi, I.; Shemer, J.Jaylor, D. N.; Hale, T.L.; Sadoff, J.C.; Pavliakova, Di; Sehneerson, R.; and robins, J.B.(1997): Double blind vaccine –controlled randomized efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults .Lancet 346: 155-159.

Cohen, D.; Block, C.; Green, M. S.; Lowell,G.; and Ofek, I.(1989): Immunoglobulin M, A, and G antibody response to lipopoly - saccharide D- antigen in symptomatic and asymptomatic *Shigella* infections .J. Clin. Microbiol. 27: 162-167.

Cohen, D.; Green, M.S.; Block, C.; Rouach, T.; and Ofek., T. (1988): Serum antibodies to lipopolysaccharide and natural immunity to Shigellosis in an Israeli military population .J Infect. Dis. 157: 1068-1071.

Cohen, D.; Green, M.S.; Block, C.; Slepon, R.; and Ofek, I.(1991): A protective study of the association between serum antibodies to lipopolysaccharide O- antigen and the attack rate of Shigellosis .J Clin. Mcrobiol. 29: 386-389.

Colman, R.(1989):The role of plasma protease in septic shock. N Engl J. Med. 320: 1207-1209.

Corthesy, B.; and Kraehenbuhl.J.P.(1999):Antibibody- mediate protection of mucosal surfaces. Curr. Top.Microbiol. Immunol. 236: 93-111.

Dawed, K.; and Al-Yas, Z.A.(1991): Statastical methods in agricultural researches: Mosul university-Iraq.

De lorenzo, V. ; Wee, S.; Herrero, M.; and Neilands, J.B.(1987): Operator sequences of the aerobactin operon of plasmid colv-K30 binding site of the ferric uptake regulation (*fur*) repressor. J Bacteriol. 169: 2624-2630.

De Silva, D.G.; Mendis , L.N.; and Sheron, N. (1993): Concentration of interleukin-6 and tumor necrosis factor in serum and stools of children with *Shigella dysenteriae* 1 infection .Gut 34 : 194-198.

Donohue-Rolfe, A.; Jacewicz, M.; and Keusch, G.T. (1989): Isolation and characterization of functional Shiga toxin subunits and renatured holotoxin. Mol Microbiol 3: 1231-1236.

Duhamel, R.C.; Talbol, P.; Grady, G.F.(1970): Production, purification and assay of cholera entero toxin.J.Infect. Dis 121:*P*585.

DuPont, H.L., and Formal. S.B.(1971): Pathogenesis of *Escherichia coli* diarrhea. N.Engl.J.Med. 285 :1-9.

DuPont, H.L.; Levine, M.M.; and Dorman, C.(1989): Inoculums size in Shigellosis and implications for expected mode of transmission .J. Infect. Dis. 159: 1126-1128.

Echeverria, P.; Sethabutr, O.; and Pitarangsi, C. (1991): Microbiology and diagnosis of infections with *Shigella* and enteroinvasive Escherichia coli .Rev. Infect. Dis. 13: 5220-225.

Elson, C.O.; Heck ,J.A.; and Strober, W.(1979): T cell regulation of murine IgA synthesis .J. Exp. Med. 149 :632-643.

Endo, Y.; Tsurugi, K.; and Yutsudo, T.(1988): Site of action of a vero toxin (VT_2) from *Escherichia Coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes .Eur J. Biochem 171 :45-50.

Fasano ,A.; Kay, B.A.; and Russell, R.G.(1990): Enterotoxin and cytotoxin production by enteroinvasive *Escherichia coli*. Infect Immun 58: 3717-3723.

Fasano, A.; Noriega, F.R.; and Maneval D.R.(1995): *Shigella* enterotoxin 1 : An enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine in vivo and in vitro .J .Clin .Invest 95: 2853-2861.

Fehinger, T.A.;Herbein ,G.; Yu, H.; Para, M.I.; and Bernestein, Z. P.O. (1998): NK cells from HIV-1 patients produce C-C chemokines and in hinit HIV infection . J. Immunol. 161: 6433-6438.

Flad, H.D.; Loppnow, H., and Rietschel, E.T.(1993): Agonists and antagonists for lipopolysaccharide induced cytokines .immunology .187: 303-316.

Fliedner, M.; Mehls, D.; Reterberg, E.W.; and Ritz, E.(1986): Urinary SIgA in children with urinary tract infection.J Pediatr. 109: 416-421.

Fontaine ,A.; Arondel, J.; and Sansonetti, P.J. (1988): Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox mutant of *Shigella dysenteriae* 1 .Infect. Immun. 56: 3099-3109.

Formal, S.B.; oaks, E.V.; Olsen, R.E.; Wingfield –Eggleston, M.; Snog, P.J.; and Cogan, J.P.(1991): The effect of prior infection with virulent *Shigella flexneri* 2a on the resistance of monkeys to subsequent infection with *Shigella sonnei* J.Infect. Dis. 164: 533-537.

Frederik, W.; Ginkel, V.A.N.; Huan, H.; Guyen, N.; and McGhee, J.R.(2000): Vaccines for mucosal immunity to combate emerging infections disease .J. National centers for infections disease. Vol. 6. No.2.

French, M.A.H(1988): Immunoglobulins in health and disease. 1st ed. MTP press limited. USA.

Garvey, J.S.; Cremer, N.E.; and Sussdrof , D.H.(1977): Methods in immunology, 3rd ed. Addison –wesley publishing Company, Inc., Reading .

Gebbers, J. O.; and Laissue, J. A. (1989): Immunological structures and functions of the gut. Sehweizer archive tierheil. 131: 221-238.

Germain, R.N. (1995): The biochemistry and cell biology of antigen presentation by MHC class I and class II molecules. Implications for development of combination vaccines. Ann NY Acad Sci. 754: 114-125.

Glauser, M.; Zanetti, G.; Baumgartner, J.; and Cohen, J.(1991): Septic shock pathogenesis. Lancet. 338 :732-736.

Goldbery, M.B.; and Sansonetti, P.J.(1993): *Shigella* subversion of the cellular cytoskeleton: A strategy for epithelial colonization .Infect. Immun. 61 :4941-4946.

Granucci, F.; and Ricciardi-casta-gnoli, P.(2003): Interaction of bacterial pathogens with dendritic cells during invasion of mucosal surfaces. Cur. Opin Microbiol. 39:237-238.

Griebel, P., Beskorwayne, T.; Van den Brocke, A.; and Ferrari, G.(1999): CD40 signaling induces B cell responsiveness to multiple members of the α-chain common cytokine family. Int. Immunol. 11: 1139-1144.

Gyles, C.L.(1986): *Shigella* In Gyles, C.L. and Thoen, C.O.(eds) Pathogenesis of bacterial infections in animals. Iowa state university press.

Hahn, T.; Levin, s.; and handzel, Z.T.(1976): Leukocyte migration inhibition factor (LIF) Production by lymphocytes of normal children and children with immunodeficiency .Clin Exp .Immunol 24: 448-453.

Haicheur, N.; Benchetrit, F.; Amessou, M.; Leclerc, C.; and Tartour, E.(2003): The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell mediated immune responses associated with a Th 1-dominant polarization. International .Immunology. 15:1161-1171.

Haicheur, N.; Bismuth, E.; Bosset, S.; Adotevi, O.; Warnier, G.; Lacabanne, Vi; Regnault, A.; Desoymard, C.; Amigorena, S.; Ricciardi- Castagnoli, P.; Goud, B.; Fridman, W.H.; Johannes, L.; and Tartour, E.(2000): The subunit of shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class 1-restricted presentation of peptides derived from exogenous antigens. J.Immunol. 165 : 3301.

Hale, T.L.(1995): *Shigella* vaccines, Molecular and Clinical aspects of bacterial vaccine development, eds Al'a Aldeen DAA, Hormaeche CE, John wiley and sons LTD, chichester, New York. 179-204.

Hale, T.L.(1998): Bacillary dysentery in : Topley and wilson's Microbiology and microbial infections, edited by Hausler WJ and Sussman M. London: Arnold, Vol.3.

Hansbrough, J.; Tenenhaus, M,; Wikstrom, T.; Braide, M.; Mennekampff,O. H.Kiessig, V.; and Bjursten, L.M.(1990): Effect of recombinant bactericidal /permeability –increasing protein on nentrophil activity in burned rats .J. Trauma 40:886-893.

Hartman, A.B.; Powell, C.J.; Schultz, C.L.; Oaks, E.V.; and Eckels ,K.H.(1991): Small animal model to measure efficacy and immunogenicity of *Shigella* vaccine strains .Infect. Immun. 59: 4075-4083.

Harty, J.T.; and Bevan, M.J.(1995): Specific immunity to *Listeria monocytogenes* in the absence of IFNY. Immunity 3: 109-117.

Harty, J.T.; Schreiber, R.D.; and Bevan, M.J.(1992): CD^{+}_{8} T cells can protect against an intra cellular bacterium in an interferon-α –independent fashion .Proc.Natl. Acad. Sci. USA 89: 11612-11616.

Hathaway, L.J.; and Kraehenbuhl, J.P.(2000): The role of M cells in mucosal immunity .Cell .Mol.Life Sci. 57: 323-332.

Henry, F.J. (1991): The epidemiologic importance of dysentery in communities. Rev Infect .Dis. 13: 5238-244.

Herias, M.V.; Mattsby-Batzer, I.; Cruz, J.;R.; and Hanson, L.A.(1997): Antibodies to Escherichia coli and *Shigella flexneri* in in milk from undernourished mothers: Studies on sodium dodecyle sulfate –polyacrylamide gel electrophoresis-separated antigen. Pediatric. Res. 42: 644-650.

Herrington, D.A.; Van Deverg, L.; Formal, S.B.; Hale ,T.L.; Tall, B.D.; cryz,S.J.; Tramont, E.C.; and Levine, M.M.(1990): Studies in volunteers to evaluate candidate *Shigella* vaccines : Further experience with a bivalent *Salmonella typhi- Shigella sonnei* vaccine

and protection cinfferred by previous *Shigella* sonnei disease. Vaccine 8: 353-357.

Hiroi, T.; Yanagita,M.; Ohta, N.; Sakaue, G.; and Kiyono, H.(2000): IL-15 and IL-15 receptor selectively regulate differentiation of common mucosal immune system –independent B-1 cells for IgA responses. J.Immunol. 165: 4329-4332.

Hiroi,T.; Fuihashi, Ki; McGhee, J.R.; and Kiyono, H.(1995): Polarized Th2 cytokine expression by both mucosal and α B T cells .Eur. J. Immunol. 25:2743-2747.

Hiroi,T.; Yanagita, M.; Iijima, H.; Iwatani, K.; Yoshida, T.; Takatsu, K.; and Kiyono, H.(1999): Deficiency of IL-5 receptor α-chain selectively influence the development of the common mucosal immune system independent IgA producing B-1 cell in mucosa- associated tissues. J Immunol. 162:821-825.

Hofmann, S.L.(1993): South western Internal Medicine conference: Shiga-like toxins in hemolytic –uremic syndrome and thrombotic thrombocytopenic purpura. Am. J. Med. Sci .306 : 398- 406.

Hornick, R.B.; Greisman, S.E.; Wood ward, T.E.; and Snyder, M. J. (1970): Typhoid fever: pathogenesis and immunologic control.N. Engl. J.Med. 283: 686-691.

Hovde, C.J.; Calder wood, S.B.; Mekalanos, J.J.; and Collier, R.J.(1988): Evidence that glutamic acid 167 is an active –site residue of Shiga-like toxin 1 .Proc Natl Acad Sci .USA 85: 2568-2572.

Huebner, J.; Czerwenka, W.; and Hale, T.L.(1993): Shigellemia in AIDs patients: Cases report and review of the literature .Infection, 21 : 122-124.

Hyde,R.M.(2000): Immunology 4th ed ., Lippinlott Williams and Wlkins, New York.

Inagaki- Ohara, K.; Nishimura, H.; Mitani, A.; and Yoshikai, Y.(1997): Interleukin-15 preferentially promotes the growth of intestinal intraepithial lymphocytes bearing T cell receptor in mice. Eur. T. Immunol. 27 :2885.

Islam, D., Veress, B.; Bardhan, P.K.; Lindbery, A. A.; and Chris tensson, B.(1995): Immunoglobulin subclass distribution and dynamics of *Shigella*-specific antibody responses serum and stool samples. Infect. Immun. 63: 2054-2061.

Islam, D.; Veress, B.; Bardhan, P. K.; Linberg, a.A.; and Christensson, B.(1997): Quantitative assessment of IgG and IgA subclass producing cells in rectal mucosa during shigellosis J.Clin. Pathol. 50: 513-520.

Islam, M.M.; Azad, K.; and Bardhan, P.K. (1994): Pathology of Shigellosis and its complications. Histopathology 24 :65-71.

Jacewicz, M.; Clausen, H.; and Nudelman, E.(1986): Pathogenesis of *Shigella* diarrhea. Isolation of a *Shigella* toxin-binding glycolipid from rabbit Jejunum and Hela cells and its identification as globotriaosylceramide. J.Exp. Med. 163:1391-1404.

Jakson, M.P.; Wadolkowski, E.A.; and Weinstein, D.L.(1990): Functional analysis of the Shiga toxin and Shiga- like toxin type II variant binding subunits by using site-directed mutagenesis .J. Bacteriol. 172:653-750.

Johansen, F.E.; Pekna, M.; and Norderhaug, I.N. (1999): Absence of epithelial immunoglobulin A transport, with increase mucosal leakiness, in polymeric immunoglobulin receptor/ secretory component –deficient mice J. Exp. Med. 190:915-922.

Johnstone, A.; and Thorpe, R.(1982): Immunochemistry in practice. Black well scientific publication.

Jung, H.C.; Eckmann, L.; Yang, S.K.; Panja, A.; Fierer, J.; Morzycka-Wroblewska, E.; and Kagnoff, M.F. (1995): A distinct array of pro-

Inflammatory cytokines in expressed in human colon epithelial cells in response to bacterial invasion .J. Clin Invest 95:55-65.

Juttner, S. and Bernhagen, J. (1998): Migration inhibitory factor induce killing of Leishmania major by macrophages: Dependence on reactive nitrogen intermediates and endogenous. Journal of Imunology 161: 2383-2390.

Kagi, D.; Ledermann, B.; Burki, K.; Hengartner, H.; and zinkernagel ,R.M.(1994): CD^{+}_{8} T- cell mediated protection against an intracellular bacterium, by perforin- dependent cytotoxicity .Eur. J. Immunol. 24: 3068-3072.

Kaisho, T. and Akira, S.(2001): Dendritic cell function in toll-like reccptor and My D_{88} Knockout mice. Trends Immunol. 22 :78-83.

Kandel , G.; Donohue-Rolfe, A.; Donowitz, A.; and Keusch, G.T.(1989): Pathogenesis of *Shigella* diarrhea. XVI . Selective targeting of Shiga toxin to villus cells of rabbit Jejunum explains the effect of the toxin on intestinal electrolyte transport. J.Clin .Invest. 84 :1509-1517.

Karnell, A.; Li, A.(1995): Safety and immunogenicity study of the auxotrophic *Shigella flexneri* 2a vaccine SFL 1070 with a deleted *araD* gene in adult Swedish volunteers . Vaccine .13 :86-99 .

Karnell,A.; Cam, P.D.; and Verma ,N.(1993): *AroD* deletion attenuates *Shigella flexneri* strain 2457T and makes it a safe and efficacious oral vaccine in monkeys .Vaccine .11: 830-836.

Kaufmann, S.H.E.; and Hahn, H.(1982): Biological function of T cell lines with specificity for the intra cellular bacterium *Listeria monocytogenes* in vitro and in vivo. J. Exp .Med. 155: 1754-1765.

Kaufmann, S.H.E.; Fensterie, J.; and Hess, J. (1999): The need for a novel generation of vaccines. Immuno biology 201 :272-2823.

Kaufmann, S.H.E.;Rodewald,H.R.;Hug,E.;and Libro,G.D.(1988):Clonal *Listeria monocytogenes* spesfic non-MHC-restricted Lyt-2+Tcells with cytotoxic and protective activity.J.Immunol.140:3173-3179.

Kaufmann, S.H.E.; Hug, E.; Vath, U.; and Libero, G.D.(1987): Specific lysis of *Listeria monocytogenes*- infected macrophages by class II-restricted L_3T_4 + T cells. Eur. J. immunol. 17 :237- 246.

Kaufmann, S.H.E.; Simon, M.M.; and Hahn, H.(1979): Specific Lyt 123 cells are involved in protection agains *Listeria monocytogenes* and in delayed type hypersensitivity to *listeria* antigens. J. Exp. Med. 150: 1033-1038.

Keusch, G.T.; Donohue-Rolfe, A.; and Jacewicz, M.(1985): *Shigella* toxin and the pathogenesis of Shigellosis .CIBA found. Symp.112: 193-214.

Keusch, G.T.; Grady, G. F.; Mata, L.G.; and McIver, J.M.(1995): Pathogenesis of *Shigella* diarrhea 1 .Enterotoxin production by *Shigella dysenteriae* type I. J. Clin 51 :1212-1218.

Kisich,K.O.; Hoggins, M.; Diamond, G.; and Heofets, L.(2002): Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophil.Journal of infection and immunity 70: 4591-4599.

Klebanoff, S.J.(1970): Myeloperoxidase in mediated antimicrobial systems and their role in leukocyte function. In Biochemistry of the pathocytic process,ed. Schulz J. Amsterdam :North Holland publishing company.

Klebanoff, S.J.(1991): Myeloperoxidase: Occurrence and Biological function, chemistry and Biology .Boca Raton, Fla CRC press.

Klimpel, G.R.; Niesel, D.w.; and Klimpel K.D.(1986): Natural cytotoxic effectors cell activity against *Shigella flexneri*- infected Hela cells. J. Immunol. 136 :1081.

Kotloff, K.L.; Nataro, J.P.; and Losonsky, G.A.(1995,b): A modified *Shigella* volunteer challenge model in which the inoculums is administrated with bicarbonate buffer: Clinical experience and implecations for Shigella infectivity .Vaccine .13: 1488-1494.

Kotloff, K.L.; Winickoff, J.P.; Ivanoff, B.; Clemons, J.D.; Swerdlow, D.L.; Sansonetti,P.J.; Adak, G.K.; and Levine, M.M.(1999): Global burden of *Shigella* infections: implication for vaccine development and implementation .Bull world health organ 77: 651-666.

Kotton,C.(2002): Encyclopedia T(Thymus derived) lymphocyte count. The University of Pennsylvania Health system.

Kroese, F.G.; Ammerlaan, W.A.; and Kantor, A.B.(1993): Evidence that intestinal IgA plasma in μ-k- transgenic mice are derived from B-1 (Ly-1B) cells. Int. Immunol. S: 1317-1320.

Kroese, F.G.; Butcher, E.C.; Stall, A. M.; Lalor, P.A; Adams, S.; and Herzenberg, L.A.(1989): Many of the IgA producing plasma cells in murine gut are derived from self- replenishing precursors in the peritoneal cavity .Int. Immunol. 1: 75-80.

Kruze, D.; Holzbecher, K.; Andrial, M.; and Bossarrt, W.(1989): Urinary antibody response after immunization with a vaccine against urinary tract infection .Urol. Res. 17: 361-366.

Kudsk, K.A.(2002): Current aspects of mucosal immunology and its influence by nutrition .Am. J. Surg. 183: 390-398.

Kumazawa, Y.; Nakatsuka, M.; Takimoto, H.; Furuya, T.; Nagumo, T.; Yamato, A.; Homma, J. Y.; Inada, K.; Yoshida, M., Kiso, M.; and Hasegawa, A.(1988): Importance of fatty acid substituents of chemically synthesized lipid A-subunit analogs in the expression of immunopharmacological activity .Infect. Immun. 56 :149-155.

LaBrec, E.H.; and Schneider, H.(1964): Epithelia cell penetration as an essential step in the pathogenesis of bacillary dysentery. J.Bacteriol .88 : 1503-1518.

Lamm, M.E.(1997): Interraction of antigens and antibodies at mucosal surfaces .Annu. Rev.Microbiol. 51: 311-340.

Lebman, D.A.; and Coffman, R.I. (1988): The effects of IL-4 and IL-5 on the IgA response by murine Peyer's patch B cell subpopulations. J Immunol. 141: 2050.

Lee, R.S.; Tartour, E.; Vander Bruggen, P.; Vantomme, V.; Joyeux, I.; Goud, B.; Fridman, W.H.; and Johannes, L. (1998): Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of Shiga-toxin .Eur. J.Immunol .28: 2726-2730.

Lefkovits, I.(1997): Immunology methods manual, the comprehensive sourcebook of techniques (Vol-2).Harcot Brace and Company, publisher.

Lehmann, J.D.; Smith, J.W.; Miller, T. E.; Barnet, J.A.; and Samford, J.P.(1968): Local immune response in experimental pyelonephritis J.Clin. Invest. 47:2541-2550.

Leive, l., and Morris, D.C. (1972): Isolation of lipopolysaccharide from bacteria. In methods in Enzymology.Vol.xxVIII(B). Edited by N: Ginsburg.PP.254- 262.

Levenson, V.I.; Egorova, T.P.; Belkin, Z. P.; Fedosova, V.G.; Subbotina, J.L.; Rukhadze., E.Z.; Dzhikidze, E.K.; and Stassilevich, Z.K.(1991): Protective ribosomal preparation from *Shigella sonnei* as parenteral candidate vaccine .Infect. Immun 59-3610-3618.

Levenson, V.I.;and Egorova, T.P.(1990):Polysaccaride nature of O-antigen in protective ribosomal preparations from

*Shigella:*experimental evidence and implications for the ribosomal vaccine.Res-Microbiol.141-720.

Levine, M.M.; McEwen, J.; Losonsky, G.; Reymann, M.; harai, I., Brow, J.E., and Taylo, D.N. (1992): Antibodies to Shiga holotoxin and to two synthetic patients with *Shigella dysenteriae* 1 dysentery .J.Clin. Microbial. 30: 1636-1641.

Li, A.; Cam, P.D.; and Islam, D.(1994): Immune response in vietnamese children after a single dose of an auxotrophic live *Shigella flexneri* Y vaccine strain SFL 124.J. Infect. Dis. 28: 11-23.

Li,A.; Rong, Z.C.; Fkwall, E.; Forsum, U.; and Lindbery, A.A.(1993): Serum antibody responses against *Shigella* lipopoly saccharides and invasion plasmid- coded antigens in *Shigella* infected Swedish patients .Scand J.Infect. Dis. 25: 569-577, 1993.

Lindh, E.(1975): Increased resistance of IgA to proteolytic degradation after binding of secretory component. J.Immunol. 114 :282-286.

Lindberg ,A.A.; Karnell, A.; Pal, T.; Sweiha, H.; Hulten by, K.; and Stocker, B.A.(1990): Construction of an auxotrophic *Shigella flexneri* strain for use as a live vaccine .Microb. Patho.8: 433-440.

Lindberg, A.A.; Karnell, A.; and Weintraub, A. (1991): The lipopolysaccharide of *Shigella* bacteria as a virulence factor .Rev. Infect. Dis. 13: 5279-5284.

Lopez, E, E.L.; diaz, M.; Nataro, J.P. (1989): Hemolytic uremic syndrome and diarrhea in Argentine children: The role of Shiga –like toxins. J. Infect. Dis. 160-469-475.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; and Randall, R.J. (1951): Protein measurement with the phenol reagent .J Biol. Chem. 193 :265-275.

Luderitz, O.; Staub, A.M.; and Westphal, O.(1966): Immunochemistry of O and R antigens of *salmonella* and related Enterobacteriaceae .Bacteriol Rev 30: 192-255.

Lycke, N. (1998): T cell and cytokine regulation of the IgA response.Chem. Immunol. 71:209-214.

Macfaddin, J.F.(2000): Biochemical tests for identification of medical bacteria 3rd ed. ,lippincott Williams and Wilkins, USA.

Mackie and McCartney (1996): Practical Medical Microbiology 14th ed., Collee, J. G.; Fraser, A.G.; Marion, B.P.; and Simmons, A., eds. Churchill Livingstone.

Mage, R.(1998): Immunology of lagomorphs.In: Handbook of vertebrate immunology. Griebel P. Pastoret P.P.; Baszin H., Govaerts A. Eds. Academic press Limited .673 pp.

Magee, D.M.; and Wing, E.J. (1988): Cloned L_3 T_4 + T lymphocytes protectmice against *Listeria monocytogenes* by secreting INF-γ. J. Immunol. 141 :3203- 3207.

Mallett, C.P.; Hale, T.L.; Kaminski, R.W.; Larsen, t.; Orr, N.; Cohen, D.; and Lowell, G.H. (1995): Intranasal or intragastric immunization with protosome –*Shigella* lipopolysaccharide vaccines protects against lethal pneumonia in a murine model of *Shigella* infection .Infect .Immun. 63: 2382-2386.

Mallett, C.P.; and VanDeverg, L.L. (1993): Evaluation of *Shigella* vaccine safety and efficacy in an intranasally challenged mouse model .Vaccine. 11: 190-196.

Mata, I.J.; Gangarosa, F.J.; Caceres, A. ; Pereta, D.R.; and Mejicanos, M.L. (1970): Shiga bacillus dysentery in central America. J. Infect. Dis. 122: 170-175.

Mathan, I. V.; and Mathan, M.M.(1991,a): Intestinal manifestation of invasive diarrheas and their diagnosis .Rev. Infect. Dis. 13, suppl 4: 5311-313.

Mathan, M.M.; and Mathan, I.V(1991,b): Morphology of rectal mucosal of patients with Shigellosis. Rev.Infect. Dis. 13: 5314-318.

Mayeux, P.R.(1997): Pathobiology of lipopolysaccharide. J.Toxicol. Environ Health 51: 415-435.

Mazanec, M.B.; Kaetzel, C.S., Lamm, M.E.; Fletcher, D.; and Nedrud, J.G.(1992): Intracellular and neutralization of virus by immunoglobulin A antibodies. Proc. Natl. Acad. Sci. 89 :6901-6905. USA.

Mazanec, M.B.; Nedrud, J.G., Kaetzel, C.S., Lamm, ME.(1993): A three –tiered view of the role of IgA in mucosal defense. Immunol. Today. 14: 430-435.

McGhee, J.R.;Mestecky, J.; Elson, C. O.; Kiyono, H.(1989): Regulation of IgA synthesis and immune response by T cells and interleukins. J.Clin .Immunol. 9: 175-179.

McIntgre, T.M.; Ogra, P.; Strober, W.; Mestecky, J.; McGhee,J.R.; Lamm, M.; Bienenstock, J.(1999): Gut-associated lymphoid tissue :regulation of IgA BE cell development, eds. Hand book of mucosal immunology 319- Academic, san Diego.

Medzhitov, R.; and Janeway, C.J.(2000): Innate immune recognition: mechanisms and pathways. Immunol Rev. 173:89-97.

Meitert, T.; and Pencu, E.(1984): Vaccine strain *S. flexneri* T_{32}- ISTRa TI. Studies in animals volunteers. Antidysentery immunoprohylaxis and immunotherapy by live vaccine. Arch. Roun. Patho. Exp. Microbiol. 43 :251-258.

Meiver, J.; Grady, G.F.; and Keusch, G. T.(1975): Production and characterization of exotoxin(s) of *Shigella dysenteriae* type 1.J.Infect. Dis. 131: 559-566.

Mel, D.M.; Terzin, A.L.; and Vuksic, L.(1971): Studies on vaccination against bacillary dysentery. 4 .Oral immunization with live monotypic and combined vaccines. Bull. W. H. O. 39 :375-380.

Menard, R.; Prevost, M. C.; Gounon, P.; Sansonetti, P.; and Dehio, C.(1996): The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells .Proc. Nah .Acad. Sci USA 94: 1254-1258.

Menard, R.; Sansonetti, P.J.; and Parsot, C.(1993): Nonpolar mutagenesis of the Ipa genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol .175: 5899-5906.

Metcalf, J. A.; Gallin, J.I.; Nauseef, W.M.; and Root, R.K (1986): Laboratory manual of neutrophil function, PP: 100-103. Raven press, NewYork.

Mielke, M.E.A.; Ehlers, S.; and Hahn, H.(1988): T-cell subset in delayed-type hypersensitivity, protection, and granuloma formation in primary and secondary *Listeria* infection in mice: superior role of Lyt-2+ cells in required immunity .Infect. Immun. 56: 1920-1925.

Mielke, M.E.A.; Niedobitek, G.; Stein, H.; and Hahn, H.(1989): Acquired resistance to *Listeria monocytogenes* is mediated by Lyt-2^{+} T cells independently of the influx of monocytes in to granulomatous lesions .J. Exp. Med .170 :589-594.

Moretta, L.; ferarine, M.; Mingari, M.C.(1977): Subpopulation of human T cells identified by receptors for immunoglobulins and mitogen responsiveness .J.Immunol 117: 2171-2176.

Morrison, D.C. and Leive, L.(1975): Isolation and characterization of two fraction of lipopolysaccharides from E. Coli O11: B4. J Biol. Chem. 250: 2911.

Mostov, K. E. (1994): Transepithdial transport immunoglubulins .Annu. Rev. Immunol. 12 :63-84.

Munder, M.; Mallo, M.; and Eichann, K.(1998): Murine macrophages secrete intereron gammaupon combined stimulation with interleukin (IL)12 and IL-18: A novel pathway of endocrine macrophages activation. J. Exp. Med. 187: 2103-2108.

Muzio, M. and Mantovani, A.(2000): Toll- like receptors. Microbes. Infect. 2:251-255.

Nataro, J.P.; Seriwatana, J.; and Fasano, A. (1993): Cloning a sequencing of a new plasmid- encoded entertoxin in enterinvasive *E.coli* and *Shigella* .In program and Abstracts of the 29th Joint conference on cholera and Related Disease. National In statutes of health, PP. 144-147.

Nataro, J.P.; Seriwatana, J.; Fasano, A. and Levine, M.M.(1995): Identification and cloning of a novel plasmid- encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains .Infect. Immun. 63: 4721-4728.

Nelson, M.R.; Shanson, D.S.; and Robin, G.(1992): *Salmonella*, *Campylobacter*, and *Shigella* in HIV seropositive patients, AIDs, 6: 1495-1498.

Neutra, M.R.(1999): M cells in antigen sampling in mucosal tissue. Curr. Top. Microbial Immunol. 236: 17-32.

Noriega, F.R.; Liao, F.M.; and Formal, S.B.(1995): Prevalence of Shigella enterotoxin 1 ($ShET_1$) among *Shigella* clinical isolates of diverse serotypes .J. Infect Dis. 172: 1408-1411.

Noriega, F.R.; Liao, F.M.; Maneval D.R.; Ren, S.; Formal, S.B.; and Levine, M.M. (1999): Strategy for cross protection among *Shigella flexneri* serotypes. Infect immun .67:782-788.

Noriega, F.R.; Wang, J.y.; Losonsky, G.; Maneval, D.R.; Hone, D.M.; and Levine, M.(1994): Construction and characterization of attenuated Δ *aroA* Δ *virG Shigella flexneri* 2a strain CVD 1203., a prototype live oral vaccine .Infect. Immun. 62: 5168-5172.

Noriega, F.R.;Losonsky, G.; landerbaugh, C.; Liao, F.M.; Wang,M. S.; and Levine, M.M.(1996): Engineered Δgua BA, Δvir G *Shigella flexneri* 2 a strain CVD 1205: construction, safety ,Immunogenicity and potential efficacy as a mucosal vaccine .Infect. Immun. 64: 3055-3061.

Oaks, E.V.; Hale, T.L.; and Formal ,S.B.(1986): Serum immune response to *Shigella* protein antigens in rhesus monkeys and humans infected with *Shigella spp*.Infect. Immun. 53:57-63.

Oberhelman, R.A.; Kopecko, D.J.; Salazar-L indo, E.; Gotuzzo, E.; Buysse, J.M.; Venkatesan, M.M Fernandez- prada, A.YC.; Guzman, M.; Leon-Barua, R.; and Sack, R.B.(1991): Prospective study of systemic and mucosal immune responses in dysenteric patients to specific *Shigella* invasion plasmide antigens and lipoplysaccharides. Infect. Immun. 59: 2341-2350.

O'Brien, A.D.; and Laveck, G.D.(1982a): Production of *Shigella dysenteriae* type 1 like cytotoxin by *Escherichia coli*.J Infect. Dis .146: 763-769.

O'Brien, A.D.; and Laveck, G.D.(1982b): Immunochemical and cytotoxic activities of *Shigella dysenteriae* 1 (Shiga) and Shiga-like toxins. Infect. Immun. 35: 1151-1154.

Ohteki,T.; Yoshida, H.; Matsuyama, T.; Duncan, G.S.; Mak, T.W.; and Ohashi, P.S.(1998): The transcription factor interferon regulatory

factor I(IRF-1) is important during the maturation of natural killer 1.1^+ T cell receptor- α / B^+ (NKl^+ T) cells, natural killer cells ,and intestinal intraepithelial T cells. T. Exp. Med. 187: 967.

Orr, N.; Robin, G.; Lowell, G.; and Cohen, D.(1992): Presence of specific immunoglobulin A- secreting cells in peripheral blood after natural infection with *Shigella sonnei*. J. Clin. Microbiol, 30: 2165-2168.

Orr,N; Robin, G., Cohen, D.; Arnon, R.; and Lowell, G..H.(1993): Immunogenicity and efficacy of oral or intranasal *Shigella flexneri* 2a and *Shigella sonnei* protosome- lipoplysaccharide vaccines in animal models. Immun. 61: 2390-2395.

Park, B.H.; Fikrig, S.M.; and Smithwick, E.M.(1968): Infection and nitroblue-tetrazolium reduction by neutrophils. Lancet 2 : 532-534.

Parrillo, J.(1993): Pathogenic mechanisms of septic shock. N. Engl J.Med. 328: 1471-1477.

Parslow, T. G., Stites, D.P.; Terr, A.I.; and Imbodem, J. B.(2001): Medical Immunology. 3rd ed. McGraw Hill Comp. USA.

Parsot, C.(1994): *Shigella flexneri* : Gentics of entry and intercellular dissemination in epithelial cells .Curr. Top. Microbiol. Immunol. 192 :217-241.

Perdomo, J.J.Gounon, P.; and Sansonett, P. J. (1994): Polymorphonuclear leukocyte transmigration promotes invasion of colonic epithelial monolayer by *Shigella flexneri.* J .Clin .Invesr 93: 633-643.

Phalipon, A.; Kaufmann, M.; Michetti, P.; Coaaillon, J. M.; Huerre, M.; Sansonetti, P.; and Kraehenbuhl, J.P.(1995): Monoclonal immunoglobutin A antibody direct against serotype- specific epitope of *Shigella flexneri* lipopolysaccharide protects against murine experimental Shigellosis .J. Exp. Med. 182 : 769-778.

Philpott, D.J.; Yamaoka, S.; Israel, A.; and Sansontti, P.J.(2000): In vasive *Shigella flexneri* activates NF-KB through an LPS-dependent

innate intracellular response and leads to IL-8 expression in epithelial cells. J .Immunol 165: 905-914.

Poltorak, A.; He,X.; and Smirnova, I.(1998): Defective LPS Signaling C_3H / HeJ and C57BL /10 scrmice: Mutations in Tlr4 gene. Sience 282: 2085-2088.

Prentice, A.; Stirling ,D.M.; Sulivan, P.B.; Northrop- Clewes, C.A.; and Lunn, P.G.(1991): Raised urinary secretory IgA in chronic diarrhea. Arch. Dis. Child. 66 : 223-226.

Prevost, M. C.; Lesourd, M.; Arpin, M.; vernal, F.; Mounier, J.; Hellio, R.; and Sansonetti, P.J.(1992): Unipolar reorganization of F-actin layer at bacterial division and bundling of actin filaments by plastin correlate with movement of *Shigella flexneri* within Hela cells. Infect .Immun. 60 : 4088-4099.

Qureshi, S.T.; Lariviere, L.; and Leveque, G.(1999):Endotoxin tolerant mice have mutations in toll-like receptor 4(T1r4). J. Exp. Med. 189: 615-625.

Raqib, R.; Lindberg, A.A.; Wretlind, B.; Bardhan, P.K.; Andersson, U.; and Andersson, J. (1995): Persistence of local cytokine production in Shigellosis in acute and convalescent stages .Infect. Immun. 63:289.

Raqib, R.; Lindbery, A.; Bjork, L.; Bardhan, P.; Wretlind, B.; Andersson, U.; and Andersson, J.(1995): Down –regulation of gamma interferon, tumor necrosis factor type I, intereukin 1 (IL-1) type I, IL-3, IL-4 ,and trans forming growth factor β type I receptors at the local site during acute phase of *Shigella* infection .Infect. Immun. 63 :3079-3087.

Raqib, R.; Ljungdahl, A.; Lindberg, A.A.; Andersson, U.; and Andersson, J.(1996): Local entrapment of interferon in the recovery from *Shigella dysenteriae* type I infection .Gut 38:328.

Raqib, R.; Tzipori, S.; Islam, M.; and Lindberg, A.A.(1993): Immune responses to *Shigella dysenteriae* 1 and *Shigella flexneri* lipopolysaccharide and polysaccharide antigens in Bangladeshi patients with Shigellosis .Serodiagn. Immunother .Infect. Dis. 1 :37-45.

Raqib, R.; Wretlind, B.; Andersson, J.; and Lindberg ,A.A. (1995): Cytokine secretion in acute Shigellosis is correlated to disease activity and directed more to stool than to plasma. J. Infect. Dis. 171 :376-384.Cam, P.D.; Pale, T.; and Lindbery, A.A.(1993): Immune response against lippolysaccharide and invasion plasmid-coded antigens of *Shigella* in Vietnamese and Swedish dysenteric patients .J.Clin. Microbiol. 31(2): 454-457.

Rasolof-Razanam parany, V,; Beraud ,A.M.S.; Ronx, J.; Sansonetti, P.J.; and phalipon, A.(2001): Predominance of serotype specific mucosal antibody response in *Shigella flexneri* infected humans living in an area of endemicity .Infect.immun .69: 5230-5234.

Reed, W.P. (1975): Serum factors capable of opsonizing *Shigella* for phagocytosis by polymorphnuclear leukocytes. Immunol. 28: 1051-1059.

Reed, W.P.; and Albright , E.L. (1974): Secretory IgA and direct killing of *Shigella* .Immunol.26 :205-215.

Reinecker, H.C.; MacDermott, R.P.; Mirou,S.; Dignass, A.; and podolsky,D.K.(1996):Intestinal epithelial cells both express and respond to interleukin 15. Gastroen terology 111:1706-1710.

Reisesousa,C.; Sher, A.; and Kaye, P. (1999): The role of dentritic cells in the induction and regulation of immunity to microbial infection. Curr Opin Immunol. 11: 392-399.

Remick, D. (1995): Applied molecular biology of sepsis. J. Crit Care 10: 198-212.

Reynolds, J.D. (1986): Peyer's patches: a role at the center of B cell ontogeny. 136: 304-312.

Ribi,E.; Haskins, W.T.; Landy, W.; and Milner, K.C.(1961): Preparation and host-reactive properties of endotoxin with content of nitrogen and lipid .J Exp .Med. 114: 647-663.

Rietschel, E.; Holst, O.; Brade, L.; Mullerloennies, S.; Mama, U.; Zahringer, U.; and Beckmann, F.(1996): Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. Curr. Top.Microbiol. Immunol. 216:39-81.

Robbins, J. B.; Chu, C.Y.; and Schneerson, R.(1992): Hypothesis for vaccine development : protective immunity to enteric diseases caused by non typhoidal *salmonellae* and *Shigella* may be conferred by serum IgG antibodies to the O specific polysaccharide of their lipopolysaccarides. Clin. Infect. Dis. 15: 346-361.

Roberts, R.S.(1966): Preparation of endotoxin .Nature (London) 209:80.

Rohde, J.E.(1984): Selective primary health care : strategies for control of disease in the developing world .XV.Acute diarrhea. Rev .Infect Dis. 6: 840-854.

Roitt, I.; Brostoff. J.; and Male, D.(2001). Immunology. 6th ed . Harcourt publisher limited, Mosby, London.

Salamatova, S.A.; Sukhoroslova, L.I.; Shalmberidze, I.A.; and Brusenina, N.D.(1993): An immuno enzyme method for determining IgA antibodies to lipopolysaccharide in secretions in the diagnosis of Shigellosis .Zh-Microbiol –Epidemiol .Immunobiol. 3: 85-89.

Samandari, T.; Kotloff, K.L.; Losonsky, G.A.; Picking ,W.D.; Sansonetti, P.J.; Levine, M.M.; and sztein, M.B.(2000): Production of IFN-gamma and IL-10 to *Shigella* invasion by mononuclear cells from

volunteers orally inoculated with a Shiga toxin- deleted *Shigella dysenteriae* type 1 strain J. Immunol. 164: 2221-2232.

Sansonetti, P.J.(2001): Microbes and microbial toxins: Paradigms for microbial- mucosal inter actions.III .Shigellosis : from symptoms to molecular pathogenesis. Am.J. Physiol. Gasterointest Liver physiol. 280: 319-323.

Sansonetti, P.J., Arondel, J.; and Sereny, B.(1995): Role of IL-1 in pathogensis of experimental Shigellosis .J. Clin Invest. 96: 884-892.

Sansonetti, P.J.; and Phalipon, A.(1999): M cells as ports of entry for enteroinvasive pathogens: Mechanisms of interaction ,consequences for the disease process, Semin .Immunol 11: 193-203.

Sansonetti, P.J.; Arnodel, J.; Huerre, M.; Harda, A.; and Mastsushima, K.(1999): Interleukin-8 controls bacterial transepithelial translocation at the coast of epithelial destruction in experimental Shigellosis .Infect .Immun. 67: 1471-1480.

Sansonetti, P.J.; Arnodel, J.;Arondel,J.;Cantey,R.J.;Prevost,M.C.;and Huerre,M.(1996):Infection of rabbit Peyer's patches by *Shigella flexneri*: effect of adhesive or invasive bacterial phenotypes on follicular –associated epithelium.Infect.Immun.64:2752-2764.

Sansonetti, P.J.; Arondel, J.; Fontaine, A.; D'Haautevill, H.; and Bernardini, M.L.(1991): OmpB(Osomo-regulation)and icsA(cell to cell spread) mutants of *Shigella flexneri.*Evaluation as vaccine candidates. Probes to study pathogenesis of Shigellosis. Vaccine 9: 416-422.

Sansonetti, P.J.; VanNhieu, G.T.; and Egile, C. (1999): Rupture of the intestinal epithelial barrier and mucosal invasion by *Shigella flexneri.* Clin. Infect. Dis. 28: 466-475.

Schaffner, T.; Mueller, J.; Hess, M.W; Cottier, H.; Sordat, B.; and Ropke, C.(1974): The follicle-associated epithelium in the sheep ileal peyer's patche and rabbit appendix. Cell immunol. 13: 304-312.

Sedgwick, J.D.; and Holt, P.G.(1983): A solid phase immunoenzymatic technique for the enumeration of specific antibody –secreting cells. J. Immunol. Methods 57: 301-309.

Sethabuter, O.; Venkatesan, M.; and Sereny ,B.(1993): Detection of *Shigella* and enteroinvasive *Escherichia coli* by amolification of the invasion plasmid antigen H DNA sequence in patient with dysentery. J. Infect. Dis. 167: 458-461.

Shnawa, I.M.S.; and Thwaini, Q.N.A.(2002): Lapin mucosal humoral versus systemic humoral and cellular immune responses post to intratesticular administration of heat killed *campylobacter fetus* bacterin.j Babylon Sci Appl. 3: 538-542.

Shotriffe, L.M. D.; Elliott, K .; and Sellers, R.G.(1989): Measurement of urinary antibodies to crude bacterial antigen in patients with chronic bacterial prostatitis. J. Urol. 141: 632-636.

Silobreic, V.; Sabioncello, A.; Mzuran, R.; Dekaris, D.; and Lovrencic ,M.(1975): A comparison of the inhibition of leukocyte migration and monocyte spreading as in vitro assays for tuberculin hypersensitivity in man. Clin.Exp .Immun. 20: 239-247.

Soborg, M.(1986): In vitro migration of peripheral human leukocytes in cellular hypersensitivity. Acta. Medica Scandonavica. 184: 135-139.

Sobory ,M.(1969): Interaction of human peripheral lymphocytes and granulocytes in the migration-inhibition reactions. Acta. Med. Scand. 185 :221.

Solvason, N.; Lehuen, A.; and Kearney, J.F. (1991): An embryonic source of lyl but not conventional B cells. Int. Immunol. 3: 543-548.

Speelman, P.; Kabir, I.; and Islam, M. (1984): Distribution and spread of colonic lesions in shigellosis : Acolonscopic study. J. infect Dis. 899-903.

Speelman, P.; McGlauglin, R.; and Tacket, C.O.(1987): Difference in clinical features and stool finding in Shigellosis and amoebic dysentery. Trans R. Soc. Trop .Med Hyg. 81: 549-551.

Stein, P.E.; Boodhoo, A.; and Tyrrell, G.J. (1992): Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E.Coli*. Nature 355 :748-750.

Stiehm, E.R.; Winter, H.S.; and Bryson Y.J.(1979): Cellular, (T cell) immunity in the human Newborn. Pediatrics 64: 814-821.

Stites, D.P.; Terr, A.I; and Parslow, T.G. (1994): Basic and clinical immunology .8th ed. Prentice-Hall international Inc. USA.

Stokes, C.R.; Soothill, j.F.; and Turner, M.W. (1975): Immune exclusion is a function of IgA. Nature. 255 :745-746.

Stoll, B.J.; Glass,R.I.; and Toledo, M. R. (1982): Epidemiologic and clinical features of patients infected with *Shigella* who attended a diarrheal disease hospital in Bangladesh. J. Infect. Dis. 146:177-183.

Strockbine, N.A.; Jackson ,M.P; and sung L.M.(1988): Cloning and sequencing of genes for shiga toxin from *Shigella dysenteriae* type I. J Bacteriol 170 :116-122.

Takeuchi, A.; Formal, S.B.; and Sprinz ,H.(1968): Experimental acute colitis in the rhesus monkey following peroral infection with *Shigella flexneri.* Am. J.Pathol. 52:503-520.

Taylor, D.N.; Trofa, A.C.; Sadoff, J.; Chiayung, C.; Bryla, C.; Shiloach, J.: Cohen, D.; Ashkenazi, S.; Lerman, Y.; Egan, W.; Schneerson, R.; and Robbins ,J. (1993): Synthesis, characterization ,and clinical evaluation of conjugate vaccines composed of the O-Specific

polysaccharide of *Shigella dysenteriae* type 1, *Shigella flexneri type 2 ,and Shigella sonnei*)*Plesiomonas shigelloides*) bound to bacterial toxoids .Infect. Immun. 61: 3678-3687.

Taylor, D.N.; Bodhidatta, L.; Brown, J.E.; Echeverria, P.; Kunanusont, C.; and Lindberg, A.A.(1989): Introduction and spread of multiresistant *Shigella dysenteria* 1 in Thailand .Am. J. Med. Hyg. 40: 77-85.

Trinchieri, G.(1995): Interleukin 12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity .Annu Rev Immunol. 13: 251-276.

Turbyfill, K.R.; Hartman, A. B.; and Oaks, E.V. (2000): Isolation and characterization of a *Shigella flexneri* invasion complex subunit vaccine. Infect. Immun. 68: 6624-6632.

Ulevitch, R.; and Tobias, P.(1995): Receptor- dependent mechanisms of cell stimulation by bacterial endotoxin. Ann thorac Surg. 57:376-382.

Vajdy, M.; Sethupathi, P.; and Knight, K.L.(1998): Life and death of B cells in peyer's patch –like structure. J. Immunol. 160: 2725-2729.

Van Deverg, L.L.; Herrington, D.A.; Bostego, J. ; Lindberg, A.A.; and Levine, M.M.(1992): Age-specific prevalence of serum antibodies to the invasion plasmid and lipopolysaccharide antigens of Shigela species in Chilean and North American populations. Infect. Dis. 166: 158-161.

Van Deverg, L.L.; Mallett, C.P.; Collins, H.H.; Larsen, T.; Hammack, C.; and Hale, T.L. (1995): Antibody and cytokine responses in a mouse pulmonary model of *Shigella flexneri* serotype 2a infection. Infect. Immun. 63:1947.

Van Furth, R. (1970): Mononuclear phagocytes. Oxford: Blackwell scientific publications.

VanNhieu, G.T.; Caron, E.; Hall, A.; and Sansonetti, P.J.(1999): IpaC induce actin polymerization and filopodia formation during *Shigella* entry into epithelial cells.E.M.B.O.J. 18: 3249- 3262.

Vicari, G.; Olitzki, A.L.; and Olitzki, Z.(1960): The action of the thermolabile toxin of *Shigella dysenteriae* on cells cultivated in vitro. Br J. Exp. Pathol 41: 179-189.

Voino- Yasenetsky, M.V.; and Khavkin T.N.(1964): A study of intraepithelial localization of dysentery causative agents with the aid of fluorescent antibodies. J.Microbiol. 12: 98-100.

Waksman, B, H. and Ozer, H. (1976): Peyer's patches structure that influences B cell ontogeny .Prog Allergy. 21: 1-113.

Wassef, J; Keren, D.F.; and Mailloux. J.L.(1989): Role of M cells in initial bacterial uptake and in ulcer formation in the rabbit intestinal loop model in Shigellosis .Infect. Immun. 57: 858-863.

Way, S.S.; Borczuk, A.C.; and Dominitz, R. (1998): An essential role for gamma interferon in innate resistance to *Shigella flexneri* infection. Infect Immun. 66: 1342-1348.

Way, S.S.; Borezuk, A.C.; and Goldberg, M. B.(1999): Adaptive immune response to *Shigella flexneri* 2a cycle in immunocompetent mice and mice lacking immunoglobulin A. Infect. Immun. 67 :2001-2004.

Weinstein ,D.L.; Jackson, M.P.; and Samuel, J.E. (1988): Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine .J.Bacteriol. 170: 4223-4230.

Wells, J.G.; and Morris, G.K. (1981): Evaluation of transport methods for isolating *Shigella* spp. J. Clin. Microbiol. 13: 789-790.

Westphal, O.; and Jann, K. (1965):Bacterial lipopolysaccharide: extraction with phenol –water and further applications of the procedure. Methods carbohydr. Chem. 5: 83.

Westphal, O.; Luderitz, O.; Eichenberger, E.; and Keiderling, W. (1952): Lipopolysaccharide extraction from bacterium with phenol-water. Z. Naturforsch. B: Anory. Chem. Org. Chem. Biochem. Biophys. Biol. 7: 536-548.

Wybran, J. Carr, M.C.; and Fudenberg, H.H.(1971): The human rosette – forming cell as a marker of a population of thymus-derived cells.J .Clin .Invest.51: 2537-2541.

Yamada, K.; Kimura, Y.; Nishimura, H.; Namii, Y.; Murase, M.; and Yoshikai, Y(1997): Characterization of CD^{+}_{4} $CD_{8\alpha\alpha}{}^{+}$ and CD^{-}_{4} $CD_{8\alpha\alpha}{}^{+}$ intestinal intraepithelial lymphocytes in rats. Int.Immunol. 11:21-27

Yamada, S.; Kai, A.; and Kudoh, Y.(1994): Serodiagnosis by passive haemagglutination test and verotoxin enzyme-linked immune sorbent assay of toxin producing E-Coli infection in patients with haemolytic –uremic syndrome. J.Clin Microbiol. 32: 955-959.

Yamanaka, T.; Straumfors, A.; and Morton, H. (2001): M cell pockets of human Peyer's patches are specialized extensions of germinal centers. Eur. J.Immunol. Today 20 :141-151.

Yang, O.O.; Kalams, S.A.; Trocha, A.; and Cao, H.(1997): Suppression of human immunodeficiency virus type 1 replication by CD8 cells. Evidence for HLA class 1 restricted triggering of cytolytic and non cytolytic mechanisms. J.Virol. 71: 3120-3128.

Zubbler, R.H.(1998): Antigens, T-dependent and independent in: Delves, R.J. and Roitt,I.(eds) .Encyclopedia in immunology Vol.1 .Academic press, London. 214-218.

Zuercher, A.W,; Coffin, S.E.; Thurnheer, M.C.; Fundova, P. ; and Cebra, J.J. (2002): Nasal-Associated lymphoid tissue IS a mucosal inductive site for virus- specific humoral and cellular immune responses.J. Immunol. 168: 1796-1803.

Zychlinsky, A.; Kenny, B.; and Menard, R, R.(1994): I paB mediates macrophage apoptosis induced by *Shigella flexneri.* Mo1 .Microbiol 11 : 619-627.sansonetti, P.J.; Arnodel, J.; and Cavailon, J.M.(1995): Role of interleukin 1 in the pathogenesis of experimental Shigellosis .J.Clin. Invest. 96: 884-892.

Zychlinsky, A.; Perdomo, J.J.; and Sansonetti, P.J. (1994): Molecular and cellular mechanisms of tissue invasion by *Shigella flexueri.* Ann NY Acad. Sci. 730 : 197- 208.

Zychlinsky, A.; Thirumalai, K.; Arondel, J.; Cantey, J.; Aliprantis, A.; and Sansonetti, P.(1996): In vivo apoptosis in *Shigella flexneri* infections.Infect. Immun. 64 :5357-5365.

الخلاصة:

تهدف هذه الدراسة للتحري عن مناعة *S.dysenteriae , S.flexneri* النوعية ، الخلطية والخلوية المضادة لسموم هذه البكتريا وفي كلا محوري المناعة الجهازية والمناعية المخاطية، والتي تم فيها تحضير السم الداخلي (Endotoxin) والخارجي (Exotoxin) وتقييم فعاليتها البايلوجية، اضافة الى تحضير مشتقات بروتينات *Shigella* من السم الخارجي. لقد تم استعمال فحص التلازن الدموي لتحديد المناعة الخلطية، في حين تم استعمال اختيار اختزال الصيغة (NBT) ، التشكيل الزهري التائي واختيار تثبيط هجرة الخلايا البيض لتقيم استجابة المناعة الخلوية.

لقد تبين ان الجرعات (1.5,2.5mg /Kg) تمثل الجرعات الآمنة و الممنعة لسم *S.dysenteriae, S.flexneri* الداخلي ، بينما مثلت الجرعات (0.5,1mg/Kg) الجرعة الآمنة والممنعة لسم *S.dysenteriae* الخارجي . أنجز العمل على (85) أرنب ذكر بالغ (وزان كل منها حوالي 2kg). قسمت الأرانب الى عشر مجاميع، من المجموعة الأولى الى المجموعة السابعة، كل مجموعة تتكون من عشرة مكررات، من المجموعة الثامنة الى المجموعة العاشرة، كل مجموعة تتكون من خمسة مكررات.

لقد ظهر ان سم *S.dysenteriae , S.flexneri* الداخلي عند الجرعات (1.5,2.5mg /Kg) قادر على تحفيز الاستجابة المناعية الخلطية والخلوية وعلى مستويي المناعة الجهازية والمناعة المخاطية وخاصة في عينات الزائدة الدودية (عينات المخاطية شملت عينات الزائدة الدودية وعينات الاثنى عشر). اذ كانت قيم وسيط الاستجابة المناعية الخلطية المخاطية 44.8,41,48,48 لسم *S.dysenteriae* الداخلي عند الجرعات (1.5,2.5 mg/Kg) ولسم *S.dysenteriae* الداخلي عند الجرعات (1.5,2.5 mg/Kg) ، على التوالي أعلى إحصائيا اذا ما قورنت بقيم وسيط الاستجابة المناعة الخلطية الجهازية لسم *S.dysenteriae , S.flexneri* الداخلي عند الجرع (1.5,2.5 mg/Kg) والتي كانت 304,336,320,304 على التوالي . كذلك ظهر ان قيم وسيط عينات الجهاز المخاطي عالية ، لا سيما في عينات

الزائدة الدودية عند تمنيع الأرانب بالسم الخارجي وبالجرعات (0.5,1mg/Kg) حيث كانت 44.8, 48، لكن قيم وسيط عينات الجهازية بدت اقل من تلك التي كانت لعينات المخاطية اذ كانت 256 ,288 ، على التوالي.

اظهر فحص اختزال الصيغة من قبل كريات الدم البيض الملتهمة قابلية عالية على اختزال هذه الصيغة في كل الجرع (1.5,2.5mg /Kg) لسم *S.dysenteriae* *S.flexneri* , الداخلي في عينات المخاطية ولا سيما في عينات الزائدة الدودية ، اذ لوحظ ان قيم الوسيط لاختزال الصيغة لـ *S.dysenteriaei* كانت 45 ,47.2 لعينات الزائدة الدودية عند الجرع (1.5,2.5mg/Kg) من السم الداخلي ، على التوالي . قيم وسيط اختزال الصيغة *S.dysenteriae* وفي كلا الجرعتين أعلاه كانت 46.3 ,49 في عينات الزائدة الدودية ولكل جرعة على التوالي . قيم الوسيط لفحص اختزال الصيغة للأرانب الممتعة بكلا الجرعتين (0.5,1mg /Kg) من السم الخارجي ازدادت أيضا ولكنها اقل من تلك التي كانت لعينات المخاطية ، خاصة في عينات الاثنى عشر ، حيث كانت 29.4 , 31.2 للجرع (0.5,1mg /Kg) على التوالي.

كذلك لوحظ ازدياد فعالية اختزال الصيغة جهازياً ، ولكنه اقل من تلك التي كانت لعينات المخاطية ، لا سيما في جرع السم الخارجي ، اذ كانت قيم الوسيط 38.4,35 لسم *S.flexneri* الداخلي ، 37.4, 38.7 لسم *S.dysenteriae* الداخلي وعند الجرع (1.5,2.5mg /Kg) ,26.4 , 27.5 لسم *S.dysenteriae* الخارجي وعند الجرع (0.5,1mg /Kg) ولكل جرعة ، على التوالي.

ظهر ان قيم وسيط عامل تثبيط هجرة الخلايا عند الجهاز المخاطي أعلى معنويا من قيم وسيط عامل تثبيط هجرة الخلايا في الجهازية لسم *S.flexneri* وسم *S.dysenteriae* , الداخلي للجرع (1.5,2.5mg /Kg) وخاصة في عينات الزائدة الدودية واقل منها في عينات سم *S.dysenteriae* الخارجي عند الجرع (0.5,1mg /Kg) اذ ان قيم الوسيط كانت 35.4, 33.5 في عينات الزائدة الدودية المخاطية لسم *S.flexneri* الداخلي و34.1 ,32.4 في عينات الزائدة الدودية

المخاطيـــــة لـــــسم .*S.dysenteriae* الـــــداخلي عنـــــد الجـــــرع (1.5,2.5mg/Kg),39.6,42.1 لـسم *S.dysenteriae* الخـارجي عنـد الجـرع (0.5,1mg /Kg) وعلى التوالي.

أظهرت قيم الوسيط لمعامل تثبيط هجرة الخلايا البيض في العينات الجهازية انخفاضاً عند استخدام السم الخارجي كمتحمس مقارنة بسم *S.dysenteriae* الداخلي في كلا الجرعتين (0.5,1mg /Kg)، حيث كانت قيم الوسيط 57, 54.8 لكل جرعة على التوالي.

لقد كانت قيم الوسيط لفحوصات التشكيل الزهري التائي في عينات الجهاز المخاطي عالية، اذ كانت 47 ,49 ,48 ,48 في عينات الزائدة الدودية منها في الجهازية والتي كانت 37.4 ,39.9 ,36.4 ,40.6 لسم *S.flexneri* الداخلي للجرعات (1.5,2.5mg /Kg) وسم *S.dysenteriae* الداخلي عند الجرعات (1.5,2.5mg /Kg)، على التوالي . جرعات السم الخارجي (0.5,1mg /Kg) ايضاً سبب زيادة في قيم وسيط التشكيل الزهري التائي ، ولكنها اقل من قيم وسيط التشكيل الزهري الثائي في عينات الجهاز المخاطي عند تمنيع الأرانب بالسم الداخلي وفي كلا الجرعتين أعلاه . هذا وان قيم الوسيط كانت 40 ,44.4 في عينات الزائدة الدودية للجرع (0.5,1mg /Kg)، على التوالي.

كانت قيم وسيط التشكيل الزهري التائي في عينات الجهازية للسم الخارجي أيضا اقل من تلك التي كانت لعينات المخاطية إذ إنها كانـــت 30.2 ,30.4 للجـــرعات (0.5,1mg /Kg) , على التوالي.

استخدم بروتين *S.dysenteriae* المحضر من السم الخارجي عند الجرعة (1mg/Kg) وســـم *S.flexneri* الـــداخلي ، الجرعـــة (2.5mg/Kg) وســـم *S.dysenteriae* الداخلي ، الجرعة (2.5mg/Kg) لتحديد الاستجابة المناعية الموضعية باستخدام اختبار الجلد.

دراسة مقارنة للاستجابة المناعية المخاطية والجهازية المتخصصة بنوعين من الجنس *Shigella* في الأرنب

أطروحة
مقدمة الى مجلس كلية العلوم – جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراه-فلسفة
في علوم الحياة / الأحياء المجهرية

من قبل
عدنان كريم عبد السلامي

1426 هـ 2005م