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Full Length Research Paper

Disease management by vaccination using *Escherichia coli* antigens

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A major challenge for health care in 21st century is the increase in level of resistance to pathogens and effective vaccines to prevent the often life threatening infections are urgently required. Pathogen has been investigated for their potential use either as therapeutic or prophylactic vaccines, in order to identify new antigens of diagnostic and vaccine potential. The present investigation was done with five different type of antigens produced from single pathogen. It also identifies the immune complex and DNA antigen as most suitable for vaccine development against pathogens. Antigens may vary within the host during the course of infection or antigenic types of parasites in the population. Antigenic variation is an important mechanism used by pathogenic microorganism for escaping and neutralizing activities of the antibody. Immune complexes and DNA antigen serve as vaccines, and it can be used to develop multi antigen. Furthermore, it is also easy to produce in a large scale. Vaccination is still the best way for prevention of bacterial diseases. The conditions for the preparation of antigens of intact natural composition and conformation from Escherichia coli (whole cell and heat killed), was determined using Swiss albino mice (Balb/C) as experimental species. A novel approach for vaccine design and production is discussed.

Key words: Immune complex, vaccine, delayed type hypersensitivity, *Escherichia coli*.

INTRODUCTION

Escherichia coli are a gram negative bacterium that is notable for the frequency and severity of infections that it causes hospitalized patients. These infections range from localized urinary tract infections to bacteremia and septic shock. In the past 20 years, there has been a dramatic increase in the incidence of noscomial staphylococcal infections: this increase parallel of the increased use of intravascular devices and invasive procedures. *E. coli* is identified as one of the three most frequent noscomial pathogens and is responsible for approximately 25% of the 2 million noscomi infections reported in US each year (Berry et al., 1986).

In an immunological survey of *E. coli* surface antigens, human acute phase serum was screened with *E. coli.* Bacterial surface display libraries with polypeptides fused to *E. coli* surface proteins (Kuklin et al., 2006).

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MATERIALS AND METHODS

A loopful of pathogenic culture of *E. coli* was streaked on nutrient agar and incubated overnight at 37°C. A single colony (taken from the streaked agar plate) is inoculated into blood heart infusion broth and incubated overnight in a shaker. Thus generated culture was used to prepare whole cell bacterial antigen.

A fraction from that culture was heat killed at 60°C for 3 h or at 80°C for 30 min in a water bath (sterility checked by restreaking on nutrient agar plate) and used to prepare heat killed bacterial antigen. Heat killed culture was centrifuged at 3,000 rpm for 30 min, supernatant discarded and pellet washed with PBS. Packed cells are resuspended to desired concentration - 25% in PBS (for mice 10^6 cells in 0.2 ml PBS after counting in haemocytometer).

Animals and treatment

Mice weighing 16.0 ± 0.1 gm (28 days old) were recruited from the acclimatized stock. The mice were grouped into five groups with six individuals in each group. Test antigens were given through the intraperitonial (i.p) injection at optimum levels with primary and secondary doses (two weeks intervals). Treatment was given to

animals for 3 - 5 days. During treatment period, pellet feed and water were given in *ad libitum*. Food consumption, general condition and other symptoms were observed daily and body weights recorded.

Experimental system

The test animals were divided into five groups including control (unimmunized). Four groups of animals stimulated with different types of antigen (pathogen, heat killed pathogen, pathogen with antiserum and heat-killed pathogen with antiserum) suspended in normal saline (0.15 M). Approximately 25 x 10^6 bacterial cells in one ml saline were administered for the primary and 50 x 10^6 cells/ml for the secondary immunization three days after the primary dose. Unstimulated (Control) mice were treated similarly except immunization with antigen. Blood samples of stimulated and non-stimulated mice were collected on the third, fourth and fifth days, following antigen exposure by cardiac puncture, after anaesthetizing the mice with chloroform. The serum was separated for each group separately and kept at – 20^0C, till analyzed. Heparin was used in collecting the whole blood and leukocyte rich plasma for lymphocyte subset enumeration.

Antibody titration

The total amount of antibody production was carried out by Log_2 titre plate method. 50 µl of physiological saline was added into all the wells of a clean microtitre plate. Then 50 µl of the antiserum is taken in a pipette and added in first well. From the first well 50 µl of the content is serially diluted till the 11th well of the microtitre plate leaving the 12th well as a negative control. 25 µl of 1% SRBC in saline was added to all the wells of the microtitre plate. The microtitre plates were hand shaken for effective mixing of reagents and incubated for an hour at 37°C and for one more hour at 10°C. The highest dilution of the serum samples, which shows detectable agglutination, is recorded and expressed in Log_2 titre of antibody.

B cell and T cell E - rosette assay (Dhasarathan et al., 2007)

5 - 10 ml of blood was collected and it was introduced into sterile conical flask containing (4 - 5) sterile glass beads. It was then continuously swirled until no sound was heard from the beads. This indicates that all the fibrins have adhered to the beads. This defibrinated blood was taken and diluted with equal volume of physiological saline. 3 ml of the lymph prep solution was taken in a centrifuge tube. The tube was kept in slanting position and 9 ml of diluted blood was slowly added along the sides of the centrifuge tube using a Pasteur pipette. Care was taken so that the FICON layer of the lymph prep solution present in the centrifuge tube was not disturbed.

The content of the centrifuge tube was then centrifuged at 1600 rpm for 20 min. The interphase (containing lymphocytes) was removed using a pipette. The cells were washed with 1 ml saline and excess FICON was removed. The sample was again washed with 1 ml of saline, after centrifugation, the supernatant was decanted by inverting the tube over a filter paper. After the saline drained; the pellet was resuspended in 300 µl of RPMI 1640 medium.

Re-suspended lymphocytes were loaded into an activated nylon wool column. The column was held vertically above an eppendorf tube, and hot saline (about 60°C) was slowly dripped into the column. The hot saline passing out of the column was collected in column. The hot saline passing out of the column was collected in the eppendorf tube and will contain T lymphocytes and 0.2 ml of 1% SRBC was added. The mixture was centrifuged for 12 min at 1600 rpm. After centrifugation, the samples were incubated at 4°C for 5 min. The pellet formed in the eppendorf tube was re-suspended by gentle flushing with a Pasteur pipette (0.2 ml of the saline was added to tube containing B and T lymphocyte). Then a drop from each of the tubes was taken in clean dry slides, B and T cells enumerated under the microscope (20x/40x) for rosettes. Number of B and T cell rosettes formed was observed per hundred lymphocytes present.

Delayed type hypersensitivity response

DTH was studied following the method described by Berry et al. (1986) for mice. The experimental mice (control and 7 days antigen exposed mice) were sensitized by a single subcutaneous application of 0.5 ml DNCB (10 mg/ml^{-1}) in vehicle consisting of acetone olive oil (4:1) mixture. Primary sensitization was done on the tenth day of intoxication and sensitized mice were challenged on the 14th day of intoxication with 0.5 ml of DNCB (10 mg/ml^{-1}). A positive response is conventionally assessed as one giving ≥ 5 mm indurations. Response can be graded with 3 - 4 mm = +; 5 - 8 mm = ++; 9 - 11 = +++; 12 mm or more = ++++.

Mice were sensitized by subcutaneous injection with 0.5 ml of Freunds complete adjuvant containing 500 mg of antigen and boosted at 6 and 8th days by an intradermal injection and sterile phosphate buffer saline (PBS), pH 7.2 served as control. Thickness of the skin was measured in all mice at regular 2 h interval up to 12 h from injection using Vernier Caliper. The increase in mean skin thickness (MST) of mice was obtained after deducting the skin thickness of the same site before challenge. Overall MST was obtained by taking the mean of individual mice in that particular group.

RESULTS AND DISCUSSION

One future which is common to several of the routine laboratory test on animal toxicity is the continuous use of pathogens to test animals and the point of death or the end of the test period which ever occurs first (usually 96 h, that is, 4 days). The number of dead animal after standard period of exposure (24, 48, 72 and 96 h) was recorded. Pathogens adversely affect the animal growth, water consumption, feed consumption and reproductively. It was shown that, pathogen decreases the body weight compared to that of normal mice, some notable changes were also noted in activity, growth, water and feed consumption and reproduction (number of offspring produced per pregnancy). It would be concludes that the pathogenic organism acts as biotic stress to the host animals.

To optimize the serum dilution with saline, control serum were loaded over a range of dilution from 1:1250, and a dilution of 1:320 appeared to be highest antibody titre (Table 1). An estimation of antibody levels in the serum after an antigenic challenge will expose the functioning of humoral immune systems. In immunodeficient animal antibody production was affected and there by humoral response against a disease causing antigen was less. But the immune complex treated animals enhance the production of antibody. In the present study, pathogen and heat killed pathogen treated mice showed an

Table 1. Antibody titre value in mice treated with different antigens.

S. no.	Animal	Antibody titre
1.	Control	5 $\log_2$ 2
2.	Pathogen	3 $\log_2$ 2
3.	Heat killed pathogen	5 $\log_2$ 2
4.	Pathogen with antiserum	8 $\log_2$ 2
5.	Heat killed pathogen with antiserum	9 $\log_2$ 2

Table 2. Enumeration of B and T cells using rosette forming assay in normal and treated mice.

S/No.	Test chemical	I week		II weeks	
		% of lymphocyte estimation		% of lymphocyte estimation	
		B cell	T cell	B cell	T cell
1.	Control	12.8	40.8	14.9	41.8
2.	Pathogen	25.6	32.8	17.6	29. 2
3.	Heat killed pathogen	32.8	41.2	24.2	36.3
4.	Pathogen with antiserum	34.6	68.8	30.6	74.4
5.	Heat killed pathogen with antiserum	36.2	71.4	36.3	75.7

antibody suppressive effect. The suppression of antibody reflects on the reduction of humoral immune response and this state subject the mice to easy infection. Immunomodulation of whole pathogen with antiserum and heat killed pathogen with antiserum treated mice showed moderate change in antibody production.

Cukrowsha et al. (1996) stated that, the damage to splenic of 1 g secreting cell, reduces antibody production in mice. According to Laufer et al. (1995) the inhibition of comple-ment protein in serum interferon with antibody production. Sreekumaran et al. (2002) found the organochlorine pesticide to damage DNA molecule. That damage to DNA molecule interferes with protein synthetic machinery, which then reduces the production of antibody. A similar observation was made in the present study too. Overall immune complex of the samples tested were immuno enhancer for antibody productions. This was suggested that when animals were exposed to immune complex, they will resist many intestinal pathogens.

Earlier studies reported cross reaction of *E. coli* with antibody to many other pathogens as *Citrobacter* species, *Brucella* and *Salmonella* species (Berry et al., 1986). B-Lymphocytes counts using rosette forming assay revealed significant decrement in pathogen exposed mice than control (Table 2). Of the two pathogens decrement in B-Lymphocyte was much pronounced in *E. coli* in the first week and *E. coli* pathogens had more or less similar impact on B-cell estimations. Present study, clearly confirms the decreement in B-cell number in mouse exposed to whole and heat killed pathogens. So the impact of whole cell and heat killed pathogenic molecules on the synthesis, proliferation and activation of lymphocytes.

Here, it is remarkably noted that enhancement in B-cell production is due to immune complex of antigens. The enhancement of this type of immune responses confirms the potential of immune complexes to be used as vaccines. Several workers (Genestripe et al., 1998; Dhasarthan et al., 2007) reported that immuno enhancive drugs enrich cell proliferations. B-cell proliferations modification depends on the exposure of antigens. But in the present study, nucleotide (DNA) produce moderate B-cell proliferations. These studies support foreign substance to be genotoxic and moderate synthesis of DNA in cell (Sreekumaran et al., 2002). The delayed type hypersensitivity reaction to tuberculin and DNCB antigen were tested in control and pathogen exposed mice. The impact of pathogens on DTH response in mice is recorded in Table 3.

A comparative analysis of DTH response in mice (in control and pathogen exposed) exhibited some interesting changes due to pathogenesity in two pathogens. From the result, it would be suggested that, disease can be cured even with the wound formation by using *Staphylococcus aureus*, immune complex as vaccine. Thus, elucidation of the mechanism involved in the progression of pathogenic disease and definition of relevant immune effectors mechanism are equally important for the identification of potential targets for beneficial intervention and subsequent vaccination, indeed the specific triggering of potent effectors mechanisms and responses without activating the immune responses.

Components leading to the pathology, together with the prevention of the escape mechanism involved by pathogens are the major idea of vaccine technology.

Table 3. DTH response in normal and treated mice.

Test animal	Test antigen	Inflammation (mm of mean skin thickness (MST))				DNCB response
		Control	Initial	After 3 h	After 6 h	
Control	Saline	0.30	0.30	0.35	0.35	++
	Pathogen	0.32	0.31	0.33	0.41	+++
	Heat Killed	0.32	0.31	0.35	0.47	++++
Pathogen treated	Saline	0.29	0.30	0.29	0.26	++
	Pathogen	0.29	0.29	0.33	0.39	-
Heat killed pathogen treated	Saline	0.29	0.29	0.32	0.32	++
	Heat Killed	0.29	0.30	0.36	0.32	-
Pathogen + Serum treated	Saline	0.29	0.29	0.27	0.29	++
	Pathogen + Serum	0.29	0.29	0.42	0.37	-
Heat killed pathogen + Serum treated	Saline	0.31	0.30	0.32	0.29	++
	Heat Killed + Serum	0.30	0.29	0.39	0.42	-

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