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Antibiotic Susceptibility and Immunomodulatory Potential of Chosen Bacterial Pathogens

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Abstract: Problem statement: Antibiotic susceptibility is still the best way for bacterial pathogen escape mechanism against immunity. **Approach:** In the present investigation, bacterial pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila*, *Klebsiella* and *Pseudomonas aeruginosa* were used to screen antibiotic susceptibility and immunomodulatory potential. **Results:** All the test pathogens were sensitive to all the test antibiotics 11±2 mm) except penicillin. The conditions for the preparation of antigens of intact natural composition and conformation from pathogens (whole cell and heat killed), were determined using Swiss albino mice (Balb/C) as experimental species. Immunomodulatory potential of test pathogens were screened using animal model. Test pathogen decreases the body weight comparing that of normal mice, some notable changes were also noted in activity, growth, water consumption, feed consumption. Antibody titre level in animal serum decreased upto 50% in whole cell pathogen and heat killed pathogen treated animals. **Conclusion:** The five pathogens administered animals, decrement in B-lymphocyte was much pronounced in *Pseudomonas aeruginosa* followed by *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* sp., *Aeromonas hydrophila* in the 5 week. Pathogen treated mice showed an IgG suppressive effect. It is found to be suppressive to T cell production, so induction in cell mediated immunity has confirmed pathogenic potential of test pathogens. All these test pathogenic strains were remarkably suppressing immune system of pathogen exposed animals.

Key words: Bacterial pathogens, immune response, antibiotic susceptibility, immunomodulatory, mice

INTRODUCTION

Pathogens expresses many potential virulence factors such as, surface proteins that promote colonization of host tissues; invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase); surface factors that inhibit phagocytic engulfment (capsule, Protein A); biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production); immunological disguises (Protein A, coagulase, clotting factor) and membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin; exotoxins that damage host tissues or otherwise provoke symptoms of disease (TSST, ET) and inherent and acquired resistance to antimicrobial agents (Lowy, 1998; Akond *et al*., 2009; Maripandi and Al-Salamah, 2010).

For the majority of diseases caused by bacterial pathogens, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in particular diseases (Shokrollah *et al*., 2009).

A better understanding of the relationship between immune responses to specific pathogens and protection is needed. This information will be required as vaccines move toward licensure. For example, what comprises a surrogate marker for protection? Is a calculated protective antibody level a reasonable surrogate marker for protection and equivalency measure in other populations than the one in which an efficacy trial was run? Perhaps *in vitro* functional equivalency could be used to make the case of antibody levels equivalency more acceptable as a surrogate marker (Lowy, 1998). *In*

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vivo protection studies in animals may be able to help interpret the significance of antibody responses. A difficulty here is the need for an animal model which closely mimics the population expected to develop an infection. In an immunological survey of bacterial pathogenic antigens, display libraries with polypeptides fused to bacterial surface proteins (Dhasarathan *et al*., 2010). Hence, in the present investigation planned to screen the antibiotic susceptibility and immunomodulatory potential of chosen bacterial pathogens.

MATERIALS AND METHODS

Antibiotic susceptibility test: The chosen pathogenic strains (*Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila*, *Klebsiella* and *Pseudomonas aeruginosa*) were tested for the sensitivity to antibiotics by the disc diffusion method. The test organism, 12-24 h culture was spread uniformly on the surface of the sterile air dried Muller-Hinton agar plated using sterile cotton swabs. The selected antibiotic (Penicillin (10 µg), Chlorampenicol (5 µg), Streptomycin (5 µg) Erythromycin (5 µg)) discs were placed on the centre of the test culture seeded plates using sterile forceps. Each antibiotic control, culture control and media control plates were maintained. The plates were incubated at 37°C for 24 h and measured the susceptibility of test strain was classified as being Sensitivity (S), Intermediate (I) or Resistance (R).

Immunological study: The five bacterial pathogens were collected from Microbial Type Culture Collection (MTCC) Institute of Microbial Technology (IMTECH) Chandigarh India. The samples were brought out the laboratory immediately and checked for their viability and culture were streaked on Muller-Hinton agar slants for storage and sub cultured on Muller-Hinton broth for analysis. The test strains were prepared into two different types of antigens like that heat killed bacterial antigens and whole cell bacterial antigens.

Preparation of heat killed antigen: Approximately 2.5×10^5 cells mL^{-1} test strains were heat killed at 60°C for 1 h in a water bath and centrifuged at 3000 rpm for 15 min. The pellet was washed with PBS for purity and resuspended into PBS and make into desired concentration.

Preparation of whole cell bacterial antigen: The test strain was centrifuged at 3000 rpm for 30 min, pellet washed with PBS and then packed cells were re-suspended to desire concentration (2.5×10^5 cells mL^{-1} for five different bacterial antigens) in PBS after counting in haemocytometer.

Animals and treatment: For the experimental study, mice weighing (20±28 g) were recruited from the acclimatized stock. Each group has 6 animals, housed in a specially designed cage with provision for systematic supply of pellets and water *ad libitum*. Test bacterial antigens were administered to the test animal and find-out LD_{50} by method described by Dhasarathan *et al*. (2010). From LD_{50} $1/10^{th}$ concentration was determined and injected into test animal through intraperitonial route with primary dose (2.5×10^5 cell mL^{-1}) and secondary doses (5×10^5 cell mL^{-1}) after successive 3 days. After treatment, Food consumption, general conditions and other symptoms were observed daily and body weights were recorded during experimental period. Blood samples were collected after antigen treatment on the 1st, 2nd and 3rd weeks of test animals by cardiac puncture. The serum was separated for each group separately and kept on-20°C till analysis. Heparin was used in collecting whole blood and leucocytes rich plasma for lymphocyte subset enumeration.

Screening of antibody titre: From the normal and treated mice, serum samples were used to screen the antibody levels. Quantification of serum antibodies were carried out by antibody titre plate technique containing respective antigens.25 µL of physiological saline was added in to the first well of microtitre plate, then 25 µL of antiserum added in the first well of microtitre plate, the antiserum was serially diluted in the well of the row till the 11th well of the microtitre plate leaving the 12 th well as positive control. Then 25 µL of 1% test antigen in saline were added to all the wells of the microtitre plate. The plate was hand shaken for the effective mixing of reagents and incubated for an hour at 37°C. After incubation period antibody titre was calculated based on the button formation in titre plate.

Enumeration of lymphocyte subset population: Five ml of blood samples were collected from test antigen treated and control mice using heparin pretreated vials. Lymphocytes were isolated using lymph prep solution and separated by Nylon wool column method described by (Dhasarathan *et al*., 2010). The separated subsets of lymphocytes were observed and enumerated by e rosette assay techniques (Dhasarathan *et al*., 2010).

Quantification of immunoglobulin: From the whole blood of normal and test animal, immunoglobulin was

separated using protease 'A' column. The eluted immunoglobulin was read at 280 nm. At 280 nm an absorbance of 1.0 is equivalent to an immunoglobulin concentration of 0.74 mg mL^{-1} which can calculate by this equation of:

Absorbance at 280 nm of sample × range of blank × number of dilution factor to be used

From the above equation the range of blank for immunoglobulin was consider as the constant (0.74).

RESULTS

In the present investigation, all the test pathogens were sensitive to all the test antibiotics, except penicillin (Table 1).

Evaluation of sub lethal concentration: 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 occurs first four days (usually 96 h).The most widely adopted measure of test sample sufficient to kill 50% of test animal within specific period. The lethal concentration of test pathogens were found *Staphylococcus aureus* was 3.1×10^5(cells mL^{-1}), *Escherichia coli* was 6.4×10^5 (cells mL^{-1}), *Pseudomonas aeruginosa* was 6.4×10^5 (cells mL^{-1}) and *Klebsiella* sp. was 6.4×10^5 (cells mL^{-1}).

Morphological analysis of normal and pathogen treated mice: Pathogens were adversely affect the animal growth, water consumption and feed consumption. This shows that pathogen decreases the body weight comparing that of normal mice, some notable changes were also noted in activity, growth, water consumption, feed consumption. It concludes, the pathogenic organism acts as biotic stress to the host animals (Table 2).

Screening of antibodies: An estimation of antibody levels in the serum after an antigenic challenge will expose the functioning of humoral immune system. In the present study, whole cell pathogen and heat killed pathogen treated mice blood samples were showed antibody suppressive effect (Table 3).

Table 1: Antibiotic susceptibility pattern of test pathogens against test antibiotics

		Zone of inhibition (mm) against test pathogens				
Antibiotic	Disc concentration (µg)	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. hydrophila*	*Klebsiella* sp.
Penicillin	10	0	0	0	0	5
Chlorampenicol	5	11	14	13	6	15
Streptomycin	5	8	12	12	9	10
Erythromycin	5	15	6	17	6	7

Table 2: Morphological changes in normal animal and animals administered with different bacterial antigens

Test organism	Test Ag	Wt. of animal (g)	Movement of the animal	Water consumption (mL day^{-1})	Feed consumption (g day^{-1})
Normal	-	25	Normal	30.00	35.0
S. aureus	Heat killed and whole cell Ag	20	Resting	22.10	24.1
		21	Abnormal	22.07	27.3
E. coli	Heat killed and whole cell Ag	20	Abnormal	20.00	27.6
		22	Abnormal	19.00	24.0
P. aeruginosa	Heat killed and whole cell Ag	20	Abnormal	9.00	9.0
		22	Abnormal	10.00	12.6
A. hydrophila	Heat killed and whole cell Ag	25	Restless	19.00	32.6
		23	Resting	18.00	29.3
Klebsiella sp.	Heat killed and whole cell Ag	28	Abnormal	12.07	24.0
		27	Wound formation	14.08	22.3

Table 3: Estimation antibody titre in control and animals exposed with different types of test antigens

Test antigen	Normal	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. hydrophila*	*Klebsiella*
Heat killed antigen						
1st week	$8Log^2_2$	$5Log^2_2$	$4Log^2_2$	$4Log^2_2$	$7Log^2_2$	$4Log^2_2$
2nd week	$9Log^2_2$	$4Log^2_2$	$5Log^2_2$	$5Log^2_2$	$7Log^2_2$	$5Log^2_2$
3rd week	$8Log^2_2$	$3Log^2_2$	$4Log^2_2$	$4Log^2_2$	$7Log^2_2$	$4Log^2_2$
Whole cell antigen						
1st week	$8\ Log^2_2$	$7Log^2_2$	$5Log^2_2$	$5Log^2_2$	$6Log^2_2$	$4Log^2_2$
2nd week	$8\ Log^2_2$	$5Log^2_2$	$6Log^2_2$	$6Log^2_2$	$7Log^2_2$	$4Log^2_2$
3rd week	$8\ Log^2_2$	$4Log^2_2$	$4Log^2_2$	$4Log^2_2$	$6Log^2_2$	$3Log^2_2$

Table 4: Estimation of B lymphocytes in animals administered with test antigens

	B-cells (%)-test antigens					
Duration	Normal	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. hydrophila*	*Klebsiella* sp.
Heat killed bacterial antigen						
1st week	33.3	23.9	23.5	20.4	28.3	27.5
2nd week	31.9	28.7	29.8	24.6	30.8	29.9
3rd week	32.8	29.9	22.6	22.7	24.5	23.9
Whole cell Bacterial antigen						
1st week	32.2	23.7	23.1	20.1	28.0	27.2
2nd week	31.7	28.1	29.6	24.3	30.7	29.9
3rd week	32.3	29.8	22.4	22.3	24.4	23.8

Table 5: Isolation of Immunoglobulin (IgG) in normal and antigen exposed animal serum samples

	Concentration of immunoglobulin (mg mL^{-1})					
Test antigen	Normal	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. hydrophila*	*Klebsiella* sp.
Heat killed Ag	0.86	0.25	0.40	0.27	0.59	0.30
Whole cell Ag	0.86	0.16	0.29	0.18	0.40	0.24

Table 6: Estimation of T lymphocytes in animals administered with test antigens

	T-cells (%)-test antigens					
Duration	Normal	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. hydrophila*	*Klebsiella* sp.
Heat killed bacterial antigen						
1st week	62.2	36.8	35.3	34.4	40.1	36.8
2nd week	61.7	37.8	37.2	36.5	45.4	38.9
3rd week	63.8	36.3	36.1	32.4	32.1	37.6
Whole cell Bacterial antigen						
1st week	62.2	33.7	34.3	32.2	38.1	36.7
2nd week	61.7	35.3	38.7	33.8	41.0	39.1
3rd week	63.8	34.4	36.3	31.2	35.3	38.3

B-lymphocyte estimation: B-Lymphocyte counts using rosette forming assay revealed significant decrement in pathogens exposed mice than control (Table 4).

Purification of Immunoglobulin: An estimation of antibody level in the serum after an antigen challenge in expose the functioning of hummoral immune system. In the present study, pathogen treated mice showed an IgG suppressive effect. Pathogen treated mice showed moderate change in IgG production (Table 5).

T-Lymphocyte estimation: T-cell is a vital component in cell mediated immune response, gets suppressed due to exposure of antigen (whole cell and heat killed antigen). It is found to be suppressive to T cell production, so induction in cell mediated immunity has confirmed pathogenic potential of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, *E. coil*, *Aeromonas hydrophila* and *Klebsiella* sp. (Table 6).

DISCUSSION

Cukrowska *et al*. (1996) stated the damage to spleenic Ig secreting cell reduces antibody production in mice administered with foreign particles. The inhibition of complement protein in antigenic challenge animal serum was observed (Karami *et al*., 2009). Dhasarathan *et al*. (2010) found that DNA molecule damage was occurred in animal administered with organochlorine pesticide. DNA molecule damage interferes with the protein synthetic machinery, which then reduces the production of antibody. A similar observation was made in the present study too. The suppression of antibody reflects on the reduction of humoral immuno response and this state subject the mice to easy infection.

Of the five pathogens administered animals, decrement in B-lymphocyte was much pronounced in *Pseudomonas aeruginosa* followed by *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* sp., *Aeromonas hydrophila* in the first week and compared to control, *Staphylococcus aureus* pathogen had more or less similar B-cell estimations in 3rd week. In present study, clearly confirm the decrement 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. Gebel *et al.* (1997) and Dhasarathan *et al.* (2010) reported the differentiation of B-counts affected

by pathogens. Muller *et al*. (1997) had reported that the immuno suppressive drug inhibits cell proliferation and T-cell cytotoxicity.

Here, it is remarkably noted that enhancement in t and B-cell production 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 Genestier *et al*. (1998) and Dhasarathan *et al*. (2010) reported that immuno enhancive drugs enrich cell proliferation. B-cell proliferations modification depends on the exposure of antigens.

CONCLUSION

In the present investigation, heat killed bacterial antigen and whole cell bacterial antigen were tested for immunomodulation. The induction of immune cells against the optimal concentration of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Klebsiella* sp. were confirmed by comparing with the several immunological assays in the normal mice. Immunomodulation to immune system was accessed directly by quantifying immunological factors that governs cell mediated and humoral immune response. All these test pathogenic strains were remarkably suppressing immune system of exposed animals.

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