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Research Article

Laboratory Development Cellular Immune Features and Immune Interference of Prototype *Escherichia Coli* and *Pseudomonas aeruginosa* Combined Bacterins in a Lapin Model

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Abstract

Bacterial vaccines, the bacterins are of both prophylactic and therapeutic potentials. Autogenous bacterins; however, are of profound importance in certain clinical settings like complicated urinary tract infections. The aim of the present work was at development, cellular immune features and immune interference of combined *E.coli* and *P.aeruginosa* in rabbits. Single *E.coli* and single *P.aeruginosa* as well as balanced [1xE-1xP, 2xE-2x P strength], and unbalanced [1xE-2xP, 2xE-1xP strength] heat killed bacterins combinations were prepared, developed and evaluated on laboratory scale. The developmental features were found; pure, safe, antigenic and immunogenic. These combined bacterins induced an increase in mitotic index of bone marrow cells; significant leukocyte inhibitory factors, lowered spleen body index. Balanced one x and two x combined bacterins induced higher IL10 mean values than normal. 2x strength bacterins combinations initiate higher IL2 concentration mean values than single bacterins and control. Both of the unbalanced bacterins combinations were raising up the TNF alpha concentration means than that of single bacterins and control. In practical sense, the immune interference in rabbits primed with the study bacterins combination lead to, either of three results as; one damps the other, one enhances the other and one doesn't affect the other. The immune interference appeared in the form of one enhances the other like that of IL2 and IL10 cytokine responses. The present findings are being novel in cases of *Pseudomonas* lung and urinary tract infections as potential experimental therapeutic bacterins.

Keywords: Bacterins; Combined bacterins; Cytokines; Immune response

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Introduction

Single and combined heat killed organismic bacterins like that of cholera and typhoid are not uncommon in human vaccinology sense [1]. *E.coli* bacterins are mostly common in veterinary and least common in human vaccination programs [2]. Pseudomonas *P.aeruginosa* bacterins are currently occupying an increasing interest among scientific workers tackling lung infections in immune compromised and cystic fibrosis patients [3-6] as well as complicated urinary tract infection [4]. Combined bacterins find wide range of applications in veterinary practice. Likewise there are some combined bacterins licensed in human vaccine programs [7-9]. Autogenously bacterins standing as an experimental laboratory scale developments [1]. The objective of the present work was to report on; 1-Development of *E.coli*, *P.aeruginosa* combined bacterins on laboratory scale settings, 2-Investigating the cellular immune features of rabbits primed with them using homologous prime-boost multi-injection protocols and 3-Probing the occurrence of immune interference between these combinations in post priming state.

Materials and Methods

Bacterins starter strains

From a series of patients with urinary tract infections, an uropathic gram negative isolates were purified and identified by vatic identification system as *E.coli* and *P.aeruginosa* [9]. They were grown in broth media and dense inocula were transferred to brain heart infusion broth tubes then layered by sterile liquid paraffin as cryo-protectant and kept at -18C in the refrigerator chest freezer till use for bacterins preparation [10].

Bacterins designations

To make ease description with in the text we adopt abbreviated designations for the developed bacterins (Table 1).

Bacterins preparation

A 0.1ml from a fresh 18hrs brain heart infusion broth cultures which constitute the seed lot of the starter bacterins strains were transferred into50 ml sterile brain heart infusion broth in 100ml size conical flasks. Then incubated at 37ºC in shaker water-bath with 60 shake per minute for 18hrs.Growth harvested into a series of sterile centrifuge tubes of 10ml size. Tubes were centrifuged at 5000 rpm for

15 minutes. Supernatants were discarded and pellets were kept. The pellets were reconstituted with sterile saline to the original volumes for triple wash at 5000 rpm for 10 minutes. Triple washed pellets were reconstituted with 5 ml sterile saline for each tube. The 5ml bacterins containing tubes were set onto test tube racks and left in water-bath at 60C for one hr. The tube containing suspensions were made in bulks. These bacterins preparations were checked for purity and ratified as one X strength 1.5x10 to eight and two X strength 3x10 to eight bacterins units per/ml. These preparations stand as a prototype bacterins. After adjustment to one and two x strength they were mixed in equal volumes to form the balanced and unbalanced combinations prior to specific immune priming of rabbits [11].

Bacterins Type	Description	Designations
Organismic heat killed *E.coli* bacterins	*E.coli* 1.5x 10 to eight [one x strength]	BEC
Organismic heat Killed P.aeruginosa bacterins	*P.aeruginosa* 1.5x 10 to eight [one x strength]	BPA
Balanced combined one x strength *E.coli, P.aeruginosa*	E.coli 1.5x10 to 8-*P.aeruginosa* 1.5 x10 to 8	X EC-X PA
Balanced two x strength *E.coli, P.aeruginosa*	E.coli 3x10 to 8-*P.aeruginosa* 3x10 to 8	2XEC-XPA
Unbalanced Onex strength *E.coli*-2X strength *P.aeruginosa*	E.coli 1.5x 10 to 8-*P.aeruginosa* 3x10 t0 8	1XEC-2XPA
Unbalanced two x strength *E.coli* one x strength *P.aeruginosa*	*E.coli* 3x10 to 8-*P.aeruginosa* 1.5x10 to 8	2XEC-XPA

Table 1: Abbreviated bacterins designations.

Purity

The final batch to be used prototype single and combined bacterins were checked for sterility in which inocula from each bacterin preparations was quadrate streaked onto nutrient agar plates and incubated for 18hrs at 37C. Presence of any contaminating bacterial growth makes preparation as unsuitable for experimentation [12].

Rabbits

A group of adult New Zealand male rabbits with three to five months old and 1-1.5 body weight was brought to the animal house, College of science, University of Babylon. These rabbits were checked for the presence of natural serum antibodies for common bacterial pathogens especially those for *E. coli* and *P.aeruginosa*. Absence of such serum antibodies make rabbits usable for this study. Rabbits were acclimatized to two weeks in housing conditions. Then categorized into four groups and marked as sham, control, safety and test as in the followings:

Sham……2 rabbits
Saline control……..5 rabbits
Safety ……7x two rabbits
BEC……5 rabbits
BPA……..5 rabbits
XEC-XPA……5 rabbits
2XEC-XPA……..5 rabbits
XEC-2XPA…….5 rabbits
XEC-2XPA……..5 rabbits

Rabbits kept during the housing condition under a dlibitum of food and drinks. They were handled and managed following the standard international rules for animal humanity regulations [13].

Safety

A volume of 0:1 ml from each should be used prototype bacterins were intra-pretoneally injected in rabbits of safety group. Then followed by follow up for five days to exclude gross and internal organ pathologies for the test and controls [14].

Homologous prime-boost protocols

A two ml amounts from each of the prototype pure bacterins were primed into each rabbit of the test groups. One ml was IM injected and second one distributed SC in sub-clavian and pelvic regions in week a part for three weeks followed by one week leave. Then bleed through cardiac puncture rout [15].

Cellular immune parameter

i) Blood Samplings

Five ml blood samples were collected into blood collecting tubes from the test and control rabbits by cardiac puncture method. Of which two ml were with anticoagulants for cellular immune assays and the remaining 3mls left clotted and sera saved at -18C at the chest freezer of refrigerator till use [16].

ii) Bone Marrow cell Mitogen city

The test and control rabbits were inoculated with colchicine and left at room temperature for one hour then thigh femurs were collected. Bone marrow film stained and examined for mitotic cell figures. Mitotic index were calculated [17] as following
Number of dividing cells/Number of cells calculated X 100.

iii) Spleen Body index

On evisceration, spleens from the test and control rabbits were removed from the abdominal cavities. The removed spleens were kept in between blotting papers, and then weighted. Body weights were made to all rabbits before evisceration. Spleen body weight was calculated as in [18]. Sham and saline groups are eligible for calculation of spleen index of control group. Likewise test groups and saline control group are eligible for calculation of spleen index of test groups.

Mean spleen weight for primed rabbit/ Mean body weight of the prime rabbits

Spleen index= ----------

Mean Spleen weight of control rabbits/Mean body weight of control rabbits

iv) Leukocyte Inhibitory Factor (LIF)

LIF was done by capillary-agar well method [19,20].

v) Serum antibody

Serum antibody titers for the agglutinins were made as in [16].

vi) Cytokines determination

Eliza test for the cytokines IL2, IL10 and TNF alpha following the methodology of the manufacturing company [Bioassay Technology Laboratory].

Biometery

Means and standard and deviations as well as P significance were made as in [21].

Results

I- Laboratory Bacterin Development

1-Purity**:** The bacterins BEC, BPA, XEC-XPA, 2XEC-XPA, XEC-2XPA and 2XEC-XPA were found on sterility check with no contaminating microbes (Table 2).

2-Safety: Safety test using 0.1 ml intra-peritoneal injections from the test single and combined bacterins with five days fallow up have shown no evident gross and internal organ pathology. Same was found on prime-boosted rabbits (Table 2).

3- Antigenicity: Prime-boost rabbits with single and combined bacterins have raised serum agglutinating antibody titers of 1280 (Table 3).

4-Immunogenicity: The battery of humeral and cellular immune function tests made on the bacterin prime-boosted rabbits proved that the test bacterins are immunogenic (Table 3).

5-Developmental features: The prototype single and combined bacterins were found; Pure, safe, antigenic and immunogenic (Tables 2 and 3).

II- Cellular Immune Function

BEC has shown an increase mitotic index, significant LIF values, increased TNF alpha, and lowered IL2, IL10 and lowered spleen body index. BPA initiated high mitotic index, significant LIF values, high IL2, IL10 and lowered TNF alpha than normal. While XEC-XPA has shown an equivocal mitotic index, high IL10 concentration means and significant LIF values. But with lowered spleen body index, lowered TNF alpha as compared to normal. 2XEC-XPA were showing high mitotic index, significant LIF values and higher IL2 values, with lowered IL10 and TNF alpha concentration means. XEC-2XPA have shown an equivocal mitotic index, significant LIF values lowered IL2 and IL10 as well as lowered spleen index and higher TNF alpha concentration mean as compared to normal. 2XEC-XPA,however,they initiate high mitotic index, significant LIF values, lowered spleen index, lower IL2 and IL10 concentration means as compared to normal (Table 3).

III-Immune-interference

The practical phenomenology of immune interference in cases of immunity to combined bacterins, will appeared in three forms as one damped the other ,one enhance the other and one not affect the other. But what is worth is the damping and/or enhancing. 2XEC-PA which contained both of BEC and BPA in 2X strength induces an increase in IL2 concentration means as compared to BEC and BPA and control. XEC-2XPA which contained one X strength EC and 2X strength PA induce higher TNF alpha than BEC, BPA and control. Other combinations were with no effect on each other (Table 3).

Discussion

P.aeruginosa bacterin studies are evidently tackled in the current literature [3-6] but at most in single bacterin formulations [7,8]. *E. coli, P.aeruginosa* combinations have been reported in urinary tract infection in this area. The combination had been exhibiting antigenic competition phenomenon [4]. The present work was aimed at: 1-developing *E. coli - P.aeruginosa* bacterin combinations, 2-Cellular immune features of rabbits prime-boosted with these bacterin combination using multisite injection protocols, and 3-probing the immune interference effects in these primed rabbit groups.

Feature [22]	BEC	BPA	XEC-PA	2XEC-PA	XEC-2XPA	2XEC-XPA
UC	UC	UC	UC	UC	UC	UC
UD	UD	UD	UD	UD	UD	UD
Prototype Bacterin	Prepared	Prepared	Prepared	Prepared	Prepared	Prepared
Purity	Pure	Pure	Pure	Pure	Pure	Pure
Safety/rabbit	Safe	Safe	Safe	Safe	Safe	Safe
Antigenicity/rabbit	Ag	Ag	Ag	Ag	Ag	Ag
Immunogenicity/rabbit	Im	Im	Im	Im	Im	Im

Table 2: Laboratory developmental Features of the test bacterins.

Note: UC=Understanding causal; Ag=Antigenic; UD=Understanding Disease; Im=Immunogenicity

Immune features	Control	XEC-PA	2XEC-PA	XEC-2XPA	2XEC-XPA	BEC	BPA
Mitotic index	54+-8.49	54.8.+3.96	60+-3.63	55.2.0+-3.89	63+-4.00	56.2+-7.01	56.2+-4.74
LIF	2.68+-9.21	1.52+-0.294	1.22+-0,886	1.92+-0.176	2.00+-0.00	1.74+-0.164	1.48+-0.164
SBI	1	0.35	0.46	0.58	0.32	o.5	0.62
IL2*	18.67+-5.37	16.9+-4,6	20.29+-3,59	15.56+-1.99	15.38+-1,22	10.18+-0.55	15.14+-0.78
IL10*	293.91+-0.16	358.16+-20.17	391.91+-15.87	266.0+-37.52	281.85+-130.54	385.52+-8.59	296.+-9.56
TNF alpha*	42.5+-13.07	54.0.09+-10.71	34.01+-7,57	54.09+-10.71	68.33+-0.36	50.+-1.37	35.49+-3.05

Table 3: The test bacterins primed-booted rabbit's cellular immune features.

Note: LIF=Leukocyte Inhibitory factor; * concentration means in pg/ml.

The laboratory scale developed single and combined bacterin forms were found pure, safe, antigenic and immunogenic (Table 2) [23,24]. The immune features of the bacterin prime-boost rabbits in post priming state (Secondary immune Response), were showing an array of immune functions such that of mitotic index of bone marrow cells , spleen body index, leukocyte inhibitory factors as well as, the cytokine response of IL2,IL10 and TNF alpha with variable degree of responses [25].

The immune responses of rabbit's models to combined bacterins both in man and laboratory animals may face some sorts of immune interference [24]. Abdul Wahid and Al Harmoosh [4] have been reported antigenic competition between *E.coli and P.aeruginosa* combined bacterins. In the present work a prove was made on the enhancing form of some cytokine responses to such combined bacterins. The cytokines IL2, IL10 and TNF alpha were found as rationally good battery for probing some aspects of lapin cytokine responses for the post- priming with gram negative bacterins [25].

Bacterin priming in human and animal models generate dendritic cells produce TH1 and Th17 cell responses through the activation of naïve T cells either to produce TGFB, IL6, IL23 and IL1B and differentiate to IL17 cells and IL17.Or to produce IL12 and IL23 and differentiated to TH1 cells [25]. Current investigations have shown that combination approaches may significantly amplify the immunogenicity thereby increasing their preventive and therapeutic potentials [5].

The significant leukocyte inhibitory factors LIF noted on rabbits prime boosted with these bacterin combinations and in single bacterin forms may shed a light on involvement of cell mediated immunity and cellular delayed hypersensitivity to these bacterin in rabbit model [19,20].

Among the main essence of combined bacterin formulations is to cover more than bacterin types and applied to the subject as single one injection in one single site followed booster dose (doses) similar to the actual in human being [7,8]. The forthcoming work will be of applying homologous prime-boost in one dose and single injection site [15]. What is to be the nature for the prime-boosted rabbit's immune response and which nature of the immune interference will be? This remained to be explored. The present study is being a novel basic contribution for laboratory scale development of *E. coli, P.aeruginosa* combined bacterins valid as an experimental bacterins for problematic combined *E. coil and P.aeruginosa* lung and urinary tract infections complicated with multidrug resistant causals [25].

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