Influence of Nano-bodies produced from Gram-negative Bacteria against infection with Pseudomonas Aeruginosa induced Bronchial Pneumonia with special references to their effect on Immune system in Male Albino Rats

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Abstract: Seventeen Gram-negative isolates were tested for Nano bodies or Membrane Vesicles production and their bacteriolytic activity against different Gram-positive and Gram-negative bacteria. The most Nano bodies producing bacterial isolates were exposed to the antibiotics Cefotaxime and Gentamycin, which induced the production of Cefotaxime membrane vesicles(c-MVs) and Gentamycin membrane Vesicles (g-MVs), respectively. The c-MVs and g-MVs are larger with higher lytic activities against the susceptible host bacteria when compared to those produced under normal growth conditions. Influence of these Nano bodies from Proteus vulgaris on the morbidity and mortality rates of albino rats was investigated and the results revealed that the animal resist the pseudomonas aeruginosa infection which induced chronic bronchial pneumonia. Also, the nano bodies from Erwinia cartovora and Proteus vulgaris were tested as vaccine in rats to protect them against Lethal (LD) and Sub-lethal (SLD) doses (acute and chronic infection) of Pseudomonas aeruginosa and surprisingly results, the animals lived till the end of the expermintal period. The vaccinated rats challenged with LD or SLD of Pseudomonas aeruginosa showed high clearance of the pathogen from Lung, Spleen, Liver and Blood when compared to the bacterial counts in control rat groups. The result of the present study proved that Nano bodies or membrane vesicles from Erwinia cartovora and Proteus vulgaris could enhance the immune response of animals and gave the protection against pseudomonas aeruginosa induced Bronchial Pneumonia. So, Nano bodies or membrane vesicles can be expressed as a new strong antigenic structure could have the ability to enhance the immune response, and also, expressed as a new hope as antibiotic,vaccine and a biological control for human and animal. Further studies on the effect of these nano bodies on other Auto-immune, cncer and different chronic diseases might be needed. [Journal of American Science 2010;6(8):64-71]. (ISSN: 1545-1003).

Keywords: Nano-bodies, Gram-negative Bacteria, Immune system.

1. Introduction

Molecular biological methods have not yet given Scientists a precise historical record of Gram-negative bacteria, but ancient stromatolites containing fossilized remained of Cyanobacteria- like Prokaryotes date back to Archaen eon-over such extraordinary periods of time (much of it when no other life existed), we can imagine that random mutation, selection and the slowly but ever-changing global environment gave rise to two fundamentally different cell wall formats in bacteria; Gram-positive and Gram-negative varieties (Beveridge, 1999).

The Gram-negative bacterium contains Lipopolysaccharids lipoprotein major components, and relatively little peptidoglycan (less than 10 % of the total cell wall) in their walls, whereas the walls of Gram-positive are mainly composed of peptidoglycan (30 – 70 % of the tota; cell wall), polysaccharids or teichoic acid (broth),or teichuronic acid (Tamm et al., 2001). So, the peptidoglycan is the only cell wall polymer common to both Gram-positive and Gram-negative, and named as basal structure (Wack, 1957), mucopeptide (Mandelstem and Rogers, 1959); glycopeptide (Strange and Kent, 1959); murein (Weidel and Pelzer, 1964) and peptidoglycan (Stromimgeret al., 1967).

Outer membrane vesicles or Nano-bodies are defined by Li et al., (1998) as bilayered membraneous particles produced by Gram-negative bacteria, into which degradative enzymes are concentrated (Autolysins, peptidoglycan hydrolase and other enzymes). It was reported that naturally produced bacterial vesicles are discrete, closed outer membrane blobs produced by growing cells (McBroom and Kuchn, 2005).

Although, outer membrane vesicles (Nano-bodies) production has been observed for more than 50 years, the machinery that allows vesicle or nano-bodies secretion while maintaining bacterial viability remains elusive. Many theories exist on the

mechanism of vesiculation based on biochemical and genetic data and are reviewed elsewhere (McBroom and Kuchn, 2005).

Nano bodies are capable of lysine a variety of Gram-positive and Gram-negative bacteria and the potency of lyses depended on the peptidoglycan chemotype of the attacked cell (Beveridge, 1999). For Gram-positive, Nano bodies adhere to the cell wall, where they break open and digest the immediate underlying peptidoglycan. It attacks the Gram-negative bacteria in much different manner. Here, it adheres to the outer membrane and rapidly fuse to it (Kadurugamuwa and Beveridge, 1996) and the luminal contents are released directly into the periplasmic space of the recipient cell. Nano bodies mediated lysis of bacteria occurs only when the recipient cells are under insufficient nutrient conditions (Beveridge, 1999).

Kadurugamuwa and Beveridge, (1995), have been reported the membrane surface-active agents such as Gentamycin could increase the production of Nano bodies. Gentamycin Nano bodies can kill Pseudomonas species, which is normally permeable to amino-glycoside antibiotics through fusing the normally impermeable outer membrane of the resistant strain and deliver gentamycin into the periplasm where it can be actively imported to the cytoplasm and inhibit protein synthesis. Another possible medical application is as vaccine agents. Nano bodies (membrane vesicles) are strongly antigenic particulate structures which also posses natural adjuvant qualities for enhancing an immune response Beveridge, (1999). This vaccine has many advantages including ease of administration and induction of a mucosal immune response at the site of infection for many pathogens.

From the above mentioned, the study aimed to produce nano bodies or membrane vesicles from different Gram-negative bacteria isolated and exposed or not exposed to antibiotics under normal growth condition. Uses of these isolate (nano bodies or membrane vesicles) against some dangerous pathogens such as Pseudomonas species, which induced Bronchial Pneumonia in Human and Animals.

2. Material and Methods

Experimental Design

Random bred male albino rats weighing approximately 110±3.71g, obtained from Animal house laboratory, National research Center, Giza, Egypt. The rats were evaluated prior to initiation of the study to ensure a sanitary Hygienic condition and acclimation to the study environment. Clinically accepted animals were randomly assigned into two major groups and each group was sub-divided into 4 groups (10rats / group).

Environmental Condition

A total of 80 rats, were housed in stainless steel wire mesh cages on a bedding of wood chips (Five animals / Cage). They were kept in an ambient temperature of 25 ± 3 oC, on a light / dark cycle of 12 / 12 hours and supplied rat chow (the diet) and fresh water ad libitum.

Diet

A basal diet (Table: 1) was formulated to meet the rat nutrient requirements as recorded by Osfor (2003).

Antibiotics:

Cefotaxime and gentamycin were purchased from the local market and they produced from T3A Pharma Group and Glaxo Wellcome Companies respectively.

Experimental design

This study was performed in two main experiments.

The first one was divided into equal 4 groups as follows:

Group I: Fed the basal diet and received the nano bodies from Erwina Caroyovora injection and served as control positive group.

Group II:Fed the basal diet and received Saline solution and challenged with Erwina Carotovora and served as control negative group.

Group III: Fed the basal diet and received the nano bodies from Erwinia Caroyovora injection and challenged with the lethal dose (2 × 105 CFU "Colony Forming Unit") of Pseudomonas Aeruginosa (after 14 days).

Group IV: Fed the basal diet and received the nano bodies from Erwina Caroyovora injection and challenged with Sub-Lethal dose (2 × 103 CFU) of Pseudomonas Aeruginosa (after 14 days).

The second experiment was also divided into equal 4 groups as follow:

Group I: Fed the basal diet and received the nano bodies from Proteus Vulgaris injection and served as control positive group.

Group II: Fed the basal diet and received Saline solution and challenged with Proteus Vulgaris injection and served as control negative group.

Group III: Fed the basal diet and received the nano bodies from Proteus Vulgaris injection and challenged with the lethal dose (2 × 105 CFU "Colony Forming Unit") of Pseudomonas Aeruginosa (after 14 days).

Group IV: Fed the basal diet and received the nano bodies from Proteus Vulgaris injection and challenged with Sub-Lethal dose (2 × 103

(CFU) of Pseudomonas Aeruginosa (after 14 days).

Bacterial Strains:

The tow main used Bacteria were chosen from 17 kinds of bacteria. They provided by Professor H.H. Martin, Institute of Microbiology, TH Darmstadt, Germany.

Culture Media:

Nutrient broth, Buffer (Phosphate & Borate) and MacConkey Agar were used for cultivation and Isolation of Bacteria from Oxford, England.

Electron Microscope Investigation:

Twenty micro-liters tested Gram-negative bacteria suspension was placed on Carbon-Coated nickel grids and stained with 2% aqueous solution of uranyl acetate for 20 Seconds. Then, examined with Zeiss EM10 transmission electron microscope operating under standard condition (60 Kilo volt).

Purification and Isolation of Nano bodies (Membrane Vesicles):

Nano bodies were purified and isolated as described by Mayrand (1986).

Clinical Observation:

The rats were observed daily throughout the experimental period to notice the morbidity and mortality rats specially after challenge with bacteria.

Clinical Pathology:

Blood samples containing EDTA as anticoagulant was used for the determination of haemoglobin content, erythrocyte and leuckocyte counts (Total and Differential).

Histopethological studies:

Immediately after sacrifice of animals, samples of Lung tissues was fixed in 10 % formal saline, dehydrated, cleared, embedded in paraffin and were sectioned at 7 µm. Paraffin sections were stained with hematozyline and eosin stain according to the method of Pearse, (1985).

Statistical Analysis:

The obtained data were statistically analyzed after Snedecor and Cochron, (1973).

3. Results and Discussion

Table (1): Composition of the basal Diet:

Ingredients	Percentage
Sorghum	39
Corn yellow	31.6
Barley	8
Meat meal	8
Corn Cobs	7.3
Vegetable Oil	4
Lysine	0.3
Methionine	0.4
Di-Calcium Phosphate	0.2
Lime Stone	0.4
Sodium Chloride	0.3
Vitamins and Mineral Mixture*	0.5
Calculated Nutrient Composition:	
Crude protein	11.99
Energy	3404.2
Crude Fibe	4.46
Ether Extract	7.51
Lysine	0.71
Methionine	0.61
Calcium	0.45
Phosphorus	0.4

*Vitamins and Minerals Mixture (g): Copper sulphate (0.05), Ferric Citrate (0.59), Zinc Carbonate (0.053), Calcium carbonate (7.25), Calcium hydrogen phosphate (11.3), Di-sodium hydrogen phosphate (6.0), Potassium Chloride (7.3), Potassium Iodide (0.003), Magnesium Chloride (2.3) and Magnesium sulphate (0.154).
Thiamine (0.3), Riboflavin (1.0), Pyridoxine (0.2), Calcium pantothinate (6.0), Nicotinic acid (20.0), Cyanocobalamine (0.005), Folic acid (0.2), Biotin (20.0), Inositol (60.0), Choline Chloride (60.0), Vitamin A (4000 IU), Vitamin E (30 IU) and Vitamin K (50 IU).

Table (2): Lytic effect of Nano bodies or membrane vesicles formed by Citrobacter Freundii and Enterobacter Cloacae on different bacteria:

Host bacteria	Clear zone (mm) caused by MV_s of Citrobacter Freundii			Clear zone (mm) caused by MV_s of Enterobacter Cloacae		
	n-MV_s	c-MV_s	g-MV_s	n-MV_s	c-MV_s	g-MV_s
Bacillus cereus	14.7±0.07	23.5±0.07	18.0±0.14	0.0	0.0	0.0
Bacillus subtilis	0.0	0.0	0.0	15.4±0.06	0.0	20.5±0.20
Enterobacter cloacae	0.0	0.0	0.0	0.0	0.0	0.0
Erwinia carotovora	0.0	0.0	0.0	17.3±0.10	0.0	26.0±0.10
Escherchia coli	17.8±0.07	24.2±0.10	22.8±0.034	17.0±0.10	0.0	25.0±0.37
Proteus vulgaris	18.7±0.23	22.0±0.12	23.1±0.12	18.1±0.14	0.0	22.6±0.06
Pseudomonas solanacearum	0.0	0.0	0.0	17.1±0.07	0.0	22.9±0.10
Pseudomonas syringae	18.6±0.01	26.0±0.10	21.5±0.35	17.6±0.06	0.0	20.6±0.28
Serratia marcescens	19.3±0.06	19.5±0.12	23.3±0.06	0.0	0.0	0.0

n-MV_s: Natural membrane vesicles

c-MV_s: Membrane vesicles produced in the presence of cefotaxime.

g-MV_s: Membrane vesicles produced in the presence of gentamycin

±: Standard error of means.

Table (3): Lytic effect of Nano bodies or membrane vesicles formed by Erwinia carotovora and Klebsiella pneumoniae on different bacteria:

Host bacteria	Clear zone (mm) caused by MV_s of Erwinia carotovora			Clear zone (mm) caused by MV_s of Klebsiella pneumoniae		
	n-MV_s	c-MV_s	g-MV_s	n-MV_s	c-MV_s	g-MV_s
Bacillus cereus	18.0±0.14	20.5±0.07	20.0±0.14	0.0	35.0±0.5	0.0
Bacillus subtilis	15.6±0.06	16.1±0.07	19.0±0.14	0.0	33.2±0.04	0.0
Enterobacter cloacae	19.5±0.07	20.2±0.11	21.8±0.04	15.5±0.07	30.0±0.12	19.5±0.07
Erwinia carotovora	0.0	23.6±0.11	21.0±0.11	0.0	31.5±0.2	0.0
Escherchia coli	19.0±0.14	19.5±0.07	21.5±0.07	19.15±0.12	31.0±0.1	23.7±0.15
Proteus vulgaris	0.0	20.5±0.07	22.2±0.24	19.97±0.20	20.3±0.15	22.5±0.07
Pseudomonas solanacearum	16.7±0.11	16.5±0.03	19.0±0.40	0.0	29.0±0.30	0.0
Pseudomonas syringae	18.5±0.07	18.8±0.11	21.6±0.03	0.0	34.0±0.14	22.0±0.10
Serratia marcescens	0.0	21.0±0.14	20.5±0.07	19.55±0.01	32.5±0.20	23.3±0.08

Table (4): Lytic effect of Nano bodies or membrane vesicles formed by Proteus vulgaris and Serratia marcescens on different bacteria:

Host bacteria	Clear zone (mm) caused by MV_s of Proteus vulgaris			Clear zone (mm) caused by MV_s of Serratia marcescens		
	n-MV_s	c-MV_s	g-MV_s	n-MV_s	c-MV_s	g-MV_s
Bacillus cereus	15.8±0.03	20.3±0.06	27.0±0.15	19.0±0.14	19.5±0.07	23.9±0.11
Bacillus subtilis	16.5±0.07	22.5±0.07	24.0±0.14	23.0±0.10	25.5±0.53	27.1±0.08
Enterobacter cloacae	18.0±0.14	27.5±0.07	26.1±0.10	0.0	0.0	0.0
Erwinia carotovora	15.6±0.06	18.4±0.10	17.6±0.06	18.3±0.07	17.3±0.06	23.1±0.12
Escherchia coli	15.8±0.03	25.0±0.14	23.4±0.07	19.0±0.28	18.5±0.07	24.25±0.04
Proteus vulgaris	0.0	0.0	0.0	16.5±0.07	20.1±0.36	21.0±0.10
Pseudomonas solanacearum	16.5±0.07	23.5±0.07	23.8±0.01	31.3±0.30	22.8±0.14	35.5±0.07
Pseudomonas syringae	0.0	0.0	0.0	30.2±0.12	24.3±0.15	23.1±0.10
Serratia marcescens	16.5±0.07	30.0±0.14	20.75±0.03	0.0	0.0	0.0

Table (5): Effect of different doses of Pseudomonas aeruginosa on mortality rate of non-immunized Animals.

Doses	First Experiment				Second Experiment			
	G-I	G-II	G-III	G-IV	G-I	G-II	G-III	G-IV
2 × 10 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2 × 10 4	25	25	50	100	100	100	100	100
2 ×10 5	50	75	100	100	100	100	100	100
2 × 10 6	100	100	100	100	100	100	100	100

4. Discussion:

Many Gram-negative bacteria produced external nano-bodies named membrane vesicles during the normal growth. The release of these vesicles or nano-bodies from the whole cell depends on the bacterial strains as well as the nutritional conditions (Ksdurugamuwa and beveridge, 1995 and Wai et al., 1995). Nano bodies (Membrane Vesicles) entrap several periplasmic components include alkaline phosphatase, phospholipase, proelastase, protease and peptidoglycan hydrolase (Kraft et al., 1998). Nano bodies may be important during the initial phases of infection, as they concentrate such factors and convey them to host tissue (Ksdurugamuwa and beveridge, 1997). This cell wall degrading enzymes could be use to lyses surrounding dissimilar bacteria in the bacterium environment, thereby releasing organic compounds for growth. Naturally produced bacterial vesicles are discrete, closed outer membrane blebs produced by growing cells, not products of cell lyses or cell death (Yaganza et al., 2004 and McBroom & Kuehn, 2005).

In the present study, 17 bacterial strains of Gram-negative bacteria were tested for their lyses or killing effect on other 17 bacterial strains of different Gram-negative and Gram-positive bacteria under suitable condition for action and production of Nano bodies (Azab, 2004). Out of the tested isolates, ten strains had high killing effect on the tested hosts.

The electron micrograph of the bacterial strains which exhibited killing effect revealed that all of these strains produced many outer membrane nano bodies different in size and amount. They were almost spherical in shape with average diameter of 25 – 200 nm, and they had a dense materials enclosed inside them.

In this study six strains of the tested bacteria exhibited high killing effects with high nano bodies' production, were exposed to Cefotaxime and Gentamycin and the bacteriolytic activities were determined. These six strains except Enterobacter cloacae produced Nano bodies with higher lytic activities against most of the recipient bacteria after exposure to cefotaxime (β-lactam antibiotics) or gentamycin (aminogylicosid antibiotics). The higher lytic activities of the Nano-bodies might be due to the formation of large vesicles filled with enzymes and cefotaxime or gentamycin which might act

synergistically with the degradative enzymes enclosed to lyse hard-to-kill hosts.

The present study was investigated the effect of intrapritoneal injection of different doses of Proteus Vulgaris on the mortality of male albino rats and it was found that all the challenged groups lived till the end of the experimental period (ten days). No illness symptoms were noticed on the rats, and they had a significant increase in the total leuckocytic counts, and percentage of neutrophils and lymphocytes which indicated that the normal immune response of the rats could manage the pathogen and overcome it. So, no bacteria were detected in the kidney, liver and spleen tissues from the challenged animal groups. Also, the used doses of proteus vulgaris did not affect the mortality of male albino rats used, and there was noticed increase in the total leuckocytic counts, neutrophil and lymphocytes percentage. This give a conclusion that this strain of proteus vulgaris is a weak pathogen and the natural immune response of the animals can over come it. The increased total leuckocytic counts and the significant increase in lymphocytes which responsible for immune response and antibody formation may explain how rats could over come the pathogen. Also, the different in the rate of increase in the total leuckocytic counts of rats may be due to the difference in the age and species. This result was in agreement with Larsson (1980) who reported that Proteus bacteremia was dominated among the very young and the elderly patients was explained by the importance of the host-parasite relationship in Proteus bacteremia.

In he present study the effect of Proteus Aeruginosa (a human pathogen isolate) on the mortality rate of albino rats was investigated. The dose 2 × 105 Colony Forming Unit (CFU) / rat was found to be the lethal dose and causing mortality level more than 75% within 2 days, and the sub-lethal dose 2 × 103 CFU / rat caused increase in the total leuckocytic counts to 9.5 × 109 / mm3 compared the untreated control, with a high significant increase in the percentage of the neutrophils and in the lymphocytes.

These findings were agreement with Johansen et al., (1995) who reported that the rise in polymorph nucleus lymphocytes is a distinctive feature of patients with cystic fibrosis suffering from the chronic infection of lung caused by Pseudomonas aerugenosa. It was revealed also from the presented results that the eosinophil and monocyte counts were not significantly changed, and thesecan be explained according to Mbajiorgu et al., (2007) who reported that the eosinophil and monocyte counts did not contribute to the change in the total leukocytes count, since neutrophils and lymphocytes have the major role in fighting foreign organisms, the variations in leukocyte counts were essentially caused by variations in neutrophil and lymphocyte numbers. Also, it was found that the lung tissue of the rat group, who received saline and challenged with Proteus Vulgaris, was damaged and filled with polymorph nucleus lymphocytes. This result was in agreement with Hoiby, (1995) and Pedersen, (1992) whom reported that patient's lung infected with Pseudomonas aeruginosa slowly progressive damage of lung parenchyma and eventually respiratory insufficiency.

The present study was investigated the immune response for the isolated Nano bodies, as a vaccine in rats protection against Pseudomnas aeruginosa lethal (LD) and sub-lethal (SLD) doses, and the result revealed that all the vaccinated rat groups with Nano bodies (Membrane Vesicles) of Erwinia carotovora showed a high resistance against the LD and SLD of Pseudomonas aeruginosa, and all the rats lived till end of the experimental period. This means that the MVs vaccine stimulated the immune response of the rats which persist for two weeks after vaccination and help the rats to overcome the infection with Pseudomonas aeruginosa as a cross-protection immune response. This may be explained by the results of Alaniz et al., (2007) who demonstrated that MVs possess important inflammatory properties as well as B ant T cell antigens known to influence the development of salmonella specific immunity to infection in vivo. Also, these authors revealed that MVs are a functional nonviable complex vaccine for salmonella by their ability to prime protective B and T cell responses in vivo.

In the present study the bacterial count was significantly decreased in target organ (lung), parenchymal and immune organs (Spleen and Liver) and Blood of the vaccinated animal groups, when compared with the infected group. These indicate the high immune response induced by the used vaccines. On the other hand, for the group injected with LD of Pseudomonas aerugenosa specially that vaccinated with membrane vesicles of Erwinia Carotovora, the immune response induced was not solid enough to give adequate elimination of the pathogen, this could be attributed to; the shared antibodies formed were not highly specific; the intra-species variability of the pathogen or it may be the dose regimen followed in this study was not adequate.

The lung of all rat groups were examined for histopathological changes and the results revealed that the lung tissues of the rat groups vaccinated with membrane vesicles of Erwinia carotovora or Proteus Vulgaris were as normal as the control one and the groups of the tow vaccines which challenged with

SLD of Pseudomonas aerugenosa showed no change and they were almost normal. These indicated that the vaccinated animals were highly protected against damaging effect of Pseudomonas aerugenosa on the lung. On the other hand, the lungs of rat group vaccinated with membrane vesicles of Erwinia carotovora after challenged with LD of Pseudomonas aerugenosa showed some inflammation and exudates in the bronchiole space and slightly damaged parenchyma cell walls when compared with the lung tissue of the infected group. The lung of the rat group immunized with membrane vesicles of Proteus Vulgaris when challenged by LD of Pseudomonas aerugenosa had some inflammation without any damages in the cells. These results could be explained according to Bertot et al., (2007) who reported that the chronic lung infection by opportunistic pathogen, such as Pseudomonas aerugenosa and Burkholderia cepacia complex, is a major cause of morbidity and mortality in patients with cystic fibrosis. Each outer membrane preparative given to the rats intraperitoneally in a single dose injection (5 micrograms / rat) protected the animals not only in homologous but also in varying intensity in heterologous systems. Evidence was obtained that this nonspecific protection is cell mediated. Moreover, Shoemaker et al., (2005) reported that native outer membrane vesicles vaccine was administered to rabbits via different routs using different primary immunization schedules. Similar high levels of serum bactericidal activity were induced regardless of route or number on immunization.

At the end, one can conclude that the membrane vesicles of Erwinia Cartovora and Proteus Vulgaris enhanced the immune response of the rats and gave a cross-protection against Pseudomonas aerugenosa, but the result of protection from membrane vesicles of Proteus Vulgaris were more effective than that induced by Erwinia Cartovora. So, nano-bodies or membrane vesicles are strong antigenic structures and could have the ability to enhance an immune response. Nano bodies or membrane vesicles are a new hope as antibiotic, vaccine, immune enhancer and as biological control for human and animal diseases.

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