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The Effect of Bacterial DNA on Phagocytic Function in Balb/c Mice Peritoneal Macrophages

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Abstract: This study was aimed to view the ways, bacterial DNA affect innate immunity, used macrophages to defeat bacterial DNA, then, evaluated the process of maturation, morphogenesis and killing activities. Cells obtained from peritoneal cavity lavage of 6-8 week old, pure Balb/c mouse, were harvested in complete tissue culture medium including RPMI, FBS 10%, penicillin, streptomycin. Cells were divided into 4 groups based on the type of received stimulator (DNAase, Bacterial DNA, FMLP 450 and Control). After 3 and 6 h from beginning of incubation period, macrophages adhered to bottom of the flasks, were studied for the number of adherent cells, morphogenesis maturation and induction of NBT, after removal of non-adherent cells. Regarding the number of adhered cells, bacterial DNA, as a rapid acting stimulator, showed the highest number of adhered cells and the most ideal morphogenesis, in 3rd h of incubation. In the 6th h of incubation, FMLP showed the most adherency and the best morphogenesis..Considering NBT reduction strength in the 3rd h, the highest percentage belonged to FMLP (42%), however, in the 6th h bacterial DNA allocated the highest rate with 95%. Can be DNA introduce as a strong stimulator with great performance over macrophage system. Besides other effects on the immunity system, it has drawn researchers attention for years and has resulted in taking beneficial steps in understanding innate immunity mechanisms. This genetic substance can be used as a lab stimulator in researches related to phagocytic system.

Key words: Bacterial DNA, peritoneal macrophages, phagocytosis

INTRODUCTION

The most important function of tissue and peripheral macrophage in mammalians is their phagocytic activity, which leads to the surrounding and killing of the particles such as infectious agent. These bone marrow derived cells have tasks beyond phagocytosis[1-4].

Macrophages have special roles in production, stimulation, activation and regulation of the effector immune cells. Therefore, transportation to peripheral tissue and differential stage have important roles in their evolutionary stages. In coordination with other cellular responses, they initiate the regulatory cascade of inflammatory reaction. Progenitors of these cells (blood monocytes), are changed into the tissue fixed macrophages in many parts of the body. At the exposure of the effective inflammatory stimulants, more monocytes migrate to the site and could act as active macrophages. The secretary and cytotoxic functions of these cells potentiate their ability to participate in inflammatory reactions. The commencement of macrophage activation is a complicated phenomenon. In most cases, nonspecific receptors for bacterial endotoxin is the important factor for the initiation of inflammatory responses in these cells[5].

Other bacterial products such as peptidoglycan and formyl-methionin are also introduced as macrophage immune stimulators. Recently, it has been proved that bacterial DNA is able to stimulate immune system. It is suggested that mammalian DNA lacks this ability. So it can be concluded that special structures or sequences in bacterial DNA may lead to this activity[6,7].

In vitro and experimental animal studies introduce this substance as a stimulant that effect on recognition between self from non-self agents. Different competent immune cell lineages have the ability to respond to this stimulant and show different functions. In the existing references and information, there are some indications of bacterial DNA as a phagocytic stimulant and their interferences in the production mechanisms of oxidants and lethal substances[7-13].

This study was intended to assess the macrophage's phagocytic response against bacterial DNA stimulation and also measure the lethal radicals in this reaction.

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Consequently, after comparing this genetic substance with the other usual lab stimulants, it can be introduced as a lab phagocytic stimulant.

The study was carried out only with one dose because of facility limitations, but we're optimistic to continue this study with more doses (< >20 μg mL^{-1}).

MATERIALS AND METHODS

This research was conducted in Department of Immunology and Molecular Biology Research Center, Faculty of Medicine, Shaheed Beheshti Medical Science University from March to September 2002.

Extraction and purification of bacterial DNA: Pathogenic Coli bacilli over the blood agar culture was used. After transferring the colonies into the Liquid Bacterial culture (LB) and incubation in the special rotatory apparatus, they were centrifuged and then divided. After being washed with PBS buffer (phosphate buffer saline) and being added 1% SDS (Sodium Dodecil Solfate), the extra bacterial proteins and RNA were eliminated using proteinase K and RNase, respectively and then the bacterial DNA was purified.

The electrophorase of bacterial DNA: After preparing the agarose gel (8% agarose in TAE buffer) and adding ethidium bromide (0.1 mg/10 mL agarose), it was transferred into the electrophorase plates.

And then DNA loading was carried out. The samples were transferred into the wells and electrophorase was done with 90 voltage and 40 mA DC for 30 min. Finally the centrifuged components over the gel, mostly conjugated derivatives of fleuroscetin were considered and observed. In this technique, the ethidium bromide which moves opposite to DNA (from anode to cathode) is placed between the two chains of the DNA and causes it to be fleuroscented before the UV lamp. The DNA band was observed with the wavelength of 254 nm, then its contents were kept in the -20°C refrigerator.

Enrichment and purification of peritoneal macrophages
Laboratory animals

Mice/Laboratory animals: A 6 week old Balb/c mouse was gone under lavage technique using cold HANKS solution after incision of its spinal cord by a standard technique (three times, each time by 4 mL HANKS). The experiment was carried out under the very complete sterilized conditions.

These mice were used as a source of macrophages. They were obtained from Research Center of Genetic and Biological Science.

Cell preparation: Mice were killed by cervical dislocation. Single cell suspensions were prepared aseptically from the pretoneal cavity lavage technique. All cell were cultured at 37°C in a 5% CO_2 humanified incubator and maintained in RPMI 1640 supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), 1.5 nM L-glutamine, 100 μg mL^{-1} of each streptomycin and penicillin. Cells (10^6 cell/mL)/200 μL/well were treated with LPS.

Culturing of the peritoneal cells: The cells obtained from lavage, were washed with 400 ng for 10 min and were counted using a neobar lams (at least 5×10^6 cells/mL). Then, their macrophagic purity was assessed by differential count: The cells were transferred into the complete cell culture (RPMI + FBS (10%) + streptomycin + L-glutamine) in a special multi plate tissue culture dish with the following order:

- Macrophage cells in resting state: They were incubated without the presence of any stimulant.
- Macrophage cells along with the stimulant of bacterial DNA (20 μg mL^{-1}).
- Macrophage cells along with a certain stimulant of FMLP (formyl methionin lusin phenylalanin) to the amount of about 240-450 μg mL^{-1} equal to: 10^6 (μM) with the volume of 10 λ mL^{-1} culture media.
- The fourth group of the cultured cells along with DNase (10 μg mL^{-1}). This was done in order to control the effect of DNase on the activity of phagocyte macrophages (Elute contaminated medium from probably environmental DNA).
- The study of the adherence and morphologic states of the cultured cells:
- Counting and determining the rate of the cells adhered to the culture plastic surface:

After the end of the incubation at 37°C and the CO_2 exposure of 50% with the intervals of 3 and 6 h and the complete exit of the supernatant fluid along with the non-adherent cells, (to remove the adherent cell) the adherent cells were counted using a contrast-phase microscope. The countings were repeated several times at all the four series of the study systems and their mean values were determined as the number of the adherent cells.

The study of the morphologic features of the adherent cells: In order to study the phagocytic and morphologic features of the macrophagic cells, the following criteria were applied using Gimsa staining:

- Geometric deformation of the cells
- Producing of the psodopeds (false feet)

- Formation of the phagocytic granules and vacoules
  All the mentioned assessments were qualitative,

which show the cells activity in the resting position along with the stimulants.
The scoring of the morphogenesis varied between + and ++++, so that:

- If 20% of the cells in the microscopic field showed the mentioned changes: (+).
- If 40% of the cells in the microscopic field showed the mentioned changes: (++).
- If 60% of the cells in the microscopic field showed the mentioned changes: (+++).
- If 80% of the cells in the microscopic cycles showed the mentioned changes: (++++).

Assessing the free oxidative radicals in the cultured cells: In order to determine the effects of the bacterial DNA, FMLP and DNase on the lethal strength of the peritoneal macrophages, NBT test was used. NBT will be reduced and its color will be changed in case that the oxidative radicals agents are produced. Its presence in the cytoplasm cells in the form of dark blue crystals called furmazan is indicative of the lethal activity of the cells. Therefore, 5 mg NBT powder was dissolved in 5 mL saline (0.9%). After incubation, the culturing plates were removed from the incubator. All non-adherent cells and the surface liquid were removed and washed using warm RPMI.

To all the samples and controls some 200λ RPMI, 100λ NBT and 100λ PMA, as the stimulant were added. The cells were incubated at 37°C for 30 min, then, once again the surface liquid was removed and the cells were washed using methanol. After the evaporation of the methanol, the cells were quickly studied by the microscope and the cells with the fourmazan crystals were counted, three times, then, their relative percent and absolute number were calculated.

It is to be noted that in case of a need to more cells, one can use more peritoneal lavages of the mice.

RESULTS

The first stage of this study includes the documentation of bacterial DNA bands in the agar by UV spectrophotometer. These bands are shown in Fig. 1. In the second stage, after cell culturing and incubation of the cells derived from peritoneal lavage, it has been shown that at the beginning of the incubation period macrophages were not adhered in both test and control groups.

In the 3rd h of the incubation period, macrophages which were incubated with bacterial DNA had increased the adherent cells compared the resting group. (about 1.55 time)

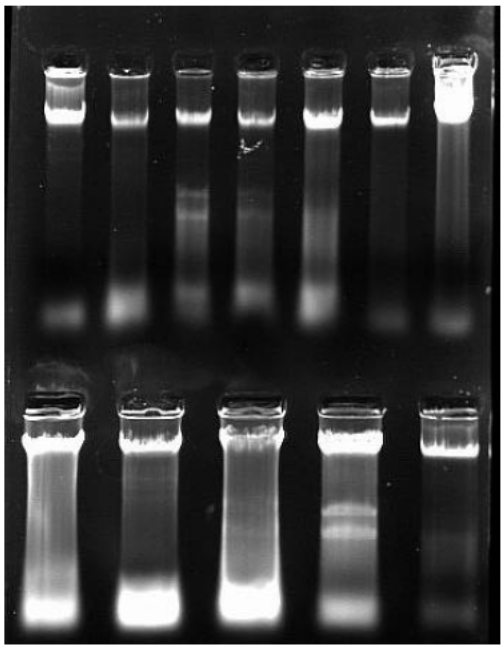

Fig. 1: Bacterial DNA bands in the agar by UV spectrophotometer

Also this differentiation was reported ++++ that is an ideal compared with the control, which was ++ (Table 1). The number of adherent cells in the stimulated group was reduced and severe apoptosis was observed in the 6th h.

Table 2 and 3 show cultured cell adherency and morphogenesis. FMLP as the most suitable macrophages stimulated had the highest rate of adherecy, but their morphologic score was about ++.

Dnase introduced itself as a macrophage stimulant which has significant difference compared with the control group in respect of adherency and morphogenesis.

Table 4 shows that the resting group has no indication of progression nor regression during 3rd and 6th h of the study.

In the 6th h, FMLP had the best adherency followed by DNase and bacterial DNA.

Wells which were added to the bacterial DNA showed apoptosis early in the 6th h, unless DNase had significant delay. Table 1-3 plot different groups versus the control.

Study of the ability of killing and NBT reduction in cells: Resting group had reduced NBT, 35 and 45% in the 3rd and 6th h of the study, respectively (Table 5).

Table 1: The comparison of the results of cultured peritoneal macrophages between two groups; control and stimulated cells by bacterial DNA

	Incubation time			
Cellular characteristics	Stimulant	Start time	3rd h	6th h
No. of adherent cells	Control	0	1292	1224
	Bac. DNA	0	2010	390
Morphogenesis	Control	0	+/++	++ Start of apoptosis
	Bac. DNA	0	++++	Storong apoptosis

Table 2: The comparison of the results of cultured peritoneal macrophages between two groups; control and stimulated cells by FMLP

	Incubation time			
Cellular characteristics	Stimulant	Start time	3rd h	6th h
No. of adherent cells	Control	0	1292	1224
	FMLP	0	1612	2720
Morphogenesis	Control	0	+/++	++ Start of apoptosis +++ Near
	FMLP	0	+++	Storong apoptosis

Table 3: The comparison of the results of cultured peritoneal macrophages between two groups; cells by DNase

	Incubation time			
Cellular characteristics	Stimulant	Start time	3rd h	6th h
No. of adherent cells	Control	0	1292	1224
	DNase	0	1306	1619
Morphogenesis	Control	0	+/++	++ Start of apoptosis
	DNase	0	+/++	+++

Table 4: The comparison of the ratio of adherent cells in stimulated to control and the comparison of the ratio of adherent cells of groups in 6th and 3rd h

	Ratio		
	No. of adherent cells of stimulated and control group		
Group	3rd h	6th h	No. of adherent cells in 6th h and 3rd h
Bac. DNA	1.55	0.31	0.19
FMLP	1.24	2.22	1.68
DNase	1.01	1.32	1.23
Control	1	1	0.23

Table 5: The comparison of percentage of NBT positive cells of groups in different incubation times

	Different times	
	NBT (%)	
Groups	3rd h	6th h
Control	35	45
Bac. DNA	29	95
FLMP	42	66
DNase	1	82

DNAse had no significant effect on the NBT reduction in the 3rd h, but in the 6th h interestingly 95% of the cells reduced NBT.

Consequently, FMLP and DNase in the 6th h and bacterial DNA in the 3rd h lead to a great cell maturation in the macrophages.

In comparison to the control group these stimulants also potentiated the ability of NBT reduction and macrophage maturation.

DISCUSSION

There is a suggestion here: Is there any homology between prokaryote and eucaryote genes?

These are tens of genes in viruses which are homologous with the mammalian genes. Bacterial genome can not enter into the mammalian genome because of their low rate of methylation. So that, they can stimulate the innate immune system. Bacterial products such as LPS and peptydoglycan have great ability in initiating the phagocytic procedure[9-11].

Takunaga *et al.*[12] showed that not only naïve bacteria but also purified bacterial elements have the ability to stimulate phagocytosis. In 1996, Stacy *et al.*[6] showed that specific bacterial DNA motifs have some effects on the mocrophage's function. Cytokines cascade includes TNFα and IL-1β which has specific role in the stimulatory process of these motifs. This role, as adjuvants, opens a new horizon in immunization and vaccination. Studying these results made us to evaluate the bacterial DNA as a non-adaptive immune system (specifically macrophages stimulant)[9-13]. Peritoneal macrophages has been used in order to avoid delay aquiring phagocytic functions. It is reported that bacterial DNA was the most effective adherence stimulant in the 3rd h. Following bacterial DNA, FMLP had the best results expected due to references. In this study, DNase which was added to eliminate any contamination with other genetic factors, showed the ability to stimulate the phagocytosis. The 6th h of the study was the best time for this substance's optimal function. There was no significant difference between DNase and the resting group in the 3rd h. There is no impression about DNase as a stimulant in the literature, but in this study it was found that it is a phagocytic stimulant with delayed function. Interestingly, it has also the ability to reduce NBT. The highest rate of cell adherence in the 6th h belongs to FMLP. This substance is a pioneer stimulant for the macrophages adherence. In respect of reducing NBT, FMLP produces less oxidative radicals in comparison to the bacterial DNA, but more oxidative radicals in comparison to the resting group.

Bacterial DNA has the ability to stimulate adherence in the earlier hours and also it reduces NBT in the latest hours in comparison to other stimulants, so that it can be

called as an early onset stimulant with great ability in reducing NBT. These findings in comparison to the resting group results show significant differences. In many of the intracellular interactions activation of DNase can trigger respiratory burst in the cell that can be the initiating of apoptosis. In this study DNase had a delayed effect. Consequently, bacterial DNA introduced as a strong and early onset stimulant for the cell adherence.

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