Influence of protein kinase C inhibitor in phagocytosis activity toward *Candida sp*

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Abstrak

Protein kinase C, yang dapat diekspresikan oleh hampir semua sel adalah protein yang penting dalam alur sinyal transduksi yang berperan dalam sejumlah aktivitas sel, seperti berbagai macam hormon, sitokin, neurotransmiter dan growth faktor. Imunitas terhadap Candida sp antara lain ditentukan oleh sel limfosit T dan makrofag. Sebagai obyek penelitian ini adalah bagaimana pengaruh inhibitor protein kinase C - bisindolylmaleimides pada aktivitas fagisitosis Candida sp oleh makrofag. Kultur makrofag peritoneal mencit BALB/c diberi perlakuan dengan memberikan bisindolylmaleimides dari kadar 5 ng/ml sampai 100 ng/ml selama 10 menit. Kemudian segera dimasukkan Candida sp dan diamati setiap 30 menit selama 120 menit. Rancangan penelitian yang digunakan adalah rancangan faktorial dan ortogonal polinomial. Data yang dikumpulkan berupa panjang pseudopodia dan banyaknya Candida sp yang difagositosis serta lamanya waktu pengamatan dianalisis dengan uji statistik Anova (satu jalan) untuk memperlihatkan perbedaan diantara perlakuan-perlakuan, Anova dua jalan untuk memperlihatkan interaksi antara perlakuan-perlakuan dan Student's t Test untuk memperlihatkan perbedaan terhadap kontrol. Test statistik memperlihatkan perbedaan yang bermakna pada panjangnya pseudopodia dan banyaknya Candida sp yang difagositosis pada pemberian berbagai macam kadar bisindolylmaleimides ($p<0,001$) dan lamanya waktu pengamatan ($p<0,001$). Interaksi antara pemberian berbagai macam kadar bisindolylmaleimides dengan lamanya waktu pengamatan sangat bermakna ($p<0,001$). Semakin tinggi kadar bisindolylmaleimides yang diberikan, semakin awal waktu pengamatan, maka semakin banyak protein kinase C yang tidak aktif dan semakin pendek panjang pseudopodia atau semakin menurun aktivitas fagositosis Candida sp oleh makrofag. Hasil penelitian menunjukkan bahwa bisindolylmaleimides dapat menghambat mobilitas dan aktivitas fagositosis Candida sp oleh makrofag. Penelitian lebih jauh tentang protein kinase C, terutama pada makrofag, sangat dianjurkan. ***(Med J Indones 2001; 10: 150-7)***

Abstract

Protein kinase C isoenzyme family that expresses in all of cells plays a pivotal role in the signal transduction pathway of a variety of hormones, cytokines, neurotransmitter, and growth factors. The immunity against Candida sp is mainly mediated and performed by the T cells and macrophages. The objective of this experiment is to know the influence the protein kinase C inhibitor - bisindolylmaleimides in phagocytosis activity toward Candida sp. The culture of peritoneal macrophage derived from BALB/c mice are treated with bisindolylmaleimides as a protein kinase C inhibitor concentration varied from 5 ng/ml to 100 ng/ml for as long as 10 minute. Then the Candida sp added is observed after every 30 minute for as long as 120 minute. As the experimental design is used the method of factorial and orthogonal polynomial. The data consisting the length of pseudopodia and the number of Candida sp which are phagocytosed are analyzed applying the Anova. One Way Anova to show the differences of each manipulation, the Two Way Anova to show the interaction of manipulations and the Student's t Test to show the differences with control. Statistical test show significant differences on the length of pseudopodia, and phagocytosed Candida sp, at different bisindolylmaleimides concentration ($p<0,001$) and different observed time ($p<0,001$). The data show a significant interaction between the bisindolylmaleimides concentration and observed time ($p<0,001$). The higher the bisindolylmaleimides concentration, the earlier the observed time, the much number the protein kinase C are going inactive and the shorter the length of pseudopodia or the lower the macrophages phagocytic activity toward Candida sp. The result of this experiment indicates that bisindolylmaleimides can inhibit the macrophage mobility and phagocytic activity toward Candida sp. Further experiment in protein kinase C, especially in macrophage, is suggested. ***(Med J Indones 2001; 10: 150-7)***

Keywords: *Signal Transduction, protein Kinase C, bisindolylmaleimides, phagocytosis*

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Signal transduction

It is essential for their survival that cells communicate with their neighbors, monitor the conditions in their environment, and respond appropriately to a host of different type of stimuli that impinge on their surface. Cells carry out these interaction by the phenomenon known as cell signalling, in which information is relayed across the plasma membrane to the cell interior and often to the cell nucleus.[1]

In most systems, cell signalling includes:

1. Recognition of the stimulus at the outer surface of the plasma membrane by a specific receptor embedded within the membrane.
2. Transfer of a signal across the plasma membrane to its cytoplasm surface.
3. Transmission of the signal to specific receptor molecules on the inner surface of the membrane or within the cytoplasm that trigger the cell's response. The response might involve a change in gene expression, an alteration of the activity of metabolic enzymes, a reconfiguration of the cyto-skeleton, a change in ion permeability, the activation the DNA synthesis, or even the death of the cell.
4. Cessation of the response as a result of the destruction or inactivation of the signaling molecule combined with a decrease in the level of the extracellular stimulus.[1]

Protein kinase C

Protein kinase C (PKC) family is a heterogeneous family of phospholipid-dependent kinases that can be divided into three categories on the basis of co-factor requirement and structure.[1-3] *Protein kinase C* as an isoenzyme plays a very pivotal role to generate the signal in the transduction pathway. *Protein kinase C* has a number of important roles in cellular growth, differentiation, metabolism, and transcriptional activation, most of which are not well understood.[1-4] Conventional PKC requires calcium and diacylglycerol (DAG) or phorbol ester as cofactor whereas novel PKC requires only DAG or phorbol ester. Atypical PKC do not require calcium or DAG for maximal activity. *Protein kinase C* consist of a single polypeptide chain that contains an amino-terminal regulatory region (20-70 kD) and a carboxy-terminal kinase domain (approximately 45 kD). The catalytic domain contains the ATP-binding and substrate-binding. This domain is separate from regulatory domain containing PS-binding and phorbol ester-binding site.[2]

Bisindolylmaleimides is a potent and selective inhibitor of Protein kinase C provides evident for the potential use of PKC inhibitor as therapeutic immunomodulators. This compound also selectively inhibited the secondary T cell mediated response in the developing adjuvant arthritis model in rats.[3] Bisindolylmaleimides was a competitive inhibitor with respect to ATP and displayed high selectivity for PKC.[3,4] Many inhibitors in two different domains have already been described: calphostin C and sphingosine which interact with the regulatory moiety and sangivamycin, the isoquinoline H7 and staurosporine which interact with the ATP-binding site.[4]

Immunity to fungi

T cell and macrophage are very importance in the immunity to fungi. T cell immunity is also implicated in resistance to other fungal infection, since resistance can sometimes be transferred with immune T cell. It is presumed that Th cells release cytokines which activate macrophages to destroy the fungi.[5-9] Little are known about the precise mechanisms involved in immunity to fungal infection, but it is thought that they are essentially similar to those involved in resistance to bacterial infections.

Macrophage

The classic studies have clearly shown that monocytes transform into macrophages and multinucleated giant cells in vitro. Incorporation of the surrounding medium and soluble molecules by invagination of the cell surface, *pinocytosis* and *macropinocytosis*, became increasingly active as the monocyte changes into the macrophage and appear to increase in activity with associated increase in metabolic activity. Phagocytosis can be divided into several phases which are attachment, ingestion, secondary lysosome formation (enzymatic degraded), and vesicle closure and disposal of degradated material.[10] As the organism move, portion of the cell surface are seen to be pushed outward by the column of the cytoplasm that flows through the interior of the cell toward the periphery. The broad, rounded protrusions formed during ameboid movement are called pseudopodia. Ingestion then occurs as the phagocyte surrounds the cell by the extension of two pseudopodia which meet to form the endocytic vesicle, or phagosome.[1]

Candida sp

Candida are a group of fungi which produce localized disease of skin and mucous membrane. In immuno-compromised hosts they can cause invasive and/or disseminated disease. They are emerging as an important nosocomially acquired pathogen and a frequent opportunistic infection in the setting of neutropenia and AIDS. Most infection is acquired endogenously, typically from the gastrointestinal tract, with disease produced either by breakdown of normal barriers or by overgrowth of normal flora. Other pathogenic factors include extra cellular enzymes (such as phospholipase and proteases) and cell wall mannans, which produce anaphylaxis and death in animal model.[11, 12]

METHODS

Cells

Peritoneal-derived macrophages were obtained by the method of Colligan et. al. with some modifications. [13] We have five male mice BALB/c from Hayati Laboratory, Gajah Mada University. 1.5 ml syringe was filled with 1.0 ml of Freund's Adjuvants Incomplete (Sigma Chem. Co. St. Louis, USA) and a 25-G needle was attached, the solution was then injected into the peritoneal cavity of each mouse. Inflammatory response was alloned for 7 days and the mice were killed by cervical dislocation. The mouse abdomens were washed with 70% alcohol to sterile the area. Midline incision was made with sterile scissors. The abdominal skin was retracted with forceps to expose the intact peritoneal wall. A 10 ml syringe was attached with 19-G needle and then filled with the RPMI-1640 medium (Sigma Chem. Co. St. Louis, USA). The syringe plunger was pushed to allow a small amount of medium to pass through the needle as the needle penetrates the peritoneum to avoid hitting the intestine. With bevelled end of needle facing up, insert needle through peritoneal wall at the midline. Inject 10-ml RPMI-1640 medium into each mouse. Using the same syringe and needle, insert needle bevelled end down into peritoneum. Raise needle slightly to cause tenting of peritoneal wall. Withdraw peritoneal fluid slowly. remove needle from syringe and dispense pooled peritoneal fluid to 50 ml polypropylene centrifuge tube on ice. 2X10000 peritoneal cells in 20 ml RPMI-1640 medium were plated into 20 mm diameter wells of a 24 well culture disk (Costar). Before plated, place 20-mm diameter coverslip into the well. For most experiments cells were plated at 1000 cells per well. After allowing 30 minutes at room temperature for the suspended cell to adhere to the dish, RPMI-1640 medium was replace with Fetal Bovine Serum (GIBCO) and streptomycine 1% and fungizone 1%, and the culture were left overnight at room temperature in incubator.

Influence of inhibitor protein kinase C

Before treatment, the culture medium were removed and the macrophage were washed extensively using PBS-10F (PD : 137 nM-NaCl, 3 nM-KCl, 7 nM-Phosphat Buffer, pH 7.4) and incubated with bisindolylmaleimides (Sigma Chem. Co., St. Louis, USA) the protein kinase C inhibitor for 10 minute at room temperature. The culture macrophages were added to the *Candida sp* and observed every 30 minutes for 120 minutes.[14]

Microscopy

For time-lapse, coverslips were put and stained with Giemsa (MERCK). Nikkon phase microscope images were collected via a 100 x objective lens every 30 minutes for 120 minutes.[15]

Experimental design

This experiment was done in the laboratory. As in-ependent variable the concentration of bisindolyl-aleimides were used. As dependent variables : (1) the length of the pseudopodia; (2) the number of *Candida sp* that phagocytosed by macrophage were used, and as controlled variable the observed time (every 30 minutes) is used. The data consisting the length of pseudoodia and the number of *Candida sp* which is phagocytosed are analyzed by applying the Anova. One Way Anova to show the differences of each manipulation, the Two Way Anova to show the interaction of manipulations and the Student's t Test to show the differences with control.[16]

RESULT

Pseudopodia

The photographs and data of the experiment about pseudopodia were shown below:

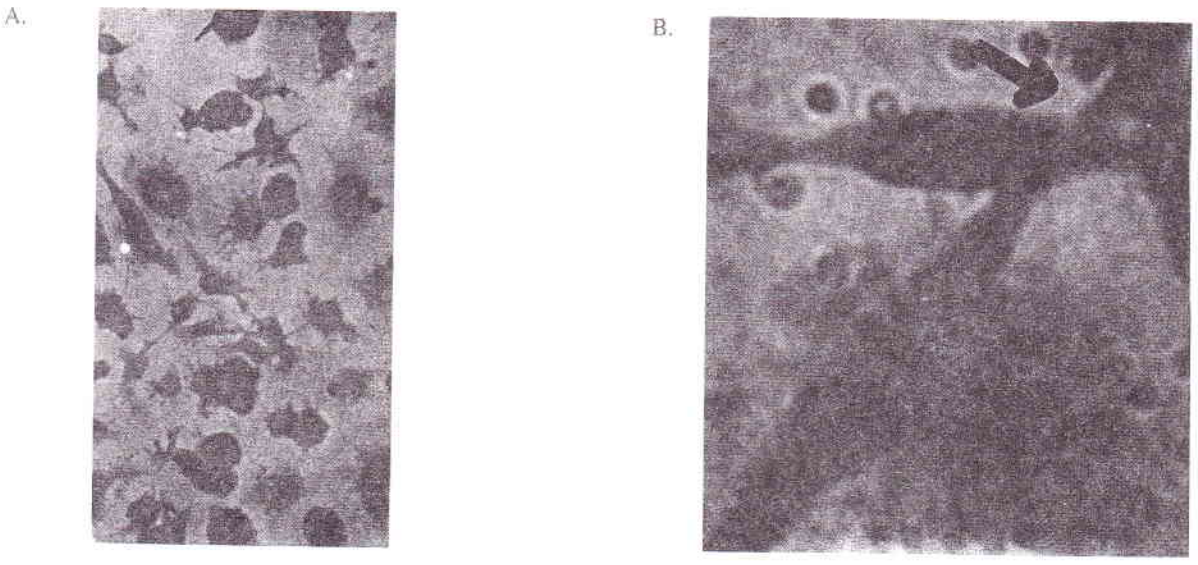


Figure 1. A. The length of pseudopodia (the arrow) before treatment with bisindolylmaleimides after 30 minutes of observation (200X). B. The length of pseudopodia (the arrow) before treatment with bisindolylmaleimides after 30 minutes of observation (1000X).

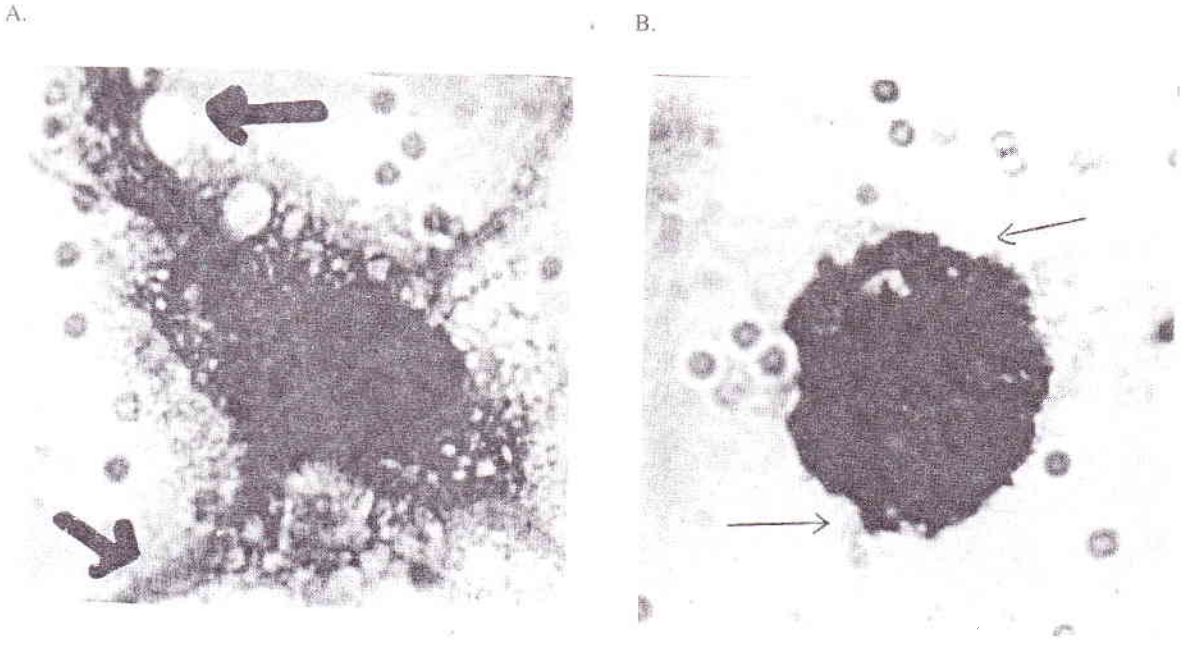


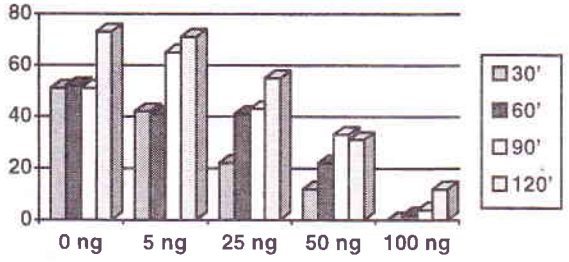
Figure 2. A. The length of pseudopodia (the arrow) after 30 minutes of treatment with 5 ng/ml bisindolylmaleimides. (1000X). B. The length of pseudopodia (the arrow) after 30 minutes of treatment with 100 ng/ml bisindolylmaleimides. (1000X).

Table 1. Mean and standard deviation of the length of pseudopodia (micrometer) in various concentration of bisindolylmaleimides.

Concentration of bisindolyl-maleimedes (ng/ml)	$\overline{X}$ (after observed 30 minutes)	SD	$\overline{X}$ - SD	$\overline{X}$ (after observed 120 minutes)
0	55,9	10,1	45,8-66	
5	***54,8****	-	-	***70***
25	40,2	-	-	***55****
50	24,5	-	-	31
100	4,5	-	-	12

Note : * : NS (not significant).
ng/ml : nanogram per mililiter.
$\overline{X}$: mean
SD : standard deviation

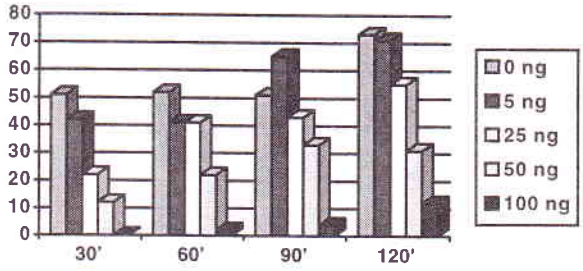
the length of **pseudopodia (micrometer).**



concentration of bisindolylmaleimides (ng/ml).

Figure 3. The length of pseudopodia in various concentration of bisindolylmaleimides. The higher the bisindolylmaleimides concentration, the shorter the length of pseudopodia.

the length of pseudopodia (micrometer).



the time observed (minute).

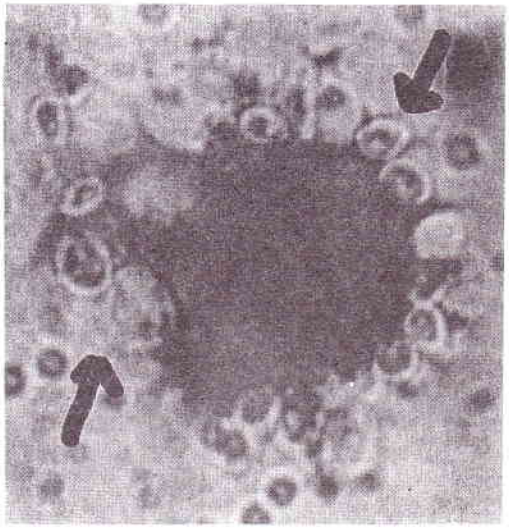
Figure 4. The length of pseudopodia in time observed. The earlier the observed time, the shorter the length of pseudopodia.

Statistical test showed significant differences on the length of pseudopodia, at different bisindolylmaleimides concentration ($p<0.001$) and different observed time ($p<0.001$). The data show a significant interaction between the bisindolylmaleimides concentration and observed time ($p<0.001$). The higher the bisindolylmaleimides concentration, the earlier observed time, the more number of protein kinase C became inactive, the shorter the length of pseudopodia. From T test were shown a significant difference for concentrations 25 ng/ml ($p=0.004$), 50 ng/ml and 100 ng/ml ($p=0.0001$), no significant difference were found at concentration 5 ng/ml.

Phagocytosis of *Candida sp*

The photographs and data of the experiment about phagocytosis of *Candida sp* were shown below:

A.



B.

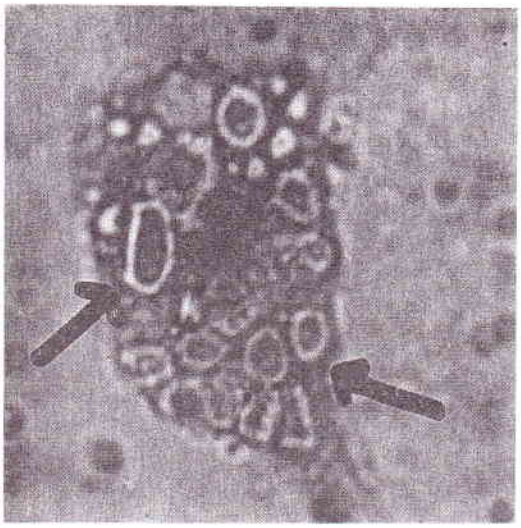


Figure 5. The number of Candida sp. phagocytosed (the arrow) before the bisindolylmaleimides treatment. A. After 30 minutes of observation (1000X). B. After 120 minutes of observation.

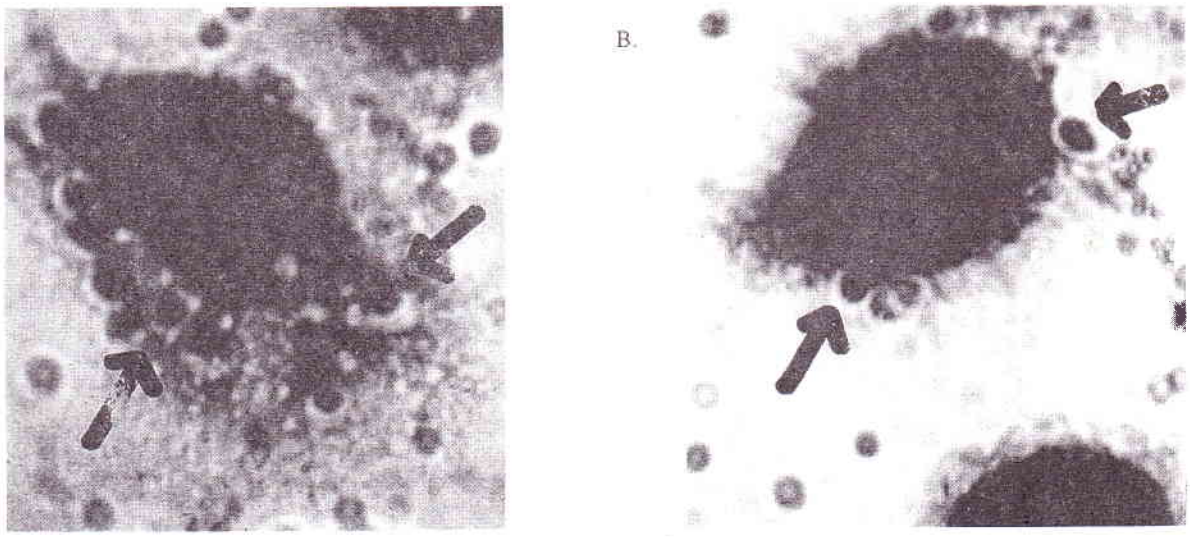


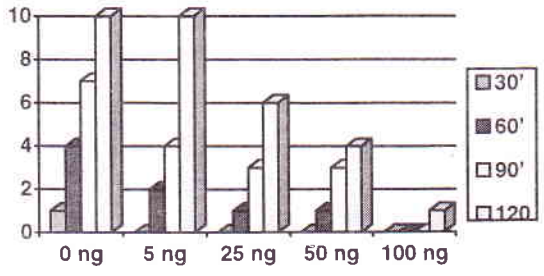
Figure 6. The number of Candida sp. phagocytosed (the arrow) after treatment with bisindolylmaleimides. A. Treatment with 5 ng/ml bisindolylmaleimides after 30 minutes of observation (1000X). B. Treatment with 100 ng/ml bisindolylmaleimides after30 minutes of observation (1000X).

Table 2. Mean and standard deviation of the number of *Candida sp* (cell) that were phagocytosed in various concentrations of bisindolylmaleimides.

Concentration of bisindolyl-maleimedes (ng/ml)	$\overline{X}$ (after observed 30 minutes)	SD	$\overline{X}$ - SD	$\overline{X}$ (after observed 120 minutes)
0	5,3	3,3	2-7,3	-
5	*4**	-	-	*9,6*
25	*2,6**	-	-	*6**
50	1,9	-	-	*4**
100	0,4	-	-	1,3

Note : * : NS (not significant)
ng/ml : nanogram per mililiter.
$\overline{X}$: mean
SD : standard deviation

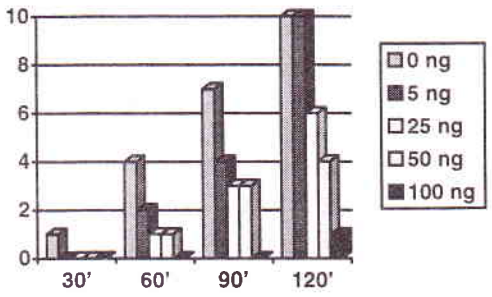
the number of *Candida sp* that were phagocytosed.



concentration of bisindolylmaleimides (ng/ml).

Figure 7. The number of Candida sp phagocytosed in various concentration of bisindolylmaleimides. The higher the concentration, the lower the number of Candida sp phagocytozised.

the number of *Candida sp* that were phagocytosed.



the length of observation time (minute).

Gambar 8. The number of Candida sp phagocytosed in time observed. The earlier the observation, the lower the number of Candida sp phagocytosed.

Statistical test showed significant differences on phagocytosed *Candida sp*, at different bisindolylmaleimides concentration ($p<0.001$) and different observation time ($p<0.001$). The data show a significant interaction between the bisindolylmaleimides concentration and observation time ($p<0.001$). The higher the bisindolylmaleimides concentration, the earlier the observation time, the more number of the protein kinase C are going inactive, and the lower the macrophages phagocytosis activity toward *Candida sp*. From T test were shown a significant difference for concentrations 25 ng/ml ($p=0.004$), 50 ng/ml and 100 ng/ml ($p=0.0001$), no significant difference were found in concentration 5 ng/ml.

DISCUSSION

Cells

Protease peptone - treated mice should yield 3-4 X 1.000.000 macrophages per mouse. Thyoglycollate - treated mice will yield 10.000.000 macrophages per mouse. But treated mice with Freund's adjuvants incomplete only have 2 X 10.000 macrophages per mouse after 5 days. Inflammatory agents recruit young, immature macrophages into peritoneum. Total cell yields and subpopulations of cells will differ depending on the irritant used. Easily digestible inflammatory agent will induce a short-lived inflammatory response and the animal will be able to return to homeostasis within days. On the other hand, agent that are more difficult to digest, such as thyoglycollate or colloidal starch, can induce an inflammatory response in vivo that lasts for 5 to 7 days.[13]

Freund's Adjuvants is water-in-oil emulsion used to stimulate immune responses. There are two forms of Freund's adjuvants, depending on the presence or absence of killed Mycobacteria. Complete Freund's Adjuvants contains Mycobacterium tuberculosis, or other strains of Mycobacteria. It induces strong Granuloma formation at the site of injection and enhances the immune response. Weak antigens may be rendered more immunogenic when incorporated in complete Freund's adjuvant.[7, 8]

Influence of inhibitor protein kinase C

Staurosporine is the most potent inhibitor of protein kinase C (PKC) described in the literature with a half maximal inhibitory concentration of 10 nM. Nevertheless, this natural product is poorly selective when assayed against other protein kinases. In order to obtain specific PKC inhibitor, a series of bisindolylmaleimides has been synthezised. GF 109203X was a competitive inhibitor with respect to ATP and displayed high selectivity for PKC as compared to five different protein kinases. In further experiment determined the potency and specificity of GF 109203X in two cellular model : human platelets and Swiss T3T fibroblast. GF 109203X – bisindolylmaleimides inhibited collagen - and alpha - thrombin - induced platelet aggregation as well as collagen - triggered ATP secretion. In Swiss 3T3 fibroblast, GF 109203X reversed the inhibition of epidermal growth factor binding induced by phorbol 12,13-dibutyrate and prevented thymidine incorporation into DNA, only when this was elicited by growth promoting agent which activate PKC. His results illustrate the potential of GF 109203X bisindolylmaleimides as a tool for studying the involvement of PKC in signal transduction pathway.[3]

The protein kinase inhibitor staurosporine has been used to design a series of selective bisindolylmaleimide inhibitors of protein kinase C (PKC). Guided by molecular graphics, conformational restriction of the cationic side chain has led to ATP competitive inhibitors of improved potency and selectivity. Two compounds have been further evaluated and were shown to inhibit PKC of human origin and prevent T-cell activation in a human allogeneic mixed lymphocyte reaction. One of this compounds was orally absorbed in mice and antagonized a phorbol ester induced paw edema in a dose-dependent manner. This compound also selectively inhibited the secondary T cell mediated response in a developing adjuvant arthritis model in rats and provides evidence for the potential use of PKC inhibitors as therapeutic immunomodulators.[4]

All of the PKCs have an amino-therminal regulatory domain containing PS-binding and phorbol ester binding site. This domain is separated from the carboxyl-terminal catalytic domain containing the ATP-binding and substrates-binding site by flexible hinge region.[1-4,15] Inhibitors of PKC could interact with the substrate-binding site or with regulatory site of PKC. The structural similarity between bisindolylmaleimides and staurosporine suggested that bisindolylmaleimides may be a competitive inhibitor with respect to ATP. The inhibition of PKC by bisindolylmaleimides was demonstrated to be highly dependent on the ATP concentration. Dixon plot data indicated that bisindolylmaleimides was a competitive inhibitor versus ATP. In this experiment bisindolylmaleimides is very significant in inhibiting the mobility and activity of the macrophages in phagocytosis toward *Candida sp* (see Fig. 3, 4, 7 and 8). The higher the bisindolylmaleimides concentration, the sooner the time observed, the more PKCs are going inactive, the shorter the length of pseudopodia the lower the mobility and activity of phagocytosis toward *Candida sp* by macrophages.

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