Original Article

DOI: 10.5582/ddt.2019.01065

Different activities of antitumor immunomodulators to induce neutrophil adherence response

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Summary **Functions of neutrophils, major participant in host defense mechanisms, are known to be regulated by various types of immunomodulators. Capacity of immunomodulators which are reported to show antitumor effect *in vivo* to induce neutorophil adherence response *in vitro* was investigated. Several bacterial immunomodulators (OK-432, *Corynebacterium parvum*, B.C.G.) and components of bacteria cell walls (lipopolysaccharide (LPS), lipid A, lipoteicoic acid, N-cell wall skelton (N-CWS), muramyl dipeptide (MDP)) and fungal polysaccharides (lentinan, zymosan A, *etc.*) were tested. Neutrophils prepared from peripheral blood of healthy men were incubated with each immunomodulator at 37°C for 60 min in 96 well plastic plates, then neutrophils adherent to substratum were stained by crystal violet and their optical density at 570 nm was measured as a parameter of neutrophil adherence. Although purified polysaccharides mainly prepared from fungi did not induce the adherent response, not only bacterial bodies and their components but also tumor necrosis factor-α (TNF-α) clearly induced it. On the base of these results, functional classification and typing of immunomodulators by different activities in neutrophil adherence was discussed.**

Keywords: Immunomodulator, neutrophil adherence, tumor necrosis factor-α, bacterial components, fungal polysaccharide

1. Introduction

Many types of immunomodulators are conceived to have antitumor activities mainly through activation of various leukocytes, such as macrophage, natural killer cells and T, and B-lymphocytes. Neutrophils have been also recognized to participate in antitumor action by bacterial immunomodulators (OK-432, *etc.*) and β-1,3-glucans (*1-3*). We reported that intraperitoneal administration of various types of antitumor immunomodulators rapidly induce neutrophils into the peritoneal cavity of mice (*4*). This suggested that many types of antitumor immunomodulators activate neutrophils *in vivo*. Neutrophils, responding to stimulus at the earliest phase in inflammatory cellular reaction, affect the following cellular reactions with host defence functions.

Clarification of antitumor immunomodulators based on neutrophil activation is important to understand their antitumor action, but it has not been studied systemically. So, we examined here the capacity of immunomodulators to activate neutrophils *in vitro*.

There are many parameters of neutrophil activation, such as chemotaxis, increased adherence, release of lysosomal enzymes, production of active oxygens and so on. Here, we used a neutrophil adherence assay to measure activation of human peripheral blood neutrophils because adherence to plastic plates is reported to be a reliable method for testing phagocytotic activity of neutrophils (*5,6*).

2. Materials and Methods

2.1. *Materials*

Samples were kindly donated as follows. Recombinant-human tumor necrosis factor (rhTNF); Asahi Chemical

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Ind.(Tokyo, Japan), anti-recombinant human TNF-α monoclonal antibody (anti-rhTNF antibody); Prof. D. Mizuno of Biotechnol. Res. Center, Teikyo Univ. (Kawasaki, Japan), OK-432; Chugai Pharmaceutical Ltd. (Tokyo, Japan), muramyl dipeptide (MDP) and purified mannoglucan polyalcohol (MGA); Daiichi Seiyaku Co. Ltd. (Tokyo, Japan) (currently, Daiichi-Sankyo Company, Limited, Tokyo, Japan), TAK (linearβ-1,3 glucan); Takeda Chemical Industries (Tokyo, Japan), lentinan; Ajinomoto Co. (Kanagawa, Japan), lipoteicoic acid; Funakoshi (Tokyo, Japan), N-cell wall skelton (N-CWS); Fujisawa Pharm. Ind. Ltd.(Osaka, Japan) (currently, Astellas Pharma Inc. Tokyo, Japan), levamisole; Kyowa Hakko, Co. (Kanagawa, Japan) (currently, Kyowa Kirin Co., Ltd., Tokyo, Japan). Another samples were purchased as follows. *Escherichia coli* derived lipid A (lipid A); Daiichi Chemical Ltd. (Tokyo, Japan), killed *Bordettela pertussis*; Chiba Serum Institute (Chiba, Japan), killed *Corynebacterium parvum*; Ribi Immunochem. Res. Inc. (Hamilton, Mont., USA), B.C.G.; Japan B.C.G. Ind. (Tokyo, Japan), *E. coli*-lipopolysaccharide (*E.coli*-LPS); Difco Laboratories (Detroit. Mich., USA), and lambda-carrageenan, dextran sulfate, zymosan A and poly(I)-poly(C); Sigma Chemicals (St. Lous, Mo., USA). Dextran 70, Ficoll-paque solution and 96-well flat-bottom culture plates were purchased from Midori-jyuji Ltd. (Osaka, Japan) (currently, Mitsubishi Tanabe Pharma, Osaka, Japan), Pharmacia Fine Chemicals INC. (Uppsala, Sweden) (currently, GE Health HyClone, USA) and Falcon Ltd. (Franklin Lakes, NJ, USA), respectively.

2.2. *Neutrophil preparation*

Neutrophil was prepared according to the method of Yakuwa *et al*. (*5*). Heparinized venous blood obtained from healthy male volunteers was mixed with an equal volume of 6% dextran/saline and kept at room temperature approximately for 30 min to allow the erythrocytes to sediment. Separated plasma fraction was layered on Ficoll soln., and centrifuged at 1,400 rpm (200 × g) for 30 min at room temperature.

Neutrophils and red blood cells sedimented at the bottom of the tube were washed with phosphate buffer saline (PBS(-)), and residual erythrocytes were lysed at first by the addition of hypotonic PBS(-) (one third of isotonic) and next by the addition of Gey's solution. The residual neutrophils were suspended with medium (10% FCS-20mM HEPES RPMI 1640).

2.3. *Neutrophil adherence Assay*

Assay was done in accordance with the method of Yakuwa *et al*. (*5*). In brief, a 10^2 μL of samples (TNF for control) was placed in a well of 96 well flat bottom culture plates, and an equal volume of neutrophil suspension (ca. 2.5×10^6 cells/mL) was added there.

After incubating at 37°C for scheduled times, non-adherent cells were removed by washing with saline. Then, each well was dried and stained with 2×10^2 μL of distilled water containing 0.5% crystal violet, 12% formalin and 10% ethanol solution. After washing the wells, attached cells stained with crystal violet were solubilized with 1% SDS and the absorbance of optical density at 570 nm with the use of a Titertek Multiskan MTP-100 (Thermo Fisher Scientific K.K., Tokyo, Japan) was measured.

2.4. *Statistics*

Each data represents the mean of 3 values. Neutrophil adherence was observed as the absorbance at 588 nm after being stained with crystal violet, and expressed as percent value of that observed with 1 U/mL of rhTNF in the same assay plate in order to compare independent experiments.

3. Results

3.1. *Time course change of LPS-induced adherence*

We examined the adherence inducing ability of various biological response modifiers (BRMs) preliminary, then found that rhTNF and *E.coli*-LPS have potent activity on neutrophil adherence (*5,6*). Concentration dependency and time course change of neutrophil adherence induced by *E.coli*-LPS and rhTNF are indicated in Figures 1 and 2, respectively. Concentration dependent adherence was observed at 1-10^3 ng/mL by *E.coli*-LPS and most potent activity was seen at 60 min. On the other hand, concentration dependent adherence was observed at 10^{-1}-10^2 U/mL in the case of rhTNF. Adherence reached almost plateau level at 10 U/mL. One U/mL of rhTNF was used as the internal standard

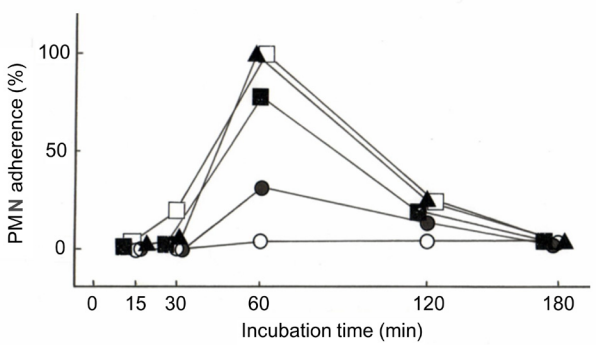

Figure 1. Time course change of neutrophil adherence to 96 well plastic plates induced by *E. coli*-LPS. Neutrophils (2.5×10^5 cells/well) were incubated with 1-10^3 ng/mL of LPS at 37°C for several time intervals. Adherent neutrophils were stained and adherence activities were measured at O.D. 588 nm. Neutrophil adherence was expressed as % of the maximum O.D. induced by 10^2 U/mL of rhTNF. Each point represents the mean of 3 values. ○, control; ●, LPS 1 ng/mL; ■; LPS 10 ng/mL; ▲, LPS 10^2 ng/mL; □, LPS 10^3 ng/mL.

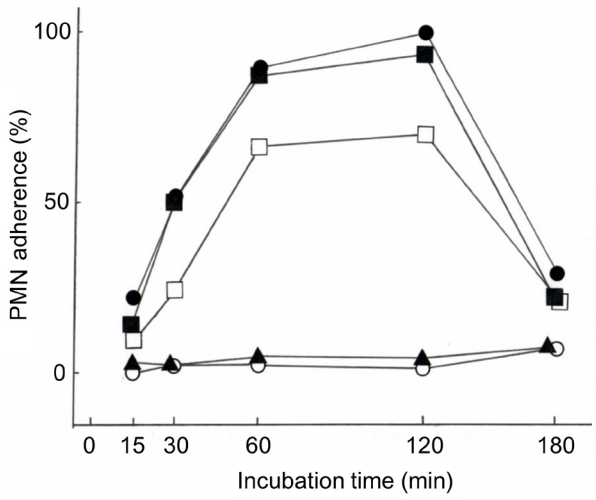

Figure 2. Time course change of neutrophil adherence to 96 well plastic plates induced by rhTNF. Neutrophil (2.5×10^5 cells/well) were incubated with 10^{-1}-10^2 U/mL of rhTNF at 37°C for several time intervals. Adherent neutrophils were stained and adherence activities were measured at O.D. 588 nm. Neutrophil adherence was expressed as % of the maximum O.D. induced by 100 U/mL of rhTNF. Each point represents the mean of 3 values. ○, control; ▲, rhTNF 10^{-1} U/mL; □, rhTNF 1 U/mL; ■, rhTNF 10 U/mL; ●, rhTNF 10^2U/mL.

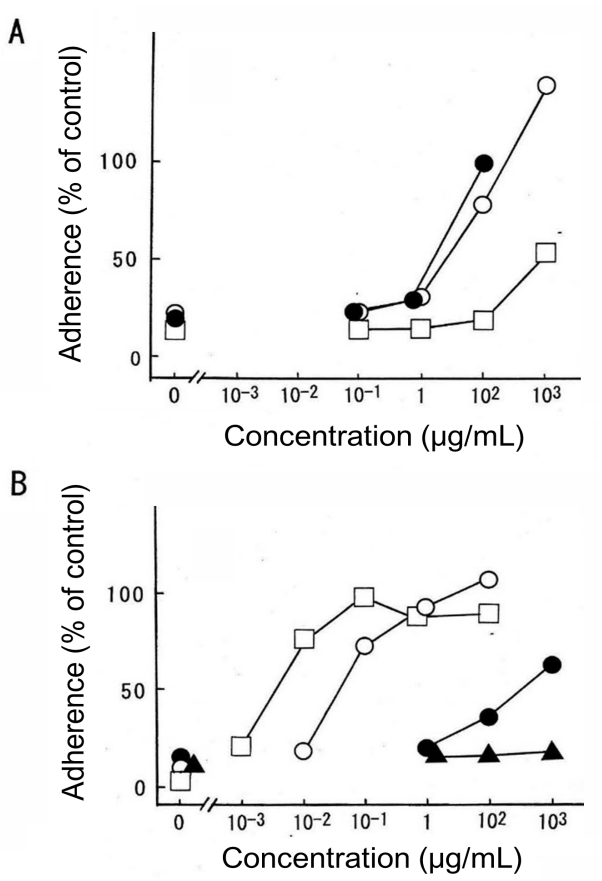

Figure 3. Neutrophil adherence activities of bacteria-BRMs (A) and cell wall components of bacteria (B). Activities was expressed as % of that of rhTNF 1 U/mL. Neutrophil (2.5×10^5 cells/well) were incubated with each sample at 37°C for 60 min. Adherent neutrophils were stained and adherence activities were measured at O.D. 588 nm. Each point represents the mean of 3 values. **A:** ●, *C. parvum*; ○, OK-432; □, B.C.G. **B:** □, lipid A; ○, *E. coli*-LPS; ●, lipoteicoic acid; ▲, MDP.

for all the experiments, because 1 U/mL of rhTNF induced significant neutrophil adherence constantly at submaximum level in several independent experiments (data not shown) and purified TNF can be supplied. On the incubation period, although maximum effect was seen at 120 min, submaximum effect was already obtained even at 60 min at rhTNF and maximum effect was observed at 60 min for LPS. Then, we adopted 60 min for incubation period for all the plenary studies to keep the consistency throughout the experiments.

3.2. *Induction of neutrophil adherence by bacteria and its components*

Neutrophil adherence activity was examined for bacteria-derived BRMs which were reported to be effective for tumor models in animals (*7-15*). All the tested bacteria BRMs (*C. paruvum*, OK-432, B.C.G.) showed neutrophil adherence activities (Figure 3A).

Adherence activities of cell wall components of bacteria were indicated in Figure 3B. LPS derived from gram-negative *E. coli* (*E. coli*-LPS) induced adherence activity in a dose dependent manner. Although the adherence response by 10^{-2} μg/mL of LPS varied in each experiment tested, responses induced by it at more than 10^{-1} μg/mL seems to show relatively constant values. In the following experiments, we tested the effect of several samples for 3 concentrations or more (10 fold difference in the adjacent 2 concentrations) which had been thought to be effective in *in vitro* experiments.

Lipid A, which is thought to be the active center of LPS, shows a prominent effect on neutrophil even at a dose of 10^{-2} μg/mL which is about one tenth of that of LPS. These concentrations of lipid A and LPS on adherence correspond well with that of migrating activity which is reported by Kotani *et al.* (*24*). Although a dose dependent increase of adherence activity was also observed in a component of gram-positive bacteria cell wall lipoteicoic acid, high concentration was necessary to show a prominent effect. Any activity was not observed in MDP even at a high dose of 10^2 μg/mL.

3.3. *Effects of polysaccharides on neutrophil adherence*

Neutrophil adherence activity was tested for polysaccharide-derived BRMs and some others which have been reported to be effective against tumors in animal models already (*16-23*) (Figure 4). Glucans such as zymosan and lentinan shows little activity even at a high dose of 10^2 μg/mL. MGA has no effect on PMN adherence also. Furthermore, TAK (linear β-1,3-glucan), which we reported to have an activating effect on neutrophil exudated in peritoneal fluid *in vitro* (*1*), did not induce adherence activity on neutrophil

in this condition (data not shown). Levamisole, a low molecular weight chemical immunomodulatory did not demonstrate any adherence activity.

3.4. *Effect of anti-rhTNF antibody on neutrophil adherence activity*

Neutrophil adherence induced by bacteria and its component might be caused by some kinds of cytokines secreted from neutrophil. In such cytokines, TNF is the most probable candidates since interleukin-1 (IL-1) and platelet activating factor (PAF), which are also secreted from neutrophil when stimulated (*25,26*), did not show any significant adherence activities on neutrophil even at high doses (IL-1 10^3 U/mL: 18.3%, PAF 10^{-4} M: 25.5%, when compared with the activity of control), then we examined whether adherence activity of BRMs might be affected by the anti-rhTNF antibodies or not. Anti-rhTNF antibody (2×10^4 fold dilution) suppress the activity of 1 U/mL of rhTNF almost completely (2.1%, when compared with the activity of control). However, the antibody did not have any effect on the activities of 0.1 KE/mL OK-432 (without antibody: 52.5%, with antibody: 51.1%, when compared with the activity of control), 1 μg/mL *E.coli*-LPS (without antibody: 52.5%, with antibody: 51.1%, when compared with the activity of control) and 100 ng/mL lipid A (without antibody: 99.0%, with antibody: 98.2%, when compared with the activity of control). These data suggest that PMN adherence induced by bacteria-derived BRMs such as OK-432, *E. coli*-LPS and lipid A are not mediated by the TNF secreted exogenously from neutrophil.

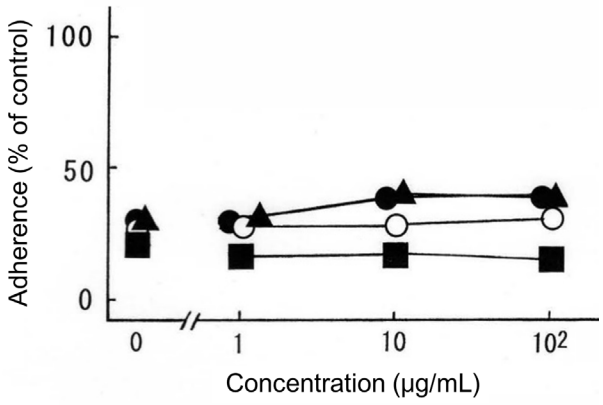

Figure 4. Neutrophil adherence activities of polysaccharide-BRMs. Activity was expressed as % of that of rhTNF 1 U/mL. Neutrophil (2.5×10^5 cells/well) were incubated with each sample at 37°C for 60 min. Adherent neutrophils were stained and adherence activities were measured at O.D. 588nm. Each point represents the mean of 3 values. ○, lentinan; ▲, C. zymosan; ■, MGA; ●, levamisole.

3.5. *Classification of various BRMs according to neutrophil adherence properly*

General classification of BRMs according to their origin are shown in Table 1. On the right column, global classification based on the quantity of neutrophil exudate into the peritoneal cavity after injecting BRMs into mice intraperitoneally, which was reported by Morikawa *et al.* (*4*), is demonstrated. This data suggests that although most bacteria and cell wall components

Table 1. Summary of neutrophil adherence activities and neutrophil-inducing pattern of various BRMs

Category	BRM	Neutrophil Adherence (A)	Neutrophil induction (B) *In vivo*
Bacteria			
G (-)	*B pertussis* (*7*)	-	
G (+)	*C. parvum* (*8*)	+	++
	OK-432 (*9*)	+	++
	BCG (*10*)	+	++
Component of bacteria			
G (-)	*E.coli*-LPS (*11*)	+	+
	Lipid A (*12*)	+	
G (+)	MDP (*13*)	-	+
	N-CWS (*14*)	++	
	Lipoteicoic acid (*15*)	+	
Polysaccharide	MGA (*16*)	-	
	Carrageenan (*17*)	-	++
	Dextran sulfate (*18*)	-	-
	Lentinan (*19*)	-	++
	β-1,3glucan (*20*)	-	++
	Zymosan A (*21*)	-	++
Others	Levamisol (*22*)	-	-
	Poly(I)-poly(C) (*23*)	-	+

BRMs showing *in vivo* antitumor effect are categorized based on their origin, and neutrophil inductive activity *in vivo* and neutrophil adherence activity *in vitro* are indicated. G(-); gram negative, G(+); gram positive. **(A)** Relative activities of neutrophil adherence were classified according to their potencies. +, adherence more than 50% of that of rhTNF 1 U/mL was observed at 10^2 μg/mL, and dose dependency was observed. -, No adherence activity was observed even at 10^2 μg/mL or 10^8 cell/mL. **(B)** Quoted from the paper of K. Morikawa *et al.* (*4*). ++, Highly potent type inducing more than 6×10^6 neutrophil/mouse, +, Relatively low neutrophil-inducing type inducing less than 6×10^6 neutrophil/mouse, -, No neutrophil-inducing activity. *In vivo* antitumor activities on each BRM are shown below.

of bacteria-derived BRMs have neutrophil adherence activity, polysaccharide and another BRMs (levamisole, poly (I)-poly (C), ET-18-OMe) do not have any such property *in vitro* although induction of PMN into peritoneal cavity are observed *in vivo* (*16-23*).

4. Discussion

In this report, we examined direct neutrophil adherence inducing properties of various antitumor BRMs. All the BRMs tested in this study are reported to have anti-tumor effect *in vivo* (*7-23*). Adherence activities of neutrophil to plastic plates were found in *E. coli*-LPS, N-CWS, bacteria and its components such as lipid A and lipoteicoic acid. In summary, bacteria-derived BRMs have direct activating properties on neutrophil. On the other hand, antitumor polysaccharide such as lentinan, zymosan and β-1,3-glucan, which show antitumor activity, did not show adherence induction on neutrophil at all.

We reported already that all of the BRMs tested in this paper (except levamisole, dextran sulfate) induce neutrophil more than 10^6 cells/mouse into peritoneal cavity within 6 h after injection (*4*). These data suggest that these BRMs must have some influence on neutrophil in the body. Considering from these data and our new data that most bacteria-derived BRMs except *B. pertussis* and MDP can induce the adherence of neutrophil and polysaccharide do not effect on neutrophil adherence at all, bacteria-derived BRMs must have an effect on neutrophil directly, but another ones (polysaccharides) must accumulate neutrophil indirectly through the activation of complements or macrophage *in vivo*. For example, zymosan is well known to activate neutrophil prominently by activating complement of alternative pathway (*27*). However, in this system, complement system does not work, because only inactivated bovine serum was used. Lack of complement system must be the reason why zymosan did not induce neutrophil adherence.

Another different point is such BRMs that directly activate the neutrophil have local antitumor effects generally (*12,28*). In other words, such BRMs are effective prominently, if they are injected locally to contact with tumor tissue. On the other hand, such agents as polysaccharides and poly(I)-poly(C), which did not induce neutrophil adherence directly, are not reported to have local antitumor activity (*28*). This relationship suggests that local antitumor effect of bacteria-derived BRM might be connected with direct activation of neutrophil and systemic antitumor effect of polysaccharide might be connected with indirect activation of neutrophil through the activation of complements or macrophages.

If TNF producing ability of BRMs are classified into 2 groups (priming agents and triggering agents), BRMs that induce the adherence of neutrophil directly (OK-432, LPS, lipoteichoic acid *et al.*) are known to be TNF triggering agents (*29-32*). On the other hand, another BRMs such as MDP, glucan and zymosan are reported to have priming activity of TNF release (*30,33*). Considering from these facts and the report that neutrophil can produce TNF in certain condition (*34*), direct induction of PMN adherence by bacteria-derived BRMs is considered to be caused by TNF released from neutrophil. Then, we tested the effect of rhTNF antibody on neutrophil adherence induced by BRMs. However, induction of neutrophil adherence by LPS, lipid A and OK-432 were not influenced at all. Then it is unprobable that TNF secreted from neutrophil by BRM mediate the adherence of neutrophil.

It is reported that increased activity of neutrophil by LPS depends on the increased expression of CD11b/CD18 *via* TLR-4 (*35,36*). On the other hand, TLR-2 is thought to mediate responses to Gram-positive bacterial protein (*37*). TLR-2 as well as TLR-4 are expressed in neutrophils in addition to macrophages (*38*). Then, stimulated adherence by *E. coli* LPS, lipid A and OK-432 may be explained by the increased expression of CD11b/CD18 through TLR4 and the increased adherence by N-CWS and lipoteicoic acid may be mediated by TLR-2 expressed in neutrophils in addition to macrophages. As activation of TLR4 and TLR2 are reported to be tumoricidal (*11,39*), antitumor mechanism of bacterial BRMs may depend on the activation of TLR4 and/or TLR2 expressed in neutrophil in addition to macrophage. So far, the mechanism of antitumor effect of BRM was explained mainly through the activation of macrophage (*40*). However, neutrophils and macrophages are considered to coordinate in immune response in several diseases (*41*). Therefore, neutrophils activated by bacterial BRMs may attack tumors directly and/or indirectly in cooperation with macrophages.

In conclusion, direct activation of neutrophil may be related with the local antitumor effect of bacteria-derived BRM to some extent.

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(Received September 11, 2019; Revised October 26, 2019; Accepted December 22, 2019)