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# Technique for Screening Immune-Enhancing Polysaccharides in Food Using 1,25-Dihydroxyvitamin D3-Differentiated HL60 Cells

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A technique for screening immune-enhancing polysaccharides in food using the phagocytotic activity of 1,25-dihydroxyvitamin D3 (VD3)-differentiated HL60 cells is presented. HL60 cells, a human acute promyelocytic cell line, can differentiate along the monocytic lineage following exposure to VD3 or phorbol-12-myristate-13-acetate (PMA). For differentiated cells along the monocytic pathway, HL60 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 120 nM VD3 for more than 1 week. VD3-differentiated HL60 cells were seeded into 48-well plates, YG-labeled microspheres and polysaccharides were added and mixed using a plate shaker at 1100 rpm for 30 s, and then the mixture was incubated overnight at 37 °C in 5% $CO_2$. The cells were fixed with 2% formaldehyde and resuspended in phosphate-buffered saline. The rate of phagocytosis was measured with a flow cytometer. VD3-differentiated cells but not non- and PMA-differentiated cells resulted in an elevation of phagocytic activity by various immune-enhancing polysaccharides in foods.

**KEYWORDS: HL60; 1,25-dihydroxyvitamin D3; immune-enhancing polysaccharides; phagocytosis**

## INTRODUCTION

Modulators of the immune system in foods were screened (*1*). Food polysaccharides in mushrooms, algae, and plants activate macrophage immune responses and lead to immunomodulation, antitumor activity, wound healing, and other effects (*2*). Phagocytosis by macrophages is one of the most important nonspecific host-defense mechanisms taking place immediately or within several hours after exposure to antigen and is used as an important indicator of the activation of immune function (*3*); therefore, the phagocytosis assay is used to assess the potential of immunostimulants (*3*, *4*).

Primary cells and cell lines from humans or animals are used to evaluate the state of the immune system such as the secretion of cytokines and chemokines and phagocytotic activity (*5*−*7*); however, human primary cells are especially difficult to obtain and cannot be used. Primary cells are also unsuitable to assay many samples because it is difficult to obtain a large amount of cells from small animals such as mice and rats at a time, so THP-1, a human monocytic cell line, and J774, a mouse macrophage-like cell line, are frequently used for the assessment of immunostimulants (*3*, *5*). HL60 cells can differentiate along the monocytic lineage following exposure to phorbol-12-myristate-13-acetate (PMA) or 1,25-dihydroxyvitamin D3 (VD3) (*6*−*8*), and it is well-known that macrophage-like HL60 cells have phagocytotic activity. In VD3- or PMA-differentiated HL60 cells, however, the enhancement of phagocytotic activity by immunostimulants has never been reported.

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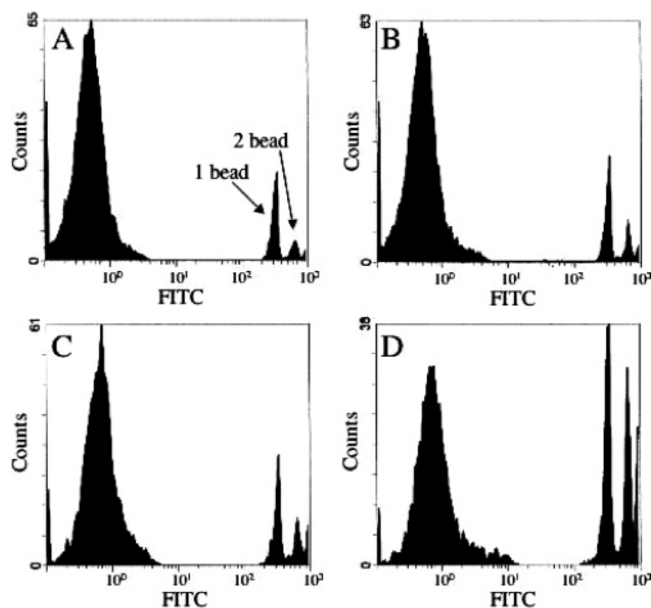


**Figure 1.** Flow cytometry histograms of fluorescent beads taken up by HL60 cells: (**A**) nondifferentiated cells; (**B**) nondifferentiated cells incubated with 1 $\mu$g/mL LPS; (**C**) VD3-differentiated cells; (**D**) VD3-differentiated cells incubated with 1 $\mu$g/mL LPS. Right peaks reflect the number of fluorescent beads taken up.

In this study, we report a method for screening immune-enhancing polysaccharides using the phagocytotic activity of VD3-differentiated HL60 cells.

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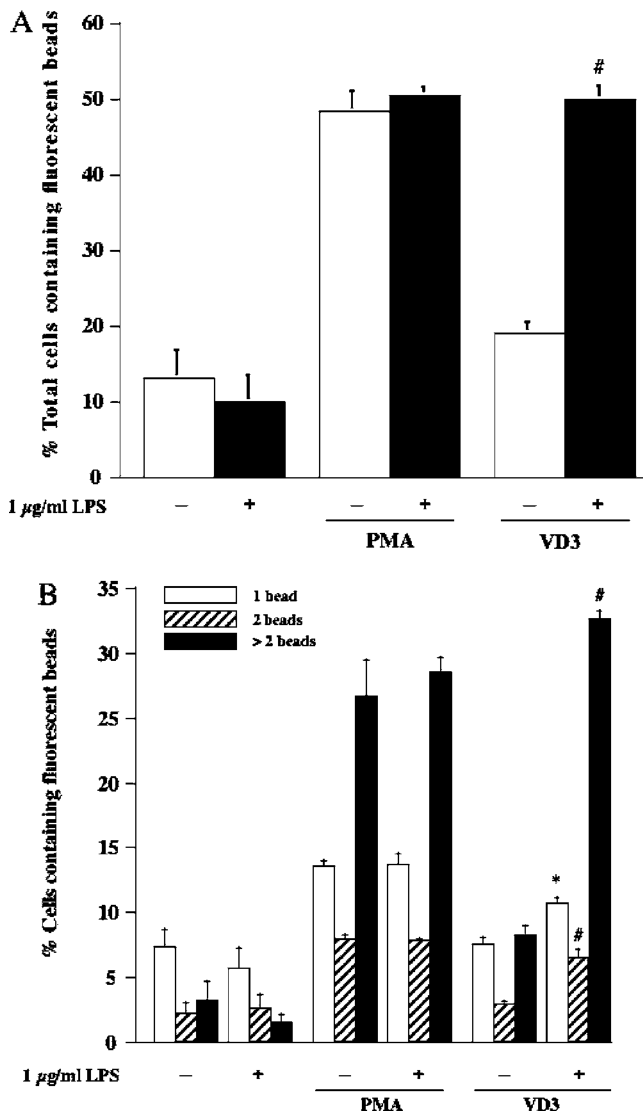


**Figure 2.** (**A**) Percentage of non-, VD3-, or PMA-differentiated HL60 cells containing beads. Non-, VD3-, or PMA-differentiated cells were incubated with beads in the presence or absence of LPS (1 $\mu$g/mL). Values are the means ± SD, $n = 3$. #, $p < 0.001$ versus the absence of LPS. (**B**) Percentage of cells containing one, two, or more beads per cell. Values are the means ± SD, $n = 3$. ∗, $p < 0.001$, and #, $p < 0.001$, versus the absence of LPS.

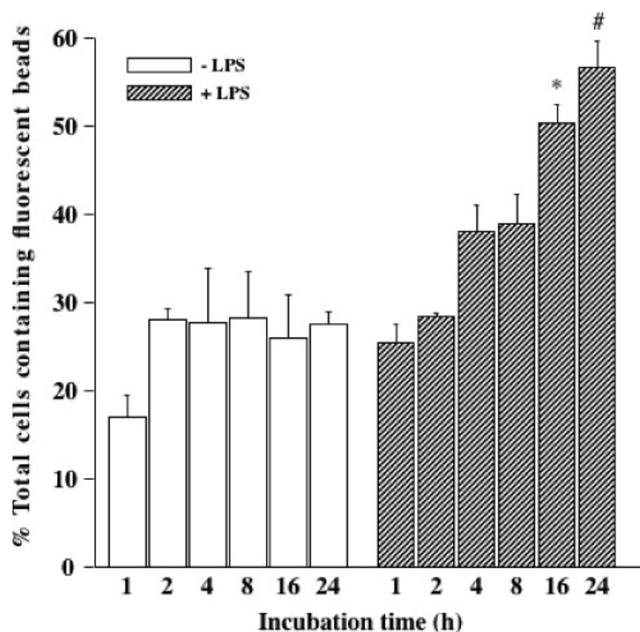


**Figure 3.** VD3-differentiated HL60 cells were incubated at various times with beads in the presence or absence of LPS (1 $\mu$g/mL). Values are the means ± SD, $n = 3$. ∗, $p < 0.01$, and #, $p < 0.001$, versus the absence of LPS.

## MATERIALS AND METHODS

**Cell Culture and Differentiation.** HL60 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Gibco-Invitrogen, Burlington, ON, Canada) supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies, Carlsbad, CA) in humidified 95% air/5% $CO_2$ at 37 °C. To differentiate cells along the monocytic pathway, the cells were seeded at densities of $(1-5) \times 10^5$ cells/mL in growth medium supplemented with 120 nM VD3 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and differentiation was allowed to proceed for more than 1 week. To differentiate cells along the macrophage pathway, the cells were seeded at a density of $1 \times 10^6$ cells/mL in growth medium supplemented with 1 $\mu$M PMA (Wako Pure Chemical Industries, Ltd.), and differentiation was allowed to proceed for 3 days (*6*).

**Phagocytosis Assay.** Differentiated HL60 cells ($2.5 \times 10^5$ cells/250 $\mu$L/well) were seeded into 48-well plates (Falcon 351178; BD Biosciences, Franklin Lakes, NJ). Cells were treated with 25 $\mu$L of lipopolysaccharide (LPS) (Calbiochem, Darmstadt, Germany), water-soluble $\beta$-glucan, curdlan, sodium alginate, and carrageenan (Wako Pure Chemical Industries, Ltd.); galactomannan, polygalacturonic acid, and amylopectin (Sigma, St. Louis, MO); chitosan 80 M (a gift from Katokichi Co., Ltd., Kagawa, Japan); galactan (Aldrich Chemical Co., Inc., Milwaukee, WI); or xylan (ICN Biochemicals Inc., Cleveland, OH); 25 $\mu$L of a 1% suspension of YG-labeled microspheres was added (458 nm excitation, 540 nm emission, Polysciences, Inc., Warrington, PA), mixed using plate shaker N-704 (Nissin Rika, Tokyo, Japan) at 1100 rpm for 30 s, and then incubated at 37 °C in 5% $CO_2$ for the indicated time. The cells were fixed with 2% formaldehyde and resuspended in phosphate-buffered saline (PBS). The rate of phagocytosis was measured with an EPICSXL-flow cytofluorometer (Beckman Coulter, Fullerton, CA).

**Cell Surface Marker.** The expression of cell surface antigen CD14 (Immunotech, a Beckman Coulter Company, Marseille, France) was determined during the course of differentiation by measuring the binding of phycoerythrin-conjugated antibodies. Differentiated HL60 cells at a density of $10^6$ cells/mL were incubated with antibody according to the manufacturer's instructions. Excess antibody was removed by washing the cells once with PBS. Specific antibody binding was measured in terms of total fluorescence of the cell population with an EPICSXL-flow cytofluorometer.

**Statistical Analysis.** All data were derived from at least three experiments. Data are expressed as the means ± standard deviation (SD). The efficiency of phagocytosis was compared using the paired *t* test. A value of $p < 0.05$ was considered to be significant. *n* represents the number of experiments.

## RESULTS AND DISCUSSION

**Differentiation and Phagocytic Activity.** The conventional procedure involves a microscopic examination of individual phagocytes. Several modifications have been introduced to facilitate counting, and rapid and simple phagocytotic assays have been developed using a fluorometer and flow cytometry (FCM) (*5*, *9*). FCM is an elegant procedure with which to evaluate phagocytotic activity because it offers multiparameter measurement of single cells (*9*, *10*); thus, in this study, the bead phagocytic assay using FCM was adopted.

The phagocytic activity of non-, VD3-, or PMA-differentiated HL60 cells was tested by incubating cells in the presence of latex beads. Cells that had ingested one bead could be clearly distinguished from cells that had ingested two or more beads

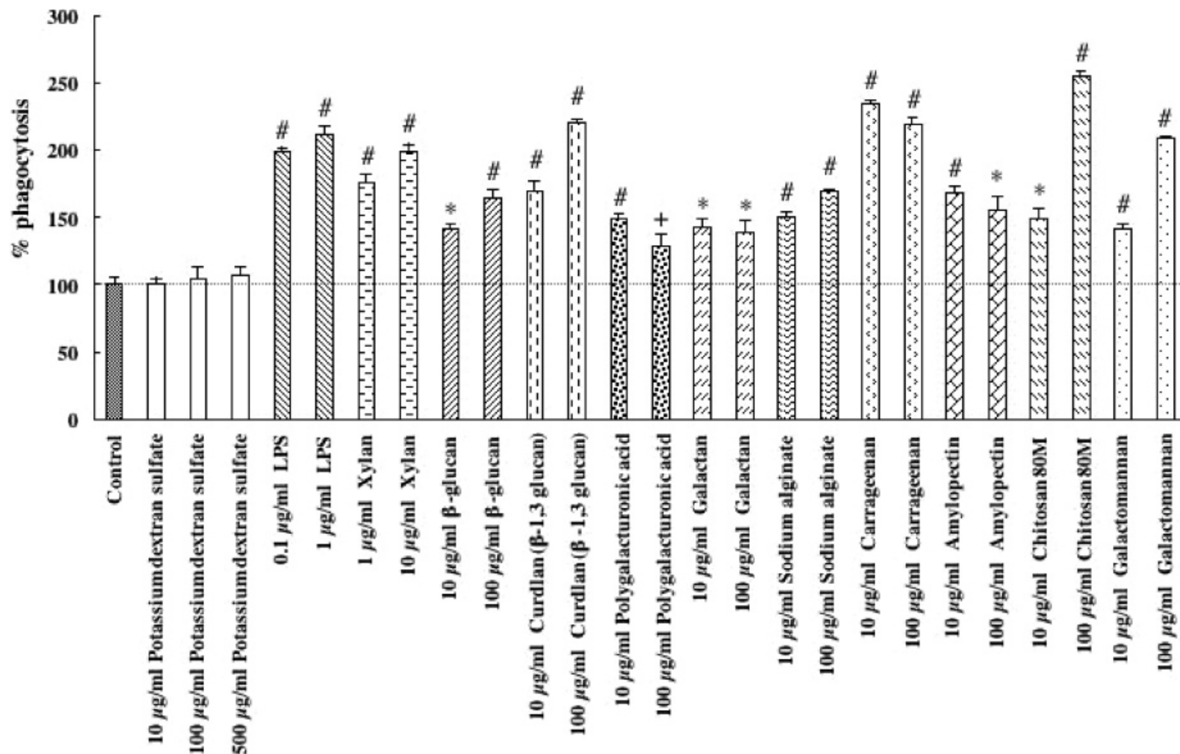


**Figure 4.** Phagocytic activities by various polysaccharides. VD3-differentiated cells were incubated with beads in the presence of polysaccharides. Phagocytosis activity in the absence of polysaccharides (control) is normalized to 100%. Values are the means ± SD, $n = 3$. +, $p < 0.05$, ∗, $p < 0.01$, and #, $p < 0.001$, versus the control.

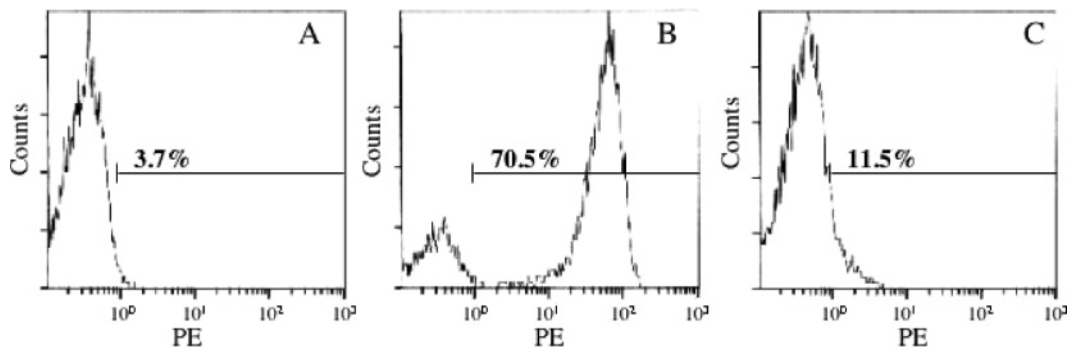


**Figure 5.** Cell surface expression of CD14 in non- (**A**), VD3- (**B**), or PMA-differentiated (**C**) HL60 cells. Histograms from one of three representative experiments are shown.

on the histogram (**Figure 1A**). VD3-differentiated cells but not nondifferentiated cells resulted in an elevation of phagocytic activity by 1 μg/mL LPS (**Figures 1** and **2**). The number of beads ingested per VD3-differentiated cell also increased by LPS stimulation (**Figure 2B**). In the absence of LPS, the phagocytic activity of beads in PMA-differentiated cells was higher than that in VD3-differentiated cells (**Figure 2**); however, PMA-differentiated cells did not result in an elevation of phagocytic activity by LPS stimulation (**Figure 2**). Thus, VD3-differentiated HL60 cells were judged to be useful for screening immune-enhancing polysaccharides.

The influence of incubation time on the phagocytic activity of VD3-differentiated HL60 cells was tested in the presence or absence of 1 μg/mL LPS. The phagocytic activity of VD3-differentiated HL60 cells without LPS stimulation was around 20−30% between 1 and 24 h of incubation (**Figure 3**). Phagocytic activity increased from 4 h after LPS stimulation, and significant difference was found 16 and 24 h after LPS stimulation (**Figure 3**). The estimation of phagocytic activity by LPS stimulation needed at least 16 h of incubation.

**Activity for Various Immune-Enhancing Polysaccharides.** The change of phagocytic activity in VD3-differentiated HL60 cells was also examined with various polysaccharides such as water-soluble β-glucan solution, curdlan suspension, potassium dextran sulfate solution, chitosan solution, galactomannan suspension, galactan solution, sodium alginate solution, polygalacturonic acid solution, amylopectin suspension, carrageenan solution, and xylan solution. Water-soluble β-glucan (*4*, *11*, *12*), curdlan (*13*, *14*), chitosan (*15*), galactomannan (*16*), galactan (*17*), sodium alginate (*18*), amylopectin (*19*), polygalacturonic acid (*20*), carrageenan (*21*), and xylan (*22*, *23*) have been reported as potential substances modulating the ability of the immune system such as the secretion of cytokines and phagocytosis reaction. VD3-differentiated HL60 cells showed an increase in the phagocytic activity of these water-soluble and insoluble polysaccharides, except for potassium dextran sulfate (**Figure 4**). Previous studies reported that dextran did not stimulate macrophages (*24*), and our results were in agreement with the results reported.

**Expression of Cell Surface Marker.** In this study, the phagocytic activity of beads in VD3- or PMA-differentiated cells was higher than that in nondifferentiated cells (**Figure 2**); however, PMA-differentiated cells did not result in a greater increase of phagocytic activity by LPS stimulation (**Figure 2**). VD3 and PMA resulted in different achievements of differentiation (*25*). In this study, VD3- but not PMA-differentiated HL60 cells increased the expression of CD14 antigen (**Figure 5**). These results are in agreement with the results of White et al. (*26*). CD14, a glycosylphosphatidylinositol-linked plasma-membrane glycoprotein (*27*, *28*), acts as a receptor that binds LPS, triggering inflammatory responses (*29*). In this study, various polysaccharides, not only LPS, up-regulated phagocytic activity in VD3-differentiated HL60 cells (**Figure 4**). Phagocytic receptors can be categorized into six different structural classes, including C-type lectin receptors (mannose receptor), integrins, Ig superfamily members (Fc$\gamma$R), leucine-rich repeat receptors (CD14), receptor tyrosine kinases (Mer), and scavenger receptors (*30*). Polygalacturonic acid and mannan are bound to CD14 (*31*). The major receptor for $\beta$-glucans is also the C-type-lectin-like receptor (Dectin-1) (*32*). The expression of phagocytic receptors might be associated with phagocytic sensitivity of immune-enhancing polysaccharides in VD3-differentiated HL60 cells. VD3-differentiated HL60 cells are judged to be useful for screening immune-enhancing polysaccharides that have at least the activity of the substances used in this study.

## ABBREVIATIONS USED

VD3, 1,25-dihydroxyvitamin D3; PMA, phorbol-12-myristate-13-acetate; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SD, standard deviation; FCM, flow cytometry.

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