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Original Article

Jackfruit Lectin: Properties of Mitogenicity and the Inhibition of Herpesvirus Infection

Nuanchawee Wetprasit*, Weena Threesangsri[1], Natapong Klamklai[1] and Montri Chulavatnatol[2]

Department of Biotechnology, Faculty of Science, Ramkhamhaeng University, Bangkapi, Bangkok 10240,
[1]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000 and
[2]Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

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SUMMARY: Jackfruit lectin (JFL) from *Artocarpus heterophyllus* has been found to exhibit inhibitory activity in vitro with a cytopathic effect towards herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), and cytomegalovirus (CMV). The 50% inhibitory dose values from plaque reduction assay (inactivation) were 2.5, 5, and 10 μg/ml of JFL for HSV-2, VZV, and CMV, respectively. Lymphocyte proliferation was significantly increased in the presence of the JFL in the concentration range of 2.5 to 50 μg/ml, but was reduced at 500 μg/ml. It was found that $CD16^+/CD56^+$ cells (natural killer cells) were induced among the primary lymphocyte subpopulations. The activity of natural killer (NK) cells was not affected by JFL in the concentration range of 5 to 500 μg/ml. These data suggest that JFL is mitogenic for NK lymphocyte ($CD16^+/CD56^+$) and also active against HSV-2, VZV, and CMV.

INTRODUCTION

Lectins are carbohydrate-binding proteins of non-immune origin that are often derived from plant seeds. They are well known to have biological activity in relation to lymphocyte proliferation (1,2), the inhibition of virus infection (3,4), and bacterial agglutination (5,6). The binding of lectins to cell or viral glycoproteins and glycolipids depends on their sugar specificities (7). Lectins have previously been demonstrated to reduce viral infectivity in experiments using cell monolayers. For example, concanavalin A (Con A) and wheat germ agglutinatinin (WGA) inactivate and reduce rubella virus multiplication (3,8). Viruses that contain glycoproteins in their envelopes would be expected to interact with lectins since lectins have a specific affinity for their carbohydrates. The inhibition of virus infection after treatment with lectins of Con A (9,10), *Limulus polyphemus* (LPA) (8), WGA (8), *Bauhinia purpurea* (BPA), and *Eranthis hyemalis* agglutinins (11) has been reported for herpes simplex virus (HSV). In addition, the replication of rabies virus is successfully inhibited by WGA and LPA (12).

Mitogenic stimulation is an effect caused by the interaction of lectins with cells. It plays a role in signal transduction at the surface of lymphocytes for growth and proliferation. Many lectins have been recognized to be mitogenic. Jacalin from *Artocarpus integrifolia* is also mitogenic to $CD4^+$ T cells (13,14) and blocks in vitro human immunodeficiency virus type 1 (HIV-1) infection to a T-lymphocyte cell line (15). Recently, jackfruit lectin (JFL), derived from the seeds of *A. heterophyllus*, which is a tropical tree of the family Moraceae found in Thailand, has been found to be similar to jacalin in being specific for *N*-acetylgalactosamine (16). This lectin is a tetramer of *Mr* 62,000 and consists of two subunits with *Mr* of 18,000 and two subunits with *Mr* of 13,000, whereas jacalin is an $\alpha 2\beta 2$ tetramer of *Mr* 39,000-54,000 (14,17-19). JFL can agglutinate human, rat, mice, sheep, and goat erythrocytes as well as human and rat sperm (16,20). Furthermore, it has also been found to agglutinate several glycoproteins in biological fluids and can be used to agglutinate several serovars of *Bacillus thuringiensis*, serovar H4 (sotto), and *Salmonella typhi*, except for *S. paratyphi*, *Escherichia coli*, *B. sphaericus*, and *Saccharomyces cerevisiae*. Although JFL is similar to jacalin, it does differ in some of its biological properties. To further establish the biological properties of JFL, the in vitro inhibitory effects of JFL towards HSV-2, varicella-zoster virus (VZV), and cytomegalovirus (CMV) were studied. Moreover, its actions on lymphocyte proliferation and natural killer (NK) activity were also investigated.

MATERIALS AND METHODS

Cells: BHK-21 (baby hamster kidney) and MRC-5 (human embryonic lung) cells were cultured at 37°C in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo) containing 1.2 g/l $NaHCO_3$ and supplemented with 100 U/ml penicillin, 50 μg/ml streptomycin, and 10% or 4% (vol/vol) fetal bovine sera (FBS) (Biowhittaker, Walkersville, Md., USA) for growth medium and maintenance medium, respectively.

Virus: The HSV-2 (LB-strain) and CMV (AD-169 strain) were propagated in BHK-21 and MRC-5 cells, respectively. Virus stocks were then obtained by freezing and thawing at -70°C and 37°C to break the cells, followed by centrifugation at 1,200 g for 10 min. The supernatant was collected as virus stock seeds and kept frozen at -70°C until use.

The VZV (Kawaguchi strain) was adsorbed to MRC-5 cells in a 75-cm^2 tissue-culture flask at 37°C for 60 min. The maintenance medium was added, and the culture was incubated

*Corresponding author: Tel/Fax: +66-2-319-4385, E-mail: nuanwee@yahoo.com

until an 80% cytopathic effect (CPE) was observed. Each infected culture was mixed with an uninfected one in a ratio of 1:4 (vol/vol) according to a trypsinization method to prepare cell-free virus, as described previously (21).

Purification of JFL: JFL was purified from the seeds of *A. heterophyllus* according to a previous study (16). Seeds were chopped and homogenized for 3 min in 3 volumes of 5 mM phosphate-buffered saline (PBS), pH 7.4, using a Waring Blender. The following steps were performed at 4°C. After the homogenization, the mixture was stirred for 2 h and then centrifuged at 3,000 × g for 15 min to remove insoluble debris. The supernatant was then collected, and ammonium sulfate was added to 60% saturation. The precipitate was collected by centrifugation at 27,000 × g for 15 min, resuspended in PBS, and dialyzed for 24 h with repeated changes of buffer. The dialyzed fraction was applied to a column (1.8 × 6.5 cm) of *N*-acetylgalactosamine-agarose (Sigma Chemical Co., St. Louis, Mo., USA) that was pre-equilibrated with PBS. After washing off the unbound material, the column was eluted with 0.03 M galactose in PBS, and the absorbance of the collected fractions was monitored at 280 nm. The galactose-eluted fractions exhibiting hemagglutination activity (using 2 [vol/vol] sheep erythrocyte suspension) were pooled and concentrated for use as JFL in this study.

Antiviral activity assay: JFL was studied for its antiviral activity by following plaque reduction under three conditions.

(1) Pre-treatment condition: BHK-21 and MRC-5 cells were pre-incubated at 37°C for 2 h with various concentrations of JFL in the maintenance medium. After the pre-treatment, the monolayer cells were washed two times with the medium and then inoculated at 37°C for 60 min with 100 plaque-forming units (PFU) of virus. Any unadsorbed virus was discarded, and the cells were washed with the medium. The monolayers were then incubated in the medium until 80% CPE was observed in the control containing no JFL. The HSV-2 CPE was measured by the neutral red assay, whereas that of VZV or CMV was measured by staining with methylene blue.

(2) Post-treatment condition: BHK-21 or MRC-5 cells were adsorbed with 100 PFU of virus at 37°C for 60 min; the unadsorbed virus was then discarded, and the cells were washed with the medium. After washing, the monolayer cells were incubated with various concentrations of JFL in the medium. The infected cell cultures were incubated until an 80% CPE was observed in the control without any treatment.

(3) Inactivation condition: 100 PFU of virus was mixed with the medium containing various concentrations of JFL and incubated at 37°C for 30 min. After incubation, 0.2 ml was inoculated into monolayer cells. The inoculum was allowed to be adsorbed to monolayer cells for 60 min at 37°C. The unadsorbed virus was then discarded, and the cells were washed with the medium. The cells were incubated with the medium until 80% CPE was observed in the control cell culture (without JFL).

In order to examine the effects of sugars on the JFL inhibition of virus infectivity, the galactose, *N*-acetylgalactosamine or glucose (Sigma), was mixed with JFL (40 μg/ml final concentration) and each virus (100 PFU). After incubation for 30 min at 37°C, the mixture was inoculated into a monolayer cell culture and processed as above.

Blood samples: Blood samples were collected from healthy Thai adults who had no history of hepatitis B infection or exposure to HIV-1, antimalarials, corticosteroids, or immunosuppressive drugs.

Preparation of peripheral blood mononuclear cells (PBMC): Mononuclear cells were separated from the heparinized blood by density gradient centrifugation using Ficoll-Hypaque (Isoprep, Robbins Scientific Corp., Sunnyvale, Calif., USA) (22). The collected mononuclear cells were washed three times with RPMI 1640 (Flow Laboratories, McLean, Va., USA) by centrifugation at 400 × g for 10 min at 4°C. The mononuclear cells were counted and adjusted to an appropriate concentration in complete RPMI 1640 (RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin G, and 100 μg/ml streptomycin containing 10% FBS).

Lymphocyte proliferation response assay: 100 μl of prepared mononuclear cells (2×10^6 cells/ml) were cultured in triplicate in 96-well microtiter plates (Costar, Cambridge, Mass., USA) with 100 μl of complete RPMI 1640 containing different concentrations of JFL or phytohemagglutinin (PHA) HA 16/17 (Murex Diagnostics Ltd., Dartford, England) at 1 μg/ml (final concentration). The cultures were incubated at 37°C with 5% CO_2 for 72 h. Eighteen hours before harvest, 20 μl of 0.5 μCi ^{3}H-thymidine (specific activity, 8.3 mCi/mg: Amersham, Buckinghamshire, UK) was added. After incubation, cells were washed with a multichannel automatic cell harvester (Dynatech Lab., Inc., Sussex, UK) onto glass-fiber filters (Whatman International Ltd., Maidstone, England). The ^{3}H-thymidine incorporation was measured by a liquid scintillation counter (Wallac Oy, Turka, Finland). The degree of activation was expressed as a stimulation index (S.I.), i.e., the ratio of ^{3}H-thymidine uptake in counts per minute (cpm) of samples with to those without lectin.

Flow cytometry analysis: The prepared mononuclear cells (1×10^7 cells/ml) were cultured with JFL or PHA in complete RPMI 1640 containing 10% FBS. After a 24-h incubation, cell pellets were harvested and washed with RPMI 1640 by centrifugation at 400 × g for 5 min at room temperature. Cells were stained using fluorescein-conjugated CD3, CD3/CD4, CD3/CD8, CD3/CD16+56, or CD3/CD19 mouse monoclonal antibodies (Becton Dickinson Immunocytometry System, San Jose, Calif., USA). After staining, the cells were fixed in PBS containing 3% formaldehyde, and the fluorescence intensity was measured by flow cytometry using a FACScan (Becton Dickinson).

Assay of NK activity: The PBMC (2×10^6 cells/ml) were washed and resuspended in complete RPMI 1640 and incubated with or without JFL or PHA at 37°C with 5% CO_2 for 18 - 24 h. K562 cells, kindly provided by Dr. Busarawan Srivanna (National Institute of Health, Department of Medical Sciences, Thailand), were used as target cells and were grown in complete RPMI 1640 containing 10% FBS. The target cells (1.5×10^6 cells) were labelled with $Na_2{}^{51}CrO_4$ (specific activity, 37.0 MBq/mg, Amersham) at 37°C, 5% CO_2 for 45 min and washed three times with RPMI 1640 containing 10% FBS.

The cytotoxicity assay was performed in U-shaped microtiter plates (Corning Inc., Corning, N.Y., USA) using 1×10^4 target cells/well and PBMC in varying ratios. After adding the target cells, the mixture was incubated for 4 h at 37°C with 5% CO_2. The plates were then centrifuged at 200 × g for 5 min. The supernatant fraction from each well (100 μl) was transferred into a tube, and the radioactivity was counted in a Gamma counter (Packard Instrumental Co., Meriden, Conn., USA). The percentage of cytolysis was calculated according to the formula: %cytolysis = (experimental release − spontaneous release) / (maximal release − spontaneous

release). Spontaneous release was measured after incubation of the target cells with the medium alone, while maximal release was measured after lysis of the target cells with 5% Triton x-100. NK cell activity was expressed as lytic units (LU)/10^7 PBMC as determined by least squares analyses of the percentages at different cell ratios. One LU was defined as the number of NK cells required for 20% specific lysis of 1×10^4 target cells.

Statistical analysis: Data were expressed as the mean ± the correspondent standard deviation (SD), and statistical analysis was carried out by employing the Student's *t*-test.

RESULTS

Antiviral activity of JFL: To determine the antiviral activity of JFL, three experimental conditions (pre-treatment, post-treatment, and inactivation) were used in the plaque-reduction assay. JFL was found to inhibit the CMV-induced plaque reduction under all three conditions. The plaque reduction by HSV-2 was not affected by pre-treatment with JFL, and the plaque formation induced by VZV was only affected in the inactivation assay with the 50% inhibitory dose of JFL 5 μg/ml (Table 1 and Figure). The inhibitory effect of JFL on the infectivity of the virus was diminished by the addition of 0.2 M galactose or *N*-acetylgalactosamine but not glucose. Each sugar without JFL showed no effect on the virus-induced plaque formation (Table 2).

Lymphocyte proliferation response and subpopulations: The mitogenic potential of JFL was investigated by using PBMC. PBMC were exposed to different concentrations of JFL before the proliferative response was measured by a DNA synthesis assay. The results are shown as S.I. in Table 3. It was found that JFL induced proliferative responses. The induction was small in the presence of 0.5 ng/ml to 0.5 μg/ml of JFL, but was significant in the range of 2.5 to 50 μg/ml of JFL ($P < 0.05$). However, at 500 μg/ml of JFL, an inhibition was observed. In contrast, PHA at 1 μg/ml stimulated PBMC proliferation by approximately 300-fold. In order to define the subpopulation among the lymphocytes

Table 1. Inhibitory dose of JFL in plaque formation in the presence of a virus

Treatment	50% inhibitory dose of JFL (μg/ml)[1]		
	HSV-2	VZV	CMV
Pre-treatment	NA[2]	NA	15
Post-treatment	35	NA	35
Inactivation	2.5	5	10

[1]Minimal non-cytotoxic concentration of JFL = 125 μg/ml
[2]NA: No activity
$n = 3$

Table 2. Inhibitory effect of JFL on the infectivity (plaque formation) of HSV-2, VZV, and CMV by inactivation assay

Final concentration of JFL (μg/ml)	HSV-2	VZV	CMV
	No. of plaques	No. of plaques	No. of plaques
0	150	100	120
40	0	0	0
40+Gal[1]	140	87	100
40+GalNAc[1]	148	95	112
40+Glu[1]	0	0	0
0+Gal	147	97	110
0+GalNAc	155	110	105
0+Glu	145	105	113

[1]Final concentration = 0.2 M
$n = 3$

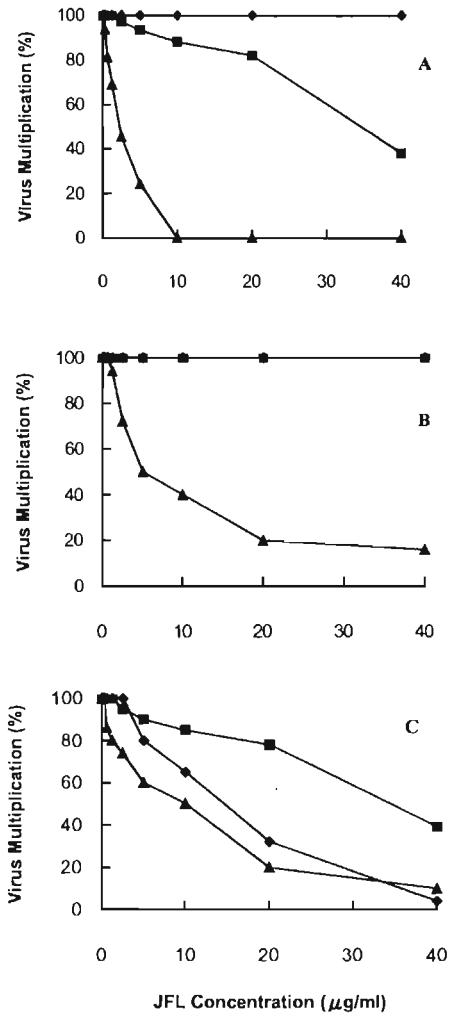

Figure. Dose-response curves of HSV-2 (A), VZV (B), and CMV (C) multiplications in various concentrations of JFL by plaque reduction assay after pre-treatment (◆), post-treatment (■), and inactivation (▲) assay.

Table 3. Effect of JFL on the proliferation of peripheral blood mononuclear cells (PBMC)

JFL	^{3}H-thymidine incorporation (cpm)[1]	Stimulation index (S.I.)[1]
None	1245±184	1
0.5 ng/ml	1415±192	1.21±0.16
5 ng/ml	1659±180	1.43±0.50
50 ng/ml	1710±160	1.46±0.57
0.5 μg/ml	1853±136	1.57±0.66
2.5 μg/ml	3398±257[2]	3.23±1.37[2]
5 μg/ml	4387±293[2]	4.66±2.31[2]
50 μg/ml	67249±3393[2]	72.99±39.07[2]
500 μg/ml	291±64[2]	0.33±0.18[2]
PHA (1 μg/ml)	238227±13863[2]	300.5±207.89[2]

[1]Each value represents mean ± SD
[2]$P < 0.05$
$n = 10$

Table 4. Effect of JFL on the percentage of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+/CD56^+$ and $CD19^+$ subpopulation cells in normal human donors

JFL	$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+/CD56^+$	$CD19^+$
None	59.4±10.44[1]	31.6±4.92	24±6.78	26.2±10.19	12.6±5.75
0.5 μg/ml	57.2±9.33	30.2±4.87	23.2±3.66	31±9.08	13.8±6.43
5 μg/ml	53±6.84	26.2±4.26	21.2±2.71	32.6±10.21[2]	18.8±6.62
50 μg/ml	51.8±5.84	22.6±5.92	21±7.01	34.6±9.83[2]	14.8±5.31

[1]Each value represents mean ± SD
[2]$P < 0.05$
$n = 5$

Table 5. Effect of PHA on the percentage of circulating $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+/CD56^+$ and $CD19^+$ subpopulation cells in normal human donors

PHA	$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+/CD56^+$	$CD19^+$
None	60.8±3.19[1]	37.8±6.72	23.2±4.35	23.8±1.94	11.4±1.74
0.2 μg/ml	58±4.05	38±6.23	22±4.56	29.8±1.47[2]	11±2.10
1 μg/ml	57.8±2.23	37.2±4.71	22.6±3.98	31.4±3.01[2]	11.2±2.56

[1]Each value represents mean ± SD
[2]$P < 0.05$
$n = 5$

Table 6. Activity of cells with natural killer (NK) function in lytic units (LU)/10^7 PBMC in the presence of JFL extract

Lectin concentrations		LU/10^7 PBMC
	None	90.56±22.94[1]
JFL	5 μg/ml	74.33±8.12
	50 μg/ml	85.71±8.82
	500 μg/ml	84.09±12.51
PHA	0.2 μg/ml	102.59±30.92
	1 μg/ml	277.13±81.45[2]

[1]Each value represents mean ± SD
[2]$P < 0.05$
$n = 5$

stimulated by JFL, an analysis of lymphocyte subsets using a flow cytometer was carried out. A major significant increase in the percentage of $CD16^+/CD56^+$ was observed at 5 and 50 μg/ml of JFL ($P < 0.05$) (Table 4), and similar significant increase was induced by the mitogenic PHA (Table 5).

NK activity: NK activity in the presence of JFL was assessed and compared with that in the presence of PHA. A significant induction of NK activity as measured in LU was demonstrated through the use of PHA-treated PBMC. In contrast, no increase in NK activity was detected using JFL-treated PBMC (Table 6).

DISCUSSION

Several plant lectins have been shown to inhibit the infectivity of viruses. For example, jacalin has been found to inhibit in vitro infection of HIV-1 without preventing the virus from binding to the host cell (15,23). In this study, the in vitro antiviral activity of JFL was demonstrated for the first time. Previously, many lectins with different specificities have been proven to inhibit viral infection. In the case of PHA, the treatment of CMV prior to the infection of cell monolayers reduced the viral infectivity by 90% and a 99% reduction in infectivity was obtained by pretreatment of cells with PHA (24). The inactivation of HSV-1 adsorption has been observed in response to Con A, succinyl-Con A, WGA, and helix pomatia agglutinin, whereas BPA and soybean agglutinin have been found to be effective against viral replication after treatment (8,25,26). The antiviral activity of JFL in response to HSV-2 and CMV either before or after viral infection of cell monolayers was observed at different doses (Table 1); this result differs markedly from the lack of effect reported for collectin, mannan-binding protein, and bovine conglutinin on HSV-2 (27). These results suggest that JFL may act either on the surface of host cells or directly on the viral envelope, thereby inhibiting viral infectivity.

This is the first report of the effects of JFL on lymphocyte proliferation. The degree of lymphocyte reactivity, S.I., which was higher than the control (Table 3), suggests that JFL can stimulate the PBMC, i.e., that JFL is a mitogen. However, at high concentrations JFL was found to decrease lymphocyte proliferation responsiveness, suggesting that JFL preparations are toxic to cells at high concentrations (500 μg/ml) due to the stimulation of PBMC. Lymphocyte proliferation under lectin stimulation has been found to produce optimal levels of mitogenic activity (28). According to our study of JFL-stimulated lymphocyte subsets, there appears to be a change in the numbers of $CD16^+/CD56^+$ lymphocyte subpopulations which were NK cells (Table 4). This finding differs from that of in vitro studies showing that jacalin stimulates the $CD4^+$ lymphocyte (14,28) and B-cell proliferation at the high concentration of 2 mg/ml (28). Other lectins purified from seeds of different *Artocarpus* species in Vietnam (*A. asperulus*, *A. heterophyllus*, *A. musticata*, *A. melinoxylus*, *A. parva*, and *A. petelotii*) have also been reported to stimulate human PBMC proliferation with variable mitogenic efficiencies, but they all stimulate only the $CD4^+$ T lymphocyte (29). Furthermore, Con A, poke weed mitogen (PWM), and PHA have been found to be mitogens that can induce B cells to proliferate in the presence of 1 to 5% of T cells (30). These lectins activate B cells to proliferate indirectly, as they induce T cells to produce mitogenic factors acting on B cells (31,32). In the present study, PHA was found to induce a significant increase in the function of NK cells, whereas JFL did not alter the NK cell activity (Table 6). The observed inhibitory effect of PHA on the cytotoxicity of NK cells may be caused by the production of some cytokine such as interleukin-4 (IL-4) (33). However, JFL is different from PHA

in this respect.

The present study has demonstrated that JFL is a new mitogen that is mitogenic toward $CD16^+/CD56^+$ cells. Hence, JFL can be used instead of other well-known mitogens such as Con A, PHA, and PWM to specifically activate lymphocyte proliferation. However, the results obtained in this study preliminarily show JFL to have the characteristic of in vitro immunomodulating activity. Further studies should be performed to determine whether JFL exhibits any other immunostimulatory activity.

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