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Comparison of Lectin from *Pseudixus japonicus* and Concanavalin A on Lymphocytes Proliferation and Cytotoxicity

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Abstract □ *Pseudixus japonicus* agglutinin (PJA) was isolated. And its characteristics were compared with those of concanavalin A (Con A). PJA is a glycoprotein composed of 49.3% carbohydrate and 50.7% protein which had relatively high percentages of glutamic acid, aspartic acid and phenylalanine residues. The hemagglutinating activity of PJA was approximately one-eighth of that of Con A when tested with mouse erythrocytes. PJA failed to stimulate the proliferation or transformation of human and mouse lymphocytes in contrast to Con A. PJA and Con A showed cytotoxicities against SNU-1 (human stomach cancer cells), SNU-C1 (human colon cancer cells) and mouse Sarcoma 180 cells when tested by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The antitumor activity of the lectin *in vivo* was also tested in Sarcoma 180 bearing mice. There was no significant difference in prolongation of life span of the mice after the treatment with PJA and Con A for 10 consecutive days.

keywords □ Lectin, concanavalin A, mitogenic activity, MTT assay, human stomach cancer, human colon cancer, mouse sarcoma 180.

Lectins derived from plants and all other categories of living things are divalent or multivalent carbohydrate-binding proteins called glycoproteins of non-immune origin. A wide range of specificities of lectins has been observed in the interaction with cells[1-3]. Lectins show selectivity in their agglutination of erythrocytes for different species and even human blood type specific. This ability to distinguish different human blood groups provided the basis for the name "lectins", coined by Boyd and Sharpleigh[4] from the Latin "legere"-to select or choose. Moreover, lectins can distinguish between lymphocyte subpopulations and identify the nature of cell surface glycoproteins with great sensitivity and selectivity.

One of the most dramatic effects of the interaction of lectins with cells is the triggering of quiescent lymphocytes into a state of growth and proliferation. Since it was known that phytohemagglutinin (PHA, the lectin from *Phaseolus vulgaris*, red kidney been) can stimulate lymphocytes to grow and divide[5], the number of mitogenic lectins reported has increased markedly. Many of them have been obtained in purified form and their various properties were clarified[6]. Different biological effects of lectins on subpopulations of lymphocytes were reported[7]. Both Con A and PHA can cause proliferation of significant fractions of T cell subpopulations in the presence of accessory cells. Pokeweed mitogen (PWM), although generally regarded as a T cell-dependent B cell mitogen, can activate T cells. Wheat germ agglutinin (WGA), is generally regarded as a non-mitogenic lectin for lymphocytes, but it can induce proliferation of both T and B cells under certain conditions[8].

However, several lectins are highly toxic to eukaryotic cells and WGA and PHA were reported to be at least, 1,000 times less toxic than ricin and abrin which have been known to be much more toxic than the lectins[9,10].

It is handed down traditonally that *Pseudixus japonicus*, one of parasitic plants, is used as an anti-

cancer drug. Some of its characteristics, specificities of hemagglutinating activities on species and blood types and specific binding carbohydrates were previously reported[11].

On the basis of these observations, the present report is concerned with the effects of PJA on lymphocytes proliferation and cytotoxicity. It was intended to clarify PJA by comparing with those of ConA, one of well known lectins.

EXPERIMENTAL METHODS

Materials

Con A which was purchased from Sigma Co., U.S.A. was diluted to 10 mg/m*l* in distilled water and further diluted, if necessary, in culture medium. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] from Sigma Co., U.S.A. was stored in 0-4C, protected from light and diluted in distilled water before use. 5-FU (5-Fluorouracil) was from Pfizer Pharm. Co., U.S.A. Plant materials (*Pseudixus japonicus*) and chemicals were from commercial sources.

Isolation & Purification of Lectin from Pseudixus japonicus

Crude lectin was isolated from *Pseudixus japonicus* and further purified through the ion exchange chromatography and gel filtration (Scheme 1)[11]. The fractions showing absorbance at 280 nm and hemagglutinating activity were pooled and dialized at 4℃ and lyophilized. The dry matter was considered as *Pseudixus japonicus* agglutinin, abbreviated to PJA, and used for experiments.

Chemical analysis

The carbohydrate composition of PJA was determined by gas chromatography using Shimadzu GC-9AM according to the method of Yoshida and Mega[12]. The amino acid composition of PJA was determined with Hitachi L-8500 high speed amino acid autoanalyzer after hydrolysis in 6N HCl at 110 ℃ for 22 hr in a sealed tube[13].

Comparison of hemagglutinating activity

Each 50 μ*l* of two-fold serial dilution of PJA (1 mg/m*l*) and Con A (1 mg/m*l*) were separately placed on microplate and mixed with equal volume of 3% (v/v) suspension of mouse erythrocytes that had been washed several times with PBS. After gentle shaking, the suspension was permitted to stand for 1 hr at room temperature. Hemagglutination titer was represented as the lowest concentration of the lectin solution at which good agglutination of erythrocytes was observed.

Preparation of lymphocytes

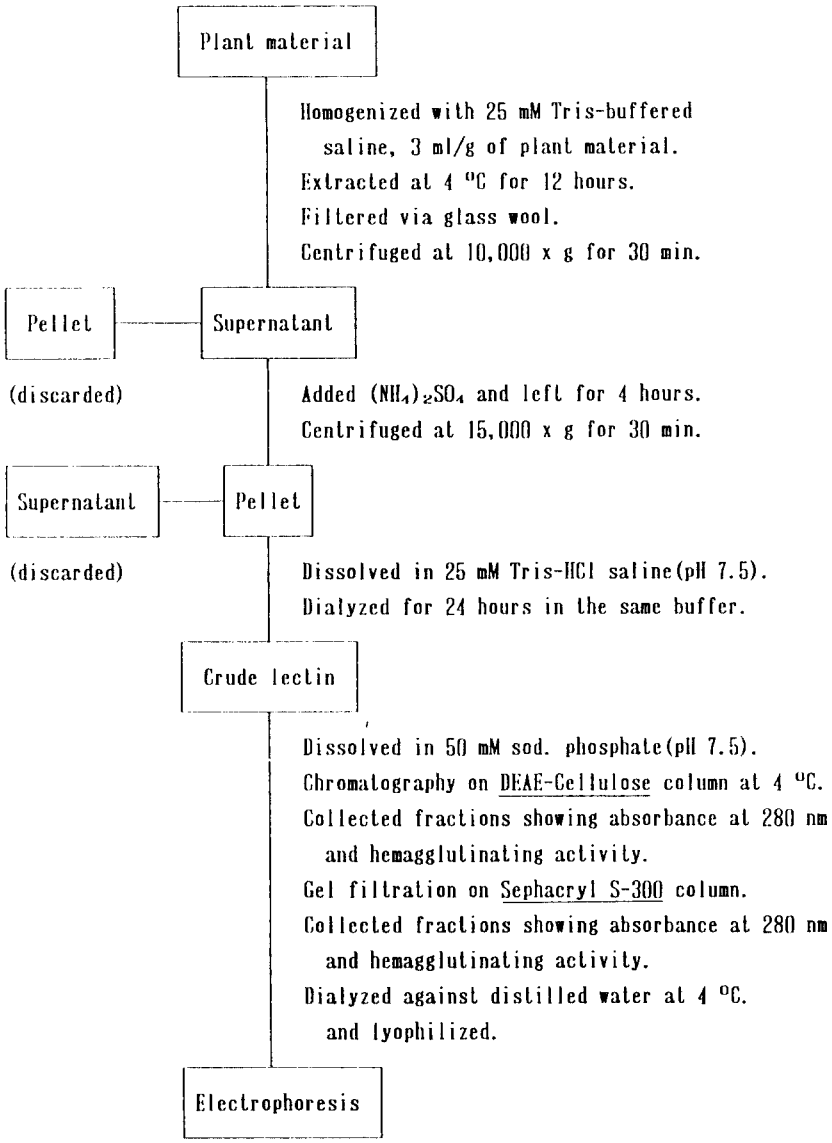
Human lymphocytes: Human lymphocytes were prepared from Isopaque-Ficoll (IF) density gradient centrifugation method which had been established by Boyum[14]. Heparinized blood, 10 m*l* was centrifuged for 10 min at 600×g. The leukocytes layer resting on the top of erythrocytes pellet was removed (1 m*l*) and mixed with 1 m*l* of the colloidal iron suspension.

After the mixture was incubated in a shaking bath at 37℃ for 30 min, the cell suspension was mixed with equal volume of PBS and 4-6 m*l* was layered on the top of IF solution, 3 m*l* and centrifuged. An almost pure suspension of lymphocytes was obtained from the interface.

Mouse lymphocytes: The spleens of ICR mice were removed and put in a Petri dish containing a few m*l* of Dulbecco's medium (phosphate-buffered saline containing 2% bovine serum). The spleens were cut into fragments of 2-3 mm with sharp scissors and gently disrupted[15]. The large aggregated cells could be partly dissociated by repeatedly sucking up and down into a Pasteur pipette. Finally, to remove the cell debris and lumps which cannot be resuspended, the suspension was filtered through a very loose pledget of absorbent cotton and the absorbent was washed through with a few m*l* of fresh medium. Erythrocytes, contaminants in the splenocytes suspension, were removed by 0.83% (w/v) ammonium chloride treatment. Most of macrophages and granulocytes were also removed by drawing the cell suspension into a syringe which had been loosely prepared with glass wool. Dye exclusion tests (Trypan blue, 0.5%) were routinely performed on cell suspension. Prepared splenocytes suspensions contained approximately 90% viable cells.

Cell culture

The prepared lymphocytes were cultured in RPMI 1640 (Sigma Co.) medium supplemented with heat-inactivated fetal bovine serum (FBS, 10%), $NaHCO_3$



Scheme 1. Flow sheet for purification of lectin from *Pseudixux japonicus*.

(0.075%) and antibiotics (100 units/ml of penicillin G and 100 μg/ml of streptomycin), which is abbreviated to R10 medium, in a humidified incubator maintained at 95% air: 5% CO_2 at 37℃. SNU-1 (human stomach cancer cells) and SNU-C1 (human colon cancer cells) were donated by Dr. J.G. Park, Seoul National University Hospital, Dept. of General Surgery. They are routinely grown in suspension culture and maintained in the medium described above for lymphocytes. Sarcoma 180 cells (mouse sarcoma cells) were donated by Dr. Y.Y. Lee, Yonsei University, College of Medicine, Dept. of Microbiology. It is grown in attached monolayer and maintained in the medium described above.

For the cytotoxic studies, cells used in all experiments were in the exponential phase of growth and maintained at 37℃ in R10 medium during the experiments.

MTT assay

As an *in vitro* system for the screening of potency on cytotoxicity of lectins, MTT colorimetric assay

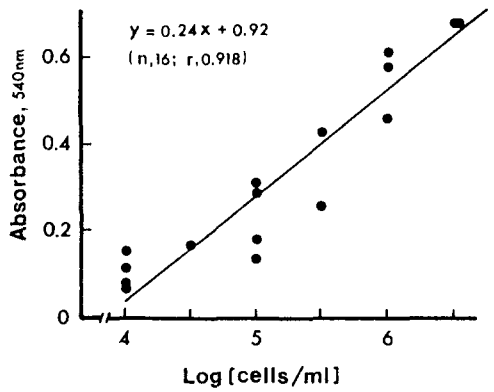


Fig. 1. Correlation between absorbance and the number of cells (SNU-C1).

was used. This is dependent on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically[16,17]. As shown in Fig. 1, there is a good correlation between absorbance by MTT assay and number of living cells (SNU-C1, $10^4-3\times10^6$ cells/ml). Single cell suspensions of floating SNU-1 or SNU-C1 were obtained by pipet disaggregation Sarcoma 180 were obtained by trypsinization. The statistically counted equal number of cells were inoculated into 96 wells in 0.19 ml of R10 medium, to which 0.01 ml of 20 ×concentration of lectin, 5-FU or PBS was added. After incubation in CO_2 incubator for programmed period, 50 μl of 2 mg/ml of MTT was added to each well and incubated at 37℃ for further 4 hr. Plates were centrifuged at 450×g for 5 min in a plate holder and then the medium was aspirated from plates. To each well 150 μl of dimethyl sulfoxide was added. The plates were placed on a shaker for 10 min to solubilize the formazan crystals and then were immediately at 540 nm on a scanning multiwell spectrophotometer (enzyme-linked immunosorbent assay reader, Biotek Instrument Inc., Burlington, VT). All data obtained were the mean of minimum of 3 wells. Percentage of control absorbance was considered to be the survivors. For the proliferation of lymphocytes, MTT assay had been intended to measure the change of the lymphocytes number following administration of lectins. However, it was almost impossible to detect the precise alteration in absorbance because minor change showed no significant difference.

Table I. Carbohydrate composition of PJA

Carbohydrate	w/w %
Mannose	38.41
Xylose	18.89
Glucose	12.25
Galactose	8.63
Fructose	8.45
Hexoamine	4.67
Unidentified	8.70

In vivo antitumor activity

In order to test *in vivo* antitumor activity of PJA and Con A, male ICR mice were used. Sarcoma 180 were harvested by brief treatment with trypsin-EDTA and then maintained in abdominal cavities of mice by intraperitoneal transplantation of 10^6 cells/mouse for every 7 to 10 day. At day-0, mice weighing 18-22g were inoculated with 10^6 Sarcoma 180 cells/0.1 ml/mouse. Twentyfour hours after transplantation (Day-1), 0.1 ml of PJA (4.5 mg/ml, PJA group), Con A (3 mg/ml, Con A group) or saline (control group) was injected i.p. once per day from Day-1 to Day-10. Thereafter, the survival time of the injected mice were recorded, antitumor activity of the PJA group was evaluated by life-span and compared with those of the control and Con-A groups.

RESULTS

PJA was found to be a glycoprotein with 49.3% carbohydrate and its carbohydrate composition is shown in Table I. And Table II shows that is has relatively high percentages of glutamic acid, aspartic acid and phenylalanine residues. Hemagglutination of PJA was inhibited by dextran. However, it was not inhibited by any of monosaccharides and several hexosamines[11].

To evaluate the potency of hemagglutination of PJA as an index of easily detectable manifestations of lectin, although it is affected by many factors, it was tested and compared with that of Con A using mouse erythrocytes. Fig. 2 shows that hemagglutinating activity of PJA was approximately one-eighth of that of Con A. This means that PJA 1 μg may have similar potency of Con A 0.125 μg.

To determine whether PJA is mitogenic to lymphocytes or not, it was estimated by counting the

Table II. Amino acid composition of PJA

Amino acid	w/w %
Glutamic acid	23.04
Aspartic acid	16.96
Phenylalnine	16.96
Alanine	6.52
Cysteine	5.65
Serine	5.22
Glycine	4.35
Isoleucine	3.92
Arginine	3.91
Methionine	3.04
Threonine	3.04
Valine	3.04
Leucine	2.61
Lysine	1.74
Tyrosine	—
Proline	—

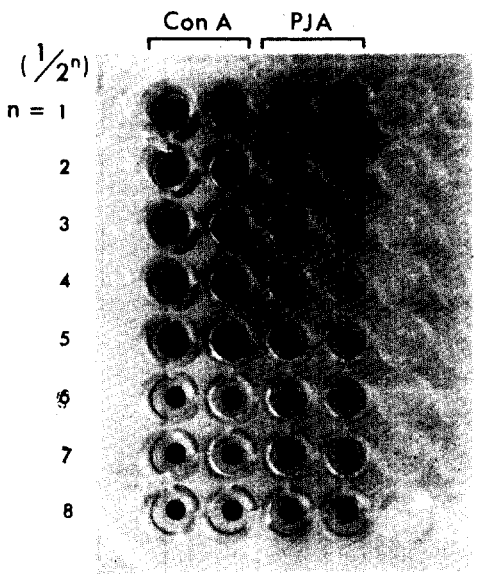


Fig. 2. Photo-presentation of comparison of hemagglutinating activity between PJA and Con A, using mouse erythrocytes. The concentrations of the top are equal 1 mg/ml of Con A and PJA. Left value means two fold-serial dilution.

number of lymphocytes under a microscope as shown in Fig. 3 and Fig. 4. In the earlier studies, the alterations in absorbance by MTT assay and ^{3}H-thymidine incorporation into human lymphocytes had been designed to measure for this purpose. They were ruled out because of no significant difference. Into each well (0.2 ml capacity) of microplate, 10^{6} cells/ml were inoculated at Day-O, with or without lectin, incubated for 4 days and counted in a hemacytometer after dye exclusion with trypan blue (0.5%) from Day-1 to Day-4. When this number of the control is considered as 1.0 for each day and lectin treated lymphocytes are expressed as a portion of the control, the ratio is considered as representative of mitogenic or other cytotoxic effect induced by lectin. At day-1, the ratios of human lymphocytes decreased regardless of PJA and Con A. Con A-treated lymphocytes were stimulated to proliferate through next 3 days of incubation dose-dependently, whereas the PJA-treated group in which the ratios were below 1.0 was not. For mouse lymphocytes, the same manner was conducted to determine effects of lectin. When mouse lymphocytes were incubated without lectins, the number of lymphocytes decreased in a similar degree with that of human's. The Con A-treated mouse lymphocytes increased after 2 days of incubation dose-depen-

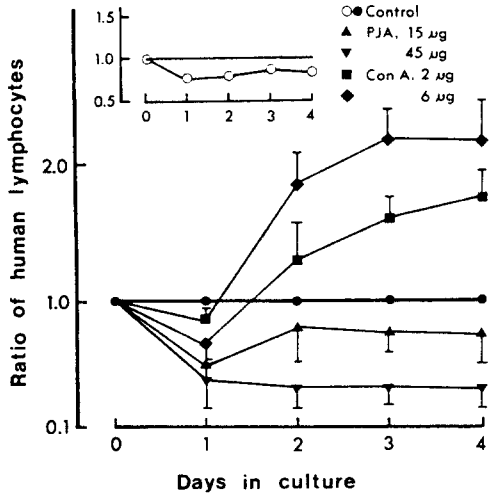


Fig. 3. Effects of PJA (15 and 45 μg) and Con A (2 and 6 μg) on proliferation of human lymphocytes in culture. Compared with that of the nontreated control group to be considered as 1.0 each day. The upper graph represents the change of number of lymphocytes of the control in culture when the number of lymphocytes in preculture (Day-0) is considered as 1.0. Each value denotes the mean± the standard error of the mean of 3-4 experiments.

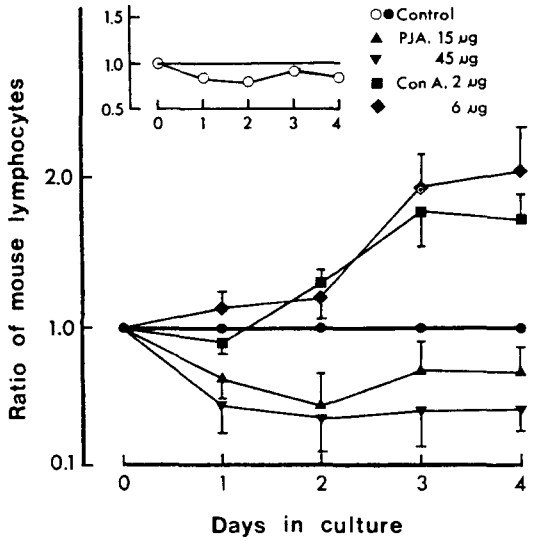


Fig. 4. Effects of PJA (15 and 45 μg) and Con A (2 and 6 μg) on proliferation of mouse lymphocytes in culture. Compared with that of the nontreated control group to be considered as 1.0 each day. The upper graph represents the change of number of lymphocytes of the control in culture when the number of lymphocytes in preculture (Day-0) is considered as 1.0. Each value denotes the mean± the standard error of the mean of 3-4 experiments.

dently, whereas numbers of PJA-treated lymphocytes were lowest at Day-2 and did not recover to the control. So, these results suggested that PJA did not seem to stimulate the proliferation or transformation of human and mouse lymphocytes in contrast of Con A.

The cytotoxicities of PJA and Con A on human cancer cells, SNU-1 and SNU-C1, were assayed by MTT colorimetric method and shown in Fig. 5. When compared with those of the control treated without any lectin, absorbances of the PJA treated (15 μg in 0.2 ml capacity of well) and the Con A treated (2 μg/well) respectively were 56.1± 9.2% and 71.8± 7.4%, representing anticancer activities of PJA and Con A against SNU-1. Those of the 5-FU treated (0.1 and 0.4 μg/well) which was administered in order to compare with the potency of lectins were 68.5± 5.0% and 60.1± 4.4%. Against another human cancer cells, SNU-C1, the PJA treated (15 μg/well) and the Con A treated (2 μg/well) showed anticancer activities, 53.9± 9.0% and 66.0± 7.2%, respectively.

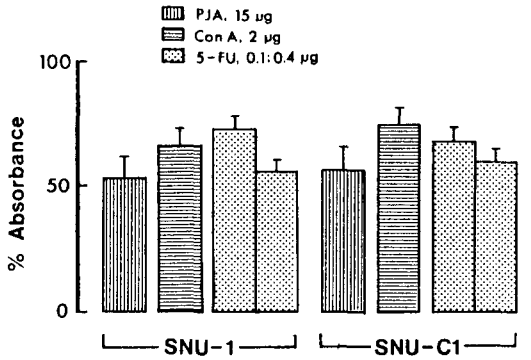


Fig. 5. Inhibition of PJA (15 and 45 μg), Con A (2 and 6 μg) and 5-FU (0.1 and 0.4 μg) on the proliferation of SNU-1 or SNU-C1. Cells were cultured for 3 days with or without lectins and followed MTT assay. Control vaule is considered as 1.0 and compared. Each value denotes the mean± the standard error of the mean of 3-4 experiments.

The 5-FU treated (0.1 and 0.4 μg/well) also showed anticancer activity against SNU-C1 as much as against SNU-1.

On the other hand, the cytotoxicities of PJA and Con A against Sarcoma 180 were shown in Fig. 6. Absorbances of the PJA treated (15 and 45 μg/well) were 22.6± 1.8% and 13.9± 3.2%. These were much less than those of the Con A treated (2 and 6 μg/well), 63.7± 3.1% and 44.8± 3.5%. And those of the 5-FU treated (0.1 and 0.4 μg/well) were 29.5± 3.5 % and 24.0± 4.3%.

Mice bearing Sarcoma 180 were given lectins by i.p. injection daily for 10 consecutive days, while the control group received saline. Fig. 7 reveals the effects of PJA and Con A in the survival of mice. The means of life span of the PHA and Con A groups were 29.6 and 22.8 days whereas that of the control was 32.3 days. But there were no significant difference, when estimated with unpaired t-test.

DISCUSSION

Lectins have a variety of biological effects on cells. Among these, the most extensively studied effects are agglutination and mitogenic stimulation. Lectins must bind to their surfaces, presumably through specific cell surface receptors.

The hemagglutination of PJA was inhibited by dextran[11]. The potency of hemagglutinaition of PJA was approximately one-eighth of that of Con A.

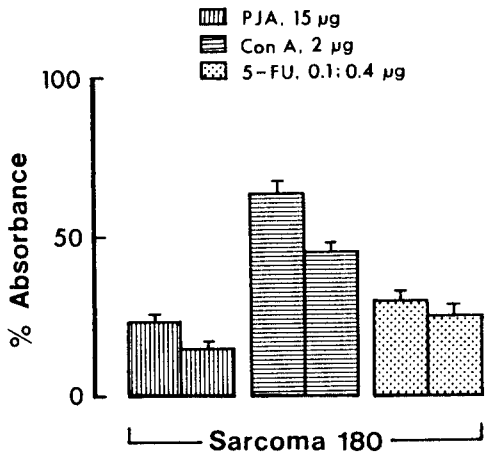


Fig. 6. Inhibition of PJA (15 and 45 μg), Con A (2 and 6 μg) and 5-FU (0.1 and 0.4 μg) on the proliferation of Sarcoma 180. Cells were cultured for 3 days with or without lectins and followed MTT assay. Control value is considered as 1.0 and compared. Each value denotes the mean± the standard error of the mean of 3-4 experiments.

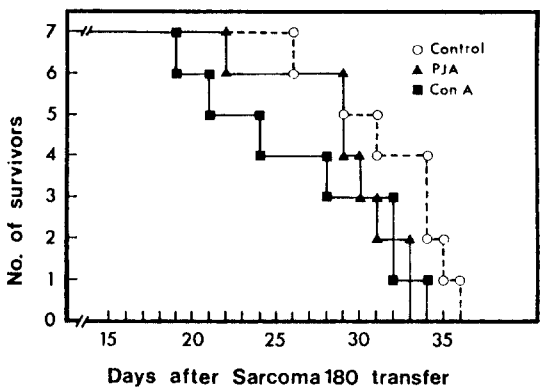


Fig. 7. Effects of PJA and Con A on the survival of mice bearing Sarcoma 180. Sarcoma cells (1×10^8) were transplanted at Day-0 and the lectins were administered intraperitoneally once per day from Day-1 to Day-10. The concentrations of lectins are: saline (○); PJA (△), 4.5 mg/kg/day; Con A (■), 3 mg/kg/day.

As the increase in agglutinability does not always mean a result of an increase in the number of lectin binding sites on the cells, the specific binding sites are not simply explained what contributes to the difference in binding strength. It may be due to the interaction with surface receptors whose affinity for the lectin is higher, multivalent interaction and furthermore higher association constant[18]. It is required to be studied precisely in this experiments. However, hemagglutinability was used as a index of easily detectable manifestations of PJA and Con A.

Studies of lymphocyte activation began with discovery by Nowell[5] that PHA can stimulate lymphocytes to grow and divide. Now, a large number of lectins is known to be mitogenic[1,6,7]. It is known that lectin-mediated lymphocytes functions are frequently induced by lectin-dependent cell-cell contact and transmembrane signaling. It was reported that lectins induce mitogenic activity though to varyihng degrees, and many of these changes occur within seconds to minutes after the addition of mitogen. On the mitogenic activity by lectins, the earliest detectable changes are in membranes, including permeabilities for glucose, amino acid and ions and rapid turnover of membrane phospholipids. Later, RNA and protein synthesis accelerate and morphological changes are observed. Through this signal transduction, the role of membrane glucoconjugate to serve as target structures in lectin mediated cellular interactions is not known[19]. How the specificity of binding carbohydrate moiety of each lectin provides the distinction seems not to have been examined yet precisely. Only it was suggested that the first step is the crosslinking of membrane proteins such as CD3[20]. In this experiment, PJA seems to be nonmitogenic although it is not defined whether this means no mitogenicity or direct cytotoxicity to lymphocytes. WGA, previously strictly as a nonmitogenic or anti-mitogenic lectin, can under appropriate conditions markedly stimulate *in vitro* synthesis and secretion of immunoglobulin (Ig) by human B lymphocytes[8]. In the case of PJA, it is not excluded that PJA can induce proliferation of subpopulation of lymphocytes under certain conditions as WGA can. To solve this, it is required to be examined whether PJA is mitogenic to specified subpopulation of lymphocytes and positive mitogenic activity dependent on the dose of administration of lectin.

In vitro, the survival of T lymphocytes is critically dependent on interleukin-2 (IL-2), a growth factor produced by helper T (TH-1) cells after exposure to antigens or polyclonal mitogens such as lectins[21]. Among these, cytotoxic T cells require exogenous

IL-2 to maintain viability. Actually, Con A induces rat splenocytes to produce IL-2[22]. At present it was not determined whether no secretion of IL-2 by PJA contributes to no mitogenic activity to lymphocytes.

When *in vitro* cytotoxicities and *in vivo* antitumor activities of PJA and Con A were compared, PJA and Con A supressed the proliferations of SNU-1 and SNU-C1 with similar degree and PJA showed the higher potency than Con A to inhibit the growth of Sarcoma 180 which grows more rapidily than SNU-1 and SNU-C1. This is in agreement with the facts that several lectins including Con A, WGA and PHA, are highly toxic to mammalian cells and some of these preferentially kill rapidly growing transformed cells[9,10].

Murine peritoneal polymorphonuclear leukocytes (PMNs) whose primary role is in phagocytosis, killing microogranism and host defense against tumors can lyse murine tumor cells in the presence of WGA, while other lectins, such as Con A, PHA and PMN did not cooperate with PMNs in tumor lysis[23]. Furthermore, it was reported that this mechanism was dependent on superoxide[24]. And it was reported that in the presence of Con A or PHA, target cell killing occurs *via* cytotoxic T lymphocytes-mediated cytotoxicity, while non-mitogenic lectins such as WGA did not mediated lectin-dependent cellular cytotoxicity[25,26]. Murine splenocytes or human blood lymphocytes on incubation in the presence of IL-2 or lectins acquired the ability to lyse a variety of fresh syngeneic murine and autologous human tumor cells[27]. These lymphokine-activated killer (LAK) cells are distinct from natural killer cells or classical cytotoxic T-cells. Okada *et al.*[28] reported that the LAK cells to exhibit higher cytotoxic activity *in vitro* exerted no effect when they were infused intravenously into mice with melanoma and LAK cells showed more marked antitumor activity *in vivo* if they responded to IL-2 and proliferated very well with a small amount of IL-2. Recent investigations showed that all commonly used plant lectins including those that are generally regarded as nonmitogenic for lymphocytes can cause a variety of other lymphocytes responses including Ca^{2+} mobilization, blast transformation, lymphokine secretion, and nonspecific inhibition of membrane protein mobilities and mitogenesis[29,30]. But, earliest reports had demonstrated that *in vivo* toxic effects and immunosuppressive effects was dependent on the administered dose of Con A and controversial[31].

When tested *in vivo* antitumor activities of PJA and Con A by estimation of prolongation in life span of Sarcoma mice, the treatment of PJA or Con A decreased the life span a little, but not significantly. On these observations, it is further required to examine the *in vivo* immunological effects of PJA and Con A.

CONCLUSION

New lectin from *Pseudixus japonicus* (PJA) is a glycoprotein with 49.3% carbohydrate and 50.7% protein. It did not show mitogenic activity on human and mouse lymphocytes in constrast to Con A. It has been revealed to have cytotoxicity against SNU-1, SNU-C1 and Sarcoma 180 *in vitro* by MTT assay. The antitumor activity *in vivo* shows no significant differences in prolongation of life span after treatment with PJA or Con A for 10 consecutive days.

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