Lectins from Wisteria floribunda Seeds and Their Effect on Membrane Fluidity of Human Peripheral Lymphocytes*

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SUMMARY

Two lectins were isolated from Wisteria floribunda seeds. One is a strong mitogen against human peripheral lymphocytes and has been purified in the previous paper (Toyoshima, S., Akiyama, Y., Nakano, K., Tonomura, A., and Osawa, T. (1971) Biochemistry 10, 4457). The other, which is a strong hemagglutinin being devoid of mitogenic activity against normal lymphocytes, has been purified in this paper by affinity chromatography on a Sepharose 6B column followed by DEAE-Sephadex column chromatography. Both lectins were found to be glycoproteins and their molecular weights were estimated to be 136,000 for the hemagglutinin and 67,000 for the mitogen by sedimentation equilibrium. The hemagglutinin is composed of four apparently identical subunits of a molecular weight of 35,000 and the mitogen is a dimer of 32,000 molecular weight subunit. Binding experiments with 125I-labeled W. floribunda mitogen revealed that the maximal incorporation of [6-3H]thymidine or 32P04 occurred when only 5.2% of the available receptor sites on normal lymphocytes were occupied by the mitogen. Furthermore, the mobility of W. floribunda lectins as well as other lectins bound to the cell receptor sites of normal lymphocytes was determined by fluorescence polarization of fluorescein-labeled lectins. The mitogenic lectins tested, W. floribunda mitogen and Lens culinaris hemagglutinin, have high mobility whereas the nonmitogenic lectins, W. floribunda hemagglutinin, Sophora japonica hemagglutinin, and eel serum anti-H hemagglutinin show relatively low mobility. However, W. floribunda hemagglutinin bound to neuraminidase-treated lymphocytes showed relatively high mobility in accord with the fact that this hemagglutinin exerted weak but definite mitogenic activity against neuraminidase-treated lymphocytes. The change of membrane fluidity upon binding of the lectins to normal lymphocytes was also measured by fluorescence polarization of fluorescent hydrocarbon, 1,6-diphenyl-1,3,5-hexatriene, embedded in the membrane. The mitogenic lectins, W. floribunda mitogen and L. culinaris hemagglutinin, increased the membrane fluidity upon binding to lymphocyte cell surface within 30 min, whereas the non-mitogenic lectins, W. floribunda hemagglutinin and S. japonica hemagglutinin, did not affect the membrane fluidity. We suggest that the increase of membrane fluidity is one of the common biochemical events in the earliest stage of lymphocyte transformation.

In the previous paper, we described the isolation of two lectins from Wisteria floribunda seeds (1). One is a strong mitogen against human peripheral lymphocytes (W. floribunda mitogen) having relatively weak hemagglutinating and leukoagglutinating activities. In contrast, the other has strong hemagglutinating activity (W. floribunda hemagglutinin), but it is devoid of mitogenic activity in ordinary conditions. The hemagglutination inhibition assays using simple sugars as hapten inhibitors have revealed that both lectins belong to the so-called galactose-specific lectins (1), but subtle difference in specificity has been observed between these two lectins when certain glycopolymers and their sequential enzyme degradation products were used, as hapten inhibitors (2). The purification of one of the lectins, W. floribunda mitogen, has already been reported (1). In this paper, we have purified the other lectin, W. floribunda hemagglutinin. Furthermore, these two W. floribunda lectins have been characterized with respect to molecular weights, subunit structure, and binding property to human erythrocytes and lymphocytes, and their effect on the membrane fluidity upon binding to human lymphocytes has also been studied.

EXPERIMENTAL PROCEDURE

Purification of Wisteria floribunda Lectins—(a) W. floribunda mitogen was purified from the seeds purchased from F. W. Schumacher (Sandwich, Mass.) according to the method previously described (1). (b) W. floribunda hemagglutinin was purified from the partially purified hemagglutinin (Fraction A), which had been prepared according to the method previously described (1), by successive chromatographies on Sepharose 6B and DEAE-Sephadex A-50 as described in the legends of Figs. 1 and 2.

Preparation of Other Lectins—LCH1 was purified from lentils (kindly provided by the Department of Medicinal Plants, Kathmandu, Nepal) by the method previously described (3). SJ-II (4) was kindly provided by Dr. T. Terao, University of Tokyo.

1 The abbreviations used are: LCH, the purified Lens culinaris hemagglutinin; WFI, the purified Wisteria floribunda hemagglutinin; WFM, the purified Wisteria floribunda mitogen; SJ-II, the purified Sophora japonica hemagglutinin II; ESH, the purified eel serum anti-H hemagglutinin; F-, fluorescein-labeled; DPH, 1,6-diphenyl-1,3,5-hexatriene.
ESH was purified from fresh eel serum according to the method of Matsumoto and Osawa (5). Molecular Weight Determination—The molecular weights of the purified lectins were measured by sedimentation equilibrium with a Hitachi model UCA-1A ultracentrifuge according to the method of Yphantis (6). The concentrations of the lectins tested were 0.2, 0.5, and 1% in 0.01 M phosphate buffer, pH 7.0, containing 0.15 M NaCl.

Disc Electrophoresis—Disc electrophoresis in polyacrylamide gels was carried out in 7.5% gels in Tris-HCl buffer, pH 8.9, according to Ornstein and Davis (7). For the molecular weight determination of subunits of the lectins, disc electrophoresis was performed with 0.25% Coomassie brilliant blue and destained electrophoretically in 7.5% acetic acid-5% methanol. The following 0.1% sodium dodecyl sulfate and 1% β-mercaptoethanol was used as standards for the molecular weight determination (molecular weights shown in parentheses): chymotrypsinogen (25,000); ovalbumin (45,000); bovine serum albumin (67,000); and human γ-globulin (160,000).

Amino Acid Analysis—Amino acids were determined after hydrolysis for 24, 48, and 72 hours in constant boiling HCl (5.7 N) at 100°C. The hydrolysate was buffered with 0.9% NaCl. Purified lymphocytes were incubated for 15 hours at 4°C with fluorescein isothiocyanate was removed by gel filtration on a Sephadex G-50. The specific radioactivity of the iodinated lectins was used to calculate the rotational relaxation time of an F-lectin by the method of Woidow et al. (16). The DPII-labeled systems temperature was adjusted to 20 or 25°C with a thermostat bath (Komatsu model CTR-120). The DPIIlabeled systems were exposed to the excitation light for less than 10 s which eliminated the possibility of reversible bleaching of DPII which presumably originated from reversible photoisomerization (25).

Preparation of F-Lectins and Their Binding to Lymphocytes—The purified lectins were iodinated with Na125I by the chloramine-T method of Hunter (18). The labeled lectin was added to lymphocytes preincubated in SNP buffer in N-2-morpholinepropane sulfonic acid buffer (pH 7.0). The suspension of lymphocytes in a concentration of 5 × 10^6 cells per ml, the fluorescence measurement was immediately performed.

Fluorescence Labeling of Lymphocytes—The fluorescent hydrocarbon, DPH (Tokyo Kasei Co., Tokyo) was used as a probe for monitoring fluidity in the cell membrane lipid layers. Labeling of cells with DPH was performed by the method of Inbar et al. (22). For the evaluation of the change in the membrane fluidity upon binding of a lectin to the cell surface, the labeled cells were incubated with a lectin for 30 min at 37°C and immediately used for fluorescence measurements.

Fluorescence Methods—Fluorescence polarization was measured by a fluorescence spectrophotometer (Hitachi model MPF-2A) with a rotating analyzer. The wavelengths of polarized bands used for excitation were 360 nm for DPH, and 435 nm for F-lectins. These bands were generated from a xenon lamp and passed through a polarizer. Fluorescence polarization and intensity were obtained by measurements of I0 and I⊥, where I0 and I⊥ are the fluorescence intensities polarized parallel (I0) and perpendicular (I⊥) to the direction of polarization of the excitation beam. The degree of fluorescence polarization (P), the fluorescence anisotropy (r), and the total fluorescence intensity (F) are defined by the following equations:

\[ P = \frac{I_0 - I_\perp}{I_0 + 2I_\perp} \]

\[ r = \frac{I_0 - I_\perp}{I_0 + 2I_\perp} \]

\[ F = I_0 + 2I_\perp \]

where \( r_0 \) is the limit fluorescence anisotropy, \( r \) is the excited state lifetime, \( T \) is the absolute temperature, \( \rho \) is the rotational relaxation time, \( \tau \) is the viscosity of the medium, and \( C(\tau) \) is a parameter which relates to the molecular shape of the fluorophore and has a specific value for each \( \tau \) (24). \( \rho \) is proportional to the viscosity (\( n \)) and inversely proportional to the absolute temperature (T).

In this study, the excited state lifetime (\( \tau \)) was measured by a phase fluorometer (JASCO model FL-10-C). Equation 4 was used to calculate the rotational relaxation time of an F-lectin and Equation 5 was used for the determination of microviscosity of the hydrocarbon region of the cell membrane as described under "Results and Discussion." In all of the fluorescence measurements temperature was adjusted to 20 or 25°C with a thermostat bath (Komatsu model CTR-120). The DPH-labeled systems were exposed to the excitation light for less than 10 s which eliminated the possibility of reversible bleaching of DPH which presumably originated from reversible photoisomerization (25).

Neuraminidase Treatment of Human Lymphocytes—To 1 ml of human lymphocyte suspension (10^6 cells per ml) in 0.01 M acetae buffer (pH 5.5) containing 0.15 M NaCl were added 25 units of Vibrio cholerae neuraminidase (Calbiochem), and the suspension was gently shaken at 67°C for 30 min. The cells were then collected and washed several times with phosphate-buffered saline (pH 7.0).

RESULTS AND DISCUSSION

Purification of Wisteria floribunda Hemagglutinin—The chromatography of the partially purified hemagglutinin (Fraction A) (1) on a Sepharose 4B column was carried out as shown in Fig. 1. After the bulk of inactive proteins was eluted near the void vol-
of the hemagglutinin was 11 mg. Hemagglutinating activity is denoted by shaded portions. The elution volumes of blue dextran (1.6 X 40 cm) equilibrated against the same buffer. Elution was carried out with the same buffer and fractions of 5 ml were collected at 6 ml per hour at 4°C. Hemagglutinating activity is denoted by shaded portions. Yield of the hemagglutinin was 11 mg.

Fig. 2 (right). DEAE-Sephadex chromatography of the hemagglutinin from the Sepharose 6B chromatography. The hemagglutinin, 10 mg, was dissolved in 1 ml of 0.025 M phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer, and then applied to a column (1 x 9 cm) equilibrated against the same buffer. Elution was carried out with the same buffer, and after a small peak was eluted out, subsequent elution was performed with 0.5 M phosphate buffer (pH 7.0) as indicated by the arrow. Fractions of 1 ml were collected at 10 ml per hour at 4°C. Hemagglutinating activity is denoted by shaded portions. Yield of the purified hemagglutinin was 5 mg.

Table I
Comparison of biological activities of Wistaria floribunda lectins

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Hemagglutinating activity*</th>
<th>Leukocytoglutinating activity*</th>
<th>Mitogenic activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction A</td>
<td>80</td>
<td>320</td>
<td>&gt;200</td>
</tr>
<tr>
<td>WFM</td>
<td>8</td>
<td>32</td>
<td>&gt;200, 100</td>
</tr>
<tr>
<td>WFH</td>
<td>5000</td>
<td>2500</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Minimum hemagglutinating dose against human group O erythrocytes.
* Minimum agglutinating dose against human peripheral lymphocytes.
a Mitogenic dose to give 5000 cpm of [6-3H]thymidine incorporation against 3 X 10^6 human peripheral lymphocytes.

A 10-fold purification from Fraction A based on the hemagglutinating titer with human erythrocytes was achieved as shown in Table I. The hemagglutinating and leukoagglutinating activities of WFH is much stronger than those of WFM, but the former is devoid of mitogenic activity against human peripheral lymphocytes. When neuraminidase-treated human peripheral lymphocytes were used, however, WFH exerted weak but definite mitogenic activity as shown in Table I.

Analytical Results—Ultracentrifugation of the purified hemagglutinin yielded a single peak during the whole of the run. Molecular weights of WFH and WFM were estimated to be 136,000 and 67,000, respectively, by means of sedimentation equilibrium at a speed of 7,640 rpm assuming a partial specific volume of 0.73 which was calculated from the analytical data for both lectins. This molecular weight of WFM is in good agreement with that (70,000) estimated by gel filtration in the previous paper (1).

Polyacrylamide gel electrophoreses of WFH and WFM in the presence of 0.1% sodium dodecyl sulfate gave a single discrete band in each case. The molecular weights of these subunits were estimated to be 35,000 for WFH and 32,000 for WFM from the curve relating the electrophoretic mobilities and the logarithm of molecular weights of various standard proteins. These facts suggest that WFH is a tetramer of the subunit of an approximate molecular weight of 35,000 and WFM is a dimer of the subunit of an approximate molecular weight of 32,000.

Results of amino acid and carbohydrate analyses of WFH were listed in Table II. About 91.6% of the dry weight of WFH could be accounted for as amino acid residues. If one adds the weights of carbohydrate moiety (5.7%), the recovery is about 97%. When these results were compared with those obtained on WFM earlier described (1), WFH has a quite similar amino acid composition to WFM, but the latter contains larger amounts of carbohydrates (11.4%) than the former (5.7%).

Binding to Erythrocytes and Lymphocytes—The binding of ^3H-labeled WFH and WFM to human group O erythrocytes and human lymphocytes was carried out and the data were plotted according to the method of Steck and Wallach (Figs. 3 and 4). From the data, the apparent association constants and the average number of molecules bound per cell were calculated as shown in Table III. No remarkable difference in the number of receptor sites on the erythrocytes was observed between WFH and WFM, whereas the association constant of WFM to the erythrocytes was much smaller than that of WFH. On the other hand, there are more receptor sites for WFM than WFH on the lymphocytes, but the association constant of WFH to the lymphocytes was approximately 4-fold that of WFM. The neuraminidase-treated lymphocytes, which could be induced to mitosis by WFM, bound more WFH with greater affinity than did the normal lymphocytes.
Fig. 3 (left). Binding of WFH to human erythrocytes and lymphocytes. Binding reactions were carried out in silicone-coated tubes presoaked overnight with 0.25% bovine serum albumin solution in 0.15 M NaCl-5 mM phosphate buffer (pH 7). The reaction mixtures contained 1 × 10^6 erythrocytes or 2 × 10^5 lymphocytes, 1.5 to 32 μg of WFH labeled WFH in a final volume of 0.2 ml of 0.25% bovine serum albumin solution in 0.15 M NaCl-5 mM phosphate buffer (pH 7). After 30 min of incubation at room temperature, the cells were washed twice with the phosphate-buffered saline, and the amount of bound WFH was determined by an Allofer Autogamma counter. The data were plotted by the method of Steck and Wallach (19). (●—●), erythrocytes; (○—○), lymphocytes.

Fig. 4 (right). Binding of WFM to human erythrocytes and lymphocytes. Binding reactions were carried out as described in the legend of Fig. 3. (●—●), erythrocytes; (○—○), lymphocytes.

These data of the binding studies cannot be directly correlated to the biological activities of the above two lectins, because only a small proportion of the receptor sites actually might be responsible for the triggering of lymphocyte transformation as revealed by the following experiments. Thus, in order to determine how many receptor sites on the lymphocyte surface should be occupied by WFM for the triggering of lymphocyte transformation, the optimum amount of [3H]WFM bound on the cell surface for the maximal incorporation of [3H]thymidine was determined. As shown in Fig. 5, the maximal incorporation of the labeled compounds occurred when only 5.2% of the available receptor sites were involved in the interaction with WFM. This value is in good agreement with the observations by Betel and Van den Berg (26) and also by Inbar et al. (27) that optimum stimulation of DNA synthesis occurred when about 5 to 6% of the available sites on lymphocytes were occupied by concanavalin A.

Degree of Mobility of Lectins on the Surface Membrane of Human Lymphocytes—A series of solutions containing an F-lectin (20 μg per ml) in phosphate-buffered saline (pH 7.0) and increasing amounts of sucrose up to 70% (w/v) were used to measure r and T values at 25°C. The results are given for F-WFH and F-WFM in Fig. 6 as l/r versus ηrT/η. η, the viscosity in phosphate-buffered saline without sucrose, was introduced in order to relate the data to rotational diffusions in phosphate-buffered saline. At the region ηrT > 0.8, where l/r increases linearly, the fluorescein depolarization reflects only the rotations of the protein molecule. The extrapolation of the line at this region gives l/ηr. Thus, the rotational relaxation time of a free F-lectin, τ, can be obtained by substituting in Equation 4 under "Experimental Procedure" the values of r and T in phosphate-buffered saline and η. Because r and T values may not change upon the binding of a lectin to the cell surface (28), these values were used for the calculation of a ρ value of a lectin bound on the lymphocytes and the values calculated were listed in Table IV. According to Shinitzky et al. (28), we have defined the parameter ρ/τ, the degree of mobility, which extends from 0 (immobilized state) to 1 (fully mobile state). As shown in Table IV, F-WFH and F-WFM bound to lymphocyte cell surface, both of which have strong mitogenic activity against normal lymphocytes, show a high degree of mobility close to that of the corresponding free F-lectin in phosphate-buffered saline, whereas F-WFH, F-SJ-II, and F-ESH, all of which are devoid of mitogenic activity against normal lymphocytes, have a relatively low degree of mobility compared to the corresponding free F-lectin. However, treatment of lymphocytes with neuraminidase caused an increase in the mobility of F-WFH bound to the cell surface, in accord with the fact that this lectin exerted weak but definite mitogenic activity against the neuraminidase-treated lymphocytes. These results are consistent with the results of Shinitzky et al. (28) that the mitogenic fluorescein-labeled concanavalin A bound to lymphocyte cell surface has a high degree of mobility which is about twice that of the non-mitogenic fluorescein-labeled wheat germ agglutinins or soy bean agglutinin. The observations described above suggest a relationship between the mobility of receptor sites on the cell surface and the mitogenic triggering.

Effect of Lectins on Microviscosity of Surface Membrane of Human Lymphocytes—Because the thermal mobility of cell surface receptors might be controlled by the fluid state of the membrane lipid layer, the change of microviscosity of the surface membrane lipid layer upon binding of lectins was determined by measuring the fluorescence polarization of DPH which can be introduced
FIG. 5 (left). Optimum amounts of WFM bound for maximal stimulation of lymphocytes. Binding reactions were carried out in Eagle's minimal essential medium using silicone-coated tubes. The reaction mixtures contained 2 × 10⁶ lymphocytes, 1 to 100 µg of [125I]-labeled WFM, and 0.1 ml of autologous plasma in a final volume of 1 ml. Assays of [32P] incorporation into DNA were carried out with the corresponding amounts of unlabeled WFM as described in the text.

FIG. 6 (right). Perrin plots of F-WFH and F-WFM at 25°C

TABLE IV
Fluorescence polarization characteristics of F-lectins at 25°C
The wavelength of polarized band used for excitation was 435 nm.

<table>
<thead>
<tr>
<th>Lectins</th>
<th>In phosphate-buffered saline (pH 7.0)</th>
<th>Bound to human peripheral lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>t</td>
</tr>
<tr>
<td>F-WFM</td>
<td>3.0</td>
<td>0.142</td>
</tr>
<tr>
<td>F-LCH</td>
<td>2.7</td>
<td>0.154</td>
</tr>
<tr>
<td>F-WFH</td>
<td>2.9</td>
<td>0.132</td>
</tr>
<tr>
<td>F-SJ-II</td>
<td>3.1</td>
<td>0.111</td>
</tr>
<tr>
<td>F-ESH</td>
<td>2.8</td>
<td>0.070</td>
</tr>
</tbody>
</table>

* Obtained with neuraminidase-treated human peripheral lymphocytes.

TABLE V
Effect of Wistaria floribunda lectins on degree of fluorescence polarization (P) and the apparent microviscosity (v) obtained with human peripheral lymphocytes labeled with DPH at 25°C
The concentration of lymphocytes was 5 × 10⁶ per ml. The wavelength of polarized band used for excitation was 366 nm.

<table>
<thead>
<tr>
<th>Lectins (µg/ml)</th>
<th>r</th>
<th>v (poise)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.305</td>
<td>4.5</td>
</tr>
<tr>
<td>WFM (13)</td>
<td>0.249</td>
<td>2.8</td>
</tr>
<tr>
<td>LCH (50)</td>
<td>0.298</td>
<td>3.1</td>
</tr>
<tr>
<td>WFH (100)</td>
<td>0.296</td>
<td>4.1</td>
</tr>
<tr>
<td>SJ-II (100)</td>
<td>0.308</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Average value of duplicate experiments.

Fig. 7. The effect of an optimum mitogenic dose of WFM on the membrane fluidity of human peripheral lymphocytes at various times after addition of the mitogen.

Fig. 8. The dose-response curves for the effect of WFM on the [3H]thymidine incorporation and the membrane fluidity of human peripheral lymphocytes. (solid line), microviscosity of the membrane; (open line), [3H]thymidine incorporation after 72 hours of incubation.

Easily into the hydrocarbon region of membrane lipid layers (25). The microviscosity of the surface membrane lipid layer was determined according to Equation 5 under "Experimental Procedure" by the methods of Shinitzky et al. (24) and Cogan et al. (29). The limit fluorescence anisotropy of DPH (r0) excited at 366 nm and the C(r) value were obtained according to Shinitzky and Barenholz (25). The results summarized in Table V indicate that the binding of a mitogenic lectin, WFM or LCH, to the membrane apparently increases the membrane fluidity, whereas the binding of a nonmitogenic lectin, WFH or SJ-II, does not significantly affect the membrane fluidity. As shown in Fig. 7, this change in the membrane fluidity showed maximal increase after a 30-min exposure to WFM. The dose-response curve shown in Fig. 8 for the increase of membrane fluidity also corresponded well with the dose-response curve for the mitogenic activity of WFM measured by the incorporation of [3H]thymidine. It should be noted that in a recent report Barnett et al. (30) have also described the increase of membrane fluidity measured by spin-labeling of membrane fatty acids within 30 min after the binding of concanavalin A to lymphocyte cell surface.

These experimental results suggest that mitogenic lectins can induce the increase of the membrane fluidity upon binding to the
cell surface receptor sites, whereas nonmitogenic lectins cannot significantly affect the membrane fluidity, and that the increase of membrane fluidity is one of the common biochemical events in the earliest stage of lymphocyte transformation. However, further investigations are necessary to know whether the increase of the membrane fluidity is one of the important prerequisites for the activation of lymphocytes or only one of the concurrent phenomena which are not on the major biochemical pathway leading to mitosis.

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