Stimulation of Mitogenesis in Normal and Leukemic Human Lymphocytes by Divalent and Tetravalent Lima Bean Lectins (phytohemagglutinin-P/cell surface receptors/valency)

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ABSTRACT Two hemagglutinating components purified from the lima bean and composed of identical 62,000-molecular-weight subunits have mitogenic activity in normal and leukemic human lymphocyte cultures. Component II (247,000 molecular weight), a tetramer with four saccharide binding sites, is several fold more active than component III (124,000 molecular weight), a dimer with two binding sites per molecule, as a mitogen for normal lymphocytes. Component II also stimulates mitogenesis in populations of cultured leukemic lymphocytes and appears to have greater activity than phytohemagglutinin-P in leukemic lymphocyte cultures obtained from some patients. Lima bean component III has minimal mitogenic activity toward leukemic lymphocytes. Components II and III appear to compete for the same cell-surface binding sites, but component II has a much greater capacity than component III to trigger DNA synthesis at low concentrations (2.5-25 μg/ml) in normal lymphocyte cultures. These results suggest that the mitogenic activity of lima bean lectin components is directly related to their valence.

The purification and characterization of hemagglutinins from the lima bean (Phaseolus lunatus) have been previously reported (1-3). Lima bean lectin specifically binds blood-group A substance, and this binding is inhibited by N-acetyl-D-galactosamine (1-3). Two active components have been identified: components II and III with molecular weights of 247,000 and 124,000, respectively. Both components are composed of identical 31,000-molecular-weight glycoproteins linked by an interchain disulfide bond to form 62,000-molecular-weight subunits. Thus, components II and III represent a tetramer and a dimer of these subunits. The specific hemagglutinating activity of component II is four times that of component III (2), and Scatchard analysis of data obtained by equilibrium dialysis indicates that component II has four saccharide binding sites per molecule, whereas component III has two binding sites (Bessler, W. and Goldstein, I. J., manuscript submitted for publication).

The purified, unfractionated lima bean lectin containing a mixture of components II and III has been reported to induce blastic transformation and mitogenesis in human lymphocytes (4). It was of interest to compare the relative activities of components II and III since these naturally occurring, chemically unmodified lectins, composed of identical subunits, would seem to offer a unique opportunity to examine the importance of molecular size and valency in the occupation of cell surface receptors by lectins which stimulate mitogenesis in lymphocytes. Gunther et al. (5) have reported that chemically modified dimeric derivatives of concanavalin A (Con A) have altered biological activities compared to the parent tetrameric compound. The observed differences in agglutination, ability to inhibit cap formation by anti-immunoglobulins, and mitogenic activity were attributed to the reduced valence of the chemical derivatives. Reichert et al. (4) found that acetylated Con A, a dimer (5), possessed about one-half the potency of tetrameric Con A as a mitogen for human lymphocytes when the lectins were compared over a concentration range of 0.5-10 μg/ml. In the present study, we have found that component II of lima bean lectin is severalfold more potent than component III as a mitogen for normal and leukemic human lymphocytes. Our results suggest that valency is an important determinant for the ability of lectins to trigger the mitogenic response in lymphoid cells.

MATERIALS AND METHODS

Blood from six adult, male, normal donors and four patients with chronic lymphocytic leukemia (CLL) (white cell count = 44 to 96 × 10⁶ per mm³) was utilized in this study. Lymphocytes were isolated from heparinized human blood samples by the carbonyl iron technique as previously described (6). The purified lymphocytes were suspended in a medium containing 50% modified McCoy's 5A and 50% Eagle's basal medium supplemented with 10% fetal-calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cell concentration was adjusted to 2 to 3 × 10⁶ cells per ml of culture medium; 4-ml aliquots were then removed and placed in Falcon flasks (25 cm² size). The cultures were incubated for 24 hr prior to the addition of lectins. Cell viabilities were determined at the time of lectin addition and at the time of the [H]thymidine pulse by dye exclusion with erythrocin B. Lectins were added in sterile saline to the cultures. Controls received an equivalent volume of saline without lectin.

DNA synthesis was determined by the addition of 1 μCi/ml of [H]methyl-thymidine (20 Ci/m mole; New England Nuclear Corp.) to the lymphocyte cultures and measurement of incorporation into acid-precipitable material as previously described (6). The cells were pulsed for 6 hr at various times after the addition of the lectins. DNA concentration was determined by diphenylamine assay (7), and the data were calculated as cpm [H]thymidine incorporated per μg of DNA. Each experimental point represents the mean of duplicate samples; variation between duplicates was routinely less than

Abbreviations: Con A, concanavalin A; PHA, phytohemagglutinin; CLL, chronic lymphocytic leukemia.
Fig. 1. Dose–response curves for the stimulation of DNA synthesis in normal lymphocytes (A = donor number 1, B = donor number 2) by lima bean lectin components II and III. All cultures were incubated for 72 hr after the addition of lectin and then pulsed for 6 hr with [3H]thymidine (1 μCi/ml). In experiment 1B, 1 or 2 mg/ml of N-acetyl-D-galactosamine (GalNAc) were added to some cultures along with 25 μg/ml of component II. In the experiment labeled “II + III”, 25 μg/ml of component III was added 1 hr prior to addition of various concentrations of component II. PHA (5 μg/ml) and control cultures were incubated for 72 hr, pulsed, and analyzed as in the case of the lima bean components. All assays were run in duplicate. Concentration (μg/ml) is plotted as a log function. (The numbers on the ordinate have been multiplied by 10⁻¹.)

±10%, based on the specific activity of the DNA in each sample. The stimulation of incorporation of [3H]thymidine into DNA by various lectins has been used as an index of their ability to induce “mitogenesis.” No attempt was made to quantitate the percentage of cells actually undergoing blastic transformation or cell division.

Phytohemagglutinin (PHA-P) was obtained from Difco Laboratories.

RESULTS AND DISCUSSION

Fig. 1 illustrates dose–response curves for the mitogenic effect of lima bean lectin components II and III with lymphocytes isolated from two normal donors. In this experiment, a 6 hr pulse of [3H]thymidine was performed 72 hr after the addition of the lectins. Component II was an active mitogen at concentrations as low as 2.5 μg/ml and was severalfold more potent than component III. Component III was active only at concentrations above 25 μg/ml. In other experiments with normal donors (not shown), it was found that the mitogenic response to component II was not linear above 100 μg/ml, but maximal stimulation was achieved at about 200 μg/ml.

The activity of III continued to increase up to concentrations of 250 μg/ml, and in lymphocyte cultures from two normal donors approached the activity of component II at this concentration. These data indicate that the ability of component III to activate cell surface receptors which trigger DNA synthesis in lymphoid cells is quite low compared to component II. N-Acetyl-D-galactosamine in concentrations of 1 or 2 mg/ml inhibited the incorporation of thymidine by 36% and 62% when added concomitantly with 25 μg/ml of component II (Fig. 1B). Reichert et al. (4) have previously shown that the stimulation of DNA synthesis produced by the un-fractionated lima bean lectin in human lymphocytes was specifically blocked by this hapten inhibitor. These results are consistent with the hemagglutination data since the binding of lima bean lectin to blood group substance was more effectively inhibited by glycosides of N-acetyl-D-galactosamine than by other saccharide haptenes (2).

In an attempt to determine if lima bean lectin components II and III compete for the same binding sites, a fixed concentration of component III (25 μg/ml), which did not stimulate [3H]thymidine incorporation, was added to lymphocyte cultures 1 hr prior to various concentrations of component II (Fig. 1B). The dose response curves for component II alone and for component II plus component III were superimposable up to 25 μg/ml of component II. At 50 and 100 μg/ml of component II there was an inhibition of thymidine incorpora-
Kinetics of stimulation of DNA synthesis in normal lymphocytes (donor number 3) by component II (25 μg/ml), component III (95 μg/ml), and PHA (10 μg/ml). Duplicate samples were pulsed for 6 hr with [3H]thymidine 24, 48, 72, 96, and 120 hr after the addition of lectin or saline (control). The numbers on the ordinate have been multiplied by 10\(^{-3}\).

The conclusion from this experiment was that at low concentrations of component II, receptor sites were still available to bind sufficient amounts of component II even in the presence of bound component III; but as greater amounts of component II were added, occupation of an increasing number of sites would have to occur in order to trigger a dose-related increase in mitogenesis. At this point, cell surface receptor blockade by component III became evident.

In another experiment (Fig. 2), a fixed concentration of component II (25 μg/ml) was incubated with normal lymphocytes in the presence of increasing concentrations of component III. The mitogenic response to 25 μg/ml of component II was diminished when 50, 100, or 250 μg/ml of component III were present in the cultures. This is consistent with the hypothesis that components II and III compete for the same cellular binding sites. The smaller mitogenic response induced by component III may be due to a lower affinity of component III for cellular binding sites which are involved in the activation of DNA synthesis or a decreased ability of the divalent component III, compared to the tetravalent component II, to produce the receptor crosslinking and clustering which may be necessary for mitogenic stimulation (5). We are currently attempting to radiolabel components II and III in order to carry out more definitive binding studies which should resolve these points.

The kinetics of stimulation of DNA synthesis in normal lymphocytes by lima bean components II and III are depicted in Fig. 3. Component II (25 μg/ml) produced an increase in rate of [3H]thymidine incorporation which appeared to peak at 48 hr after lectin addition. This was in contrast to the stimulation of DNA synthesis by PHA, which peaked at 72 hr after PHA was added to the cultures. Typically, normal lymphocytes exhibit peak DNA synthesis 72 hr after PHA addition (8). Component III (95 μg/ml) produced much less of an enhancement of thymidine incorporation.

The mitogenic activity of components II and III was also examined in lymphocyte cultures prepared from patients with chronic lymphocytic leukemia. As in the case of the normal lymphocyte cultures, component II was an active mitogen, but component III had essentially no activity even in concentrations up to 125 μg/ml (Fig. 4). Thus, leukemic lymphocytes appeared to be even less responsive to component III than lymphocytes from normal donors. Moreover, in the leukemic cultures component II was apparently more active than PHA as a mitogen. In general, leukemic lymphocyte cultures have a reduced response to mitogenic lectins, and the time to maximal stimulation of DNA synthesis after lectin addition is delayed (8). This is presumably due to the fact that the relative amount of B-type lymphocytes is expanded in the peripheral blood of most patients with CLL, and the proportion of T-lymphocytes is diminished. The observation that component II could achieve a greater effect than PHA as a mitogen in leukemic lymphocytes, whereas both lectins produced about the same maximal response in normal lymphocytes (5 μg/ml of PHA was the optimal concentration for normal cells), may indicate that II has a greater ability to activate leukemic lymphocytes than PHA and (or) may have some activity towards B cells. However, whether our observations reflect real differences between leukemic lymphocytes and normal lymphocytes remains to be established, since there is a report that the majority of leukemic lymphocytes are inherently incapable of responding to the

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**Fig. 3.** Kinetics of stimulation of DNA synthesis in normal lymphocytes (donor number 3) by component II (25 μg/ml), component III (95 μg/ml), and PHA (10 μg/ml). Duplicate samples were pulsed for 6 hr with [3H]thymidine 24, 48, 72, 96, and 120 hr after the addition of lectin or saline (control). (The numbers on the ordinate have been multiplied by 10\(^{-3}\).)

**Fig. 4.** Dose-response curves for stimulation of DNA synthesis by lima bean components in leukemic lymphocytes (A = patient number 1, WBC = 44,000/mm\(^3\); B = patient number 2, WBC = 96,000/mm\(^3\)). [3H]Thymidine pulses were performed 72 hr after lectin addition. (The numbers on the ordinate have been multiplied by 10\(^{-3}\).)
mitogenic effects of PHA and that only normal cells present in the population of peripheral lymphocytes from CLL patients will respond (9). The decreased and delayed mitogenic response of CLL cultures was reported to be due simply to the low concentration of normal cells in the lymphocyte population (9).

The kinetic data shown in Fig. 5 further illustrate that populations of leukemic lymphocytes are responsive to the mitogenic effects of component II, virtually unresponsive to component III, and somewhat more responsive to component II than to PHA. These data are from two separate patients and reflect the delayed responses of leukemic cell populations mentioned above. In one case, the time to peak response was 120 hr (Fig. 5A), and in the other it was 96 hr (Fig. 5B). In both cases, however, the time of maximal thymidine incorporation was the same for component II and for PHA.

It should be noted that in all the above experiments the viability of the various lymphocyte preparations was 90–98% at the time of lectin addition and >75% at the time of the [3H]thymidine pulse. There were no significant differences between control and lectin-treated cultures nor among the cultures containing component II, component III, or PHA. Thus the observed mitogenic effects were not due to alterations in cell viability produced by the various lectin treatments.

The results of this study indicate that (i) purified hemagglutinating components of lima bean (Phaseolus lunatus) have mitogenic activity toward human lymphocytes, (ii) the tetrameric, tetravalent component II is severalfold more potent than the dimeric, divalent component III as a mitogen for normal and leukemic human lymphocytes, and (iii) lima bean component II appears to be more active than PHA as a mitogen for some leukemic lymphocyte populations. These data suggest that the number of available binding sites possessed by a lectin molecule is an important determinant of the ability of the lectin to initiate DNA synthesis in lymphoid cells. Recently, Myron Leon (personal communication) has also shown that lima bean lectin component II is more potent than component III as a mitogen for normal human lymphocytes. However, neither component, in concentrations up to 400 μg/ml, stimulated DNA synthesis in mouse spleen cells. Additional studies are being initiated to determine the nature of the binding of the lima bean components to various types of lymphocytes and the relationship of binding to the triggering of DNA synthesis and cell proliferation.

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