Opposing Effects of Mitogenic and Nonmitogenic Lectins on Lymphocyte Activation

EVIDENCE THAT WHEAT GERG AGGLUTININ PRODUCES A NEGATIVE SIGNAL*

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In an effort to clarify the mechanism by which certain plant lectins induce lymphocyte activation, we have studied amino acid ([14C]isobutyric acid) uptake in purified human lymphocytes exposed to mitogenic and nonmitogenic lectins. In confirmation of earlier work, mitogenic lectins (concanavalin A and two phytohemagglutinins) produced a dose-related, 2- to 10-fold increase in aminoisobutyric acid transport. Changes occurred as early as 2 hours and reached a maximum after 18 hours. The stimulation by concanavalin A was inhibited by α-methyl-p-mannoside but not by other selected monosaccharides, indicating that the effect is modulated through specific carbohydrate receptors.

In contrast to the stimulation with concanavalin A and phytohemagglutinin, the nonmitogen wheat germ agglutinin inhibited aminoisobutyric acid transport, both in the presence and absence of the mitogenic lectins. The inhibition was seen over a broad wheat germ agglutinin dose range, was prevented by N-acetylglucosamine, a known inhibitor of wheat germ agglutinin binding, and did not appear to be associated with cytotoxicity. Comparative binding studies with radiolabeled concanavalin A and wheat germ agglutinin demonstrated that differences in transport occurred in cells containing comparable numbers of bound lectin molecules indicating that the failure of wheat germ agglutinin to stimulate a response was not a result of ineffective binding. The lack of stimulation by wheat germ agglutinin was not due to its inability to interact multivalently with membrane receptors since this lectin is divalent, produces capping and agglutination, and continues to inhibit aminoisobutyric acid transport even after the minimum valence was increased to 4 by cross-linking with glutaraldehyde. In contrast both divalent and tetravalent concanavalin A produced stimulation. Competitive binding studies with soluble wheat germ agglutinin or lectin attached to 300 A latex spheres revealed little or no competition for binding sites with radiolabeled concanavalin A, suggesting the two lectins are interacting with different receptors. This was further suggested by kinetic studies of aminoisobutyric acid transport which indicated that wheat germ agglutinin was probably affecting both the Vmax and Km of transport, whereas concanavalin A affected only the Vmax. While the mechanism by which concanavalin A and wheat germ agglutinin produce opposing effects on amino acid transport is not clear, since the two lectins appear to be interacting with different surface receptors we would suggest that they are perturbing microanatomically and functionally different domains on the lymphocyte plasma membrane.

Lymphocyte activation (1) is characterized by the selective uptake of a variety of simple molecules including ions (2-5), sugars (6, 7), nucleosides (8), and amino acids (9, 10). Removal or omission of amino acids in the incubation medium results in inhibition of mitogenesis (11-14) suggesting an important requirement for exogenous amino acids at one or more stages in the transformation response. When studies are performed during the first 2 to 8 hours of lectin stimulation, increases in amino acid uptake are confined to amino acids transported by a Na+-dependent mechanism (e.g. asparagine, glutamine, and glycine) (15). The nonmetabolizable amino acid, aminoisobutyric acid, interacts with monosaccharides, shares this transport system and can be used to examine Na+-dependent transport as an isolated response uninfluenced by subsequent alterations in intracellular amino acid metabolism (16). In murine lymphocytes, the magnitude of the early increase in aminoisobutyric acid transport is closely correlated with eventual changes in DNA synthesis (17).

Much of the previous work on aminoisobutyric acid transport in lymphocytes has been conducted with animal cells and concerned with the response to three mitogenic lectins, purified
erythroagglutinating phytohemagglutinin, a mixture of leu-
koagglutinating and erythroagglutinating phytohemagglutinins (9), and concanavalin A (10). In this paper the effects of these lectins on aminoisobutyric acid uptake will be examined in greater detail and the response compared with that of wheat germ agglutinin, a nonmitogenic lectin. Evidence will be pre-
sented that in contrast to concanavalin A, E-PHA,1 and P-PHA, wheat germ agglutinin inhibits the aminoisobutyric acid response even though it binds effectively to cells and perturbs the cell membrane sufficiently to produce receptor capping and increases in intracellular cyclic AMP.

**Materials and Methods**

**Reagents and Sources—**Concanavalin A (Miles-Yeda), α-methyl-β-
mannoside, and N-acetylglucosamine (Sigma) were dissolved and diluted in 0.1 M NaCl on the day of the experiment. P-PHA (Difco), E-PHA (Burroughs Wellcome), and wheat germ agglutinin (Miles-Yeda) were dissolved in 0.1 M NaCl and stored in small aliquots at -20°C or -80°C for not more than 14 days. Dimeric concanavalin A was prepared by the method of Young (18). Briefly, 200 mg of tetrameric concanavalin A was dissolved in 15 ml of 0.05 M barbital buffer (pH 8.0). Then 100 mg of solid maleic anhydride were added in 10-mg aliquots with continuous stirring at 4°C. The pH was maintained at 8.0 by sequential addition of 0.2 M NaOH. After a total incubation time of 1 hour the solution was dialyzed extensively against distilled water and lyophilized. The sample was then dissolved in phosphate-buffered saline (0.15 M NaCl, 0.01 M phosphate, pH 7.4) and applied to a Sephadex G-75 (Sigma) column. Retained protein was washed extensively with phosphate-buffered saline and eluted with 0.1 M α-methyl-
β-mannoside. The overall recovery, based on absorbance at 280 nm, was 80%. The product was shown to have a molecular weight of 50,000 to 60,000 by molecular sieve chromatography on Bio-Gel P-100 (Bio-Rad), in accord with the predicted molecular weight for dimeric concanavalin A of 54,000. Aggregated wheat germ agglutinin was prepared by a method similar to that described by Lotan et al. for soybean agglutinin (19) which involves a cross-linking reaction with glutaraldehyde. Wheat germ agglutinin, 1 mg, trace labeled with 3 x 10^6 cpm of 125I, labeled wheat germ agglutinin (see below) was dissolved in 0.05 ml of phosphate-buffered saline, pH 7.4, and applied to a Sephadex G-75 (Sigma) column. Retained protein was washed extensively with phosphate-buffered saline and eluted with 0.1 M α-methyl-
β-mannoside. The overall recovery, based on absorbance at 280 nm, was 80%. The product was shown to have a molecular weight of 50,000 to 60,000 by molecular sieve chromatography on Bio-Gel P-100 (Bio-Rad), in accord with the predicted molecular weight for dimeric concanavalin A of 54,000. Aggregated wheat germ agglutinin was prepared by a method similar to that described by Lotan et al. for soybean agglutinin (19) which involves a cross-linking reaction with glutaraldehyde at neutral pH. Wheat germ agglutinin, 1 mg, trace labeled with 3 x 10^4 cpm of 125I, labeled wheat germ agglutinin (see below), was dissolved in 0.08 ml of phosphate-buffered saline and reacted with 0.03% (v/v) glutaraldehyde for 1 hour at room tempera-
ture. The reaction mixture was chromatographed on Sephadex G-75 (Sigma) and the protein excluded from the resin was stored at 4°C. The cross-linked wheat germ agglutinin represented approximately 10% of the total eluted radioactivity and absorbance at 280 nm. The estimated molecular size of the product was 64,000 as determined by molecular sieving on Bio-Gel P-100 (Bio-Rad).

Latex spheres of 300 Å average diameter containing reactive carboxy-
ly and hydroxyl groups were generously provided by Dr. Alan Reina-
baum and Dr. William Dreyer at the California Institute of Technology. The spheres were reacted with ε-aminoacrylate and coupled with wheat germ agglutinin using a water-soluble carbodiimide in the first step and glutaraldehyde in the second step as described for the binding of goat anti-rabbit Igs to these particles by Molday et al. (20).

Concanavalin A and wheat germ agglutinin were labeled with 35S by the method of Hunter and Greenwood (21) at final chloromine-T concentrations of 0.33 and 0.19 mg/ml, respectively. Unconjugated radioiodine was removed by gel filtration on Bio-Gel P-10 (Bio-Rad) and the 35S-concanavalin A was further purified by affinity chromatography on Sephadex G-25 (Sigma) followed by elution with 0.1 M α-methyl-β-mannoside and dialysis. The final specific activities ranged between 2.7 and 10.0 rCi/mg for 125I-concanavalin A and 81.0 to 100.0 μCi/mg for 35S-labeled wheat germ agglutinin.

Solutions of unlabeled aminoisobutyric acid (Sigma) for adjusting the final total concentration of this amino acid were prepared in 0.1 M NaCl and stored at 4°C. The cell incubation medium was Eagle’s minimal essential medium with glutamine supplemented with Earle’s salts (Gibco) containing 2% (v/v) heat-inactivated (56°C for 30 min) AB serum. The pH of the incubation medium was adjusted to 7.4 with 0.05 M NaOH.

**Purification of Lymphocytes—**Human peripheral lymphocytes were purified from heparinized venous blood of normal volunteer donors by dextran sedimentation and isopycnic centrifugation over a Ficoll-
Hypaque mixture as previously described (22). On the average 70% of the original lymphocyte cell number was recovered, and by morphologic criteria 92 to 98% of the nucleated cells were lymphocytes and of those 20 to 25% were stained with fluorescein-labeled rabbit anti-human γ-globulins and were therefore considered to be B cells. Platelets normally numbered less than 3/lymphocyte; cell viability was rou-
tinely greater than 98% as estimated by trypan blue exclusion (23). In selected experiments essentially pure lymphocytes (<1% contaminating nucleated cells) were prepared by passing Ficoll/Hypaque-
purified lymphocytes over a short nylon wool column as previously described (22). Of these cells, 1 to 5% stained positively with fluorescein-labeled rabbit anti-human γ-globulin; therefore, this cell population was considered to be primarily composed of T cells.

**Aminoisobutyric Acid Transport—**Aminoisobutyric acid transport was measured over various time periods during the final 2 to 60 min of a 1- to 7-hour incubation at 37°C using a modification of the techniques of Mendelsohn et al. (9) and Van den Berg and Betel (10). All experimental conditions were examined in triplicate. The various stimulants or 0.1 M NaCl were added to glass test tubes (10 x 100 mm) in a total volume of 100 μl. The lymphocyte cell suspension (400 μl) at a density of 12.5 x 10^6/ml of incubation medium was added to each tube. The cells were incubated at 37°C without agitation in a 95% O2/5% CO2 atmosphere for the desired period of time under the described conditions. As a rule, aminoisobutyric acid was used at a final concentration of 0.2 mM. The incubation was stopped by addition of 2 ml of ice-cold phosphate-buffered saline containing unlabeled aminoisobutyric acid (0.15 M NaCl, 0.01 M phosphate, and 0.01 M aminoisobutyric acid, pH 7.4, Buffer A). The cells were centrifuged at 1200 x g for 5 min and washed with an additional 2 ml of Buffer A. The cell pellets were resuspended in 500 μl of distilled water and disrupted by sonication. Samples were then quantitatively transferred to scintil-
lation vials with 10 ml of counting solution (Instagel, Hewlett-Packard) and assayed in an automatic liquid scintillation counter (Searle).

**Cyclic Nucleotide Measurements—**Cyclic AMP and cyclic GMP were analyzed in lymphocyte extracts by radiomnnoassay as previously described (24, 25). In most experiments measurements were made on extracts of boiled and sonicated cells. In selected experiments the cyclic nucleotides were purified prior to assay by extraction of the entire cell suspension with 0.5% perchloric acid followed by neutralization with 3 M Tris base, adsorption with neutral alumina (Sigma), and chromatography on Ag 2-8X (Bio-Rad).

**Capping—**Wheat germ agglutinin receptor capping was evaluated using a fluorescein-labeled lectin preparation (Miles-Yeda, substitu-
tion ratio = 0.7) at a final concentration of 25 μg/ml. Lymphocytes (3 x 10^6/ml incubation medium) were added to small glass tubes (13 x 100 mm) in a total volume of 100 μl of Gey’s solution, pH 7.4. The cells were incubated for 60 min at room temperature in the presence of the fluorescein-labeled wheat germ agglutinin. The cells were then placed on ice and layered over heated fetal calf serum gradients and centrifuged at 2000 x g for 1 min. The wash procedure was repeated, followed by resuspension of the cell pellets in 1 drop of heated fetal calf serum, transfer to glass microscope slides, and evaluation of fluorescent patterns under a Leitz ortholux fluorescent microscope.

**Lectin Binding—**Concanavalin A and wheat germ agglutinin bind-
ing to lymphocytes was studied by the method of Phillips et al. (26). Briefly, 100 μl of a lymphocyte suspension (12.5 x 10^5 cells/ml incubation medium) were added to small glass tubes containing desired concentrations of radiolabeled lectins in a volume of 40 μl. The cells were incubated for varying periods of time at 37°C. Unbound lectin was removed by centrifuging the cells at 12,000 x g for 30 s through 200-μl gradients of heated fetal calf serum formed in standard microfuge tubes (Beckman). Cell-associated radioactivity was assayed by counting the cell pellets in an automatic gamma counter (Searle).

**Viability Studies—**Cell viability in purified lymphocytes exposed to various concentrations of radiolabeled lectins was measured by trypan blue (index of cell death) (23) and the number of cells which successfully deacylated fluorescein diacetate (an index of cell viability) (27).

**Error Analysis—**When appropriate, the means of triplicate determinations were compared using Student’s t test for unpaired data (28). Unless otherwise stated, error bars in all figures indicate standard deviations obtained from triplicate determinations.

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1 The abbreviations used are: PHA, phytohemagglutinin; E-PHA, erythroagglutinating phytohemagglutinin; P-PHA, mixture of leukoagglutinating and erythroagglutinating phytohemagglutinin.
RESULTS

On the basis of previous studies in human and animal lymphocytes it has been concluded that mitogenic lectins increase both the initial rate of aminoisobutyric acid uptake and the eventual steady state level, both being about 3-fold higher than in resting cells. This effect appears to be through changes in the $V_{max}$ rather than the $K_m$ of aminoisobutyric acid transport (9, 10). In accord with the earlier results, when cells were incubated for 2 hours or longer with E-PHA, P-PHA, or concanavalin A and then exposed to 0.2 mM amino-$[^{14}C]$-isobutyric acid for 30 min, there was a severalfold increase in cell-associated radioactivity (Fig. 1). A representative time course of aminoisobutyric acid uptake in concanavalin A-stimulated and control lymphocytes is shown in Fig. 2. In this and other experiments cell-associated radioactivity increased in an essentially linear fashion over a 20- to 30-min time period both in the presence and absence of lectin with less rapid increases thereafter. Mendelsohn et al. have previously reported that in PHA-stimulated human lymphocytes the increase in aminoisobutyric acid uptake was linear for greater than 20 min (9). A steady state was not approached until the incubation had been allowed to proceed in the presence of aminoisobutyric acid for at least 60 min. Based on an estimated average cell diameter of 7.5 μ and a water content of 75% of the cell volume, the calculated intracellular to extracellular concentration ratios after achievement of a steady state were 8 to 12:1 for lectin-stimulated cells and 2 to 3:1 for control cells. In accord with the results of other investigators (9, 10, 15), both stimulated and unstimulated lymphocytes incubated at 4° or at 37° in the presence of metabolic inhibitors (KCN or NaN₃) accumulated very little aminoisobutyric acid (<10% of the normal response at 37°), indicating minimal effects due to simple diffusion. Preincubation with unlabeled aminoisobutyric acid at concentrations as high as 8 mM did not alter the rate of aminoisobutyric acid uptake, either in the presence or absence of PHA, indicating that relatively little exchange diffusion was taking place. Nonetheless, in most experiments, including the one shown in Fig. 2, zero time values obtained by extrapolation were slightly above the origin, possibly due to low levels of diffusion or contamination with extracellular aminoisobutyric acid. Since these effects were small no correction was applied and in most subsequent experiments aminoisobutyric acid uptake was examined at either 10 min, in the middle of the initial linear uptake phase, or 30 min, at or just after the completion of this phase.

With all three lectins maximal stimulation of amino-$[^{14}C]$-isobutyric acid uptake occurred at lectin concentrations in the 10 to 50 μg/ml range (shown for concanavalin A, Fig. 3), somewhat above the usual optimal mitogenic concentrations for these agents (2 to 25 μg/ml, depending on the culture conditions) (Fig. 3); however, substantial stimulation of transport was observed at these lower mitogenic concentrations.

Changes in aminoisobutyric acid uptake were not demonstrated until the cells had been in contact with mitogenic lectin for at least 60 to 120 min as illustrated in the experiment with concanavalin A shown in Fig. 4. The response increased with longer periods of incubation eventually peaking at 18 hours and declining at 48 and 72 hours. These slowly developing changes in transport are not solely due to increasing numbers of lectin molecules being bound as the culture is continued although
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Concanavalin A binding does continue to increase between 30 and 240 min in some experiments (see below).

In accord with the studies of Van den Berg and Betel in rat lymphocytes (10), the increase in aminoisobutyric acid transport observed with concanavalin A was partially blocked by 50 mM α-methyl-D-mannoside (up to 90% inhibition), a known inhibitor of concanavalin A binding (29) (Fig. 5A). Since other monosaccharides were noninhibitory, it can be assumed that α-methyl-D-mannoside is producing its inhibition by competing with cell surface receptors for the specific carbohydrate binding sites on concanavalin A. The inhibition was most marked when α-methyl-D-mannoside was present throughout the entire incubation period (Fig. 5B). Nearly maximal inhibition was observed with additions of the monosaccharide inhibitor at any time during the first 2 hours of incubation.

Later additions of α-methyl-D-mannoside resulted in only partial inhibition of the response with stimulation values plateauing at the level present in cells at the time of addition of inhibitor. Thus once increases in aminoisobutyric acid transport have occurred the response is not immediately reversed by α-methyl-D-mannoside although further increases in transport are prevented and the response returns to control levels within several hours (not shown). The delay in reversal of concanavalin A stimulation is probably due to a progressive diminution in the ability of the monosaccharide to release concanavalin A from the cell surface, since approximately the same quantity of radiolabeled concanavalin A remained on the cell after elution with the sugar at 4 hours as after elution at 1 hour (Table I).

Table I

<table>
<thead>
<tr>
<th>Total incubation time</th>
<th>Time of addition of</th>
<th>Concentration of</th>
<th>Molecules of concanavalin A bound</th>
<th>Inhibition of binding</th>
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<tr>
<td>min</td>
<td>α-methyl-D-mannoside</td>
<td>μg/ml</td>
<td>(× 10^9)/cell</td>
<td>%</td>
</tr>
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<td>50</td>
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<td></td>
</tr>
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<td>1.0</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>60</td>
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<td>0.4</td>
<td>36</td>
<td></td>
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<tr>
<td>60</td>
<td>30</td>
<td>12.5</td>
<td>53</td>
<td></td>
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<td>50</td>
<td>2.4</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>12.5</td>
<td>1.1</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>210</td>
<td>0.6</td>
<td>68</td>
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</table>

Fig. 4. Time course of concanavalin A-stimulated aminoisobutyric acid (AIB) transport. Five million lymphocytes were incubated at 37° with 0.1 M NaCl or concanavalin A (25 μg/ml) for time periods noted on the abscissa and then exposed to amino[A^14C]isobutyric acid (0.2 mM final concentration) for an additional 30 min at 37°.

Fig. 5. A, concanavalin A-stimulated aminoisobutyric acid (AIB) transport in the presence of 0.05 M α-methyl-D-mannoside, 0.01 M N-acetylglucosamine, or 0.15 M NaCl. The sugars or 0.15 M NaCl were present throughout the 4.5-hour incubation and amino[A^14C]isobutyric acid (0.2 mM AIB final concentration) was added for the final 30 min. α-Methyl-D-mannoside alone did not affect the aminoisobutyric acid response; however, concentrations of N-acetylglucosamine in excess of 0.02 M did produce modest stimulation. B, time course of α-methyl-D-mannoside (0.05 M) inhibition of concanavalin A (Con A)-induced changes in aminoisobutyric acid transport. All conditions were examined for aminoisobutyric acid accumulation after 4.5 hours of incubation with concanavalin A (25 μg/ml) at 37° with labeled amino acid present during the final 30 min. α-Methyl-D-mannoside was added at varying times during this incubation as noted along the abscissa. Inhibition is expressed as a percentage of the response observed in matched controls which received 0.15 M NaCl instead of 0.05 M α-methyl-D-mannoside. C, inhibition of aminoisobutyric acid transport with the nonmitogenic lectin wheat germ agglutinin. Lymphocytes were incubated for 4.5 hours at 37° with varying concentrations of wheat germ agglutinin and 0.15 M NaCl, wheat germ agglutinin and 0.05 M α-methyl-D-mannoside, or wheat germ agglutinin and 0.05 M N-acetylglucosamine. Modest stimulation (~50%) of aminoisobutyric acid transport with N-acetylglucosamine is seen at low wheat germ agglutinin concentrations. No stimulation and complete blocking of the wheat germ agglutinin inhibition is observed at 0.01 M concentrations of this sugar.
In contrast to the increase in aminoisobutyric acid transport observed with the three mitogenic lectins, wheat germ agglutinin, a nonmitogenic lectin (31), failed to stimulate aminoisobutyric acid transport and in fact produced a marked inhibition of the response over a wide range of aminoisobutyric acid concentrations (Fig. 5C). This inhibitory effect persisted despite an extension of the incubation period to 8 hours or challenge of cells with a broad range of wheat germ agglutinin concentrations. The diminution in amino acid uptake was blocked by N-acetylglucosamine but not by α-methyl-D-mannoside (Fig. 5C). Since N-acetylglucosamine is bound selectively by wheat germ agglutinin (32) and concentrations of N-acetylglucosamine as low as $1 \times 10^{-4}$ M partially reversed the effect of wheat germ agglutinin on transport, it seemed likely that the monosaccharide was acting by inhibiting wheat germ binding. This was confirmed by direct binding studies with radiolabeled wheat germ agglutinin. N-Acetylglucosamine alone produced a 1.5- to 2.0-fold stimulation of aminoisobutyric acid uptake but only at concentrations in excess of $2 \times 10^{-3}$ M. The basis for the increase in aminoisobutyric acid uptake at high concentrations of N-acetylglucosamine is currently under investigation.

The wheat germ agglutinin binding study further indicated that the failure of wheat germ agglutinin to stimulate aminoisobutyric acid uptake was not due to ineffective binding to cells. At wheat germ agglutinin concentrations of 25 μg/ml, lymphocytes contained an average of $3 \times 10^7$ wheat germ agglutinin molecules/cell, well above the level of lectin binding required to produce marked stimulation in the concanavalin A system. Nor was the inhibition due to a failure to obtain sustained wheat germ agglutinin binding since at 25 μg/ml there was still $> 2.5 \times 10^6$ lectin molecules/cell after 4 hours at 37° and 85% of the cell-bound radioactivity was elutable with N-acetylglucosamine, indicating that most of the wheat germ agglutinin was still localized on the cell surface (not shown).

When lymphocytes were exposed to wheat germ agglutinin and concanavalin A in combination, the mitogen-induced transport response was markedly diminished (Fig. 6). Additions of wheat germ agglutinin as late as 3 hours after initiation of concanavalin A stimulation resulted in significant decreases in the concanavalin A response (Table II).

Since wheat germ agglutinin as it normally exists in neutral aqueous solution is divalent (33, 34) and concanavalin A is tetravalent (30) one possible explanation for their different effects on aminoisobutyric acid transport is through differences in valence. Divalent concanavalin A was used to evaluate this possibility. As shown in Fig. 7, divalent concanavalin A produced marked stimulation of aminoisobutyric acid uptake demonstrating that the inhibition is not due to the difference in valency per se. Interestingly, divalent concanavalin A did not produce a high dose diminution in stimulation similar to that seen with tetravalent concanavalin A. Similar observations have been made in studies of concanavalin A-induced mitogenesis where tetravalent and divalent preparations both are stimulatory but only tetravalent concanavalin A produces high dose inhibition (35).

Changes in cell surface charge density in areas of lectin binding might conceivably exert important effects on plasma membrane transport thereby explaining the difference in wheat germ agglutinin and concanavalin A action. Wheat germ agglutinin contains 40 glutamyl and aspartyl residues and only $5 \times 10^8$ cells/30 min.

Lymphocytes were incubated for 270 min at 37° with aminoisobutyric acid (0.2 mM final concentration) added in the final 30 min.

### Table II

<table>
<thead>
<tr>
<th>Concanaualin A</th>
<th>Wheat germ</th>
<th>Time of</th>
<th>Promomoles</th>
</tr>
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<tbody>
<tr>
<td>μg/ml</td>
<td>μg/ml</td>
<td>addition</td>
<td>of cell-associated</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>0</td>
<td>aminoacidic acid/</td>
</tr>
<tr>
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<td>50</td>
<td>0</td>
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<tr>
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<td>518 ± 51</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>240</td>
<td>1100 ± 21</td>
</tr>
</tbody>
</table>

![Fig. 6. Aminoisobutyric acid (AIB) transport in lymphocytes exposed to concanavalin A (Con A) and wheat germ agglutinin (WGA) or combinations of these lectins. Lymphocytes were incubated for 4.5 hours at 37° with lectin, N-acetylglucosamine (N-Ac-GluN, 0.01 M), or 0.1 M NaCl present throughout the experiment and aminoisobutyric acid (0.2 mM final concentration) added in the final 30 min.](http://www.jbc.org/content-pdf/4021/F6)

![Fig. 7. Dose response curve of aminoisobutyric acid (AIB) transport with dimeric and tetrameric concanavalin A. Dimeric concanavalin A was prepared as described under "Materials and Methods." Aminoisobutyric acid transport was measured during the final 30 min of a 270-min incubation with tetrameric concanavalin A, dimeric concanavalin A, and dimeric concanavalin A + 0.05 M α-methyl-D-mannoside.](http://www.jbc.org/content-pdf/4021/F7)
15 lysyl, histidyl, and arginyl groups (36) making it a highly negatively charged protein at neutral pH (30). Concanavalin A is electronegative at neutral pH (37) but is considerably less so than wheat germ agglutinin. However, since divalent concanavalin A, which is extensively substituted with maleyl groups and is even more negatively charged than wheat germ agglutinin, was stimulatory, differences in charge density per se do not appear to provide the basis for wheat germ agglutinin inhibition.

Since divalent concanavalin A and wheat germ agglutinin have different molecular structures and carbohydrate binding specificities, the possibility was considered that wheat germ agglutinin might be unable to stimulate amino-isobutyric acid uptake because of an inability to cross-link carbohydrate residues on the cell surface. This might occur because of an unfavorable steric relationship between the two carbohydrate binding sites of wheat germ agglutinin or because of peculiarities in the distribution of specific receptors for this lectin on the cell membrane. There is substantial evidence that cross-linking of membrane receptors is necessary to evoke the capping response (38-40) providing a means of evaluating the ability of lectins to interact multivalently with cells. In the present study wheat germ agglutinin was observed to produce both patching and capping of appropriate receptors in human lymphocytes making it very probable that these lectin molecules undergo bivalent interactions at the surface of these cells as well. Approximately 90% and 40% of the lymphocytes exhibited patched and capped membrane receptors, respectively, under the conditions described. The capping but not the patching response was blocked by the metabolic inhibitor valinomycin (10^-4 M).

Despite the apparent ability of the usual molecular form of wheat germ agglutinin to interact bivalently with the lymphocyte surface, it seemed possible that polymerized preparations of this lectin might perturb the lymphocyte membrane differently changing a negative to a positive stimulus. This proved not to be the case. Wheat germ agglutinin, which had been extensively cross-linked with glutaraldehyde, agglutinated lymphocytes much more readily than dimeric wheat germ agglutinin, but still produced a diminution in amino-isobutyric acid uptake (Table III). Furthermore, this diminution was blocked by N-acetylglucosamine (10^-4 M). Taking this and the capping data together it would appear that the qualitatively different amino-isobutyric acid responses to wheat germ agglutinin and concanavalin A are not due to differences in the way in which the binding sites are arranged on the two lectins.

As a further means of elucidating why wheat germ agglutinin and concanavalin A affect amino-isobutyric acid transport differently and even are mutually antagonistic when both lectins are present, the physical relationship of concanavalin A and wheat germ agglutinin binding sites on one another on the lymphocyte plasma membrane was studied. Since carbohydrate residues accessible to lectins are presumably limited to small segments of glycoprotein molecules protruding from the lipid bilayer, if concanavalin A and wheat germ agglutinin were binding to the same glycoproteins they would probably compete with one another for binding. When wheat germ agglutinin was used in an attempt to block concanavalin A binding to lymphocytes either by simultaneous mixing or preincubation experiments, little or no competition was found suggesting that they are indeed acting through different glycoproteins (Fig. 8). Since neither concanavalin A nor wheat germ agglutinin contains carbohydrate (33, 34, 41) the failure to observe inhibition should not be due to concanavalin A binding to cell-bound wheat germ molecules in a “piggy back” fashion. Concanavalin A binding to wheat germ agglutinin was further excluded by mixing radiolabeled wheat germ agglutinin and unlabeled concanavalin A in aqueous solution in the absence of cells and evaluating for formation of complexes. By gel filtration on Bio-Gel P-100, no evidence for an altered distribution of radioactivity was found. Since wheat germ agglutinin has a molecular weight of 36,000 (34, 41) and concanavalin A is 110,000 (42), a partial redistribution of radioactivity should have occurred if association was taking place. In addition, when cells were preincubated with mixtures

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>+0.15 M NaCl</th>
<th>+ N-Acetyl-gluco-</th>
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</thead>
<tbody>
<tr>
<td>Wheat germ agglutinin (10 μg/ml)</td>
<td>34,600 ± 5,100</td>
<td>59,900 ± 10,200</td>
</tr>
<tr>
<td>Wheat germ agglutinin (20 μg/ml)</td>
<td>39,800 ± 2,000</td>
<td>61,000 ± 1,400</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>47,200 ± 1,000</td>
<td>55,900 ± 6,700</td>
</tr>
<tr>
<td>Cross-linked wheat germ agglutinin</td>
<td>38,500 ± 4,800</td>
<td>55,900 ± 6,700</td>
</tr>
<tr>
<td>Cross-linked wheat germ agglutinin</td>
<td>39,800 ± 2,000</td>
<td>61,000 ± 1,400</td>
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TABLE III

Amino-isobutyric acid transport response to cross-linked wheat germ agglutinin

Amino-[14C]isobutyric acid transport was measured during the final 30 min of a 240-min incubation as described under "Materials and Methods" with the exception that the final reaction volume was 150 μl instead of 500 μl, accounting for the increase in the absolute number of cell-associated counts. N-Acetylglucosamine (10^-2 M) has been shown to produce little or no stimulation of the amino-[14C]isobutyric acid response. Data are expressed as the mean number of counts per min of amino-[14C]isobutyric acid obtained from triplicate determinations ± S.D.
of concanavalin A and wheat germ agglutinin using a radiolabel on one but not both of the lectins, N-acetylglucosamine eluted up to 85% of the wheat germ agglutinin radioactivity but less than 15% of the concanavalin A radioactivity (not shown). As expected, α-methyl-D-mannoside eluted 50 to 75% of the 125I-concanavalin A radioactivity and only 10% of the 125I-labeled wheat germ agglutinin radioactivity in these experiments.

The relationship between the wheat germ agglutinin and concanavalin A binding sites was further examined by conjugation of the lectins to latex particles 300 Å in diameter. If the concanavalin A and wheat germ agglutinin binding sites are on the same receptors, then the introduction of the latex particle coupled to lectin might sterically interfere with the binding of the other lectin. However, no competition was observed between the wheat germ agglutinin-latex conjugate and soluble 125I-concanavalin A (Table IV). In contrast, the wheat germ agglutinin conjugates produced inhibition of soluble 125I-labeled wheat germ agglutinin binding. This is presumptive evidence for a substantial spatial separation of the major binding sites for these two lectins, although a limited level of binding to common surface receptors cannot be entirely excluded. Moreover, it must be kept in mind that soluble wheat germ itself inhibits binding so the inhibition by wheat germ latex particles does not necessarily involve interference by the particles themselves.

As a further means of comparing the effects of wheat germ agglutinin and concanavalin A on amino acid transport the kinetics of aminoisobutyric acid transport were studied (Fig. 9). Tetrameric concanavalin A increased the V max of transport for aminoisobutyric acid while not changing the K m in accord with earlier studies (9, 10) (Fig. 9A). Furthermore the K m and V max of transport determined were 2.0 mM and 1.0 nmol/10 6 cells/10 min (uncorrected for nonlinearity, see above) both of which are in close agreement with the studies of Mendelsohn et al. in human lymphocytes (9). Not unexpectedly, concanavalin A acted in the same way. Thus, the magnitude but not the mechanism of the transport response was altered by these lectins. Wheat germ agglutinin appears to increase the K m of aminoisobutyric acid transport (Fig. 9B) although since the changes are small and the assay is not completely linear, more studies are needed to establish this conclusively. Thus concanavalin A and wheat germ agglutinin seem to be acting by different mechanisms lending indirect support to the argument that the concanavalin A and wheat germ agglutinin receptors are on physically and functionally distinct glycoproteins.

Cyclic Nucleotide Measurements—The results of cyclic AMP measurements are given in Fig. 10. Both wheat germ agglutinin and concanavalin A produced significant increases in intracellular cyclic AMP with little or no change in cyclic GMP. The stimulation of cyclic AMP accumulation by wheat germ agglutinin and concanavalin A followed a similar time course (Table V). The wheat germ agglutinin response was blocked by N-acetylglucosamine (Fig. 10) suggesting that it is being modulated through specific cell surface receptors. The response was seen in the same concentration range at which effects on aminoisobutyric acid are seen (Table V). In addition, the cyclic AMP response to combinations of wheat germ agglutinin and concanavalin A appeared nearly additive sug-

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**TABLE IV**

<table>
<thead>
<tr>
<th>Lectin-latex conjugates and soluble lectin binding</th>
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<tr>
<td>One million lymphocytes in incubation medium (see “Materials and Methods”) were preincubated for 30 min at 37°C with wheat germ agglutinin-latex conjugate (~25 μg of wheat germ agglutinin/mI) or phosphate-buffered saline and then with soluble 125I-concanavalin A (~20,000 cpm/tube) or 125I-labeled wheat germ agglutinin (~30,000 cpm/tube). Derivatized latex beads possessed an average diameter of 300 Å and were conjugated to wheat germ agglutinin as described under “Materials and Methods.” Cells were subsequently layered over heated fetal calf serum gradients formed in microfuge tubes and centrifuged at 12,000 × g for 30 s. Cell-associated radioactivity was determined by assay of the cell pellets.</td>
</tr>
<tr>
<td>125I-Concanavalin A</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Wheat germ agglutinin-latex</td>
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</tbody>
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**Fig. 9.** Kinetic analysis of aminoisobutyric acid (AIB) transport with concanavalin A molecules differing in valency (A) or with wheat germ agglutinin alone and in combination with concanavalin A (B). Uptake of amino[14C]isobutyric acid was studied after a 270-min incubation with the label present for the final 10 min. In A the cells were stimulated with 0.15 M NaCl, dimeric concanavalin A (25 μg/ml), or tetrameric concanavalin A (35 μg/ml). In B the cells were stimulated with 0.1 M NaCl, concanavalin A (25 μg/ml), wheat germ agglutinin (50 μg/ml), or combinations of concanavalin A and wheat germ agglutinin.
gesting the molecules are activating or inhibiting different enzymes (Table V).

**DISCUSSION**

In this study we have demonstrated that the nonmitogenic lectin, wheat germ agglutinin, inhibits aminoisobutyric acid uptake in human lymphocytes, in contrast to the stimulatory effects of the two mitogens, phytohemagglutinin and concanavalin A. Since differences are seen during the first several hours of exposure to lectin, it is apparent that a commitment in regard to stimulation, nonstimulation, or inhibition is made very early in the response. This is in accord with the results of studies of early changes in Ca$^{2+}$ uptake where wheat germ agglutinin again is nonstimulatory but phytohemagglutinin and concanavalin A both produce a positive response.

In addition to confirming the ability of PHA and concanavalin A to produce early increases in aminoisobutyric acid uptake in human lymphocytes (Fig. 1), our studies provide new information on the time course and modulation of the response by lectin. In concanavalin A-stimulated cells maximal increases in the rate of aminoisobutyric acid uptake were seen at about 6 hours, the monosaccharide produced near maximal inhibition even when it was absent during the first 1 to 2 hours of exposure to lectin (Fig. 5A). Thus, despite the relative rapidity with which increases in aminoisobutyric acid uptake occur in the course of lectin stimulation, it is apparent that concanavalin A must remain on the lymphocyte surface for at least 1 to 2 hours in order for an increase to occur. The need for sustained stimulation by lectin in the aminoisobutyric acid response is similar to what has been observed for concanavalin A stimulation of DNA synthesis. Maximal stimulation of DNA synthesis is not obtained unless the lectin is present in the culture for at least 18 to 20 hours after 6-methyl-D-mannoside is added (45). These observations argue against a mechanism of lectin action in which a transient initial stimulus programs the cell for a complex series of metabolic alterations culminating in increased DNA synthesis 24 hours or more later.

In contrast to the sustained requirement for lectin when the aminoisobutyric acid response was initiated, once the response was well underway (addition of the inhibitory sugar after 3 or 4 hours) an increase in the rate of aminoisobutyric acid uptake continued at its previous levels for at least several hours before inhibition is seen. This is understandable if it is assumed, as the data of Van den Berg and Betel suggest, that new protein synthesis is necessary for the expected increase in aminoisobutyric acid uptake to occur (10). If the proteins being synthesized are part of the aminoisobutyric transport system and they persist for some time after the stimulus to increased aminoisobutyric transport is withdrawn, there could be a considerable delay in the appearance of the 6-methyl-D-mannoside inhibition. Alternatively, it is possible that a subpopulation of cell-bound concanavalin A molecules becomes progressively more difficult to displace with 6-methyl-D-mannoside as the time of incubation is increased. However, the binding data suggest that this is probably not the case (Table I).

While the basis for the inhibitory effects of wheat germ agglutinin on aminoisobutyric acid uptake is not presently clear, they do not appear to be due to overt toxicity. The prevention or reversal of the inhibition by N-acetylglucosamine suggests that wheat germ agglutinin must interact selectively with cell surface receptors before it can initiate its effects, but we cannot exclude a mechanism in which wheat germ agglutinin aggregates cell surface receptors, is taken into the cell by endocytosis, and produces its inhibitory effects somewhere in the cytoplasm or nucleus. Whatever the mechanism, the effects of wheat germ agglutinin are not limited to aminoisobutyric acid transport. Wheat germ agglutinin, alone or in combination...
Opposing Effects of Lectins on Lymphocyte Activation

with mitogenic lectins, also inhibits other parameters of cell activation including DNA synthesis (46, 47), phosphatidylinositol turnover (48), and membrane protein phosphorylation. Taking the results of the binding and metabolic studies together, it is apparent that wheat germ agglutinin interacts to a significant extent with the lymphocyte plasma membrane but for some reason produces a qualitatively different response than E-PHA, P-PHA, or concanavalin A. Interestingly, preliminary studies indicate a second nonmitogenic plant lectin isolated from Agaricus bisporus (a generous gift from Dr. H. Sage) also inhibits aminoisobutyric acid transport both alone and in combination with concanavalin A or E-PHA. Thus, inhibition of amino acid transport and other early parameters of lymphocyte activation may be a property of certain other nonmitogenic lectins in addition to wheat germ agglutinin.

It will be of interest to determine whether receptors for negative modulatory lectins such as wheat germ or mushroom agglutinin or positive modulatory lectins such as E-PHA or concanavalin A are randomly dispersed or fall within common domains. While random dispersion would seem to be predicted by the present lipid bilayer models for membrane structure (49), our data raise the interesting possibility of a mosaic membrane organization in which positive and negative modulation of lymphocyte function is exerted through discrete stimulatory and inhibitory domains on the lymphocyte surface.

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Opposing effects of mitogenic and nonmitogenic lectins on lymphocyte activation. Evidence that wheat germ agglutinin produces a negative signal.
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