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INHIBITION OF HUMAN LYMPHOCYTE PROLIFERATION BY THE NONMITOGENIC LECTIN WHEAT GERM AGGLUTININ

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DNA synthesis in human lymphocytes stimulated with mitogenic lectins (purified phytohemagglutinin, Con A, or pokeweed mitogen) or antigen (streptokinase-streptodornase) is markedly inhibited by the nonmitogenic lectin wheat germ agglutinin (WGA). In contrast, WGA fails to inhibit DNA synthesis in cells stimulated with a calcium ionophore, A23187, or heavy metal mitogen, mercuric chloride. The inhibition by WGA in lectin or antigen stimulated cells is: 1) reversible with *N*-acetyl-D-glucosamine (NAG), a known inhibitor of WGA binding, 2) not associated with excessive cellular cytotoxicity, 3) dose related, occurring at concentrations greater than 15 micrograms/ml, 4) effective only with additions of WGA during the initial 24 to 48 hr of culture, and 5) present in cell populations highly enriched for either T or B cells. Interestingly, Con A-activated lymphocytes elaborate a soluble immune suppressor supernatant (SISS) that contains a factor(s) of m.w. 30 to 45,000 that binds to the same lymphocyte surface receptors as WGA and functionally recapitulates many of the inhibitory effects of WGA, including decreased mitogen or antigen-induced proliferation. These data suggest the presence of distinct glycoprotein membrane receptors for suppressor signals that contain NAG residues. The interaction of appropriate ligands with these receptors produces an effective intracellular signal that negatively modulates the immune response.

Plant lectins have served with great utility in immunobiology as highly specific probes of the cell surface (1, 2), potent agglutinating agents (3), and polyclonal stimulants of lymphoid cells (4–6). These lectins have been classified as mitogens or nonmitogens, based on their ability to augment DNA synthesis and produce blastic transformation in isolated lymphocyte populations (7, 8). The mitogens, purified phytohemagglutinin (P-PHA)[2] and concanavalin A (Con A), and nonmitogens, wheat germ agglutinin (WGA) and *Agaricus bisporus* lectin, each participate in high affinity, multivalent interactions with defined receptors on the external plasma membrane of lymphocytes (9–11). In contrast to nonmitogens, binding of mitogenic lectins stimulates a generalized activation of the cell surface as reflected in early enhanced transport of cations (12, 13), sugars (14), amino acids (15, 16), increased cyclic nucleotide accumulation (17, 18) and membrane protein phosphorylation (19), and later as changes in protein (20), RNA (21), and DNA synthetic rates (7, 8, 22). The biochemical ineffectiveness of the nonmitogenic lectins suggested that these plant proteins interact with the cell surface in a neutral manner; however, this is certainly not the case. Prior studies with WGA by the authors (16, 23) and others (24, 25) have demonstrated that this lectin alone and in combination with the mitogens Con A and P-PHA produces marked inhibition of early parameters of lymphocyte activation, including amino acid transport, phospholipid turnover, and membrane protein phosphorylation. In this study we report marked inhibitory effects of WGA on DNA synthesis in stimulated human peripheral blood lymphocytes. In addition, we suggest these effects of WGA are of considerable biologic relevance in understanding mechanisms of immunoregulation, since activated human lymphocytes in culture elaborate a soluble immune suppressor supernatant (SISS) containing a factor(s) that binds to the same membrane receptor as WGA and produces similar inhibition of DNA synthesis.

MATERIALS AND METHODS

P-PHA (Difco), Con A (Miles-Yeda), affinity-purified WGA (Miles-Yeda), pokeweed mitogen (PWM, GIBCO), and streptokinase-streptodornase (SKSD, Lederle) were each prepared in diluent consisting of RPMI 1640 (GIBCO) supplemented with penicillin (50 units/ml), streptomycin (100 μg/ml), and L-glutaminine (2.0 mM) and stored at −20°C for not more than 2 weeks before used. Mercuric chloride (Sigma) was dissolved in diluent as needed. *N*-acetyl-D-glucosamine (NAG, Sigma) and α-methyl-D-mannoside (Sigma) were prepared as 500 mM solutions in diluent and stored at 4°C. The calcium ionophore, A23187 (Calbiochem), was dissolved in dimethylsulphoxide (Sigma) at a concentration of 10 mg/ml and stored at −20°C with further dilutions in diluent on the day of use. The mushroom lectin obtained from *Agaricus bisporus* was a generous gift from Dr. Harvey Sage.

SISS was prepared by incubation of human peripheral blood mononuclear cells in medium containing 10% (v/v) heat-inactivated (56°C for 30 min) pooled human A+ serum at 37°C for 48 hr in a humidified atmosphere in the presence of Con A (10 μg/ml). The supernatants of these cultures were collected, centrifuged at 1200 × G, passed through 0.45-μ filters (Millipore), aliquoted, and stored at −80°C until used. It should be noted that these supernatants almost certainly contain a complex array of biologically active factors, including migration

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[2] Abbreviations used in this paper: WGA, wheat germ agglutinin; P-PHA, purified phytohemagglutinin; SKSD, streptokinase-streptodornase; PWM, pokeweed mitogen; NAG, *N*-acetyl-D-glucosamine; SISS, soluble immune suppressor supernatant.

inhibitory factor, interferon, and leukocyte inhibitory factor. We have used the acronym SISS only to identify and emphasize an activity in this supernatant that appears to be quite different from those previously described.

Residual Con A in the SISS preparations was partially removed by two approaches in selected experiments. Serial absorption of SISS with packed Sephadex G-50 (1:1 v/v) for 30 min at 37°C with continuous rocking) produced an 85% reduction in Con A after six absorptions as indicated in experiments with ^{3}H-Con A (New England Nuclear, specific activity 73.5 Ci/mM). Similarly, an approximate 85% reduction in residual Con A was obtained by replacing the supernatant of cultures with fresh medium at 24 hr and continuing the incubation for an additional 24 to 48 hr. Both of these procedures resulted in suppressive supernatants that contained less than 1.6 μg/ml of Con A. Addition of equivalent amounts of soluble Con A to P-PHA proliferation assays produced no inhibition and in fact was routinely stimulatory. Furthermore, SISS effects in P-PHA proliferation were not affected by α-methyl-D-mannoside, an inhibitor of Con A binding. Therefore, the suppressive effects of SISS were not due to the Con A remaining in the supernatant.

Purification of lymphocytes. Human peripheral blood lymphocytes were purified from heparinized (10 units/ml, preservative free, Upjohn) venous blood of healthy adult volunteers obtained on the day of experimentation by a modification of the method of Boyum (26). Briefly, venous blood was diluted 1:1 (v/v) in a minimal essential medium prepared without added calcium or magnesium and overlayed on Ficoll-diatrizoate discontinuous gradients formed in sterile conical 15-ml plastic tubes (Falcon). Gradients were centrifuged at 1500 × G for 30 min at room temperature followed by removal of the lymphocyte-enriched interface with a sterile pipet. Lymphocytes were subsequently washed three times, counted, and suspended in diluent supplemented with 20% (v/v) heat-inactivated (56°C for 30 min) pooled human A+ serum at a cell density of 2×10^6/ml. Cell viability was routinely greater than 98% as assessed by a supravital dye exclusion technique (27).

Long-term T cell (MOLT-4, CEM) and Epstein-Barr-transformed B cell (HB-3, Schiff) lines were kindly provided by Dr. T. Uchiyama. Sezary cells were prepared as described above from venous blood of a patient with Stage IV-B Sezary Syndrome (greater than 90% leukemic cells) admitted to the National Cancer Institute for therapy.

Lymphocyte proliferation. One hundred-microliter aliquots of the cell suspension were dispensed into individual flat bottom microtiter wells (Falcon) containing desired additives or diluent. Microtiter plates were incubated for 3 to 7 days at 37°C. in a humidified atmosphere containing 5% CO_2. During the final 5 hr of incubation, 1 μCi of ^{3}H-methyl-thymidine (New England Nuclear, specific activity 25 Ci/mM) was present in each well. Cells were harvested onto fiberglass filters with a multiple channel automated cell harvester and washed repeatedly with buffer. Cell-associated radioactivity was measured by transfer of filters to glass vials containing 10 ml of scintillant (Biofluor) and counting in an automated counter (Beckman). In each experiment all experimental and control conditions were performed in at least triplicate. Error bars appearing in all figures represent standard errors of the arithmetic means. All figures and tables shown present results obtained in representative experiments performed at least three times.

^{3}H-WGA binding. Two hundred thousand lymphocytes suspended in diluent were incubated for 30 min at 37°C in microtiter wells in the presence of P-PHA (2.5 μg/ml) dilutions of SISS, NAG (50mM), or diluent, followed by addition of ^{3}H-WGA (New England Nuclear, specific activity 18.7 Ci/mM, approximately 1×10^5 cpm/well) in the presence of unlabeled WGA at a final concentration of 10 μg/ml. Cells were incubated for an additional 30 min at 37°C and harvested as described above. Filters obtained from wells containing no cells retained less than 0.05% of added radioactivity.

RESULTS

As shown in Figure 1, proliferation of human lymphocytes exposed to optimal concentrations of the three mitogenic lectins P-PHA, Con A, and PWM (not shown) and the antigen SKSD was markedly inhibited in the presence of the nonmitogenic lectin WGA. These inhibitory effects occurred in 41 of 41 experiments with lymphocytes obtained from several unrelated donors on different days. In the presence of these agents, WGA (20 to 50 μg/ml) routinely produced greater than 50% inhibition of stimulated thymidine uptake obtained in cells incubated with mitogenic lectin or antigen alone. The inhibition by WGA was reversed by 80 to 95% when NAG (50 mM) was added to the cultures in accordance with the known saccharide binding specificity of WGA (28).

In contrast to the results obtained with mitogenic lectins and antigen that initiate activation of lymphocytes through defined

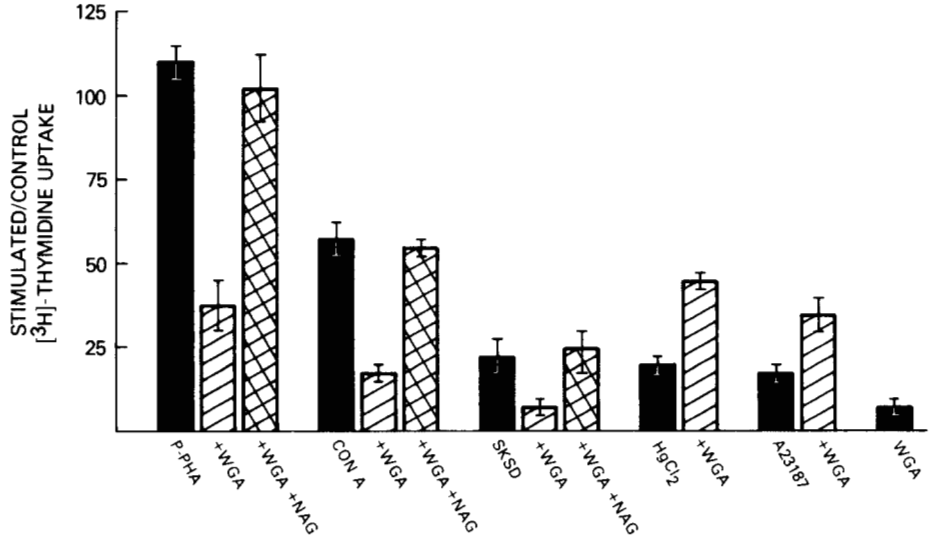

Figure 1. Wheat germ agglutinin effects on DNA synthesis in lymphocytes stimulated with various activating agents. Lymphocytes were incubated at 37°C with optimal mitogenic concentrations of P-PHA (2.5 μg/ml), concanavalin A (25 μg/ml), SKSD (100/25 units/ml), mercuric chloride (2.5×10^{-5} M), and the calcium ionophore A23187 (0.25 μg/ml) alone and in combination with wheat germ agglutinin (WGA, 20 μg/ml) with and without *N*-acetyl-D-glucosamine for 3 to 7 days followed by measurement of DNA synthesis as described in the *Materials and Methods* section. Uptake of radiolabeled thymidine is shown as a ratio of values obtained in stimulated and control cells.

surface receptors, WGA failed to inhibit, and in fact augmented, proliferation in cells stimulated with the calcium ionophore A23187 or heavy metal mitogen mercuric chloride (Fig. 1). These agents, although effective in producing early and late biochemical changes characteristic of lymphocyte activation (29–34), do not appear to act through classical surface membrane receptors.

WGA inhibition was not associated with increased cellular cytotoxicity. In cultures of cells exposed to combinations of P-PHA (2.5 μg/ml) and WGA (25 μg/ml) and assayed for trypan blue positivity at 12, 24, 48, and 72 hr, the percentage of dead cells was 1%, 2%, 4%, and 10%, respectively. These changes were essentially identical to those found in cells exposed to P-PHA alone. Furthermore, the incubation of ^{51}Cr-labeled human P-PHA-induced lymphoblasts for 6 hr with WGA (25 μg/ml) failed to produce excess chromium release compared with cells incubated without WGA (not shown). In addition to measurements of radiolabeled thymidine uptake in 72-hr cultures of cells with WGA (25 μg/ml) and P-PHA, cell counts were performed after thorough washing of the microtiter wells. The number of cells recovered from cultures receiving WGA was routinely 30 to 60% of that obtained with cultures receiving only P-PHA; however, this number was 85 to 120% of the cell number initially added to each culture, suggesting a cytostatic rather than cytotoxic effect for WGA.

As seen in Figure 2, concentrations of WGA greater than 5 to 10 μg/ml were required for effective inhibition of P-PHA-induced DNA synthesis (20 of 22 experiments). The effect of WGA was mediated through its saccharide binding site as shown by the near complete reversal with NAG (50 mM) but not with equimolar concentrations of α-methyl-D-mannoside. Similar WGA dose-response relationships were observed with Con A, PWM, and SKSD (not shown). In several but not all experiments, low concentrations of WGA (1 to 5 μg/ml) produced augmentation of mitogen response that was also blocked in the presence of NAG. A similar augmentation of proliferation with low WGA concentrations in rat thymocytes have been reported by Karsenti and co-workers (24).

Limited experiments with a second nonmitogen, *Agaricus bisporus* lectin, suggested similar inhibition of mitogen-induced proliferation. Although possessing a different saccharide specificity, this lectin appears to bind to at least some of the same membrane glycoproteins recognized by WGA in porcine lymph node cells (35), and competes with WGA for binding to the major WGA receptor present on human peripheral blood lymphocytes (Greene, W. C., unpublished data).

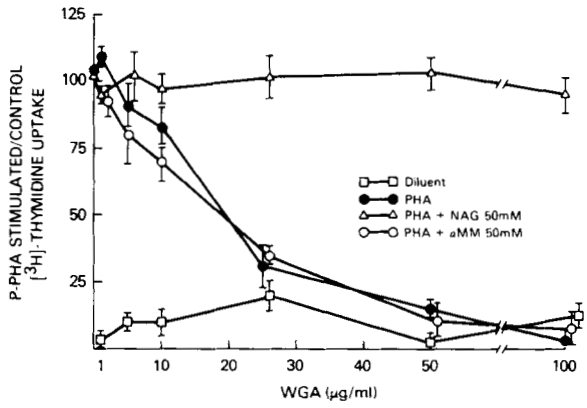

Figure 2. Dose-response relationship and saccharide specificity for WGA inhibition of lymphocyte proliferation. Lymphocytes were cultured with varying concentrations of WGA alone (□——□), WGA + P-PHA (2.5 μg/ml, ●——●), and WGA + P-PHA and *N*-acetyl-D-glucosamine (50 mM, △——△) or alpha-methyl-D-mannoside (aMM, 50 mM, ○——○) as described above. Uptake of radiolabeled thymidine is shown as a stimulation ratio.

Inhibition of DNA synthesis by WGA occurred at concentrations of P-PHA (20 to 100 μg/ml) or Con A (50 to 200 μg/ml) in excess of optimal mitogenic doses, suggesting that inhibition was not secondary to displacement of bound mitogenic molecules from surface receptors by WGA (three of three experiments, not shown). Furthermore, prior studies have shown that WGA and Con A interact with different membrane receptors that are spatially separated by at least 300 Å (16).

In contrast to the inhibitory effects of WGA obtained in combination with mitogenic lectins or antigen, WGA alone failed to reproducibly inhibit thymidine uptake below basal levels (14 of 14 experiments, Fig. 2). This result was unexpected in view of prior studies in human lymphocytes showing inhibition by WGA alone of phospholipid turnover (23), amino acid transport (16), and membrane protein phosphorylation (25). In rodent thymocytes, very high concentrations of WGA (50 to 200 μg/ml) were required to inhibit basal DNA synthesis (24). In contrast to WGA, the second nonmitogen, *Agaricus bisporus* lectin, produced approximately 50% inhibition of basal DNA synthesis. In the present study, WGA produced only 2- to 25-fold increases in DNA synthesis compared with 100- to 2000-fold changes with mitogenic lectins. Notwithstanding, this stimulation was quite reproducible and inhibited in the presence of NAG. Other investigators have identified subpopulations of lymphocytes that bear differing numbers of WGA receptors with differing affinities (36–39). It is quite possible that WGA stimulates one subset of the cells while inhibiting others. In this regard, we have recently obtained preliminary evidence that low concentrations of WGA serve as an effective B cell mitogen and can stimulate mixtures of T and B cells to produce large amounts of immunoglobulin (Greene, W. C., and Waldmann, T. A., unpublished data).

As shown in Figure 3, WGA effectively inhibited P-PHA-induced increases in DNA synthesis only if present during the initial 24 to 48 hr of a 72-hr proliferation assay (six of seven experiments). Additions of WGA after 48 hr were routinely without significant effect. These data suggest that WGA interferes with a step in the activation sequence that occurs moderately early or alternatively generates a negative signal for proliferation that requires 24 to 36 hr for expression. The data certainly do not suggest a cytotoxic mechanism of action or direct effects on the intracellular transport of thymidine.

In order to assess whether cells once exposed to WGA were irreversibly inhibited, preincubation-washout experiments were performed (Table I). Briefly, lymphocytes were preincubated for 24 hr at 37°C in the presence of inhibitory concentrations of WGA (25 or 50 μg/ml) or diluent and subsequently washed repetitively with medium or medium supplemented with NAG (50 mM) to remove membrane-associated WGA. Previous studies with radiolabeled WGA indicate this procedure in the presence of NAG removes approximately 90% of the surface-bound radioactivity (Greene, W. C., unpublished data). Washed lymphocytes were subsequently stimulated with P-PHA for 72 hr, and DNA synthesis was measured as described above. As seen in Table I, the inhibitory effects of WGA were entirely abrogated by washing in the presence of NAG (three of three experiments). These cells, however, remained responsive to the effects of WGA, since, as shown, addition of WGA produced the expected inhibition of DNA synthesis in the presence of P-PHA. These data indicate that WGA effects are reversible, not

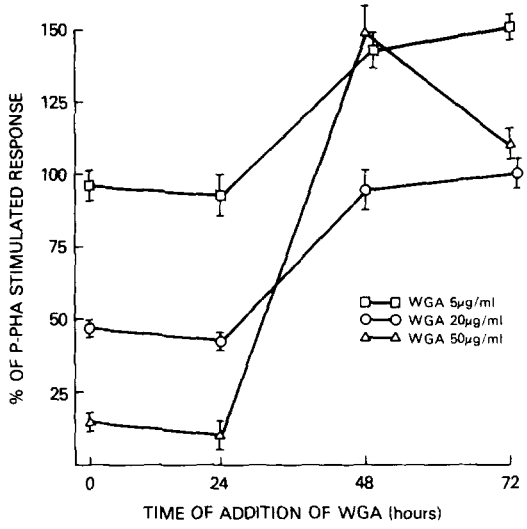

Figure 3. Time course of delayed additions of varying concentrations of WGA in P-PHA stimulated lymphocyte proliferation. Lymphocytes were incubated for 72 hr at 37°C in the presence of P-PHA (2.5 μg/ml) with additions of WGA (50 μg/ml, △——△); (20 μg/ml, ○——○); and (5 μg/ml, □——□) at time periods noted on the abscissa.

associated with cell death, and provide further support for WGA action at the level of the plasma membrane rather than through internalization and remote effects on intermediary metabolism as has been described for the toxic lectin, ricin (40).

Although large numbers of WGA receptors have been demonstrated on each of the principal cells participating in the immune response (T cells, B cells, and macrophages) (39), thereby presumably providing for a direct mechanism of action, the possibility that WGA interacted with a subset of cells to produce a separate inhibitory factor was investigated. Supernatants of cells incubated for 12 to 48 hr with varying concentrations of WGA were examined in the presence of NAG for inhibitory effects on DNA synthesis. No inhibitory activity was found, (three of three experiments, data not shown); however, these findings must be qualified, in that the authors have recently identified a suppressor factor (SISS) produced by human lymphocytes that is subject to inhibition by NAG. Activity of this and similar factors would not have been detected in this assay system.

A second series of experiments with delayed additions of varying concentrations of NAG to cells stimulated with combinations of P-PHA (1 μg/ml) and WGA (25 μg/ml) are shown in Figure 4. Additions of the inhibitory saccharide at any time within the initial 24 hr of culture blocked WGA effects, whereas additions at periods later than 48 hr produced only minimal reversal (five of five experiments). NAG was effective over a broad concentration range (5 to 100 mM) and in selected experiments, enhanced DNA synthesis was obtained in the presence of low concentrations of NAG (1–5 mM).

To further characterize the nature of WGA interaction with lymphoid cells, two T cell lines (MOLT-4, CEM), two virally transformed B cell lines, and Sezary leukemia cells were studied with WGA alone or in combination with P-PHA (Table II). In the four long-term transformed cell lines, WGA alone or with mitogen failed to produce marked changes in DNA synthesis in contrast to its effects on normal peripheral blood lymphocytes. The lack of effect was not due to ineffective lectin binding, since these cells were readily agglutinated by WGA and in addition other authors have identified WGA receptors on the surface of MOLT-4 cells (41). Unlike the cell lines examined, lectin-stimulated DNA synthesis in Sezary cells was negatively modulated in the presence of WGA; however, in contrast to normal cells, WGA alone produced substantial proliferation. These findings are currently under further investigation with cells from other patients with this cutaneous T cell leukemia. The lack of inhibition in the presence of binding obtained with both the B and T cell lines again argues against a cytotoxic mechanism of WGA inhibition or alterations in thymidine transport.

In view of recent reports by Boldt *et al.* (36, 39), Hellstrom *et al.* (37), and Brouet and Chevalier (38) reporting WGA-agarose affinity techniques to isolate functionally distinct subpopulations of lymphocytes, further experiments with highly purified T and B cells were performed. As shown in Figure 5, T cells demonstrated a WGA dose-response relationship for WGA inhibition of mitogen-stimulated DNA synthesis essentially identical to that found in peripheral blood lymphocytes (five of six experiments). Furthermore, WGA alone produced considerably less thymidine uptake in these cells than was obtained in peripheral blood lymphocytes. In contrast, as shown in Table III, WGA in low concentration produced significant proliferation of highly enriched B cell preparations (three of five experiments). Similarly, as noted above, low concentrations of WGA alone can stimulate lymphocytes to produce large quantities of immunoglobulin. Interestingly, combinations of B cells and irradiated T cells (2000 R; able to provide help for B cells but unable to divide (42)) stimulated with P-PHA remained responsive to negative modulation by WGA, which was most pronounced at high WGA concentrations (Table III). As was found in proliferation, mixtures of T and B cells activated to produce immunoglobulin with the T-dependent polyclonal stimulant, PWM, are inhibited by high concentrations of WGA, whereas low concentrations have variable effects, with enhancement of immunoglobulin production occurring in several experiments. Taken together, these data suggest substantial differences in cellular responses depending upon WGA concentration.

TABLE I

Reversibility of WGA effects in DNA synthesis[a]

Preincubation	Wash	Culture	^{3}H-Thymidine Uptake (cpm)
Medium	Medium	Diluent	180 ± 80
		P-PHA	153,837 ± 9,889
		P-PHA + WGA (50)	365 ± 21
		P-PHA + WGA (25)	1,160 ± 66
		WGA (50)	277 ± 71
		WGA (25)	1,942 ± 367
Medium	NAG	Diluent	343 ± 79
		P-PHA	125,607 ± 4,836
		P-PHA + WGA (50)	92,127 ± 2,187
		P-PHA + WGA (25)	165,023 ± 17,051
WGA (50)	NAG	Diluent	1,008 ± 240
		P-PHA	148,306 ± 6,877
WGA (25)	NAG	Diluent	491 ± 21
		P-PHA	199,823 ± 21,644

[a] Lymphocytes were preincubated in medium or medium-containing WGA (50 μg/ml (50), 25 μg/ml (25)) at 37°C for 24 hr, then washed three times in medium or medium containing NAG (50 mM). Cells were then further incubated under wash conditions with diluent, P-PHA (2.5 μg/ml) alone or in combination with WGA for an additional 72 hr before assay of ^{3}H-thymidine uptake over a 5-hr period. Data shown represent mean cpm of triplicate determinations ± S.E.M.

Since high- and low-affinity WGA receptors have been described on lymphoid cells (37, 39), and since stimulatory effects of WGA are largely obtained at low lectin concentrations that would select for high-affinity interactions, it is possible that WGA generates a positive signal via the high-affinity receptor and a negative signal through its low-affinity interactions.

Recently, increasing interest has focused on an array of soluble mediators that function to amplify or suppress cellular and humoral immunity (43-48). Of particular interest to the authors has been the identification of a soluble factor or factors (SISS) present in the supernatant of human mononuclear cells cultured with Con A that inhibits T cell proliferative responses to antigens and mitogens. Careful studies have shown that the inhibitory effects of SISS on T cell proliferation are blocked in part by NAG but not by a variety of other monosaccharides, including α-methyl-D-mannoside, L-fucose, gentiobiose, L-rhamnose, and cellobiose. It seemed plausible that both WGA and SISS were interacting with the same lymphocyte surface receptors and that this receptor-ligand interaction negatively modulated T cell proliferation. As shown in Table IV, SISS effectively competed with WGA in a dose-related manner for binding sites on the cell surface (five of six experiments). Similar competitive binding data were obtained in cells incubated at 4°C, in which SISS would not induce artifacts in WGA binding secondary to effects on capping, endocytosis, or receptor shedding. Mock suppressor supernatants prepared in the absence of con A produced no change in WGA binding, nor did they consistently produce inhibition of DNA synthesis or immunoglobulin biosynthesis.

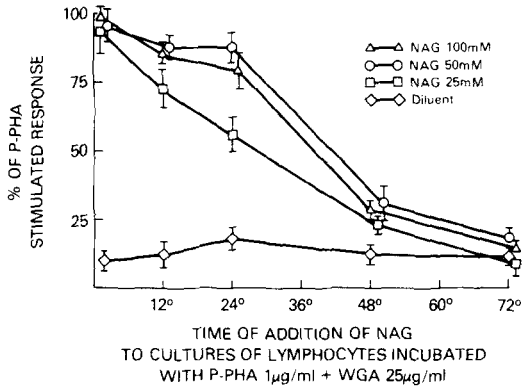

Figure 4. Time course for delayed additions of *N*-acetyl-D-glucosamine in lymphocytes stimulated with P-PHA (1 μg/ml) and WGA (25 μg/ml). Reversal of WGA effects by NAG (100 mM, △——△); (50 mM, ○——○); and (25 mM, □——□) and lack of effect of diluent (◇——◇), is expressed as a percentage of the response obtained in cells incubated with P-PHA (1 μg/ml). Additions of NAG over a similar concentration range did not substantially affect the P-PHA response (not shown).

DISCUSSION

When stimulated with mitogenic lectins or antigens, human peripheral blood lymphocytes undergo a complex sequence of early and late biochemical alterations that collectively comprise lymphocyte activation (4). This sequence is initiated at the level of the plasma membrane and then proceeds by not-well-understood mechanisms to involve the remainder of the cell culminating after several rounds of DNA synthesis and cell division in the emergence of clones of specialized effector cells mediating cellular and humoral immunity. Recent interest has focused on mechanisms through which this activation process is regulated. Immunoregulatory functions for cyclic nucleotides (4, 17, 18, 25), calcium (13, 32, 34), microtubules (49), and microfilaments (50, 51) have each been proposed.

In this study we report interesting negative modulatory effects of WGA on DNA synthesis in stimulated human lymphocytes. WGA is a nonmitogenic (or weakly mitogenic) lectin of m.w. 36,200 (52) with binding specificity for NAG residues and polymers of this saccharide (28, 53). The inhibitory effects of WGA on DNA synthesis were dose related, reversed by NAG, not associated with cytotoxicity, seen only if the lectin was present at time periods moderately early in the culture period, and did not occur in lymphocytes stimulated with A23187 or mercuric chloride.

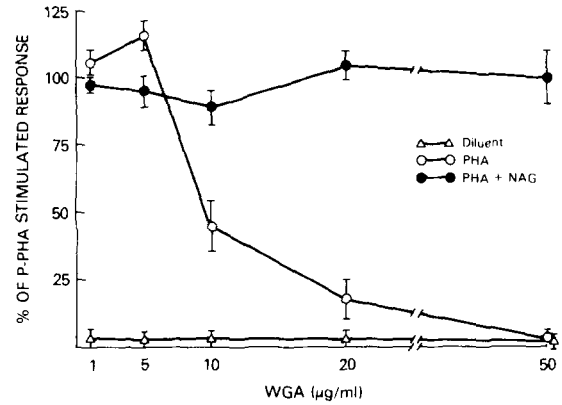

Figure 5. Wheat germ agglutinin dose-response relationships with and without *N*-acetyl-D-glucosamine in highly enriched human T cells. T cells were prepared as previously described (see 68). Briefly, peripheral blood lymphocytes were passed over a Sepharose-Fab' anti-human immunoglobulin immunoabsorbent column. Nonretained cells were subsequently incubated with washed sheep red blood cells and rosetted cells recovered by density centrifugation. This cell population consisted of greater than 98% T cells. Retained cells were eluted with soluble human immunoglobulin, thoroughly washed, and used as a highly enriched B cell population (see Table III). T cells were incubated with WGA alone (△——△), P-PHA (1 μg/ml) + WGA (○——○), or P-PHA + WGA + NAG (50 mM, ●——●). Results are expressed as a percentage of thymidine transport obtained in cells stimulated with P-PHA.

TABLE II

WGA effects on long term B and T cell lines and Sezary leukemic cells[a]

	MOLT-4	CEM	Sezary	HB-3	Schiff
Diluent	184,496 ± 17,208	242,856 ± 4,100	239 ± 20	48,459 ± 3,740	92,569 ± 6,165
WGA (20)	140,064 ± 8,589	259,599 ± 6,226	19,748 ± 1,534	37,048 ± 8,682	74,045 ± 3,860
WGA (10)	188,980 ± 12,577	260,025 ± 14,033	7,239 ± 307	41,016 ± 3,705	85,902 ± 6,763
P-PHA (2.5)	147,936 ± 489	244,090 ± 29,077	25,902 ± 1,595	46,824 ± 2,987	71,380 ± 780
P-PHA + WGA (20)	168,973 ± 7,076	180,666 ± 13,708	12,076 ± 589	45,445 ± 2,301	60,123 ± 12,109
P-PHA + WGA (10)	158,117 ± 10,252	242,254 ± 6,592	17,514 ± 2,605	41,699 ± 2,799	75,334 ± 7,828

[a] Lymphocytes from two long-term T cell lines (MOLT-4, CEM), two Epstein-Barr transformed B cell lines (HB-3, Schiff), and Sezary leukemia cells were incubated for 72 hr with combinations of agents as shown above and assayed for DNA synthesis. Parentheses enclose microgram/milliliter concentrations of reagents employed. Data shown represent mean cpm of triplicate determinations ± S.E.M.

TABLE III

Wheat germ agglutinin effects on human T and B cell proliferation[a]

	^{3}H-Thymidine Uptake		
	B cells	T cells	B cells + irradiated T cells + P-PHA
	cpm		
Diluent	422 ± 186	326 ± 48	39,043 ± 920
WGA 50 μg/ml	2,875 ± 733	N.D.[b]	227 ± 16
WGA 20 μg/ml	14,795 ± 767	3,272 ± 514	1,330 ± 184
WGA 10 μg/ml	35,632 ± 716	1,391 ± 111	4,857 ± 919
WGA 5 μg/ml	30,941 ± 4,274	259 ± 28	28,819 ± 4,118
WGA 1 μg/ml	6,145 ± 738	127 ± 21	35,157 ± 3,889
WGA 0.1 μg/ml	760 ± 131	128 ± 22	37,201 ± 1,868

[a] B and T cells were prepared as described in the legend to Figure 5 and proliferation assayed as described in the *Materials and Methods* section. Irradiated T cells were prepared by exposure to 2000 R. Proliferation was measured at 7 days for B cells, 3 days for T cells, and 5 days for B + irradiated T cells. Prolongation of T cell incubations to 5 and 7 days failed to result in increased thymidine incorporation (not shown). B cell + irradiated T cell experiments were conducted with 5×10^4 cells of each type in the microtiter wells in the presence of P-PHA (1 μg/ml). Irradiated T cells cultured alone incorporated 202 ± 8 cpm. Data shown represent mean cpm of ^{3}H-thymidine uptake ± S.E.M.

[b] Not determined.

Several features of the inhibition by WGA of lymphocyte proliferation seem clear. First, WGA does not appear to produce inhibition by stimulation of a discrete subpopulation of cells to secrete inhibitory factors of cellular proliferation as has been described for cells activated with mitogenic lectins (45, 47). Inhibitory activity was not detected in the supernatants of lymphocytes incubated for varying times with several concentrations of WGA. Similarly, there is no evidence for the induction of suppressor cells as is seen with Con A, since lymphocytes pulsed with WGA for 48 hr do not suppress immunoglobulin biosynthesis of lymphocyte co-cultures in a PWM-driven reverse hemolytic plaque assay (Fleisher, T. and Greene, W. C., unpublished data). Furthermore, the striking effects of WGA on early biochemical events in activation, many of which occur within minutes after exposure to this lectin, suggest a direct rather than inducing mechanism of action for WGA.

Secondly, WGA effects on lectin-stimulated DNA synthesis do not occur through inhibition of mitogen binding. In this study we have shown inhibitory effects of WGA on proliferation induced by three lectins with differing carbohydrate specificities and a soluble antigen. It is quite unlikely that WGA inhibits binding of each of these differing agents to their respective membrane receptors. In direct binding studies by the author, soluble WGA failed to inhibit radiolabeled Con A or P-PHA binding to lymphocyte surface receptors (16). Additionally, WGA complexed via a water soluble carbodiimide reaction to 300 Å latex beads was ineffective in producing effects on Con A binding, suggesting substantial spatial separation of these receptors on the lymphocyte membrane (16). Furthermore, in guinea pig and mouse lymphocytes, Con A and WGA receptors do not co-cap togehter (54, 55); and in Novikoff tumor cells, separate glycoprotein receptors for WGA and Con A have been identified (56).

A direct effect exerted beyond the level of binding appears to represent a likely mechanism of WGA action. WGA produces inhibitory effects on a variety of early biochemical changes occurring in mitogen-stimulated cells. These include reduced phospholipid turnover (23), amino acid transport (16), membrane protein phosphorylation (25) and calcium transport (32). Interestingly, in regard to calcium uptake, WGA negatively modulates ^{45}Ca uptake stimulated with mitogenic lectins, however, fails to affect increased transport of this cation obtained with A23187 (32). These findings parallel the results reported in this paper regarding DNA synthesis.

Despite opposing effects of the mitogenic lectins and WGA on most biochemical events occurring in lymphocyte activation, both classes of agents produce early increases in whole cell cAMP levels (16). Furthermore, in lymphocytes stimulated with combinations of Con A and WGA, additive increases in cAMP are obtained, suggesting the possibility of increased activity of separate adenylate cyclases. In most systems studied, cyclic nucleotides exert their biologic effects through activation of protein kinases, which in turn mediate phosphorylation of critical regulatory proteins (57). When phosphorylation of high m.w. membrane proteins is evaluated in human lymphocytes stimulated with con A, P-PHA, N_6-monobutyryl cAMP, and WGA, the mitogens and cAMP analogue induce rapid phosphorylation of several discrete proteins, whereas WGA produces rapid dephosphorylation of similar or identical membrane proteins (25). An attractive hypothesis to explain these differing effects is the selective activation of a cAMP-dependent phosphatase by WGA and a cAMP-dependent protein kinase by the mitogenic lectins; however, direct enzymatic measurements have not been performed to test this hypothesis.

Substantial data exists supporting rather refined compartmentalization of adenylate cyclases in the nucleus, cytoplasm, and plasma membrane of human peripheral blood lymphocytes (4, 58). Stimulation of the adenylate cyclases associated with these different compartments produces contrasting effects on activation and growth of these cells (4). Mitogenic lectins appear to produce a preferential activation of the plasma membrane-associated adenylate cyclase, whereas the nuclear and cytoplasmic compartments are selectively stimulated by epinephrine and prostaglandin E_1, respectively. In contrast to the mitogenic lectins, these latter agents produce inhibition of cell growth and division in lymphocytes (4). It is not yet known which cAMP compartment is stimulated in the presence of WGA. WGA could conceivable produce changes in cytoplasmic or nuclear cAMP levels by virtue of sophisticated membrane-transducing structures that would convey binding information to these compartments as opposed to mitogens that produce increases in cAMP localized within or near the plasma membrane. If such membrane-transducing structures exist, microfilaments and microtubules do not appear to be involved, since

TABLE IV

Soluble immune suppressor supernatant (SISS) inhibition of WGA binding[a]

	Molecules of WGA × 10^5 Bound/Cell	% Inhibition of WGA Binding
WGA (10 μg/ml)	3.49 ± 0.24	
WGA + NAG (50 mM)	0.11 ± 0.03	97.1
WGA + SISS (1:2)	1.76 ± 0.09	49.6
WGA + SISS (1:4)	2.06 ± 0.10	41.0
WGA + SISS (1:8)	2.49 ± 0.17	28.7
WGA + SISS (1:20)	3.27 ± 0.24	6.4
WGA + SISS (1:200)	3.60 ± 0.10	−3.2

[a] Lymphocytes (2×10^5 cells/microtiter well) were incubated for 30 min at 37°C in the presence and absence of varying dilutions of the SISS (prepared as described in the *Materials and Methods* section), NAG (50 mM), or diluent. Cells were incubated for an additional 30 min with radiolabeled WGA (10 μg/ml) and harvested as described above. Data shown represent number of molecules bound/cell assuming a m.w. for WGA of 36,200 (52).

WGA effects persist in the presence of cytochalasin B and colchicine, agents that selectively interfere with the function of these cytoskeletal structures (Greene, W. C., unpublished data).

Alternatively, it seems more likely that WGA exerts local effects at the level of the plasma membrane. If true, then there must exist a considerable degree of membrane organization to explain the striking functional differences obtained with the mitogens and WGA. Although the precise manner in which surface receptors, integral membrane proteins, and phospholipids are organized within the membrane remains unresolved, it is clear that previous membrane models of proteins floating randomly in the lipid bilayer require modification (59). Membrane proteins appear to be subject to a variety of long- and short-ranged constraints to lateral mobility, including effects secondary to microtubules, microfilaments, and intermolecular interactions (60–62). In support of increased lymphocyte membrane organization are recent studies demonstrating a nonrandom distribution of surface immunoglobulins on these cells (63, 64); however, it should be noted that artifacts secondary to fixation and processing have not been entirely excluded (65).

We have previously proposed a model for lymphocyte plasma membrane organization consisting of a highly ordered microanatomic mosaic of inhibitory and stimulatory domains associated with mitogenic and nonmitogenic receptors, respectively (16). We would suggest that interaction with each class of receptor results in a rise in intracellular cAMP; however, since these changes are occurring in different domains within the membrane, functionally opposite cellular responses may result. The studies of Horwitz *et al.* (66) in 3T3 cells suggesting that Con A and WGA receptors reside in separate domains of differing lipid composition provides further support for this mosaic model of lymphocyte membrane organization.

The data presented in this paper are in agreement with this model of stimulatory and inhibitory domains within the lymphocyte membrane; however, a modification is required, since it appears that low doses of WGA can produce stimulatory effects. We would suggest that high-affinity WGA receptors may mediate this phenomenon and that these receptors may be segregated into stimulatory rather than inhibitory domains. Furthermore, since WGA appears to preferentially stimulate DNA synthesis in B cells and Sezary leukemic cells, we would suggest that these cells may bear either greater numbers of high-affinity receptors or fewer low-affinity receptors, or alternatively that the nonrandom distribution of WGA receptors is less rigidly maintained in these cells. In this regard, studies are currently underway to investigate high- and low-affinity WGA binding kinetics in purified T, B, and Sezary leukemic cells.

Further studies will also be required to investigate WGA enhancement of A23187 and mercuric chloride induced proliferation. These agents are clearly not subject to negative modulation by WGA, suggesting that they produce activation of cells either by a different mechanism or alternatively stimulate biochemical changes required for activation occurring beyond the site of WGA action. With regard to the mechanism of augmented DNA synthesis, it will be of interest to investigate the effect, if any, of these agents on the number of high-affinity WGA receptors present on the cell surface.

Finally, of considerable biologic interest, we have demonstrated the presence of a factor(s) present in the SISS secreted by activated human lymphocytes or monocytes that binds to the same receptor as recognized by WGA. Preliminary physicochemical evidence indicates that the suppressive activity in SISS is nondialyzable, noncytotoxic, heat labile (50% loss of activity when heated at 56°C for 30 min), and of m.w. 30 to 45,000 as determined by molecular sieve chromatography. SISS produces 40 to 70% inhibition of mitogen-induced proliferation and 70 to 95% inhibition of antigen (SKSD, tetanus toxoid) stimulated DNA synthesis. As is true with WGA, SISS also inhibits proliferation in mixed lymphocyte cultures. SISS-induced inhibition of mitogen proliferation is partially reversed by NAG, and this inhibitory activity is largely retained on chromatographic material composed of NAG immobilized on Agarose bead supports or chitin columns (polymerized NAG obtained from clam shells). SISS activity is detectable in Con A-activated cell supernatants within 24 hr after the addition of lectin, whereas maximal activity is obtained in cultures incubated for 48 to 72 hr. Elaboration of SISS appears to persist in cells irradiated with 500 and 2000 R, respectively, but is abrogated after 6000 R treatment. In addition, adherent cells appear to be required for the production of the inhibitor of proliferation.

In summary, we have reported that the lectin WGA and human suppressor factor(s) SISS have similar and pronounced inhibitory effects on lymphocyte metabolism. Furthermore, data is provided demonstrating that SISS and WGA exert their effects through interaction with the same plasma membrane glycoprotein receptors. Interestingly, increasing evidence for defined saccharide specificities for a number of soluble immunoregulatory molecules has appeared, including fucose and rhamnose inhibition of MIF (migration inhibitory factor) activity, and *N*-acetyl-D-galactosamine inhibition of LIF (leukocyte inhibitory factor) activity (67). It seems reasonable to conclude that studies with lectins of defined saccharide specificity will continue to provide valuable insights into the mechanisms of immunoregulation in human lymphocytes, since these proteins interact with critical receptor molecules also recognized by a variety of soluble lymphokines and cytokines.

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