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Lectin-Mediated Induction of Human Neutrophil Chemotaxis, Chemokinesis, and Cap Formation

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Six lectins, including concanavalin A, phytohemagglutinin P, castor bean I, wheat germ agglutinin, peanut agglutinin, and pokeweed mitogen, were studied for their ability to stimulate human neutrophil locomotion and cap formation. Five of these lectins with known monosaccharide specificities, including concanavalin A, phytohemagglutinin, P, castor bean I, wheat germ agglutinin, and peanut agglutinin, were found to stimulate human neutrophil migration in a modified Boyden assay. Pokeweed mitogen showed negligible activity in the locomotion assay as compared with other lectins. Tests were performed to determine if the observed neutrophil migration in response to lectins was directional, and it was found that concanavalin A, phytohemagglutinin P, and peanut agglutinin were both chemokinetic and chemotactic, whereas castor bean I was only chemokinetic. Wheat germ agglutinin could not be declared chemotactic or chemokinetic due to its tendency to agglutinate neutrophils. Studies with fluoresceinated lectins demonstrated that lectins which stimulate neutrophil migration also bind to neutrophil surfaces. Preincubation with specific monosaccharide ligands blocked both stimulated locomotion and fluorescence, suggesting that an available lectin-binding site was required both for lectin binding and the stimulation of migration. Additional experiments indicated that fluoresceinated concanavalin A, phytohemagglutinin P, castor bean I, wheat germ agglutinin, and peanut agglutinin all induce cap formation on the neutrophil.

The attraction of neutrophils to an inflammatory focus is an important step in host defense against bacterial infection. This attraction requires both the chemotactic factor and cells capable of directional migration in response to this factor. The mechanism of this directional locomotion and the nature of the interaction between the chemotactic factor and the cell membrane is poorly understood.

In general, the regulation of cell function by external factors is mediated by the interaction of these factors with the cell surface (17). A binding of chemotactic factors such as formylmethionine-leucine-phenylalanine (f-met-leu-phe) and casein to cell membrane receptors has been described (23, 26) and is probably responsible for the initiation of directional locomotion. One theory of chemotaxis suggests that the chemotactic factor binds to cell surfaces and initiates a complex series of events which culminate in the mobilization or polarization of chemotactic receptors to the posterior of the migrating cell (19). This process resembles the phenomenon of cap formation originally described with lymphocytes (22) and subsequently shown with neutrophils (15). The movement of cell membrane-bound chemotactic factors has been demonstrated with f-met-leu-phe (J. Niedel, I. Kahane, and P. Cuatrecasas, Fed. Proc. 38:4538, 1979), where factors aggregate and are internalized, as well as with the chemotactic fucose-binding *Lotus* lectin (24), where cap formation is observed.

In a previous study by Van Epps and Tung, fucose-binding lectin from *Lotus tetragonolobus* seeds was found to bind to and induce a chemotactic response in human neutrophils (24). Both chemotaxis and binding were abrogated when *Lotus* lectin was preincubated with L-fucose, its specific monosaccharide ligand. Other lectins such as concanavalin A (ConA) have been shown to induce neutrophil hexose monophosphate shunt activity (20), microtubule assembly (10), lysozomal enzyme release, and chemotactic deactivation (6), much like those changes observed with other common chemotactic factors (2, 6, 7, 18). Recently, Till et al. demonstrated that ConA and phytohemagglutin P (PHA) were chemotactic for human monocytes, rat macrophages, and to a lesser extent, rabbit neutrophils (20).

In this study, we used six different lectins, including ConA, PHA, wheat germ agglutinin (WGA), castor bean I (CBI), peanut agglutinin (PNA), and pokeweed mitogen (PWM), to determine whether various lectins with different

monosaccharide specificities can stimulate human polymorphonuclear leukocyte (PMN) migration and whether lectins capable of stimulating migration also bind to the cell membrane and induce cap formation.

MATERIALS AND METHODS

Neutrophil preparation. Blood from healthy human volunteers was collected in heparinized syringes (10 U/ml). PMNs were isolated by Plasmagel (HTI, Buffalo, N.Y.) sedimentation of erythrocytes (1), followed by Ficoll-Hypaque density gradient centrifugation (3). The resulting cell pellet contained greater than 95% PMNs and was adjusted to 5×10^6 cells per ml in Hanks balanced salt solution (pH 7.2) supplemented with 5% fetal calf serum.

Migration assays. A total of 0.4 ml of a suspension of 5×10^6 PMNs per ml was placed in the upper compartment of a modified Boyden chamber, and 0.25 ml of lectin at various concentrations in the lower chamber was used to stimulate migration. A 5-μm-pore size filter with a thickness of 130 μm (Sartorius, South San Francisco, Calif.) was used to separate the two compartments. Chambers were incubated at 37°C for 1 h in a humidified atmosphere. Filters were fixed in formaldehyde, stained with hematoxylin, and cleared in isopropyl alcohol and xylene (27). Migration was measured with the leading front method, which measures the distance that cells move into a filter (distance to the leading three cells) with the microscope micrometer (27). Neutrophil migration was expressed as a migration index, which is the absolute PMN migration in micrometers observed in response to lectin present in the lower compartment minus the PMN migration in response to Hanks balanced salt solution present in the lower compartment.

In blocking studies, various monosaccharides were incubated with the lectin preparations at room temperature for 30 min, and the mixture was subsequently added to the lower compartment of the chemotaxis chamber. Any direct inhibitory effect of the monosaccharides on PMN movement in general was assessed by adding similar concentrations of monosaccharide to a chemotaxis system mediated by the chemotactic C5 fragment. Chemokinesis studies were performed with the lectin in the upper or lower compartment of the Boyden chamber or with equal concentrations in both compartments.

Reagents. ConA, CBI, WGA, and PNA were obtained from Sigma Chemical Company, St. Louis, Mo. PHA was obtained from Difco Laboratories, Detroit, Mich., and PWM was obtained from GIBCO Laboratories, Grand Island, N.Y. Lectin solutions were prepared in Hanks balanced salt solution without further purification for all assays.

Fucose, fructose, *N*-acetyl-*d*-galactosamine, *N*-acetyl-*d*-glucosamine, *d*-galactose, glucose, and α-methyl-*d*-mannoside (Sigma) were dissolved in Hanks balanced salt solution.

Fluorescein-labeled lectin assays. Fluoresceinated lectins were prepared by incubating lectins (10 mg of lectin per ml) with 8 μg of fluorescein isothiocyanate (FITC) per mg of protein. The solution was made 10% with 0.5 M carbonate buffer (pH 9.5) so that the pH of the FITC-lectin mixture was greater than 9.3. This preparation was then incubated at room temperature for 1 h and dialyzed against phosphate-buffered saline (pH 7.4) (5). This procedure gave fluorescein/protein ratios ranging from 1.6 to 3.6.

In experiments with fluorescein-labeled lectins, 2×10^6 PMNs were incubated for 5 min at room temperature with a subagglutinating dose of FITC-lectin. Cells were then fixed in 2% paraformaldehyde, washed with cold phosphate-buffered saline, and read as wet mounts by fluorescence microscopy. In each case, 100 cells were counted, and the number of cells positive for fluorescence was determined.

Lectin-binding specificity was determined with FITC-lectin in a similar manner except that each preparation of lectin was incubated at room temperature for 30 min with 0.005 M or 0.015 M concentrations of various monosaccharides before incubation with PMNs.

In some instances, the development of fluorescent caps on PMNs incubated with FITC-lectin was evaluated. In these studies, FITC-lectin was incubated with 2×10^6 PMNs in 0.4 ml for 5 min at 4°C, followed by 25 min at 25°C. An aliquot of cells was removed at zero time and every 5 min after the initiation of the 25°C incubation and fixed with 2% paraformaldehyde. These aliquots were washed with cold phosphate-buffered saline and read as wet mounts. A total of 100 cells was observed, and the numbers of cells showing linear patterns of fluorescence, fluorescent caps, or granular staining (fluorescein concentrated in patches and granules) were counted.

RESULTS

Migration studies. The ability of various lectins to stimulate neutrophil migration was evaluated by titrating ConA, PHA, WGA, CBI, PNA, and PWM in the lower compartment of the chemotaxis chamber. The results of these studies are illustrated in Fig. 1 and are expressed as the mean migration index ± the standard error of the mean. PHA, ConA, CBI, WGA, and PNA produced peak responses at 3, 8, 15, 15, and 15 μg/ml, respectively. Neutrophil migration in response to PWM was minimal, with a maximum response of 13 μM at the very highest concentration (125 μg/ml). The titration curves obtained with each lectin were different both in the magnitude of response and the shape of the curve. PHA, ConA, and WGA stimulated neutrophil migration most effectively (mean peak migration indexes of 31, 28, and 25 μm, respectively), whereas CBI and PNA stimulated average peak responses of only 18 μm over the control. PHA and ConA both produced a relatively sharp peak of activity which tapered off rapidly at higher concentrations. Other lectins (CBI, WGA, and PNA) showed less amplitude but tapered off more gradually at higher concentrations. In most cases, higher concentrations of lectin (greater than 125 μg/ml) resulted in de-

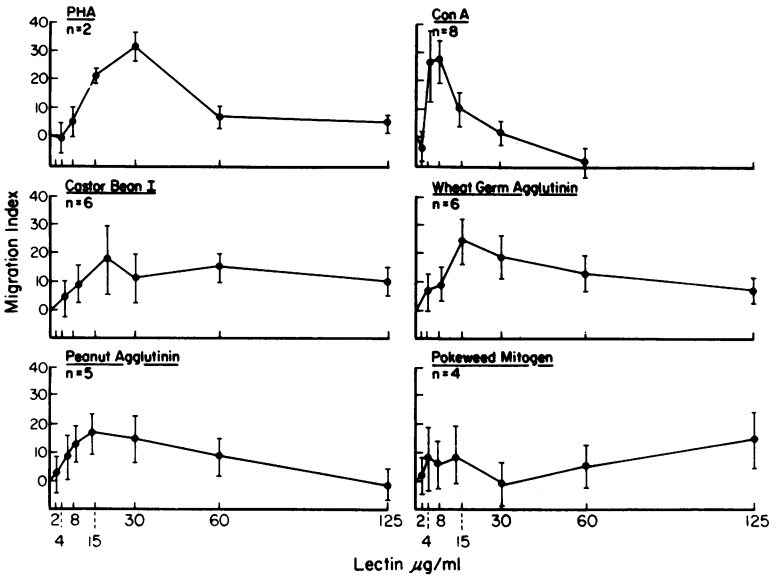

FIG. 1. *Neutrophil migration in response to ConA, PHA, WGA, CBI, PNA, and PWM. The data are presented as the mean migration index ± the standard error of the mean. The lectin in each case was present in the lower compartment of the chemotaxis chamber at the concentration indicated, and n refers to the number of experiments from which each point was derived.*

creased chemotaxis. In all cases, similar titration curves were observed with fluoresceinated lectins.

Demonstration of directional locomotion. Chemotaxis (directional migration) requires a chemotactic factor gradient, whereas chemokinesis (enhanced random migration) does not. For determining if lectin-stimulated migration was directional, neutrophil migration stimulated by various concentrations of lectin in the upper compartment or both compartments of the chemotaxis chamber was measured and compared to neutrophil migration in response to lectin in the lower compartment. The results of these studies (Fig. 2) indicate that ConA, PHA, and PNA can induce directional migration in PMNs because the peak migration observed in the titration curves with lectin present in the lower compartment was always greater than that observed with lectin present in the upper compartment or both compartments. These studies indicating directional locomotion to PNA, PHA, and ConA were confirmed by the Zigmond and Hirsch (27) checkerboard assay method, as shown in Table 1, in which migration in the presence of a positive gradient (below the diagonal) exceeds the theoretical migration due to chemokinesis alone (numbers in parentheses). Similarly, by the checkerboard assay, actual migration in the presence of a negative gradient is generally below the calculated theoretical values for chemokinesis. In these studies, PWM and WGA were not shown because the former produced a minimal response and the latter was found to agglutinate cells when added to the upper compartment. Results show that PHA, ConA, CBI, and PNA are also chemokinetic as indicated by their ability to enhance PMN migration when present in the upper compartment or both compartments. Studies also show that CBI is chemokinetic but not chemotactic because migration in response to lectin in the lower compartment did not exceed that with lectin present in both compartments.

Specificity of lectin-stimulated migration. Experiments were conducted to determine if chemotaxis or chemokinesis was dependent upon an available monosaccharide-binding site on each lectin. In each case, lectins were preincubated with their specific monosaccharide ligands (Table 2) and then tested for activity in the migration assay. If stimulated locomotion is mediated by the binding of lectin to the cell surface through its monosaccharide-binding site, the addition of the appropriate monosaccharide should effectively decrease or eliminate the migration in response to lectin. The results of these studies are shown in Fig. 3 and demonstrate that preincubation of each lectin with its specific monosaccharide ligand significantly reduced the migration of PMNs in response to that lectin. Inhibition was most efficient with the monosaccharide for which each lectin is known to have the greatest affinity (Table 3). The lack of a cell

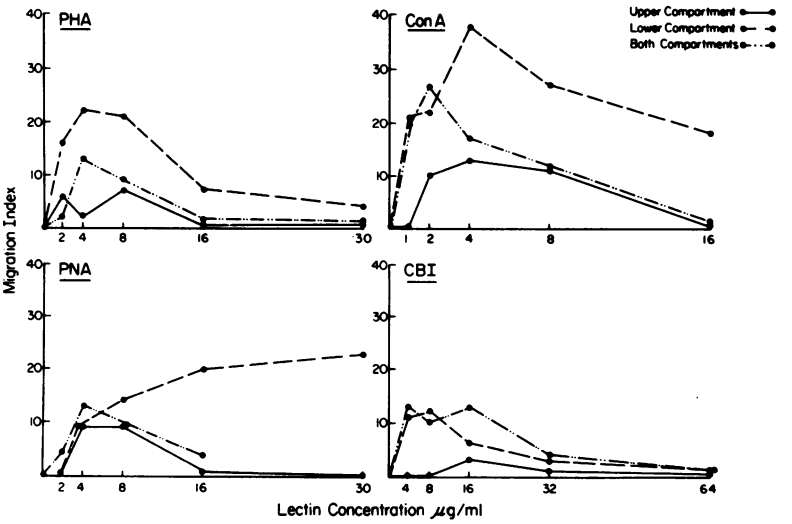

FIG. 2. *Neutrophil migration in response to ConA, PHA, PNA, and CBI when the lectin was present in the lower compartment, the upper compartment only, or both compartments. Neutrophil migration is expressed as a migration index.*

TABLE 1. *Checkerboard assays to determine if PMN migration in response to lectins is chemotactic or chemokinetic*

Lectin in lower compartment	Concn of lectin in lower compartment (μg/ml)	Response to lectin concn (μg/ml) in the upper compartment[a]						
		0	1	2	4	8	16	32
PNA	0	55						
	4				62	69 (65)	60 (83)	62 (87)
	8				70 (65)	74	69 (72)	73 (85)
	16				71 (78)	85 (80)	89	73 (86)
	32				83 (77)	98 (82)	103 (84)	81
PHA	0	43						
	2			55	52 (55)	62 (61)	65 (61)	
	4			69 (55)	55	61 (58)	63 (58)	
	8			71 (62)	62 (64)	66	62 (64)	
	16			68 (58)	63 (60)	78 (57)	56	
ConA	0	76						
	1		84	74 (85)	77 (93)	62 (91)		
	2		89 (85)	86	79 (86)	85 (90)		
	4		93 (90)	89 (91)	94	83 (92)		
	8		101 (91)	89 (88)	98 (91)	88		

[a] Upper compartment lectin was PNA, PHA, and ConA when lower compartment lectin was PNA, PHA, and ConA, respectively. The response is the distance of migration measured in micrometers. Numbers in parentheses represent the theoretical migration attributed to chemokinesis alone as calculated by the method of Zigmond and Hirsch (27).

response to lectin in the presence of the monosaccharide was not due to a direct effect of the monosaccharide on PMN migration because no suppression was observed with any monosaccharides in a chemotactic system mediated by a preparation of the chemotactic C5 fragment. In these studies, PHA produced the most specific response in that *N*-acetyl-*d*-galactosamine blocked lectin-induced migration by 100%, whereas no other monosaccharides inhibited the response. With other lectins some low-grade inhibition of lectin-stimulated migration was observed with more than one monosaccharide. However, the monosaccharide known to have the greatest affinity for each particular lectin was always the most effective inhibitor. These

studies indicate that the lectin must have a free combining site to stimulate PMN migration.

Binding of fluoresceinated lectins to PMNs. Whether chemotactic lectins actually bind to the surfaces of PMNs was studied with fluoresceinated lectins. PMNs were incubated with FITC-lectin for 5 min at room temperature, fixed with 2% paraformaldehyde, washed with phosphate-buffered saline, and observed for fluorescence. The percentage of cells binding FITC-lectin in each case was calculated. The results of these studies (Table 4) demonstrate that ConA, PHA, WGA, CBI, and PNA all bind to neutrophil surfaces to varying degrees. PWM was the only negative lectin, consistent with its negligible activity in the migration assay.

TABLE 2. *Lectins used in this study along with their monosaccharide ligands*

Lectin	Specific monosaccharide ligand
ConA	α-Methyl-*d*-mannoside
PHA	*N*-Acetyl-*d*-galactosamine
WGA	*N*-Acetyl-*d*-glucosamine
CBI	*N*-Acetyl-*d*-galactosamine
PNA	*d*-Galactose
PWM	(Not determined)

The blocking of fluorescence with each lectin was performed by preincubation of FITC-lectins with various ligands before incubation with PMNs. If the lectin-combining site is essential for binding, as it appears to be for stimulated locomotion, fluorescence should be effectively blocked by the specific monosaccharide for each lectin. The results of these studies are shown in Table 4 and demonstrate that lectin binding to the PMN surface was blocked by monosaccharides with an affinity for each lectin. As with the migration assay, some decrease in fluorescence occurred with more than one monosaccharide with certain lectins. However, the suppressive

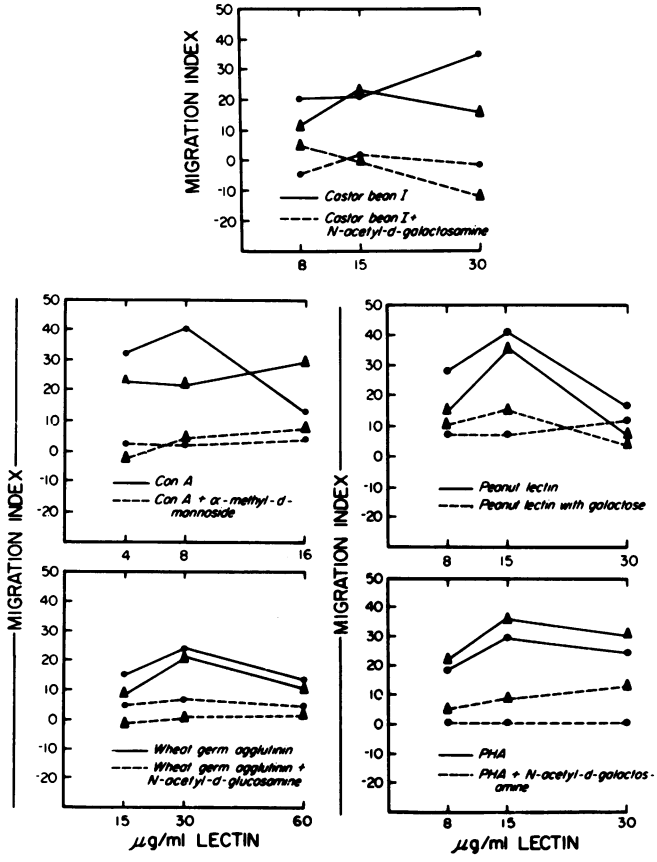

FIG. 3. *Neutrophil migration in response to ConA, PHA, CBI, WGA, and PNA (solid lines) or the same lectins preincubated with the appropriate monosaccharide (dashed lines). ConA, PHA, CBI, and WGA were blocked with a 0.005 M concentration of monosaccharide, and PNA was blocked with a 0.015 M concentration of monosaccharide. Three lectin concentrations were chosen to test blocking by monosaccharides. Duplicate experiments with two different cell preparations were performed as indicated by the different symbols.*

TABLE 3. *Suppression of lectin-stimulated migration by various monosaccharides*

Lectin (μg/ml)[a]	% Inhibition[b] of stimulated migration by:						
	Fucose	Fructose	NA Gal	NA Gluc	Gal	Gluc	αMM
PHA (15)	0	0	**100**	0	0	0	0
ConA (8)	0	0	12	0	28	20	**88**
CBI (15)	37	21	**74**	26	21	0	0
WGA (30)	45	30	45	**100**	25	50	35
PNA (16)	43	68	50	32	**100**	0	64
C5a	0	0	0	0	0	0	0

[a] Concentration of lectin in the lower compartment of the chemotaxis chamber.

[b] NA gal, *N*-Acetyl-*d*-galactosamine; NA gluc, *N*-acetyl-*d*-glucosamine; Gal, galactose; Glu, glucose; αMM, α-methy-*d*-mannoside. The boldfaced numbers indicate the inhibition with the particular monosaccharide known to have the highest affinity for that lectin.

TABLE 4. *Inhibition of FITC-lectin fluorescence by various monosaccharides*

Lectin	% of cells binding FITC-lectin	% Inhibition[a] of fluorescence in the presence of various sugars						
		αMM	NA Gluc	NA Gal	Gluc	Gal	Fucose	Fructose
PHA	84	5	67	**69**	15	10	5	0
ConA	54	**100**	0	0	26	19	0	22
CBI	84	0	6	**100**	0	76	0	0
WGA	100	0	**100**	5	0	0	0	0
PNA	67	7	54	0	13	**100**	0	0
PWM	0	ND[b]	ND	ND	ND	ND	ND	ND

[a] See Table 3, footnote *b*.

[b] ND, Not determined.

effect was always substantially greater with the monosaccharide known to have the greatest affinity for each particular lectin.

Lectin-mediated cap formation. It has been suggested that cap formation and mobilization of surface receptors may be involved in chemotaxis (19). Studies to determine if lectins stimulate cap formation were performed by incubating neutrophils and lectin for 5 min at 4°C, followed by 25 min at 25°C, with periodic sampling and fixing of cells. The results are shown in Fig. 4 and indicate that in the first 5 to 10 min, rim fluorescence predominates, followed by cap formation after 10 to 20 minutes and more intense granular fluorescence (fluorescein concentrated in patches or granules) after 20 to 30 min. The latter would suggest that some of the lectin may be internalized into vacuoles or patched on the surface. This was observed with PHA, ConA, PNA, CBI, and WGA. Figure 5 shows an example of the appearance of these cells at each stage with FITC-WGA to stimulate cap formation. The relationship between cap formation and chemotaxis is not known, although these studies clearly demonstrate that five out of six lectins tested here are capable of stimulating human neutrophil migration as well as cap formation.

DISCUSSION

In this study, we reported that ConA, PHA, WGA, CBI, and PNA bind to human neutrophil surfaces, stimulate migration, and induce neutrophil cap formation. PWM failed to show binding by fluorescence microscopy and elicited only a minimal response in the neutrophil migration assay. Titration curves with each lectin showed that the response to individual lectins varied in both amplitude and the shape of the curve. A recent study by Till et al. demonstrated that both ConA and PHA could stimulate a chemotactic response with rat macrophages, human monocytes, and rabbit neutrophils (20), although the response of the latter was quantitatively less. Results here show that ConA, PHA, and PNA can stimulate a chemotactic response with human neutrophils, whereas CBI stimulates migration, but without direction. All lectins with the exception of PWM produced a titration curve similar to other chemotactic factors, showing dose dependency at low concentrations and inhibition at high concentrations. An additional factor seen with the lectin-mediated system is cell agglutination, which was observed with all lectins at high concentrations and with WGA present in lower concentrations in the upper compartment. Whether this accounts for the high-dose inhibition observed in lectin titration experiments or whether suppression is due to degranulation shown to occur with lectins such as ConA (6) is undetermined at this time.

Studies with FITC-lectins indicate that lectins which stimulate neutrophil migration also bind to PMN surfaces. PWM, the only lectin which failed to stimulate a significant response in our studies, also failed to show binding as determined by fluorescence microscopy. This supports the contention that binding to the cell surface is essential to stimulate migration. In addition, inhibition of both neutrophil migration and lectin binding by appropriate monosaccha-

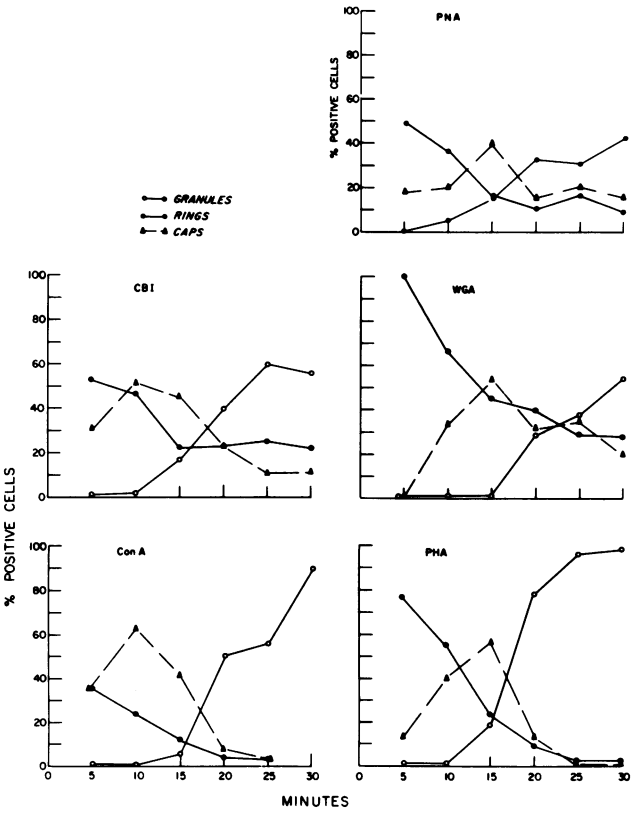

FIG. 4. *Lectin-mediated neutrophil cap formation. Results are expressed as the percentage of positive cells with ring fluorescence, cap fluorescence, or granular fluorescence (fluorescein concentrated in patches or granules). The initial 5-min incubation was at 4°C. The following incubation was indicated on the horizontal axis was at 25°C.*

rides supports the concept that binding is essential to stimulate migration.

Although the ligands reported in the literature as specific for each lectin (8, 13, 20) are the most efficient blockers of the lectin-neutrophil interaction, some inhibition of migration did occur with other monosaccharides. For example, glucose and fructose have been reported to be inhibitory for ConA, along with α-methyl-*d*-mannoside (13), and likewise also show some inhibition of fluorescence. The reasons for the low-grade inhibition of chemotaxis and fluorescence by several monosaccharides, seen most strongly with WGA and CBI, could be due to either a low-affinity binding of other monosaccharides to lectin or a consequence of cell surface carbohydrate stereochemistry. If the latter is true, low-grade inhibition of binding by other monosaccharides may be an indication of less efficient binding of lectins to cell surfaces than to isolated monosaccharides, and thus lectin may be easily dissociated from the cell surface by a number of monosaccharides.

Lectins do appear to bind in varying degrees to PMN surfaces. These differences may be a reflection of the efficiency of fluoresceination or of the number of binding sites on the cell surface or on the lectin. The former is not likely because the fluorescein/protein ratios for labeled lectins were all between 1.6 and 3.6, and no parallel existed between the percentage of fluorescent cells and the rank order of the fluorescein/protein ratio for each lectin. The question of how many available lectin-binding sites are necessary to stimulate cell migration has not been addressed in this study, although it is known that ConA, CBI, PNA, and PHA are tetramers (8, 9, 11, 13), WGA is a dimer (12), and PWM is thought to be a single polypeptide chain (14). Till et al. have indicated in their work that dimeric ConA will stimulate chemotaxis (20), and Goldman et al. (8) have shown that the valency of lectins has an effect on the ability of lectins to stimulate vacuolation in macrophages. It is feasible that the number of lectin-binding sites may be important in stimulating both cap

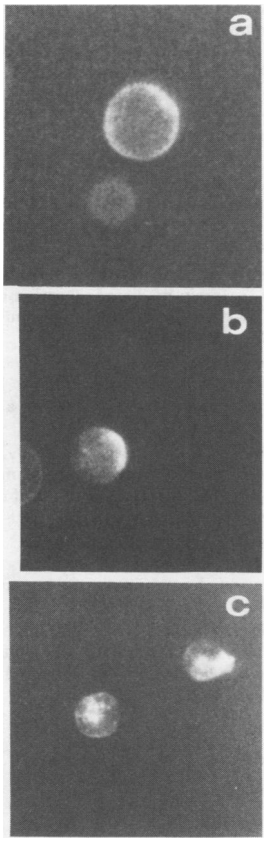

FIG. 5. *Photographs of human neutrophil cap formation induced by FITC-WGA. (a) Neutrophils showing linear or ring fluorescence after a 5-min incubation with FITC-WGA at 4°C. (b) Partially capped neutrophil after 15 min of incubation with FITC-WGA (5 min at 4°C and 10 min at 25°C). (c) Neutrophils showing patched or granular fluorescence after 30 min of incubation with FITC-lectin (5 min at 4°C and 25 min at 25°C). Similar results were obtained with PHA, ConA, CBI, and PNA.*

formation and cell migration, although direct evidence would require the use of monovalent lectins.

Our studies demonstrate that lectin mediated-migration in response to PHA, ConA, and PNA is directional because migration in the presence of a positive gradient is increased over that obtained with lectin in the upper compartment or both compartments of the chemotaxis chamber. This directional response to PHA, PNA, and ConA was confirmed by the checkerboard assay system of Zigmond and Hirsch (27), showing that either system demonstrates directionality. Furthermore, these studies show that lectins are chemokinetic when placed in the cell compartment in the absence of a positive chemotactic gradient and that some lectins, CBI in particular, are chemokinetic without being chemotactic.

The model of chemotaxis proposed by Stossel (19) implies that the initiation of a chemotactic response involves the binding of the chemotactic factor to the cell surface, followed by the mobilization of the receptor-chemotactic factor complex to the posterior of the cell. B lymphocyte locomotion in response to anti-immunoglobulin (16) is consistent with this concept because anti-immunoglobulin G also stimulates cap formation with these cells. A similar parallel can be found with PHA and ConA, which are chemotactic for lymphocytes (25) and can stimulate cap formation as well. Furthermore, a previous report with neutrophils has shown that the fucose-binding lectin from *L. tetragonolobus* seeds stimulates both cap formation (21) and chemotaxis (24) in these cells. The data presented here with several lectins indicate that under the conditions of study, cap formation and cell locomotion may be related.

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