Interaction of pokeweed mitogen with monocytes in the activation of human lymphocytes

H. C. STEVENSON, P. J. MILLER, M. J. WAXDAL, B. F. HAYNES, C. A. THOMAS & A. S. FAUCI *Biological Therapeutics Branch, Biological Response Modifiers Program, National Cancer Institute–Frederick Cancer Research Facility, Frederick, Maryland and Laboratory of Immunoregulation and Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.*

Summary. The present study examines the role of monocytes in the *in-vitro* activation of human T cells and B cells by pokeweed mitogen (PWM). The T cell-dependent PWM-induced B-cell activation process was found to be monocyte dependent. Fluorescence-activated cell sorter (FACS) analysis revealed that upon addition to peripheral blood mononuclear cells, fluoresceinated PWM, at concentrations that provided optimal B-cell and T-cell activation, bound predominantly to human monocytes. The binding of PWM to monocytes was reversible and could be displaced within the first few hours of binding by oligomers of *N*-acetylglucosamine (GlcNAc). As a functional correlate of the binding studies, it was shown that PWM-pulsed monocytes could induce B lymphocytes to become plaque-forming cells (PFC) and T lymphocytes to undergo proliferation. In contrast, markedly reduced PFC and blastogenic responses were observed when monocyte-depleted B lymphocytes and T lymphocytes were respectively pulsed with PWM and washed, followed by the addition of non-PWM-pulsed monocytes to the cultures. Thus, the initial event in the PWM-induced activation of human lymphocytes, for both *in-vitro* T-lymphocyte blastogenic responses and B-lymphocyte Ig secretion, appears to be binding of the mitogen to sugar residues on the surface membrane of the monocyte, followed by subsequent interaction with the appropriate lymphocytes. The process of PWM binding to monocytes did not appear to affect the baseline production of interleukin-1 (IL-1) by human monocytes, nor could soluble factors from PWM-pulsed monocytes substitute for intact cells in the initiation of the lymphocyte-activation process.

INTRODUCTION

The details of how pokeweed mitogen (PWM) induces immunoglobulin (Ig) secretion by human B cells has been an area of particular interest, since this mixture of

lectins (Waxdal, 1974) is one of the most effective and commonly employed T cell-dependent B-cell activators in man (Fauci, 1979). We have previously described an *in-vitro* system for assessing normal human B-cell function in response to PWM stimulation (Fauci, Whalen & Burch, 1980b), and we have delineated the regulation of human *in-vitro* antibody production by helper T cells (Fauci, Pratt & Whalen, 1976), suppressor T cells (Haynes & Fauci, 1978), monocyte products (Dimitriu & Fauci, 1978), and the modulation of this system by pharmacologic manipulations such as treatment with alkylating agents (Stevenson & Fauci, 1980a) and corticosteroids (Haynes & Fauci, 1979).

Recent studies have demonstrated an absolute requirement for monocytes in the PWM-induced activation of human B cells to Ig secretion (deVries *et al.*, 1979; Rosenberg & Lipsky, 1979; Fauci *et al.*, 1980a; Thiele & Lipsky, 1982). We have employed a negative-selection technique for monocyte and lymphocyte purification, known as counter-current centrifugation elutriation (Contreras *et al.*, 1980), that has allowed us to extend investigations into the mechanisms of monocyte dependence in the PWM-stimulated B cell-activation system (Fauci *et al.*, 1980a) because of the marked purity of the monocytes and lymphocytes produced. The present study describes investigations which focus on the interaction of PWM with human monocytes and the functional significance of that interaction for the subsequent lymphocyte activation process.

MATERIALS AND METHODS

Cell suspensions
UFMNL suspensions were obtained by Ficoll–Hypaque gradients and were further fractionated by an elutriation procedure that has recently been applied to the isolation of human monocytes and lymphocytes as previously described (Stevenson & Fauci, 1980b).

Thymus-derived T cell-enriched (TCE) suspensions were purified by sheep erythrocyte (E) rosetting as previously described (Stevenson & Fauci, 1980b). The T cell-depleted (TCD) suspensions thus obtained contained 0–1% E rosette-positive cells, whereas the TCE suspensions were 95–100% E rosette-positive cells.

Cell identification procedures
Monocyte purity was confirmed by morphology on Wright's-stained cytocentrifuge preparations, by non-specific esterase staining (Yam, Li & Crosby, 1971), and by the ability to ingest latex bead particles and IgG-coated ox red blood cells. Viability was determined by trypan blue dye exclusion. Percentage of B cells in the lymphocyte preparations was measured by assaying the cells for the presence of surface membrane Ig (Winchester & Fu, 1976).

Plaque-forming cell (PFC) assay
The total number of B cells secreting Ig after 6 days of culture in the presence of PWM was assayed with a staphylococcal protein A indirect PFC assay as previously described (Fauci, 1979).

Mitogens employed
PWM (Gibco, Grand Island, NY) was directly conjugated to fluorescein isothiocarbamyl by a previously described method (Nairn, 1976) for subsequent use in FACS analysis. In certain experiments, fluorescein-conjugated PWM (Fl-PWM) was used directly to stimulate lymphocyte responses. In these experiments, Fl-PWM was found to have 83% of the stimulatory capabilities of unconjugated PWM (on a per-mg-protein basis) for both T-cell proliferation and B-cell activation.

Culture conditions for induction of PFC responses
The culture conditions for the generation of PFC after PWM stimulation of peripheral-blood (PB) lymphocytes have been previously described in detail (Fauci *et al.*, 1980b). The duration of culture for the measurement of PFC responses was 6–7 days. The optimal stimulating dose for T-cell proliferation was found with 1:200 final dilution of PWM and 1:160 final dilution of Fl-PWM.

Measurement of tritiated thymidine incorporation by T lymphocytes
Cultures were set up for 5 days in triplicate in microtitre plates in RPMI + 10% foetal calf serum (FCS) and harvested as previously described (Fauci & Dale, 1975). Optimal stimulatory doses for B cell activation were found with 1:200 final concentration of PWM and 1:160 final dilution of Fl-PWM.

Fluorescence-activated cell sorter (FACS) analysis
FACS analysis was performed as described by Loken & Herzenberg (1975) using a fluorescence-activated cell sorter, FACS-II (Becton Dickinson, FACS Systems, Mountain View, CA).

Fl-PWM binding to mononuclear cells

For all cell preparations, the concentration was adjusted to 2×10^7 cells/ml in Hanks's balanced salt solution supplemented with 0·1% human albumin (Cutter Labs, Berkeley, CA). Cells (5×10^6) were incubated with RPMI 1640 in a 1:160 final dilution of Fl-PWM for 30 min at 37°. Cells were washed three times after incubation with Hanks's balanced salt solution +0·1% human albumin and then resuspended in 0·4 ml of the same buffer for FACS analysis.

Pulsing studies

Cell preparations were pulsed with PWM (Gibco, Grand Island, NY) by suspending 5×10^6 cells in 1 ml of RPMI 1640 and incubating them for 30 min at 37° with a 1:160 final dilution of Fl-PWM or 1:200 final dilution of PWM. Cells were then washed three times with RPMI 1640 and recounted for use in subsequent culture experiments.

Removal of PWM from lymphocytes and monocytes

A competitive binding solution to PWM was prepared from soluble oligomers of *N*-acetyl-D-glucosamine (GlcNAc). The concentration of GlcNAc used was approximately 0·1 N and the solution was adjusted to 300 mOsmol with PBS. The displacement of cell-bound PWM or Fl-PWM from lymphocytes or monocytes was achieved by incubating the cells in the pGlcNAc solution for 15 min at 37°. These cells were then washed three times in RPMI 1640 prior to culture or FACS analysis.

Monocyte supernatant experiments

Elutriator-purified monocytes were assayed for their ability to secrete mitogenic factors while unstimulated in culture or following pulsing with PWM or Fl-PWM. Leucocytes were cultured at a density of 2×10^5 cells/ml and supernatants were harvested at 4, 8, 12, 24, and 36 hr. Supernatants from unstimulated and PWM-exposed monocytes from each time point were added to purified T lymphocytes, with and without additional PWM (1:200 final dilution), at concentrations ranging from 10 to 50% and tritiated thymidine incorporation was measured on days 4 to 6. Duplicate samples of these supernatants were assayed for interleukin-l (IL-l) by the human fibroblast and mouse thymocyte proliferation assays (Schmidt *et al.*, 1982; Mizel, Oppenheim & Rosentreich, 1978).

RESULTS

Characteristics of cell suspensions employed

Counter-current centrifugation elutriation of UFMNL resulted in monocyte fractions that were an average of 92% pure by Wright's stain, latex ingestion, and esterase stain and lymphocyte fractions that were an average of 99·5% pure by the same criteria (Table 1). T-lymphocyte suspensions were 96·3% sheep erythrocyte receptor positive; monocyte contamination was assumed to be at the same level observed in the lymphocytes or less since the same starting cell population was used.

Dependence of PWM-induced B-cell activation on monocytes

As previously reported, PWM-driven T-lymphocyte proliferation and Ig production by B lymphocytes were found to be dependent on the presence of monocytes. Figure 1a demonstrates that PWM-induced B-cell responses fell to 17·1% in the monocyte-depleted lymphocyte preparations when compared to an equal number of B cells present in unfractionated mononuclear leucocytes. The reconstitution of cultures with graded numbers of monocytes (from $1-5 \times 10^5$ monocytes per 5×10^5 lymphocytes) restored the capacity of the cultured lymphocytes to generate optimal numbers of PFC. Figure 1b demonstrates that T lymphocyte-proliferative responses to PWM were similarly monocyte-dependent.

FACS-II analysis of the cellular binding of Fl-PWM

When the ability of various cell populations in UFMNL to bind Fl-PWM was evaluated by FACS-II analysis, a selective binding to monocytes was noted. Figure 2a demonstrates that only 14·9% of UFMNL were able to bind to Fl-PWM. Using fractionated cells, Fig. 2b demonstrates that only 3·7% of all lymphocytes are able to bind Fl-PWM. Figure 2c reveals that most (92·9%) of monocytes bound Fl-PWM. The binding of Fl-PWM to monocytes was sugar-specific, since oligomers of GlcNAc were able to completely displace all bound Fl-PWM from the surface of monocytes.

The ability of PWM-pulsed monocytes to activate autologous B cells

When human monocytes were pulsed with PWM for

Table 1. Characteristics of cell suspensions employed in experiments

| Cell suspension	Wright's stain analysis			Sheep erythrocyte receptor+	Esterase staining	Latex ingestion	Surface Ig+
	Lymphocytes	Monocytes	Others				
UFMNL	83·6±7·2*	14·5±4·5	1·9±1·1	ND	ND	ND	11·2±1·1
CCE lymphocytes	99·5±0·2	0·5±0·1	0·0	ND	0·3±0·1	0·2±0·1	12·0±1·3
T lymphocytes	99·4±0·1	0·7±0·2	0·0	96·3±0·9	ND	ND	ND
CCE monocytes	3·5±2·0	92·8±4·3	3·7±2·1	1·1±0·2	90·0±2·0	92·0±2·1	0·3±0·1

* Mean %±SEM of 8 separate experiments.
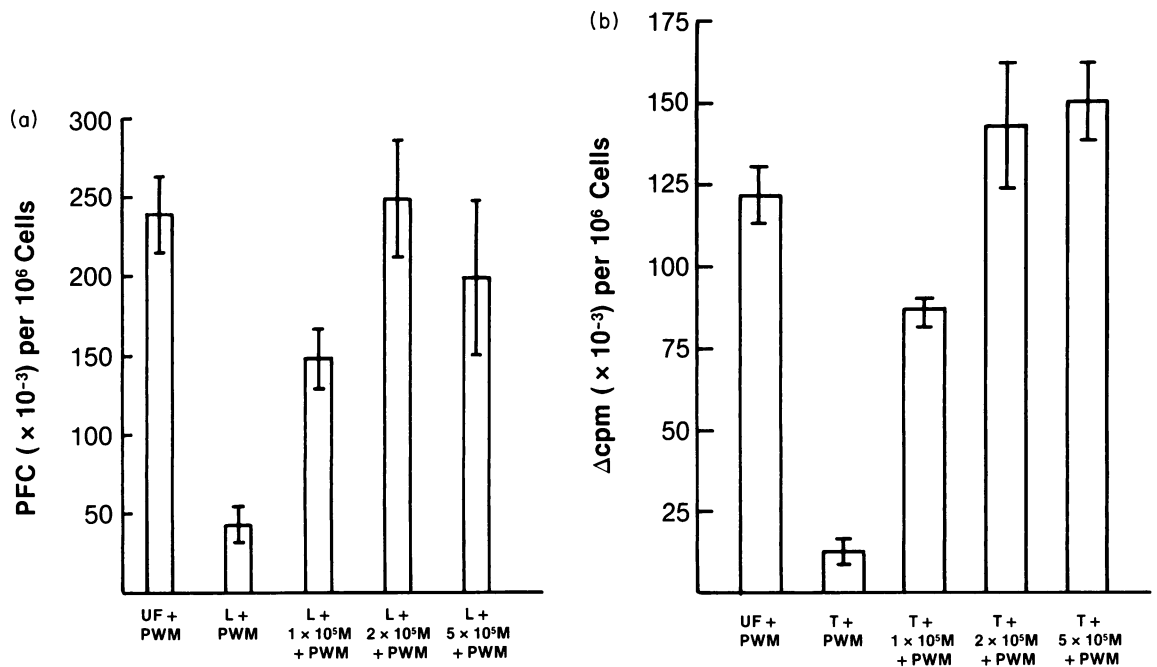
Figure 1. Dependence on monocytes of pokeweed mitogen-induced PFC and T-lymphocyte proliferation. Leucocytes were cultured with PWM for 6 days in the PFC assay and 5 days in the proliferation assay. Data represent means and standard errors of eight experiments.

30 min at 37° and then washed three times with RPMI 1640, they displayed virtually no PFC or proliferative responses after 6 days in culture. However, when these PWM-pulsed monocytes were added to autologous lymphocytes, the responder lymphocytes generated PFC at a level comparable to that seen when UFMNL were cultured with PWM continuously for 6 days (Fig. 3a). The treatment of PWM-pulsed monocytes with oligomers of GlcNAc virtually abolished their ability to stimulate B lymphocytes to secrete Ig. The dose of PWM used in the pulsing studies was a 1:200 final

dilution of the stock PWM solution. A 1:160 final dilution of the stock Fl-PWM solution was of equal potency. Figure 3b demonstrates an identical pattern of T-lymphocyte proliferation in response to PWM-pulsed monocytes to that demonstrated for the B-lymphocyte activation process.

The ability of soluble factors of PWM-pulsed monocytes to activate autologous lymphocytes

Cell culture supernatants were harvested from un-

(a)

(b)

(c)
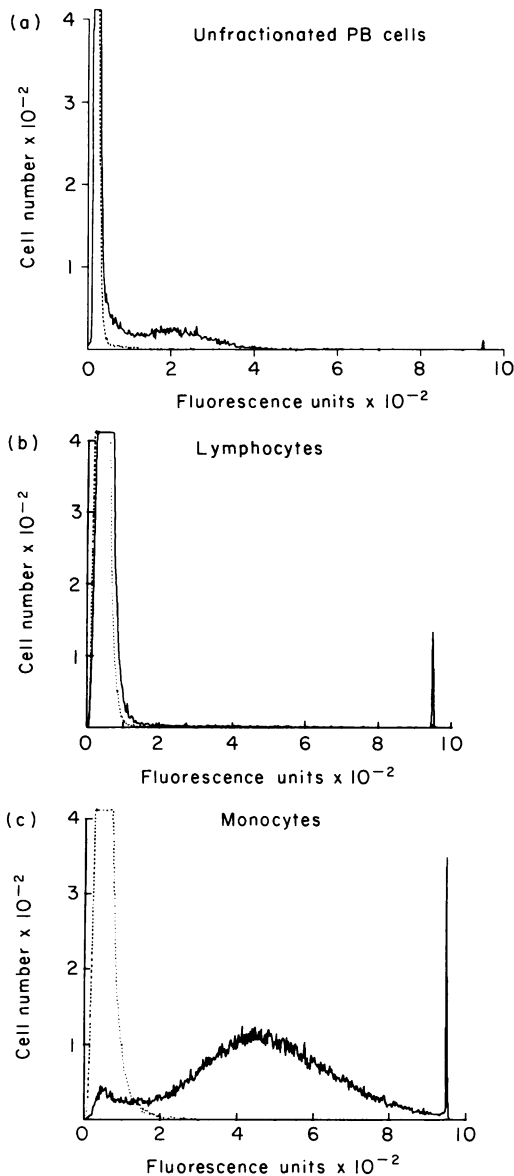
Figure 2. FACS-II analysis of the binding patterns of Fl-PWM to leucocyte subsets in UFMNL. Leucocytes were exposed to Fl-PWM for 30 min; after 1–12 h of culture at 37°, displacement of PWM by pGlcNAc took place in 15 min.

pulsed and PWM-pulsed monocytes after 4, 8, 12, 24, and 36 hr of culture. These supernatants were added to fresh autologous T lymphocytes with and without PWM at concentrations ranging from 10 to 50% and T-cell proliferation was measured on days 4, 5, and 6

of culture. Neither unpulsed nor PWM-pulsed monocytes produced a soluble factor capable of stimulating T-lymphocyte proliferation with or without additional PWM (data not shown). Similarly, there was no significant difference in the amount of IL-1 produced by PWM-pulsed monocytes as compared to unpulsed monocytes in the above cited conditions. Maximal IL-1 concentrations in supernatants were seen following 36 hr of culture, but supernatants from both PWM-pulsed and control monocytes produced equal amounts.

DISCUSSION

The present study demonstrates that PWM binds selectively to human monocytes at the concentrations that optimally stimulate T-lymphocyte proliferation and B-lymphocyte Ig secretion. PWM has been demonstrated to bind predominantly to human monocytes in a functional sense as well, since PWM-pulsed monocytes can initiate the B-lymphocyte and T-lymphocyte activation processes at a level comparable to unfractionated mononuclear cells exposed to PWM for the duration of culture. Of interest is the finding that, after human monocyte membranes bind PWM, pokeweed appears to remain on the surface for prolonged periods of time, since it can be reversibly displaced by oligomers of GlcNAc, resulting in abrogation of the lymphocyte-stimulating capability of the monocytes. This observation is in contrast to the observation that antigen processing by monocytes requires an early endocytosis step (Rosenthal, 1980).

The actual mechanism whereby PWM-pulsed monocytes are able to stimulate the responding lymphocytes is unclear at present. Since soluble factors from PWM-pulsed monocytes do not seem capable of entirely substituting for intact cells, it is possible that there is merely a selective binding of the lectin to the surface of the monocyte, following which the lectin is then made physically available to responding lymphocytes which bind the lectin rather poorly. Since previous studies have shown that monocytes are destroyed by a lymphokine produced by PWM-stimulated lymphocytes within 3 days of culture (deVries *et al.*, 1980), it would appear that the monocyte–lymphocyte interaction that leads to lymphocyte activation must be an early culture event. Analogies to the well documented antigen-presentation function of monocytes (Rosenthal, 1980) must await further research into this phenomenon, but it appears that substantial
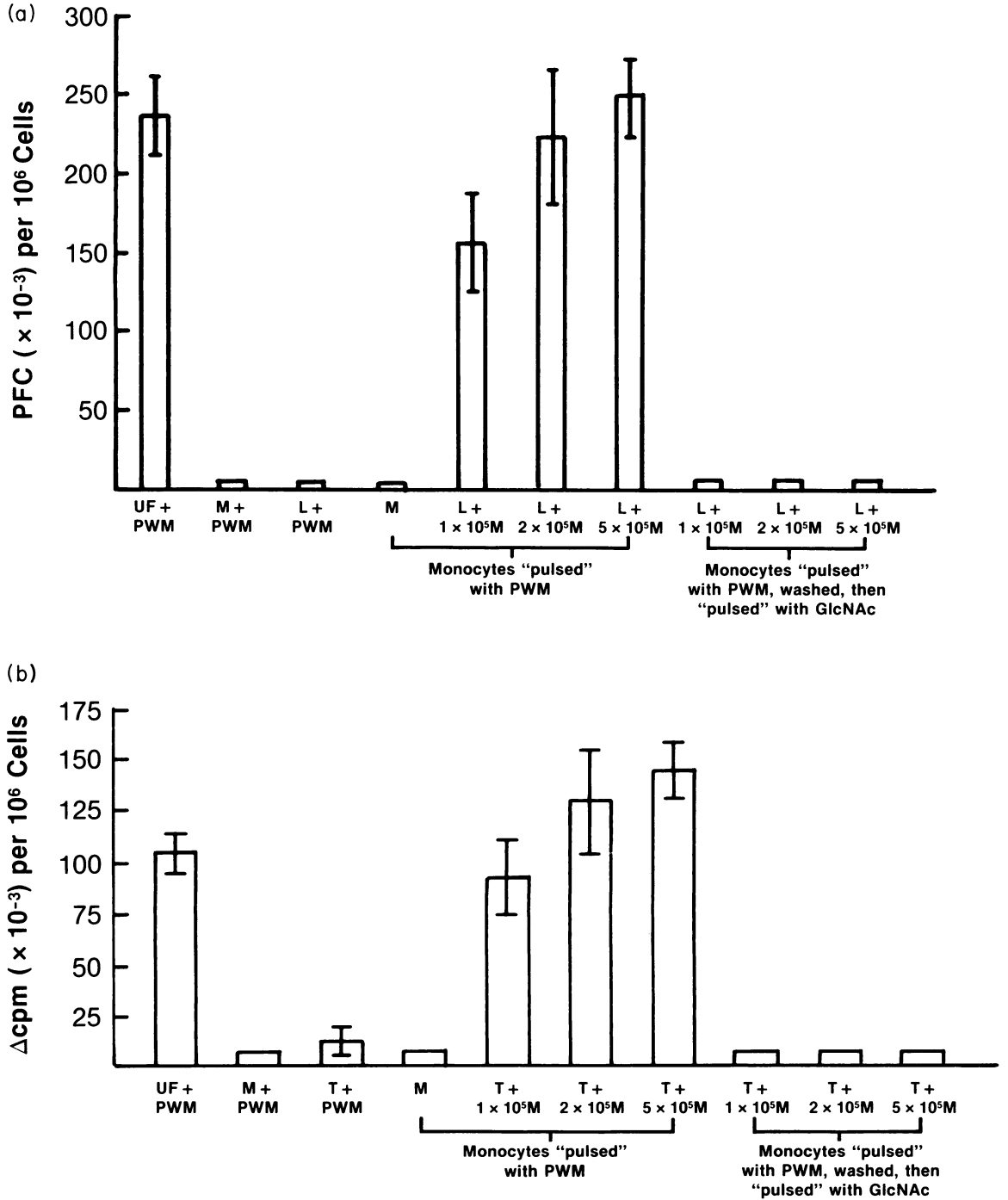
Figure 3. Ability of PWM-pulsed monocytes to activate T and B lymphocytes with and without PGlcNAc competitive displacement. Pulsing and displacement experiments were performed under the same conditions used in the FACS analysis. Data represent means and standard errors of eight experiments.

differences exist in the way monocytes handle antigens and PWM.

We do not currently know which specific cell-membrane determinants on the monocyte membrane serve as binding sites for PWM. Studies by Yokoyama, Terao & Osawa (1978) have established that Pa-2, one of the lectins in PWM, has binding specificity for particular glycoproteins, notably the core structure of di-*N*-acetylchitobiose moities. This same specificity was observed in our experiments by competitive inhibition, demonstrated by the addition of oligomers of GlcNAc. Previous research performed in murine systems (Waxdal, Nilsson & Basham, 1976; Nilsson & Waxdal, 1978) indicated that PWM was able to bind directly to a variety of H-2 antigens, including Ia antigens; similar experiments have not yet been performed in man. Further investigation, to localize the precise membrane determinants on human monocytes to which PWM binds and whereby it effects polyclonal activation of responder lymphocytes, is in progress.

ACKNOWLEDGMENTS

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