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CELL-SURFACE CHANGES DURING MITOGENIC STIMULATION OF LYMPHOCYTES ASSESSED BY THE BINDING OF WHEAT-GERM AGGLUTININ AND OTHER PLANT LECTINS

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Lymphocytes from murine lymph node, cultured in the presence of an optimally mitogenic dose of phytohaemagglutinin, were stained with fluoresceinated lectins and analysed by flow cytometry. A marked increase in the ability of lymphocytes to bind wheat-germ agglutinin was observed that is particularly pronounced for the blast cells, reaching a maximum at about 40 h, when they are 5.5-times brighter than cells at zero time. The corresponding intensification of the small cells is 2-fold. Much smaller increases in binding accompanying blast transformation were observed when fluoresceinated concanavalin A or *Lens culinaris* haemagglutinin were used. Polyacrylamide gel electrophoresis of plasma membranes followed by treatment of the gels with radioactively labelled lectins and autoradiography also showed a very distinct increase in the binding of wheat-germ agglutinin to membranes from mitogen-stimulated porcine lymphocytes. Less marked changes in the binding of concanavalin A, *Lens culinaris* heamagglutinin and *Ricinus communis* agglutinin 120 were also noted. The apparent multiplicity of glycoproteins that bind each lectin, suggests that in each case the sites are heterogeneous. We conclude that lymphocytes stimulated by the T-cell mitogen phytohaemagglutinin expose new glycoprotein receptors for wheat-germ agglutinin that are most abundant on blast cells at 40 h. Attempts to characterize the receptor biochemically suggest that the carbohydrate moiety recognised by wheat-germ agglutinin is present on a glycoprotein of approx. 120 kDa molecular mass and also possibly on glycoproteins of 170–190 kDa.

Introduction

Lymphocyte activation leads to important changes in the biosynthesis and glycosylation of proteins and lipids of the plasma membrane [1–5]. In an earlier study we showed that the membranes of mitogen-stimulated murine lymphocytes develop strong binding for the lectin wheat-germ agglutinin before DNA synthesis commences [6]. Knowledge of the components that are affected has relevance for the understanding of the events which precede cell division, but our own interest was focussed on the possibility of identifying one or more major components associated with this process so that relevant monoclonal antibodies able to identify activated lymphocytes, and perhaps having a therapeutic potential, could be raised. Accordingly, in order to define the wheat-germ agglutinin phenomenon more precisely and to identify the cells predominantly affected, the binding of conjugated lectin to polyclonally activated cells was reassessed using flow cytometry. In addition, membrane proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis were treated with radioactively labelled wheat-germ agglutinin in an attempt to characterize the major glycoprotein(s) concerned.

Materials and Methods

Lectin labelling

Purified phytohaemagglutinin was obtained from Wellcome Laboratories Ltd., wheat-germ agglutinin from Pharmacia (Great Britain) Ltd., concanavalin A from I.C.N. Nutritional Biochemicals, *Lens culinaris* haemagglutinin from B.D.H. Chemicals Ltd. and *Ricinus communis* agglutinin 120 from Miles Laboratories Ltd. Fluorescein conjugation was achieved by adding 500 μg of wheat-germ agglutinin or 1 mg of *Lens culinaris* haemagglutinin to 25 μl of 2 mg/ml fluorescein isothiocyanate (FITC) in 0.25 M bicarbonate buffer at pH 9.0. The solution was agitated gently at 4°C overnight before removal of unconjugated FITC by gel filtration on Sephadex G-25. Concanavalin A was poorly labelled by this method and therefore the following procedure was used. 4 mg of concanavalin A in 400 μl of the same bicarbonate buffer were added to 10 mg of FITC-Celite (1 : 10 ratio, B.D.H. Chemicals Ltd.) and mixed for 30 min at 37°C. The solid phase was then removed by centrifugation and free dye by Sephadex gel filtration. *Ricinus communis* agglutinin was not fluoresceinated.

Lectins were labelled with ^{125}I using iodogen (Pierce Chemicals Ltd., U.K.) by an adaptation of the method described by Fraker and Speck [7]. Iodogen was dissolved in dichloromethane at 100 μg/ml and 25 μl aliquots were dispensed into the bottom of microfuge tubes. The solvent was allowed to evaporate, leaving the iodogen coated on the tubes. Lectins (0.5 nM) in 0.1 M sodium phosphate buffer (pH 7.0) (25 μl) were added to the coated tubes followed by 1 mCi of $Na^{125}I$ and incubated for 10 min at room temperature with gentle mixing at 1-min intervals. Free ^{125}I was separated from the labelled lectins by gel filtration on Bio-Gel P-30 in 0.15 M NaCl/0.05 M Tris-HCl (pH 7.0)/0.1% (w/v) haemoglobin.

Cell preparation and staining

Mixed lymph node cells, isolated from BALB/c mice, were cultured in Linbro plates with or without an optimally mitogenic dose of phytohaemagglutinin (0.006 mitogenic units/ml). Each well contained $5 \cdot 10^6$ cells in 2 ml of RPMI medium supplemented with glutamine (2 mM), gentomycin (0.1%) and heat-inactivated foetal calf serum (20%, v/v). After harvesting, cells were washed twice and then assessed for viability by observing ethidium bromide exclusion microscopically. For flow cytometry, staining was carried out by resuspending $2 \cdot 10^6$ cells in 60 μl of phosphate-buffered saline solution (Dulbecco 'A', pH 7.3) containing azide (0.1% w/v) and fluoresceinated lectin. The cell concentration was then adjusted to $1 \cdot 10^6$/ml with phosphate-buffered saline/azide and the samples kept on ice until use. The lectin dilution in each case was sufficient to produce optimal cell fluorescence as observed by microscopy.

Preparation of lymphocyte plasma membrane

Phytohaemagglutinin-stimulated and resting lymphocytes from mouse lymph node were used initially for plasma membrane preparation. However, in order to increase the quantity of isolated membrane, lymphocytes from the domestic pig were also studied. Mesenteric porcine lymph nodes were obtained immediately after the animal was killed and kept on ice until the cell suspensions were prepared. Dead cells were removed using a modified Ficoll-Hypaque technique [8]. The lymphocytes were then cultured in the RPMI medium described above in the presence of 0.04 mitogenic units/ml phytohaemagglutinin (optimally mitogenic dose) for 40 h using 500 ml flasks. Once again, dead cells were removed and the remainder were washed three times in ice-cold RPMI before disruption for membrane preparation. This was achieved using a Stansted Cell Disruptor (Model A0612, Stansted Instruments, Essex, U.K.) at a disruption pressure of 5520 kPa and 3750 kPa for resting cells and phytohaemagglutinin-stimulated cells, respectively. Plasma membrane fractions were separated from the broken cells by differential and sucrose density gradient centrifugation [9] and stored in 10 mM Tris-HCl (pH 7.4) at −70°C. The purity of the plasma membrane preparation prepared in this way from resting and phytohaemagglutinin-stimulated cells as assessed by horseradish peroxidase-*Lens culinaris* haemagglutinin binding [10] was greater than 85%.

Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis

was performed in a 1 mm thick, 5–15% (w/v) polyacrylamide gradient slab gel which was prepared with a three-channel peristaltic pump and subjected to electrophoresis in the Laemlli buffer system exactly as described by Thorpe et al. [11]. Membrane samples in 5% (w/v) sodium dodecyl sulphate (SDS), 40 mM 2-mercaptoethanol, 5% (w/v) sucrose, 0.0005% (w/v) Bromophenol blue and 125 mM Tris-HCl (pH 6.8) were prepared for electrophoresis by incubation for 3 min at 100°C. Normally, 40–50 μg of membrane protein were loaded and the gels were run at a constant 190 V with fan-driven air cooling.

Lectin staining of gels

After electrophoresis, the gels were fixed and stained with radioactively labelled lectins by a combination of previously described procedures [12,13]. The gels were incubated in 250 ml of 50% (v/v) methanol for 10 min. 0.5 ml of 25% (w/v) glutaraldehyde was then added and after 90 min the gels were removed and placed in either 0.05 M Tris-HCl/0.15 M NaCl buffer containing 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 20 μg/ml $NaBH_4$ (for *Lens culinaris* haemagglutinin and concanavalin A) or 33 mM sodium phosphate buffer containing 0.1 M NaCl (pH 8.0) and 20 μg/ml $NaBH_4$ (for *Ricinus communis* agglutinin). The solution was changed after 30 min and left overnight. After removal of the borohydride solution and equilibration in relevant buffer, the gels were placed in 0.1% (w/v) haemoglobin in the same medium for 1 h to reduce nonspecific binding. Labelled lectins ($5 \cdot 10^6$ cpm per μg) were then applied in 30 ml of buffer containing 0.1% (w/v) haemoglobin. The gels, in this medium, were sealed in plastic bags and left on a rotating shaker for 10–20 h. They were then washed with several changes of buffer over a period of 24 h and after the final rinse were stained with 0.2% (w/v) Coomassie brilliant blue R in methanol/water/acetic acid (5 : 5 : 1, v/v) for 15 min and destained in methanol/water/acetic acid (3 : 6 : 1, v/v) until a relatively colourless background was obtained. The labelled, Coomassie blue-stained gels were dried on filter paper in a Bio-Rad slab gel drier (Model 224) and then placed on Kodak X-ray film for autoradiography.

Immunoprecipitation of ^{3}H-labelled cell surface glycoproteins with wheat-germ agglutinin and anti-wheat-germ agglutinin antibody

For these studies, phytohaemagglutinin-stimulated and resting lymphocytes from mouse lymph node were used. Cells were cultured with phytohaemagglutinin for 48 h as described above. A proportion were then assessed for viability and FITC-wheat-germ agglutinin binding. The surface sialic acid residues on the remaining cells were labelled with ^{3}H by exposure to sodium periodate followed by treatment with NaB^3H_4 [14]. $2.5 \cdot 10^7$ of these cells were then added to 1 ml of phosphate-buffered saline containing wheat-germ agglutinin and kept on ice for 30 min. Three concentrations of lectin were used: 0.1 μg/ml, 1.0 μg/ml, 100 μg/ml and a phosphate-buffered saline control. After washing the cells twice, the labelled surface membrane (with bound lectin) was solubilized in 1.0 ml of 1% (w/v) NP40 detergent containing 1 mM phenylmethylsulphonyl fluoride (to inhibit proteolysis) at 4°C for 30 min. The nuclei were then removed by centrifugation at $2000 \times g$ for 15 min. The labelled, solubilized membrane material was next subjected to $12000 \times g$ to remove any particulate matter. The wheat-germ agglutinin-^{3}H-labelled glycoprotein complexes were treated with 10μl of rabbit anti-wheat-germ agglutinin, kept on ice for 30 min and finally mixed with 100μl (w/v) formalin-fixed *Staphylococcus aureus*. These bacteria had previously been washed three times in 1% NP40. After a further 30 min at 0°C the suspension was washed three more times in NP40 ($12000 \times g$). The bound complexes were finally eluted in 0.125 M Tris-HCl (pH 6.8)/5% SDS. The eluted material was analysed by gradient polyacrylamide gel electrophoresis (5–15% polyacrylamide) and scintillant was incorporated into the gel so that the tritiated glycoprotein could be located fluorographically by exposing Kodak X-ray film to the dried gel [15]. Identical procedures were used with resting lymph node cells.

Flow cytometry

Analyses were performed with a laboratory-built instrument developed from the cytometer described by Nash et al. [16]. This instrument is of modular design and for these measurements incorporated a stream-in-air flow system fitted with a

100 μm diameter nozzle. The cell stream was illuminated by an argon laser operating at 488 nm (200 mW). The geometry of the forward scatter collection was adjusted to allow discrimination between viable and non-viable cells [17]. Fluorescence was collected at 90° by a 32×/0.6 NA microscope objective (E. Leitz Instruments Ltd.) and passed to a photomultiplier tube. In order to eliminate scattered laser light, a low-fluorescence filter (Schott KV 520) and a long-pass filter (OG 515) were included in this path. Single-parameter, pulse-height distributions were collected in the pulse-height analyser and then stored on the disk of a Digico M16E computer. Hardcopy was produced subsequently by a Calcomp 565 incremental plotter. Glutaraldehyde-fixed chicken erythrocytes, stored under refrigeration, were used as test particles to align the instrument and as a calibration standard throughout the course of these experiments.

The mean fluorescence intensity, in pulse-height analyser channel numbers, of a population of cells is given by

$$F = \frac{1}{N_{\text{scat}}} \sum_{i=1}^{i=255} in_i,$$

where i is the channel number, n_i is the number of cells classified in channel i and N_{scat} is the total number of cells analysed. The mean cell fluorescence is then given by $F(\text{sample}) - F(\text{control})$. (This quantity represents a measure of the average fluorescence of all the cells analysed and not just those that are positive.) Electronic volume sensing was carried out using a Coulter counter Model B as described previously [16]. A 70 μm diameter aperture, 84 μm thick, was used.

Results

Flow cytometry

In order to investigate the time-course of wheat-germ agglutinin receptor expression during polyclonal stimulation, murine lymphocytes were cultured with or without phytohaemagglutinin for 0, 23, 45, 67 and 95 h. While cells in the presence of phytohaemagglutinin survived well, in its absence there was a gradual decline in viability. Before cytometric analysis the cell fluorescence was assessed by microscopy. Fig. 1 shows a typical time-course of the proportion of positive cells estimated in this way. This assessment takes no account of cell brightness or size, but the dead cells have been excluded. Since the observed fluorescence is initially rather weak, it is uncertain whether the increase around 40 h is due to a general increase in the number of binding sites per cell or to an increase in the number of cells that bind wheat-germ agglutinin or to both of these factors.

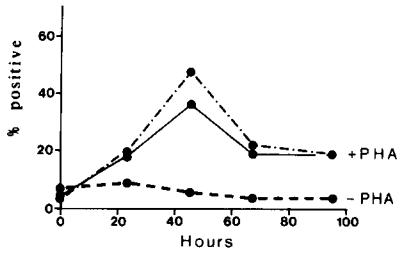

Fig. 1. Percentage of wheat-germ agglutinin-positive cells in cultures of murine lymphocytes determined by fluorescence microscopy at successive times after stimulation with mitogen (+PHA, duplicate cultures) and control cells cultured without mitogen (−PHA). (PHA, phytohaemagglutinin).

When cell fluorescence was measured by flow cytometry, light scatter gating was used to exclude the contribution from dead cells and debris. As an example, Fig. 2 shows the scatter distribution from cells harvested at 23 h. The small peak at channel 60 is due to dead cells and the truncated peak to the left is caused by debris. The arrows indicate

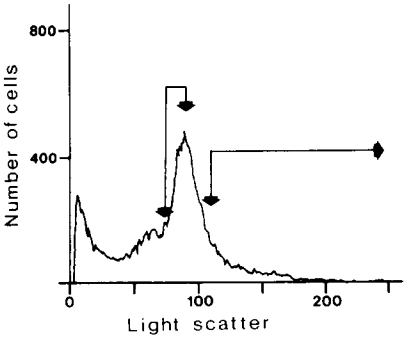

Fig. 2. Light-scatter intensity distribution of murine lymphocytes cultured in the presence of phytohaemagglutinin for 23 h. The two selected regions, indicated by arrows, specify the small and large cell windows used for scatter gating. Units on the abscissa are channel numbers.

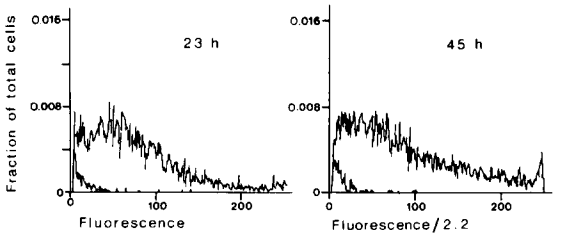

Fig. 3. Light-scatter-gated fluorescence intensity distributions of murine lymphocytes cultured with phytohaemagglutinin for 23 and 45 h and stained with FITC-wheat-germ agglutinin. The data in the right-hand panel were collected with an amplifier gain in the fluorescence channel reduced by a factor of 2.2 The lower traces were obtained from unstained cells from the same cultures. Each distribution is normalized to the same number of scatterers and the units on the abscissa are channel numbers.

the two regions from which fluorescence information was collected. The leftmost region consists of scatter signals from small lymphocytes and the rightmost region contains signals from lymphoblasts (no upper discriminator level was set). These gating conditions were selected in each case to give the best exclusion of dead cells and the least overlap between small and large lymphocytes. Discrimination between small cells and non-viable cells and debris was somewhat less efficient at long culture times. Typical fluorescence distributions for the large cells are shown in Fig. 3. The range of intensities is very wide and the cells harvested at

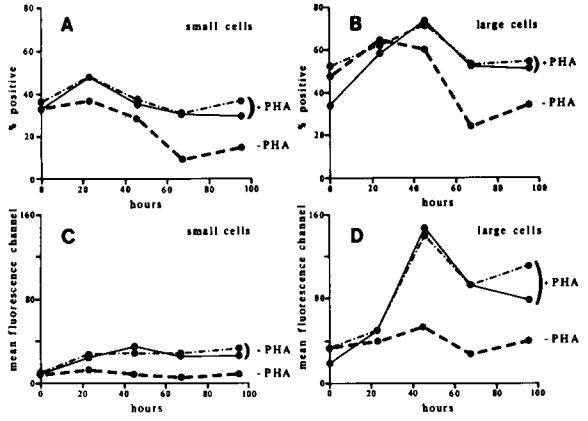

Fig. 4. Fluorescence time-course data obtained by flow cytometric analysis of murine lymphocytes cultured with mitogen (+PHA (phytohaemagglutinin), duplicate cultures) and without (−PHA) and subsequently stained with FITC-wheat-germ agglutinin. Panels A and B: percentage of positive cells in the small and large cell populations, respectively. Panels C and D: mean fluorescence of the small and large cells, respectively.

45 h are much brighter than those obtained at 23 h. The lower trace in each panel was obtained from unstained controls. In order to reduce the information in these distributions to a single number, we may either calculate the percentage of cells that are positive with respect to the unstained control or we may calculate the mean cell fluorescence of the whole population (selected by the scatter gates). The time courses of these parameters are shown in Fig. 4. The following points may be noted.

(1) In all cases the percentage of positive cells estimated from the cytometric data is greater than that estimated by microscopy (Fig. 1).

(2) In the presence of phytohaemagglutinin, there is an increase in the proportion of positive cells at about 40 h in the large cell population. However, there is also a smaller increase in the proportion of positive cells in the culture without phytohaemagglutinin, (possibly due to mitogenic components present in foetal calf serum).

(3) The percentage of positive small cells varies much less with time than that of the large cells.

(4) The mean fluorescence of the small cell population increases to a steady value after 20–40 h that is 2–3-times that of the unstimulated cells.

(5) The mean fluorescence of the large cells increases dramatically with respect to the unstimulated control at 45 h, but subsequently falls.

In order to confirm that these increased emissions are due to specific binding of wheat-germ agglutinin, cells harvested at 45 h were stained in the presence of either *N*-acetylglucosamine (50 mM), fucose (50 mM) or unconjugated wheat-germ agglutinin (250 μg/ml). The ability of these reagents to block the binding of fluoresceinated wheat-germ agglutinin was then assessed by comparing fluorescence histograms and by microscopy (data not shown). Unconjugated wheat-germ agglutinin reduced the mean cell fluorescence by approximately 70% and the competing sugar, *N*-acetylglucosamine also produced a reduction of approximately 40%. Fucose, a sugar that is unrelated to the structure of the wheat-germ agglutinin binding site, did not significantly reduce the fluorescence.

In order to quantitate the increased binding of wheat-germ agglutinin and to investigate the binding of other lectins to stimulated lymphocytes,

TABLE I

INCREASE IN LECTIN BINDING TO MURINE LYMPHOCYTES AFTER 40 h

Each value is a ratio of mean cell fluorescence, i.e., the ratio relative to small cells at zero time. Mean ± S.D. (n = three experiments). WGA, wheat-germ agglutinin; LcH, *Lens culinaris* haemagglutinin; Con A, concanavalin A.

	WGA	LcH	Con A
Small cells	2.08 ± 0.55	0.97 ± 0.11	1.55 ± 0.10
Large cells	5.45 ± 0.24	2.17 ± 0.42	2.12 ± 0.59

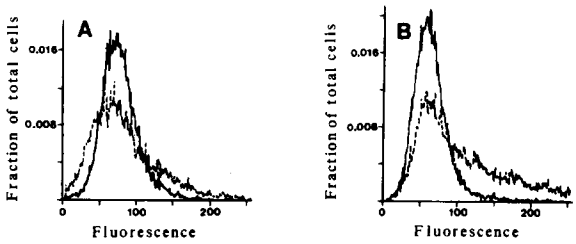

Fig. 5. Fluorescence of murine lymphocytes cultured in the presence of phytohaemagglutinin. Panels A and B show the normalized distributions obtained from viable cells not cultured (solid lines) and cultured for 40 h (broken lines) and stained with FITC-*Lens culinaris* haemagglutinin and FITC-concanavalin A, respectively.

experiments were carried out in which cells, cultured with phytohaemagglutinin for 40 h, were compared with freshly prepared cells treated with phytohaemagglutinin but kept on ice (zero time). Aliquots of cells from both of these treatments were then stained with either FITC-wheat-germ agglutinin, FITC-*Lens culinaris* haemagglutinin or FITC-concanavalin A, or left unstained. Typical fluorescence distributions are shown in Fig. 5. The emission from the *Lens culinaris* haemagglutinin and concanavalin A-stained specimens at zero time is very bright (20- to 30-times that of wheat-germ agglutinin-stained cells) and all of the cells are positive. Nonetheless, it can be seen that there is also increased binding of these lectins at 40 h. The intensification of the small cells, expressed as a ratio of the mean emission at 40 h to that at time zero, is indicated in Table I. A measure of the intensification that accompanies blast transformation is then given by the ratio of the mean fluorescence of the large cells at 40 h to that of the small cells at time zero. This is also shown in Table I. In summary, the large cells at 40 h have twice the ability to bind *Lens culinaris* haemagglutinin and concanavalin A compared with cells at zero time, while the wheat-germ agglutinin binding is increased more than 5-fold. Small lymphocytes also bind twice as much wheat-germ agglutinin at 40 h, while *Lens culinaris* haemagglutinin-binding is not increased and concanavalin A-binding increases by only 50%.

An approximate estimate of the volume increase of cells transformed by phytohaemagglutinin was made using Coulter analysis. Volume distributions from resting lymph node cells and cells harvested at 40–42 h were compared and the volume distribution of the transformed population was calculated by simple subtractive stripping, although the presence of cell debris and dead cells limits the accuracy of this method to some extent. The ratio of the median sensed volume of the transformed population to that of the resting cells was 1.84 (mean of three experiments, S.D. = 0.06). Raising this value to the 2/3 power then gives an approximate value for the surface area ratio for the two cell types of 1.5.

Characterization of lectin-binding components

(1) SDS-polyacrylamide gel electrophoresis and lectin staining. The glycoprotein components present in the plasma membranes of resting and mitogen-stimulated pig lymphocytes were investigated by SDS-polyacrylamide gel electrophoresis using autoradiography to locate the components that bind radiolabelled lectins (Fig. 6). The autoradiographs, shown in panel A of Fig. 6, reveal the glycoproteins that bind wheat-germ agglutinin, although the pattern is somewhat diffuse. A distinct band corresponding to a molecular mass of 120 kDa (indicated by an arrow), a set of bands between 170 kDa and 190 kDa (indicated by an asterisk) and a diffuse region of high molecular mass material (greater than 200 kDa) show a marked increase in binding in stimulated cells (lane b). The membrane from resting cells (lane a) appears to bind very little wheat-germ agglutinin. The protein-staining patterns of the gel in panel B show that comparable amounts of total membrane protein were loaded. The electrophoretic patterns obtained with radiolabelled *Lens culinaris* haemag-

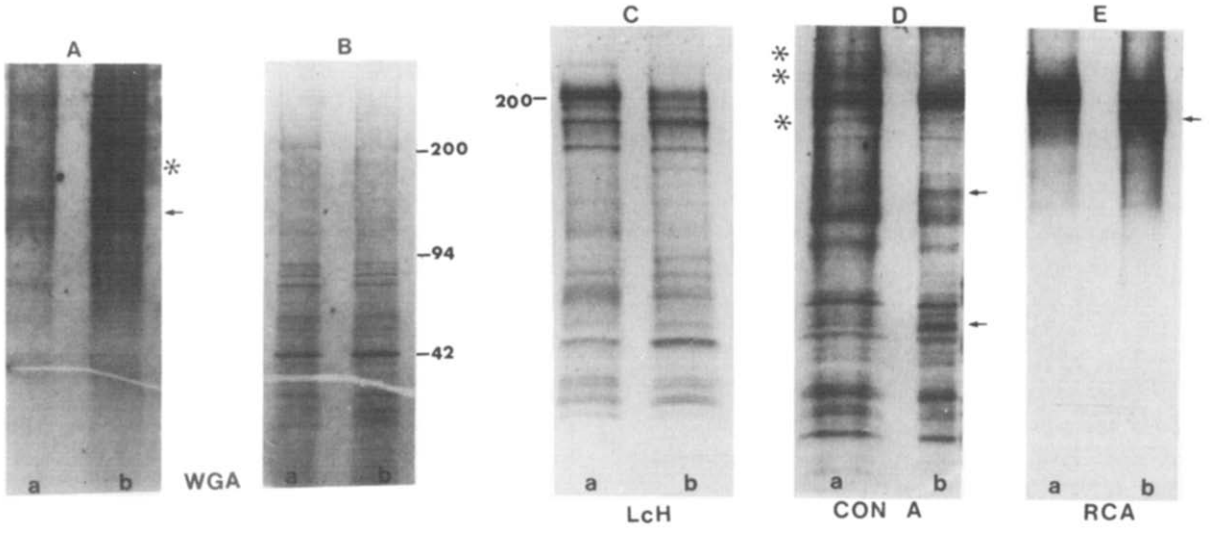

Fig. 6. SDS-polyacrylamide gel electrophoresis of plasma membrane components from resting (lane a) and stimulated (lane b) porcine lymphocytes. Panel B shows a gel treated with radiolabelled wheat-germ agglutinin and stained for protein with Coomassie brilliant blue. Panel A is the corresponding autoradiograph. Panels C, D and E are autoradiographs obtained from gels after treatment with radioactively labelled *Lens culinaris* haemagglutinin, concanavalin A and *Ricinus communis* agglutinin, respectively; (protein-stained gels not shown). Molecular masses are indicated in kDa. These autoradiographs have been positioned so that the 200 kDa components are aligned. They are not otherwise directly comparable due to differences caused by the drying procedure.

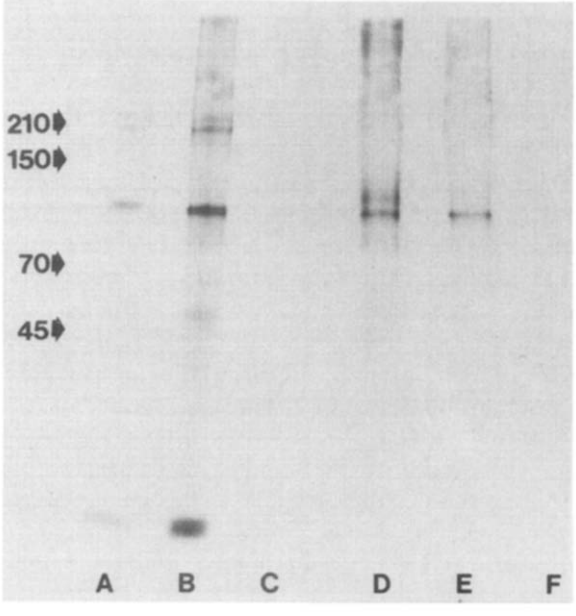

Fig. 7. Fluorograph of an SDS-polyacrylamide gel of membrane components from ^{3}H-labelled blast cells, immunoprecipitated with various concentrations of wheat-germ agglutinin as described in the text. Lanes A and B show the total sialic acid-containing, cell surface glycopeptides labelled by periodate oxidation and $NaB^{3}H_{4}$; approx. 6000 and 30000 cpm were loaded in each lane, respectively. Lanes C, D and E are immunoprecipitates from 0.1 μg, 1.0 μg, and 100 μg wheat-germ agglutinin and with approx. 5000, 14000 and 7000 cpm loaded, respectively. Lane F represents the control where wheat-germ agglutinin was omitted. Approx. 3000 cpm were loaded. The film was developed after fluorography for 60 days at −70°C.

glutinin, concanavalin A and *Ricinus communis* agglutinin are also shown in Fig. 6 (panels C, D and E, respectively). The corresponding Coomassie blue-stained gels indicate that comparable amounts of membrane protein were loaded for resting and stimulated cells (data not shown). 15–20 glycoprotein components binding concanavalin A can be clearly resolved in both the membrane preparations ranging in molecular mass from approx. 20 to 250 kDa (panel D). Although most of these bands are present in both resting and stimulated cells (lanes a and b, respectively), certain glycoproteins bind more concanavalin A after stimulation (for example at 120 kDa and 45 kDa, indicated by arrows) while others bind less (for instance two components with molecular

masses greater than 200 kDa and one at 170 kDa, indicated by asterisks). A similar result is seen with *Lens culinaris* haemagglutinin (panel C), although the disparities between the resting and stimulated cells are considerably less marked (lanes a and b). *Ricinus communis* agglutinin, on the other hand, reveals fewer binding glycoproteins than concanavalin A or *Lens culinaris* haemagglutinin and increased binding after stimulation is only evident for the 180 kDa component (indicated by an arrow in lane b of panel E).

(2) Characterisation of wheat-germ agglutinin-binding components by immunoprecipitation of ^{3}H-labelled surface glycoproteins. Membrane components binding wheat-germ agglutinin were precipitated by antibody and the glycoprotein-binding components detected by autoradiography of SDS-polyacrylamide gels as described above. The results are shown in Fig. 7. At a concentration of 1.0 μg/ml (lane D), wheat-germ agglutinin co-precipitated blast cell membrane components running at 120–130 kDa and 100 kDa together with some higher molecular mass material. At 100 μg/ml (lane E), only the 100 kDa band may be seen. No band was detected at 0.1 μg/ml (lane C), in controls lacking wheat-germ agglutinin (lane F), nor in wheat-germ agglutinin immunoprecipitates obtained from ^{3}H-labelled, resting lymph node cells (not shown).

Discussion

The enumeration of wheat-germ agglutinin-binding cells by fluorescence microscopy yielded data similar to those obtained by Robinson et al. [6] in that there appears to be an increased incidence of positive cells at approx. 40 h after the addition of mitogen. At time zero, the percentage of wheat-germ agglutinin-positive cells is typically less than 10% (Fig. 1). The difficulty with this assessment is that cells that are stained weakly are hard to see and tend to be scored as negative. Furthermore, estimating the incidence of positive cells does not provide information about the abundance of receptors, since cell brightness cannot be accurately assessed by eye. For these reasons we have used flow cytometry to provide quantitative intensity data. A principal advantage of this technique is that light-scatter signals may be used to select, on a size basis, two populations of cells for fluorescence analysis (Fig. 2). In these experiments, the bright light-scatter population consists mainly of transformed lymphocytes, while the dimmer scatter window contains signals from small lymphocytes that have not increased in size. Using these scatter criteria to gate the collection of fluorescence data, we find that at zero time there is significant wheat-germ agglutinin-binding to resting lymphocytes, a large proportion of which would not be scored as positive by fluorescence microscopy. Following phytohaemagglutinin-stimulation there is a marked increase in the intensity of the larger cells that peaks at about 40 h. Accompanying this, there is a less marked intensification of the small cells. If we make the reasonable assumption that fluorescence intensity is proportional to dye content [18] and consequently to the number of binding sites, then the blast cells at 40 h possess 5.5-times the number of sites that exist on small lymphocytes at time zero. Since this binding is inhibited by *N*-acetylglucosamine, we conclude that it is specific.

The binding of the lectins *Lens culinaris* haemagglutinin and concanavalin A is also increased for cells that have been exposed to phytohaemagglutinin for 40 h. However, the extent of the increased uptake is considerably less than that observed with wheat-germ agglutinin (Table I). Cells that undergo blast transformation become enlarged, increasing their surface area. In so doing they may reveal binding sites that were previously inaccessible. Alternatively the glycoproteins that bind certain lectins could be synthesized at the same rate as the new plasma membrane, perhaps because they form an integral part of its structure. Either of these possibilities might account for the increased binding of *Lens culinaris* haemagglutinin and concanavalin A to stimulated cells which could occur without an appreciable increase in receptor surface density. Coulter analysis reveals that the transformed cells at 40 h have increased their surface area by approx. 50%, assuming that the cells are spherical and that any contribution made by microvilli does not affect the calculation unduly. If the fluorescence is proportional to the number of binding sites, then the surface density of the receptors for both *Lens culinaris* haemag-

glutinin and concanavalin A has increased by approx. 45%. This is a relatively modest figure compared with a factor of 3.6 for wheat-germ agglutinin. (Unfortunately, we are not able to measure Coulter volumes directly in the flow cytometer in order to relate individual cell fluorescence and surface area.)

In order to characterize the components involved in these lectin-binding changes, isolated membranes were run on SDS-polyacrylamide gels and stained with radioactively labelled lectins. It was necessary to use pig lymphocytes for these studies to provide the quantity of membrane required. In agreement with the fluorescence data, the membranes from phytohaemagglutinin-stimulated blasts bind considerably more wheat-germ agglutinin than do resting cell preparations, the main increases being associated with bands corresponding to 120, 170–190 and over 200 kDa. The results observed with the other lectins are less dramatic, but concanavalin A does pick out components at 45 and 120 kDa which are more strongly stained in the blast cell preparations. Although the autoradiographs obtained with labelled *Lens culinaris* haemagglutinin and concanavalin A show discrete bands, the pattern seen with wheat-germ agglutinin is rather diffuse and more difficult to analyse. The reason for this is unclear, since wheat-germ agglutinin staining of human erythrocyte membranes by identical techniques gives sharp bands corresponding with Band III and the glycophorins (unpublished observations). Less equivocal results were obtained by tritiating directly the sialic acid residues on murine lymphocyte membranes and co-precipitating the wheat-germ agglutinin-binding molecules with rabbit antiserum. This reveals dominant bands at 100 and 120 kDa (and possibly some higher molecular mass material) which are not detectable in preparations from resting cells treated similarly. At much higher concentrations of wheat-germ agglutinin, only the 100 kDa band is visible; it may be that the 120 kDa wheat-germ agglutinin 'receptors', perhaps because of different affinity, form complexes with the lectin which strip from the surface and are lost on washing. It is of interest here that Andersson et al. [19,20] have demonstrated the expression on the cell surface of a 130 kDa glycoprotein in activated T cells [19] and also other, lower molecular mass, components in both T and B lymphoblasts [20].

Our results indicate that only a relatively restricted group of glycoproteins are altered markedly with respect to their affinity for wheat-germ agglutinin on polyclonal stimulation of small lymphocytes. They also provide information that will allow further studies to isolate and characterize these components with a view to identifying blast-specific molecules which could act as possible targets for immunological attack. Furthermore, the changes in the sugar residues on the surface of lymphocytes are likely to be related to function. While the details of the mechanism by which growth factors control transformation and proliferation are not yet understood, activation by mitogenic lectins clearly requires a glycoprotein binding site to initiate the response. A recent report has shown that surface sugars are also required at a later stage during stimulation by concanavalin A or lipopolysaccharide, since inhibition of membrane protein glycosylation by tunicamycin abrogates cell proliferation [21]. A possible immunoregulatory role for the glycoprotein receptors that bind wheat-germ agglutinin has been proposed [22] whereby a positive signal is transmitted by high-affinity sites and a negative signal by low-affinity sites [23]. The weakly staining cells that we observe at time zero may be those that can be stimulated to both proliferate and secrete immunoglobulin upon treatment with low doses (2–10 μg/ml) of wheat-germ agglutinin. If the additional wheat-germ agglutinin receptors that appear after stimulation by phytohaemagglutinin correspond to the low-affinity sites, the transformed cells might then be susceptible to control by soluble suppressor factors, like those reported by Greene et al. [24] which function by binding to the same receptors that are recognized by wheat-germ agglutinin.

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References

1 Udey, M.C. and Parker, C.W. (1981) J. Immunol. 126, 1106–1113
2 Thomas, B.D. (1971) Nature 233, 317–321
3 Feizi, T. (1981) Trends Biochem. Sci. 6, 333–335
4 Gruner, K.R., Van Eijk, R.V.W. and Muhlradt, P.F. (1981) Biochemistry 20, 4518–4522
5 Van Eijk, R.V.W., Rosenfelder, G. and Muhlradt, P.F. (1979) Eur. J. Biochem. 101, 185–193
6 Robinson, P.J., Anderton, B.H. and Roitt, I.M. (1976) Exp. Cell Res. 102, 431–434
7 Fraker, P.J. and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. 80, 849–857
8 Davidson, W.F. and Parish, C.R. (1975) J. Immunol. Methods 7, 291–300
9 Allan, D. and Crumpton, M.J. (1970) Biochemical J. 120, 133–143
10 Banga, J.P.S., Penfold, P.L. and Roitt, I.M. (1981) Biochem. J. 198, 421–423
11 Thorpe, R., Delacourte, A., Ayers, M., Bullock, C. and Anderton, B.H. (1979) Biochem. J. 181, 275–284
12 Robinson, P.J., Bull, F.G., Anderton, B.H. and Roitt, I.M. (1975) FEBS Lett. 58, 330–333
13 Burridge, K. (1978) Methods Enzymol. 50, 54–58
14 Gahmberg, C.G. and Andersson, L.C. (1977) J. Biol. Chem. 252, 5888–5894
15 Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335–341
16 Nash, G.B., Tatham, P.E.R., Powell, T., Twist, V.W., Speller, R.D. and Loverock, L.T. (1979) Biochim. Biophys. Acta 587, 99–111
17 Loken, M.R. and Herzenberg, L.A. (1975) Ann. N.Y. Acad. Sci. 254, 163–171
18 Kerker, M., Van Dilla, M.A., Brunsting, A., Gray, J.W. and Langlois, R.C. (1981) Cytometry 2, 108
19 Andersson, L.C., Gahmberg, C.G., Kimura, A. and Wigzell, H. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3455–3458
20 Gahmberg, C.G., Hayry, P. and Andersson, L.C. (1976) J. Cell Biol. 68, 642–653
21 Harrison, M.L. and Villemez, C.L. (1981) J. Immunol. 127, 2616–2617
22 Greene, W.C. and Waldmann, T.A. (1980) J. Immunol. 124, 2979–2987
23 Greene, W.C., Goldman, C.K., Marshall, S.T., Fleisher, T.A. and Waldmann, T.A. (1981) J. Immunol. 127, 799–804
24 Greene, W.C., Fleisher, T.A. and Waldmann, T.A. (1981) J. Immunol. 126, 1185–1191