Membrane lectins on human monocytes

Maturation-dependent modulation of 6-phosphomannose and mannose receptors

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Freshly isolated human monocytes, which do not contain cell-surface mannose-specific receptors, bind mannose 6-phosphate and actively endocytose mannose 6-phosphate-bearing neoglycoproteins (6-P-Man-F-BSA). Three days after isolation, human monocytes endocytose very actively 6-P-Man-F-BSA as well as Man-F-BSA, and the endocytosed neoglycoproteins are rapidly degraded. These results were obtained in quantitative flow cytofluorometry by using a panel of fluoresceinylated sugar-substituted serum albumins (neoglycoproteins). Thus, in contrast to mannose receptors which appear only after maturation, mannose 6-phosphate receptors are already present on freshly isolated human monocytes.

Lectin *Monocyte* *Endocytosis* *Flow cytofluorometry*

1. INTRODUCTION

Mannose-specific receptors have been found on a variety of macrophages, including alveolar macrophages [1], peritoneal macrophages [2,3] and macrophages derived from bone-marrow [1]. These receptors (membrane lectins) mediate endocytosis of their specific macromolecular ligands. Efficient internalisation of biological response modifiers (BRMs), such as muramyldipeptide (MDP) linked to a mannosylated serum albumin, has been shown to occur in the case of murine alveolar and peritoneal macrophages and to cause these macrophages to become tumoricidal in both in vitro and in vivo experiments [4,5], thereby allowing efficient treatment of lung metastases [5]. The lack of mannosyl receptors on freshly isolated human monocytes [6] precluded the use of such a carrier for activation of monocytes in vivo. Because circulating monocytes are the first cells that a systemically administered BRM carrier could encounter and because they are precursors of tissue macrophages such as alveolar macrophages, it seemed worthwhile to examine freshly isolated monocytes for the presence of specific receptors able to mediate endocytosis. Using fluoresceinyl neoglycoproteins and a quantitative flow cytofluorometric method [7] we demonstrate here that freshly isolated monocytes express 6-phosphomannose receptors and thus allow internalization of 6-phosphomannosylated serum albumin.

2. MATERIALS AND METHODS

Bovine serum albumin (BSA) was purchased from IBF-Reactifs, Pointet-Girard (Villeneuve-la-Garenne, France). Alkaline phosphatase was obtained from Sigma (St. Louis, MO). Fluoresceinyl isothiocyanate (isomer I) was from Molecular Probes (Junction City, OR). *p*-Nitrophenyl-α-D-mannopyranoside and leupeptin (*N*-propionyl-L-leucyl-L-leucyl-L-arginal) were from Sigma. Monensin was obtained from Calbiochem (La Jolla, CA), a 25 mM stock solution in ethanol being prepared and stored at $-20°C$.

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2.1. *Synthesis of* p-*nitrophenyl-6-phospho-α-D-mannopyranoside*

The title compound was prepared by a direct phosphorylation method according to Sowa and Ouichi [8] for the preparation of 5′-ribonucleotides from ribonucleosides. Briefly, *p*-nitrophenyl-α-D-mannopyranoside (3 g, 10 mmol) was dissolved by adding pyridine (4 ml, 50 mmol), acetonitrile (10 ml, 190 mmol) and water (0.4 ml, 22 mmol). To this solution, phosphoryl chloride (4 ml, 44 mmol) was added and the mixture was stirred for 1 h at 0°C. The reaction mixture was poured onto 120 g ice and adjusted to pH 7.0 by slowly adding 2.5 M NaOH. The neutralized solution was evaporated to dryness. The solid material was dissolved in 150 ml water. The solution was concentrated under reduced pressure at 35°C in a rotary evaporator to a final volume of 3–4 ml. The concentrated solution was kept at 4°C overnight to complete the crystallisation. The crystals were filtered and washed with 10 ml absolute ethanol. The compound was recrystallised from a 10 ml water/100 ml ethanol mixture resulting in 3 g material.

Our procedure is faster than the previously described method [15]. Characterization of the product was performed as follows:

Thin-layer chromatography on silica gel G (Merck, Darmstadt): R_f, 0.37 (solvent $CHCl_3$/CH_3OH/H_2O, 13:8:2, v/v); absorption spectroscopy, at 310 nm $\epsilon_{M,1\ cm}$ = 9800; at 410 nm $\epsilon_{M,1\ cm}$ = 30, in 0.1 M NaOH).

In the ^{31}P-NMR spectrum obtained with a Bruker AM300WB spectrometer operating at 121.513 MHz, a triplet at 1.61 ppm (J=6 Hz) characteristic of a phosphate bound to a primary alcohol function; upon proton noise decoupling, the signal was a singlet. ^{31}P chemical shifts are reported relative to trimethyl phosphate;

Digestion with alkaline phosphatase followed by thin-layer chromatography analysis: the R_f 0.37 compound was quantitatively converted into an R_f 0.68 compound which comigrated with an authentic *p*-nitrophenyl-α-D-mannopyranoside sample.

2.2. *Neoglycoproteins*

Neoglycoproteins were obtained by allowing phenylisothiocyanate glycoside to react with bovine serum albumin [7]. Neoglycoproteins were purified by gel filtration on Ultrogel ACA 202 or GF05 (IBF-reactifs) in distilled water (or in phosphate-buffered saline for 6-P-Man derivatives). Neutral sugar content was determined by using the resorcinol sulfuric micromethod [9] and protein concentration according to Bradford [10]. Fluoresceinyl neoglycoproteins were prepared as in [7] and the average number of fluorescein residues bound to each neoglycoprotein was determined after pronase treatment [11]. Here, fluoresceinyl neoglycoproteins contained 3 ± 0.5 fluorescein residues and 20 ± 3 sugar residues per molecule.

2.3. *Monocyte purification*

Whole blood was drawn from healthy donors (Centre de transfusion sanguine, Orléans) on citrate as anticoagulant. After centrifugation (300 × *g*, 15 min to remove the platelet-rich plasma), mononuclear leukocytes were isolated using centrifugation on Ficoll Paque (Pharmacia, France) [12]. Cells ($2–3\times10^6$/ml) in RPMI supplemented with 10% decomplemented horse serum (IBF-Réactifs) were further plated on petri dishes precoated with fetal calf serum. After 60 min incubation at 37°C in a humidified atmosphere (5% CO_2, 95% air), non-adherent cells were removed by 3 vigorous washes with phosphate buffer (PBS) and adherent cells were released by incubation for 10 min at 37°C in PBS containing 0.02% EDTA. Adherent cells were washed by centrifugation and plated in RPMI containing 10% decomplemented horse serum, (5×10^5 cells/well), in 24-well plates, replenished after a day with MEM medium supplemented with 10% horse serum.

2.4. *Flow cytofluorometry analysis*

After overnight incubation (day 1) or after 2 or 3 days in culture (day 3, 4), the supernatant was discarded and the plated cells incubated with 100 μg/ml fluoresceinyl neoglycoproteins in complete PBS (1 mM $CaCl_2$, 0.5 mM $MgCl_2$) supplemented with 1% bovine serum albumin (fraction V, Sigma). Incubation was performed for 2 h at 4°C, or at 37°C to measure endocytosis, in the absence or presence of protease inhibitor (200 μM leupeptin). Cells were then collected by using a rubber policeman, washed twice with cold PBS and sheath fluid [134 mM NaCl, 0.96 mM Na_2EDTA, 3.75 mM KCl, 1.9 mM KH_2PO_4, 16.53 mM Na_2HPO_4, 15.25 mM NaF, 0.2% (v/v) 2-phenoxyethanol]. Fluorescence of the cell suspension was analyzed

using an FACS analyzer (Becton Dickinson, Sunnyvale, CA). Size and fluorescence (λ_{exc} 485 ± 10 nm, λ_{em} 530 ± 15 nm) intensity were recorded at a rate of 200–300 cells/s. The fluorescence of cells incubated at 4 and 37°C was analysed either immediately or after 30 min incubation at 4°C in the presence of 50 μM monensin. The FACS analyzer was standardized by using fluorescent polystyrene-sulfonate beads as in [7]. Monocytes were identified by using specific monoclonal antibodies Mo2, (Coulter Clone, Coulter Electronics, Hialeah, FL) labeled with fluoresceinyl-goat anti-mouse immunoglobulin (Nordic, Tilburg, The Netherlands).

3. RESULTS

Freshly isolated human monocytes are known to be devoid of mannose-binding receptors [6]. Upon culturing for a few days, monocytes differentiate and concomitantly mannose-specific receptors appear [6]. Neoglycoproteins bearing either neutral sugars of *N*-acetylglucosamine (fig.1) failed to be bound and/or internalize significantly to freshly isolated monocytes. Knowing that monocytes derive from the same embryonic tissue as fibroblasts, i.e. mesenchyme, and that fibroblasts [13] as well as rabbit alveolar macrophages [14] express a 6-P-Man-specific receptor, we decided to check for the presence of such a membrane receptor in freshly isolated human monocytes. Neoglycoproteins and their fluorescein-substituted derivatives were prepared as in [7].

The 6-phosphorylmannosylated serum albumin (6-P-Man-BSA) used here contained about 20 6-phosphorylmannose residues per molecule neoglycoprotein as determined by the resorcinol-sulfuric acid method [9].

Mononuclear cells isolated by centrifugation through Ficoll-Paque and adhesion in petri dishes were plated and the presence of membrane lectin was investigated after an overnight incubation in complete medium or after 3 or 4 days in cultures. Monocytes were identified by using anti-monocytes (Mo2) and corresponded to the cells having the highest volume.

Binding of neoglycoproteins was measured by incubating plated cells for 2 h at 4°C with 100 μg/ml fluoresceinylated neoglycoproteins. The fluorescence intensity of the cells was only slightly higher than that of cells incubated with sugar-free

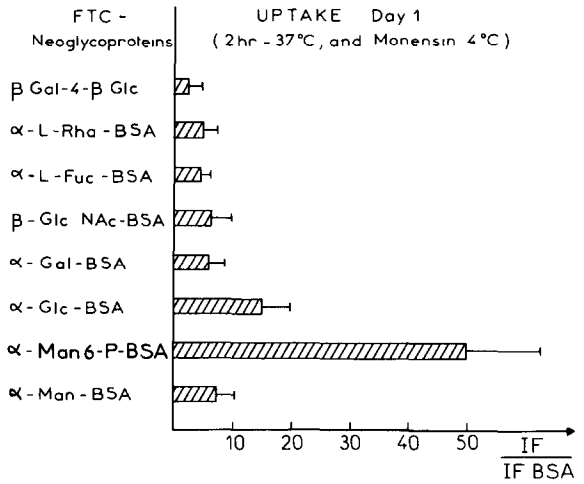

Fig.1. Uptake of fluorescein-labeled neoglycoproteins by freshly isolated monocytes. Monocytes, isolated by centrifugation through Ficoll-Paque and adherence on petri dishes, were plated in 24-well plates (5×10^5 cells) in complete RPMI medium. Adherent cells were incubated for 2 h at 37°C after overnight incubation (day 1) or after 3 days in culture (day 4), in the presence of 100 μg/ml fluorescein-labeled neoglycoproteins in PBS containing 1% BSA, washed and then incubated for 30 min at 4°C in the presence of 50 μM monensin. The cell-associated fluorescence was expressed as $(F_N - F_C)/F_C$ where F_N is the cell-associated fluorescence of cells incubated in the presence of fluorescein-labeled neoglycoproteins and F_C in the presence of fluorescein-labeled serum albumin. Monocytes were identified by monoclonal antibody Mo2. The values shown are the average of 5 independent experiments; bars indicate SD.

F-BSA, except upon incubation with 6-P-Man-F-BSA in which case the cells gave significant labeling (table 1). The fluorescence intensity of cells incubated for 2 h at 37°C with F-neoglycoproteins was higher than after 4°C incubation; however, it did not reflect the actual amount of cell-associated neoglycoproteins. Indeed, the fluorescence of fluorescein-labeled proteins or neoglycoproteins is very dependent on pH [16]; at low pH, the fluorescence intensity is almost quantitatively quenched. As internalized fluorescein derivatives are associated with endosomes and lysosomes (the pH of which is close to 5) the fluorescence of cells after internalizaton of F derivatives is much lower than that of an equivalent amount bound to the cell surface. To circumvent this pitfall, monensin was used as follows. Monensin, a proton-sodium iono-

Table 1

Cell-associated fluorescence of monocytes incubated in the presence of fluoresceinylated neoglycoproteins

	Fluorescence intensity[a]					
	4°C, (−)		37°C, (−)		37°C, (+)	
	Day[b] 1	Day 4	Day 1	Day 4	Day 1	Day 4
F-BSA	0.1	0.1	0.15	1.5	0.2	4
α-Man-F-BSA	0.15	2	1.5	105	3	240
6-P-Man-F-BSA	3	3	15	120	30	270
α-Glc-F-BSA	0.5	0.5	9	120	15	230

[a]The mean fluorescence intensities were standardized with fluorescent beads [7]

[b]Day 1, freshly isolated monocytes after overnight incubation in culture medium; day 4, monocytes which had been cultured for 3 days

Plated monocytes were incubated either at 4 or 37°C for 2 h in the presence of 100 μg/ml fluorescein-labeled neoglycoproteins and further incubated 30 min at 4°C in the presence (+) or absence (−) of 50 μM monensin in phosphate-buffered saline

phore, is known to equilibrate the internal and external pH [17] and so allows a more accurate determination of the amount of F-neoglycoproteins endocytosed by the cells. After incubation at 37°C, in the presence of F-neoglycoproteins, the cells were washed and further incubated at 4°C in the presence of 50 μM monensin (30 min). These conditions were optimized by varying the concentration of monensin and the incubation times in preliminary experiments.

As shown in table 1, the uptake of 6-P-Man-F-BSA by freshly isolated monocytes was much higher than that of Man-F-BSA. Among the neoglycoproteins used to label cultured monocytes, 6-P-Man-F-BSA was the only one (with the exception of Glc-F-BSA) to give uptake significantly higher than that of Man-F-BSA (fig.1). All the F-neoglycoproteins had a similar number of sugars and a similar number of fluorescein molecules per serum albumin molecule; only slight fluorescence labeling was obtained with sugar-free-F-BSA and Man-F-BSA which is known not to be internalized by freshly isolated monocytes as shown by Shepherd et al. [6]. A neoglycoprotein bearing a large amount of negative charge, such as succinylated Man-F-BSA, was endocytosed to the same extent as Man-F-BSA; furthermore, the uptake of 6-P-Man-F-BSA was inhibited by incubation in the presence of the fluorescein free 6-P-Man-BSA (not shown). Thus, 6-P-Man receptors are able to bind and internalize 6-P-Man derivatives and appear on monocytes before mannose receptors, and before in vitro maturation or transformation into macrophages. However, the amount of 6-P-Man-F-BSA endocytosed by freshly harvested monocytes was lower than that endocytosed by monocytes cultured for 3 days (table 1). The level of endocytosis of 6-P-Man-F-BSA by matured monocytes is 10-times higher than the level of endocytosis by freshly isolated monocytes and is in the range of that of Man-F-BSA.

The capacity of monocytes to digest internalized fluorescein-labeled neoglycoproteins was investigated. The fluorescence intensity of neoglycoproteins containing 3 fluorescein residues is about one-third of that of the same fluorescein-labeled neoglycoproteins fully digested by pronase [11]. Thus, the extent of digestion of internalized fluorescein-labeled neoglycoproteins could be assessed by comparing the cell-associated fluorescence of cells incubated in the absence and presence of a lysosomal protease inhibitor such as leupeptitin [18]. It was first determined that 200 μM leupeptin does not impair fluorescein-labeled

Table 2

Effect of leupeptin on the cell-associated fluorescence of monocytes incubated at 37°C for 2 h in the presence of fluorescein-labeled neoglycoproteins

	Fluorescence intensity[a]			
	Day[b] 1		Day 4	
	(−)	(+)	(−)	(+)
F-BSA	0.1	0.1	4	2
α-Man-F-BSA	3	3	240	140
6-P-Man-F-BSA	30	28	270	120
α-Glc-F-BSA	9	9	230	90

[a]The mean fluorescence intensities were standardized by using fluorescent beads

[b]Day 1, freshly isolated monocytes after overnight incubation in culture medium; day 4, matured monocytes which had been cultured for 3 days

Plated monocytes were incubated at 37°C for 2 h with 100 μg/ml fluorescein-labeled neoglycoproteins in the presence (+) and absence (−) of 200 μM leupeptin and further incubated at 4°C for 30 min in the presence of 50 μM monensin in phosphate-buffered saline

neoglycoprotein uptake. As shown in table 2, leupeptin has almost no effect on the cell-associated fluorescence of freshly isolated monocytes incubated for 2 h in the presence of fluorescein-labeled neoglycoproteins. Conversely, the cell associated-fluorescein of monocytes cultured for 3 days, and then incubated in the presence of both 6-P-Man-F-BSA and leupeptin, is about one-half that of cells incubated in the absence of leupeptin, showing that matured monocytes are able to degrade internalized neoglycoproteins.

4. DISCUSSION

Freshly isolated human monocytes contain cell-surface receptors which mediate the uptake of 6-phosphomannose-terminated glycoconjugates and to a lower extent that of α-glucose-terminated glycoconjugate. The endocytosed material was in an acidic environment, since the cell-associated fluorescence increased upon post-incubation at 4°C in the presence of 50 μM monensin. A similar effect of monensin was observed with membrane lectins of tumor cells such as L1210 and Lewis lung carcinoma cells (3LL) which bind and internalize fucosylated and glucosylated serum albumin, respectively [7,11]. The 6-phosphomannose serum albumin was not digested by fresh monocytes as shown by the absence of the expected effect of leupeptin addition in the incubation medium; this is in agreement with the low content of lysosomal hydrolases.

Matured monocytes obtained after 3 days in culture are able to mediate the uptake not only of mannose-terminated glycoprotein [1] but also to a similar extent of α-glucose-terminated glycoproteins and 6-phosphomannose-terminated glycoproteins. The difference between fresh and matured monocytes is more important for mannose than for 6-phosphomannosyl receptors, since the fluorescence of Man-F-BSA internalized by 3 days cultured monocytes is 100-times higher than the amount internalized by monocytes 1 day after isolation. The material endocytosed by matured monocytes is in an acidic environment and is mostly digested as shown by the enhancement of cell-associated fluorescence upon post-incubation at 4°C in the presence of 50 μM monensin and the lower cell-associated fluorescence upon incubation in the presence of 200 μM leupeptin, respectively.

Thus, matured monocytes are clearly able to digest the major part of the endocytosed fluorescein-labeled neoglycoproteins upon 2 h incubation at 37°C.

The presence of 6-P-mannosyl receptors on human monocytes, mediating internalization of the specific ligand, opens the possibility of targeting immunostimulants to human monocytes, even if the internalized neoglycoprotein is not, or only partially digested. Immunostimulants linked to a 6-phosphomannose-terminated carrier via a pH-sensitive bond could be released inside monocytes leading to the specific activation of monocytes and monocyte-derived macrophages.

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