Influence of Spacer Length on Interaction of Mannosylated Liposomes with Human Phagocytic Cells

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Purpose. To improve target specificity and uptake of liposomes by macrophages, one can improve high-affinity receptor binding to mannose determinants with their 175-kDa mannose receptor (MR), which is mainly influenced by the length and flexibility of the spacer between the carbohydrate head group and liposome surface. Liposomes containing alkylmannosides with hydrophilic spacers 0 to 8 ethyleneoxy units (EO) long (Man0...Man8) were used to investigate systematically the effects of spacer length on liposome–cell interactions.

Methods. Concanavalin A (ConA)-induced liposome aggregation was studied by turbidity measurement and cell uptake using PMA-induced HL-60 cells or native human macrophages by determining 6-CF after cell lysis or NBD-fluorescence with flow cytometry. Detection of MR in native cell populations was carried out by an antibody assay using flow cytometry; MR-representing cells were selected analytically.

Results. Liposomes containing mannosides with more than one EO spacer length were specifically aggregated by ConA, indicating accessibility of the carbohydrate ligands of these derivatives. Increase in EO spacer units of incorporated mannosides (two or more EO) led to suppression of cellular uptake of mannosylated liposomes by phagocytes lacking MR (HL60, U937). The extent of suppression increased with spacer length. Liposome uptake by native macrophages expressing MR was, on the contrary, improved, particularly by Man6 and Man8.

Conclusions. Uptake of liposomes modified with Man6 or Man8 by native cells was enhanced but did not reach an optimum. Thus, Man6, Man8, and mannosides with even longer spacer arms are of potential use in receptor-mediated targeting.

KEY WORDS: liposome uptake; mannose receptor; spacer length; macrophages; targeting; long-chain alkyl mannosides.

INTRODUCTION

In recent decades liposomes have attracted a great deal of interest as drug carriers. The nonspecific uptake of liposomes by macrophages can be used as a tool for drug delivery. As a result of their major role in physiological and pathophysiological functions, macrophages are also interesting target cells for the therapeutic use of liposome-associated immunomodulators, administration of antiinflammatory steroids, efficient treatment of diseases linked to macrophage-resident microorganisms and parasites, as well as optimization of the immune response to liposomal vaccines (1–3). To improve target specificity and uptake, the interaction of liposome surface determinants with macrophage membrane receptors can be exploited. Among the different membrane receptors of human macrophages, the 175-kDa mannose receptor is attractive for liposome targeting.

A variety of substances have been used to create MR-directed targeting devices. These include compounds of natural origin such as yeast mannan, mannosylated glycoproteins from *Leishmania,* and mycobacteria-derived lipo(arabino) mannan (1,4,5) as well as partially altered compounds of natural origin such as neoglycoproteins and mannosylated serum albumins (6). Enhancement of endocytosis by 100% to 150% compared to plain liposomes has been reported (7–10). Synthetic mannosylated liposomes have been prepared by coupling mannose determinants to preformed phosphatidylethanolamine-containing vesicles (neomannosylated liposomes) (11–13). Such mannosylated liposomes showed a 60% increased uptake by the liver relative to nonmannosylated liposomes (12) and have been used to reduce *Leishmania* parasites in the spleens of mice (14). Inhibition of liver metastasis in mice was observed by Sakai *et al.* (15) following targeting of immunomodulators using mannosylated liposome carriers. Mannosylated cationic liposomes (16–18) are also used in MR-mediated gene transfer to macrophages. Muller *et al.* achieved an improvement of liposome uptake by macrophages of between 200% and 800% *in vitro* (depending on cell type and liposome label) using mannose residues conjugated with a hydrophilic spacer arm (19). High-affinity binding to the MR has been investigated by other groups using polymers conjugated with short hydrophilic spacers and mono-, bi-, or trivalent carbohydrate determinants (20,21). Furthermore, polymannosylated conjugates have been used by a number of groups to improve receptor interaction to a greater or lesser extent *in vitro* (22) and *in vivo* (23). Biessen *et al.* (24) have reported very high-affinity binding to MR using lysine-based oligomannosides containing short spacer arms and suggested that this high affinity was a result of optimal configuration of the mannose determinants.

The work reported so far is quite heterogeneous. Because of the variability of mannosylation methods, varying numbers and densities of mannose residues, diversity of the parameters under investigation *in vitro* and *in vivo* (such as uptake of liposome marker, persistance of microorganisms, plasma clearance of the targeting devices or coinjected inhibitors, drug effect, or rate of survival of test animals), it is difficult to make a meaningful comparison.

However, it can be deduced that a certain number of mannose residues and an appropriate spatial extension along with a certain flexibility of the spacer arms are required for high-affinity binding. The current study was undertaken to investigate the influence of spacer length and flexibility on liposome–phagocyte interaction by stepwise elongation of the

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ABBREVIATIONS: 6-CF, 6-carboxyfluorescein; ConA, concanavalin A; DPPG, dipalmitoyl phosphatidylglycerol; EO, ethyleneoxy unit; FCS, fetal calf serum; H, 9:1 mol% HSPC:DPPG; HSPC, hydrated soy phosphatidylcholine; Man0...Man8, alkylmannosides with 0...8 EO spacer units; MR(−) cells, MR-lacking cells; MR(+) cells, MR-expressing cells; MR, mannose receptor; NBD-PE, dipalmitoyl phosphatidylethanolamine-(7-nitro-benz-2-oxa-1,3-diazo-4-yl); PcM, pericardial monocytes/macrophages; PMA, phorbol 12-myristate 13-acetate; PtM, peritoneal macrophages; RT, room temperature.

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spacer arm between the sugar moiety and the lipid anchor. To do this, a number of alkylglycosides were synthesized for incorporation into liposomes. The uptake of carbohydrate-modified liposomes by phagocytic cells expressing [MR(+) cells] or lacking mannose receptors [MR(−) cells] was studied in order to optimize receptor-mediated liposome targeting.

MATERIALS AND METHODS

Synthesis of Mannolipids

Alkyl mannosides were synthesized with or without spacers (Man^0...Man^8) according to previously published methods (25) using $FeCl_3$ as a catalyst. All spectral data of the synthesized alkyl mannosides were in accordance with that reported in the literature (26). Purity of the mannosides was shown to be 99.9% by HPLC.

Preparation of Liposomes and Fluorescence Labeling

Hydrated soy phosphatidylcholine (HSPC) was purchased from Lipoid KG (Ludwigshafen, Germany), dipalmitoyl phosphatidylglycerol (PG) from Sygena Ltd. (Liestal, Switzerland), dipalmitoyl phosphatidylethanolamine-(7-nitro-benz-2-oxa-1,3-diazo-4-yl) (NBD-PE) from Avanti Polar Lipids (Alabaster, AL), and 6-carboxyfluorescein (6-CF) from Serva (Heidelberg, Germany). The substances were used without further purification. All salts and buffers were of analytic grade. Lipids in chloroform/methanol (9:1, v:v) were combined as follows: a mixture of 9:1 mol% HSPC:PG (H) was taken as the basic lipid matrix for all liposomes, 7:3 mol% H:ManX was used for the lectin binding assay, and 9:1 mol% H:ManX for cell uptake studies. Liposomes prepared for native cell uptake studies using flow cytometry were labeled with 0.5 mol% NBD-PE. After solvent evaporation, the lipid film was suspended either in 0.15 M PBS, for lectin binding and native cell uptake studies, or in 0.1 M 6-CF solution (pH 7.4), for cell line uptake studies. The resulting preparations (15 μmol total lipid/ml) were extruded five times through 200-nm polycarbonate membrane (Nucleopore). Liposome size was determined by dynamic light scattering using a Malvern IIC Autosizer. Excess 6-CF was removed by dialysis against PBS. 6-CF fluorescence emission was determined using a Shimadzu RF-5001 PC spectrofluorophotometer. Because 6-CF was encapsulated at a self-quenching concentration, nonencapsulated 6-CF in the liposome preparation was determined by direct measurement. Encapsulated 6-CF was quantified following lysis of liposomes with 1% solution of Triton X-100 (Serva).

Cell Culture

HL-60 cells (ATCC, Rockville, MD) were cultured in suspension in IMDM (Gibco BRL, Grand Island, NY) supplemented with 20% heat-inactivated FCS (c.c. pro, Neustadt/W. Germany). Differentiation into macrophage-like cells was induced by incubation for 72 h with 100 nM Phorbol-12-myristate-13-acetate (PMA) (Calbiochem, Bad Soden, Germany) in the culture medium, according to Rovera *et al.* (27). After induction, cells were matured in PMA-free medium for 48 h before use. Cell viability was tested by trypan blue exclusion (0.4% solution) (Sigma, Deisenhofen, Germany).

Pericardial monocytes/macrophages (PcM) were collected from fresh pericardial fluid from patients undergoing open-heart surgery, and peritoneal macrophages (PtM) from fresh patient-derived peritoneal dialysis solution. After centrifugation, each of the cell types was washed twice with PBS and diluted with RPMI 1640 to 10^6 cells/ml.

Characterization of Native Cells by Flow Cytometry

Patient-derived monocytes/macrophages were identified using a Simultest™ IMK Plus-Kit (Becton Dickinson, Heidelberg, Germany) as follows: 5 μl LeucoGATE antibody solution (CD45-FITC/CD14-PhE) was added to 100 μl cell suspension and incubated for 15 min at room temperature (RT), followed by treatment with BD-Lyse (Becton Dickinson) for 10 min (RT). After being washed twice with PBS at 6–8°C, cells were fixed with 0.5 ml 2% paraformaldehyde in PBS for 5 min at RT and analyzed using a FACScan flow cytometer (Becton Dickinson) to define a forward/sideward light scatter gate for monocyte/macrophage identification.

Detection of MR was carried out by a two-step assay using a monoclonal antibody (mouse) against human MR (mAb-MR 15-2, a generous gift from D. Rijken, Gaubius Lab., Leiden, Holland) as described in the literature (28). Briefly, 50 μl cell suspension was incubated with 5 μl mAb-MR (10 μg/ml) for 30 min at 4°C and washed twice with cold (4°C) PBS. After incubation with antimouse IgG (PhE-labeled) for 30 min at 4°C, cells were washed twice with cold PBS, fixed by paraformaldehyde treatment (see above), and analyzed using a FACScan. Setting an analytic window in the FSC/FL2 diagram allowed mainly MR(+) cells to be selected.

The viability of the cells was tested separately by propidium iodide exclusion (Sigma; 0.5 mg/ml; 1 μl/100 μl cell suspension).

Estimation of Liposome Uptake

Cell Line HL-60

Each well of the culture plates was incubated with 20 μl liposome dispersion in 500 μl FCS-free IMDM. After incubation, cells were washed three times with cold PBS (4°C) to stop endocytosis and to remove excess liposomes. Finally, cells were lysed with Triton X-100 (1% in PBS), and 6-CF fluorescence was determined. All cell experiments were carried out both at 37°C and 4°C in parallel to distinguish between cellular uptake and adhesion of liposomes. Liposome uptake was calculated as the difference between fluorescence data obtained after treatment at 37°C (F $37°C_{cells}$) and 4°C (F $4°C_{cells}$). Furthermore, cell-free culture wells were treated in the same way to quantify nonspecific adhesion on plastic for every liposome preparation (F $37°C_{plastic}$; F $4°C_{plastic}$).

Calculation

Fluorescence data of the lysates (F_{lysat}) were corrected (F_{corr}) with the 6-CF encapsulation capacity of the liposomes (E%) to eliminate alteration of cell uptake data by altered 6-CF encapsulation of each individual liposome preparation: $F_{lysat}/E\% = F_{corr}$. Corrected fluorescence data were used for calculation of cellular uptake (F_{uptake}), as described above:

$$(F\ 37°C_{cells} - F\ 37°C_{plastic}) - (F\ 4°C_{cells} - F\ 4°C_{plastic}) = F_{uptake}$$

Native Cells

Approximately 10^5 cells were incubated with 20 μl liposome dispersion (3 μmol/ml) in 500 μl FCS-free RPMI 1640 at 37°C and 4°C in 12 × 75 mm Falcon® polystyrene test tubes (Becton Dickinson). Cell-associated fluorescence was analyzed (FACScan) after certain time spans. Liposome uptake was calculated using the difference in cell-associated fluorescences at 37°C and 4°C.

Lectin Binding Assay

Concanavalin A (ConA)-induced liposome aggregation was carried out by adding 10 μl ConA solution (25 mg/ml, Boehringer Mannheim, Germany) to 25 μl liposome preparation in 1 ml HEPES-buffer (1 mM Ca^{2+}, Mn^{2+}) and was monitored by turbidity measurement using a Shimadzu UV 120-02 spectrometer at 360 nm.

RESULTS

Mannolipids and Liposome Characteristics

Alkylmannoside derivatives with constant hydrophobic regions, i.e., hexadecyl anchor, and hydrophilic head group containing PEG spacers with increasing length between carbohydrate and lipid anchor moieties (Fig. 1) were incorporated into liposomes.

To achieve a sufficient density of the carbohydrate ligand, a glycolipid content of 10 mol% was used. Based on the surface area characteristics of the lipids and the measured liposome size, a density of 10–30 × 10^3 mannose ligands/liposome was calculated.

The stability of all liposome preparations was more than 90% retention of encapsulated marker under conditions necessary for cell uptake studies (37°C, cell culture medium, 1 h), which was assumed to be sufficient.

Lectin-Induced Liposome Aggregation

A ConA-binding study was carried out to estimate the accessibility of the mannose ligands to receptor proteins. The requirement of a minimum distance from the carbohydrate head group to the liposome surface has already been reported for galactosylated vesicles, but differing results were found for the spacer length necessary to allow lectin binding (29–31).

No binding was found with pure HSPC/PG-vesicles or with Man0- and Man1-modified liposomes. Lectin binding took place with mannosides containing at least 2 EO spacer-units, indicating sufficient exposure of the mannose residues to allow carbohydrate–protein interaction (Fig. 2). Aggregate formation was completely reversible through the addition of free mannose, indicating a specificity for mannose ligands. It can be concluded from this that a minimum spacer length of 2 EO units is required for mannoside binding.

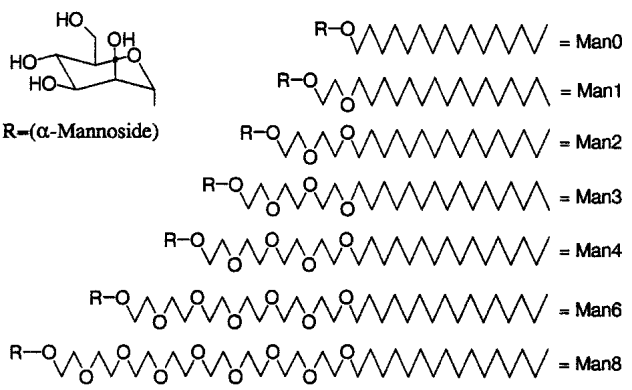

Fig. 1. Structures of alkyl mannosides.

Liposome–Cell Interactions: MR(–) Cells

Human macrophage-like cells (differentiated HL-60) that lack the MR were used to investigate nonspecific interactions between mannoside-modified liposomes and phagocytic cells. Phagocytosis of plain and Man-modified liposomes by differentiated HL-60 cells is shown in Fig. 3, where the extent of uptake of liposomes containing mannosides with short spacers (Man0, Man1) can be seen to be greater than that of unmodified vesicles.

On the other hand, liposomes containing mannosides with from 2 spacer units onward showed suppression of uptake. Suppression increased with spacer length. Similar results were obtained with differentiated U937 cells (unpublished data). Also, results obtained using glucoside, galactoside, and cellobioside derivatives showed an identical trend (unpublished data), indicating that suppression of liposome uptake is independent of sugar head group. An insight into the mechanism by which spacer length influences nonspecific cellular uptake can be gained by distinguishing between effects on adhesion and effects on total cell-associated fluorescence. At 4°C, where phagocytosis is completely suppressed, the tendency of the liposomes to adhere to the cell culture plates was found to correspond closely to liposome adhesion to the cells (Fig. 4, top).

At 37°C, where liposome internalization takes place, total cell-associated fluorescence and liposome adhesion are enhanced to the same extent in the cases of Man0 and Man1. Because liposome adhesion is the initial step for phagocytosis, the enhanced uptake observed is assumed to be related to the increased adhesion of these short-spacer liposomes, and not to an enhancement of uptake *per se*. Mannosides with longer spacers (Man2–4) inhibited uptake to a far greater extend than they inhibited adhesion (Fig. 4, bottom). This indicates

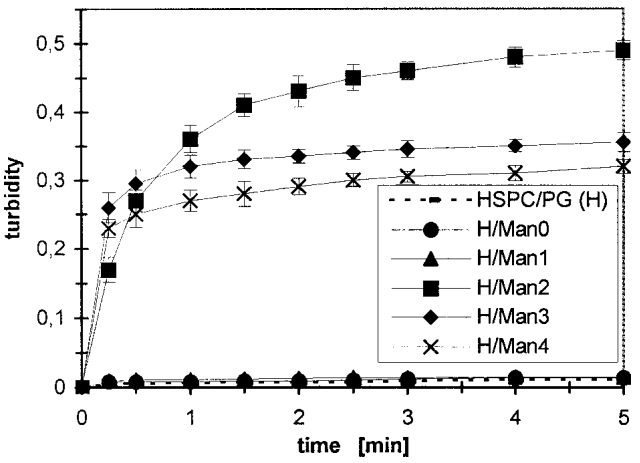

Fig. 2. ConA-induced aggregation of pure HSPC/PG (control) and Man liposomes.

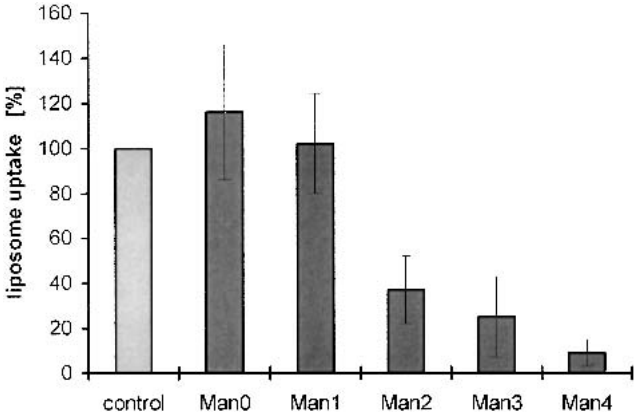

Fig. 3. Uptake of HSPC/PG (control) and Man liposomes by differentiated HL-60-cells after 60 min.

that these longer derivatives act as a barrier not only to adhesion but also to nonspecific uptake.

Liposome–Cell Interactions: MR(+) Cells

To investigate specific receptor-mediated interactions between mannoside-modified liposomes and phagocytic cells, native human cell populations of macrophages/monocytes were identified by flow cytometry and used. By use of a monoclonal antibody specific for the human 175-kDa mannose receptor (28), MR(+)-cells were selected analytically as shown in Fig. 5.

In the FSC/FL2-diagram (Fig. 5B), MR(+)-cells (red) appear in region 2 (R2). The corresponding representation of the FSC/SSC scan (Fig. 5A) shows an inhomogeneous distribution of MR(+) cells within the PcM total population (R1), which is shifted from smaller monocytes toward bigger macrophages. The cluster marked in blue represents MR(−)-cells. Setting an analytic window R3 in FSC/SSC-diagram (Fig. 5C) leads to mainly MR(+)-cells being selected, as can be seen in the corresponding FSC/FL2 diagram (Fig. 5D, green) compared to FSC/FL2-diagram (Fig. 5B, red).

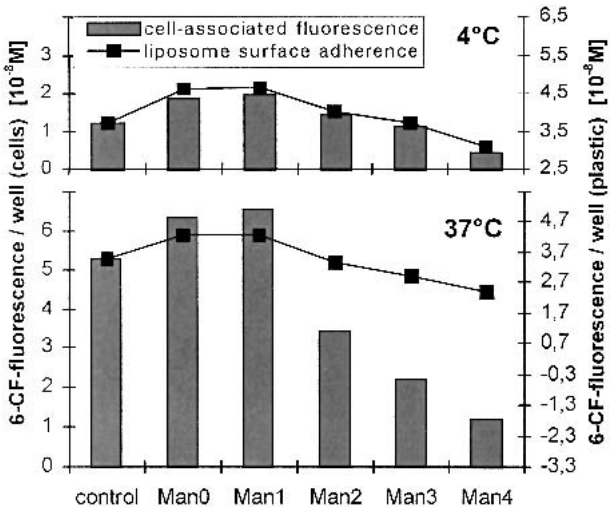

Fig. 4. Adhesion behavior of liposomes and cell-associated fluorescence after 60 min at 4°C (top) and 37°C (bottom) using differentiated HL-60-cells.

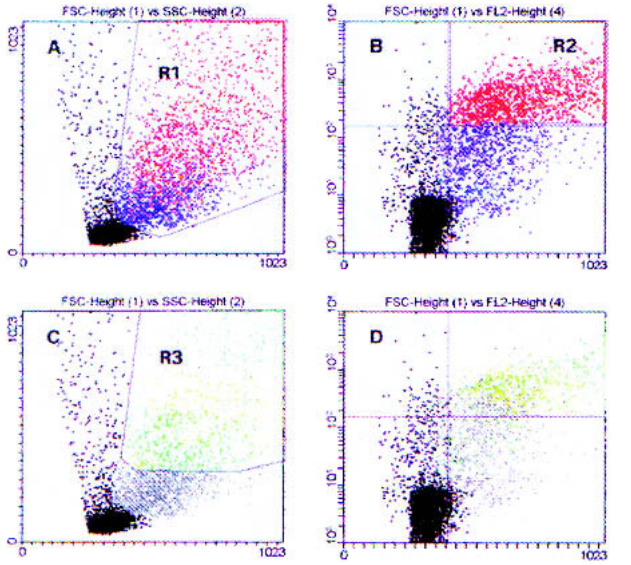

Fig. 5. Distribution of MR(+) cells in a typical PcM entire population.

It was found that the proportion of cells representing the MR varied from 15% to 57% of the entire macrophage/monocyte cell population, depending on the patient.

The liposome uptake by such selected cells (MA-gate) along with a comparison of the uptake by the entire population of PcM (MO-gate) is shown in Fig. 6. For these cell

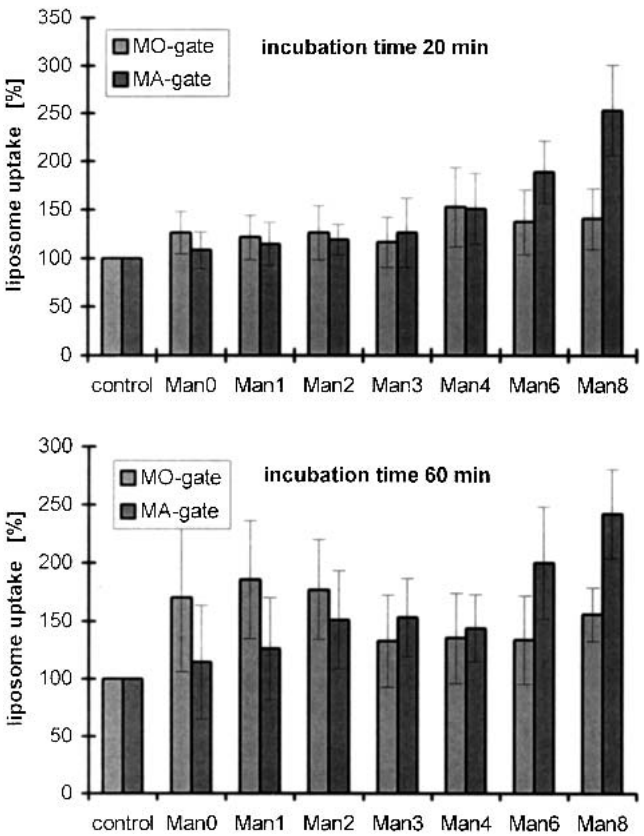

Fig. 6. Uptake of HSPC/PG (control) and Man liposomes by PcM after 20 min and 60 min. MO-gate, entire population; MA-gate, selected MR(+) cells.

uptake studies, mannosides with longer spacer arms (Man6, Man8) have been inserted.

It is obvious from Fig. 6 that the uptake of Man-modified liposomes by MR(+) cells (MA-gate) is different from that by the entire mixed cell population (MO-gate). Maximum uptake by MR(+) cells, indicating receptor-mediated endocytosis, was obtained with liposomes modified by mannosides with 6 or 8 EO spacer units, detectable already after 20 min incubation time. Conversely, uptake of liposomes modified by Man2, Man3 and Man4, which were recognized by ConA (see Fig. 2), was comparatively small. These findings might be due to the fact that binding of different receptor proteins are rarely comparable. Interestingly, after 1h, endocytosis of liposomes modified with short spacer mannosides was enhanced by the mixed cell population (MO-gate), for which mainly MR(−)-cells were responsible. This finding is in good agreement with the results obtained using HL-60-cells (which also lack the MR).

Investigation of human peritoneal macrophages containing mainly MR(+)-cells gave similar results (Fig. 7), proving that of all the modified insertions, Man6 and Man8 are of most potential use in receptor-mediated liposome targeting.

DISCUSSION

The above study was undertaken to investigate the influence of sequentially increasing spacer length on receptor-mediated uptake of Man-modified liposomes. Initially, ConA agglutination experiments were carried out to estimate the binding ability of mannosides incorporated into the liposome membrane. The results clearly show that the mannose residues of derivatives with at least 2 EO units spacer length were accessible to the soluble lectin and are likely, therefore, to be sufficiently exposed to partake in interactions with cellular receptor proteins. To determine nonspecific effects of the liposome surface modification, uptake studies with phagocytes lacking the MR were carried out. The surface adhesion of the liposomes was found to vary with spacer length, and this was shown to be of importance in distinguishing receptor-mediated and nonspecific liposome uptake. Adhesion of liposomes containing short-spacer mannosides (Man0, Man1) was increased. Previous work (unpublished data) in our group has shown that membrane rigidity is increased by the incorporation of glycolipids of up to 1 EO spacer-unit, and that liposome adhesion is affected by membrane rigidity. It is proposed, therefore, that the increase in adhesion is caused by enhanced membrane rigidity as a result of Man0 and Man1. Interestingly, Man derivatives that were accessible to the lectin suppressed nonspecific uptake by these cells. A similar trend of uptake suppression was obtained using glucosides, galactosides, and cellobiosides, all showing suppression with 2 EO spacer-units or more. The extent of suppression was found to increase with sugar head group size and spacer length, showing steric hindrance to the endocytosis similar to that known from long-chain PEG-modified liposomes (32). A nonspecific adhesion effect of short-spacer mannosides, which led to improved uptake, could also be seen using native human phagocytes.

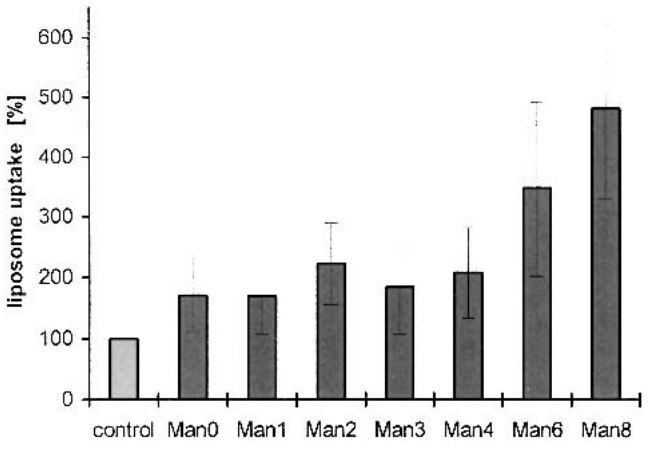

Fig. 7. Uptake of HSPC/PG (control) and Man liposomes (10mol% Man) by PtM after 60 min; entire population containing mainly MR(+) cells.

The use of mAb-MR, developed by Noorman *et al.* (28), proved excellent for detecting and quantifying MR expression and allowed analytic selection of MR(+) cells in the material used for the current study. The selection of MR(+) cells was found to be of fundamental importance because the uptake of Man-modified liposomes by the entire monocyte/macrophage population differed substantially from that by the selected cells (see Fig. 6). In quite a few of the studies cited above, the state of activation of the cells used, and so also the extent of MR expression, remained unknown. This makes meaningful comparison of results regarding receptor-mediated targeting success even more difficult.

In the present study, a spacer length of 6 or 8 EO units was required for MR-mediated endocytosis by native cells. Although Man2, Man3, and Man4 were recognized by the lectin, interaction with cellular MR could not be detected. This might be because of the different molecular properties of the MR with repeating carbohydrate recognition domains (CRDs) compared to other C-type lectins like ConA. First the insertion of longer derivatives (Man6, Man 8) allowed interaction with cellular MR, which still did not reach an optimum. Therefore, further studies should focus on derivatives with even longer spacer arms. However, the limited transferability of results obtained from studying the binding of different carbohydrate receptor proteins was also reported by others (33). From detailed studies using lectins of plant origin (34,35) it is known that multivalent carbohydrate ligands improve receptor affinity manyfold. Comparable results have been reported for mammalian cell membrane lectins (12,23,36,37). It is, however, not clear whether a number of ligands being able to bind to a single receptor protein (38,24) or the interaction of complex ligands with more than one receptor (39,40) is more important for effective receptor binding. Furthermore, it has been reported that a limited flexibility of the ligands is necessary to attain high-affinity binding (20,21), and glycosides with highly flexible spacer arms (PEG1000, PEG2000) were not recognized (41). It has also been reported that local concentration of the ligands in the membrane (through lateral diffusion and/or cluster formation) may be of importance for the binding affinity of carbohydrate receptor proteins (42,43), which might explain the glycolipid concentration effects in dependence on the spacer length reported by Orr *et al.* (44).

The results obtained here clearly show that carbohydrate head group exposure alone is not sufficient for MR-mediated uptake. In a review, Monsigny *et al.* concluded (23) that an "appropriate arrangement" as a result of exposure of the car-

bohydrate ligands and the flexibility of the spacer might lead to high-affinity binding, which is different for different receptor proteins. Similar conclusions have been drawn, as already mentioned, by Biessen *et al.* (24). The molecular structure of the MR is well characterized, defining ligand selectivity, and binding properties of the CRDs as functions of the complexity of the carbohydrate-bearing structure have been addressed (45,46). Nevertheless, alkylmannosides representing monovalent ligands embedded in a fluid phospholipid bilayer are assumed to be able to delocalize to a certain degree by lateral diffusion and thus, from a certain spacer length onward, to partake in an appropriate arrangement for receptor interaction.

When all these findings are taken into consideration, high-affinity binding to meet the requirements of each specific receptor seems to be the result of a complex of certain properties of the carbohydrate-bearing structure. These properties include the multiplicity, spacial exposure, lateral diffusion, and possible cluster formation of ligands as well as the flexibility of the ligand-bearing moieties. Further studies to characterize such requirements for Man-modified liposomes are currently in progress.

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