Glycoconjugate J (1989) 6:241-255

Muramyl Dipeptide Bound to Poly-L-Lysine Substituted with Mannose and Gluconoyl Residues as Macrophage Activators

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Received February 6/April 5, 1989.

Keywords: drug carrier, targeting, macrophage membrane lectin, endocytosis, macrophage activation.

Poly-L-lysine modified with mannose derivatives, the residual cationic charges of which being neutralized by *N*-acylation, were synthesized and used as carriers of a macrophage activator (*N*-acetylmuramyl dipeptide, MDP). The influence of the acylating agent on the targeting efficiency was investigated: a hydrosolubilizing group such as a gluconoyl moiety led to very efficient carrier conjugates, while an acetyl group did not. The effect of sugar and acyl content of the polymers was assessed using these compounds as inhibitors of red blood cell agglutination by Concanavalin A. The binding and specific endocytosis of poly-L-lysine substituted with several mannose derivatives and gluconoyl residues ($GlcA_x$-, Man_y-PLK) have been determined by a quantitative flow cytometry analysis. MDP bound to these conjugates was much more efficient *in vitro* than free MDP in macrophage cytostasis assays.

A low molecular weight drug has usually a low efficiency, whatever the mode of injection. To increase the therapeutic efficiency of drugs, macromolecular carriers can be used to target cells selectively. Reticuloendothelial cells, including free circulating monocytes and macrophages, as well as macrophages in various tissues can be activated and rendered tumoricidal by targeting immunostimulating drugs such as *N*-acetylmuramyl dipeptide (MDP). Free MDP upon intravenous injection is rapidly removed from the circulation [1, 2] and is inefficient in rendering macrophages tumoricidal in *in vivo* experiments.

Abbreviations: MDP, *N*-acetylmuramyl dipeptide; PLK, poly-L-lysine; BSA, bovine serum albumin; BOC, *t*-butyloxycarbonyl; DMF, dimethylformamide; DCHU, *N,N'*-dicyclohexylurea; DCCI, *N,N'*-dicyclohexylcarbodiimide; TEA, triethylamine; Su, succinimidyl; DMSO, dimethylsulfoxide; FITC, fluoresceinyl isothiocyanate; RPMI, Roswell Park Memorial Institute; PBS, phosphate buffered saline; Fl, fluoresceinyl; GG, glycyl-glycyl.

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Liposome-entrapped MDP [3, 4] is captured by monocytes and macrophages *in vitro* and *in vivo* and allows their activation, leading to eradication of lung metastases in tumor mice models. However, the use of liposomes in human therapy is not yet feasible: repeated injections of liposomes can impair the reticuloendothelial cell clearance function; moreover, many anti-tumor drugs encapsulated into liposomes have also cytotoxic effects on reticuloendothelial cells [5]. Pitfalls due to the use of liposomes can be overcome by taking into account the fact that macrophages have sugar binding receptors (also called membrane lectins) specific for mannose residues [6, 7]. These membrane lectins specifically bind and internalize mannosylated neoglycoproteins (Man_x-BSA). On this basis, we prepared serum albumin substituted with mannose and MDP residues (Man_x-,MDP_y-BSA), and showed that these conjugates were much more efficient than free MDP in activating macrophages to a tumoricidal state in both *in vitro* and *in vivo* experiments [8, 9]. MDP bound to Man_x-BSA is as efficient as MDP encapsulated into liposomes in eradicating lung metastases and to increase the survival time of tumor bearing mice [10]. However, serum albumin derivatives cannot be used in human therapy; serum albumin and its derivatives indeed induce the production of antibodies and undesirable side effects occur by repeated injections.

Many other macromolecular systems have been proposed as drug carriers. Various polymers are in use as drug vehicles (for reviews see [11,12]). Among the imposed and desirable properties of polymers used as carriers, biocompatibility, sterilizability, water solubility, bioresorbability and low immunogenicity are required qualities. Homopolymers such as poly-L-lysine (PLK) are known to be non-immunogenic in most animal species [13,14], and may therefore be used as drug carriers. In experimental cancer therapy, linkage of various toxic drugs to polycationic polypeptides has already been described [15-17]. However, the use of polycationic polypeptides lead to a strong non-specific binding to cells because the cell surface contains a large number of electronegative charges and thus such polypeptides have a cytotoxic activity [18].

Poly-L-lysine can be easily substituted both with biological response modifiers such as MDP, and with sugar derivatives such as mannoside or phosphomannoside (Man6P) to allow a targeting of biological response modifiers to macrophages or monocytes; this targeting will be cell specific and usable *in vivo* providing that no residual positive charges are left. The remaining ε-amino groups of lysine residues can easily be substituted by acyl groups.

In this paper, we describe the preparation of several mannosylated polymers substituted with MDP and present a study of the efficiency of the polymers with regards to their capacity to inhibit red blood cell agglutination by a specificity-related lectin, to bind to and to be internalized by macrophages and to render macrophages tumoricidal.

Materials and Methods

Chemicals

Poly-L-lysine.HBr (30,000-50,000) containing about 190 lysine residues and *N*-BOC-Glycyl-Glycine (*N*-BOC-Gly-Gly-OH) were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). D-Gluconic acid δ-lactone was obtained from Roquettes Frères

(Lestrem, France). Bovine serum albumin (BSA) was purchased from IBF Biotechnics (Villeneuve-la-Garenne, France). Fluoresceinylisothiocyanate (isomer 1) was from Molecular Probes (Junction City, OR, U.S.A.). Polymyxin B was from Sigma (St. Louis, MO, U.S.A.). Monensin was obtained from Calbiochem (La Jolla, CA, U.S.A.), a 25 mM stock solution in ethanol was prepared before use. Anhydrous and amine-free dimethylformamide (DMF) was freshly prepared by distillation on fluoro-2,4-dinitrobenzene (0.2 mg/ml) and $NaHCO_3$ (0.1 mg/ml). *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP) was synthesized according to the method of *Merser et al.* [19]. All other chemicals used were of the highest purity available and were used without further purification. The purity of small molecular weight compounds and of their reaction products was checked by TLC on Silica gel G plates (Merck, Darmstadt, F.R.G.), with solvent A; $CHCl_3/CH_3OH/CH_3COOH/H_2O$, 10/10/1/1 by vol: with solvent B; $CHCl_3/CH_3OH/H_2O$, 6/6/1 by vol: or with solvent C; $CHCl_3/CH_3OH/H_2O/CH_3COOH$ 8/1/1/1 by vol.*p*-Isothiocyanato phenyl-α-D-mannopyranoside and fluorescein labeled neoglycoproteins were prepared as previously described [20].

Synthesis of p-(Carboxymethyl)phenyl-α-D-mannopyranoside

p-(Carboxymethyl)phenyl-tetra-acetyl-α-D-mannopyranoside was synthesized according to the procedure of Helferich and Schmitz-Hillebrecht [21]. D-Mannose pentaacetate (20 g, 59 mmol), $ZnCl_2$ (1 g, 7.3 mmol), and methyl-*p*-hydroxyphenylacetate (20 g, 120 mmol) were added in a flask and the mixture was heated in an oil-bath up to 160°C. After melting, the mixture was cooled at 25°C, and 200 ml of toluene were added. The solution was washed twice with 200 ml of water at 4°C, six times with 200 ml of 1 N NaOH at 4°C and twice with 200 ml of water at 4°C yielding a colourless organic solution. Then the organic solvent was evaporated under reduced pressure and the residue was dissolved in ethanol. Ten equivalents of 1 N NaOH were added and the mixture was stirred at 25°C for 2 h; completion of the reaction was checked by TLC using solvent A ($R_F = 0.7$). The solution was adjusted to pH 8 by adding 10% acetic acid, and freeze-dried. The sodium salt of *p*-(carboxymethyl)-phenyl-α-D-mannopyranoside was purified by crystallization from ethanol. The *p*-(carboxymethyl)phenyl-α-D-mannopyranoside (100 mg, 0.32 mmol) was obtained by ion exchange chromatography through a Dowex 50W-X8 (H^+ form, 100-200 mesh) column (20 x 1.5 cm).

Synthesis of Substituted Polymers

Preparation of $GlcA_x$-,Man_y-,MDP_z-PLK. Poly-L-lysine hydrobromide (2 g in 200 ml water) was passed through an anion exchange column, (Dowex 2-X8, OH^- form, 20-50 mesh, 20 x 3 cm), then the effluent solution was neutralized with 10% *p*-toluene sulfonic acid in water and freeze-dried to give the *p*-toluenesulfonate salt of poly-L-lysine which is soluble in DMF. The ε-amino groups of poly-L-lysine were substituted with MDP and mannosides upon activation of the carboxyl group of the isoglutamyl residue of MDP and of the carboxyl group of the *p*-(carboxymethyl) phenyl-α-D-mannopyranoside. The remaining free amino groups of the conjugates were finally substituted by acylation (gluconoyl residues, GlcA).

In a typical experiment, to the *p*-toluenesulfonate polylysine (100 mg, 1.85 μmoles i.e. 350 μmoles of ε-NH_2 residues) in 4 ml of DMF at 4°C, triethylamine (TEA, 49 μl, 350 μmol) was added. MDP (36 mg, 74 μmol), hydroxybenzotriazole (HOBt, 10 mg, 74 μmol) and *N,N'*-dicyclohexylcarbodiimide (DCCI, 18 mg, 89 μmol) were then added. The mixture was

stirred at 25°C for 24 h; the reaction being monitored by TLC using solvent B [MDP, $R_F = 0.3$, MDP-PLK, $R_F = 0$]. After 24 h the solution was cooled to 4°C and *p*-(carboxymethyl)phenyl-α-D-mannopyranoside (17 mg, 56 μmoles), HOBt (7 mg, 56 μmol) and DCCI (13 mg, 67 μmol) were added. The mixture was stirred for a further 24 h at 25°C; the reaction being monitored by TLC using solvent B [(carboxymethyl)phenyl mannopyranoside, $R_F = 0.3$]. Insoluble dicyclohexylurea (DCHU) was removed by centrifugation at 2,000 x *g* for 10 min at 4°C. The polymer was then precipitated in cold isopropanol, centrifuged and the precipitate was washed once with isopropanol, twice with diethylether and finally dissolved in water; the aqueous solution was freeze-dried. Free amino groups of the polymer (about 120-140 residues) were substituted by acylation with D-gluconic acid δ-lactone: the polymer containing MDP and mannose residues, Man_y-,MDP_z-PLK (110 mg, 1.85 μmol), was solubilized in 4 ml DMF in the presence of 180 μl TEA (1.3 mmol) at 25°C; to this solution, δ-gluconolactone (178 mg, 1 mmol) and 400 μl water (10% final volume) were added. The mixture was stirred at 60°C for 24 h. The polymer $GlcA_x$-,Man_y-,MDP_z-PLK was precipitated as described above, further purified by gel filtration on a Trisacryl GF 2000 (IBF Biotechnics) column (20 x 2.5 cm) equilibrated in H_2O/*n*-butanol,95/5 by vol, and analyzed to determine the content of MDP and sugar residues. $GlcA_{140}$-,Man_{30}-,MDP_{20}-PLK (100 mg, 80% yield) was obtained after freeze-drying.

Preparation of Ac_x-,Man_y-,MDP_z- PLK. Acetic anhydride was used instead of gluconolactone to block residual free amino groups by an acetyl group (Ac). Man_y-,MDP_z-PLK (110 mg, 1.85 μmol) was solubilized in 4 ml DMF; to this solution 54 μl (390 μmol) TEA and 24 μl (260 μmol) acetic anhydride were added. The mixture was stirred at 25°C for 30 min. Then the polymer Ac_{140}-,Man_{30}-, MDP_{20}-PLK was purified as described above.

Preparation of $GlcA_x$-, $Man.GG_y$-,$MDP.GG_z$-PLK. The glycyl-glycyl polymer was prepared by substituting poly-L-lysine with *N*-BOC-Gly-Gly-OH; then *N*-BOC groups were released. The active ester of *N*-BOC-Gly-Gly-OH [22] was prepared as follows:
N-BOC-Gly-Gly-OH (22 mg, 95 μmol) and *N*-hydroxysuccinimide (9.5 mg, 95 μmol) were dissolved in 1 ml DMF. To the solution, cooled at 4°C, DCCI (23 mg, 114 μmol) was added. The solution was stirred at 25°C for 24 h, then the DCHU precipitate was removed by centrifugation. In order to obtain poly-L-lysine substituted with 90 glycyl-glycines, the solution of *N*-BOC-Gly-Gly-OSu was added to poly-L-lysine.HBr (40 mg, 1 μmol) dissolved in 2 ml of DMF/DMSO, 1/1 by vol, in the presence of TEA (14 μl, 95 μmol). The mixture was stirred for 2 h at 25°C. The reaction was monitored by TLC using solvent C (BOC-Gly-Gly-OH $R_F = 0.42$, BOC-Gly-Gly-OSu $R_F = 0.74$). Then D-glucono-δ-lactone (67 mg, 380 μmol), TEA (70 μl, 500 μmol) and water (10% final volume) were added to neutralize the unsubstituted ε-NH_2 (100) of the BOC-glycyl-glycyl polymer. The polymer was purified as described above, by precipitation in dichloromethane, washing with methanol, solubilization in water and freeze-drying. The *N*-BOC groups were removed by action of trifluoroacetic acid: a solution of $(N\text{-BOC-Gly-Gly})_y$-,$GlcA_x$-PLK (42 mg, 1 μmol) in trifluoroacetic acid (4 ml) was kept at 25°C for 1 h. The polymer was precipitated in ten volumes of dry diethylether. The precipitate was dissolved in water and the solution was stirred for 30 min at 25°C to hydrolyze any trifluoroacetic ester of gluconoyl groups, then the polymer was precipitated by adding 10 volumes of a mixture of ethyl acetate/*n*-butanol (1/1 by vol), the precipitate was dissolved in water and freeze-dried. The polymer was first passed through an anion exchange column, (20 x 2.5 cm) Dowex W2-X8 (OH^- form, 20-50 mesh), the

solution was neutralized with 10% *p*-toluene sulfonic acid in water and freeze-dried to give the *p*-toluene sulfonate derivative $GlcA_x$-,GG_y-PLK tosylate which is soluble in DMF. The addition of MDP and mannose residues was performed as described above for $GlcA_x$-,Man_y-,MDP_z-PLK. In order to block any residual free amino groups of lysine or glycine, 20 µmol acetic anhydride were added to the polymer in a DMF solution, in the presence of 30 µmol TEA. The mixture was stirred at 25°C for 30 min, and the polymer was purified as described above.

Preparation of Fluoresceinylated Conjugates. Fluoresceinylated polymers bearing 4-7 fluorescein residues were prepared. Fluorescein isothiocyanate (FITC) isomer I (5.9 mg, 15 µmol) dissolved in 100 µl DMF was added to the *p*-toluene sulfonate derivative of PLK (100 mg, 1.85 µmol) in the presence of TEA (49 µl,350 µmol) and allowed to react for 1 h at 25°C under stirring. Then, *p*-isothiocyanatophenyl-α-D-mannoside (17.8 mg, 55 µmol) was added and allowed to react for 1 h at 25°C. Remaining free amino groups were substituted by reaction with gluconolactone and the conjugate was precipitated in dichloromethane, washed with methanol, freeze-dried and purified by gel filtration on Trisacryl GF05 in H_2O/*n*-butanol (95/5 by vol) to remove all free or adsorbed fluorescent molecules [23].

Characterization of the Conjugates

The amount of free amino groups in any of the polymers was determined by using the trinitrobenzene sulfonic acid assay [24] and was found to be less than ten free amino groups per molecule i.e. less than 5% of the total number of lysyl residues.

Mannose content was determined by a microassay [25] adapted from the resorcinol sulfuric acid method [23] and was found to be about 95% of the mannose residues introduced in the reaction mixture. The number of MDP residues bound to a polymer was estimated by the *p*-dimethylaminobenzaldehyde method [26] and was found to be about 50% of the number of MDP introduced in the reaction mixture. The number of glycyl-glycine residues was determined with an amino-acid autoanalyser (Biotronik, Puchheim-Bahnhof, F.R.G.) after 5.6 N HCl acid hydrolysis at 105°C for 72 h; the coupling yield was 95%.

Animals

Wistar rats and (C57BL/6 x Balb/c) F1 hybrid mice were obtained from CSEAL (CNRS Orléans, France) and DBA/2 mice from IFFA-CREDO (Lyon, France).

Cell Culture

Mouse peritoneal macrophages were obtained by washing the peritoneal cavity of mice that had received an intraperitoneal injection of 2 ml thioglycolate medium (Institut Pasteur, Paris, France) four days earlier. Macrophages, identified by measuring neutral red uptake, were washed in serum-free RPMI medium and plated at 37°C for 24 h. Non-adherent cells were removed by washing, and macrophages were then incubated with fluoresceinylated conjugates or stimulated with MDP conjugates, in 24 well plates or in microtest plates (Falcon, Becton Dickinson, Grenoble, France.).

Rat alveolar macrophages were obtained by tracheobronchial lavage with sterile phosphate balanced saline (PBS: 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4) containing 0.02% disodium ethylenediaminetetraacetate (PBS-EDTA) at 37°C as described by Holt [27].

DBA/2 lymphoma L1210 cells (kindly given by Dr I. Gresser, Villejuif, France) adapted to grow in suspension were collected from exponential growth culture in Minimum Essential Medium with Earle's salts supplemented with 10% heat inactivated horse serum (IBF Biotechnics).

DBA/2 mastocytoma P815 cells (kindly provided by Dr Le Floch, Rhône-Poulenc Recherches, Vitry-sur-Seine, France) were passaged weekly in syngeneic animals.

All cell lines were regularly checked for mycoplasma contamination by using bis-benzimid H33258 (Serva Feinbiochemica, Heidelberg, F.R.G.) [28].

Flow Cytofluorimetry Analysis

Thioglycolate-elicited mouse peritoneal macrophages in phosphate balanced saline (PBS) supplemented with Ca^{2+} (10^{-3}M) , Mg^{2+} (10^{-3}M) and 1% BSA, were incubated with the fluoresceinylated conjugates. At the end of the incubation period, cells were washed with 0.5% BSA in PBS, then adherent cells were harvested with a rubber policeman, and resuspended in 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% (by vol) 2-phenoxyethanol].

The fluorescence intensity of single cells was analyzed at 4°C with a FACS Analyzer (Becton Dickinson, Sunnyvale, CA, U.S.A.). The volume and the fluorescence intensity of each cell were simultaneously recorded at a rate of 200 cells/sec (excitation wavelength 485 ± 10 nm; emission wavelength 520 ± 10 nm). Standardization of the flow cytofluorimeter was achieved by using fluorescent polystyrene sulfonate beads [20]. The cells which had been incubated at 37°C were analyzed before and after post-treatment at 4°C for 30 min in the presence of 50 µM monensin [20, 29, 30] in order to restore the fluorescence of fluorescent material contained in acidic vesicles (endosomes or lysosomes) [31].

Cytostatic Activation of Murine Macrophages

Adherent rat alveolar macrophages or thioglycolate-elicited peritoneal macrophages were incubated for 24 h at 37°C in a humidified atmosphere (5% CO_2, 95% air) with conjugates in serum free RPMI medium supplemented with 2 mM L-glutamine, antibiotics, and 10 µg/ml polymyxin B. Then the immunostimulating conjugates were removed; tumor cells in medium supplemented with 5% fetal bovine serum (FCS, Gibco, Renfrewshire, U.K.) were added and co-cultivated for a further 24 h. To determine growth inhibition [^{3}H]-thymidine (17.5 kBq; specific activity 0.74-1.1 TBq/mmol; CEA, Saclay, France) was added 4 h before harvesting. Cells were collected and washed on Whatman glass fiber filters and radioactivity was measured in aqueous counting scintillant (ACS, Amersham, U.K.) in a β-scintillator counter (Beckman, Geneve, Switzerland). The percentage of cytostatic activity was expressed as growth inhibition index: GI% = (R-S) / R x 100, where R is the radioactivity

incorporated in tumor cells cultivated on unstimulated macrophages and S is the radioactivity incorporated in tumor cells cultivated on stimulated macrophages.

Inhibition of Hemagglutination

Hemagglutination titers were assayed in microtest plates [32] using 3% rabbit red blood cells suspension. The concentration of Man-conjugates required to inhibit 50% of the hemagglutination induced by Concanavalin A was determined.

Results and Discussion

Characteristics of the Polymers

Poly-L-lysine (or partially substituted poly-L-lysine) is a polycationic macromolecule. It binds to the cell surface of red blood cells and of any mammalian cells [33]. It induces the agglutination of cells in suspension, and is currently used to coat plates in order to facilitate cell adhesion or to isolate cell surface membranes. In *in vitro* experiments, poly-L-lysine partially substituted with toxic drugs [34] or with oligonucleotides [35] has been used to facilitate the internalization of these compounds because upon adsorption on the cell surface (by ionic interactions) poly-L-lysine or their derivatives are readily internalized. According to the polycationic character of poly-L-lysine and to the above cited statement these polymers cannot be used *in vivo*.

On the basis of the results previously obtained by using neoglycoproteins as carriers of macrophage activators or of anti-tumor and antiviral drugs [10,36-39], it appears that it should be possible to use poly-L-lysine as carrier when the following conditions are fulfilled: i) that all the ε-amino groups are substituted by acylation to avoid the non-specific adsorption on the surface of any type of cells; ii) poly-L-lysine carries, in addition of a drug or a biological response modifier, a ligand specific of a surface receptor of the target cell; and iii) the linkage between the substituents (drug, biological response modifier or ligand) and the poly-L-lysine is cleavable by hydrolases leading to an unmodified free drug and/or to one of its active metabolites.

Poly-L-lysine was selected because it can be enzymatically degraded to the naturally occurring amino-acid (L-lysine) and because as shown by Ryser and Shen [34] poly-L-lysine, but not poly-D-lysine is a good carrier of toxic drugs in *in vitro* experiments. With the aim of targeting macrophage activators, mannoside is an accurate ligand for the mannose specific membrane lectin of macrophages [7, 40]. It is easy to substitute poly-L-lysine with a variety of mannosyl derivatives such as: i) imidate derivatives [41] leading to an amidine linkage, or ii) phenylisothiocyanates [20, 23] leading to a thiourea linkage which are resistant to naturally occurring hydrolases. Oligosaccharides may also be linked to poly-L-lysine by reductive amination [42]; Fiume *et al.* [39] used this approach to prepare neoglycoprotein as an antiviral drug carrier.

In order to fulfill the previously mentioned requirements, *p*-(carboxymethyl)phenyl-α-D-mannopyranoside and *p*-(carboxyethyl)phenyl-α-D-mannopyranoside were synthesized.

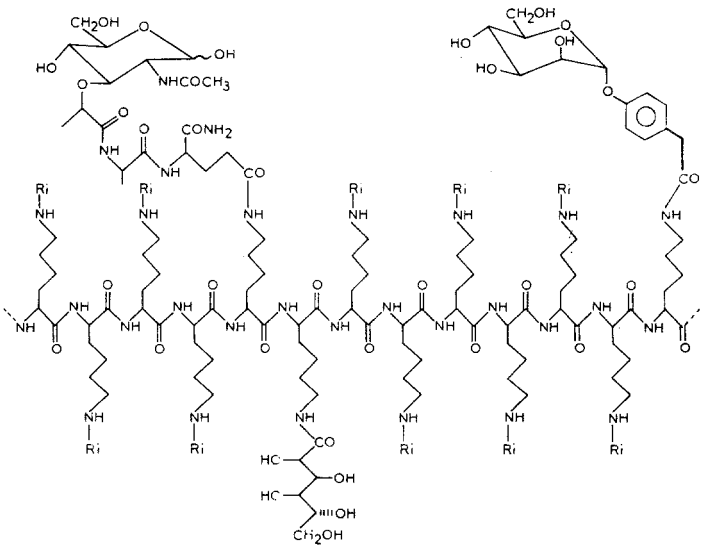

Figure 1. Chemical structure of poly-L-lysine substituted with muramyl dipeptide, mannose residues and gluconoyl groups.
Ri= MDP, GlcA or Man residue.

These compounds upon enzymatic hydrolysis by α-mannosidase give free mannose and *p*-hydroxyphenylacetate (or phenylpropionate) which is a tyrosine metabolite. These mannose derivatives were coupled to poly-L-lysine, leading to an amide linkage hydrolysable by an amidase.

N-acetylmuramyl dipeptide was coupled as previously described by activating the γ-carboxyl group of the isoglutamyl residue of MDP leading to an other amide linkage hydrolysable by an amidase.

The poly-L-lysine partially substituted by MDP and *p*-(carboxymethyl)phenyl-α-D-mannopyranoside [or *p*-(carboxyethyl) phenyl-α-D-mannopyranoside] is still polycationic. The remaining cationic groups (amines) can be removed by acylation: for instance by acetylation, succinylation, maleylation or glyconoylation. Acetylation and glyconoylation were selected because the former is one of the easiest ways to acylate an amine and the latter increases the hydrophilicity of the polymer. On this basis, polymers carrying MDP, the schematic structure of which are given in Fig. 1, were prepared. In order to get optimal macrophage activation the following parameters were studied : the number of mannose and of MDP residues bound to the polymer, the nature of the acylating agent, the presence of an additional peptide spacer (Gly-Gly) between MDP (or mannoside) and L-lysine side chains.

Table 1. Inhibition of hemagglutinin activity of Concanavalin A by neoglycoproteins and polylysine substituted with mannose derivatives.

Compound [a]	Concentration required for 50% inhibition		α-Me-Man/ "conjugate-bound Man" ratio
	μM[b]	μM[c]	
α-Me-Man	2600	2600	1
Man_{20}-BSA	0.8	16	160
Fl_4-,Man_{20}-BSA	0.8	16	160
$GlcA_{175}$-,Man_{15}-PLK	6	100	26
Ac_{143}-,MDP_{17}-,Man_{30}-PLK	3	100	26
$GlcA_{143}$-,MDP_{17}-,Man_{30}-PLK	0.6	20	130
$GlcA_{113}$-,MDP_{17}-,Man_{60}-PLK	0.2	12	200
$GlcA_{100}$-,$MDP.GG_{17}$-, $Man.GG_{30}$-,$Ac.GG_{43}$-PLK	0.17	5	500
$GlcA_{100}$-,$MDP.GG_{17}$-, $Man.GG_{60}$-,$Ac.GG_{13}$-PLK	0.08	5	500

[a] The number of each substituent is given : i.e. Fl_4-,Man_{20}-BSA means that BSA has been substituted with four fluoresceinyl residues, and with 20 mannoside residues.

For the poly-L-lysine conjugates (PLK), Man indicates a *p*-(carboxymethyl)phenyl-α-D-mannopyranoside residue.

[b] Expressed as conjugate concentration.

[c] Expressed as mannose concentration.

Starting with a poly-L-lysine containing in average 190 amino acid residues, the various polymers obtained have a molecular mass ranging from 58,000 to 78,000 depending on the number and on the nature of the substituents. These molecular masses are close to that of serum albumin (M_r = 68,000) and of neoglycoproteins (M_r = 80,000). The number of substituents of such a poly-L-lysine is higher than that of bovine serum albumin which contains 57 lysines and poly-L-lysine is quite easily substituted in organic medium.

Efficiency of the Polymers Bearing Mannose Derivatives as Inhibitors of Concanavalin A Induced Hemagglutination

It was shown previously [20] that neoglycoproteins containing about 20 sugar residues were optimally recognized by lectins. Based on the efficiency in inhibiting the agglutination of red blood cells such neoglycoproteins are much more effective than the corresponding free sugar (Table 1). With poly-L-lysines substituted with both α-D-mannopyranoside derivatives and acetyl groups directly linked to the polymer core, the optimal number of sugars per molecule is about 60. However, when the mannose derivative is bound to the polymer core through a glycyl-glycyl spacer, the optimal number of sugars is about 30. Such polymers bearing mannose derivatives have even a higher inhibitory power than neoglycoproteins. Therefore, the peptide spacer (which was initially designed to enhance the efficiency of

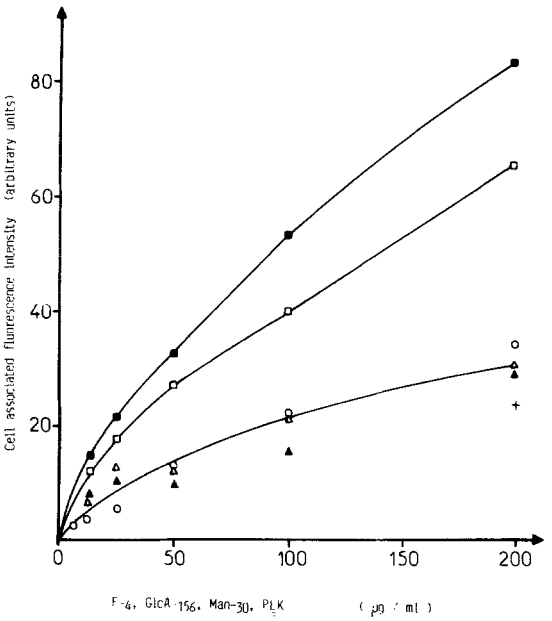

Figure 2. Binding and endocytosis of poly-L-lysine bearing mannose residues by mouse peritoneal macrophages. Plated thioglycolate-elicited peritoneal macrophages were incubated at 4°C (○) or at 37°C (□, ■, △, ▲) for 2 h in complete PBS containing 1% BSA, in the presence of various concentrations of Fl_4-,$GlcA_{156}$-,Man_{30}-PLK, in the absence (○, □, ■), or in the presence (△, ▲) of an inhibitor of endocytosis (NaF). Cells were washed, harvested in cold PBS containing 0.5% BSA and resuspended in cold sheath fluid. The cell fluorescence intensity was measured by flow cytofluorimetry directly (○, □, △) and after a post-incubation at 4°C for 30 min in the presence of 50 µM monensin (■, ▲).

targeted drugs bound to a carrier as shown by Monsigny *et al.* [43] and confirmed by Trouet *et al.* [44] and more recently by Ryser and Shen [34] because the spacer increase the releasability of the drug upon hydrolase action) is also useful to increase the accessibility of a ligand to its receptor. Surprisingly two poly-L-lysine derivatives containing the same number of sugars (30 Man/mol) and the same number of acylating agents have different inhibition activity: the poly-L-lysine bearing mannose residues and acetyl residues being five-fold less efficient than the same polymer substituted with gluconoyl groups (Table 1). This difference cannot be accounted for by a specific effect of the gluconoyl residues because: i) polymers containing the same number of mannose units but the remaining amino groups being substituted with ribonic acid or with glyceric acid have an efficiency identical to that of the analogues substituted with gluconic acid (data not shown) and because ii) a poly-L-lysine fully substituted with gluconic acid and therefore containing no mannose residue was inactive even at very high concentration (11.6 mg/ml, 200 µM). The low efficiency of the polymers substituted with acetic acid may be related to the hydrophobicity of the ε-acetyllysine residue which decreases the solubility of the conjugate.

According to this approach, the most efficient poly-L-lysine conjugates contain at least 30 sugar residues bound to the polymer core through a peptidic spacer, the remaining amino groups being substituted with gluconoyl residues but not with acetyl groups.

Table 2. Specific inhibition of cell-associated fluoresceinylated ligands by unlabeled conjugates.
Flow cytofluorimetry analysis of mouse thioglycolate peritoneal macrophages pre-incubated 30 min with 5 mg/ml of fluorescein-free ligand before incubation with 50 µg/ml of fluoresceinylated ligand (Fl-,Man-BSA or Fl-,GlcA-,Man-PLK) at 37°C for 2 h. The % inhibition was expressed as $(F_o - F)/F_o \times 100$ where F_o and F are the fluorescence intensity of macrophages incubated with fluoresceinylated ligand in the absence (F_o) or in the presence (F) of an unlabeled ligand. Results are identical whether the fluorescence intensity is measured with or without a post-incubation step in the presence of monensin at 4°C for 30 min.

Unlabeled conjugates	Man_{20}-BSA	$GlcA_{190}$-PLK	$GlcA_{160}$-,Man_{30}-PLK
Fl_4-,Man_{20}-BSA	80 ± 5	0	70 ± 5
Fl_4-,$GlcA_{156}$-,Man_{30}-PLK	52 ± 4	0	75 ± 5

Binding and Uptake of Poly-L-lysine Substituted with Mannosides and Gluconoyl Residues

The amount of ligands bound or internalized by macrophages incubated at 4°C or 37°C can be assessed by quantitative flow cytofluorimetry, when fluoresceinylated ligands with a given number of bound fluoresceinyl residues (3 to 5 residues/macromolecule) are used and when the cytofluorimeter has been standardized with calibrated fluoresceinylated beads [20, 30]. Because fluorescein does not fluoresce in an acidic environment, the emitted fluorescence intensity of the fluoresceinylated polymers bearing mannose derivatives internalized into endosomes or lysosomes which are acidic compartments, is lower than that of the same polymers in neutral solution, on a molecular basis. The fluorescence intensity can be restored by post incubation at 4°C in the presence of monensin, an ionophore allowing equilibration of the pH of the medium, the cytosol and the luminal compartment of the intracellular organelles [20, 30, 45]. It appears that the amounts of serum albumin bearing mannose derivatives or poly-L-lysine substituted with mannose residues and gluconoyl groups bound to rat alveolar macrophages or mouse thioglycolate elicited peritoneal macrophages were nearly identical a few pg/cell). As shown in Fig. 2, Fl_4-,$GlcA_{156}$-,Man_{30}-PLK is also endocytosed into macrophages upon incubation at 37°C; the enhancement of cell fluorescence intensity is not due to an increase of cell affinity since the fluorescence of macrophages incubated with the fluoresceinylated ligand at 37°C in the presence of the metabolic inhibitor sodium fluoride, which prevents the endocytotic process, is the same as the fluorescence of macrophages upon incubation at 4°C. Furthermore, a post incubation at 4°C in the presence of monensin induces an increase of the fluorescence intensity of cells which were previously incubated at 37°C (without NaF) for 2 h. These data demonstrated that this ligand has been endocytosed into acidic compartments. Neoglycoprotein and poly-L-lysine bearing mannose derivatives were probably internalized *via* the same membrane lectin [40], since pre-incubation with an excess of one or the other unlabeled ligand inhibits 50-80% of the endocytosis of the Fl_4-,Man_{20}-BSA or Fl_4-,$GlcA_{156}$-,Man_{30}-PLK (Table 2).

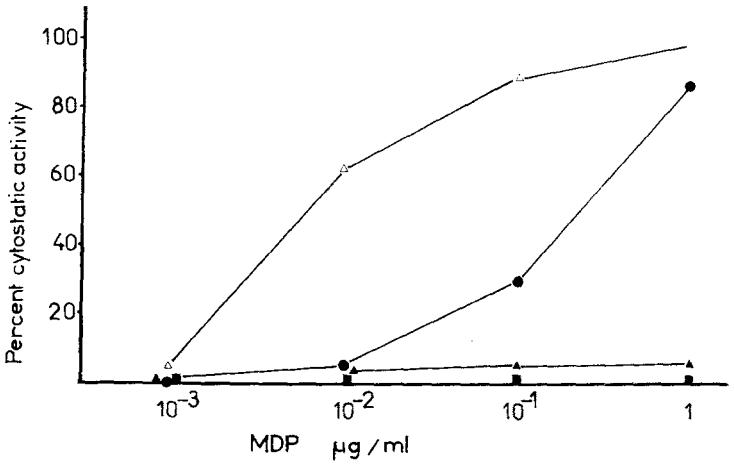

Figure 3. *In vitro* cytostatic activation of rat alveolar macrophages by *N*-acetyl muramyl dipeptide, free and bound to polymers.
2×10^5 adherent rat alveolar macrophages were incubated in the presence of various concentrations of free MDP (●),or MDP bound to Man-PLK ($GlcA_{110}$-,Man_{60}-,MDP_{20}-PLK, △) or MDP bound to PLK ($GlcA_{170}$-,MDP_{20}-PLK, ■) or with $GlcA_{190}$-PLK as control (▲), in serum free RPMI medium supplemented with L-glutamine, antibiotics and 10 µg/ml polymyxin B. After 24 h, plated macrophages were washed and further co-cultivated with 10^4 L1210 cells in Dulbecco medium supplemented with 5% FCS. At 4 h before harvesting, [^{3}H]-thymidine (37 kBq) was added. Results are mean values from triplicate cultures; the standard deviation was less than 10%.

In Vitro Macrophage Activation by MDP Bound to Polymers

In vitro, free MDP is known to activate various types of macrophages [46] to a cytostatic or cytotoxic activity against tumor cells. MDP-polymer ($GlcA_{110}$-,Man_{60}-,MDP_{20}-PLK) was more active than free MDP (Fig. 3). Furthermore MDP bound to a sugar-free poly-L-lysine ($GlcA_{170}$-,MDP_{20}-PLK) as well as MDP-free polymer ($GlcA_{130}$-,Man_{60}-PLK) were inactive. According to the type of macrophages the physiological state of the mice and the nature of the conjugates used, the concentration of bound MDP giving 50% cytostatic activation varies from 1 to 100 ng/ml. As shown in Table 3, MDP bound to poly-L-lysine substituted with gluconoyl and mannosyl residues was nearly as efficient as MDP bound to serum albumin substituted with mannosyl residues [8, 9]. The conjugate with acetyl groups (Table 3) was less efficient than the conjugates with gluconoyl groups in rendering macrophages cytostatic. This result is consistent with a lower capacity of the former conjugate to inhibit agglutination of red blood cells by Concanavalin A (Table 1).

Table 3. Relative cytostatic activity of free MDP and of carrier bound MDP on rat alveolar macrophages.

Compound	Free MDP/Bound MDP [a]
MDP	1
MDP_{20}-BSA	1
Man_{20}-,MDP_{7}-BSA	25 ± 3
Man_{20}-,MDP_{20}-BSA	30 ± 3
$GlcA_{190}$-PLK	-[b]
Ac_{140}-,Man_{30}-,MDP_{20}-PLK	4 ± 1
$GlcA_{140}$-,Man_{30}-,MDP_{20}-PLK	20.± 3
$GlcA_{100}$-,Man.GG_{60}-,MDP.GG_{20}-,Ac.GG_{10}-PLK	70 ± 30

[a] Ratio of the concentration of free and bound MDP required to give 50% cytostatic activity.
[b] No activation at a concentration corresponding to 100 times the concentration of any active MDP conjugate.

The specific activity of the conjugates did not increase when the number of bound MDP residues to the polymer varied from 10 to 20 providing that the conjugate bears a sufficient number (25-30) of mannose residues, the activity being expressed on the basis of the MDP content.

In the case of *in vitro* macrophage activation the introduction of the Glycyl-Glycyl spacer arm carrying MDP and mannose residues did not improve significantly the efficiency of the poly-L-lysine conjugates. These results clearly show that MDP linked to neutral, biodegradable, poly-L-lysine substituted with mannosides is able to activate macrophages *in vitro*; however further investigations are required to assess the efficiency of these macrophages activators *in vivo*; by comparison with free MDP it is expected that the longer lifetime of such conjugates (unpublished results) may allow their therapeutic use.

Conclusion and Perspectives

Specific receptors such as membrane lectins that induce endocytosis upon ligand binding to the surface of monocytes and macrophages are quite suitable to deliver immune response modifiers. Polypeptides substituted with glycosyl groups, providing that they are not polycationic, are interesting carriers for the following reasons:
- *in vivo* they are specifically taken up by a limited number of cells,
- they are much less immunogenic than the related neoglycoproteins,
- they are as efficient as neoglycoproteins to carry immunomodulators,
- they are not expensive and are easily prepared in an excellent yield because the chemistry is conducted in organic solvents.

New developments along these lines include the use of other monosaccharides or oligosaccharides bound to the polypeptide in order to target drugs to other cells, and the use of other activators or biological response modifiers.

Acknowledgements

The authors thank Marie-Thérèse Bernèdes and Philippe Bouchard for their skilful technical assistance in cell culture works and in chemical synthesis, respectively.
This work was partially supported by grants from "Institut National de la Santé et de la Recherche Médicale" (INSERM No. 842016) and from "Association pour la Recherche sur le Cancer". Annie-Claude Roche is "Directeur de Recherche INSERM" and Patrick Midoux is "Chargé de Recherche INSERM".

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