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Endocytosis of α_1-Acid Glycoprotein Variants and of Neoglycoproteins Containing Mannose Derivatives by a Mouse Hybridoma Cell Line ($2C_{11-12}$). Comparison with Mouse Peritoneal Macrophages

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Macrophages from various origins are known to express membrane lectins that mediate the endocytosis of mannose-bearing glycoconjugates. Most macrophage tumor cell-lines lack such receptors. In this paper we show by flow cytometry analysis that a newly generated macrophage hybridoma ($2C_{11-12}$), which displays several macrophage characteristics, also expresses mannose membrane lectins, resulting in the internalization of fluoresceinylated neoglycoproteins into acidic compartments.

Thioglycolate elicited mouse peritoneal macrophages and the $2C_{11-12}$ hybridomas were compared by flow cytometry with regard to the binding and endocytosis of α_1-acid glycoprotein (AGP) variants separated by affinity chromatography on immobilized concanavalin A. AGP C eluted specifically with methyl α-mannopyranoside, which contains two bi-antennary oligosaccharides, was endocytosed as mannosylated serum albumin (Man-BSA). In both types of macrophages, the fluoresceinylated ligands were internalized in acidic compartments as demonstrated by the fluorescence intensity increase upon monensin post-incubation. However the behaviour of the internalized ligands was found to be quite different. AGP C and Man-BSA were rapidly degraded by thioglycolate elicited peritoneal macrophages and excreted in the medium as small peptide fragments; conversely they remained a longer time in the $2C_{11-12}$ hybridoma.

Macrophages of various origins express cell surface receptors which bind and internalize glycoconjugates that bear oligosaccharides with terminal mannose [1]. Macrophages do not constitute a homogeneous population and their heterogeneity constitutes a limitation in

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many studies; furthermore when differentiated macrophages remain viable for several weeks they fail to proliferate under normal culture conditions. Macrophage cell lines (J774, U937, P388D1) growing in culture or in mice lack such receptors [2, 3]. Diment *et al.* [4] recently selected a J774 clone that is mannose receptor positive. This clone appeared to be quite useful to study mannose-specific macrophage membrane lectins.

Recently, a long term culture cell line of murine macrophages exhibiting mannose receptors has been established [5]. Several macrophage hybridomas have been established by somatic cell fusion between exudate macrophages and various tumor cell-lines [6-8]. These continuous macrophage-like cell lines possess major characteristics attributed to macrophages, such as a typical macrophage morphology, esterase positivity and secretions of lysozyme, interleukin 1 and active oxygen components.

So far, the presence of mannose receptor on macrophage hybrids has not been reported. The $2C_{11-12}$ macrophage hybridoma [7] which mediates cytostatic and cytolytic activities upon activation with LPS and lymphokines [9] was chosen and compared with thioglycolate elicited peritoneal macrophages with regard to its capacity to express mannose receptors. Lysosomal glycosidases (e.g. β-glucuronidase) [1], neoglycoproteins [10-13] and oligosaccharides [14] have been used to define the specificity of the receptors and to study their capacity to mediate endocytosis. Radiolabeled ligands were often used in the study of endogenous lectins, but fluorescent ligands are also suitable either for spectrofluorimetric analysis [12, 15] or for flow cytometry analysis [16, 17]. A quantitative flow cytometry method recently developed in our laboratory [18-20] allows a detailed study of endocytosis. Beside serum albumin substituted with mannosyl residues (Man-BSA), variants of α_1-acid glycoprotein (AGP) were also used to investigate the presence of membrane lectins on macrophages mediating an endocytosis process.

Human AGP is an acute-phase glycoprotein containing 43% carbohydrate distributed on 5 *N*-linked glycan chains per molecule [21] as bi-, tri- and tetra-branched glycan structures [22]. AGP can be separated into non reactive (AGP A), weakly reactive (AGP B) and reactive (AGP C) fractions by concanavalin A (Con A)-Sepharose chromatography [23]. The percentage of each molecular form was approximately 40.6% (AGP A), 43.7% (AGP B) and 15.6% (AGP C). Bierhuizen *et al* [24] have shown the absence of a di-antennary glycan on AGP A and the presence of one and two di-antennary glycans on AGP B and AGP C, respectively; tri- and tetra-antennary chains occurred on the remaining glycosylation sites. They also showed that 90% of the bi-antennary glycans of both AGP B and AGP C were disialylated. The nature of the oligosaccharide structures probably determines the biological effects of AGP. The comparison of the three forms of AGP with regard to their capacity to be internalized by macrophages seemed particularly interesting.

In the present paper we demonstrated by flow cytometry the presence of a mannose receptor mediating endocytosis in the $2C_{11-12}$ macrophage hybridoma cell-line. Moreover, we showed that AGP variant (AGP C) bearing two disialylated bi-antennary structures was better internalized by $2C_{11-12}$ cells and macrophages than the variants bearing one or no bi-antennary structures (AGP A or AGP B). We also provided evidence that the intracellular half life of the internalized ligands and the degrading capacity of the two types of macrophages were quite different while they both express mannose receptors.

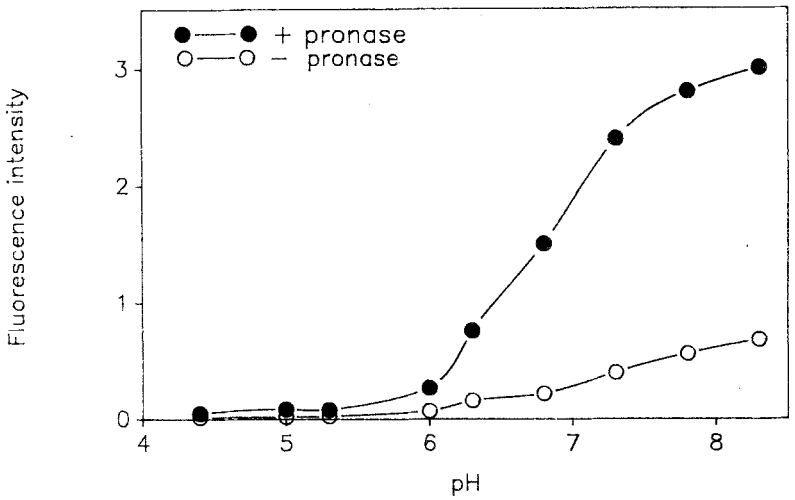

Figure 1. pH Dependence of the emission fluorescence intensity of native and digested F-AGP.
○; Fluorecence intensity of 1 µg fluoresceinylated AGP C in 1 ml 0.05 M phosphate citrate buffer at different pH:
●; Fluorescence intensity of 1 µg fluoresceinylated AGP C in 1 ml 0.05 M phosphate citrate buffer at different pH upon digestion by pronase (100 µg/ml for 1 h at 37°C).
The fluorescence intensities (excitation wavelength 495±5 nm; emission wavelength 520±5 nm) were expressed relative to the fluorescence intensities of a 2.55 x 10^{-7} M solution of quinine solution in 0.1 N H_2SO_4 (excitation wavelength 345±5 nm; emission wavelength 460±5 nm).

with pronase [20]. Fluorescence intensities of each fluoresceinylated protein were determined by spectrofluorimetry (Shimadzu R-F 5000 recording spectrofluorophotometer, Kyoto, Japan: excitation wavelength 495±5 nm, emission wavelength 520±5 nm) before and after proteolytic digestion with pronase.

Flow Cytometry Analysis

Binding and uptake of fluorescein-labeled glycoconjugates by macrophages and $2C_{11\text{-}12}$ cells were studied by flow cytometry using a FACS analyser (Becton Dickinson, Sunnyvale, CA, USA) which was calibrated with fluoresceinylated polystyrene sulfonate beads [18]. The size 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±15 nm).

Binding and Endocytosis of Fluoresceinylated Ligands

$2C_{11\text{-}12}$ cells and macrophages (TpM) were incubated in suspension (2 x 10^5 cells/200 µl) with fluoresceinylated proteins (20 µg/ml) in complete PBS either at 4°C for 1 h to study the binding of the ligands on the cell surface or at 37°C for 2 h to study the uptake of the ligands. Cells were then washed with cold complete PBS to remove unbound ligands and were then

Materials and Methods

Materials

Bovine serum albumin (BSA) was purchased from IBF Biotechnics, (Villeneuve la Garenne, France); Pronase (non-specific protease isolated from *Streptomyces griseus* Pronase B grade 45,000 PUK/g) from Calbiochem (La Jolla, CA, USA); and fluoresceinylisothiocyanate isomer I (FITC) from Molecular Probes (Junction City, OR, USA). Leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-arginal) was from Sigma (St Louis, MO, USA). Monensin was obtained from Calbiochem and a stock solution in ethanol (25 mM, 17.5 mg/ml) was prepared before use.

α_1-Acid glycoprotein (AGP) was obtained from Sigma. Con A-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Bovine serum albumin containing mannose residues (Man-BSA) was prepared as previously described [18] by allowing the *p*-isothiocyanatophenyl-α-D-mannopyranoside to react with BSA. Mannose content was determined by using a resorcinol sulfuric acid micro-method [25].

Preparation of AGP Variants

AGP was first purified from sera by immunoaffinity on an anti-AGP Sepharose 4B column [26]. Native AGP was then separated in three fractions by chromatography on a Con A-Sepharose column; a non-reactive fraction (AGP A), a weakly reactive fraction (AGP B) and a reactive fraction (AGP C) were obtained. To remove traces of Con A, a last step of purification was performed on an α-methylmannoside-agarose column. Purity was checked by sodium dodecylsulfate polyacrylamide gel electrophoresis.

Cells

Thioglycolate-elicited peritoneal macrophages (TpM) used in this study were harvested from (C57BL/6 x Balb/c)F1 mice, injected i.p. four days earlier with 2 ml thioglycolate medium (Institut Pasteur Production, Paris, France), by peritoneal lavages with RPMI 1640. The cells were centrifuged and resuspended in phosphate balanced saline pH 7.4 supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1% BSA (complete PBS).

The $2C_{11\text{-}12}$ macrophage hybridoma was obtained by somatic cell hybridization [7]: cell fusion between mouse peritoneal macrophages and a fusogenic cell-line derived from the murine lymphosarcoma J774-2 (J774-C2E2-HAT) generated macrophage hybridomas exhibiting a variety of macrophage functions. This cell line was maintained in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10% heat inactivated fetal bovine serum) supplemented with 10% NCTC (Gibco, Renfrew, UK). Cells were grown in tissue culture dishes in a humidified atmosphere (5% CO_2, 95% air) [7, 9].

Preparation and Characterization of Fluorescein-labeled Proteins

BSA, Man-BSA and AGP were fluoresceinylated with fluoresceinylisothiocyanate isomer I, as previously described [15, 18]. The number of bound fluorescein residues per protein molecule (F/P) was determined from the absorbance at 495 nm after proteolytic digestion

Table 1. Characteristics of fluorescein-labeled glycoconjugates: α_1-acid glycoprotein variants and neoglycoproteins.

Compounds[a]	M_r[b]	*N*-Glycans[c,d]	$I_f/\mu g$[e]	$I_f.d/\mu g$[f]	$I_f.d/I_f$	F/P[g]
AGP A	43,500[d]	0 diant. 5 tri/tetra	0.9	2.7	3	2.3
AGP B	42,300[d]	1 diant. 4 tri/tetra	0.4	2.4	6	2.2
AGP C	41,200[d]	2 diant. 3 tri/tetra	0.6	2.8	5	2.6
F-,BSA	68,000	-	0.35	2.4	6.8	2.8
F-,Man-BSA	77,400	-	0.4	2.5	6.2	3
F-,Man-BSA	74,300	-	0.6	4	7	5

[a] AGP A,B,C = variants of α_1-acid glycoprotein separated on Con A-Sepharose. F-,Man-BSA = bovine serum albumin substituted with fluorescein molecules and 25±5 mannose residues.
[b] M_r = Molecular weight of unlabeled glycoproteins.
[c] *N*-Glycans = number of di-, tri-, and tetra-antennary structures.
[d] according to Bierhuizen *et al*, [24].
[e] I_f = fluorescence intensity of 1 µg fluoresceinylated glycoconjugates in 1 ml PBS.
[f] $I_f.d$ = fluorescence intensity of 1 µg fluoresceinylated glycoconjugates in 1 ml PBS upon digestion by pronase.
[g] F/P = number of fluorescein molecules per protein molecule (calculated from the absorbance at 495 nm upon proteolytic digestion, Midoux *et al*, [20].

resuspended in sheath fluid [20]. Fluorescence intensities associated to the cells were measured by flow cytometry before and after a 30 min post-incubation at 4°C in the presence of 50 µM monensin [20]. All cell fluorescence intensities values were corrected with the fluorescence of cells incubated in F-glycoprotein-free medium (autofluorescence).

Time Course of the Endocytosis of Fluoresceinylated Ligands Bound at the Cell Surface

Cells (2 x 10^5/200 µl) were first incubated at 4°C with F-AGP variants or F-,Man-BSA (100 µg/ml) for 1 h in complete PBS. Then cells were washed to remove unbound ligands, resuspended in complete PBS in the absence or presence of 200 µM leupeptin (leupeptin was used to inhibit hydrolysis of intracellular proteins by lysosomal enzymes) and warmed up to 37°C to allow internalization of bound ligands. At various times, cold sheath fluid was added to stop the endocytosis process and cell fluorescence intensities were analyzed by flow cytometry, before and after a 30 min post-incubation with 50 µM monensin.

Spectrofluorimetric Analysis of Extracellular Medium

In order to measure the release by the cells of fluorescent material in the medium after endocytosis, the supernatants of 10^6 cells which had been incubated at 4°C with the F-ligands (100 µg/ml), washed, resuspended in 1 ml of complete PBS and incubated at 37°C were collected at various times. Fluorescence intensities were measured by spectrofluorimetry before and after proteolytic digestion at 37°C for 2 h in the presence of 100 µg/ml pronase.

Results and Discussion

Fluorescence Characteristics of Fluoresceinylated Ligands and Flow Cytometry Analysis

Flow cytometry is a convenient method to study the binding of fluoresceinylated ligands on the cell surface. Previous investigations from our laboratory have demonstrated that flow cytometry can also be used to evaluate the endocytosis and subsequent degradation of the internalized fluoresceinylated ligands [17-20]. For clarity, some of the fluorescence properties of fluorescein must be discussed to avoid possible misunderstanding. First, compartments encountered by the internalized ligands are acidic organelles and the fluorescence quantum yield of fluorescein free or bound to proteins decreases rapidly from pH 7 to 4 (Fig. 1). Hence, monensin, a proton sodium ionophore, is used to equilibrate the pH between the organelle lumens and the external environment in order to allow the evaluation of the fluoresceinylated ligands internalized in acidic compartments. Second, the fluorescence quantum yield of fluorescein bound to a protein is lower than that of free fluorescein, even at neutral pH (Fig. 1, Table 1). So, upon proteolytic digestion by pronase, the fluorescence intensities of serum albumin conjugates (F-BSA and F-,Man-BSA) and AGP variants increase as the result of the release of small fluoresceinylated peptides. The ratio between the fluorescence intensities of degraded and undegraded conjugates ranges from 5-6 for 2-3 fluorescein residues bound to BSA, Man-BSA or AGP B or C molecules. For AGP A this ratio is only three. The intrinsic fluorescence intensity of each compounds is slightly different, probably as a consequence of the fluorescein environment. Hence, when the endocytosed ligands are digested in endosomes and lysosomes, their fluorescence intensity will be greater than that of the same amount of undigested ligands either endocytosed or bound to the cell surface. Accordingly, it is possible to measure the extent of the degradation by flow cytometry using a permeant protease inhibitor such as leupeptin. To compare the binding of serum albumin substituted with mannose residues (Man-BSA) and the three AGP variants, conjugates bearing a similar number of fluorescein molecules (F/P = 2-3) were used (Table 1).

Binding and Uptake of F-AGP and F-,Man-BSA by Macrophages and $2C_{11\text{-}12}$ Cells

Cells incubated at 4°C with 20 µg/ml of F-ligands did not yield a high cell fluorescent signal, whatever macrophage cell type and whatever conjugate (F-,Man-BSA, F-AGP A, F- AGP B or F-AGP C) were used (Table 2). Incubation with a higher concentration (up to 200 µg/ml) did not significantly increase the cell surface labeling. As previously reported by Stahl *et al.*[1-3], macrophages express mannose receptors on their surface but the amount of

Table 2. Binding and uptake of fluoresceinylated neoglycoproteins or α_1-acid glycoprotein variants by mouse peritoneal macrophages and the $2C_{11-12}$ hybridoma cell-line.

Harvested $2C_{11-12}$ cells and thioglycolate-elicited mouse peritoneal macrophages were incubated for 1 h at 37°C in complete PBS containing 1% BSA in the presence of fluoresceinylated derivatives (20 µg/ml). Cells were then washed and resuspended in 0.5 ml cold sheath fluid. The cell fluorescence intensities were measured by flow cytometry using a FACS analyser. Fluorescence associated with the cells incubated at 37°C was measured before and after 30 min incubation at 4°C with 50 µM monensin. The cell fluorescence intensities were the cell-associated fluorescence minus fluorescence of cells incubated in F-glycoprotein-free medium (autofluorescence). All values are standardized with reference to the fluorescence of calibration beads.

	Cell fluorescence intensities					
	$2C_{11-12}$			Macrophages		
	4°C	37°C monensin		4°C	37°C monensin	
		-	+		-	+
F-BSA	2	5	9	1	1	1
F-,Man_{30}BSA	8	44	176	8	35	60
F-,Man_{20}BSA	2	22	74	n.d.[a]	n.d.	n.d.
F-AGP A	2	5	13	2	3	3
F-AGP B	2	19	55	2	4	8
F-AGP C	3	38	105	3	10	21

[a] n.d. = not determined.

receptor is low as compared to the internal pool. However, since the mannose receptor allows endocytosis of the ligands [1, 12, 27], incubation of macrophages was performed at 37°C and the cell fluorescence was analyzed by flow cytometry before and after incubation with monensin. As expected, thioglycolate-elicited peritoneal macrophages (TpM) internalized Man-BSA in acidic compartments as demonstrated by the enhancement of the cell fluorescence intensity following treatment with monensin (Table 2).

The $2C_{11-12}$ cell line could also take up F-,Man-BSA (Table 2). After monensin post-incubation the fluorescence intensity of $2C_{11-12}$ cells was three times greater than that of TpM. These results suggest that the fate of the mannosylated serum albumin internalized by the mannose receptors expressed on the two type of macrophages might be quite different.

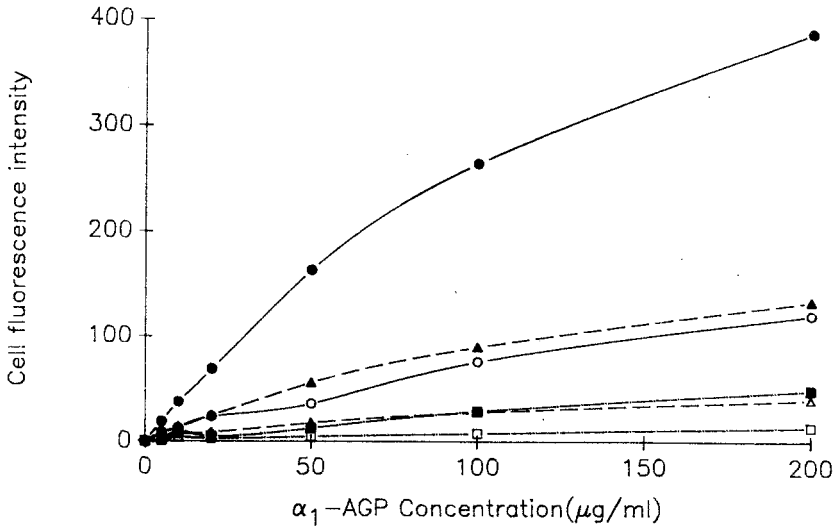

Figure 2. Uptake of AGP variants by $2C_{11-12}$ hybridoma cell-line.
Cells were incubated for 2 h at 37°C in complete PBS/1% BSA containing various concentration of F-AGP. Cells were then washed and resuspended in cold sheath fluid. The cell fluorescence intensities were measured by flow cytometry.
Fluorescence intensities of cells incubated at 37°C with F-AGP A (□), F-AGP B (△), F-AGP C (○): Fluorescence intensities of cells incubated at 37°C with F-AGP A(■), F-AGP B (▲), F-AGP C (●), and then at 4°C for 30 min in the presence of 50 μM monensin.

The capacity of the three molecular forms of AGP to be internalized by TpM and $2C_{11-12}$ was also analyzed. The fluorescence intensities of TpM and $2C_{11-12}$ incubated at 37°C with F-AGP A, the variant which lacks di-antennary glycans, were very low and not significantly different from the fluorescence of cells incubated with F-BSA alone, even after incubation with monensin (Table 2). F-AGP B was slightly internalized by $2C_{11-12}$ but poorly by TpM. Among the AGP variants, AGP C with two bi-antennary glycans was found to be the best AGP ligand for $2C_{11-12}$ and TpM (Table 2). The low level of internalization with AGP A variant as demonstrated by a slight increase of fluorescence intensity after monensin post-incubation is not related to the intrinsic fluorescence properties of fluorescein-labeled AGP A, but is more probably due to the structure of the glycan moieties (absence of di-antennary structures). The good affinity of AGP C for the receptor of $2C_{11-12}$ was confirmed by incubating the cells at 37°C with increasing concentrations of each variant as shown in Fig. 2. The cell-associated fluorescence showed a saturation occurring between 100 and 200 μg/ml of F-AGP indicating that the internalization is receptor mediated. The better affinity of AGP C variant could be related to a better accessibility of the mannose residues.

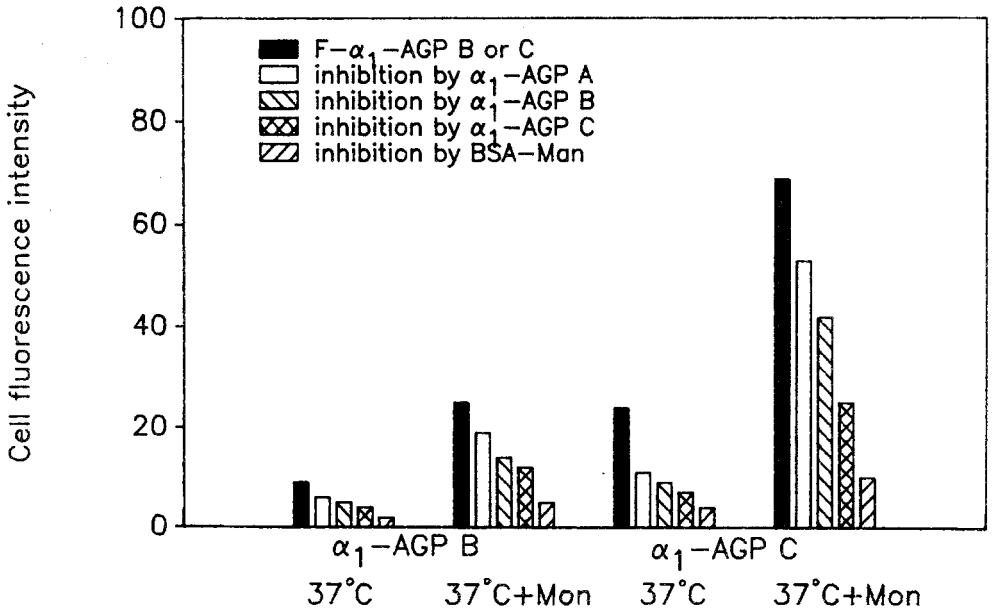

Figure 3. Inhibition of fluorescein-labeled AGP uptake by AGP variants and mannosylated BSA in the $2C_{11-12}$ cell-line.

Cells were first incubated for 1 h at 37°C in the presence of unlabeled glycoprotein: Man-BSA and AGP variants (A, B or C), 1 mg/ml. Then F-AGP B or F-AGP C were added (20 µg/ml, final concentration) and cells were further incubated for 1.5 h at 37°C. Cells were washed and resuspended in cold sheath fluid and fluorescence intensities were analyzed by flow cytometry directly and after post-incubation with monensin (Mon).

In order to test whether the endocytosis of AGP B and AGP C was mediated *via* binding to a putative mannose receptor, inhibition experiments were performed. As shown in Fig. 3 the fluorescence intensities of $2C_{11-12}$ cells pre-incubated with an excess of unlabeled ligand and subsequently with F-AGP B or F-AGP C were strongly reduced when AGP C or Man-BSA were used as inhibitors, and the most marked inhibition was observed with Man-BSA. These results indicate that AGP B, AGP C and Man-BSA are internalized *via* the same receptor.

Time Course for the Degradation of Man-BSA and AGP C

Next it was of interest to compare the half life of the conjugates following internalization by the two types of macrophages. As mentioned above, quantitation of the endocytosed material, both degraded and undegraded, inside cells is possible by measuring the cell fluorescence intensities of cells incubated at 37°C in the absence or presence of an inhibitor of proteolysis. Membrane-associated cathepsin D is found in endosomes of macrophages [28] and could be responsible for the pre-lysosomal degradation of Man-BSA by macrophages [29]. Pepstatin was found to be the best inhibitor of cathepsin D contained in

Table 3. Time course of the uptake and degradation of fluoresceinylated glycoproteins by $2C_{11-12}$ hybridoma cell-line, in the presence or in the absence of a proteinase inhibitor.

$2C_{11-12}$ cells were incubated at 4°C for 1 h in the presence of 100 µg/ml F-,AGP-C or F-,Man-BSA. The cells were then washed, resuspended in complete PBS/1% BSA and warmed at 37°C in the absence or in the presence of 200 µM leupeptin. Cells were collected at indicated time intervals and cell fluorescence intensities were measured after a 30 min treatment at 4°C with 50 µM monensin. The cell fluorescence intensities were the cell-associated fluorescence minus the autofluorescence of control cells.

	Cell fluorescence intensities			
	F-AGP C		F_5-,Man_{20}-BSA	
Incubation time (min)	Leupeptin		Leupeptin	
	-	+	-	+
0	7	7	16	16
30	19	17	52	33
60	52	32	151	73
120	119	63	222	121
180	134	75	215	139

endosomes and lysosomes but did not easily cross the cell membrane. Leupeptin, a permeant inhibitor of intracellular proteases was internalized but unfortunately in the case of macrophages, was not efficient enough to inhibit intracellular degradation totally; indeed it gave only 40% inhibition with isolated endosomes [29]. Nevertheless, the effect of leupeptin on ligand degradation by $2C_{11-12}$ cells was tested. F-AGP C and F-,Man-BSA were bound to $2C_{11-12}$ cells at 4°C for 1 h, then cells were washed to remove free ligands and incubated at 37°C to allow internalization of the bound ligands. As shown in Table 3, cell fluorescence intensities increased after 30 min incubation at 37°C. This fluorescence increase resulted from the digestion of the ligands in small peptides. The fluorescence intensities of cells incubated in the presence of leupeptin were about half of those incubated without inhibitor, when AGP C or Man-BSA were used. These results suggest that the intracellular degradation of the two compounds, partially abrogated by leupeptin, takes place in compartments containing proteases. Because the cell fluorescence intensity of cells incubated in the presence of leupeptin was greater than that bound to the cell surface (time 0), it is clear that leupeptin could not prevent the total intracellular degradation of F-AGP C and F-,Man-BSA and that degradation started very rapidly upon internalization. So, these experiments did not allow a complete quantitative estimation of degradation in contrast with data previously reported for endocytosis of serum albumin substituted with glucose by 3LL membrane lectin [20] in which degradation was totally abrogated by incubation in the presence of leupeptin.

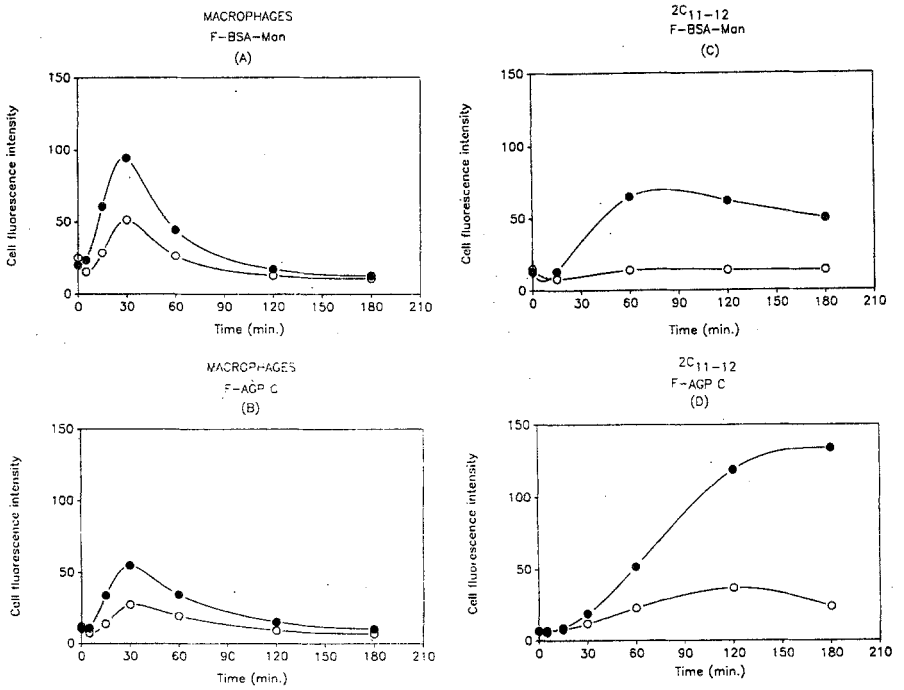

Figure 4. Time course of the uptake of F-glycoproteins by macrophages and $2C_{11-12}$ cell-lines.
Macrophages (A, B) and $2C_{11-12}$ (C, D) were incubated at 4°C for 1 h in the presence of 100 µg/ml F-AGP C or F-,Man_{20}-BSA. Cells were washed with complete PBS/1% BSA, resuspended in the same buffer and warmed up to 37°C. The cells were collected at various times and cold sheath fluid was added. The cell fluorescence intensities were measured by flow cytometry at 4°C before and after a 30 min post-incubation with 50 µM monensin. All values are corrected by the cell fluorescence intensity of cells incubated with F-BSA containing the same amount of fluorescein.
○; Cell fluorescence intensities of cells incubated at 37°C with F-AGP or F-,Man-BSA: ●; Cell fluorescence intensities of cells incubated at 37°C with F-AGP or F-,Man_{20}-BSA and post-incubated with monensin.

The time course experiments, made for comparing $2C_{11-12}$ and TpM with F-,Man-BSA and F-AGP C as ligands, showed clear-cut differences (Fig. 4). Cytofluorimetry analysis was made before and after post-incubation with monensin, and showed a more rapid fluorescence increase with TpM than with $2C_{11-12}$. The maximum was reached after 30 min in the case of TpM both with F-,Man-BSA and F-AGP C, while the fluorescence intensity of $2C_{11-12}$ increased until 120 min. Furthermore, as the cell fluorescence of $2C_{11-12}$ remained nearly constant during further incubation, the cell associated fluorescence in TpM decreased rapidly after 30 min and was very low after 2 h incubation. In the case of macrophages,

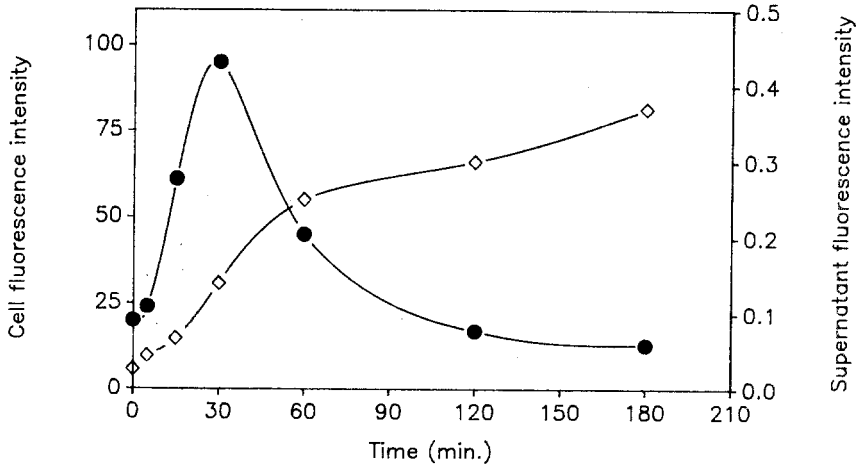

Figure 5. Time course of the uptake of F-,Man-BSA by macrophages and release of fluorescent material into the medium.
Cells were first incubated at 4°C with F-,Man-BSA (100 μg/ml) for 1 h. Cells were then washed, resuspended in complete PBS and warmed up to 37°C. At various times, cells were centrifuged; supernatants were collected and fluorescence intensities were analyzed with a spectrofluorimeter (excitation wavelength; 495±5 nm, emission wavelength: 520±5 nm). Cells were resuspended in sheath fluid and cell-associated fluorescence was measured by flow cytometry after monensin post-treatment. All measurements were done in triplicate.
◇; Fluorescence intensity of the supernatants measured by spectrofluorimetry: ●; Cell fluorescence intensities after monensin post-incubation measured by flow cytometry.

mannosylated glycoproteins (β-glucuronidase) have been shown to be transferred from endosomes to secondary lysosomes 15-20 min after the induction step of internalization [13], where proteolysis occurred. However,subcellular fractionation indicated a pre-lysosomal degradation [29]. This statement could explain the rapid degradation occurring in macrophages.

By spectrofluorimetric analysis of the extracellular medium collected at different times after cell incubation at 37°C, we have checked the release of fluoresceinylated compounds. The fluorescence recovered in the supernatants of TpM increased after 30 min as soon as the cell-associated fluorescence decreased (Fig. 5); the fluorescence intensity of the supernatants did not change after pronase treatment (data not shown) indicating that the released fluoresceinylated components were in a micromolecular form. The appearance of fluoresceinylated material into the incubation medium corresponds to the loss of intracellular ligand and might result from lysosomal digestion, as previously reported by Hoppe and Lee [11] and Wileman *et al.* [13], using radiolabeled ligands.

It thus appears that $2C_{11-12}$ cells differ from macrophages in their capacity to exocytose degraded materials, since after 2 h the fluorescence intensity of cells incubated at 37°C remained constant (Fig. 4) and a slight decrease only started after 3 h incubation. The internalized Man-BSA or AGP C were totally degraded as suggested by the fluorescence increase, but were not secreted as in the case of macrophages. Such kinetics may explain some of the data presented in Table 2. Indeed in the case of $2C_{11-12}$ cells the enhancement of the cell fluorescence intensity upon monensin treatment was greater than that obtained with macrophages. This indicates that fluorescent material ingested into $2C_{11-12}$ cells was contained in more acidic vesicles than those in macrophages. Hence, it is possible that due to the weak exocytotic capacity of $2C_{11-12}$ cells, fluorescent degraded compounds were accumulated in lysosomes (probably secondary lysosomes). In contrast, in the case of macrophages, fluorescent materials were released rapidly upon their degradation in endosomes or lysosomes.

Concluding Remarks

Peritoneal mouse macrophages manifest the capacity to bind a serum glycoprotein devoid of mannose in a terminal non-reducing position. In this respect, the macrophage lectin behaves similarly to Con A, a plant lectin, because AGP A is not retarded on immobilized Con A and does not bind to macrophages, AGP B is retarded on Con A and slightly binds to macrophages and AGP C tightly binds to immobilized Con A and to macrophages. The binding of AGP C to macrophage lectin is inhibited quite well by mannosylated BSA (Man-BSA) and therefore there is no evidence that this glycoprotein binds to a sialic acid receptor; conversely, it most probably binds to the mannose receptor.

The $2C_{11-12}$ hybridoma cell-line which shares many properties in common with macrophages also expresses the mannose receptor as shown by binding and uptake of Man-BSA and also binds AGP C. Therefore, this new cell line is of considerable interest because in contrast to many other macrophage cell lines, it expresses a mannose-specific lectin which readily undergoes the endocytosis of its ligand. However, there is a striking difference between $2C_{11-12}$ hybridoma cells and macrophages since this line which actually degrades the endocytosed material does not allow secretion of the degraded material.

In conclusion, the $2C_{11-12}$ hybridoma cell line appears to be a suitable tool to study the function of the mannose-specific lectin and may be used to elucidate the mechanisms of secretion of lysosomal degradation products. Furthermore, the presence of membrane lectins on a macrophage hybridoma cell line opens the possibility to study the efficiency of glycoconjugates as carriers of immunoactivating and antiviral drugs; with this aim, the α_1-acid glycoprotein C variant may be of particular interest.

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