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Modifications of glycosylation patterns in macrophages upon activation

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Activated macrophages, in contrast to inflammatory and resident macrophages, are able to inhibit the growth of intracellular pathogens and tumor cells. In order to understand the adaptative changes which allow macrophages to express antitumor activity, we compared, among several parameters, the glycoproteins of cytotoxic and non-cytotoxic macrophages. After activation of mouse peritoneal macrophages by two stimuli applied in a sequence (trehalose dimycolate in vivo, lipopolysaccharide in vitro), we observed that: (1) surface sialic acid residues (labeled by tritiated borohydride after treatment of intact cells in culture by periodate) were reduced by 37%; (2) total sialic acid, as measured by an adaptation to HPLC of the thiobarbituric assay, was reduced by 30%. Variations in the intensity of the labeling after periodate/borohydride treatment were especially pronounced for a few high-molecular-weight glycoproteins. Analysis of glycopeptides indicated that the reduction of sialylation was accompanied by a slight increase in the relative importance of high mannose-type oligosaccharides (glycopeptides sensitive to endoglycosidase H or retained on concanavalin A-Sepharose) but did not affect the ratio of the various anionic species separated on QAE-Sephadex. A reduced sialylation of glycans after activation may facilitate interactions of macrophages with microbes and tumor cells.

Introduction

Macrophages, when appropriately activated, selectively lyse neoplastic cells in a non-phagocytic process. Binding of tumor cells to the macrophage surface has been described as an essential part of the cytolytic reaction [1–4]. In fact, the interactions between macrophages and tumor cells may govern several different phenomena: selection of neoplastic targets for destruction [5], triggering the release of toxic factors [6], concentration and protection of lytic effectors in a close space; they may also directly support the cytolytic mechanism [7,8]. However, the recognition structures on macrophage surfaces have not yet been identified. Since properties such as the ability to bind tumor cells and cytotoxicity are expressed only in populations of macrophages that have received specific stimuli [1,2,9,10–12], it was of interest to characterize components of the macrophage surface that are altered during activation to cytotoxicity.

The degree of sialylation of glycoconjugates may be an important parameter, since sialic acid is

Abbreviations: Con A, concanavalin A; endoglycosidase H, endo-β-*N*-acetylglucosaminidase H; FCS, fetal calf serum; LPS, lipopolysaccharide; MEM, minimum essential medium, PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TDM, trehalose dimycolate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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mainly located in the surface membrane, is externally oriented and is negatively charged. The involvement of membrane sialic acids in the regulation of a great variety of biological processes such as binding and transport of cationic compounds [13,14], infection of host cells by microorganisms [15], prevention of cell–cell interactions [16], metastatic capacity and tumorigenicity [17,18] have recently been receiving increasing attention. Furthermore, many transformed and neoplastic cells are more sialylated than their normal counterparts [19].

Acquisition by macrophages of the ability to destroy tumor cells is a multi-stepped process; young inflammatory macrophages are primed in vivo by immunomodifiers, such as pyran copolymers, trehalose dimycolate (TDM) [20] and interferon γ (effective also in vitro), to respond to low doses of a second signal such as lipopolysaccharide (LPS) or muramyl dipeptide [9,21–23].

Primed macrophages, in contrast to inflammatory or resident macrophages, are characterized by a high expression of Ia and LFA-1 antigens and have the capacity to produce high quantities of H_2O_2 and to bind tumor cells [10,23–25]. Lipopolysaccharide treatment of primed macrophages induces the expression of a tumoricidal potential (probably related, in part, to the secretion of soluble cytotoxins), increases considerably the rate of hexose consumption and induces the formation of citrulline and nitrite/nitrate from arginine [23,26,27].

In order to study the modifications caused by the activation process in macrophage glycans, we compared the sialic acid content and the importance of glycoproteins containing high levels of mannose in various macrophage populations: resident cells, inflammatory macrophages elicited by thioglycolate broth, macrophages primed by trehalose dimycolate and macrophages activated by the sequential action of trehalose dimycolate and lipopolysaccharide.

By direct labeling of sialic acid residues on the membrane of intact cells in culture, we found marked differences in the number of sialic acid residues expressed at the surface of macrophages depending on their activation state; after priming and activation, fewer sialic acid residues were accessible to labeling and total sialic acid, quantified by HPLC, was reduced. Reduction of sialylation observed in activated macrophages was especially pronounced in a few glycoprotein species; it cannot be explained entirely by the slight increase of N-linked carbohydrates of the high mannose (or hybrid) type which was also detected in activated macrophages. We have previously reported that for some secreted glycoproteins, the forms produced by primed and activated macrophages were less acidic than the species released by resident macrophages [28].

Part of this work was presented in a preliminary form at the International Symposium on Glycoconjugates, July 1987, Lille.

Materials and Methods

Materials. Trehalose dimycolate, prepared from *Mycobacterium tuberculosis,* strain Peurois, was obtained from Biosys (Compiègne, France) and suspended in water according to the method of Kato [29]. Lipopolysaccharide (prepared from *Salmonella enteritidis* by the method of Westphal) was purchased from Difco (Detroit, MI), dissolved in saline at 1 mg/ml and boiled for 30 min.

Endotoxin-negative fetal calf serum (FCS) (endotoxin < 0.1 ng/ml) was purchased from Gibco (Parsley, U.K.); [^{3}H]thymidine, uniformly ^{14}C-labeled amino acids (a mixture of 15 amino acids; reference CB 51) and D-[^{14}C]glucosamine (300 mCi/mmol) were obtained from C.E.A. (Saclay, France), D-[2-^{3}H]mannose (10–20 Ci/mmol) was from Amersham International (Amersham, U.K.). [^{3}H]Sodium borohydride (reference Tmm-131 B, 16–36 Ci/mmol) was from C.E.A.

Endoglycosidase H (endo-β-*N*-acetylglucosaminidase H, from *Streptomyces plicatus*) was obtained from Miles Laboratories (Elkhart, IN); α-mannosidase (α-D-mannoside mannohydrolase, from jack bean) was from Sigma (St. Louis, MO, U.S.A.) neuraminidase (1 U/ml, from *Vibrio cholerae*) and pronase (91 000 PUK/g, from *Streptomyces griseus*) was from Calbiochem-Behring (La Jolla, CA, U.S.A.). Sephadex G-50, Con-A-Sepharose and QAE-Sephadex A-25 were obtained from Pharmacia (Uppsala, Sweden) and Bio-Gel P 10 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Macrophages. Peritoneal exudate cells were obtained by lavage of the peritoneal cavity of female mice (C57B1/6 × DBA/2) F1 (BDF1) from Iffa Credo (France). Peritoneal exudate cells were adjusted to $1.2 \cdot 10^6$ macrophages/ml (macrophages being identified after neutral red uptake) in minimum essential medium (MEM, from Pasteur Production, France) supplemented with antibiotics and 5% heat-inactivated FCS and plated in 35-mm or 60-mm dishes (Nunclon, Denmark). After 4 h at 37° C, under an atmosphere of 5% CO_2, non-adherent cells were washed out and macrophage monolayers were processed for biosynthetic or surface labeling.

The functional state of macrophages depends upon the in vivo treatment received by the animals and upon the in vitro culture conditions: resident macrophages were obtained from untreated animals, inflammatory macrophages were obtained 4 days after intraperitoneal (i.p.) injection of thioglycolate broth (1.5 ml, from Pasteur Production, France), primed macrophages were obtained 7 days after i.p. injection of a suspension of trehalose dimycolate (50 μg); resident, inflammatory and primed macrophages were cultured in endotoxin-free medium. To activate macrophages for tumor cytotoxicity, trehalose dimycolate-primed macrophages were cultured for 4 h in the presence of 10 ng/ml lipopolysaccharide [23].

Macrophage-mediated cytostasis. In order to verify the functional state of the various macrophage populations, their capacity to inhibit P815 mastocytoma cell growth was measured, using a [^{3}H]thymidine-cumulative incorporation assay [23]. P815 cells, plated at $0.2 \cdot 10^6$ cells/cm^2, were cocultured for 20 h with macrophages ($0.8 \cdot 10^6$/cm^2), in the presence of [^{3}H]thymidine (5 μM, 1 Ci/mmol). The radioactivity incorporated by P815 cells cocultured with activated macrophages was reduced to 1.2 ± 0.8% (mean of 19 experiments) of the radioactivity incorporated into tumor cells cultured in the absence of macrophages. In contrast, the residual incorporation was 48 ± 6%, 68 ± 17% and 60 ± 9% for tumor cells cultured in the presence of resident, inflammatory and primed macrophages, respectively.

Periodate: Na B^3H_4 labeling. The method described by Van Lenten and Ashwell [30] for labeling of sialo-proteins was used to label sialic acid residues directly on the surface membrane of cells in monolayers, as proposed by Flowers and Glick [31].

Macrophage monolayers ($2 \cdot 10^6$ macrophages, $0.2 \cdot 10^6$ macrophages/cm^2), were treated with 2 mM $NaIO_4$ in 1 ml of phosphate-buffered saline (PBS, 130 mM NaCl/2.68 mM KCl/0.68 mM KH_2PO_4/6.42 mM Na_2HPO_4, $12H_2O$ (pH 7.2)) for 10 min at 25° C, in the dark. The cells were washed five times with 2 ml of PBS and incubated in the presence of 500 μCi NaB^3H_4 for 5 min at 4° C in 2 ml of PBS. Monolayers were washed five times with PBS and lysed in 0.1% Triton X-100. Lysates were used for protein assay following the method of Folin and for measurements of radioactivity; total radioactivity was determined on an aliquot dissolved in Aqualyte (Baker, The Netherlands). TCA-precipitable radioactivity was measured on aliquots of cell lysates added to a large volume of 5% TCA/0.1% phosphotungstic acid.

After heating (15 min at 50° C) TCA-precipitable material was collected on glass fiber discs (GF.F from Whatman, U.K.). These conditions were selected for their maximal precipitation and recovery of ^{3}H label. Filters were incubated for 1 h at 37° C in Lipofluor/ Solulyte/H_2O (5 : 0.5 : 0.1, v/v) and counted.

Quantitation of thiobarbituric acid-reactive sialic acid by HPLC. The HPLC adaptation of the periodate-TBA assay proposed by Powell and Hart [32] was used with slight modifications. After adherence and washing, macrophage monolayers ($5.2 \cdot 10^6$ macrophages) were lysed in 1 ml H_2O. Lysates were made 0.1 M in HCl and hydrolysis was performed at 80° C for 60 min under N_2. Samples were neutralized, lyophilized and resuspended in 100 μl H_2O by vigorous vortexing. The residue was eliminated by centrifugation in a Beckman microfuge (2 min at 2000 × *g*) and the TBA assay was performed on a 40 μl aliquot of the supernatant. The final volume of the reaction mixture was 760 μl. HPLC analysis was carried out on a Waters Novapak C18 column (5 μm spherical silica, 150 × 3.9 mm). The device included the Waters 600 multisolvent delivery system and the Waters 481 variable wavelength detector set at 549 nm. 25 μl of the reaction mixture was injected and the signal was monitored using

the Waters 740 data module which performed simultaneous integration of the peak areas. A standard solution of sialic acid (from *Escherichia coli,* Type VI from Sigma) was used to calibrate the system. The percentage of methanol in the solvent was decreased to 24% to optimize the isocratic elution of the sialo-TBA chromophore in our system.

Biosynthetic labeling of glycoproteins. Macrophages ($(6-7) \cdot 10^6$ cells) were cultured for 40 h in MEM supplemented with 5% FCS and antibiotics and containing 0.2 μM D-[2-^{3}H]mannose or 18 μM [^{14}C]glucosamine.

When they receive the last activation signal, delivered by lipopolysaccharide in vitro, macrophages increase 3-fold their rate of consumption over that of glucose or mannose [23]; thus, the labeling of activated macrophages glycoproteins by D-[2-^{3}H]mannose was routinely found to be 6-fold higher than that of resident or primed macrophages due to the increase of intracellular mannose-specific radioactivity (Grand-Perret, T. and Lemaire, G., unpublished data).

Endoglycosidase H digestion. Estimation of endoglycosidase H susceptible carbohydrates was made essentially as described by Varki and Kornfeld [32].

D-[2-^{3}H]Mannose-labeled macrophage monolayers were lysed in 1.5 ml of PBS containing 0.2% SDS. The lysates were heated for 10 min at 100 °C and filtrated through a column of Sephadex G-50 (1 × 55 cm) equilibrated with 0.1 M ammonium bicarbonate/0.2% SDS/0.01% NaN_3. Fractions corresponding to the high-molecular-weight glycoproteins (87% of the radioactivity) eluting in the void volume of the column were pooled and chilled on ice; 1.5 mg of BSA was added as a carrier and 9 vol. of ice-cold acetone was added slowly; the mixture was allowed to stand at −20 °C for 20 h. Precipitates were collected by centrifugation (10 min at 15000 × *g*), washed, dried and resuspended in 0.3 ml of 0.1 M sodium citrate buffer/0.2% SDS (pH 5.5).

1 mU of endoglycosidase H was added and samples were incubated at 37 °C for 48 h. An additional 1 mU of enzyme was added after 20 h. The digestion was stopped by addition of 0.1 ml of 10% SDS and heating for 5 min at 100 °C. Samples were filtrated again through Sephadex G-50. The high-mannose oligosaccharides released by endoglycosidase H (elution volume, 29 ml) were separated from the glycoproteins eluting in the void volume (15 ml) and the radioactivity recovered in both peaks was compared.

Con A-Sepharose fractionation of glycopeptides. Fractionation of glycopeptides on Con A-Sepharose was conducted as described by Cummings and Kornfeld [34].

D-[2-^{3}H]Mannose-labeled macrophages were lysed in 0.1 M Tris-HCl buffer (pH 8)/1 mM $CaCl_2$/0.02% SDS/0.1% Triton X-100. Pronase (previously incubated for 30 min at 60 °C) was added at 1.3 mg/ml and the mixture was incubated for 24 h at 60 °C. Digestion was stopped by boiling for 10 min and digests were applied on a Bio-Gel P-10 column (2 × 25 cm) equilibrated in 0.1 M ammonium bicarbonate. Glycopeptides containing fractions were lyophilized and dissolved in 1 ml of 10 mM Tris-HCl buffer (pH 8), containing 0.15 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$ (TBS buffer) and applied to 1.5 ml columns of Con A-Sepharose equilibrated in TBS buffer. The columns were washed sequentially with 10 ml of TBS buffer, 10 ml of 10 mM α-methyl-D-glucoside in TBS buffer and 10 ml of 0.5 M α-methyl-D-mannoside. The radioactivity eluted in each step was measured.

QAE-Sephadex chromatography. To compare the amount of sialylation of the glycoproteins of various macrophage populations, pronase digests after filtration on Sephadex G-50 were chromatographed on QAE-Sephadex [33,35]. After lyophilization, the glycopeptides of pronase digests were dissolved in 2 mM Tris base and passed over 1 ml columns of QAE-Sephadex equilibrated in 2 mM Tris base. Columns were eluted with increasing concentrations of NaCl (20 mM, 70 mM, 100 mM and 140 mM; 10 ml of each concentration); 1 ml fractions were collected and the radioactivity was monitored.

Other procedures. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was carried out on 7.5% acrylamide slab gels according to the method of Laemmli [36] and fluorography was prepared as proposed by Bonner and Laskey [37]. Estimation of glycopeptide molecular weight was made by reference to marker proteins and to ^{14}C-labeled polypeptides of mac-

rophages lysates run in parallel. The molecular weight of macrophage cellular polypeptides obtained after biosynthetic labeling with ^{14}C-labeled amino acids was determined separately.

Data are presented as mean ± S.D. of n independent experiments. Results obtained for TDM + LPS-activated macrophages and resident macrophages were compared in each set of experiments by a Student's paired t-test.

Results

Variations in cell-surface sialic acid upon macrophage activation

Cell-surface sialic acid was first estimated in resident macrophages and in TDM + LPS-activated macrophages by the labeling method introduced by Van Lenten and Ashwell [30]: the two distal exocyclic carbon atoms of terminal sialic acid residues are selectively cleaved by mild periodate oxidation and the resulting aldehyde is reduced with tritiated borohydride. As shown in Table I, the labeling of macrophages was reduced (×0.63) after activation ($P < 0.01$). Less labeling of activated macrophages compared to resident cells was observed, whatever the parameter considered: (1) total radioactivity incorporated (Table I); (2) TCA-precipitable radioactivity (in this case, the ratio of activated macrophages/resident macrophages was 0.60 ± 0.07 (mean of four experiments) ($P < 0.01$); (3) methanol/chloroform (1 : 2) -extractable radioactivity (ratio of activated macrophages/resident macrophages was 0.63 (two experiments). Accessible sialic acid residues from both glycoproteins and glycolipids are reduced after activation.

SDS-PAGE revealed several interesting findings (Figs. 1 and 2): (1) the radioactivity was incorporated exclusively into polypeptides of high molecular mass (above 40 kDa); (2) in the absence of periodate treatment, no labeled species could be detected on polyacrylamide gels; (3) the labeling was considerably reduced when macrophages were pretreated (1 h at 37°C) with neuraminidase (3.3 mU/10^6 macrophages in MEM) before exposure to periodate and borohydride (Fig. 1): (4) as shown in Fig. 2, the reduction of labeling observed in the case of activated macrophages, was not equivalent for all the radioactive polypeptides, indicating that variations in sialylation or in the expression of sialylated glycoproteins were more pronounced in the case of some peculiar protein species (polypeptides of molecular mass 110 kDa; 96 kDa; 90 kDa; 86 kDa; 60 kDa and 58 kDa, named e, f, g, h, l and m, respectively, in Fig. 2).

Total sialic acid in various macrophage populations

In order to determine whether the variations observed in cell-surface sialic acid upon macrophage activation were due to a modification of the extent of sialylation or to differences in the accessibility of sialic acid residues, total sialic acid present in macrophage lysates was quantitated by an HPLC adaptation of the TBA assay [32]. The advantage of the HPLC technique is that it allows simultaneous separation of the various chromophores and their quantitation; in spectrophoto-

TABLE I

SIALIC ACID RESIDUES EXPOSED AT THE SURFACE OF MACROPHAGES ARE REDUCED UPON ACTIVATION

The results of a representative experiment are shown for borotritide labeling: $1 \cdot 10^9$ dpm of borotritide was introduced per dish. Each dish containing $3 \cdot 10^6$ macrophages (approx. 200 μg cellular protein). The means of eight independent experiments ± S.D. are shown for the macrophage activation effect.

Macrophage populations	Borohydride labeling (10^6 dpm/100 μg protein)			Macrophage activation effect
	without periodate treatment	with periodate treatment	specific labeling	
Resident	1.95	9.04	7.09	100%
Activated	1.33	5.77	4.44	63 ± 19%

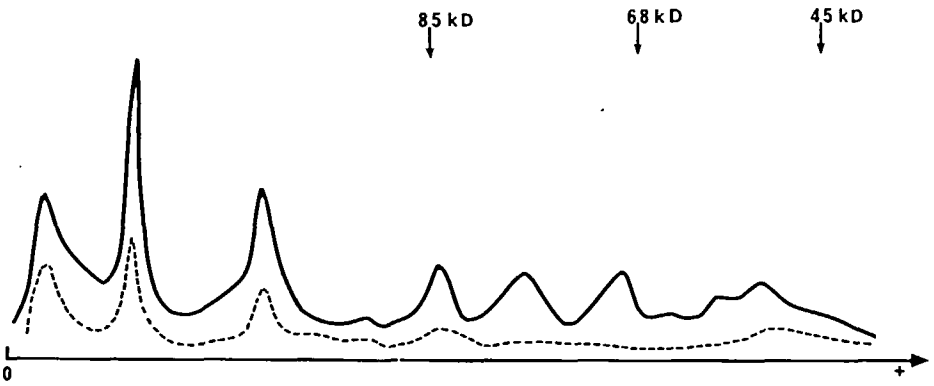

Fig. 1. Pretreatment with neuraminidase considerably reduced the labeling of macrophage glycopeptides after periodate/borohydride treatment. Resident macrophages were labeled by periodate/borohydride either directly (———) or after treatment with neuraminidase (- - - - -). Macrophages in MEM were pretreated with neuraminidase, from *Vibrio cholerae* (Sigma), added at 10 mU/ml, for 1 h at 37 °C. Polypeptides were separated on 7.5% SDS-polyacrylamide gels; densitometer scans of fluorograms are presented. kD, kilodalton.

metric or fluorometric assays conducted on cell lysates, contamination by 2-deoxyribose requires either a prior purification of sialic acids via ion exchange or measurements at several wavelengths.

As shown in Table II, TDM + LPS-activated macrophages had a lower sialic acid content than resident macrophages ($P < 0.05$). An analysis of macrophages in various functional states are pre-

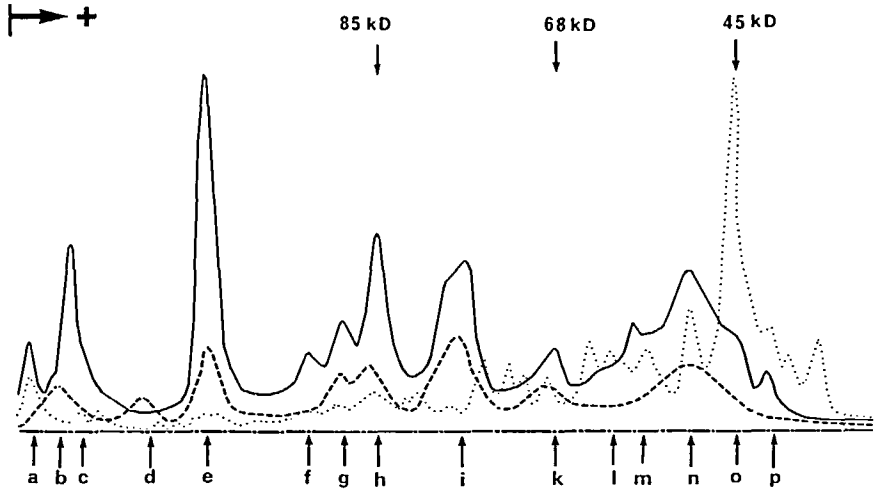

Fig. 2. Electrophoretic patterns of polypeptides labeled after periodate/borohydride treatment of trehalose dimycolate-activated or resident macrophages. After labeling by NaB^3H_4 (conducted on macrophage monolayers), cells were lysed and polypeptides were separated on 7.5% SDS-polyacrylamide gels. Fluorograms were prepared and scanned. The peak height ratios were measured for TDM + LPS-activated macrophages (- - - - -) and resident macrophages (———): after correction for the exact amount of protein measured in the lysates, the peak height ratios for TDM + LPS-activated macrophages, compared to resident macrophages, were found to be less than 0.5 for several peptides: 0.46 for peptide e (110 kDa), 0.35 for peptide g (90 kDa), 0.46 for peptide h (86 kDa) and 0.35 for peptide m (58 kDa). Some peptides of resident macrophage lysates could not be quantified in activated macrophage lysates (peptides a (more than 140 kDa), peptide f (96 kDa), peptide l (60 kDa). The labeling of a few other polypeptides was not modified (the peak height ratio was 0.83 for peptide i (76 kDa) and 0.94 for peptide k (66 kDa)). The electrophoretic pattern of polypeptides biosynthetically labeled after culture of macrophages in the presence of a mixture of ^{14}C-labeled amino acids (· · · · · ·) is given for comparison; if periodate treatment was omitted, polypeptide labeling by NaB^3H_4 (· – · – ·) was negligible.

TABLE II

TOTAL SIALIC ACID IN VARIOUS MACROPHAGE POPULATIONS AS MEASURED BY AN HPLC ADAPTATION OF THE TBA ASSAY

After adherence or after culture in vitro, macrophage monolayers were lysed in water and sialic acid released by hydrolysis in 0.1 M HCl for 60 min at 80 °C under N_2, was quantified by the HPLC-TBA assay described in Ref. 32. Results are from three independent experiments.

Macrophages	Treatment in vitro	Sialic acid (μg/100 μg protein)
Resident	0	1.87±0.3
TDM	0	1.24±0.42
TDM	LPS, 10 ng/ml, 4 h	1.29±0.26
Thioglycolate	0	4.42±0.31
Thioglycolate	LPS, 10 ng/ml, 4 h	4.22±1.44
Thioglycolate	LPS, 50 ng/ml, 24 h	3.35±0.92

sented for comparison: trehalose dimycolate-primed macrophages had a reduced level of sialic acid compared to resident macrophages. Inflammatory macrophages, elicited by thioglycolate medium, had an especially high sialic acid level; after 24 h in vitro, in the presence of 50 ng/ml lipopolysaccharide, under experimental conditions corresponding to a partial activation, the sialic acid content of thioglycolate macrophages was reduced. The especially high level of sialic acid in thioglycolate macrophages (2.3-fold the level found in resident macrophages) indicates that a low degree of sialylation is not a property common to all elicited macrophages. Priming in vivo by trehalose dimycolate or activation in vitro by lipopolysaccharide yields macrophages with a reduced sialic acid content.

Proportion of lactosaminoglycans in macrophage glycoproteins

Since in *N*-glycosylproteins, sialic acid residues are incorporated only in complex (or lactosaminoglycan) -type oligosaccharides, we investigated whether the proportion of high-mannose to complex-type oligosaccharides was modified upon activation. The relative importance of high-mannose type oligosaccharides in resident macrophages and activated macrophages was evaluated by analysis of N-linked glycopeptides of macrophage cellular proteins, either by chromatography

TABLE III

SENSITIVITY OF MACROPHAGE GLYCOPROTEINS TO ENDO H

Cellular glycoproteins were biosynthetically labelled with D-[2-^{3}H]mannose. Macrophage lysates (200 μg of proteins) were treated with endoglycosidase H (Endo H) and the ^{3}H-label released as oligosaccharides was evaluated after filtration of digests on a Sephadex G-50 column: the elution volume of the glycoprotein fraction was 15 ml; the elution volume of the oligosaccharides liberated by endoglycosidase H was 29 ml. Due to a higher rate of hexose uptake, biosynthetic labeling of TDM+LPS activated macrophages was always more efficient than the labeling of resident macrophages (see Materials and Methods and Ref. 23).

Macrophages	[^{3}H]Mannose recovered after endo H action (cpm)		Fraction digested by Endo H (%)	Ratio TDM/resident
	in glycoproteins	in oligosaccharides		
Expt. 1				
Res	98500	76700	44	
TDM	318000	381000	54	
				1.22
Expt. 2				
Res	15440	7620	33	
TDM	77840	75780	49	
				1.48
Expt. 3				
Res	26080	17240	40	
TDM	98280	116200	54	
				1.35
Mean				1.35±0.13

on Con A-Sepharose or by treatment with endo-β-*N*-acetylglucosaminidase H.

As shown in Table III, glycoproteins present in macrophage lysates were slightly more sensitive to endoglycosidase H in the case of activated macrophages: the percentage of oligosaccharides released by action of endoglycosidase H was 1.35 ± 0.13-fold higher in experiments carried out on TDM + LPS-activated macrophages compared to those on resident macrophages ($P < 0.05$); these oligosaccharides must have a high mannose content.

Several studies [34,38,39] have documented the fact that glycopeptides with tri- and tetraantennary oligosaccharides elute in the void volume fractions when chromatographed on immobilized concanavalin A, while those with biantennary oligosaccharides are eluted with 10 mM α-methylglucoside; hybrid-type and high-mannose oligosaccharides containing glycopeptides are eluted with α-methylmannoside, (or in greater than 1 bed volume of methylglucoside).

The validity of this elution scheme was confirmed under our conditions (1) by the chromatography of doubly labeled glycopeptides obtained from macrophages grown in the presence of both D-[2-^{3}H]mannose and [^{14}C]glucosamine and (2) by treatment of fractions eluted in each peak by mannosidase and isolation of liberated mannose by paper chromatography: both tests indicated that unbound glycopeptides contained complex-type oligosaccharides with a high glucosamine/mannose ratio and that material eluted with methylglucoside was heterogenous and, besides complex-type structures, must contain hybrid or oligomannosidic glycans (data not shown). Fig. 3 presents the distribution of glycopeptides obtained after pronase treatment of resident macrophage and TDM + LPS-activated macrophage lysates (an experiment representative of four). The radioactivity not bound by Con A-Sepharose was 1.23 ± 0.03 fold higher in resident macrophages ($P < 0.05$); as fractions passing through the column contained glycans insensitive to α-mannosidase and presenting a ratio [^{14}C]glucosamine/[^{3}H]mannose, in the case of double-label experiments, of 2.9, we concluded that tetra- and triantennary oligosaccharides were more abundant in resident macrophages than in activated macrophages. In contrast, fractions eluted with 10 mM α-methylglucoside corresponded, in the case of activated macrophages digests, to a large asymmetric peak (in which 26% of the radioactivity was eluted, compared to 17% for resident macrophage lysates). Susceptibility to endoglycosidase H and binding to concanavalin A indicated that the relative importance of high-mannose or hybrid-type structures increased in macrophage glycoproteins upon activation: however, since D-[2-^{3}H]mannose was used to detect the glycopeptides, we estimated that this increase might be less than 20%.

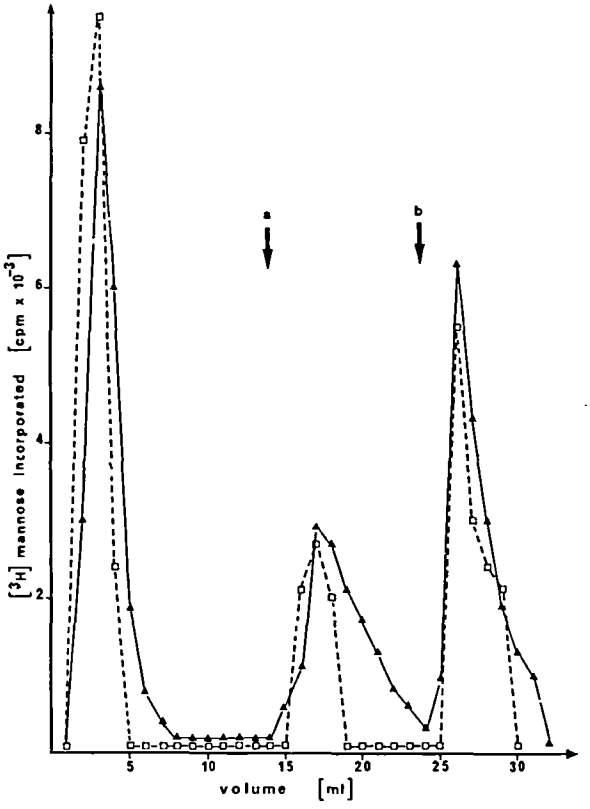

Fig. 3. Separation of macrophage glycopeptides on Con A-Sepharose. Resident-macrophages, □; TDM + LPS-activated macrophages, ▲. Macrophages ($8 \cdot 10^6$), biosynthetically labeled with D-[2-^{3}H]mannose, were lysed and treated with pronase. Glycopeptides were chromatographed on Con A-Sepharose (1.5 ml columns) in TBS (TBS, 10 mM Tris-HCl buffer (pH 8)/0.15 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$); after elution of unbound material, columns were washed sequentially with 10 ml of 10 mM α-methyl-D-glucoside (a) and 10 ml of 0.5 M α-methyl-D-mannoside in TBS (b).

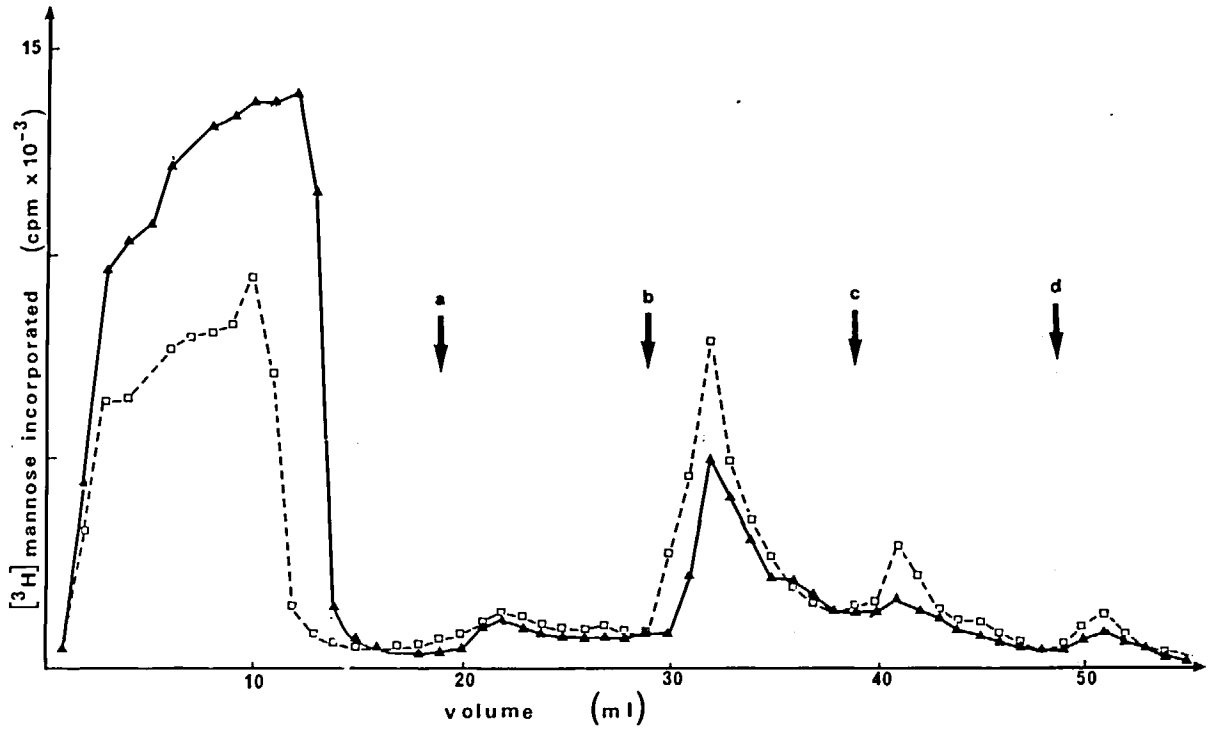

Fig. 4. Separation of macrophage glycopeptides on QAE-Sephadex. Resident macrophages, □; TDM + LPS-activated macrophages, ▲. Glycopeptides were prepared, as described in Fig. 3, from $4 \cdot 10^6$ macrophages and chromatographed on QAE-Sephadex (1 ml columns) equilibrated in 2 mM Tris base. Anionic glycopeptides were progressively eluted by increasing concentrations of NaCl added in 2 mM Tris base: (a) 20 mM, (b) 70 mM, (c) 100 mM, (d) 140 mM.

Analysis of anionic oligopeptides on QAE-Sephadex

The number of negative charges of oligosaccharides or glycopeptides can be determined by chromatography on QAE-Sephadex and stepwise salt elution [33,35]. Fig. 4 presents elution patterns observed for glycopeptides prepared from resident macrophages and TDM + LPS-activated macrophages (a representative experiment). We checked that after mild acidic treatment to eliminate sialic acid residues, the radioactivity bound ot the column was negligible. In Table IV, the data ob-

TABLE IV

SEPARATION OF ANIONIC GLYCOPEPTIDES ON QAE-SEPHADEX: COMPARISON OF VARIOUS MACROPHAGE POPULATIONS

Chromatography on QAE-Sephadex of D-[2-^{3}H]mannose-labeled glycopeptides was conducted as proposed in Refs. 33 and 35. Anionic peptides are bound to QAE-Sephadex and progressively elute with increasing concentrations of NaCl according to their number of negative charges.

Macrophages	Treatment in vitro	Radioactivity eluted (% of bound radioactivity)			
		20 mM NaCl	70 mM NaCl	100 mM NaCl	140 mM NaCl
Resident	–	13.2	57.2	21.3	8.3
TDM	–	32	47.2	14	6.8
TDM	LPS	17	54.8	19.8	8.4
Thioglycolate	–	17.3	53.1	27.3	2.3
Thioglycolate	LPS	13.8	52.7	19	14.5

tained for glycopeptides prepared from macrophages in various functional states are summarized; in all cases, the most abundant species corresponded to glycopeptides devoid of a negative charge and glycopeptides eluted by 70 mM NaCl (probably containing two negative charges). Anionic peptides represented 44% of the radioactivity in resident macrophage lysates compared to 25% in TDM + LPS-activated macrophages; treatment of thioglycolate macrophages by lipopolysaccharide also induced a reduction of the importance of anionic glycopeptides (from 32% to 16%); however, variations among macrophage populations may have several causes (variations in the specific radioactivity of incorporated mannose, modification in high-mannose-type glycan level and in non-anionic glycopeptide content), thus, unbound radioactivity was not further analyzed and we compared the relative importance of the radioactivity eluted at each NaCl concentration. As shown in Table IV, the percentage of radioactivity eluted at each step (compared to bound radioactivity) was constant in all the macrophage populations tested. The lower degree of sialylation observed in activated macrophages must concern, to the same extent, all the complex-type oligosaccharides and cannot be explained by the disappearance of highly sialylated glycopeptides.

Discussion

The reduction of sialylated glycans in macrophages upon activation was demonstrated by several methods. (1) Labeling of surface sialic acid residues by periodate/borohydride was reduced by 37% in activated macrophages compared to resident cells. (2) Total sialic acid, assayed by an HPLC/TBA method, was reduced by 30%. (3) We have previously shown that some glycoproteins secreted by activated macrophages exhibited, on two-dimensional gel electrophoresis, a less acidic charge than the corresponding species produced by resident macrophages [28].

The decrease in sialylated species seems to affect both glycolipids and glycoproteins. In the case of glycoproteins, of the two possible explanations – a decreased synthesis of sialylated proteins or a reduced sialylation – we favor the latter, since after biosynthetic labeling or Coomassie staining the only cellular polypeptides whose expression was reduced upon activation were a 68 kDa and a 34 kDa band (Lemaire, G. and Bouchahda, A., unpublished results); in secreted proteins reduced sialylation was clearly observed [28]. For cellular glycoproteins (this report), as for secreted glycoproteins [28], the reduction of the sialic acid content seems to occur during the priming step.

These data are in opposition to the conclusions drawn by Filho et al. [40] from cell electrophoresis experiments in which they observed that activated macrophages (from *Trypanosoma cruzi*-infected animals) had a higher negative surface charge and possessed more sialic acid residues sensitive to neuraminidase from *Clostridium perfringens*. However, they are in agreement with observations made by several other groups. Haimovitz et al. [41] observed that maturation of macrophages was accompanied by masking of the PNA receptor by sialic acid. Maddox et al. [42] observed, after activation, a slight decrease in the binding of the lectin from *Limulus polyphemus* (specific for sialic acid residues) and the appearance of the capacity to bind *Griffonia simplicifolia* IB_4 isolectin (specific for α-D-galactose). These observations may be related to those made by Mercurio and Robbins [43] that high-molecular-weight protein bound lactosaminoglycans terminated in a Gal-α-Gal linkage are found specifically in BCG-activated and thioglycolate-elicited macrophages; however, the methodology they used did not allow the determination of the degree of sialylation of these glycans.

Ganglioside changes have also been demonstrated upon activation. Yohe and Ryan [44] found an increase in sialic acid content of gangliosides obtained from lipopolysaccharide-induced macrophages, but there was no indication that these macrophages were activated to tumor cytotoxicity. In contrast, Wiltrout et al. [45] observed that activation of macrophages result in an increased expression of the As-GM_1 ganglioside. Such conflicting results may originate from the use of different macrophage populations and of different criteria to establish the macrophage activation state.

However, the hypothesis that interactions between macrophages and other cells may be regulated by the presence of sialic acids residues,

masking specific recognition sites [46], is attractive. From our data it can be speculated that, after activation, macrophages presented more mannose (since high-mannose-type glycans are increased) and galactose (since sialylation is decreased) external residues; this may modify their capacity to interact with parasites or tumor cells.

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References

1 Piessens, W.F. (1978) Cell. Immunol. 35, 303–317.
2 Marino, P.A. and Adams, D.O. (1980) Cell. Immunol. 54, 26–35.
3 Adams, D.O. and Marino, P.A. (1981) J. Immunol. 126, 981–987.
4 Hamilton, T.A. and Fishman, M. (1982) Cell. Immunol. 72, 332–339.
5 Marino, P.A. Whisnant, C.C. and Adams, D.O. (1981) J. Exp. Med. 154, 77–87.
6 Johnson, W.J., Whisnant, C.C. and Adams, D.O. (1981) J. Immunol. 127, 1787–1792.
7 Schlager, S.I., Meltzer, M.S. and Madden, L.D. (1983) Lipids 18, 483–488.
8 Stallcup, K.C., Liu, Y.N., Dorf, M.E. and Mescher, M.F. (1986) J. Immunol. 136, 2723–2728.
9 Meltzer, M.S. (1981) J. Immunol. 127, 179–183.
10 Johnson, W.J., Marino, P.A., Schreiber, R.D. and Adams, D.O. (1983) J. Immunol. 131, 1038–1043.
11 Torres, B.A. and Johnson, H.M. (1985) Biochem. Biophys. Res. Commun. 131, 395–400.
12 Krammer, P.H., Kubelka, C.F., Flak, W. and Ruppel, A. (1985) J. Immunol. 135, 3258–3263.
13 Masters, V.M., Webster, J. and Cook, G.M.W. (1980) Biochem. Pharmacol. 20, 3189–3201.
14 Harding, S.E. and Halliday, J. (1980) Nature 286, 819–821.
15 Glasgow, L.R. and Hill, R.L. (1980) Infect. Immun. 30, 353–361.
16 Schauer, R. (1982) Adv. Carbohydr. Chem. Biochem. 40, 131–234.
17 Yogeeswaran, G. and Salk, P.L. (1981) Science 212, 1514–1516.
18 Berthier-Vergnes, O., Portoukalian, J. and Doré, J.F. (1985) J. Natl. Cancer Inst. 75, 605–611.
19 Van Beek, W.P., Smets, L.A. and Emmelot, P. (1973) Cancer Res. 33, 2913–2922.
20 Lemaire, G., Tenu, J.P., Petit, J.F. and Lederer, E. (1986) Med. Res. Rev. 6, 243–274.
21 Adams, D.O., Johnson, W.J., Marino, P.A. and Dean, J.H. (1983) Cancer Res. 43, 3633–3637.
22 Weinberg, J.B., Chapman, H.A. and Hibbs, J.B. (1978) J. Immunol. 121, 72–80.
23 Grand-Perret, T., Lepoivre, M., Petit, J.F. and Lemaire, G. (1986) Eur. J. Immunol. 16, 332–338.
24 Steeg, P.S., Moore, R.N., Johnson, H.M. and Oppenheim, J.J. (1982) J. Exp. Med. 156, 1780–1793.
25 Strassmann, G., Springer, T.A. and Adams, D.O. (1985) J. Immunol. 135, 147–151.
26 Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1987) Science 235, 473–476.
27 Stuehr, D.J. and Marletta, M.A. (1987) J. Immunol. 139, 518–525.
28 Grand-Perret, T., Petit, J.F. and Lemaire, G. (1986) J. Leukocyte Biol. 40, 1–19.
29 Kato, M. (1970) Arch. Biochem. Biophys. 140, 379–390.
30 Van Lenten, L. and Ashwell, G. (1971) J. Biol. Chem. 246, 1889–1894.
31 Flowers, H. and Glick, M.C. (1980) Cancer Res. 40, 1550–1557.
32 Powell, L.D. and Hart, G.W. (1986) Anal. Biochem. 157, 179–185.
33 Varki, A. and Kornfeld, S. (1983) J. Biol. Chem. 258, 2808–2818.
34 Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11235–11240.
35 Pierce, M. and Arango, J. (1986) J. Biol. Chem. 261, 10772–10777.
36 Laemmli, U.K. (1970) Nature, 227, 680–685.
37 Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
38 Cowan, E.P., Cummings, R.D., Schwartz, B.D. and Cullen, S.E. (1982) J. Biol. Chem. 257, 11241–11248.
39 Quill, H. and Schwartz, B.D. (1984) J. Immunol. 132, 289–298.
40 Silva Filho, F.C., Santos, A.B.S., de Carvalho, T.M.O. and De Souza, W. (1987) J. Leukocyte Biol. 41, 143–149.
41 Haimovitz, A., Fuks, Z., Galili, N. and Treves, A.J. (1982) J. Reticuloendothelial Soc. 31, 187–192.
42 Maddox, D.E., Shibata, S. and Goldstein, I.J. (1982) Proc. Natl. Acad. Sci. USA 79, 166–170.
43 Mercurio, A.M. and Robbins, P.W. (1985) J. Immunol. 135, 1305–1312.
44 Yohe, H.C. and Ryan, J.L. (1986) J. Immunol. 137, 3921–3927.
45 Wiltrout, R.H., Santoni, A., Peterson, E.S., Knott, D.C., Overton, W.R., Heberman, R.B. and Holden, H.T. (1985) J. Leukocyte Biol. 37, 597–614.
46 Schauer, R. (1985) Trends Biochem. Sci. 10, 357–360.