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Ganglioside receptor of rat macrophages. Modulation by enzyme treatment and evidence for its protein nature

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Summary. Previous experiments have shown that rat macrophages (Mϕ) bind specifically sheep erythrocytes (E) coated with various gangliosides (EG). To study the nature of this receptor-like structure, Mϕ were treated with proteinases, and their capacity to bind EG and/or E was analyzed in a rosette assay. Within 10 min of incubation with appropriate doses of enzymes, a clear enhancement of EG-binding activity was observed. In addition, enzyme-treated Mϕ bound uncoated E. Inhibition studies with gangliosides and carbohydrates, and enzyme treatment of E showed that this binding is mediated by the same Mϕ ganglioside receptor. The kinetics of the modulation of binding activity of Mϕ during trypsin treatment were similar for both E and EG. At optimal enzyme concentration a triphasic effect was noted. Enhancement of EG-binding and appearance of E-binding activity after 10–20 min was followed by a reduction of rosette-forming cells (RFC) with a minimum at about 1 hr and then by an increase of both E-RFC or EG-RFC up to 5 hr. Simultaneous incubation of Mϕ with trypsin and cycloheximide abrogated the second rise of binding activity and abolished binding on prolonged incubation. When these cells were washed and further incubated in fresh medium, they regained their initial E- and EG-binding capacity after 4–5 hr incubation. Taken together, these results are consistent with the idea that rat Mϕ bear a ganglioside receptor-like structure which seems to be a membrane protein and which is modulated by enzyme treatment.

Abbreviations: AMϕ, alveolar macrophages; BBG, bovine brain gangliosides; E, sheep erythrocytes; EG, E coated with gangliosides; ELP, elastase-like protease; FCS, foetal calf serum; GalNAc, *N*-acetyl-galactosamine; Mϕ, macrophages; NANA, *N*-acetyl-neuraminic acid; PMϕ, peritoneal macrophages; RFC, rosette-forming cells; SL, sialyllactose.

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INTRODUCTION

Several lectin-like receptors, i.e. sugar-specific proteins in the cell membrane, have been described on macrophages (Mϕ). A D-mannose/*N*-acetyl-D-glucosamine-specific receptor was shown to be responsible for pinocytic uptake of glycoproteins (Stahl *et al.*, 1978) and for the binding and phagocytosis of certain yeasts (Warr, 1980). Desialylated rat erythrocytes are rapidly cleared from the circulation by a D-galactose specific receptor on Kupffer-cells (Kolb & Kolb-Bachofen, 1978; Kolb, Friedrich & Süss, 1981) and a similar receptor has been demonstrated on peritoneal macrophages (Nagamura & Kolb, 1980). When sialic acid of sheep erythrocytes (E) is removed or degraded to heptulosonic acid derivatives, the E are ingested by human monocytes, probably through a lectin-like receptor (Czop, Fearon & Austen, 1978). The adherence and ingestion of various bacteria by Mϕ is also mediated by lectin-like receptors (Ögmundsdóttir &

Weir, 1976), their specificity, however, seems less well defined (Weir & Ögmundsdóttir, 1980). In addition to bacteria, tumour cells may also be bound to Mϕ via this receptor (Weir & Ögmundsdóttir, 1980).

Recently, we have shown that rat alveolar (AMϕ) and peritoneal (PMϕ) macrophages bind E treated with gangliosides (Riedl *et al.*, 1982). This binding was inhibited by gangliosides, sialyllactose or neuraminic acid, suggesting that a receptor-like structure in the Mϕ membrane recognizing the carbohydrate part of the ganglioside is responsible for this binding. To learn more about the nature of this receptor, rat Mϕ were incubated with various proteolytic enzymes and their capacity to bind ganglioside-treated E (EG) or untreated E was studied. Surprisingly, enzyme-treated Mϕ were found to bind untreated E. It will be shown that this binding is also mediated by an interaction of a Mϕ receptor of the same specificity as that which reacts with gangliosides. Therefore, results obtained in E-binding as well as in EG-binding studies could be interpreted as evidence for a protein nature of the ganglioside receptor which is easily modulated by enzyme treatment and rapidly resynthesized after tryptic digestion.

MATERIALS AND METHODS

Macrophages

AMϕ were isolated from adult male rats (200–300 g) of a Sprague-Dawley derived strain (HIM:OFA(SPF), Himberg, Austria) by bronchial lavage with Hanks's balanced salt solution containing 0·25% Na_2-EDTA (Boltz-Nitulescu & Förster, 1979). PMϕ were harvested by rinsing the peritoneal cavity 4 days after proteose-peptone stimulation. The Mϕ were purified by adherence to plastic petri-dishes (Boltz-Nitulescu & Förster, 1979; Boltz-Nitulescu & Spiegelberg, 1981). The adherent cells consisted of more than 95% Mϕ as tested by morphology, phagocytosis of latex particles, and non-specific esterase staining (Boltz-Nitulescu & Förster, 1979; Boltz-Nitulescu, Bazin & Spiegelberg, 1981). The adherent cells were detached with rubber policemen, washed and resuspended in HEPES-buffered RPMI-1640 (Flow Labs, Irvine, Scotland, U.K.) to a concentration of 2×10^6/ml. The viability of the cells was higher than 90% as determined by trypan blue dye exclusion.

Erythrocytes

Erythrocytes were collected under sterile conditions in Alsever's solution and stored for 1 day to 2 weeks at 4°. Before use, E were washed three or four times in phosphate-buffered saline and resuspended to 1·0% in the same medium as the Mϕ.

Enzymes and inhibitors

The following preparations were used: bovine pancreatic trypsin, TPCK-treated, lyophilized, 3·5 U/mg; bovine pancreatic α-chymotrypsin, TLCK-treated, lyophilized, 45 mU/mg; pronase E from *Streptomyces griseus*, lyophilized, 70,000 PUK/g: all three enzymes were obtained from Merck, Darmstadt, F.R.G.; elastase-like protease (ELP) from polymorphonuclear leucocytes was kindly donated by Dr K. Havemann, Marburg/Lahn, F.R.G.; *Vibrio cholerae* neuraminidase was purchased from Behringwerke, Marburg, F.R.G.; cycloheximide from Sigma Chemical Co., St. Louis, MO, U.S.A.

Gangliosides

Monosialogangliosides (G_{M1}, G_{M2}, G_{M3}), disialogangliosides (G_{D1a}, G_{D1b}) and trisialoganglioside (G_{T1b}) were purchased from Seromed, Munich, FRG. Mixed bovine brain gangliosides (BBG), containing mainly G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, were purchased from Sigma Chemical Co., and Supelco Inc., Bellefonte, PA, U.S.A.

Other reagents

Sialyllactose (SL), *N*-acetyl-neuraminic acid (NANA), *N*-acetyl-galactosamine (GalNAc), *N*-acetyl-glucosamine (GlcNAc), lactose, L-fucose, D-mannose, α-methyl-glucosamine, α-methyl-galactosamine, and fibronectin were obtained from Sigma Chemical Co. Rabbit IgG (Cohn fraction II, Pentex Inc., Kankakee, IL) was aggregated by heating for 18 min at 63°.

Enzyme treatment of macrophages and E

Suspensions of AMϕ and PMϕ (2×10^6 cells/ml) and of 1% E were made in HEPES-buffered RPMI-1640 medium without FCS and treated with enzymes as previously described (Boltz-Nitulescu & Förster, 1982). At the end of the incubation period, the reaction was terminated by addition of 15–20 volumes of ice-cold RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS, Seromed). The cells were washed three times with cold medium, resuspended to 2×10^6 cells/ml and used for the rosetting reaction.

Digestion and resynthesis of ganglioside receptors

Macrophages were incubated simultaneously with 1·0 mg/ml trypsin and 100 μg/ml cycloheximide as previously described (Förster & Boltz-Nitulescu, 1982). To study receptor resynthesis, the enzyme and inhibitor were removed after 2 hr incubation and the Mϕ were further incubated at 37° in fresh medium RPMI-1640 containing 10% FCS. At different times of incubation Mϕ were analysed for their capacity to bind E or EG.

Rosette assays

Gangliosides were attached to E as previously described (Riedl *et al.*, 1982). Briefly, 1% E were coated with gangliosides (sonicated before use for 5 min on a 50 W MSE ultrasonic power unit) by incubation for 1 hr at 37°. The rosette assay and rosette inhibition procedure have been described elsewhere (Riedl *et al.*, 1982).

RESULTS

Binding of ganglioside-coated erythrocytes to trypsinized macrophages

To study the proteinase sensitivity of the putative ganglioside receptor, rat AMϕ were incubated with 100 μg/ml trypsin in presence or absence of 100 μg/ml cycloheximide. In a representative experiment, around 40% of AMϕ bind E coated with 100 μg/ml BBG (E-BBG) before enzyme treatment. However, the percentage of RFC dropped to about 25% after 20–30 min preincubation of cells at 37° in RPMI-1640 medium lacking FCS. After this preincubation period, trypsin and/or cycloheximide, alone or in combination, were added to the cell suspension. Incubation of AMϕ with trypsin alone up to 5 hr showed a triphasic effect (Fig. 1). An increase of the percentage of RFC after 10–20 min was followed by a decrease at 30–60 min and a second increase up to 5 hr. Simultaneous incubation of AMϕ with trypsin and cycloheximide for 10–20 min showed the same initial increase of rosette formation as trypsin alone. However, during prolonged incubation of cells with enzyme and cycloheximide, this binding activity was further decreased and disappeared after 4–5 hr incubation. Cycloheximide *per se* did not affect the capacity of non-enzyme-treated (native) AMϕ to bind E-BBG.

This pattern was typical for all such experiments, although the level of the initial binding (40–55% RFC) and the drop of the percentage of RFC in FCS-free

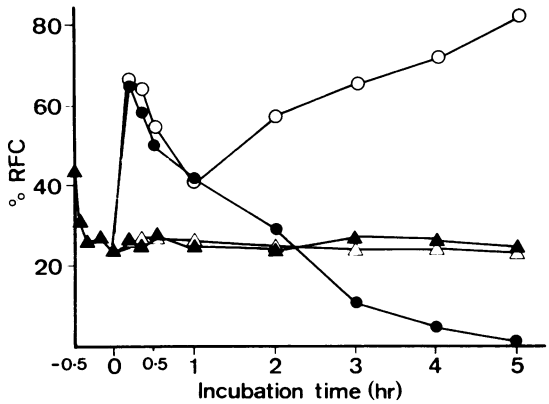

Figure 1. Effect of trypsin and cycloheximide treatment on the ability of rat alveolar macrophages to form E-BBG rosettes. Mϕ were preincubated at 37° in serum-free medium for 30 min prior addition of enzyme and/or inhibitor. Used concentrations: (○——○) 100 μg/ml trypsin; (●——●) 100 μg/ml trypsin and 100 μg/ml cycloheximide; (△——△) 100 μg/ml cycloheximide; (▲——▲) none.

medium was variable and seemed to depend on the activation status of the macrophages, on the batch of FCS and possibly on other undefined factors.

Since two peaks of increased E-BBG-binding to rat AMϕ were observed during trypsin treatment, one at 10 min and one at 300 min incubation, the binding of E coated with defined gangliosides was studied at these two time points. The results depicted in Table 1 show that native as well as trypsinized AMϕ bind E coated with G_{M2}, G_{D1a}, G_{D1b} and G_{T1b}. The extent of binding, however, was increased by pretreatment of AMϕ with

Table 1. Percentage of rat AMϕ forming rosettes

	AMϕ treated with 100 μg/ml trypsin for		
Indicator cells*	0 min	10 min	300 min
E-G_{M1} 100	1·9±1·1†	39·8±4·9	77·4±5·5
E-G_{M2} 100	78·2±5·6	85·2±5·5	83·6±6·5
E-G_{M2} 50	65·4±6·7	79·6±4·9	86·3±3·1
E-G_{M3} 100	1·1±1·3	39·3±5·0	80·4±6·1
E-G_{D1a} 100	60·1±6·2	74·4±5·9	86·8±3·0
E-G_{D1b} 100	63·6±3·6	75·9±4·4	85·6±4·5
E-G_{T1b} 100	48·8±5·3	71·8±4·3	85·4±5·1
E	0·4±0·7	38·9±5·3	78·6±5·2

* E were coated with indicated ganglioside concentrations (μg/ml 1% E).

† Results expressed as mean % RFC±SD of three experiments.

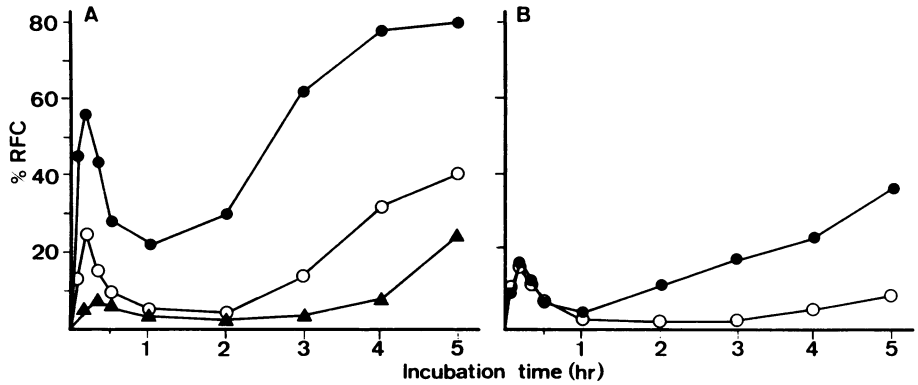

Figure 2. Binding of sheep erythrocytes to enzyme-treated rat alveolar (Fig. 2A) and peritoneal (Fig. 2B) macrophages. Enzyme concentrations: (●——●) 2·5 mg/ml trypsin; (○——○) 2·5 mg/ml α-chymotrypsin; (▲——▲) 125 μg/ml elastase-like protease.

100 μg/ml trypsin for 10 or 300 min. G_{M1}- and G_{M3}-treated E were bound by trypsinized Mϕ only to about the same extent as untreated E (see below).

Binding of E to enzyme-treated Mϕ

Native rat AMϕ did not bind uncoated E. However, when AMϕ were trypsinized for 10 or 300 min, 38·9% or 78·6%, respectively, were found to bind E (Table 1). This finding was unexpected, and therefore further studies were devoted to the elucidation of this binding process. At first, kinetic studies were performed with rat AMϕ and PMϕ treated with trypsin, α-chymotrypsin and ELP. In general, similar to EG-binding of trypsin-treated AMϕ, a triphasic change of E-binding activity was observed (Fig. 2). The extent of E-binding was dependent on the type of enzyme and Mϕ source. With all enzymes studied the percentage of PMϕ binding E was lower than that of AMϕ.

In further experiments the influence of various trypsin or pronase concentrations on E-binding capacity of Mϕ was studied. As shown in Fig. 3 the effect is dose-dependent.

When AMϕ were incubated simultaneously with 100 μg/ml trypsin and 100 μg/ml cycloheximide the initial appearance of E-binding activity was not influenced. However, during prolonged incubation of AMϕ with enzyme and inhibitor their E-binding capacity decreased slowly and disappeared after 4–5 hr incubation in a fashion similar to the reduction of EG-binding shown in Fig. 1. Cycloheximide *per se* did not induce any E-binding activity to native AMϕ.

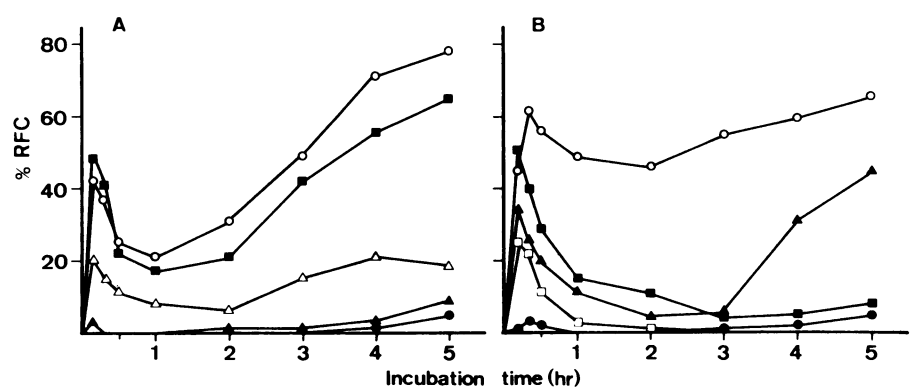

Figure 3. Effect of various concentrations of trypsin (Fig. 3A) and pronase (Fig. 3B) on E-binding capacity of rat alveolar macrophages. Enzyme concentrations: (●——●) 1 μg/ml; (▲——▲) 10 μg/ml; (○——○) 100 μg/ml; (■——■) 1·0 mg/ml; (□——□) 5·0 mg/ml; (△——△) 10·0 mg/ml.

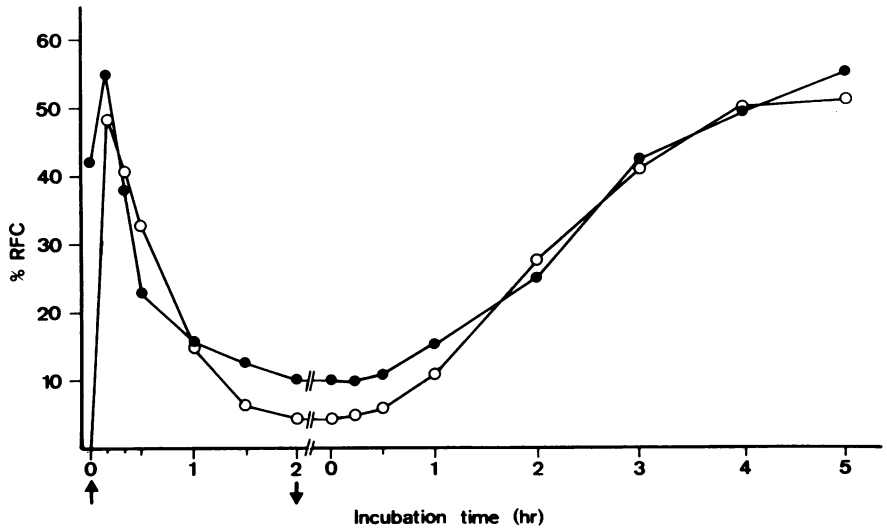

Figure 4. Digestion and resynthesis of (O——O) E- or (●——●) EG-binding activity of rat alveolar macrophages. After 2 hr incubation of AMϕ with 1·0 mg/ml trypsin and 100 μg/ml cycloheximide the cells were washed and further incubated for 5 hr in fresh medium containing 10% FCS.

Reappearance of E- and EG-binding activity after tryptic digestion

AMϕ were incubated for 2 hr at 37° with 1·0 mg/ml trypsin and 100 μg/ml cycloheximide to abolish their capacity to bind E or EG. After this treatment the cells were washed and further incubated in fresh RPMI-1640 medium supplemented with 10% FCS. As shown in Fig. 4, AMϕ regained completely their E- or EG-binding capacity within 4–5 hr of incubation. Furthermore, the structure responsible for binding of E or EG was digested and resynthesized at similar rates.

Specificity of E-binding to trypsinized AMϕ

Inhibition by gangliosides. The results depicted in Table 2 show that G_{M2}, G_{M3}, G_{D1a}, G_{D1b} and G_{T1b} inhibited the binding of E to AMϕ pretreated with 100 μg/ml trypsin for 10 or 300 min in a dose-dependent fashion. Monosialogangliosides (G_{M2} and G_{M3}) have stronger inhibitory capacity than disialogangliosides (G_{D1a} and G_{D1b}) or trisialoganglioside (G_{T1b}). Monosialoganglioside G_{M1}, which does not induce rosette formation with native AMϕ and does not inhibit the binding of E coated with other gangliosides (Riedl *et al.*, 1982; Förster, Boltz-Nitulescu, Riedl, Ortel, Fellinger, Travniczek and Bernheimer, unpublished), also failed to inhibit binding of E to trypsinized AMϕ.

Table 2. Inhibition of E-binding to trypsin-treated rat AMϕ by gangliosides

		AMϕ treated with 100 μg/ml trypsin for	
Inhibitor	(μg/ml)	10 min	300 min
G_{M1}	500	3·2±2·1 (3)*	6·9±1·9 (3)
	50	2·4±1·7 (3)	3·8±2·5 (3)
G_{M2}	500	65·5±11·5 (3)	66·1±5·9 (3)
	50	46·7±4·9 (4)	46·1±6·7 (4)
G_{M3}	500	76·5±5·8 (3)	74·6±4·0 (3)
	50	45·9±8·2 (4)	46·3±6·1 (4)
G_{D1a}	500	55·8±9·9 (6)	52·2±13·5 (5)
	50	29·1±5·3 (6)	31·3±7·5 (6)
G_{D1b}	500	51·4±13·1 (5)	59·9±14·4 (5)
	50	28·2±11·9 (4)	27·2±11·6 (5)
G_{T1b}	500	55·4±3·3 (4)	56·2±7·0 (4)
	50	23·5±7·3 (5)	25·2±9·7 (5)

* Results expressed as mean % rosette inhibition ± SD. No. of experiments in parentheses.

Although binding of G_{M3}-coated E to AMϕ does not occur, G_{M3} strongly inhibited E-binding to trypsinized AMϕ.

Inhibition by carbohydrates. Preincubation of trypsinized AMϕ (100 μg/ml for 10 or 300 min at 37°) with

Table 3. Inhibition of E-binding to trypsin-treated rat AMϕ by carbohydrates

Inhibitor		AMϕ treated with 100 μg/ml trypsin for 10 min	300 min
Sialyllactose	10^{-2} M	67·2±2·6*	72·5±2·8
	10^{-3} M	52·4±4·1	54·4±5·7
	10^{-4} M	12·6±6·8	14·7±6·9
NANA	10^{-2} M	30·7±4·2	39·7±2·1
	10^{-3} M	19·2±7·6	28·1±6·0
	10^{-4} M	3·8±3·9	6·2±5·7
GalNAc	10^{-2} M	37·8±9·4	41·8±3·1
	10^{-3} M	22·3±3·5	30·5±5·5
	10^{-4} M	6·7±2·2	5·6±4·8

* Results expressed as mean % rosette inhibition ±SD of three experiments.

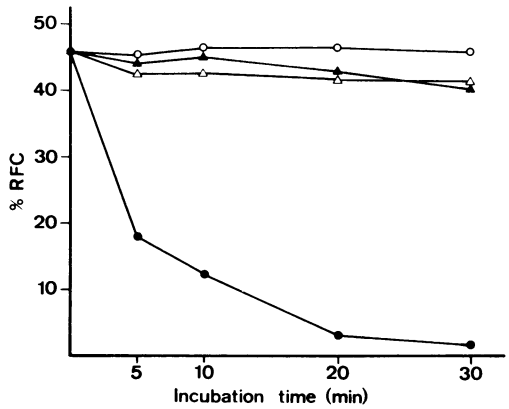

Figure 5. Binding of proteinase or neuraminidase treated sheep erythrocytes to trypsinized rat AMϕ (100 μg/ml for 10 min). Erythrocytes treatment: (●——●) 0·1 U/ml neuraminidase; (○——○) 2·5 mg/ml trypsin; (△——△) 2·5 mg/ml α-chymotrypsin; (▲——▲) 1·5 mg/ml pronase.

SL, NANA or GalNAc led to a dose-dependent inhibition of E-binding (Table 3). At similar concentrations SL has a stronger inhibitory capacity than the other two sugars. Lactose, D-mannose, L-fucose, GlcNAc, α-methyl-glucosamine, and α-methyl-galactosamine, even at 10^{-2} M concentration, failed to inhibit E-binding (data not presented).

Effect of enzyme treatment of E on their binding to trypsinized AMϕ

Vibrio cholerae neuraminidase cleaves NANA from the non-reducing terminal of the carbohydrate chains of certain gangliosides. In a previous publication it has been demonstrated that treatment of BBG or G_{D1a}-coated E with neuraminidase abolished their binding to rat AMϕ (Riedl *et al.*, 1982). Similarly, E lost their capacity to bind to trypsin-treated AMϕ after 20–30 min preincubation with *Vibrio cholerae* neuraminidase (Fig. 5). In contrast, incubation of E over 30 min with trypsin, α-chymotrypsin or pronase did not evidently affect their attachment to trypsinized AMϕ.

DISCUSSION

The aim of the present report was to study the nature of the ganglioside receptor of rat Mϕ which was described recently (Riedl *et al.*, 1982). Assuming that the putative receptor is a membrane protein, Mϕ were treated with trypsin for several hours. In the course of these studies it was found that enzyme treatment of rat Mϕ not only initially increased EG-binding, but also induced their capacity to bind native E. The attachment of these untreated E to trypsinized Mϕ was mediated by an interaction of gangliosides on the E-surface with receptor(s) on the Mϕ membrane. This conclusion can be drawn from several observations.

(i) The time course of rise and fall of E-rosette formation of Mϕ during trypsinization with or without simultaneous suppression of protein biosynthesis by cycloheximide parallels exactly that of EG-RFC. Moreover, when after tryptic digestion the cells are washed and incubated in fresh medium without trypsin and cycloheximide, E- and EG-binding activity reappear at the same rate and up to the same level within the next 4–5 hr.

(ii) E-rosette formation with trypsinized Mϕ is inhibited by preincubation with purified gangliosides in the same order as EG-rosettes (Riedl *et al.*, 1982; Förster *et al.*, unpublished).

(iii) E-rosette formation is inhibited by certain carbohydrates occurring in gangliosides. These include SL, NANA, and GalNAc, sugars which at the same concentrations inhibit EG-binding to native Mϕ in a similar fashion (Riedl *et al.*, 1982). These inhibitory effects seemed to be caused by specific binding of the sugar to the receptor(s), since other sugars of similar structure or charge were not inhibitory, and the inhibitory sugars showed this effect at rather low concentrations (10^{-3} M).

(iv) When E are preincubated with neuraminidase

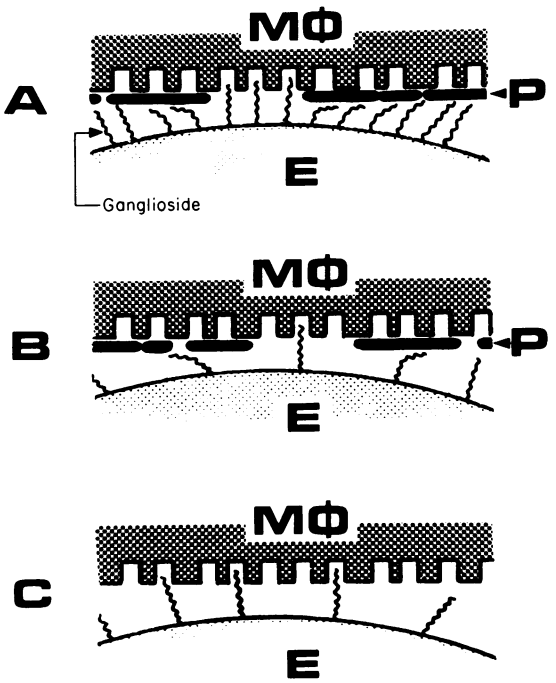

Figure 6. A possible explanation for E- and EG-binding to macrophages.

(A) Sheep erythrocytes (E) were coated with gangliosides. The density of gangliosides on E membrane is high and allows multiple binding to the free ganglioside receptors on native rat macrophages.

(B) Uncoated E have a low density of naturally occuring gangliosides on their surface. A protein (P) attached to macrophage surface inhibits multiple binding of E to macrophages.

(C) The hindering protein has been removed by proteinases. Additional ganglioside receptors are now available allowing multiple interactions with gangliosides which occur naturally on the E surface.

they lose their capacity to form rosettes with enzyme treated Mϕ, similarly to E coated with susceptible gangliosides, e.g. G_{D1a}.

Why do Mϕ not bind untreated E, if the same receptor(s) is responsible for binding of both E and EG? This may be the consequence of a low binding affinity and/or a low density of receptors on the Mϕ on one hand and of ganglioside ligands on the E on the other hand. Too low a number of binding structures on both sides may give an insufficient overall strength of binding, and therefore, E-binding cannot be visualized (Fig. 6B).

A striking feature is the triphasic course of receptor activity occuring during prolonged incubation of Mϕ with low to moderate concentrations of proteolytic enzymes. The early appearance of E-binding activity or enhancement of EG-binding may be due to the removal of an easily digestable protein from the Mϕ surface and thus, hidden binding sites may now be exposed (Fig. 6C). Alternatively, the binding affinity may be increased by this treatment. The decrease of the number of cells binding EG or E, respectively, as seen during the second phase of incubation, may be caused by digestion of the receptor structure.

Finally, an increase of E- or EG-binding was noted after 4–5 hr incubation of cells with low to moderate enzyme concentrations. This second increase which could not be seen using higher enzyme concentrations, or when protein biosynthesis was blocked by cycloheximide, may be attributed to the expression of new receptors, either from a receptor pool, or most likely after *de novo* synthesis. All these observations suggest that a protein is essential for the binding activity of the ganglioside receptor, either the receptor itself being a protein or a neighbouring protein structure of the cell membrane influencing the binding activity of the receptor.

In contrast to the mannose/*N*-acetyl-glucosamine receptor (Stahl *et al.*, 1978), the C3b receptor (Bianco, Griffin & Silverstein, 1975) or fibronectin receptor (Bevilacqua *et al.*, 1981), which are rapidly lost during trypsinization, the expression of ganglioside receptors may ultimately be enhanced by low to moderate concentrations of proteinases.

Foetal calf serum seems to contain a factor(s) which enhances ganglioside binding to Mϕ, as indicated by the reduction of RFC in FCS-free medium. Preliminary experiments show that binding may be restored by readdition of FCS but not by bovine serum albumin, bovine gamma globulin or fibronectin.

Although the biological significance of this ganglioside receptor is unknown, it has been shown that—similar to receptors of resident Mϕ for C3b (Mantovani, Rabinovitch & Nussenzweig, 1972) and fibronectin (Bevilacqua *et al.*, 1981)—it does not mediate phagocytosis (Riedl *et al.*, 1982). However, it may be envisaged that the ganglioside receptor is involved in the interaction between lymphocytes and Mϕ. Such interactions may be required for the mitogen stimulation of T cells (Rosenstreich & Oppenheim, 1976), and T cell-dependent antibody formation (Pierce & Kapp, 1976). There is some evidence *in vitro* that lymphocytes may be activated through gangliosides inserted in their membrane (Sela, 1981; Sela, Raz

& Geiger, 1978; Spiegel, Ravid & Wilchek, 1979; Spiegel & Wilchek, 1981). Binding of lymphocytes to a Mϕ ganglioside receptor may trigger their stimulation *in vivo*. During inflammation, Mϕ themselves may be a source of proteolytic enzymes (Davies & Allison, 1976), thereby leading to an autoregulation of their ganglioside receptor activity and possibly to a modulation of immune responsiveness.

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