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INTERACTIONS OF GANGLIOSIDE G_{M1} WITH HUMAN AND FETAL CALF SERA

FORMATION OF GANGLIOSIDE-SERUM ALBUMIN COMPLEXES

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The interactions of ganglioside G_{M1} with human and fetal calf sera were studied, the following main results being obtained: (a) G_{M1}, upon incubation with both sera gave origin to two G_{M1}-protein complexes, which also occurred after interaction of G_{M1} with the albumin fractions prepared from the same sera. Instead no complex formation occurrred using the albumin-free fractions. Therefore G_{M1} appeared to specifically bind serum albumin and to form G_{M1}-albumin complexes. (b) G_{M1} binding to serum albumin started at ganglioside concentrations surely micellar (above 10^{-6} M), was time and concentration dependent, and resulted in a relevant degree of G_{M1} complexation (up to 80% of total G_{M1} in human serum and up to 18% in fetal calf serum). (c) the binding kinetics appeared, in both serum and the correspondent albumin fraction, to be biphasic: in the first phase, occurring till about $2 \cdot 10^{-4}$ M G_{M1}, the ratio between bound and total G_{M1} increased linearly with increasing G_{M1} concentration; in the second phase, occurring above $2 \cdot 10^{-4}$ M, the ratio remained practically constant. After these findings it should be expected that G_{M1}, when present in serum containing systems, forms complexes with albumin. This should be appropriately considered when studying the effects of exogeneous G_{M1} in in vivo and in vitro (tissue cultures) systems.

Introduction

Gangliosides, sialic acid-containing glycosphingolipids of differing chemical structure [1], are normal components of mammalian cell plasma membranes and are particularly abundant in neuron plasma membranes [2]. Asymmetrically located in the outer membrane surface, gangliosides are assumed to play a role in a variety of cell surface events, such as receptor and recognition phenomena [3–6], ion binding and release [7], and biotransduction of membrane-mediated information [8].

The addition of gangliosides to in vitro and in vivo systems, followed by recording induced modifications of membrane-linked functional parameters, is a commonly used approach for exploring the biological role of gangliosides. Exogenous gangliosides were found to affect development of the neuromuscular junction in mixed cultures of skeletal muscle and spinal cord [9], and to markedly stimulate sprouting of cultured neuroblastoma cells and primary cultures of sensory ganglia [10]. In vivo treatment with ganglioside induced an increased rate and degree of functional recovery during nerve regeneration [11], and enhancement of motor neuron sprouting during reinnervation

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[12]. Exogenous gangliosides injected in mouse and rat were shown to reach peripheral organs and tissues, brain included, and to undergo locally metabolic processing [13–15].

In in vitro and in vivo experiments, exogenous gangliosides are in contact with serum (fetal calf serum is a basic component of tissue culture media) and are expected to form complexes with serum proteins. In fact G_{M1} ganglioside is known to produce complexes with pure bovine serum albumin [16]. On the other hand, it is becoming clear [17] that the reproducibility and quality of the above-mentioned effects are dependent more on the amount of exogenous ganglioside which remains associated to the membrane, than to that present in the medium. In this respect the physico-chemical form in which ganglioside is present in the medium (monomer, micelle, protein complex) may be extremely important.

Aiming at clarifying this matter, we studied the ability of gangliosides to bind serum proteins, and the specificity and kinetics of the binding. Human serum, fetal calf serum and ganglioside G_{M1}, which is commonly used in vitro and in vivo experiments, were employed.

Materials and Methods

Commercial chemicals were of analytical grade or of the highest purity available. Solvents were distilled before use. The water routinely used was freshly distilled using glass apparatus. Bovine serum albumin and *N*-acetylneuraminic acid were obtained from Sigma (St. Louis, MO, U.S.A.); γ-globulins (bovine immunoglobulins, dried; human immunoglobulins, lyophilized) from Behringwerke (Marburg, F.R.G.); galactose oxidase (EC 1.1.3.9) (from *Polyporus circinatus*, 98 I.U./mg protein) from KABI (Stockholm, Sweden); sodium boro[^{3}H]hydride (7.5 Ci/mM) from the Radiochemical Centre (Amersham, U.K.); Ultrogel AcA 34 from L.K.B. (Bromma, Sweden); Affi-Gel Blue (50–100 mesh) from BioRad Laboratories (Richmond, CA, U.S.A.); materials for cellulose acetate electrophoresis from Helena Laboratories (Beaumont, TX, U.S.A.); the preblended liquid scintillation solution, Instagel, from Packard Instruments (Downer Grove, IL, U.S.A.); fetal calf serum from Miles Laboratories (Stoke Poges, Slough, U.K.); dialysis tubing (1/4 inch width) from A. Thomas (Philadelphia, PA, U.S.A.); the filtration apparatus from Sartorius Membranfilter (Model M. 2634) (Göttingen, F.R.G.).

Preparation and labelling of ganglioside G_{M1}

A sample (500 mg) of mixed calf brain gangliosides, isolated according to Tettamanti et al. [18], was exhaustively digested with *Vibrio cholerae* sialidase by the method of Ghidoni et al. [19], in order to transform polysialogangliosides into the monosialoganglioside G_{M1}*. At the end of the digestion, ganglioside G_{M1} was isolated, purified and assayed as described by Sonnino et al. [20]. Chromatographic analysis indicated that the purity of G_{M1} was over 99%. The content of sphingosine bases, determined according to the method of Carter and Gaver [21], was found to be: $C_{18:0}$, 8.1%; $C_{18:1}$, 32.4%; $C_{20:0}$, 5.4%; $C_{20:1}$, 54.1%. The fatty acid moiety was predominantly (98%) octadecanoic acid.

A micellar size of 532000 ± 30000 was found for this preparation of ganglioside G_{M1} [22], as expected on the basis of its sphingosine-base and fatty acid composition, and approx. 350 monomer molecules were present in each micelle.

Tritiated G_{M1} was obtained by oxidation of the terminal galactose by galactose-oxidase followed by reduction with sodium boro[^{3}H]hydride using the method of Suzuki and Suzuki [23] as modified by Ghidoni et al. [24]. Because of the use of sodium boro[^{3}H]hydride of very high specific radioactivity (7.5 Ci/mmol) the procedure employed gave ganglioside G_{M1} with a specific radioactivity of 1.5 Ci/mmol. The radiochemical purity of ^{3}H-labelled ganglioside G_{M1} was over 99%. ^{3}H-labelled ganglioside G_{M1} was stored at 4°C in *n*-propanol/water (2:1, by vol.) and was found to be stable for at least six months.

Electrophoresis

Serum proteins and serum protein fractions were analyzed by cellulose acetate electrophoresis (in 0.05 M Tris-barbiturate buffer, pH 8.6) using a

* This paper follows the ganglioside nomenclature of Svennerholm [28] and the IUPAC-IUB Recommendations (Lipids (1976) 12, 455–465); G_{M1}, II^{3}NeuAc-GgOse$_4$Cer.

Helena (Beaumont, TX, U.S.A.) electrophoresis apparatus.

Preparation of the 'albumin fraction' and the 'albumin-free fraction' (all operations were done at 4°C)

Separation and purification of albumin from samples (1–3 ml) of fetal calf and human sera were accomplished by Affi-Gel blue chromatography according to Travis and Pannell [25]. A completely pure albumin fraction was obtained after 5–6 chromatographic cycles, purity being assessed by cellulose acetate gel electrophoresis. The final pure albumin fraction was concentrated without stirring on a Sartorius Membranfilter apparatus at a protein concentration of 15–20 mg/ml and dialyzed overnight against 500 vol. of 50 mM sodium acetate buffer, pH 7.0. All eluates and washings not containing albumin were pooled, concentrated, dialyzed as above, and constituted the 'albumin-free fraction'. This fraction contained only a portion of the γ-globulin present in the starting serum, the remainder being retained by the Affi-Gel blue column. Fresh preparations of albumin, and albumin-free fractions were employed for G_{M1} binding experiments.

Binding of ganglioside G_{M1} to serum proteins

To 8–100 mg of (cold) ganglioside G_{M1}, dissolved in water, 1.5–1.8 μCi of ^{3}H-labelled ganglioside G_{M1} was added and the mixture lyophilized. The residue was dissolved in 2–20 ml of chloroform/methanol (2:1, by vol.). The solution was evaporated to dryness. The residue was taken up with 1.8 ml of 50 mM sodium acetate buffer, pH 7.0, and allowed to stand overnight at 20°C. A 0.2 ml sample of serum (or of a serum fraction), previously dialyzed (overnight, at 0–4°C) against 500 ml of 50 mM sodium acetate buffer, pH 7.0, was added to the ganglioside solution and the mixture incubated at 37°C for the desired time.

Separation and isolation of G_{M1}-serum protein complexes

The separation of G_{M1}-serum protein complexes from G_{M1} micelles and serum proteins was accomplished by the method of Tomasi et al. [16] with some modifications. Briefly, the incubation mixture (2 ml) was submitted to gel filtration on a Ultrogel AcA 34 column (2.6 × 100 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 7.0, and maintained at a constant temperature of 37°C. The contained molecules were eluted with the same buffer (flow rate: 1.5 ml/min), 3-ml fractions being automatically collected. All fractions were analyzed for ganglioside, as bound *N*-acetylneuraminic acid and/or radioactivity, and for protein by continuous absorbance recording at 278 nm. The fractions containing both protein and ganglioside were pooled and concentrated (at 5–10 mg protein/ml) by filtration on a Sartorius Membranfilter apparatus.

Kinetics of ganglioside G_{M1} binding to serum proteins

Incubation mixtures were prepared containing 8–10 mg (as albumin) of total serum, or 'albumin fraction' (from either human or fetal calf origin) and various amounts of ganglioside G_{M1} (from 1 to 10 mg/mg albumin, carrying 1.5 μCi of ^{3}H label).

Each mixture, after incubation for a given time (varying from 15 min to 20 h) was submitted to gel filtration on Ultrogel AcA 34 column and formed G_{M1}-protein complexes were isolated. The ganglioside content of the complexes was measured.

Interactions of ganglioside G_{M1} with purified bovine and human γ-globulins

A mixture containing, in a final volume of 2 ml, 50 mM sodium acetate buffer, pH 7.0, 1–1.5 mg of γ-globulins (of bovine or human origin) and 0.5–10 mg of ganglioside G_{M1} (containing 1.8 μCi of ^{3}H-G_{M1}) was incubated at 37°C for up to 20 h. At the end of the incubation, the mixture was poured on the top of an Ultrogel AcA 34 column (1.6 × 100 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 7.0, elution being carried out with the same buffer. Protein and ganglioside contents in the eluate were determined as specified above.

Other methods

N-Acetylneuraminic acid was determined by the method of Svennerholm [26]. Protein was determined according to Lowry et al. [27], bovine serum albumin being used as the reference standard. Since ganglioside G_{M1} caused interference

with color development, probably preventing the dye from binding to the protein, standard curves were made with pure albumin and appropriate amounts of ganglioside. Serum content of albumin was established by densitometric scanning of cellulose acetate electrophoretic strip, after determination of total proteins.

Results

Binding of G_{MI} ganglioside to serum proteins and separation of G_{MI}-serum protein complexes

The elution profiles from Ultrogel AcA 34 columns of total serum, 'albumin-free fraction', and 'albumin fraction', of both human and fetal calf origin, are reproduced in Fig. 1. Human and fetal calf profiles were almost identical. The profiles of total serum were characterized by the presence of three main and separate peaks (see Fig. 1A): the first, fast moving, containing α_1-, α_2-globulin and β-globulin (in fetal calf) or γ-globulin (in human); the second, intermediate, containing albumin and; the third, retarded, containing residual α- and β-globulins. The 'albumin fraction' provided a single peak (see Fig. 1C), corresponding to the intermediate one of the total serum and containing only albumin. The albumin-free fraction displayed three peaks similar to those showed by total serum (see Fig. 1B), which cumulatively contained α_1-, α_2-globulin, β-globulin (in human), and a portion of the γ-globulin present in the starting serum.

When G_{MI} was added to either serum, or 'albumin-free fraction', or 'albumin fraction', and the mixtures immediately poured on Ultrogel AcA 34 columns (see Figs. 2A, 2B, 2C, full lines) the protein profiles remained unchanged, while an additional peak appeared between the first and second protein peaks, corresponding to G_{MI} micelles. As shown in Fig. 2A (dotted lines) the elution profiles of both human and fetal calf total sera underwent marked changes after 4-h incubation in the presence of G_{MI}. Two new, fast moving, peaks appeared carrying protein and ganglioside (as radioactivity and *N*-acetylneuraminic acid), with concomitant decrease of the second protein peak and of the G_{MI} micelle peak. These two new peaks, correspond to G_{MI}-serum protein complexes. The albumin fraction of both human and fetal calf origin provided, after incubation with G_{MI}, the same two peaks carrying protein and G_{MI} (see Fig. 2B, dotted lines). Instead the 'albumin-free fraction' of either serum showed no change in both the protein and G_{MI} micelle elution profiles (see Fig. 2C, dotted lines), indicating no formation of G_{MI}-protein complexes. The fractions containing G_{MI}-protein complexes were collected, pooled and concentrated (5–10 mg protein/ml). The concentrated solutions (from total serum and from the 'albumin fraction') of fetal calf origin were pooled; the same was done for those of human origin. Each of the resulting mixtures was resubmitted to Ultrogel AcA 34 column. In each case two peaks were eluted having the same chromatographic behaviour as the corresponding ones obtained with total serum and the 'albumin fraction'. This indicates that G_{MI}-protein complexes formed with total serum and the 'albumin fraction' were identical.

Under the used experimental conditions a clearly defined G_{MI} micelle peak was observed starting from $1 \cdot 10^{-6}$ M G_{MI}. At lower concentrations the elution profile of G_{MI} was less clear, as already reported by Tomasi et al. [16], who employed similar conditions. The lowest G_{MI} concentration at which G_{MI} protein complexes (with the previously described characteristics) could be detected was $2 \cdot 10^{-6}$ M for human serum and $4 \cdot 10^{-6}$ M for fetal calf serum. The same results were obtained using the 'albumin fraction' of either serum.

Dynamics of the formation of G_{MI}-serum protein complexes

The process of G_{MI} binding to serum proteins was studied as a function of G_{MI} concentration and incubation time. At fixed (4–5 mg/ml) protein concentration and before reaching saturation the process appeared to follow biphasic kinetics in both human and fetal calf sera and as a function of increasing G_{MI} concentration (Fig. 3). In the first phase, occurring till about $2 \cdot 10^{-4}$ M G_{MI}, the ratio between bound and total G_{MI} increased almost linearly with increasing G_{MI} concentration; in the second phase, occurring at G_{MI} concentrations above $2 \cdot 10^{-4}$ M (and at least up to $12 \cdot 10^{-4}$ M) the ratio remained practically constant. In this second phase the proportion of bound

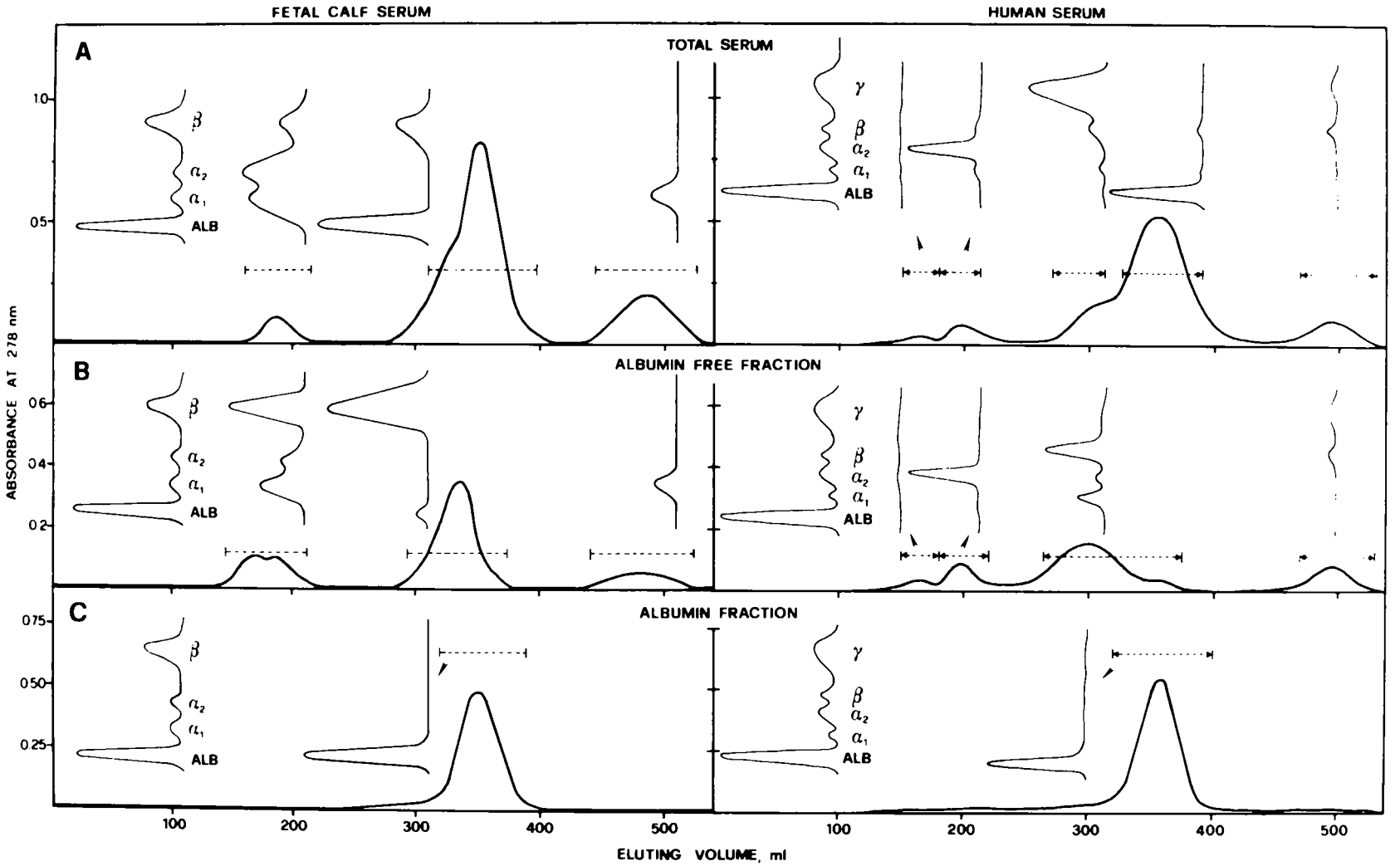

Fig. 1. Elution profile from Ultrogel AcA 34 columns of total serum, albumin-free fraction, and albumin fraction, of human and fetal calf origin. Each eluted peak was concentrated by filtration in a Sartorius Membranfilter apparatus, and submitted to electrophoresis on cellulose acetate strips. For details see Materials and Methods.

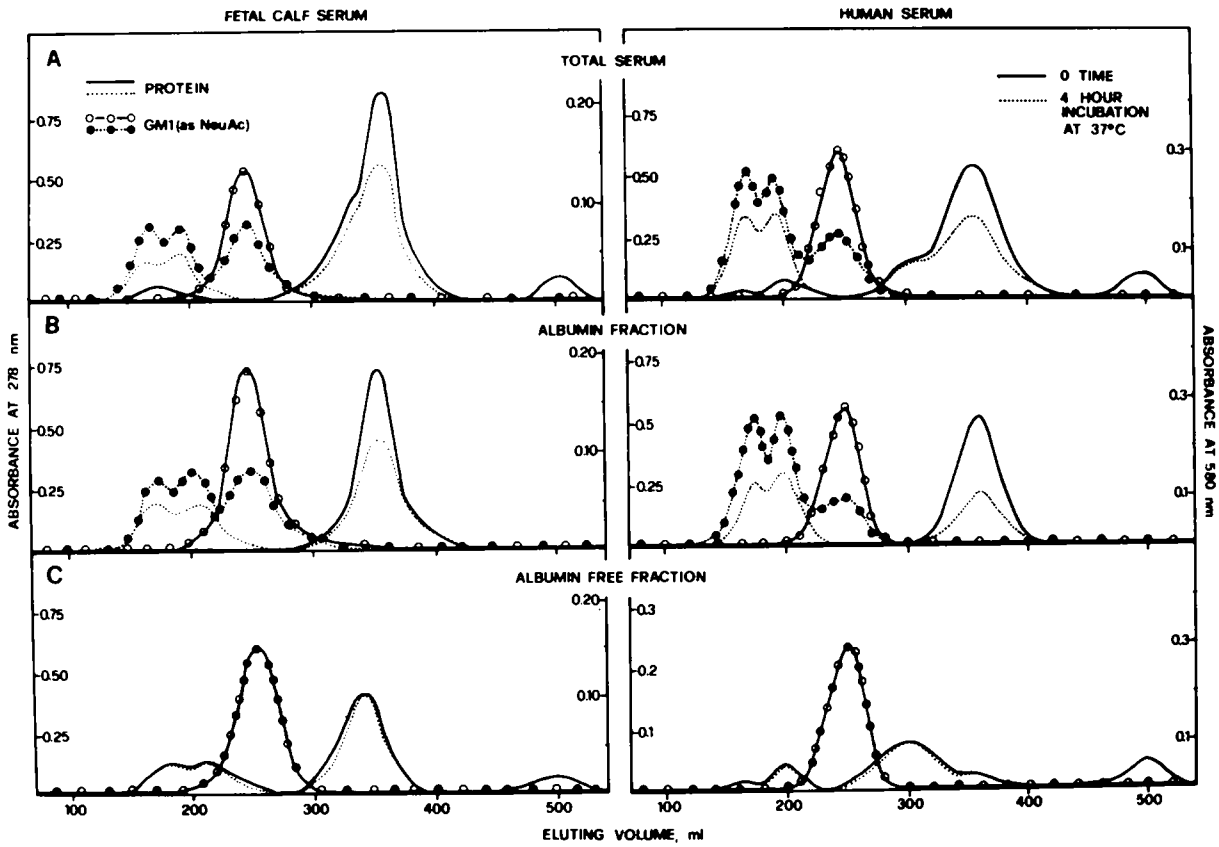

Fig. 2. Elution profile from Ultrogel AcA 34 columns of total serum, albumin-free fraction and albumin fraction (of human and fetal calf origin) before and after 4-h incubation at 37°C in the presence of ganglioside G_{M1} (0.33 mM). Protein was monitored by recording absorbance at 278 nm; ganglioside was assayed as bound *N*-acetylneuraminic acid. For details see Materials and Methods.

over total G_{M1} was 55–58% for human serum and 14–15% for fetal calf serum. Saturation was reached (see also Fig. 4) at about $20 \cdot 10^{-4}$ M G_{M1}; at this point the ratio of bound over total G_{M1} tended, as expected, to diminish. The same phenomenon was observed using the 'albumin fraction' of both human and fetal calf sera. The G_{M1} binding capability of total serum (or 'albumin fraction'), at G_{M1} concentrations over 10^{-4} M, was 3–4-times greater in human than in fetal calf serum (see Fig. 4). Under the same conditions, G_{M1} binding, expressed per mg albumin present, was practically identical regardless of the fact that total serum or the 'albumin fraction' was used in the binding experiments. The progress curves of G_{M1} binding, at a fixed ($3 \cdot 10^{-4}$ M) G_{M1} concentration, are shown in Fig. 5. The binding appeared to be complete after 20 h of incubation and occurred at a slower rate in fetal calf materials: after 1 h incubation 70% of maximal binding was achieved in human serum, with only 40% in fetal calf serum. G_{M1}-binding progress curves, expressed per mg albumin present, were practically overlapping, regardless of the material (total serum or albumin fraction) used in the binding experiments.

G_{M1} binding to purified bovine and human γ-globulins

Purified bovine and human γ-globulins after incubation with G_{M1} under a wide range of experimental conditions (incubation time from 1 to 20 h; γ-globulin/G_{M1} ratio, w/w, from 2:1 to 1:10) were fractionated on Ultrogel AcA 34 column chromatography. The radioactivity carried by G_{M1} was always completely retained on the micelle

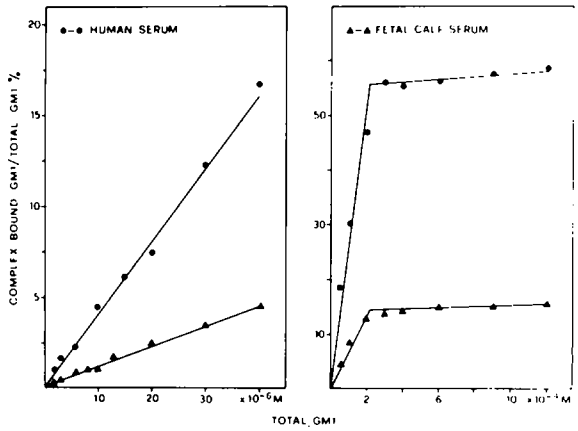

Fig. 3. Kinetics of G_{M1} binding to human and fetal calf serum proteins. The ratio between protein-bound G_{M1} and total G_{M1} is expressed as a function of G_{M1} concentration (from $2 \cdot 10^{-6}$ M). 2 ml mixtures, containing 8–10 mg protein and the specified amount of G_{M1} (carrying 1.5 μCi of ^{3}H label) were incubated (90 min) at 37°C. The formed G_{M1}-protein complexes were isolated on Ultrogel AcA columns and G_{M1}-bound radioactivity counted. For details see Materials and Methods.

peak, which was eluted in an area devoid of protein. In particular, no radioactivity was measured in the peaks corresponding to eluted γ-globulins; on the other hand, the elution pattern of γ-globulins was not modified by incubation with G_{M1}. This means that bovine and human γ-globulins did not bind G_{M1}.

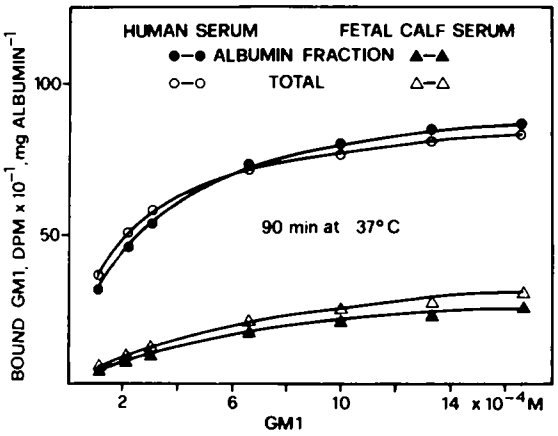

Fig. 4. Saturation curves of G_{M1} binding to total serum and to the 'albumin fraction' of human and fetal calf origin. G_{M1} concentrations above $1 \cdot 10^{-4}$ M are reported. Proteins: 4.5 mg/ml. Protein-bound G_{M1} is expressed per mg albumin. For details see Materials and Methods.

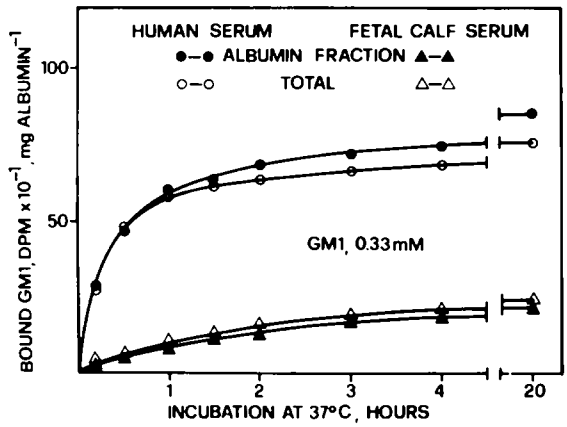

Fig. 5. Time-course of G_{M1} binding to total serum and to the 'albumin fraction' of human and fetal calf origin. G_{M1} was used at a fixed (0.33 mM) concentration. Proteins: 4.3 mg/ml. Protein-bound G_{M1} is expressed per mg albumin. For details see Materials and Methods.

Discussion

It was recently [16] demonstrated that pure bovine serum albumin binds ganglioside G_{M1} producing two complexes of different molecular weight. The smaller complex, constituted by one albumin molecule and one G_{M1} micelle, gives rise, by dimerization, to the bigger one. The present paper shows that incubation of ganglioside G_{M1} with serum, of human and fetal calf origin, is followed by the formation of two G_{M1}-serum protein complexes. The same two complexes appeared after interaction of G_{M1} with the pure albumin fractions prepared from the above sera; instead no complex formed using the correspondent albumin-free fractions. Moreover, kinetic studies showed that the process of G_{M1} binding in serum and in the corresponding albumin fraction occurred in a strictly parallel way, from both the qualitative and quantitative points of view. All this leads to the conclusion that the G_{M1}-protein complexes formed in both human and fetal calf sera actually are G_{M1}-albumin complexes.

It should be noted that the albumin-free fraction prepared from human serum had a markedly low content of γ-globulin (fetal calf serum alone is almost devoid of γ-globulin), probably retained by the Affi-Gel blue used for removing albumin. In

the case in which γ-globulin could bind G_{M1}, the observed inability of the albumin-free fraction to bind G_{M1} would give a falsely negative result. However, purified γ-globulin of human and bovine origin failed to bind G_{M1}. Thus albumin appears to be the protein selectively able to bind ganglioside G_{M1}.

The process of G_{M1} binding to serum albumin, which starts being visible at ganglioside concentrations (above 10^{-6} M) surely micellar, is time and concentration dependent and may result in a relevant degree of G_{M1} complexation (up to 70% in human and to 18% in fetal calf serum). These and other observations (the formation of two complexes; the apparent stability of the complexes upon rechromatography) are consistent with the hypothesis that G_{M1} binds to albumin in human and fetal calf sera in a way similar to crystalline bovine serum albumin [16].

As a practical consequence of these findings it should be expected that G_{M1}, when present in serum-containing systems, at concentrations exceeding 10^{-6} M, forms complexes with albumin. Therefore, it occurs in three different physical forms, monomers, micelles and albumin complexes. Which of these forms is the most suitable for G_{M1} binding to cell plasma membranes, and whether the physical form determines or influences the kind of binding is not known. However, it is clear that when studying the effects of exogeneous G_{M1} on cells or tissues, preliminary care should be taken in order to acknowledge which physical form of ganglioside is actually interacting with cells and which kind of interaction is occurring. It would be also interesting to know whether albumin binds other gangliosides besides G_{M1}. Studies in these directions are already in progress in our laboratory.

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