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Fatty acid acylated Fab-fragments of antibodies to neurospecific proteins as carriers for neuroleptic targeted delivery in brain

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A method for targeted delivery of neuroleptics from blood in brain based on using Fab-fragments of antibodies to antigens of brain glia cells (acid gliofibrillar antigen and α_2-glycoprotein) is suggested. The essence of the technique is that the molecule of neuroleptic (trifluoperazine) is conjugated with Fab-fragments of these antibodies. The conjugate thus obtained is modified by stearoylchloride in the system of Aerosol OT reversed micelles in octane. The study of the distribution of ^{125}I-labelled conjugates in the rat organism after intracordial introduction is performed. On the contrary to the nonmodified conjugates and conjugate, containing fatty acylated Fab-fragments of antibodies, nonspecific to the rat brain, the conjugate of trifluoperazine with stearoylated Fab-fragments of antibodies to neurospecific antigens accumulate in brain tissues. The drastic increase of the neuroleptic activity of trifluoperazine resulting from its coupling with stearoylated Fab-fragments of antiglial antibodies is observed.

Drug targeting; Stearoylated Fab-fragment; Reversed micelle; Neuroleptic; Neurospecific antibody

1. INTRODUCTION

The problem of transporting low and high molecular weight biologically active substances through the blood-brain barrier (BBB) into the brain is of great interest to medicine. Low permeability of the barrier with respect to nonpolar substances considerably restricts possibilities of application of antibiotics (and other drugs) during the therapy of tumours and brain regional infections, as well as the use of neuroleptics during psychiatric diseases, etc. [1–3]. Therefore, it is little wonder that many laboratories are engaged in the active search for ways of drug targeting through the BBB [3–6]. In particular, we have recently developed a method for directed delivery in the brain of neuroleptic, based on incorporation of the latter in microcontainers formed by the micelles of a polymeric surfactant, presumably capable of transcytosis through the BBB [5]. Here we describe another method for neuroleptic-targeted delivery in the brain glia cells using the Fab-fragments of antibodies to the antigens of glia cells [4]. The essence of this technique is that the molecule of a neuroleptic is conjugated with Fab-fragments of these antibodies. In order to enhance the efficiency of the transport of the thus obtained conjugate into the brain, Fab-fragments are chemically modified by fatty acid residues. It was reported recently that attachment of a hydrophobe to a protein molecule via its artificial fatty acylation results in an increase in protein binding with a cell membrane and, at least in some cases, in enhancement of protein penetration within the cell [7].

In this paper we demonstrate the possibility of targeted delivery from the blood to the brain of trifluoperazine conjugated with fatty acylated Fab-fragments of rabbit polyclonal antibodies against human acid gliofibrillar antigen (GFA) [8] and human brain α_2-glycoprotein (α_2-GP) [9].

Abbreviations: Aerosol OT, AOT, sodium bis(2-ethylhexyl)sulfosuccinate; GFA, acid gliofibrillar antigen; α_2-GP, α_2-glycoprotein; trifluoperazine,stelazine,10-[3-(4-methylpiperazin-1-yl)-propyl]-2-(trifluoromethyl)-10H-phenothiazine; TNBS, 2,4,6-trinitrobenzenesulfonic acid

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2. MATERIALS AND METHODS

2.1. *Antibody Fab-fragment isolation*

Antisera against human brain GFA, α_2-GP, and α_1- and α_2-globulins were obtained from chinchilla rabbits immunized with purified preparations of these antigens [8–10]. Specific antibodies were separated by immunosorption using the antigens immobilized on the CNBr-activated Sepharose 4B (Pharmacia) [11]. F(ab)$_2$ and Fab-fragments were prepared from the purified antibodies by limited proteolysis with bovine trypsin (Sigma) as described elsewhere [4,12].

2.2. *Epoxytrifluoroperazine synthesis*

In order to synthesize the epoxytrifluoroperazine, further used for conjugation with antibody Fab-fragments, the trifluoroperazine molecule was modified by epichlorhydrin. This reaction (quaternization of the tertiary nitrogen in the neuroleptic piperazine ring) was conducted in alkalized aqueous ethanol (pH 9.6) at 40°C during 4 h. The excess of epichlorhydrin and the major part of the solvent were evaporated. Epoxytrifluoroperazine was precipitated from ethyl acetate, dissolved in water and lyophilized. The presence of the epoxy

group in the thus obtained compound was confirmed using the reaction of the latter with sodium hyposulfite which was registered by IR-spectroscopy. The overall yield of the epoxytrifluoroperazine was 60%.

2.3. *Modification of antibody Fab-fragments*

In order to prepare the conjugate of antibody Fab-fragments with trifluoroperazine a solution of 5 mg of anti-GFA antibody Fab-fragments and 5 mg of anti-α_2-GP antibody Fab-fragments in 2 ml of 0.01 M sodium phosphate buffer (pH 7.5) was mixed with a solution of 20 mg of epoxytrifluoperazine in 2 ml of the same buffer. The reaction system was incubated at room temperature for 2 h. The conjugate obtained was purified by immunosorption [11]. The same procedure was used for the preparation of epoxy trifluoperazine conjugates with Fab-fragments of antibodies to human brain α_1- and α_2-globulins. The amount of trifluoperazine molecules linked to one Fab-fragment was determined via titration of the free amino groups of the protein with TNBS [13].

The conjugates obtained were modified by stearoylchloride in Aerosol OT reversed micelles in octane using the procedure described elsewhere [14,15]. The number of fatty acid residues introduced per Fab-fragment was determined via free protein amino group titration by TNBS. The ^{125}I-labelling of the conjugates was performed according to the method [16].

2.4. *In vivo distribution of the conjugates*

Samples, containing 1.2 mg of ^{125}I-labelled conjugates of antibodies Fab-fragments with trifluoroperazine (1 mCi/mg protein) in 100 μl of physiological solution were injected intracordially in the body of white mongrel rats (body weight 200 ± 10 g). Samples of liver, brain, kidney, lung, spleen, heart, and blood were collected from the animals decapitated under hexenal at various times after injection. The one gram of tissue samples or 1 ml of whole blood were homogenized with 2 ml of 0.05 M sodium phosphate buffer pH 7.4. ^{125}I-radioactivity was measured in homogenates using γ-counter Compu Gamma (LKB). Each experimental point presented in this work gives a radioactivity value, averaged for the group of 8–10 rats.

2.5. *Biological activity of trifluoperazine and of its conjugates with antibody Fab-fragments*

For the evaluation of the biological activity of trifluoperazine and its conjugates with antibody Fab-fragments the toxicity (LD_{100}) of these drugs was determined. At the same time the development of specific neuroleptic symptoms (closing symptom, extremity tractions, other extrapyramidal disorders, seizures) was registered according to the previously described criteria [17]. In these experiments 100 μl of the drug in physiological solution were intracordially injected in rat. The drug doses were varied in the interval 0.1–2 mg of trifluoperazine per kg of body weight (free trifluoperazine) or 0.1–2 mg of protein per kg of body weight (trifluoperazine–antibody Fab-fragment conjugate).

3. RESULTS AND DISCUSSION

It is well known that the BBB shows very low permeability to neuroleptics [2], and extremely high doses of these drugs, often causing severe complications, are usually applied to achieve therapeutic effects [1]. The targeted delivery of the drug in the brain may represent a way to considerably decrease the doses necessary for treatment and, hence, avoid these complications.

The Fab-fragments of antibodies to neurospecific proteins, namely, human GFA and α_2-GP, are used as vector parts of the trifluoperazine delivery system described in this work. In immunodiffusion tests, these antibodies (as well as their Fab-fragments) demonstrate a high cross-reactivity with the corresponding proteins from the rat brain (data not shown). At the same time the immunofluorescence study, performed using the cuts of various rat organs, revealed that these antibodies selectively interacted only with brain tissues (data not shown).

The Fab-fragments of antibodies to human α_1 and α_2-globulins, which revealed no cross-reactivity with the corresponding rat proteins, were used as Fab-fragments nonspecific to rat brain tissue.

The covalent binding of the drug to the Fab-fragments of antibodies was achieved through a reactive epoxy group which was inserted in the trifluoperazine molecule as described above. We suppose that the conjugation proceeds preferentially through a reaction between epoxy groups of the modified drug and free amino groups of a protein. According to the titration data of free amino groups by TNBS, each Fab-fragment in the conjugate obtained was linked with 5–10 trifluoperazine molecules. The attachment of the modified trifluoperazine did not influence the immunospecifity of the Fab-fragments since the conjugates preserved their ability to interact with specific immunosorbents. Only those conjugate fractions which were additionally purified by an immunosorption on the columns with immobilized antigens, have been used throughout all experiments described below.

Introduction of a hydrophobe in the biopolymer molecule was proposed for increasing biopolymer binding with cell membranes [18] and enhancement of its internalization in the cell [7,19]. In order to modify the conjugates with stearoylchloride we used the recently developed method for protein, modification with water insoluble reagents in the system of Aerosol OT reversed micelles in octane [14]. According to the TNBS titration data thus obtained, stearoylated conjugates contained from 1 to 3 fatty acid residues per protein molecule.

We have studied the effect of fatty acylation on the distribution in the rat organism of intracordially administered trifluoperazine–Fab-fragment conjugates. As can be seen in Fig. 1, the conjugates nonmodified with stearoyl residues containing Fab-fragments both specific and nonspecific to the proteins of rat brain are detected mostly in blood, liver, spleen and kidneys. Modification of Fab-fragment conjugates by fatty acid residues leads to a considerable decrease in their content in blood. Three days after administration of fatty acylated nonspecific Fab-fragments conjugate it accumulate mostly in the liver. This can be explained by the presence in the liver of large amounts of a fatty acid binding protein [20]. Quite another phenomenon is observed in the case of fatty acylated conjugates, containing Fab-fragments of antibodies specific to rat brain that accumulate mostly in brain tissues (Fig. 1). (Their localization index in the brain is maximal at the third day after administration.)

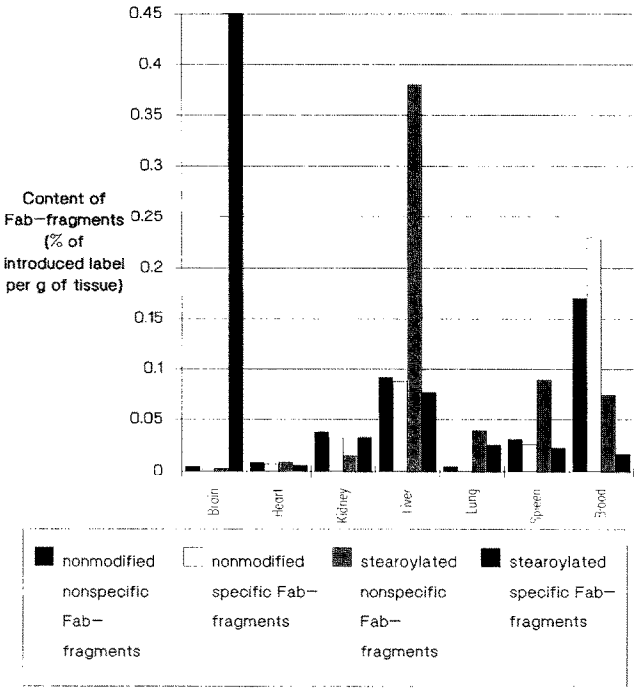

Fig. 1. Distribution of ^{125}I-labelled nonmodified and stearoylated conjugates containing Fab-fragments of nonspecific and brain tissue specific antibodies, in the rat organism at the 3rd day after intracordial administration.

What is the reason for the observed effect of *targeted transport* of fatty acylated conjugates into the brain? Interaction of Fab-fragments of antibodies with GFA and α_2-GP-antigens located in brain glial cells [8,9] is only possible after penetration of the former through the BBB. At the same time, it should be taken into consideration that we used polyclonal antibodies that are probably capable of interacting with antigens located on the external side of the BBB (e.g. in the sites of endothelial cell contacts). In this case the effective accumulation of the fatty acylated conjugates in the brain may be the result of the presence in their molecules of two sites of binding with BBB membranes, namely, the hydrophobic anchor and the antigen recognizing site. Introduction of the fatty acid residue in this case results in enhancement of conjugate binding with the membranes (including these of BBB) which in its turn provides for increase in the apparent constant of Fab-fragment binding with the antigen.

In any case the observed phenomena are of great interest in the development of systems for drug targeting into the brain. The results of the evaluation of the biological activity of the trifluoperazine and its conjugates with Fab-fragments of antibodies to neurospecific proteins are presented in Table I. These results are compared with data from animals treated with the same protein preparations not conjugated with the drug. It is obvious that the last preparations are not toxic for rats and did not cause any symptoms of neurolepsy. In contrast, for the fatty acylated conjugates, a drastic increase in toxicity associated with typical neuroleptic symptoms is observed. This effect cannot be explained by increased toxicity of the modified drug itself since the applied method of chemical modification does not essentially alter the biological activity of the trifluoperazine (data not shown).

The data obtained indicate that the conjugates of neuroleptics with fatty acylated Fab-fragments of antibodies to neurospecific proteins are capable of penetrating through the BBB and reaching biological targets of the brain. It is highly probable that the transfer of fatty acylated Fab-fragment conjugate across BBB is accomplished via transcytosis [21].

We assume, that the use of fatty acylated Fab-fragments of antibodies to neurospecific proteins opens perspectives for creating principally new drugs for the therapy of various brain diseases.

Table I

Neuroleptic activity of trifluoperazine and its conjugates with Fab-fragments of antibodies against neurospecific antigens after intracordial administration in rat

Compound	$^{LD}100$ (mg/kg body weight)	Symptoms of neurolepsy*			
		1	2	3	4
Trifluoperazine	10	+ + + +	+ + + +	+ + + +	+ + + +
Conjugate of trifluoperazine with stearoylated Fab-fragments	0.025**	+ + + +	+ + + +	+ + + +	+ + + +
Conjugate of trifluoperazine with nonmodified Fab-fragments	25**	+	+ +	+ +	+ +
Stearoylated Fab-fragments	non-toxic	-	-	-	-
Nonmodified Fab-fragments	non-toxic	-	-	-	-

*1 = Closing symptom; 2 = extremity traction symptom; 3 = extrapyramidal disorders; 4 = convulsions.
**The conjugate concentration is presented.

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