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ON THE POSSIBILITY OF THE UNIFICATION OF DRUG TARGETING SYSTEMS

STUDIES WITH LIPOSOME TRANSPORT TO THE MIXTURES OF TARGET ANTIGENS

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Abstract—In order to make the drug targeting system more effective, simple and technological, we suggest creation of drug-bearing conjugates capable of simultaneous binding with different antigenic components of the target via specific antibodies. It is supposed that the targeted therapy should include sequential administration of the mixture of modified antibodies (or other specific vectors) against different components of affected tissue and, upon antibody accumulation in the desired region, administration of modified drugs or drug carrying systems which can recognize and bind with the target via accumulated antibodies due to the interaction between vector modifier and carrier modifier. Using as a model system monolayers consisting of the mixture of extracellular antigens and appropriated antibodies, it was shown that the treatment of the target with the mixture of biotinylated antibodies against all target components and subsequent binding with the target of biotinylated liposomes via avidin permits high liposome accumulation on the monolayer. The binding achieved is always higher than in the case of the utilization of single antibody-bearing liposomes. Besides, the system suggested is very simple and its components can be easily obtained on technological scale in standardized conditions.

The idea of targeted drug transport in the organism is based on the use of drugs or drug-containing carriers (microreservoirs) modified with some vector molecule possessing specific affinity towards definite molecular structures in the affected area of target organ [1–3]. Being injected into the blood the conjugate should recognize and bind the target thus increasing local drug concentration. In several studies it is also suggested to use for drug or carrier modification a mixture of vector molecules (usually antibodies) specific towards a set of different components of a target [4–6].

To obtain targeted preparations one should perform a complex process of binding (mainly covalent) vector molecules with therapeutic preparation. Protein vectors (antibodies) are widely used for drug, polymer, liposome or cell ghost modification [7] every time a complicated task arises to modify (to immobilize) protein with complete preservation of its specific activity. The process of binding becomes even more uncomfortable when attempting to immobilize several vector molecules on the same therapeutic preparation simultaneously.

In order to simplify the obtaining of targeted preparations with high efficiency and to make this process more technological, we suggest a new approach for the unification of targeted systems which enables addressing of the universal preparation to several antigens of the target organ simultaneously. Instead of vector molecules immobilized on a drug molecule (or drug-containing microreservoir), we introduce bifunctional "bridge" molecules possessing two types of affinity—towards the affected organ and towards the unified therapeutic preparation. As bridge molecules, natural polyvalent macromolecules can be used, capable of binding two or more ligands, or synthetic conjugates obtained from molecules possessing two different types of affinity.

Thus, an optimal targeted system should consist of two principal components. They are: (1) vectors to different components of a target modified with some chemical compound (X), the nature of which does not depend on the nature of a target, and (2) drug carrier which, in turn, is modified with the compound (Z) capable of firm and specific binding with the target-bound derivatized vector via compound (X). It is evident that the same couple (X–Z) can be used for derivatization of any vector and any drug or drug carrier. In this case in the progress of targeted therapy the injection of modified vectors ("bridge" molecules) should precede the administration of derivatized therapeutic preparation. According to the scheme suggested (Fig. 1) the unified drug-containing preparation can bind vector molecules of any type.

To prove our suggestion experimentally we used the model system, consisting of the plastic adsorbed monolayers of protein antigens (collagen, laminin, fibronectin, fibrinogen and some others), appropriate biotinylated antibodies as vector molecules and biotinylated liposomes as drug carriers. The protein monolayer have been treated with the mixture of modified antibodies, then with avidin (glycoprotein capable of high affinity binding of four biotin molecules) and then with biotinylated liposomes (in this particular case the conjugate antibody-

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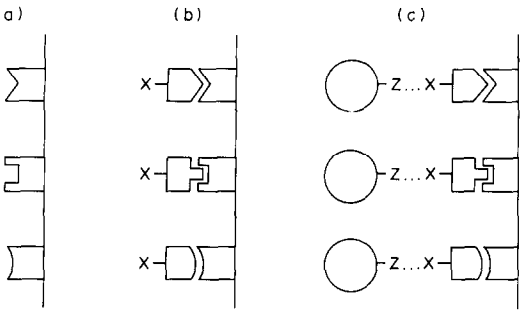

Fig. 1. Scheme of targeted delivery of unified containers to a set of target antigens: (a) exposed target antigens: (b) preliminary treatment with "bridge" molecules: (c) specific binding of unified drug carrier systems.

biotin avidin serves as a "bridge" between antigen and biotinylated liposome). Avidin–biotin systems have been already successfully used in traditional drug-targeting systems based on the use of liposomes [8] and red blood cells [9] and one type of antibodies.

MATERIALS AND METHODS

The following reagents were used: egg yolk lecithin from the Kharkov plant of bacterial preparations (U.S.S.R.), dipalmitoyl phosphatidyl ethanolamine and cholesterol from Sigma (U.S.A.), D-biotin *N*-hydroxysuccinimide ester from Calbiochem (Switzerland), avidin with specific activity 13 units/mg from Serva (F.R.G.), ^{14}C-cholesterol oleate with specific radioactivity 58 mCi/mmol from Amersham (U.K.). The antigens used—fibronectin, fibrinogen, low density lipoproteins (LDL)—were purified from the donor's blood according to refs 10–12, respectively. Human type I collagen and rabbit anticollagen antibodies were kindly donated by Dr S. Domogatsky (U.S.S.R. Cardiology Research Center), human laminin and antilaminin antibodies were kindly provided by Drs G. Martin and H. Kleinman (NIH, Bethesda, MD). Other antibodies were purified from the plasma of immunized rabbits on the columns with appropriated immunosorbents according to [13]. The cross-reactivity of all the antibodies used was negligible.

Other reagents and components of buffer solutions were analytical grade preparations from Reakhim (U.S.S.R.).

Biotinylated phosphatidyl ethanolamine have been obtained by the interaction of D-biotin *N*-hydroxysuccinimide ester with phosphatidyl ethanolamine according to [14].

Liposomes were obtained from the mixture of lecithin, biotinylphosphatidyl ethanolamine and cholesterol in 6:1:3 molar ratio by the sonication of lipid film (obtained by rotor evaporation of lipid solution in chloroform) using ultrasound desintegrator. Five microCuries of ^{14}C-cholesterol oleate per mg lipid mixture have been used as radioactive label.

Two different mixtures of antigens were used for target monolayers preparation in experiments of liposome targeting. Mixture A consisted of human plasma fibrinogen, fibronectin and LDL, Mixture B—human laminin, collagen type I and fibronectin.

To prepare model antigenic monolayers, the solution of the antigenic mixtures A or B in 0.05 M carbonate buffer, pH 9.5 was placed into wells of 96-well microplate Titertek (U.S.A.) and incubated there for 18 hr at 4°. The concentration of each component in initial solution varied from 0.5 to 5.0 μg/ml. The plate was then washed several times with phosphate buffered saline containing 2 mg/ml of bovine serum albumin (PBS-BSA). The surface concentration of each monolayer component was determined by enzyme immunoassay with the use of the avidin–biotin system [15].

In the experiments on targeted liposome transport immunoglobulins were biotinylated with D-biotin *N*-hydroxysuccinimide ester according to [16]. It was shown that the reaction leads to the modification of 60–70% surface amino groups of protein globule (titration with trinitrobenzenesulfonic acid [17]) and does not affect specific antigen-binding properties of immunoglobulins (according to the data of enzyme immunoassay).

Wells coated with the mixture of protein antigens were supplied with 100 μl of antibody solution in PBS-BSA and incubated for 1 hr at room temperature. Antibody concentration was 0.01 mg/ml (in the case of the mixture of antibodies this was the total immunoglobulin concentration). The concentration of antigens in absorption mixtures was chosen in such a way that each kind of antibody gave an identical signal in the enzyme immunoassay system which means that biotin residues immobilized on different antibodies possess identical availability for avidin.

After being washed five times with PBS-BSA, wells were supplemented with avidin solution (10 μg/ml), incubated for 10 min, again washed with PBS-BSA, and at least 100 microliters of ^{14}C-labelled liposomes (50,000 dpm) were added into each well. Liposomes were incubated with antigenic monolayers for 30 min at room temperature with mild orbital shaking. Then wells were washed five times with 200 μl of PBS-BSA, then plate was cut and firmly bound radioactivity in each well was determined on scintillation spectrometer (Rackbeta, LKB, Sweden).

RESULTS AND DISCUSSION

Biotinylated antibodies preserve the ability to bind simultaneously appropriate antigens in the monolayer and avidin. This ability can be estimated quantitatively utilizing enzyme immunoassay. The typical data on the titration of all three antibodies binding on the surface of monolayer prepared from mixture A are shown in Fig. 2.

The composition of mixture A was conditioned mainly by the availability of pure antigens. Mixture B to some extent is considered as a model of extracellular matrix underlying normal endothelial cells in the blood vessel wall. The subendothelial layer is very attractive aim for targeted drug therapy because it is known that endothelial cell damage leading to the exposition of extracellular subendothelial structures to the circulating blood causes the development of many pathological processes,

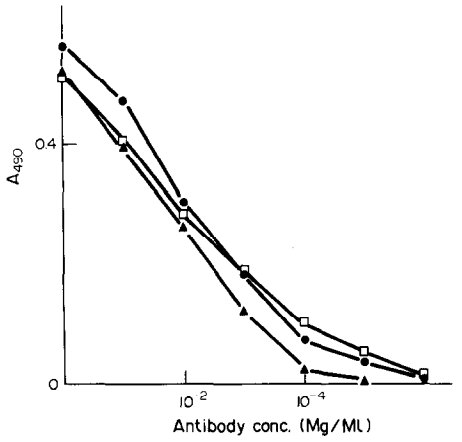

Fig. 2. Typical titration curves of the separate antigens on antigen mixture A adsorbed surface—signals of enzyme immunoassay. ▲, biotinylated antifibronectin; ●, biotinylated antifibrinogen; □, biotinylated anti-LDL

thrombi and atheroma formation among them [18]. Antibodies against different components of the subendothelial layer have already been successfully used for targeted delivery of erythrocytes and liposomes [4, 19] to vascular damage.

The ability ot biotinylated liposomes to bind avidin was proved following the turbidity increase (i.e. agglutination of liposomes) of diluted liposome suspension (0.01–0.05 mg of lipid per ml) upon free avidin addition. The liposomes obtained completely preserved their ability to bind avidin for at least 3 months after preparation when stored at 4° in the darkness.

The results of experiments on targeted liposome transport to antigenic monolayers are shown on Fig. 3. It follows from the data submitted that liposomes can be successfully bound with the target via antibody–biotin–avidin bridge. Of special importance is the fact that the simultaneous use of the mixture of antibodies is 30–50% more effective than the use of a single antibody at optimal concentration. It is evident that additional antibodies form new binding sites for liposomes on the target. The maximal liposome binding (0.4–0.5 μg of lipid per well) is close to the complete coating of the well surface with liposomes [20].

As already mentioned, the simultaneous use of several vector molecules capable of interaction with different structures of the target seems to be highly promising. The information about the antigenic structure of affected organs or tissues can be obtained from the results of different immunomorphological studies. Nevertheless, the relative amount of antigenic determinants of each type easily accessible for targeted drug-carrying systems often remains unknown. One can think that the use of maximally possible number of "bridge" molecules can drastically increase efficacy of modified drug delivery even without detailed studies of relative availability of target antigens. To prove this we prepared a monolayer from mixture A in conditions when one of its components (LDL) was discovered in the enzyme immunoassay system six times worse than the sum of all three components. It follows from the data of Fig. 4 that in this system the use of the mixture of three biotinylated antibodies provides three times higher liposome binding than the use of only anti-LDL-antibody. Again, additional antibodies formed new binding sites for liposomes.

We describe here only the principal idea of targeted drug transport as a two-step procedure. It is not necessary that the avidin–biotin–liposome system will possess sufficient efficacy *in vivo*. It is evident that another type of "bridge" molecules can be suggested, e.g. chimeric $(Fab)_2$ possessing double affinity. However, even the simplest system described by us can also find practical application. The concentration of endogenous biotin in the blood is rather low (*ca* 5 ng/ml) and is regulated by avidin entering the organism [21]. Plasma components do not prevent avidin–biotin complex formation. On the other hand, the immunoglobulin molecule acquires the ability to bind avidin effectively upon the incorporation of only few biotinyl residues [16]. This procedure affects the antibody structure very slightly. Biotinylation does not alter specificity and

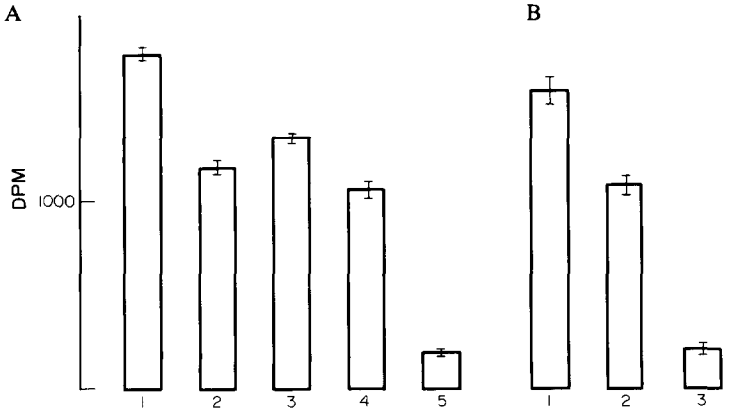

Fig. 3. ^{14}C-labelled liposome binding to plastic absorbed antigen mixtures A and B. Mixture A: (1) mixture of all three biotinylated antibodies added: (2) biotinylated antifibronectin added; (3) biotinylated antifibronogen; (4) biotinylated anti-LDL; (5) mixture of three non-biotinylated antibodies. Mixture B: (1) mixture of three biotinylated antibodies; (2) biotinylated antifibronectin; (3) mixture of non-biotinylated antibodies.

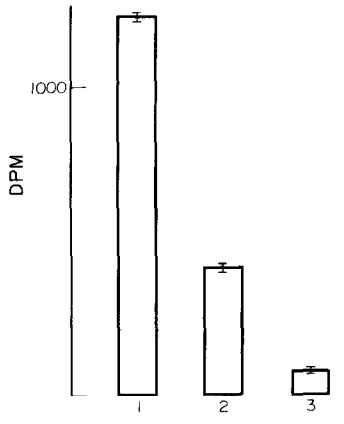

Fig. 4. ^{14}C-labelled liposome binding to antigen mixture A with low LDL content: (1) mixture of three biotinylated antibodies; (2) biotinylated anti-LDL; (3) mixture of three non-biotinylated antibodies.

practically does not decrease the affinity of antibodies. This can be confirmed by the numerous immunohistochemical data obtained using the avidin–biotin system *ex vivo* [22, 23]. It is likely that the limiting step of a therapy in this case will be antibody accumulation in target area and not avidin–biotin interaction which is very rapid ($K_{ass} = 7 \cdot 10^7 M^{-1} sec^{-1}$) [9]. Additionally, avidin, as a protein with a molecular weight of about 70,000 daltons, should not be excreted via kidneys too fast (e.g. the half-life of Fab-fragments with molecular weight about 50,000 daltons is 2–3 hr) [24]. Finally, the *in vivo* application of liposomes is also possible, because there exist already many methods which allow an increase in the half-life of liposomes in the blood up to 16–20 hr [25].

In our opinion the approach described has the following advantages:

1. It does not require the use of complex chemical methods of vector (antibody) immobilization on the drug molecule or on the drug carrier. The intermediate "bridge" molecule can easily be obtained by rather simple chemical modification.

2. Being applied to liposomes it enables us to obtain antibody-free targeted liposomes possessing higher half-life in the circulation [5].

3. The system seems to be acceptable from a technological point of view. Its components, vectors, "bridge" molecules and drugs can be easily prepared in individual form and can be stored separately for a long time.

4. The approach is quite universal and can be applied to any drug carriers (polymers, liposomes, red blood cells) and any vector molecules.

5. The further search for new affinity couples can probably produce much more effective systems than the system described in the present paper.

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