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CYTOTOXIC ACTIVITY OF DAUNORUBICIN OR VINDESIN CONJUGATED TO A MONOCLONAL ANTIBODY ON CULTURED MCF-7 BREAST CARCINOMA CELLS

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Abstract—Conjugates were constructed between daunorubicin or vindesin and a monoclonal antibody to human milk fat globule membrane associated antigen. This antibody recognizes a high molecular weight glycoprotein present at the cell surface of human normal and tumour epithelial cells; after specific binding to plasma membrane of cultured MCF-7 human breast carcinoma cells, it is endocytosed and gains access to lysosomes, wherein it is broken down (Aboud-Pirak *et al.*, *Cancer Res* **48**: 3188–3196, 1988).

Covalent linkage of daunorubicin (through a succinylated tetrapeptide arm) or of vindesin (through a hemisuccinate arm) yields conjugates with maximal molar ratios (drug molecule/specific IgG under monomeric form, i.e. unaggregated) or 2.0 and 4.5 respectively. The conjugate with daunorubicin inhibits the binding of the ^{3}H labelled antibody to MCF-7 cells as efficiently as the native unconjugated antibody, whereas the conjugate with vindesin inhibits it only by 56%. Both conjugates are entirely stable in plasma and serum; after 24 hr incubation at pH 4.8 in the presence of rat liver lysosomal enzymes, 60 and 33% of daunorubicin and vindesin respectively are released from the conjugates.

Adherent non-confluent cultures of cells recognized (MCF-7) or not (Hep-G2, human hepatocarcinoma cells) by the antibody were incubated from 1 hr to 6 days with different concentrations of daunorubicin or vindesin, free or conjugated to the specific or to a control monoclonal antibody. LD_{50}, defined as the drug concentration required to reach 50% of the amount of cell associated protein obtained in the absence of drug were determined at the end of 6 days continuous incubation or after shorter incubation followed by reincubation in drug free medium up to 6 days. Both cell lines are almost equally susceptible to the free drugs.

The conjugate between daunorubicin and the antibody appears inactive, even at saturating concentrations of antibody. This could result from the extrusion out of the cells of daunorubicin molecules released from the conjugate, impairing the drug to reach the intracellular concentration required for cytotoxicity. In contrast, conjugation of vindesin to the specific but not to a control antibody restricts the activity of the drug to cells selectively recognized by the specific antibody. However, even after corrections for the loss of immunoreactivity and for the incomplete release of vindesin from the conjugate, cytotoxicity is achieved at higher concentrations or requires longer exposure to the conjugated than to the free drug.

Nevertheless, these results clearly indicate that drug targeting relying on conjugates with monoclonal antibodies selectively recognized and endocytosed by target cells, covalently bound to a drug by a linkage stable in extracellular medium but hydrolysed within lysosomes, is valid. The pharmacological properties of the drug at the cellular level appear, however, to be also very important.

Our approach for drug targeting relies on conjugates of drugs and macromolecular carriers, such as glycoproteins or monoclonal antibodies (MAb)‡ selectively recognized by the target cells. The linkage between the drug and the carrier is constructed to be stable in the extracellular environment but hydrolysed by the lysosomal enzymes of the target cells after endocytosis of the conjugate and its access to lysosomes [1–4].

Recently, we have described experiments which allowed to select a MAb to human milk fat globule membrane. In MCF-7 cells, this MAb specifically immunoprecipitates glycoproteins with O-linked oligosaccharides and an apparent molecular weight of 350–400.000 [5]. In addition, this MAb recognizes all the breast carcinomas and deriving metastases tested, as well as other normal and tumoral tissues from epithelial origin active in secretion [6]. This MAb, labelled with ^{3}H, also binds selectively to antigens exposed at the cell surface of human cultured breast carcinoma cells (MCF-7 line), is endocytosed and gains access to lysosomes, wherein

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‡ Abbreviations used: DNR, daunorubicin; HPLC, high performance liquid chromatography; LD_{50}, lethal dose for 50% of the cells; MAb, monoclonal antibody.

it is broken down into small fragments soluble in trichloroacetic acid. Finally, a continuous supply of antigenic molecules to the cell surface allows a sustained uptake of the MAb by the cells and their delivery to lysosomes [5, 7].

This MAb is probably not completely suitable for *in vivo* drug targeting in human patients: (i) it is not tumour specific and, therefore, it should not allow to entirely restrict the access of the drug to the tumour cells; (ii) it is from murine origin and could therefore be immunogenic in man; (iii) it was isolated from mouse ascites and is then most probably heavily contaminated both biologically and chemically. However, it provides an appropriate model for studying drug targeting, relying on the lysosomotropic concept and for better understanding the cell pharmacological problems which could be encountered.

We have therefore considered the use of this MAb as a carrier for antitumoral drugs. Two molecules, widely used in cancer chemotherapy were selected for conjugation to the MAb: daunorubicin (DNR) an antrhacycline, toxic for rapidly proliferating cells and for cardiac muscle and vindesine (VDS), a vinca alcaloïd toxic for proliferating cells and central nervous system. DNR and VDS have been linked covalently to different poly- and monoclonal antibodies using different chemical strategies; both the pharmacological and immunological activities were partially or entirely preserved *in vitro* and *in vivo* [8–18].

In this paper, we report on the biological characterization and the pharmacological activity of conjugates constructed with these two drugs, using methods already reported [19, 20] and the MAb previously described [5]. The immunoreactivity of the two conjugates was tested on cultured MCF-7 cells. The conjugates, shown to be stable in the presence of human plasma or calf serum, release at least partially free DNR or VDS upon incubation in the presence of rat liver lysosomal enzymes. Finally, the cytotoxic activity of these conjugates, as well as that of conjugates with control MAb and of free drugs was compared on MCF-7 cells, which expose the antigen recognized by the specific antibody and on cultured human hepatocarcinoma Hep-G2 cells which do not bind specifically the MAb [7].

MATERIALS AND METHODS

Cells. MCF-7 cells were cultured as previously described [5]. They were cloned in order to obtain a line with a homogeneous surface expression of the antigen recognized by the specific MAb. Suspensions containing an average of *ca.* 2.5 cells/ml were harvested in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and supplemented with 1.6 μg/ml of insulin, 30 mM $NaHCO_3$, 10 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid, pH 7.4, and seeded in 96 well microplates (150 μl/well). Plates were incubated at 37° in a humidified incubator under 10% (v/v) CO_2 in air. Colonies were visually detected after 1–2 weeks; a second subcloning was then carried out. The homogeneity of antigenic expression at the cell surface was tested by using an indirect immunocytochemical assay [5].

Hep-G2 were obtained from Dr B. Knowles (Wistar Institute, Philadelphia, PA) and cultured in Minimum Eagle's Medium supplemented with non essential amino acids and 10% (v/v) fetal calf serum.

Monoclonal antibodies. Culture of the hybridoma secreting the specific MAb, as well as its production, purification and labelling have been previously described [5]. Specificity of this MAb was described elsewhere [6]. A MAb with irrelevant specificity of IgG_1 subclass, used as control, was a gift of Dr J. Van Snick (Unité de Médecine Expérimentale, Université Catholique de Louvain).

VDS-MAb conjugates. The conjugates were synthetized as previously described for conjugates to serumalbumin [19]. In brief, VDS obtained from Omnichem S.A. (Louvain-la-Neuve, Belgium) and labelled with ^3H at Amersham International (Amersham, U.K.) was condensed with succinic anhydride and the resulting hemisuccinate derivative was activated by reaction with isobutyl chloroformate. The mixed carbonic anhydride was then condensed with the MAb dissolved in 0.15 M NaCl.

Unconjugated drug was then separated by dialysis and gel filtration on Sephadex G25 (Pharmacia Belga, Bruxelles, Belgium) eluted with PBS. Conjugates were analysed by HPLC using a Protein PAK 1-300 column (Waters Associates, Bruxelles, Belgium) for the absence of contaminating aggregates and/or free drug. Drug/IgG molar ratios of 4.5 and 6.2 for conjugates with specific and control MAb respectively were used for cytotoxicity experiments.

DNR-MAb conjugates. The conjugates were synthetized as previously described [20]. In brief, DNR (obtained from Rhône Poulenc, Paris, France) was first converted into a L Leu-L Ala-L Leu-L Ala derivative, then succinylated and finally condensed with the MAb.

Unconjugated drug as well as aggregated IgG were separated by gel filtration on Ultrogel ACA 3-4 (LKB, Villeneuve-la-Garenne, France) eluted with PBS. DNR–MAb conjugates were treated with active charcoal (25–30%; w/w). Antibody-conjugated DNR was quantified by HPLC and fluorimetry as previously described [20]. In brief, the DNR-conjugate was incubated for 30 min at 80° with 40% (v/v) formic acid and then neutralized with 25% (v/v) NH_4OH. The DNR aglycone which is released from the conjugate is separated by reverse phase HPLC and quantified by fluorimetry. Conjugates with molar ratios of 2.0 and 3.5 for respectively the specific and the control MAb were used for the cytotoxicity experiments.

Immunoreactivity of the conjugates. Confluent cultures of MCF-7 cells (*ca.* 2×10^6 cells in 25 cm^2 flasks, Becton Dickinson, Lincoln Park, NJ) were incubated for 90 min at 4° in 2.5 ml of culture medium containing or not ("standard") different concentrations of either the native unlabelled specific MAb or of the drug–MAb conjugate. At the end of this incubation, the cells were washed once at 4° with culture medium and three times with PBS and then reincubated for 90 min at 4° with 2.5 ml of culture medium containing 1 μg/ml of ^3H labelled specific MAb. Cells, preincubated in the absence of

unlabelled MAb were used to evaluate the maximal binding capacity of ^3H labelled MAb by the cells.

At the end of the experiment, cells were washed once with culture medium and three times with PBS, solubilized with 1 ml of 1% (w/v) sodium deoxycholate adjusted to pH 11.3 followed by disruption by sonication (Sonifier B12, Branson Sonic Power Co, CT) and assayed for protein content using bovine serum albumin as standard [21]. Cell associated radioactivity was determined after dispersion of the samples in aqualuma cocktail (Lumac Systems, Basle, Switzerland) in a Tri Carb 460 CD scintillation counter (Packard Instruments, San Diego, CA) with automatic corrections for sample quenching.

In vitro stability of the conjugates. Drug-specific MAb conjugates, at a final concentration of 2.8 μg/ml of DNR or 13.9 μg/ml VDS were incubated at 37° in the presence of 1 mg/ml of lysosomal enzymes purified from rat liver [22], in 0.1 M acetate pH 4.8 containing 10 mM cysteine. After different durations, aliquots were analysed. For the DNR conjugate, the amount of DNR released during the incubation was determined by fluorimetry after extraction of the free drug at pH 9.2 with chloroform–methanol (6:1; v/v) and separation of native drug and fluorescent metabolites by HPLC. For the VDS–MAb conjugate, protein was first precipitated by 15% (w/v) trichloroacetic acid; supernatants were then assayed for the presence of radioactive drug. As a control, similar experiments were carried out in the absence of lysosomal enzymes.

Cytotoxicity tests. MCF-7 or Hep-G2 cells were seeded at 5×10^5 cells/25 cm^2 flask; after 24 hr culture, they were incubated for different durations at 37° in 2 ml of culture medium containing or not different concentrations of drug–MAb (specific or control) conjugates or of the equivalent amount of free drug or native unconjugated MAb. At the end of the incubation, cells were washed three times with PBS and, for some experiments, reincubated for different durations in fresh culture medium and then washed as above. After 6 days, at the end of incubation(s) and washing(s), cells which, in the absence of drug, reach near confluency were harvested in Na deoxycholate, homogenized by sonication and assayed for protein content as above. Results are presented in the form of percentage of cell associated protein for drug and/or MAb treated cells over that of cells incubated in the absence of drug and/or MAb.

RESULTS

Selection of clones from MCF-7 cells

The cytochemical analysis of the presence of the antigens recognized by the specific MAb, indicates that five clones derived from the parental MCF-7 line express homogenously the epitope, after two cycles of cloning (Table 1). Among these clones, only slight differences were detectable and the picture observed was similar to those previously reported [5].

In parallel, cells at the same passage were used to compare the uptake of the ^3H labelled MAb. The results presented in Table 1 indicate that, for the five subclones, comparable values were observed for cell accumulated labelled material and labelled digestion products in culture medium and, therefore, for the resulting uptakes; these values were systematically higher than those obtained for the parental line.

On the basis of these data showing that the uptake of the MAb is slightly higher by cells of clone 3 as well as for cell culture easiness, this particular clone was selected and systematically used for all the other experiments reported in this paper.

Conjugation of drug to MAb

VDS and DNR were covalently linked to MAbs, either, for DNR, as already published [20, 23] or, for VDS, as adapted from a method described for serumalbumin [19]. In both cases, molar ratios lower or equal to 3.5 (DNR) or to 6.2 (VDS) were attained. Raising the excess of drug over the lysine contents of IgG does not increase drug/antibody ratios and,

Table 1. Expression of the antigen and uptake of the MAb by parental MCF-7 cells and deriving clones

		Parental line	Clones				
			1	2	3	4	5
% of positive cells* (cytochemistry)		94.2 ± 1.2	99.2 ± 1.2	100 ± 0.0	99.7 ± 0.3	97.7 ± 1.8	100 ± 0.0
Uptake of the MAb† (μg/mg cell protein)							
	accumulation	3.11 ± 0.68	3.77 ± 0.28	3.68 ± 0.60	4.55 ± 0.05	2.53 ± 0.04	3.34 ± 0.10
	digestion	0.70 ± 0.58	2.07 ± 0.16	2.12 ± 0.05	1.74 ± 0.14	2.71 ± 0.16	1.87 ± 0.16
	uptake	3.81 ± 1.26	5.85 ± 0.45	5.81 ± 0.46	6.29 ± 0.19	5.24 ± 0.21	5.17 ± 0.26

* Confluent MCF-7 (2×10^6 cells in 25 cm^2 flasks) were incubated for 24 hr at 37° in the presence of 10 μg of the specific MAb ml of culture medium, washed three times with PBS, fixed with 4% (v/v) formaldehyde in PBS and treated for 4 min with Triton X100 1% (v/v) in PBS at room temperature. The cells were reincubated for 3 hr at room temperature with rabbit anti mouse IgG conjugated to peroxidase and the peroxidase activity was revealed as previously described [5]. Antigenic expression was evaluated by counting the proportion of stained cells in five different optical fields (minimum 100 cells/field).

† Confluent MCF-7 cells were incubated for 24 hr at 37° in the presence of 10 μg of the specific ^3H labelled MAb/ml of culture medium, washed and analysed as previously described [5]. At the end of the incubation, the amounts of cell associated labelled material (accumulation), labelled material soluble in trichloracetic acid present in the culture medium (digestion) and sum of these two values (uptake) were determined. Mean results of three independent experiments ± SD.

furthermore, resulted upon gel filtration in lower yields of monomeric IgG-drug conjugates. These results indicate that above a number of *ca.* 6–7 VDS/IgG or 3–4 DNR/IgG, the MAb easily aggregates.

Although no direct evidence is available, the covalent linkages between the drugs and the IgG molecule should localize within the Fc part of the molecule; molar ratios drug/immunoreactive Fab fragment are indeed much lower (unpublished results).

Immunoreactivity of the drug–MAb conjugates

In order to evaluate whether the immunoreactivity of the specific MAb has been preserved after conjugation to DNR or VDS, MCF-7 cells were incubated in the presence of different concentrations of either unconjugated or conjugated MAb and then reincubated with ^{3}H labelled unconjugated MAb. The results presented in Fig. 1A indicate that the DNR–MAb conjugate inhibits the binding of the ^{3}H labelled MAb as well as the native unconjugated and unlabelled MAb. This strongly suggests that the conjugate maintains entirely its immunoreactivity for the epitope exposed at the cell surface of the MCF-7 cells.

In contrast, for the VDS-MAb conjugate, as illustrated in Fig. 1B, the maximal inhibition of the binding of the ^{3}H labelled MAb reaches only 56% of that obtained with the unconjugated MAb. This strongly suggests that the chemical conjugation of VDS to the MAb induces a partial loss of the antibody immunoreactivity.

In vitro *hydrolysis of drug–MAb conjugates*

In the first set of experiments, both conjugates were incubated for different durations at 37° in the presence of lysosomal enzymes at acidic pH. The amounts of drug released from the conjugates as extractable fluorescent material separated by HPLC (DNR) or as ^{3}H label soluble in trichloracetic acid (VDS) were determined. As illustrated in Fig. 2, for the DNR–MAb conjugate, 60% of the drug covalently attached to the MAb at the beginning of the incubation are released after 24 hr in a process which is proportional to the duration of the incubation up to 6 hr but which then progressively slows down; released material consists almost exclusively of intact DNR. For the VDS–MAb conjugate, 33% of the radioactivity are released after 6 hr incubation but no further hydrolysis takes place thereafter; the specific radioactivity of VDS was too low to establish whether it is associated with native VDS and/or labelled metabolites. As control, it was established that no detectable amounts of both drugs are released in the absence of lysosomal enzymes.

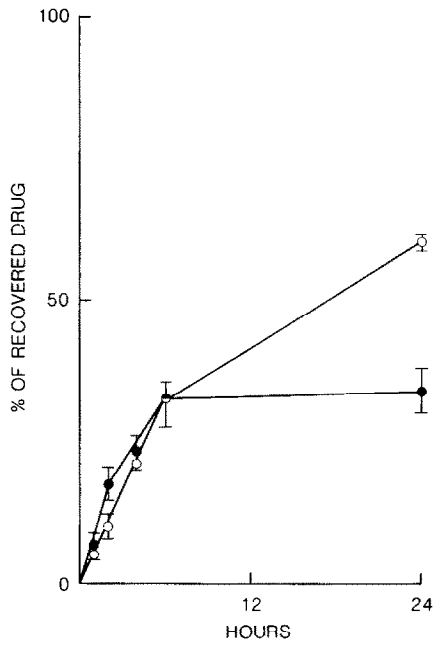

Fig. 2. Hydrolysis of the drug-MAb conjugates by lysosomal enzymes. The MAb conjugated either to DNR (○) or to VDS (●) were incubated for different durations at 37° in the presence of purified rat liver lysosomal enzymes. At the end of the incubations, the amounts of drug released by the enzymes were determined. Experimental procedures are described in Material and Methods. Mean results of three independent experiments ± SD.

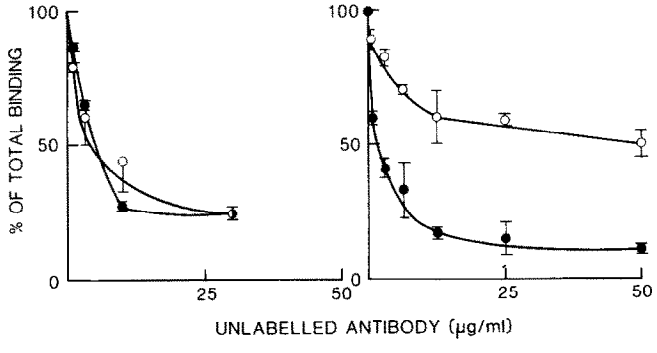

Fig. 1. Immunoreactivity of the drug–MAb conjugate. Confluent cultures of clonal population of MCF-7 cells were first incubated in the presence or absence of different concentrations of the unlabelled specific MAb unconjugated (●) or conjugated (○) to DNR (left) or VDS (right) and then reincubated with 1 μg/ml of ^{3}H labelled unconjugated MAb. Experimental procedures are described in Material and Methods. Results are expressed as the percentage of label associated with cells preincubated with unlabelled MAb (conjugated or not) over that associated with cells not preincubated with the unlabelled antibody ("standard"). Mean results of three independent experiments ± SD.

In a second set of experiments, both conjugates at final drug concentrations of respectively 3.1 μg/ml (DNR) or 15.4 μg/ml (VDS) diluted in PBS were incubated for different durations in 90% (v/v) of either fetal calf serum or fresh human plasma. No significant amounts of free DNR or VDS can be detected up to 6 days incubation at 37° (not illustrated).

Cytotoxic activity of the conjugates

In a first set of experiments, a clonal population of MCF-7 cells which express homogeneously the epitope recognized by the specific MAb and Hep-G2 cells which do not [6] were incubated for different durations ranging from 1 hr to 6 days with free DNR or VDS at concentrations ranging from 0.1 ng to 1 μg/ml. LD_{50}, defined as the free or MAb conjugated drug concentration in the culture medium required to decrease, after 6 days of culture, the amount of cell protein to 50% of that of cells incubated, in the same experiment, in the absence of free or MAb conjugated drug are given in Table 2. In addition, results are illustrated for one experimental condition for each drug in Fig. 3. Results indicate that LD_{50} range from 1.1 to 100 ng/ml according to the experimental conditions. During 6 days continuous incubation, LD_{50} of DNR and VDS are comparable on the two cell types, VDS being, however, from 13 to 62 times more cytotoxic than DNR.

In a second set of experiments, MCF-7 and Hep-G2 cells were incubated for 6 days in the presence of different concentrations of DNR or VDS conjugated to the specific MAb. As indicated in Table 2 and Fig. 4, the DNR–MAb conjugate has no cytotoxic activity on both cell lines till a drug concentration of 360 ng/ml of culture medium. In

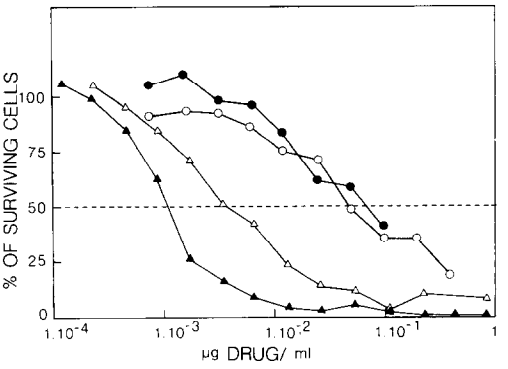

Fig. 3. Cytotoxicity of free DNR or VDS on MCF-7 and Hep-G2 cells. Adherent non-confluent cells were incubated for 6 days with different concentrations of free DNR or VDS. Experimental procedures are described in Material and Methods. Results are expressed as percentage of cell associated protein at the end of the incubation over that of cells incubated in the same conditions but in the absence of drug. Mean results of three independent experiments. (○), DNR on Hep-G2 cells; (●), DNR on MCF-7; (△), VDS on Hep-G2 cells; (▲), VDS on MCF-7.

contrast, the VDS–MAb conjugate exhibits a selective cytotoxic activity against MCF-7 cells (LD_{50} of 52 ng/ml), whereas it is not active on Hep-G2 cells even at a drug concentration of 872 ng/ml.

To further investigate this cytotoxic effect, MCF-7 cells were incubated either for different durations ranging from 1 hr to 3 days with the conjugate, followed by reincubation in a fresh conjugate-free medium up to a total incubation time of 6 days or for 6 days with VDS conjugated to a non-specific

Table 2. Cytotoxicity of free or MAb conjugated DNR or VDS on MCF-7 or Hep-G2 cells

Drug/MAb	Duration of incubation*/ reincubation† (hr)	LD_{50}‡ (ng/ml)		
		Hep-G2	MCF-7	
			experimental	corrected§
DNR/–	144/–	44	68	68
DNR/specific	144/–	>360	>360	>360
VDS/–	1/143	—	100	100
	3/141	—	62	62
	6/138	—	38	38
	24/120	—	19	19
	144/–	3	1	1
VDS/specific	3/141	—	>1000	>185
	6/138	—	800	148
	24/120	—	98	18
	72/72	—	45	8
	144/–	>872	52	10
VDS/control	24/120	—	>1000	>185
	72/72	—	>1000	>185
	144/144	—	>1000	>185

* Duration of exposure to the drug.

† Duration of reincubation in drug-free medium.

‡ LD_{50}: drug concentration in the culture medium required to decrease the amount of cell associated protein to 50% of that obtained in the same experiment for cells incubated in the absence of free or MAb conjugated drug.

§ LD_{50} value corrected by assuming that only 56% of the VDS-MAb conjugate keeps immunoreactivity (Fig. 1) and that only 33% of VDS can be released by lysosomal enzymes (Fig. 2).

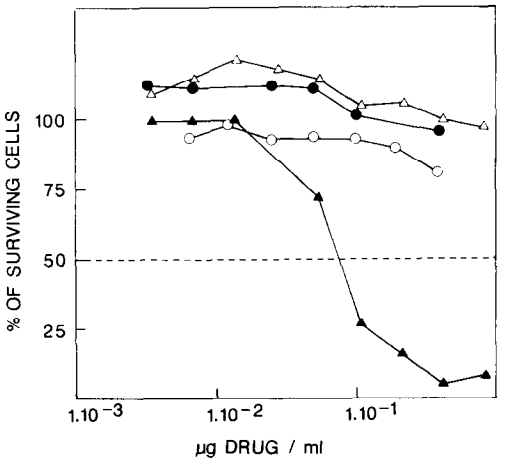

Fig. 4. Cytotoxicity of DNR or VDS conjugated to the specific MAb on MCF-7 and Hep-G2 cells. Same experimental protocol as in Fig. 3. ○, DNR-MAb on Hep-G2 cells; ●, DNR-MAb on MCF-7 cells; △, VDS-MAb on Hep-G2 cells; ▲, VDS-MAb on MCF-7 cells. Mean results of three independent experiments.

MAb or with the unconjugated specific MAb. LD_{50} values for all these experiments are given in Table 2 and for some of them illustrated at Fig. 5. These results indicate that neither VDS conjugated to the control MAb nor the unconjugated specific MAb are cytotoxic for the MCF-7 cells. In addition, they also show that the LD_{50} of the VDS-MAb conjugate is related to the duration of the exposure to the cells, 1 day being required to reach a significant effect.

DISCUSSION

The aim of this work was to investigate whether the covalent conjugation of an anticancer drug to a MAb could restrict its access to cells exposing the epitope recognized by the antibody, while preserving the cytotoxic activity. However, before performing the cytotoxic assay, it was necessary to verify the fulfilment of several conditions required by the lysosomotropic concept of drug targeting.

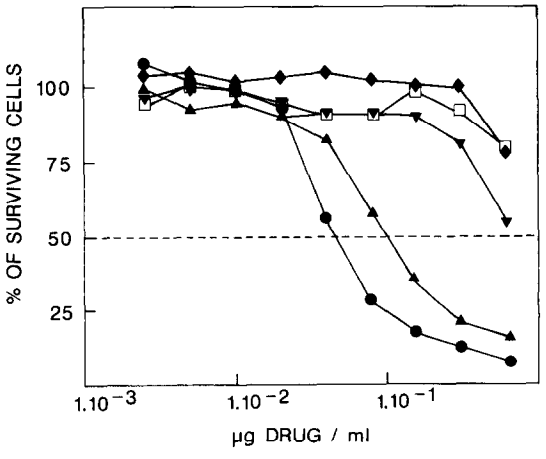

Fig. 5. Cytotoxicity of VDS conjugated to the specific or the control MAb on MCF-7 cells. Adherent non-confluent cells were incubated for 3 hr (◆), 6 hr (▼), 1 day (▲) or 3 days (●) with VDS-specific MAb or for 6 days (□) with VDS–control MAb. At the end of incubation with VDS–MAb, cells were washed and reincubated up to a final duration of 6 days in conjugate-free medium. Otherwise, experimental protocol as in Fig. 3. Mean results of three independent experiments.

First, every target cell should expose at its plasma membrane the epitope recognized by the MAb; otherwise, cells unable to bind the MAb would escape the detrimental effect of the conjugate. Occurrence of subpopulations within tumour cells has been described and, in particular, heterogeneity in the antigenic expression has been reported for MCF-7 cells [24]. To overcome this possible problem, MCF-7 cells were cloned and a subpopulation characterized by homogeneous expression of the epitope as well as by high uptake of the ^3H labelled specific MAb was selected (Table 1).

Secondly, after chemical linkage of the drug to the MAb, the conjugate should, on one hand, remain under the form of monomer and, on the other hand, preserve its immunoreactivity. The chemical reactions were optimized in order to provide the best yields of monomers with drug/IgG ratios as high as possible; maximal ratios, however, do not exceed 4 in the case of DNR and 7 for VDS. Binding assays (Fig. 1) indicate that the DNR–MAb conjugate behaves as the native MAb, strongly suggesting that it is still able to recognize the epitope. In contrast, the VDS–MAb conjugate inhibits the binding of the labelled unconjugated MAb by 56%, suggesting a partial loss of immunoreactivity after linkage to VDS.

Thirdly, according to our conceptual approach, the conjugate should, on one hand, be stable within extracellular medium and, on the other hand, be hydrolysed in the presence of lysosomal enzymes within lysosomes. Whereas both conjugates appear entirely stable in the presence of plasma or serum, part of the drug can be released after *in vitro* incubation in the presence of lysosomal enzymes at acid pH (Fig. 2). It is, however, unclear at the present time, why only a maximum of only 33% of VDS can be detached from the conjugate.

Finally, to obtain a clear interpretation of the *in vitro* assay of cytotoxicity, the conjugates should not be contaminated by even trace amounts of the free unconjugated drugs. To rule out this possibility, the activity of the conjugates was assayed in parallel on cells not recognized by the MAb but almost equally sensitive to the drugs.

The results of the cytotoxic assays (Table 2, Figs 3 and 4) show that whereas free DNR is active on both MCF-7 and Hep-G2 cells, the DNR–MAb conjugate does not exhibit any activity, even at an extracellular concentration of the MAb which allows saturation of the binding sites at the plasma membrane of MCF-7 cells [5] and which corresponds to a drug concentration largely higher than the LD_{50} of free DNR.

These results seem surprising since examples of active conjugates of DNR to antibodies have been reported. Furthermore, our data show that the conjugate keeps its immunoreactivity for MCF-7 cells, that DNR can be released from the conjugate under

the action of lysosomal enzymes and is not destroyed by these hydrolases [4]. The lack of activity could first result from the absence of endocytosis of the conjugate; however, the fact that DNR conjugated to galactosylated serum albumin is endocytosed by cultured hepatocytes or Hep-G2 cells [23] as well as the native neoglycoprotein does not support this hypothesis.

The absence of cytotoxic effect could also be due to the inability of the DNR–MAb conjugate to bring enough drug molecules within the cells; however, at the highest concentration of the conjugate (i.e. 50 μg MAb/ml corresponding to 360 ng DNR/ml), one should expect, on the basis of experiments with unconjugated MAb [5], that cells have taken up *ca.* 10×10^6 antibody molecules per cell allowing the release of *ca.* 20×10^6 DNR molecules per cell. Experiments with free DNR have, however, indicated that the accumulation of *ca.* 10×10^6 DNR molecules per cell give the LD_{50} during a 24 hr exposure (unpublished observations).

The absence of cytotoxicity of the conjugate could also result from the inability of DNR conjugated to the MAb to reach intracellular drug concentrations high enough to achieve cytotoxicity. Endocytosis and lysosomal hydrolysis of the conjugate are, indeed, slow processes, as compared to diffusion of free DNR through cellular membranes and, in addition, would be opposed by an active extrusion mechanism for anthracyclines [25]. Finally, although DNR is generally proposed to be active by intercalation between DNR bases resulting in a distortion of the double helix [26], it has recently been suggested that anthracycline cytotoxicity could be mediated by a membrane dependent mechanism [27]. In such a case, the use of a DNR-conjugate taken up by endocytosis should be inappropriate. It is, however, clear that these hypotheses remain largely speculative. Cell fractionation of cells incubated with the conjugate should allow us to establish the subcellular distribution of the MAb and the drug or related metabolites and provide the basis for an explanation to the lack of pharmacological activity.

In contrast, the VDS–MAb conjugate demonstrates cytotoxicity (Table 2, Figs 3–5). This activity is selective since it is restricted to cells able to take up the MAb [5, 6] and to a conjugate between the drug and the specific MAb. In addition, the possibility of a cytotoxic effect related to the presence of trace of free VDS contaminating the preparation may be ruled out: at the highest concentration used for the experiments (1 μg/ml of conjugated VDS), one may calculate that less than 0.3% contamination by free VDS should have been sufficient to reach LD_{50} for Hep-G2 cells.

The activity of the VDS—MAb conjugate appears lower than that of the free VDS (Table 2); figures should, however, be corrected for the actual drug concentration, on the basis of the immunoreactivity of the conjugate and of the proportion of the drug which can be released *in vitro* by lysosomal enzymes. Taking into account these corrections, results indicate that after long exposure of the cells to the conjugate (24 to 144 hr), its cytotoxicity becomes comparable to that of free VDS.

Embleton *et al.* [16] have described the cytotoxicity of a conjugate between VDS and specific MAb for the human osteogenic sarcoma 791 T. After 24 hr continuous exposure of the cells to the conjugate, the incorporation of ^{75}Se methionine was inhibited by 50% in the presence of 10 ng/ml of free VDS and 20 ng/ml of conjugated drug. The authors have calculated, at the LD_{50}, the accumulation of 15×10^9 antibody molecules per cell corresponding to 100×10^9 drug molecules. Assuming 2.2×10^6 epitopes per cell [28], they concluded that cytotoxicity resulted from non-specific uptake of the conjugate at supersaturating level.

In the case of our VDS–MAb, we may calculate that cytotoxicity is achieved at drug concentrations ranging from 45 to 98 ng of conjugated VDS/ml which correspond to MAb concentration ranging from *ca.* 2–4 μg/ml and to concentration of immunoreactive MAb ranging from *ca.* 1–2 μg/ml. Assuming that the VDS conjugated MAb behaves as the native one, on the basis of previous data [5], at these antibody concentrations there should only be specific uptake. On the basis of a molar ratio drug/MAb of 4.5 and to a release of 33% of the drug, this should correspond to the uptake ranging from *ca.* 0.5–1.0×10^6 MAb molecules per cell, i.e. of *ca.* 0.75–1.50×10^6 VDS molecules per cell.

Previous work from this laboratory has shown that the lysosomotropic model of drug targeting [2] is valid in the case of primaquine linked to asialofetuin [29] or of DNR linked to galactosylated serum-albumin [24]. The results reported in this paper clearly extend the validation of this approach to a conjugate between VDS and a MAb. A covalent drug–MAb conjugate fully stable in plasma or serum, partially hydrolysed by lysosomal enzymes and keeping partial immunoreactivity is able to kill entirely a clonal population of MCF-7, homogeneously recognized by the MAb. Conjugation of VDS to a MAb, although increasing the drug concentration required to achieve cytotoxicity or the duration of exposure of the target cells to the drug, entirely restricts the access of VDS to cells exposing the epitope recognized by the MAb. Nevertheless, it should be noted that a conjugate between DNR and the same MAb, which keeps entirely the immunoreactivity of the antibody and allows a better release of DNR is inactive. This points out that the pharmacological properties of the drug are very important and that drug targeting should probably be reserved to anticancer drugs active at low intracellular concentrations and which do not rapidly leak out of the cells.

Obviously, these results should be confirmed, on one hand, with conjugates constructed in such a way that well preserves the immunoreactivity and allows complete release of the drug. On the other hand, they should be extended to *in vivo* models of animals bearing tumors. *In vivo*, additional problems could result from the presence of anatomical barriers which could limitate the access of the conjugate to the epitope; however, higher plasmatic concentration of the drug could be maintained much easier in the case of the conjugate than with the free drug. In the case of positive results, such conjugates could be considered for the use in patients bearing tumors. However, in such a case, the use of human MAb

raised to epitopes whose presence is restricted at the accessible surface of the tumor cells, should probably be required.

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