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CHARACTERIZATION OF CYTOTOXIC ACTIVITY OF SAPORIN ANTI-GP185/HER-2 IMMUNOTOXINS

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The oncogene HER-2/*neu* encodes a trans-membrane receptor of 185 kDa with tyrosine-kinase activity. Over-expression of this molecule has been reported in a significant proportion of human breast and ovarian carcinomas, characterized by a poor clinical prognosis. Two monoclonal antibodies (MAbs), recognizing distinct epitopes of the gp 185 extracellular domain, have been utilized in the present study for the production of immunotoxins (ITs) by conjugation to the type-I RIP (ribosome-inactivating protein) plant toxin saporin 6 (SAP). These ITs have been shown to retain tumor-specificity and specifically to inhibit protein synthesis in the gp185$^{HER-2(+)}$ SK-BR-3 breast-carcinoma cell line with IC_{50} values lower then 1 nM. Kinetics of the cytotoxic activity of the ITs are characterized by a slow rate, since incubation times ranging from 24 to 60 hr, depending on the different degree of expression of the receptor, are required to determine >90% inhibition in the incorporation of radiolabeled leucine. However, the cytotoxic activity of these ITs, as evaluated by a more sensitive clonogenic assay, appears highly potent, since we have observed that 3 to 4 logs of cells are killed upon exposure to the ITs for short times at concentrations ranging from 1 to 5×10^{-8} M.

HER-2/*neu* is a proto-oncogene of the epidermal-growth-factor (EGF) family of tyrosine-kinase receptors. Similarly to the EGF receptor, the HER-2/neu gene product gp185^{HER-2} consists of 3 major domains characteristic of a trans-membrane receptor (Stern *et al.,* 1985). Indeed, a candidate ligand for gp185^{HER-2} has been reported (Peles *et al.,* 1992). Over-expression of the HER-2 oncogene with or without gene amplification has been shown to be associated with poor prognosis in patients bearing breast (De Potter *et al.,* 1990; Slamon *et al.,* 1989) and ovarian (Slamon *et al.,* 1989) carcinomas. Tissue-distribution studies performed with polyclonal or monoclonal antibodies to gp185^{HER-2}, have shown restricted expression of gp185^{HER-2} in normal human adult tissues (Press *et al.,* 1990; Natali *et al.,* 1990). Of major interest, these studies have demonstrated that expression of gp185^{HER-2} in primary tumors is almost invariably accompanied by the expression of the receptor in concomitant or subsequent metastases (Iglehart *et al.,* 1990). These findings clearly indicate that the gp185^{HER-2} may be a target of immunotherapeutic strategies.

To verify the feasibility of such an approach, we have conjugated 2 MAbs recognizing 2 distinct epitopes of the gp185^{HER-2} extracellular domain (Digiesi *et al.,* 1992), with the plant toxin saporin 6 from *Saponaria officinalis,* which requires less cumbersome purification procedures, is safer to handle, and is less cytotoxic both *in vivo* and *in vitro* (Stirpe *et al.,* 1983). This type-I RIP is endowed with advantageous properties such as absence of carbohydrate residues, extremely high pI (>9.5), which is likely to be effective in preventing degradation of the toxin in the endolysosomal compartment, absence of specific receptor structures on animal cells, and optimal stability and pharmacokinetic properties in the conjugated form (Stirpe *et al.,* 1983; Lappi *et al.,* 1985). Furthermore, saporin has been successfully utilized for the production of ITs to hematologic and solid tumors (Thorpe *et al.,* 1985; Siena *et al.,* 1988; Bregni *et al.,* 1988; Barbieri *et al.,* 1989; Tecce *et al.,* 1991*a,b*), which have shown potent cytotoxic properties associated with elevated target specificity. The results of a Phase-I clinical trial by Falini *et al.* (1992) which evaluated the effect of the systemic administration of an immunoconjugate with Saporin in patients with Hodgkin's disease have demonstrated a rapid anti-tumor effect and no serious toxicity, suggesting a potential therapeutic role for immunoconjugates using saporin 6 as toxin for clinical purposes.

We report the development of 2 ITs to gp185^{HER-2} and the analysis of their *in vitro* cytotoxic activity.

MATERIAL AND METHODS

Cell lines

The human breast-adenocarcinoma cell lines SK-BR-3, MDA-MB-365 and MCF7 were obtained from the ATCC (Rockville, MD). The T9-4 cell line derived from NIH 3T3 mouse fibroblast transfected with a plasmid containing a full-length HER-2/neu human cDNA (Di Fiore *et al.,* 1987) was kindly supplied by Dr. S.A. Aaronson (NCI, Bethesda, MD). All cell lines were grown in medium D-MEM (Flow, Irvine, UK) supplemented with 10% heat-inactivated FCS containing 2 mM L-glutamine.

Construction of immunotoxins

The 2 murine MAbs of the IgG_1 isotype, W6/800E6 and W6/900H1, recognizing 2 distinct epitopes of the gp185^{HER-2} extracellular domain, were obtained by immunization of BALB/c mice with the T9-4 cell line as described (Digiesi *et al.,* 1992). The 2 reagents, displaying an affinity of 7.68×10^9 and 3.26×10^8 respectively, were purified from ascitic fluids by DEAE Affi-gel blue chromatography as described by Tecce *et al.* (1991*a*). Fractions containing purified antibody, as judged by SDS-PAGE analysis, were dialysed against PBS, pH 7.2, and stored at −20°C. All protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL).

SAP, purified from seeds of *S. officinalis* (soapwort) by the method of Stirpe *et al.* (1983) with modifications (Lappi *et al.,* 1985), was kindly provided by Dr D. Trizio (Farmitalia, Nerviano, Italy). Chemical conjugation of W6/800E6 and W6/900H1 MAbs to SAP was carried out according to methods described by Thorpe *et al.* (1985) using the heterobifunctional cross-linker N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions. The degree of derivation was 2.04 (W6/800E6) and 1.72 (W6/900H1) moles SPDP/mol MAb, while the degree of derivation of SAP was 1.16. Conjugation was performed following reduction of derived SAP with 20 mM DTT and recovery of thiol derivative by gel filtration on Sephadex G25. The conjugation reaction was monitored by measurement of O.D. absorbance at 343 nm. Immunotoxin molecules were purified by gel chromatography on a Sephacryl S-300 column and their purity assessed by SDS-PAGE; ITs were visualized as 2 protein bands of apparent molecular weight of about 185 KDa and 215 KDa respectively, and of almost equal intensity (data not shown).

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Ribosome inactivation in presence of ITs was evaluated by measuring incorporation of radiolabeled methionine in a nuclease-treated rabbit reticulocyte lysate (N150, Amersham, Aylesbury, UK) using tobacco-mosaic-virus RNA as standard message. Serial dilutions of unreduced IT were added to incubation mixtures containing 2.5 μCi ^{35}S-methionine (Amersham). Samples were incubated for 45 min at 30°C and then processed according to the manufacturer's instructions.

Two different irrelevant ITs containing the saporin emitoxin, the first one, Ep2/SAP, directed to a melanoma associated antigen and the second one, LAM3/SAP, directed to a monocyte-specific determinant, have been utilized as toxin-matched controls of cytotoxic specificity and have been described in detail elsewhere (Tecce *et al.*, 1991*a,b*).

Binding assay

To compare the degree of expression of gp185^{HER-2} in the different cell lines, the MAb W6/800E6 was radiolabeled with ^{125}I using the chloramine-T method at a specific activity of 1.6×10^6 cpm/μg MAb. HER-2 gp185 $^{(+)}$ or $^{(-)}$ cells (2×10^5) were plated in U-bottom 96-well microtiter plates and incubated for 45 min at 4°C with 0.15 μg radiolabeled MAb. At the end of the incubation, cells were washed 7 times in PBS and counted in a gamma counter. Results were expressed as mean cpm from triplicate samples.

Serological studies

Reactivity of purified MAbs and ITs with transfected and human tumor cell lines expressing various levels of gp185^{HER-2} was analyzed by indirect immunofluorescence (IIF) on cell suspensions using as second antibody an affinity-purified FITC-labeled F(ab')$_2$ rabbit antiserum to mouse IgG (Cappel, Teknika, Tuznhout Belgium). Fluorescence intensity and distribution were evaluated by flow cytometry using FACStar equipment (Becton Dickinson, Mountain View, CA). Statistical analysis of quantitative data obtained by flow cytometry was performed utilizing PHARMA/PCS software (Springer, New York).

Cytotoxicity assay

Cells were seeded (2×10^4/well) in flat-bottom 96-well microtiter plates (Costar, Cambridge, MA) and 24 hr later serial 10-fold dilutions in complete medium of MAbs, the corresponding immunotoxins and free saporin were added in triplicate wells and incubated 4 hr at 37°C. Plates were then washed twice with PBS containing 5% FCS, and the cells were incubated in complete medium for an additional 20 hr, after which 1 μCi ^{3}H-leucine (Amersham) was then added to each well. After 18-hr incubation and extensive washing with culture medium, cells were harvested following EDTA treatment, spotted onto 3 MM (Whatman, Maidstone, UK) chromatography paper dishes processed as described (Tecce *et al.*, 1991*a*) and counted in a liquid scintillation counter. Results were expressed as mean cpm from triplicate wells. Analysis of the kinetics of the protein synthesis inhibition by ITs was evaluated following the same protocol and incubating cells with IT for different time periods. At the end of the incubation time, cells were pulsed for 1 hr with leucine-free medium containing 5 μCi/well ^{3}H-leucine, then chilled immediately on ice and processed as indicated above.

Clonogenic assay

SK-BR-3 cells grown at confluence were cultured for 1.5 hr in presence of different concentrations of W6/800E6, W6/900H1, the corresponding immunotoxins, the irrelevant immunotoxins Ep2/SAP and LAM3/SAP, and free saporin. At the end of the incubation, cells were washed with medium, detached from flasks with EDTA 0.01 M, re-suspended in complete medium and counted in a cell counter (Coulter ZM, Luton, UK). Control and treated cells were then plated in T25 flasks (4×10^3 cells/flask) and grown in complete medium. After 15 days the medium was aspirated and colonies were stained with 1% methylene blue for 3 min and counted. Treated cells were plated in triplicate, while 6 cultures of untreated control cells were plated as well. To obtain a quantitative assessment of the cytotoxic activity of the IT at the higher doses tested, treated cells were plated, always in triplicate, at higher densities ranging from 4×10^4 to 1×10^6/flask.

RESULTS

Serological reactivity of ITs

A preliminary radio-binding assay on live cells using ^{125}I-labelled MAb W6/800E6 to the extracellular domain of the oncogene product was performed to select cell targets expressing different levels of gp185^{HER-2}. High binding values were observed on the HER-2-transfected murine cells T9-4. Among the breast-carcinoma cell lines analyzed, SK-BR-3 cells displayed the highest binding; MDA-MB-365 cells gave low binding values, while the MCF7 were consistently unreactive (Table I). On the basis of this information, purified preparations of W6/800E6 MAb and the corresponding W6/800E6-SAP IT at different equimolar concentrations were assayed comparatively by FACS analysis on SK-BR-3 cells (Fig. 1). By linear regression analysis of the binding curves, a slope coefficient value of 0.886 for W6/800E6 MAb and 0.965 for W6/800E6-SAP IT was calculated. A parallelism test between the binding curves indicated a non-significant t value of 0.832 ($t95\% = 4.3$), demonstrating that the chemical conjugation of the MAb to the toxin does not alter its binding properties. Analogous results were obtained with the other MAb-IT pair (data not shown).

Inhibition of protein synthesis in breast-carcinoma cell lines by anti-HER-2 gp185 ITs

Figure 2 summarizes the dose-response curves of the cytotoxic activity of ITs as measured by ^{3}H-leucine incorporation in the cell targets differing for the degree of expression of gp185^{HER-2}. While the cell lines T9-4 and SK-BR-3 (Fig. 2*a,c*) with high density of the receptor were sensitive to the cytotoxic activity of the ITs, no significant inhibition of protein synthesis was observed with cell lines MDA-MB-365 and MCF-7 (Fig. 2*b,d*) expressing low or no level of gp185^{HER-2}. Furthermore, no inhibition of ^{3}H-leucine incorporation was detected in any of the cell lines incubated with unconjugated MAbs, at concentrations as high as 1×10^{-7} M (not shown). The cytotoxic activity of both ITs was partially blocked by co-incubation with a 10-fold molar excess of unconjugated antibody; as an example, in the T9-4 cell line MAb W6/800E6 reduced protein synthesis inhibition from 94% to 51% at an IT concentration corresponding to 7×10^{-8} M (Fig. 2*a*). Complete inhibition occurred at 100-fold molar excess of antibody, as exemplified in the SK-BR-3 cell line, where protein synthesis inhibition was reversed from 72% to 9% at a W6/800E6-SAP concentration of 1×10^{-8} M (Fig. 2*c*). Moreover, slightly higher cytotoxic activity (a 3-fold difference in IC_{50} value) was observed in cell line T9-4 with IT W6/800E6-SAP endowed with higher affinity ($IC_{50} = 4.3 \times 10^{-10}$ M), as compared with IT W6/900H1-SAP ($IC_{50} = 1.1 \times 10^{-9}$ M). Although cell lines

TABLE I – BINDING OF ^{125}I-LABELLED ANTI-gp185^{HER-2} MAb W6/800E6 TO DIFFERENT CELL LINES

Cell lines	(cpm × 10^{-5}/10^5 cells)[1]
T9-4	102.2
SK-BR-3	58.7
MDA-MB-365	5.5
MCF-7	1.4
NIH 3T3	1.2

[1]Values represent the mean of triplicate samples.

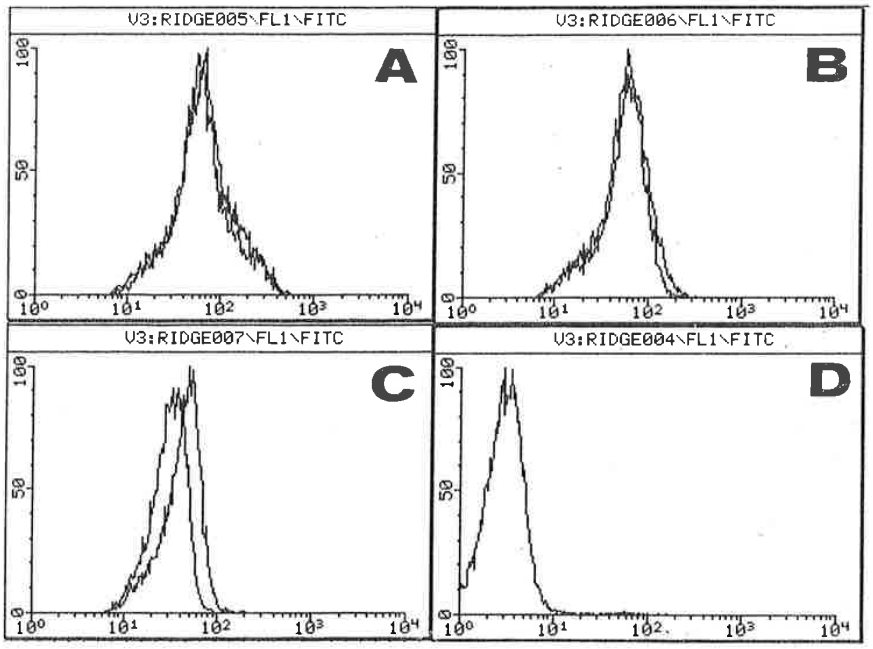

FIGURE 1 – Serological reactivity of W6/800E6/SAP and of parental antibody W6/800E6 with human breast-carcinoma SK-BR-3 cell line at different concentrations: *(a)* 0.50 μg/ml; *(b)* 0.10 μg/ml; *(c)* 0.02 μg/ml, evaluated by FACS analysis. Fluorescence profiles of MAb and corresponding IT coincide in *(a)* and in *(b)*, while in *(c)* MAb shows slightly greater reactivity than the IT. Fluorescence profile of SK-BR-3 cells not incubated with MAb or IT is represented in *(d)*.

T9-4 and SK-BR-3 displayed comparable sensitivity to free saporin and were both intoxicated by MAb-saporin conjugates, a complete block of protein synthesis was observed in the T9-4 cell line transfected with HER-2 gene (Fig. 2*a*) at an IT concentration of 7×10^{-8} M, while at the same dose a residual 26 to 28% ^{3}H-leucine incorporation was observed in the breast-carcinoma cell line SK-BR-3 (Fig. 2*c*).

Inhibition of anchorage-dependent growth by anti-HER-2 gp185 ITs assay

Because in the conventional 24-hr cytotoxicity assay the residual ^{3}H-leucine incorporation observed in SK-BR-3 cells could be indicative of incomplete killing of a cell subpopulation, we investigated the long-term effects of ITs by a different and more sensitive assay. Since anchorage-dependent *in vitro* growth of SK-BR-3 colonies was not influenced by the presence of unconjugated antibodies at doses ranging from 0.1 to 5.0 μg/ml (data not shown), we performed a clonogenic assay to measure the cytotoxic effects of the conjugate by evaluating colony growth following plating of a variable number of cells, ranging from 4×10^3 to 1×10^6. Results of this analysis are reported in Figure 3; it should be noted that the log of colony-number reduction has been determined by a conservative estimate, assuming that absence or reduction of *in vitro* growth (as observed and quantified experimentally) had to be corrected taking in account a plating efficiency value corresponding to 1 colony/400 cells seeded, even if in untreated control cells this value was >1/10 when plating >100 cells/flask. By these criteria we calculated that 1.5-hr treatment of SK-BR-3 cells with W6/800E6-SAP at a concentration of 5×10^{-8} M is capable of killing >3.65 log cells, while a 5-fold lower dose of W6/900H1-SAP kills 2.39 log of cells. This highly efficient toxicity is specific since (i) exposure to the unrelevant ITs Ep2/SAP and LAM7/SAP (1×10^{-8} M) or to free saporin 6 (1×10^{-7} M) had no significant effect on colony growth in SK-BR-3 cells and (ii) pre-incubation of the cells with a 100-fold molar excess of the unconjugated antibody resulted in almost complete suppression of cytotoxic activity of ITs.

Kinetics of immunotoxin activity

Because of the somewhat discordant results obtained on SK-BR-3 cell lines in the short-term protein-synthesis-inhibition assay and in the long-term clonogenic assay, we have analyzed the cytotoxic activity of the anti-gp185^{HER-2} ITs by evaluating the kinetics of protein-synthesis inhibition in the gp185$^{HER-2\ (+)}$ cell lines over a wide range of exposure times (Fig. 4). This analysis indicated that both cell targets were intoxicated at a relatively slow rate by the 2 ITs. While at 6 hr in the T9-4 cell line a residual 30% incorporation of ^{3}H-leucine was observed at 1×10^{-8} M IT concentration, 24-hr incubation indicated complete inhibition of protein synthesis at this same concentration (not shown). By contrast, in the SK-BR-3 cell line, longer incubation times (36 to 60 hr) in the presence of IT at the same molar concentration were required to attain complete inhibition of radionuclide incorporation (Fig. 5). In this cell line, protein synthesis inhibition proceeded very slowly during the first 6 hr of IT exposure, and 47% and 32% of reduction was attained in the presence of W6/800E6-SAP and W6/900H1-SAP IT respectively (Fig. 4). These slow kinetics, however, resulted in complete inhibition of protein synthesis, with no evidence of a minor fraction of cells escaping cell death, as likewise indicated by the clonogenic assay. It is noteworthy that at the same molar concentration employed with ITs, *i.e.*, 1×10^{-8} M, free saporin was able to reduce protein synthesis by only 50%, and this maximal cytotoxic activity was observed after 12 hr from exposure, while at longer incubation times no further increase in cytotoxicity was measured (Fig. 5).

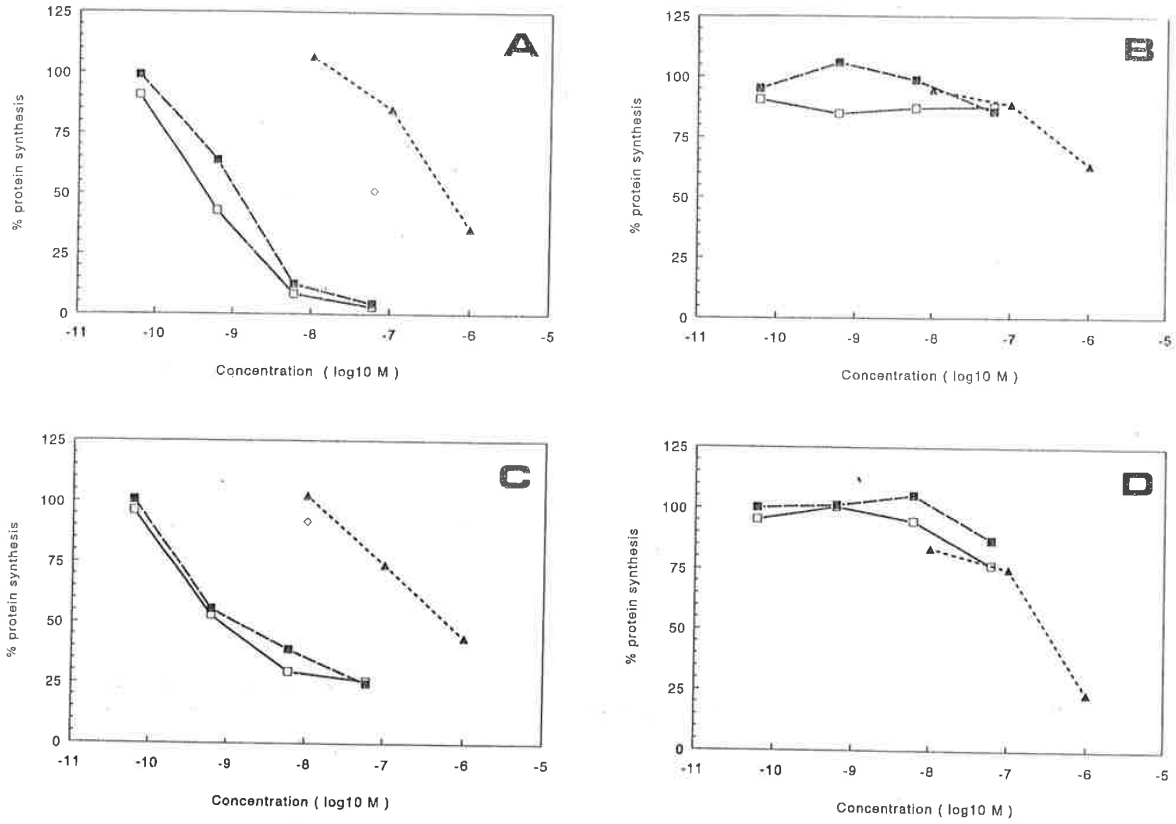

FIGURE 2 – Inhibition of protein synthesis by immunotoxins in *(a)* T9-4-transfected, *(c)* SK-BR-3, *(b)* MDA-MB-365 and *(d)* MCF-7 breast-cancer cell lines. Cells were incubated with different concentrations of W6/800E6/SAP in the absence □ or presence ◇ of *(a)* 10-fold or *(c)* 100-fold excess of unconjugated antibody, W6/900H1/SAP, ■, and SAP, ▲. Mean values of triplicate samples are expressed as percentage of controls.

DISCUSSION

The multidisciplinary approach to cancer treatment clearly justifies the current effort in developing therapeutic agents such as immunotoxins, which act through mechanisms different from conventional chemotherapeutic and radiotherapeutic regimens, may be highly tumor specific, and do not require host accessory factors.

This field has received renewed interest with progress in (i) the area of tumor radiolocalization demonstrating the ability of MAbs to act as specific carriers, (ii) improved technology in antibody production, and (iii) the emergence of valuable alternatives to the native A chain of ricin toxin, such as single-chain type-I RIP toxins (*e.g.,* saporin 6, gelonin and momordin).

A number of immunotoxins to breast and ovarian carcinoma utilizing different toxic moieties and different MAbs have been generated (Pirker *et al.,* 1985; Bjorn *et al.,* 1985, 1988; Yu *et al.,* 1990). The major constraints of these immunotoxins have been (i) limitations in the potential *in vivo* application due to their broad reactivity with normal tissues, as in the case of the anti-transferrin receptor immunotoxins (Pirker *et al.,* 1985), (ii) their reactivity with a low percentage of tumors (Bjorn *et al.,* 1985) and (iii) low levels of cytotoxicity despite prolonged incubation times (Bjorn *et al.,* 1988; Yu *et al.,* 1990).

The choice of saporin-6-like toxins as suitable for clinical uses has been demonstrated by Falini *et al.* (1992), who have been able to achieve a clinically objective response in a phase-I clinical trial conducted in Hodgkin patients using CD30-saporin IT.

The 2 antibodies to gp185^{HER-2} (Digiesi *et al.,* 1992), recognizing 2 distinct epitopes of the extracellular domain and lacking cell-growth-inhibiting activity, were selected in the present study for conjugation with saporin 6 taking into account a 10-fold difference in their affinity constants (*i.e.,* 7.68×10^9 mol/L for MAb W6/800E6 and 3.26×10^8 mol/L for MAb W6/900H1).

The conjugation procedure employed the heterobifunctional reagent SPDP at a low degree of substitution. This has allowed the generation of ITs that retain unaltered the binding properties of their parental MAbs.

Both ITs possess highly specific and potent cytotoxic activity by determining a 400 to 1000 fold increase in toxicity of unconjugated saporin 6. While in HER-2-transfected T9-4 fibroblasts, protein synthesis could be inhibited > 97%, in the breast carcinoma SK-BR-3 a residual 25% incorporation of radiolabelled leucine was observed at comparable concentrations of ITs, despite the fact that the 2 cell targets were shown by FACS analysis to express homogenously the gp185^{HER-2} in about 98% of the cell population. This has raised the question as to whether the incomplete block in protein synthesis could reflect a heterogeneous response of the tumor cells to IT exposure and whether this would result in major limitation of the potential therapeutic applications of the 2 ITs. In order to explore this possibility, 2 experimental approaches were uti-

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