Eur J Cancer Clin Oncol, Vol. 25, No. 4, pp. 633–640, 1989.
Printed in Great Britain

0277–5379/89$3.00 + 0.00

Doxorubicin–Anti-carcinoembryonic Antigen Immunoconjugate Activity *In Vitro*

VERNON J. RICHARDSON,* CHRISTOPHER H.J. FORD, GEORGIA TSALTAS and M.E. GALLANT

Oncology Research, Memorial University of Newfoundland and The Newfoundland Cancer Treatment and Research Foundation, Health Sciences Centre, St John's, Newfoundland, Canada A1B 3V6

Abstract—*An* in vitro *model consisting of a series of* 11 *human cancer cell lines with varying density of expression of membrane carcinoembryonic antigen (CEA) has been used to evaluate conjugates of doxorubicin (Adriamycin®) covalently linked by a carbodiimide method to goat polyclonal antibodies and mouse monoclonal antibodies to CEA. Conjugates were produced which retained both antigen binding and drug cytotoxicity.* IC_{50} *values were determined for free drug, free drug mixed with unconjugated antibodies and for the immunoconjugates. Cell lines that were very sensitive to free drug* (IC_{50} < 100 ng/ml) *were also found to be highly sensitive to conjugated drug and similarly cell lines resistant to drug* (IC_{50} > 1000 ng/ml) *were also resistant to conjugated drug. Although there was no correlation between CEA expression and conjugates efficacy, competitive inhibition studies using autologous antibody to block conjugate binding to cells indicated immunoconjugates specificity for the CEA target.*

INTRODUCTION

DOXORUBICIN is an extremely potent anti-cancer drug with response rates in a range of human tumours including lung, stomach, breast, sarcomas, some leukaemias and lymphomas. However, like all anti-cancer drugs it is not selective for just cancer cells and its use is limited by its toxicity to normal tissues such as bone marrow, mucous membranes and the muscle tissue of the heart. The drug's effect on the first two of these tissues is usually only temporary and they soon recover; however, the toxic effects on the heart are much more serious because they are cumulative and chronic. These effects are all dose dependent and severely restrict the maximum amount of drug that can be administered [1].

Two methods of approach are being used to try and overcome the toxic limitations imposed by this drug: firstly, the drug has been chemically modified in a search for less cardiotoxic derivatives [2] and secondly, drug carrier molecules are being tested which alter the tissue distribution of the drug and reduce the quantity of drug reaching the heart [3]. We have opted to use the latter approach and have selected anti-CEA antibodies as our carrier system, not just to potentially reduce the cardiac uptake of the drug but also to increase specificity by targeting the drug to the cancer cells which express CEA on their surface membranes. CEA is a glycoprotein found associated with many cancers including those of the lung, breast, colon, stomach and ovaries [4] (i.e. many that are also sensitive to doxorubicin).

Anthracyclines have been covalently conjugated to immunoglobulin carriers using a number of techniques: directly [5], indirectly via small spacer molecules [6] or indirectly via secondary carrier macromolecules [7–9]. We chose to initiate our studies using a direct conjugation method using a carbodiimide linkage procedure [5]. We have linked doxorubicin to both goat polyclonal (Pab) and to mouse monoclonal (Mab) (11-285-14) anti-CEA antibodies by this technique. This monoclonal antibody has been well characterized by us and by other groups and has been used for immunoscintigraphy in animals [10], cancer patients [11] and as a drug carrier *in vitro* [12] and *in vivo* with human tumour xenografts in nude mice [13].

The objective of the present study has been to evaluate the *in vitro* characterisics and efficacy of doxorubicin–anti-CEA antibody immunoconjugates on human tumour cell lines with a range of expression of the target antigen, CEA.

MATERIALS AND METHODS

CEA

CEA was affinity purified from colonic carcinoma liver metastases as previously reported [14].

Anti-CEA antibodies

Mouse monoclonal (11-28514) anti-CEA antibodies were produced from mouse ascites and pur-

Accepted 3 November 1988.
*Corresponding author.

ified using protein A Sepharose affinity chromatography as previously described [12]. The polyclonal antibody was obtained by ammonium sulphate precipitation of the serum of a goat which has been repeatedly immunized with purified CEA and was not absorbed with NCA. This antibody was kindly provided by J.A. Griffin, Surgical Immunology Unit, Birmingham, U.K.

Conjugation preparation

Doxorubicin used in the preparation of the conjugates was the pharmaceutical preparation Adriamycin. HCl (Farmitalia Carlo Erba, Milan, Italy) which contained lactose. Drug was conjugated to antibody using 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide, which links the sugar amine group of the drug to carboxyl groups on the antibody resulting in amide linkages [5]. Conjugated drug was separated from unconjugated drug on a Sepharose CL4B gel filtration column equilibrated with phosphate buffered saline (PBS) (pH 7.4). Conjugate fractions were pooled and concentrated by Amicon membrane filtration and filter sterilized through Millex GV (0.22 μm) filters (Millipore, Bedford, MA). Ultraviolet absorption at 280 nm and absorption at 495 nm was determined to estimate protein and drug concentrations respectively.

Enzyme linked immunosorbent assay (ELISA) testing of antibodies and conjugates

Antibody and immunoconjugate binding to CEA coated plates was compared using an ELISA as described previously [12]. Rabbit anti-goat IgG linked to horseradish peroxidase (HRPO) (Miles Scientific, Naperville, IL) was used in the assay of Pab and Pab-conjugates; rabbit anti-mouse immunoglobulin-HRPO (Dako Corporation, Santa Barbara, CA) was used in the assay of Mab and Mab-conjugates.

HPLC testing of conjugate

Conjugates were tested for free drug contamination using an Altec Econosphere 300 (5u) C18 reverse phase column (4.6 × 240 mm) with an isopropanol/water mobile phase containing 0.15% phosphoric acid and a gradient of 25–50% isopropanol over 20 min at a flow rate of 0.5 ml/min. Detection was either at 280 nm u.v. or by fluorescence at 470 nm exitation and 560 nm emission.

Tumour cell lines

Eleven human tumour cell lines were studied: colon carcinomas, COLO201, SW1116, WIDR, HT29, LOVO, LS174T and SKCO1; lung carcinomas BENN, CALU-3 and CALU-6; cervical carcinoma C33A. BENN was obtained from Dr M. Ellison at the Ludwig Institute for Cancer Research (Sutton, U.K.) all other cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown as monolayers in medium containing 10–16% foetal calf serum (FCS). Media used were Eagle's MEM (SKCO1, LS174T, WIDR, CALU3 and C33A), Dulbecco's Modified Eagle Medium (CALU6), RPMI 1640 (COLO201), L15 (SW1116), Medium 199 (BENN), McCoys (HT29) and Hamm's F10 (LOVO).

Characterization of CEA expression

The characterization of these cell lines for CEA expression by immunohistochemistry and binding studies using ^{125}I-labelled Mab has been reported previously [12].

Microcytostasis assay

A terminal uridine assay was used to determine the sensitivity of the cell lines to drug, conjugate or antibody [12]. Cell lines were grown until they were sub-confluent then trypsinized, washed in PBS and resuspended in fresh media. 10^4 viable cells in 100 μl of medium were plated out per well into 96-well microtitre plates. These were allowed to attach to the plates for 24 h at 37°C in a 5% CO_2 atmosphere. Doxorubicin, dissolved in sterile saline at 1 mg/ml, was used as the free drug control in each assay after sterile filtration through 0.22 μm Millipore filters. Because the drug binds to the sterilizing filters a small volume was removed aseptically, diluted in PBS (pH 7.4) and the drug concentration determined from the absorbance at 495 nm. Drug dilutions were prepared in culture medium (1–10,000 ng/ml). Medium was removed from the cells and drug dilutions were added in 100 μl of fresh medium. Tests were performed with 10 control wells (no drug treatment) and five wells for each drug dilution. Cells were exposed to the drug for 24 h at 37°C in 5% CO_2. Medium was removed and cells washed with PBS three times. One hundred microlitres of fresh medium was added to each well followed by a 24 h recovery period. Cells were then incubated for 3 h with 1 μCi of [^{3}H]uridine in 50 μl of medium and cell survival was determined from measurements of uridine uptake.

Competitive inhibition

For competitive inhibition studies a constant amount of autologous antibody was mixed with the conjugate dilutions. With Pab-conjugates, 2 mg/ml of Pab was added to the conjugate before incubation with the cells. With the Mab-conjugates, 1 mg/ml Mab was added to the conjugate before incubation with the cells. Because of the conjugate ratio and the concentration of antibodies available, maximum

ratios of antibody to conjugate of 30:1 and 2:1 were achieved at the IC_{50} values for the Pab- and Mab-conjugates respectively.

Analysis of data

Data were compared using the drug and conjugated drug 50% inhibitory concentrations (IC_{50}), which were determined for each of the cell lines from Probit plots of the survival curves [15]. Probit analysis linearizes survival curves at the region either side of the IC_{50} value. Then using linear regression analysis one can predict more accurately the IC_{50} concentration.

RESULTS

Separation of the conjugates from free drug was achieved with maximum drug to antibody molar ratios of 4.2 and 1.2 for Pab and Mab respectively. HPLC analysis revealed that there was no contamination of conjugates with free drug. Loss of antibody binding following conjugation was minimal, for both Pab- and Mab-conjugates as measured by ELISA.

Cell lines were divided into high, medium and low expressors of CEA from determinations of their membrane CEA. High expressors (SKCO1, LS174T, BENN and SW1116) had greater than 10^5 molecules of 11-285-14 binding per cell. Low expressors (C33A and CALU3) had less than 104 molecules of 11-285-14 binding per cell and medium expressors (LOVO, HT29, CALU6, WIDR and COLO201) had values between 10^4 and 10^5 molecules of 11-285-14 binding per cell [12].

A summary of the free doxorubicin IC_{50} values and ranges for the individual cell lines is shown in Table 1. Values ranged from 7 ng/ml to 10 μg/ml. For convenience of analysis cell lines were divided into three groups based on their sensitivity to doxorubicin: very sensitive lines with IC_{50} values below 100 ng/ml, sensitive lines with IC_{50} values between 100 and 1000 ng/ml and resistant lines with IC_{50} values above 1000 ng/ml. At the concentrations tested (2 mg/ml for Pab; 1 mg/ml for Mab) unconjugated antibody had no effect on any of the cell lines, and when mixed with drug the doxorubicin IC_{50} values were unaltered (data not shown).

The IC_{50} values for conjugated and free drug for 11 cell lines are summarized in Table 2. Cells were more sensitive to the free drug than to the conjugated drug with the exception of CALU6 which was found to be slightly more sensitive to the Pab-conjugated drug than to free drug. SW1116 proved to have the greatest resistance to both free and conjugated drug. The sensitivity of the cell lines to conjugated drug was always compared at the same time with free drug. Representative survival curves (probit plots) for cell lines with increasing resistance to doxorubicin (SKCO1 < LOVO < BENN) are illustrated in Fig. 1.

The kinetics of the probit survival curves indicates that, relative to the free doxorubicin curves, most of the cell lines are less sensitive to conjugate at high concentrations (i.e. they have diverging free and conjugated drug survival curves), e.g. LOVO (Fig. 1C, 1D). The exceptions were: SKCO1 with both conjugates (Fig. 1A, B) and CALU3 with the monoclonal conjugate (not illustrated) which all had converging survival curves, and BENN which had a converging survival curve with the polyclonal conjugate and a parallel survival curve with the monoclonal conjugate (Fig. 1E, F).

Scatter plots of the IC_{50} values for free doxorubicin vs. Pab-conjugate or Mab-conjugate IC_{50} values are shown in Fig. 2. A strong correlation between free drug and conjugated drug sensitivity of the cell lines was found. However, comparison of the slopes of the two curves does indicate that cells were more sensitive to the Mab-conjugate than to the Pab-conjugate. Scatter plots with the free drug or conjugated drug IC_{50} values and cell membrane CEA expression indicated no correlation between CEA expression or drug or conjugate sensitivity.

Competitive inhibition studies with unconjugated antibody are illustrated in Fig. 3A for Pab-conju-

Table 1. *Doxorubicin sensitivity of cell lines*

Cell line	Mean IC_{50} (ng/ml)	(Numbers of determinations)	Range (ng/ml)
BENN	2800	(5)	190–10,000
SW1116	1030	(2)	858–1200
CALU6	260	(3)	200–322
WIDR	217	(6)	27–450
HT29	157	(4)	16–350
LS174T	83	(19)	12–360
LOVO	60	(3)	22–110
C33A	51	(3)	32–60
COLO201	44	(5)	30–360
CALU3	38	(3)	7–100
SKC01	27	(12)	8–88

Table 2. Conjugate sensitivity of cell lines

Cell line	Doxorubicin IC_{50} (ng/ml)*					
	Passage†	Free	Pab-conjugated	Passage†	Free	Mab-conjugated
BENN	36	330	5000	66	190	800
SW1116	68	1200	10,000	—	—	—
WIDR	47	300	1000	52	450	1700
CALU6	9	200	150	9	260	620
HT29	27	80	500	14	350	620
LOVO	29	22	100	31	48	350
COLO201	16	84	220	24	360	1000
COLO201	19	30	60	27	150	340
LS174T	114	12	20	115	37	130
LS174T	119	38	54	115	57	100
LS174T	119	38	75	—	—	—
LS174T	120	300	610	—	—	—
LS174T	121	250	800	121	130	320
LS174T	122	360	1100	—	—	—
CALU3	—	—	—	119	7	15
C33A	—	—	—	14	60	750
SKC01	44	23	83	53	8	35
SKC01	46	27	73	55	8	39
SKC01	43	34	105	55	13	110
SKC01	—	—	—	58	17	205
SKC01	—	—	—	60	52	380

*Single determinations unless indicated. Free drug and conjugate tested in same assay.
†Passage numbers given for comparison, see text for details.

gate and Fig. 3B for the Mab-conjugate. In both a shift to increased cell survival was obsrved when competing antibody was mixed with the conjugate during the incubation period. The effect was greatest for the Pab-conjugate where a doubling of conjugate IC_{50} value was observed.

DISCUSSION

While our [12, 13] and other [16] work with vindesine–11-285–14 conjugates has been encouraging we wished to evaluate a drug which had a wider spectrum of activity in patients. We wanted a drug which was relevant to and active against tumours which express CEA, was clinically acceptable, potent and yet capable of benefiting from tissue specific targeting to reduce non-specific tissue toxicity. Doxorubicin fulfilled all of these criteria. We already had an *in vitro* model for screening efficacy and specificity of such conjugates and also a human tumour xenograft model for screening conjugates *in vivo*. This model consists of human tumour cell lines from lung, breast, colon and cervix with various densities of expression of membrane associated CEA. Our model has been well characterized in terms of quantification of target antigen, binding of carrier Mab and sensitivity to vindesine–11-285-14 conjugates [12, 13].

Using the carbodiimide method we were unable to obtain a conjugation ratio for the monoclonal antibody higher than 1.2 (moles drug per mole immunoglobulin) (Ig) without inactivation of binding to CEA. However, for the polyclonal antibody we did achieve a conjugation ratio of 4.2 which was similar to values previously reported by others for anthracyclines and Ig [5, 6].

The intra-assay variability of the microcytostasis assay was usually less than 10% although certain cell lines, noticeably COLO201, were found to give higher variations. Between experiments with the same cell line, but at different passage numbers, there was variation in the reproducibility of IC_{50} values (Table 1). For some lines (C33A, CALU6) these were small, while for others (e.g. COLO201) they were greater. The greatest variation in doxorubicin IC_{50} values was found with BENN cells, which may have been related to the difficulty in obtaining single cell suspensions for the microcytostasis assay. Also, with LS174T, sensitivity to doxorubicin appeared to decrease over 10 passages (see Table 2).

There was a very strong correlation between drug IC_{50} and conjugated drug IC_{50} values (Fig. 2) indicating that conjugation was not affecting the relative sensitivity or resistance of the cell lines.

Our data suggest that *in vitro* the conjugates were all less active than the free drug under the assay conditions employed. This does not appear to be unusual when our data are compared with other reports with vinca alkaloids [12, 16, 17] or anthracyclines [6]. However, while this might be disappointing at first glance it is not necessarily a reflection of *in vivo* potential. For example, while vindesine

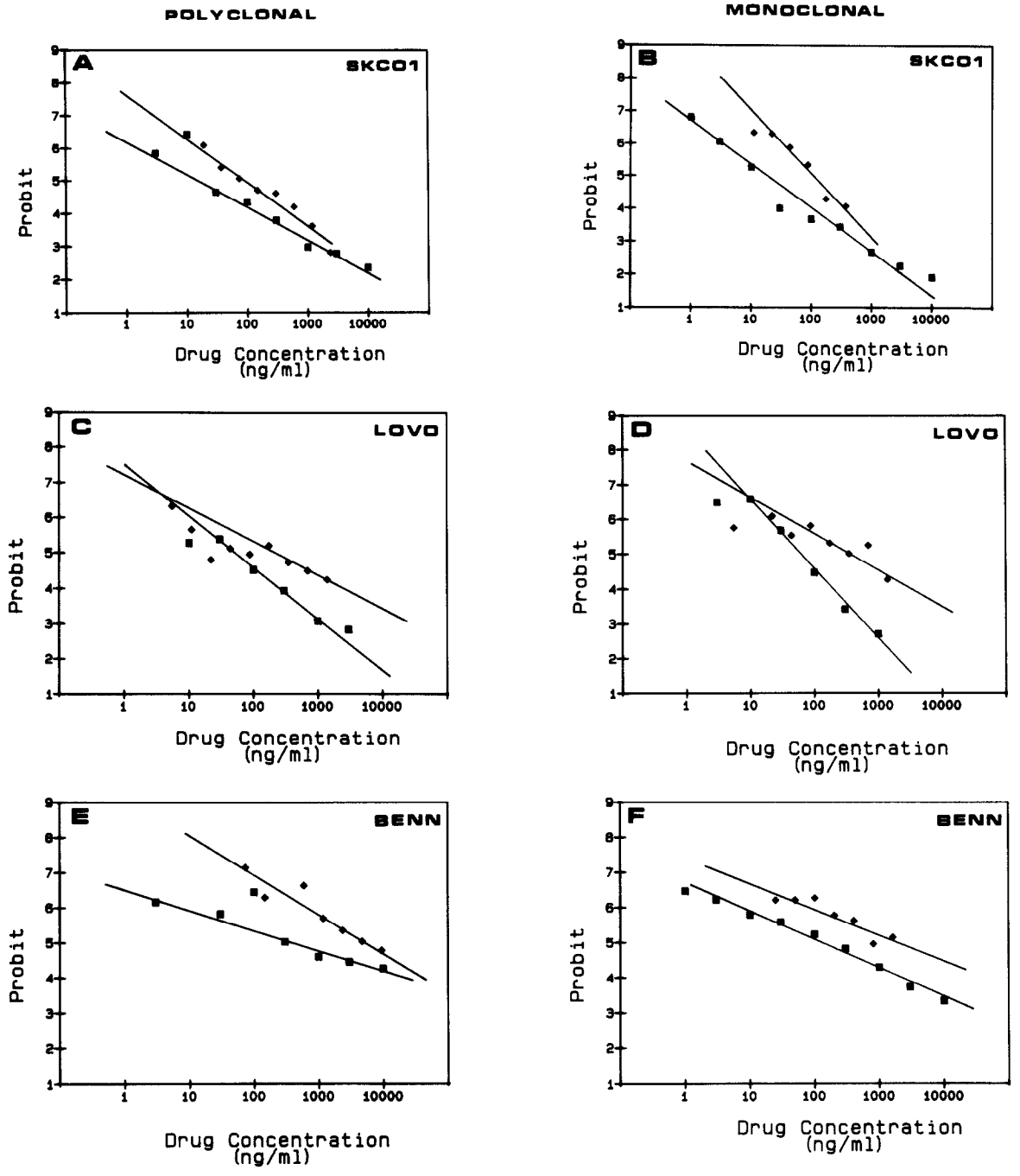

Fig. 1. Survival curves (probit scale) for SKCO1, high CEA expressor, very drug sensitive line (A,B); LoVo, medium CEA expressor, drug sensitive line (C,D); BENN, high CEA expressor, resistant line (E,F). ■ *Free doxorubicin;* ♦ *conjugated doxorubicin. A probit value of 5 is equivalent to 50% survival.*

conjugates have been shown to be less active *in vitro* than free drug [12], *in vivo* studies with the conjugates have demonstrated much less toxicity than free drug and ability to inhibit tumour xenograft growth [13, 16]. Methods of doxorubicin conjugation have been reported that yield conjugates which have been found to be more effective than free drug. These had an intermediate drug carrier molecule such as dextran [7], poly-L-aspartate [9] or the acid sensitive linker *cis*-aconitic anhydride [18] and had higher drug to antibody conjugation ratios than in our study. However, there have been no reports of direct linkage of doxorubicin to antibody and subsequent greater efficacy of immunoconjugates in comparison with free drug.

Data have been published showing that anti-CEA antibodies bind to target antigen on cell membranes both *in vitro* and *in vivo* and that this gets internalized [19]. The mechanism by which conjugated drug becomes active has not been fully evaluated, but, presumably drug is released from the carrier for cytotoxicity to occur, although some reports suggest that doxorubicin may be cytotoxic without having to enter cells [20, 21]. Recent data suggest that a

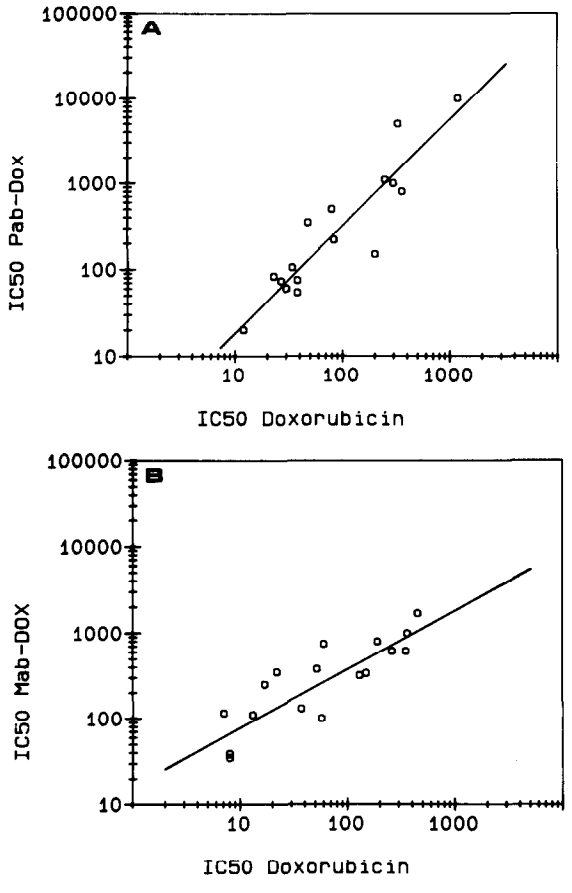

Fig. 2. Scatter plots of IC_{50} *free drug vs.* IC_{50} *conjugated drug. A. Pab, coefficient of correlation* 0.9210. *B. Mab, coefficient of correlation* 0.8475. *For both* $P < 0.0001$.

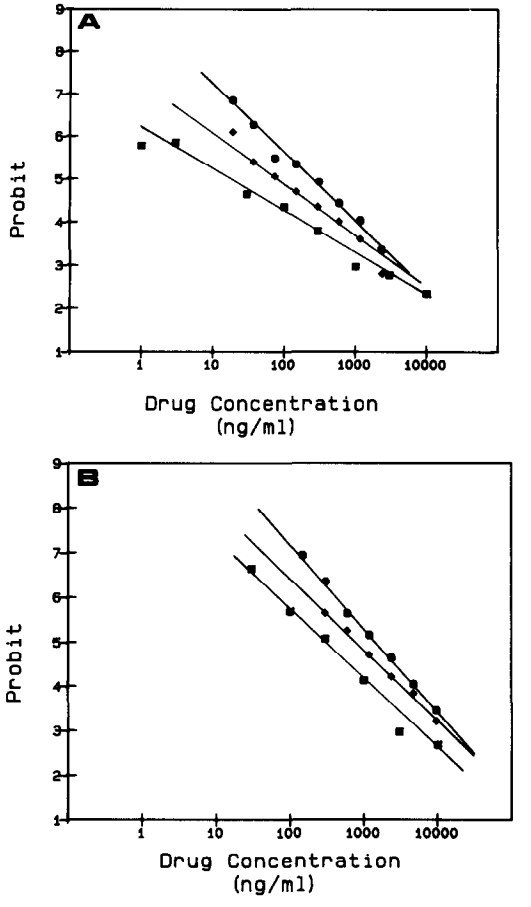

Fig. 3. A. Competitive inhibition (LS174T)—Pab-conjugate. ■ *Free doxorubicin;* ♦ *Pab–doxorubicin-conjugate;* ● *Pab–doxorubicin-conjugate* + 2 mg/ml *Pab. B. Competitive inhibition (SKC01)—Mab-conjugate.* ■ *Free doxorubicin;* ♦ *Mab–doxorubicin–conjugate;* ● *Mab–doxorubicin-conjugate* + 1 mg/ml *Mab.*

doxorubicin *cis*-aconityl linked monoclonal anti-melanoma conjugate does get internalized and that doxorubicin can be detected in the nucleus of treated cells within 2 h [18]. Evidence for the selectivity of conjugates in the present study comes from competitive inhibition results with both Pab- (Fig. 3A) and Mab- (Fig. 3B) conjugates where unconjugated antibody displaced the binding curves and resulted in higher IC_{50} values, indicating that conjugate efficacy requires target antigen binding.

Comparison of the two conjugates with free doxorubicin (Fig. 2A and 2B) indicates that the monoclonal conjugate is much more efficient than the polyclonal conjugate and is more effective against cell lines which are relatively doxorubicin resistant. This may be due to the greater specificity of the Mab-conjugate in comparison to the Pab-conjugate or could be due to different mechanisms of conjugate processing. However, the data also indicate that cell lines which have a very low expression of CEA, but which are highly sensitive to doxorubicin, appear to be highly sensitive to the conjugated drug. This suggests that either the conjugate is taken up, even by low CEA expressors, to a sufficient level for cytotoxicity to occur, that drug is released from the conjugate before being taken up by the cells or that doxorubicin is cytotoxic without having to enter the cells. This latter theory is not very likely as similar findings have also been observed with vindesine conjugated to 11-285-14 [12] and vindesine has not been reported to be effective without cell uptake. The kinetics of the survival curves which indicate that conjugated drug is, in most cases, not active by the same mechanism as the free drug makes the second mechanism less probable. The first explanation is therefore the most plausible one at present.

It is premature to try and interpret the varied types of survival curve kinetics for the different cell lines from our present data. However, we are addressing this by assessing some of the qualitative and quantitative parameters which influence the surface binding, internalization and shedding of the conjugate; the rate of turnover or re-expression of membrane CEA and the mechanism of processing and release of drug from the conjugate.

Surprisingly, antigen expression by the cell lines did not appear to influence immunoconjugate sensitivity directly under the assay conditions employed in this study, but, this may be better resolved by using shorter incubation periods with the conju-

gates. This may then permit specific and non-specific responses to be more clearly defined. Similar results were found *in vitro* with an anti-human T-cell monoclonal antibody, T101, linked via dextran to doxorubicin [22]. However, in that study difficulty in demonstrating *in vitro* specificity did not preclude demonstration of *in vivo* specificity of the immunoconjugates.

We have demonstrated *in vitro* Pab- and Mab-doxorubicin–anti-CEA immunoconjugate activity directly related to the drug sensitivity of the cell lines tested, but no correlation between sensitivity to the conjugated drug and the level of expression of membrane CEA. However, evidence to support the hypothesis of target specified drug carrier activity has been obtained in competitive inhibition studies where immunoconjugate cytotoxicity was inhibited by the use of autologous competing antibody. While our data are indicative of a targeting mechanism, further work is clearly warranted to elucidate more fully the mechanism(s) by which doxorubicin–anti-CEA immunoconjugates are effective and to determine whether use of an intermediate carrier will result in a higher drug to antibody conjugation ratio which will translate into greater efficacy.

Acknowledgements—These studies were supported by grants from the Faculty Research and Development Committee, Faculty of Medicine, the Newfoundland Cancer Treatment and Research Foundation and the Newfoundland Lung Association. We thank Ms. Gen Wight for assistance with the production of the manuscript.

REFERENCES

1. Wiernik PH. Current status of Adriamycin and daunorubicin in cancer treatment. In: Crooke ST, Reich SD, eds. *Anthracyclines. Current Status and New Developments*. New York, Academic Press, 1980, 273–294.
2. Johnson BA, Cheang MS, Goldenberg GJ. Comparison of Adriamycin uptake in chick embryo heart and liver cells and murine L5178Y lymphoblasts *in vitro*: role of drug uptake in cardiotoxicity. *Cancer Res* 1986, **46**, 218–223.
3. Rahman A, Kessler A, More N *et al.* Liposomal protection of Adriamycin-induced cardiotoxicity in mice. *Cancer Res* 1980, **40**, 1532–1537.
4. Shively JE, Beatty JD. CEA-related antigens: molecular biology and clinical significance. *CRC Crit Rev Oncol* 1985, **2**, 355–397.
5. Hurwitz E, Levy R, Maron R, Wilcheck M, Arnon R, Sela A. A covalent binding of daunomycin and Adriamycin to antibodies, with retention of both drug and antibody activities.*Cancer Res* 1975, **35**, 1175–1181.
6. Gallego J, Price MR, Baldwin RW. Preparation of four daunomycin–monoclonal antibody 791T/36 conjugates with anti-tumour activity. *Int J Cancer* 1984, **33**, 734–744.
7. Rowland GF. Effective antitumour conjugates of alkylating drug and antibody using dextran as the intermediate carrier. *Eur J Cancer* 1977, **13**, 593–596.
8. Garnett MC, Embeleton MJ, Jacobs E, Baldwin RW. Preparation and properties of a drug-carrier–antibody conjugate showing selective antibody-directed cytotoxicity *in vitro*. *Int J Cancer* 1983, **31**, 661–670.
9. Tsukada Y, Kato Y, Umemoto N, Takeda Y, Hara T, Hira H. An anti fetoprotein antibody–daunorubicin conjugate with a novel poly-L-glutamic acid derivative as intermediate drug carrier. *J Natl Cancer Inst* 1984, **73**, 721–728.
10. Macdonald F, Crowson MC, Allum WH, Life P, Fielding JWL. *In vivo* studies on the uptake of radiolabelled antibodies by colorectal and gastric carcinoma xenografts. *Cancer Immunol Immunother* 1986, **23**, 119–124.
11. Allum WH, Macdonald F, Anderson P, Fielding JWL. Localisation of gastrointestinal cancer with a ^{131}I labelled monoclonal antibody to CEA. *Br J Cancer* 1986, **53**, 203–210.
12. Ford CHJ, Bartlett SE, Casson AG, Marsden CH, Gallant ME. Efficacy and specificity of vindesine monoclonal anti-carcinoembryonic antigenic conjugate with nine human cancer cell lines. *NCI Monog* 1987, **3**, 107–116.
13. Casson AG, Ford CHJ, Marsden CH, Gallant ME, Bartlett SE. Efficacy and selectivity of vindesine monoclonal anti-carcinoembryonic antigen antibody conjugates on human tumor cell lines grown as xenografts in nude mice. *NCI Monog* 1987, **3**, 117–124.
14. Ford CHJ, Macdonald F, Griffin JA, Life P, Bartlett SE. Immunoadsorbent purification of carcinoembryonic antigen using a monoclonal antibody: a direct comparison with a conventional method. *Tumor Biol* 1987, **8**, 241–250.
15. Litchfield JT, Wilcoxon F. A simplified method of evaluating dose–effect experiments. *J Pharm Exp Ther* 1949, **96**, 99–113.
16. Rowland GF, Simmonds RG, Gore VA, Marsden CH, Smith W. Drug localisation and growth inhibition studies of vindesine–monoclonal anti-CEA conjugates in a human tumour xenograft. *Cancer Immunol Immunother* 1986, **21**, 183–187.
17. Embleton MJ, Rowland GF, Simmonds RG, Jacobs E, Marsden CH, Baldwin RW. Selective cytotoxicity against human tumour cells by a vindesine–monoclonal antibody conjugate. *Br J Cancer* 1983, **47**, 43–49.
18. Yang HM, Reisfeld RA. Doxorubicin conjugated with a monoclonal antibody directed to a human melanoma-associated proteoglycan suppresses the growth of established tumor xenografts in nude mice. *Proc Natl Acad Sci USA* 1988, **85**, 1189–1193.

19. Rosenthal KL, Tompkins WAF, Rawls WE. Factors affecting the expression of carcinoembryonic antigen at the surface of cultured human colon carcinoma cells. *Cancer Res* 1980, **40**, 4744–4750.
20. Tritton TR, Yee G. The anticancer agent Adriamycin can be actively cytotoxic without entering cells. *Science* 1982, **217**, 248–250.
22. Tokes ZA, Rogers KE, Rembaum A. Synthesis of Adriamycin-coupled polyglutcraldehyde microspheres and evaluation of their cytostatic activity. *Proc Natl Acad Sci USA* 1982, **79**, 2026–2030.
22. Dillman RO, Shawler DL, Johnson DE, Meyer DL, Koziol JA, Frincke JM. Preclinical trials with combinations and conjugates of T101 monoclonal antibody and doxorubicin. *Cancer Res* 1986, **46**, 4886–4891.