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Immunochemotherapy of human colon carcinoma xenografts in nude mice using combinations of idarubicin–monoclonal antibody conjugates

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Summary Tumour cell heterogeneity is probably a principal cause of treatment failure and represents a formidable barrier for effective antibody-targeted chemotherapy. Idarubicin (Ida), a more potent and less cardiotoxic analogue of daunomycin, has been demonstrated to specifically target and eradicate homogeneous, cloned, murine tumour cell populations *in vitro* and *in vivo* when coupled to monoclonal antibodies (MoAb); however, the antitumour activity of Ida–MoAb conjugates against human tumour xenografts remains to be established. In this study, the value of cotargeting conjugates to different human tumour-associated antigens within a solid tumour has been assessed by comparing the effects of combinations of Ida–anti-colon carcinoma MoAb conjugates with any one Ida–anti-colon carcinoma MoAb conjugate used alone. Individual Ida–MoAb conjugates have previously been evaluated for their specific binding and cytotoxicity to one of two different human colon carcinoma xenografts (Colo 205 or LIM2210) *in vitro*, although their efficacy alone or in combination required assessment *in vivo*. Combinations of the most effective Ida–MoAb conjugates were demonstrated to enable a greater number of complete tumour regressions than the most efficacious Ida–MoAb conjugate administered alone *in vivo*; some combinations inhibited control tumour growth by up to 95%. This study suggests that Ida–MoAb conjugates can be effective against subcutaneous human tumours in nude mice, although it is unlikely that any single conjugate will eradicate all the tumour cells in a solid tumour, and the value of 'cocktails' of drug-MoAb conjugates against some xenografts (i.e. LIM2210) appears to be limited.

Key words: idarubicin, immunochemotherapy, immunoconjugates, immunotoxins, monoclonal antibody, xenografts.

Introduction

Monoclonal antibody (MoAb)-recognizing antigens expressed selectively on human colon cancers are currently being evaluated for their potential to target diagnostic and therapeutic agents.[1] Many tumours, however, appear to be phenotypically unstable and consist of subpopulations of cells with significant heterogeneity in their antigen expression.[2] However, successful therapeutic antibody-mediated targeting of drugs to combat colorectal carcinoma depends on the stability and heterogeneity of antigen expression within the tumour. As heterogeneity and quantitative differences in antigen expression have been observed in primary tumours,[3] cloned tumour cells[4] and metastases,[5] it is important to establish the antigenic repertoire of tumour cells when designing effective antibody-targeting regimens.

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For small experimental tumours growing subcutaneously (s.c.), drug–MoAb complexes are particularly effective but large tumours can not be eradicated.[6] Some of the major problems lie in the inaccessibility of some areas of large solid tumours and the level of antigen expression of the tumour cells. Several studies using MoAb-targeted drugs have arrested the growth of human tumours in athymic nude mice,[7] however the constant trend of all tumours to produce phenotypic variants[8] has probably prevented their complete eradication. Immunological therapy of human breast tumours implanted in nude mice has previously demonstrated that 'cocktails' of MoAb were more effective in inhibiting the growth of tumours than any one MoAb alone.[9] It was therefore of interest to use a panel of several Ida–MoAb conjugates[6] targeted at a number of tumour-associated antigens on different human colon carcinoma cells to examine the efficacy of immunoconjugate therapy. In previous studies, Ida–MoAb conjugates were demonstrated to be effective in eradicating small murine tumours growing subcutaneously in inbred mice but not large tumours. We now report on the effects of several Ida–MoAb conjugates on human colon cancer xenografts.

Materials and methods

Tumour cells

The human colon carcinoma cell line Colo 205 was maintained *in vitro* in RPMI, supplemented with 10% heat-inactivated newborn calf serum (Flow Laboratories, Sydney, Australia), 2 mmol/L glutamine (Commonwealth Serum Laboratories [CSL], Sydney, Australia), 100 μg/mL streptomycin (Glaxo, Melbourne, Australia) and 100 IU/mL penicillin (CSL).[10] The murine thymoma ITT(1) 75NS E3 (E3)[11] was maintained in Dulbecco's modified Eagles medium (DME) with the same additives. For *in vivo* experiments, Colo 205 and LIM2210 (a fresh colon carcinoma recently excised from a patient at the Royal Melbourne Hospital, Australia, and established as a xenograft model in nude mice) were maintained by passage of s.c. tumours in Swiss nude mice. Mice were purchased from the Animal Resources Centre (Perth, Australia) and both female and male nude mice were grafted with human colon tumours at 7–8 weeks of age (bodyweight 16–25 g). Colo 205 tumours cells were injected s.c. and LIM2210 tumour were implanted s.c. (approximately 30 mm^3 pieces) into the abdominal wall and were allowed to develop into palpable tumours before commencing treatment. Mice were then subjected to a series of intravenous (i.v.) treatments, and the size of tumours was measured regularly with a caliper square measuring along the perpendicular axes of the tumours. The data were either recorded as mean tumour size (cm^2, two diameters) or mean tumour volume (cm^3, three diameters) ± s.e.m. Individual mice were monitored for their tumour growth and response to treatment. Groups of 8–10 mice, all of the same sex and age, were used in each experiment.

Monoclonal antibodies

The MoAb used were: (a) 250-30.6 (mouse IgG2b), which recognizes an antigen present on normal and malignant human gastrointestinal epithelium;[12] (b) 24-17.1 (mouse IgG2a) reactive with an antigen present on human colon and breast tumours;[13] (c) JGT-13 (mouse IgG1) reactive with carcinoembryonic antigen (CEA) on colon carcinoma but not with normal tissues; (d) 27.1 (mouse IgG1) reactive with human milk fat globule antigen (HMFG) on a number of colon tumours; (e) I-1 (mouse IgG1) reactive with CEA on many colon carcinomas but negative on normal tissues; and (f) anti-Ly-2.1 (IgG2a) used as a non-specific control antibody.[16] JGT-13, 27.1 and I-1 were made by J.G. Teh and C.H. Thompson (Research Centre for Cancer and Transplantation, Melbourne, Australia) and satisfy classification as anti-colon carcinoma MoAb. The MoAb were isolated from ascites fluid by precipitation with 40% ammonium sulfate, dissolution and dialysis in phosphate-buffered saline (PBS) and purified on an Affi-gel Blue column (Bio-Rad Laboratories, Sydney, Australia) and eluted with PBS. MoAb were then dialysed against PBS, aliquoted and stored at −70°C and tested for activity by rosetting with sheep anti-mouse immunoglobulin.[14] It should be noted that

none of these MoAb were cytotoxic *in vitro* or *in vivo*.

Preparation of Ida–MoAb conjugates

Intact MoAb (2–3 mg/mL) in 0.05 mol/L Borate buffer (pH 7.8–8.0) were mixed with a 10–20 mol/L excess of 14-bromo-4-demethoxydaunomycin (Br-Ida) dissolved in (*N*,*N*)-dimethylformamide (DMF) at 10 mg/mL. The reaction was maintained at room temperature for 4 h before centrifuging (400 *g* for 5 min) to remove any precipitate. Free Br-Ida and other unreacted starting materials were removed by gel filtration chromatography using a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden), and the conjugates were then passed through a column of Porapak Q to remove any adsorbed drug.[15] The amount of Ida incorporated in the conjugate (3–6 molecules of Ida/molecule MoAb) was determined by absorbance spectrophotometry at 483 nm ($\varepsilon_{483} = 3.4 \times 10^3$/mol/L per cm) and protein estimation;[16] further details are provided elsewhere.[6]

Drug activity

In a 24 h cytotoxicity assay, 100 µL of Colo 205 tumour cells ($2–5 \times 10^6$/mL) were added to a 96-well flat-bottomed microtitre plate and incubated for 1 h at 37°C.[16] Free Ida (prepared by dissolution in PBS) and Ida–MoAb conjugates were filtered sterile through a 0.22 µm Millipore filter and diluted in PBS; 50 µL of Ida or Ida–MoAb were added to the cells in duplicate; control wells received 50 µL of PBS and the cells were cultured at 37°C in a 7% CO_2 atmosphere for 24 h. In a 30 min inhibition assay, 200 µL of tumour cells ($2–5 \times 10^6$/mL) were collected in sterile Eppendorf tubes, resuspended in sterile conjugate and mixed for 30 min at 37°C. Cells were then centrifuged (400 *g* for 5 min), resuspended in growth medium, and 100 µL aliquots were seeded into a microtitre plate using quadruplicate wells/sample before an incubation period of 16–24 h. After the incubation period in both assays, 50 µL of medium containing 1 µCi of [^{3}H]-thymidine (specific activity 5 Ci/mmol; Amersham International Ltd, Amersham, England) was added, and the plates were incubated for 2–4 h; cells were then harvested onto a glass fibre filter paper using a cell harvester, dried for 10 min at 80°C and the individual samples were separated and counted on a β-scintillation counter. The incorporation of [^{3}H]-thymidine was expressed as a percentage of the inhibition of incorporation of controls; the standard error was generated by multiple determinations and did not exceed 5% for any given experimental point.

Flow cytometry

The reactivity of different MoAb against human colon carcinoma was assessed by flow cytometry using fluorescein isothiocyanate (FITC)-labelled MoAb and freshly prepared Colo 205 or LIM2210 tumour cells. Briefly, 80 µL of FITC solution (1 mg/mL in acetone)/mg of MoAb were coated as a fine layer in a glass bijou bottle. The MoAb (0.5 mol/L bicarbonate buffer, pH 9.0) was added and incubated in the dark at room temperature for 1 h. Free FITC was removed by gel filtration (PD-10, Pharmacia) and FITC–MoAb conjugates were quantitated spectrophotometrically.[17] LIM2210 and Colo 205 tumour cells were prepared from an excised s.c. tumour by gently breaking the tumour into small pieces, aspirating with a fine needle to a single suspension and washing three times in PBS with 1% bovine serum albumin (BSA). These cells (5×10^5) were added to Eppendorf tubes and incubated with 100 µL (10 µg MoAb) FITC–MoAb (3–4 mol/L FITC/mol MoAb) at 4°C for 1 h. Unbound antibody was removed by three washes with PBS (9000 *g* for 5–10 s) before being finally resuspended in 500 µL of fresh PBS with 1% BSA. Samples were run on a FACScan (Becton Dickinson, Mountain View, CA, USA), and data were recorded as the percentage of cells bound relative to a negative control (anti-Ly-2.1) MoAb (murine IgG2a antibody reactive with the murine Ly-2.1 alloantigen) ± the standard error (quadruplicate determinations) as calculated by a HP310 system (Hewlett Packard Australia Ltd, Melbourne, Australia).

Immunoperoxidase analysis

Analysis of MoAb reactivity was also assessed by immunoperoxidase staining of fresh

(250-30.6 and 24-17.1) and formalin-fixed (27.1, I-1 and JGT-13) LIM2210 tumour.[12] Purified antibodies were used at a concentration of 1 μg/mL.

Results

Previous *in vitro* (blocking with free MoAb) and *in vivo* (conjugates of irrelevant specificity) studies have demonstrated that the antitumour effects of Ida–MoAb conjugates depended primarily upon the antigen-binding specificity of the antibody.[6] The aim of these studies was to demonstrate that a combination of Ida–MoAb conjugates using different tumour-reactive antibodies could be more effective against an established tumour than any one tumour-reactive Ida–MoAb conjugate alone. Initially, therefore, a panel of Ida–MoAb conjugates had to be established with varying cytotoxicities and reactivities to the tumours of interest. The 24 h cytotoxicity assay was used to estimate the relative cytotoxicity of two different Ida–MoAb conjugates to Colo 205; however, this assay was not feasible using LIM2210 cells as the line cannot be established *in vitro*. Consequently, the relative reactivities of different MoAb with the LIM2210 tumour were tested using immunoperoxidase and flow cytometry to establish the various combinations of Ida–MoAb conjugates to be assessed *in vivo*.

Coupling of Ida to MoAb

Five conjugates composed of Ida and different MoAb were prepared at drug : antibody molar ratios of 3–6 : 1 following the establishment of suitable reaction conditions using different molar excesses of Br-Ida. At least 50% of MoAb activity[14] was maintained in all the conjugates containing less than six molecules of Ida/molecule of MoAb. For example, Ida-24-17.1 conjugates of four and six molecules of Ida/molecule of MoAb retained between 50 and 70% of the original unmodified 24-17.1 antibody activity, while the antibody activity was reduced at an Ida incorporation ratio of 15 (Fig. 1). Therefore, Ida–MoAb conjugates that were used *in vitro* and *in vivo* had between three and six molecules of Ida/molecule of MoAb.

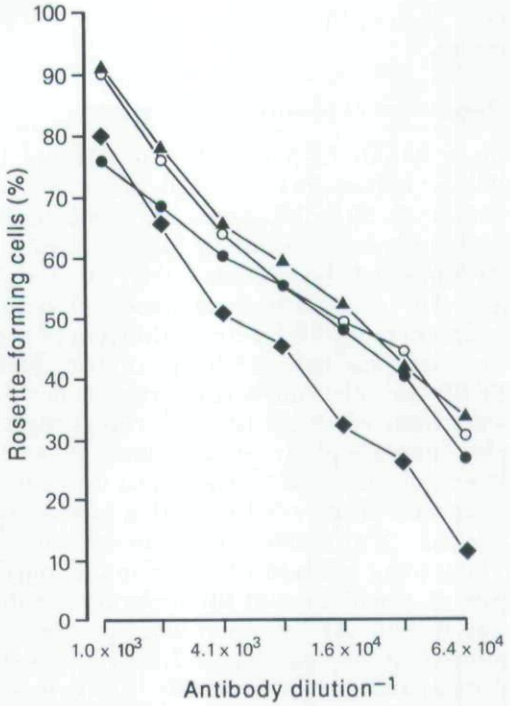

Fig. 1. Antibody titre (% rosette-forming cells) *vs* antibody dilution of 24-17.1 conjugates on Colo 205 target cells. Serial dilutions were performed upon a 1.0 mg/mL solution of either 24-17.1 (▲) or Ida-24-17.1, four molecules Ida/molecule conjugate (○) or Ida-24-17.1, six molecules Ida/molecule conjugate (●) or Ida-24-17.1, or 15 molecules Ida/molecule conjugate (◆).

Cytotoxicity of Ida–MoAb conjugates to Colo 205 in vitro

The *in vitro* cytotoxicity of Ida and Ida–MoAb conjugates were measured in the 24 h cytotoxicity assay, and IC_{50} values were determined on the reactive Colo 205 and non-reactive E3 tumour cell lines (Table 1). The IC_{50} for the Ida-24-17.1 conjugate was 11 times greater and four times greater for Ida-250-30.6 than that of free Ida. The selective cytotoxicity of both of these Ida–MoAb conjugates for antigen-positive Colo 205 cells was demonstrated by their 5–9-fold lower cytotoxicity on antigen-negative E3 cells. Therefore, both Ida-250-30.6 and Ida-24-17.1 conjugates had satisfactory *in vitro* cytotoxicity to use against human colon carcinoma Colo 205 xenografts in nude mice. The combined cytotoxicity of

Table 1. Cytotoxicity of idarubicin and idarubicin-MoAb conjugates: IC_{50} determinations using a 24 h cytotoxicity assay

Tumour	Ida	Mean IC_{50}(mol/L) Ida-250-30.6	Ida-24-17.1	Ida-anti-Ly-2.1
Colo 205	8.0×10^{-8}(5)	3.0×10^{-7}(3)	9.0×10^{-7}(3)	3.3×10^{-6}(3)
E3	1.0×10^{-7}(5)	2.6×10^{-6}(2)	4.5×10^{-6}(1)	4.3×10^{-7}(3)

IC_{50} = 50% inhibition of [^{3}H]-thymidine incorporation of controls. Parentheses indicate the number of preparations tested.

Ida-250-30.6 and Ida-24-17.1 conjugates was compared with that of either conjugate alone using a 30 min cytotoxicity assay (Fig. 2). Given that the Ida-24-17.1 conjugate ($IC_{50} = 6.0 \times 10^{-6}$ mol/L) was 6–7 times less cytotoxic than the Ida-250-30.6 conjugate ($IC_{50} = 9.2 \times 10^{-7}$ mol/L) against the Colo 205 cell line, the cytotoxic effect of a mixture of these two conjugates was at least additive. Indeed the IC_{50} of an equimolar mixture of Ida-250-30.6 and Ida-24-17.1 conjugates (1.5×10^{-6} mol/L) was comparable to that predicted by the sum of the individual conjugate growth inhibition curves ($IC_{50} = 2.0 \times 10^{-6}$ mol/L).

MoAb binding to colon cancer cells

Flow cytometry indicated that both 250-30.6 and 24-17.1 MoAb were reactive with the Colo 205 colon carcinoma binding 99 ± 1% and 94 ± 4% of the tumour cells, respectively. Quantitative analysis, however, demonstrated that the level of 250-30.6 antigen expression was approximately five times higher than 24-17.1 antigen expression on Colo 205 cells

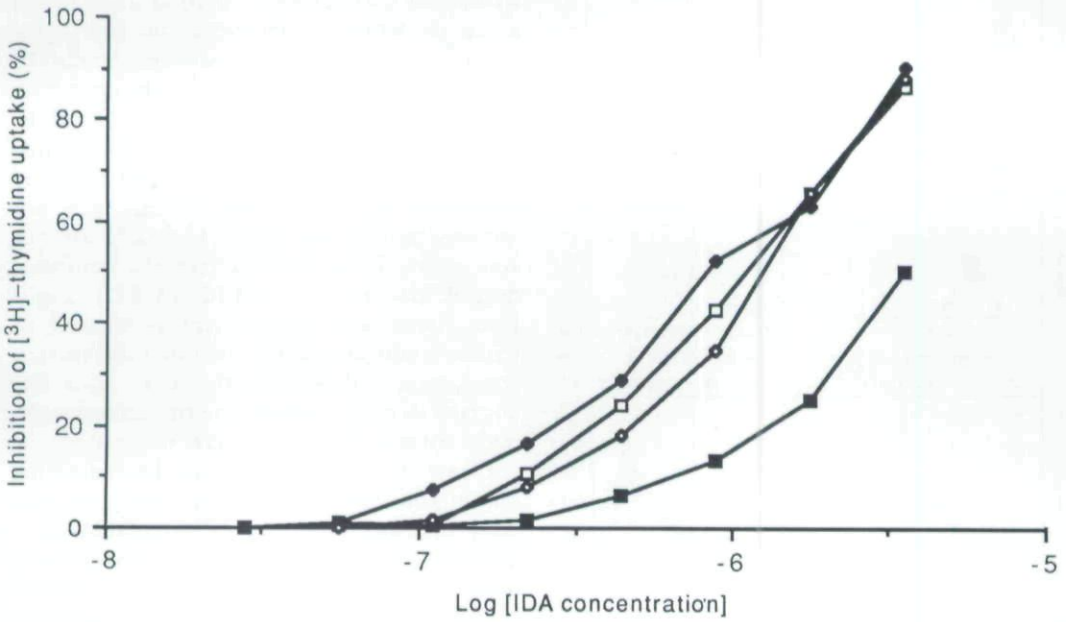

Fig. 2. The inhibitory effect of Ida-24-17.1, 5 mol Ida/mol conjugate (■), Ida-250-30.6, 5 mol Ida/mol conjugate (♦) or an equimolar mixture of Ida-24-17.1 and Ida-250-30.6 (□) on Colo 205 cells in a 30 min inhibition assay. For reference the predicted inhibitory effect of the mixture has been calculated (◇) and demonstrated presuming that the conjugate's cytotoxicities are additive.

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