A partner monoclonal antibody to Moab 730 kills 100% of DU145 and PC3 androgen-independent cancer cells

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A number of therapeutic options are available for patients with prostate carcinoma till the time that the tumour is hormone dependent. However, no fully effective therapy is available for the treatment of androgen-independent prostate carcinomas. Antibodies directed at epitopes unique to or overexpressed on the cancer cells could be of therapeutic utility. A monoclonal antibody (Moab) 2C4 has been generated, which binds with cells of two androgen-independent prostate cancers, DU145 and PC3, and does not bind to peripheral blood leukocytes (PBLs) of healthy donors. This antibody, along with the previously developed Moab 730, kills 100% of both DU145 and PC3 cells in the presence of complement and does not have a deleterious effect on PBLs of healthy males. The anti-tumour action of the two antibodies prevents the establishment of DU145 cell tumour in nude mice *in vivo*. Moab 2C4 in combination with 730 has potential for use as therapy for androgen-independent cancers.

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1. Introduction

While surgery, radiation therapy, treatment with luteinizing hormone-releasing hormone (LHRH) antagonists and other drugs are available for patients with prostate carcinoma up to the stage that the tumour is hormone dependent, no fully effective therapy is available for the treatment of androgen-independent hormone-refractory prostate carcinoma (HRPC). With the idea of developing a therapeutic antibody directed at such cancers, we made a monoclonal antibody (Moab) that causes lysis of DU145 androgen-independent carcinoma prostate cells in the presence of complement (Talwar *et al.* 2001). This antibody could, however, kill only about 80% of these cells, as the epitope recognized by this antibody was present only on about 80% of cells and not on all. Given that micro-heterogeneity exists in such cancers, an attempt was made to raise a complementary Moab.

We report a new monoclonal 2C4 which, in combination with the previously developed antibody, causes 100% killing of not only DU145 but also PC3 androgen-independent prostate carcinoma cells. This antibody does not bind to the peripheral blood cells of healthy donors of blood.

2. Materials and methods

2.1 *Cell lines*

Androgen-independent human prostate cells DU145 were obtained from the American-type cell culture (ATCC No.

Keywords. Antibody binding by FACS; complement-mediated lysis; cytotoxicity; differing epitopes; MTT

Abbreviations used: ADCC, antibody-dependent cell cytotoxicity; CDC, complement-dependent cytotoxicity; CFA, complete Freund adjuvant; DMEM, Dulbecco minimum essential medium; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; FITC, fluorescein isothiocynate; hCG, human chorionic gonadotrophin; HRPC, hormone-refractory prostate carcinoma; i.p., intraperitoneally; IFA, incomplete Freund adjuvant; Ig, immunoglobulin; LHRH, luteinizing hormone-releasing hormone; Moab, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBL, peripheral blood leukocyte; PBS, phosphate buffered saline; US FDA, US Food and Drug Administration

HTB-81). These cells were originally derived from a human prostate adenocarcinoma that metastasized to the brain by Stone *et al.* (1978). The cells are epithelial, grow in isolated islands on plastic Petri dishes, and form colonies in soft agar suspension culture. Another cell line of androgen-independent prostate cancer PC3 was also obtained from ATCC (ATCC No. CRL-1435). DU145, PC3 and myeloma fusion partner SP2/o were cultured in Dulbecco minimum essential medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotic–antimycotic mixture (penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin-B 250 ng/ml) (Gibco-BRL Life Technologies). The cells were subcultured before they reached 70% confluency. Myeloma cells SP2/o were treated with BM-Cycline (Roche Applied Sciences) for three weeks to ensure that these were free of *Mycoplasma*. Cells were harvested by treatment with trypsin–EDTA (0.05% trypsin, 0.02% EDTA), followed by centrifugation at 1250 rpm at room temperature for 5 min.

2.2 *Complement*

Prescreened class 1 rabbit complement obtained from Cedar Lane Laboratories, USA was reconstituted in MQ water followed by filtration through a filter with a 0.22 μm pore size. The concentration of complement for optimal lysis in the presence of excess of antibody was determined.

2.3 *Immunization of animals*

Cells, after washing with 10 mM phosphate buffered saline (PBS), pH 7.2, were resuspended at 10^6 cells/ml. BALB/c inbred mice were immunized intraperitoneally (i.p.) with DU145 cells (1×10^6 cells/ mouse) emulsified with complete Freund adjuvant (CFA). After 2 and 4 weeks, boosters comprising the same number of cells were given i.p. with incomplete Freund adjuvant (IFA). Animals that developed high antibody titres against the cells were injected intravenously with 10^6 cells in PBS 3 days prior to harvesting spleen cells for fusion.

2.4 *Hybridoma generation*

The splenocytes separated on Ficoll–Hypaque density gradient (Amersham-Pharmacia Biotech) were washed with PBS and fused with the SP2/o mouse myeloma cells at a 1:1 ratio in 50% (w/v) solution of polyethylene glycol 1500 (Sigma, St Louis) as per Kohler and Milstein (1975). The hybridized cells were selected in 96-well tissue culture plates in HAT medium (DMEM supplemented with 20% FCS, 1% antibiotic–antimycotic mixture, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine; Sigma, St Louis). Hybrid cells were screened for immunoreactivity with DU145 cells by solid-phase enzyme-linked immunosorbent assay (ELISA) as described below. These cells were cloned by limiting dilutions and sub-cloned three to four times to obtain stable cell lines secreting Moabs.

2.5 *FACS*

DU145 cells were harvested by treatment with trypsin–EDTA, centrifuged at 1250 rpm at room temperature for 5 min. The cell pellet was washed once with 10 mM PBS. 1×10^5 cells/tube was taken in 100 μl of FACS buffer (1% BSA, 0.2% sodium azide dissolved in 10 mM PBS). Culture supernatant of the hybrids was added and the tubes were incubated for 1 h at 4°C. Cells were washed with FACS buffer and stained with fluorescein isothiocynate (FITC)-conjugated goat anti-human immunoglobulin (Ig)G Fcγ (Jackson ImmunoResearch Laboratories) for 1 h at 4°C. Cells were washed and suspended in 0.5% paraformaldhyde solution in PBS and fluorescence assorted cell sorter (FACS) carried out in a Becton Dickinson LSR Model (Becton Dickinson, San Jose, CA). The data were analysed using the WinMdi software (version 2.9).

2.6 *Antibody isotyping*

Antibody 2C4 was classified for its isotype using the mouse Ig isotyping ELISA kit (BD Pharmingen™, BD Biosciences, USA).

2.7 *Antibody purification and quantification*

Purification of the Moab was carried out by affinity column chromatography. The culture supernatant was loaded on a Protein-A affinity column as per the manufacturer's instructions (Pharmacia Inc. Stockholm, Sweden). Elution was carried out with 0.1 M glycine buffer pH 2 and immediately neutralized to pH 7 using 1 M Tris pH 12. The eluate was dialysed against 10 mM PBS and concentrated in an Amicon ultrafiltration chamber using a 60 kDa cut-off membrane. The protein concentration was determined by the Micro BCA Kit, (Pierce [Thermo], USA).

2.8 *Western blot*

Lysates of DU145 and PC3 cells were prepared in buffer containing the detergent NP-40 and a protease inhibitor cocktail (Calbiochem, USA). SDS-PAGE was carried out by standard procedures and antibody-reactive moieties were visualized by enhanced chemiluminescence (Biological Industries, Israel), subsequent to western blotting on Hybond™ membranes (Millipore, USA).

2.9 *Immunofluorescence*

DU145 and PC3 prostate cancer cells (10^4/well) were seeded in a cell culture plate. Adherent cells were washed thrice with PBS and incubated with 10 μg/100 μl of purified antibodies for 1 h at 4°C. After washing with PBS, the cells were stained with FITC-conjugated goat anti-human IgG Fcγ (Jackson Immuno Research Laboratories, USA) for 1 h at 4°C. The cells were washed with FACS buffer and suspended in 0.5% paraformaldehyde. Images were recorded in bright field as well as by using a blue filter under a fluorescence microscope (Nikon, Eclipse TE2000-U, Japan).

2.10 *Cytotoxicity assay*

The intrinsic property of the antibody to lyse the cells in the presence of complement was measured by the colorimetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell suspensions of 4×10^4 DU145 or PC3 cells/100 μl were taken in DMEM supplemented with 10% FCS in 96-well microtitration plates. The plates were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 for the cells to adhere to the plate. The supernatant was removed and the purified antibodies, alone or in combination, dissolved in culture medium at a concentration ranging from 2.5 to 10 μg/100 μl/well, were added. The plates were incubated for 1 h at 37°C. Thereafter, 100 μl of medium containing 12% rabbit complement was added. Cells in fresh medium without antibody or complement were kept as control. Each assay was carried out in triplicate. The supernatant was removed and 25 μl of MTT reagent in PBS was added. The plates were further incubated for 2–4 h till crystals formed. To dissolve the crystals, 75 μl of lysis buffer was added per well. Per cent cytotoxicity (X') was calculated by the formula:

$$X' = (1 - X / R1) \times 100$$

Where X = optical density of the test wells; R1 = optical density of the control wells containing culture medium.

2.11 In vivo *testing of antibodies against DU145 tumour cells*

Studies were carried out in athymic nude mice. Tumour was induced by injecting 3×10^6 DU145 cells subcutaneously in the lower flanks of mice. Twenty microgram each of filter-sterilized Moab 2C4 and Moab 730 dispensed in 100 μl of 10 mM PBS, pH 7.2 were injected thrice weekly at the tumour site; controls received an equal volume of PBS. Tumour growth was measured by Vernier calipers at 10-day intervals. As tumour is a spherical mass, the volume was calculated using the formula $4/3\pi r^3$ where r = mean length measured by Vernier calipers in millimeters/2.

3. Results

3.1 *Generation of monoclonal antibody and isotyping*

Four fusions of splenocytes derived from DU145-immunized mice with SP2/o mouse myeloma cells were carried out. Six hybrid cell clones were found to be positive for antibodies reacting with DU145, as observed by flowcytometry. The monoclonal stage was attained after sub-cloning thrice by dilution each time to single-cell level. Moab 2C4 was selected for the cytotoxicity assay, alone and in combination with Moab 730.

3.2 *Reactivity of Moab 2C4*

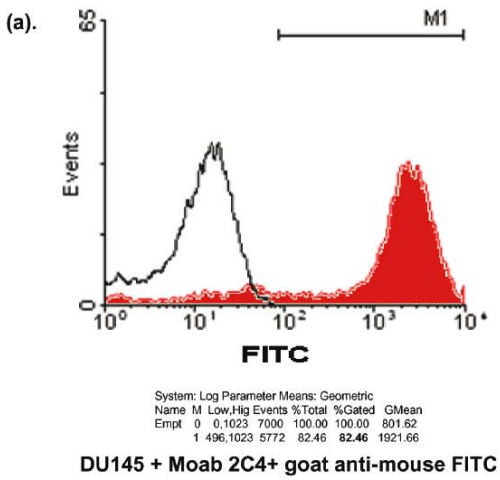
Moab 2C4 bound to live DU145 as well as PC3 cells, as seen by flowcytometry (figure 1a and 1b). However, it did not bind at all with freshly isolated PBLs isolated from normal healthy donors (figure 1c).

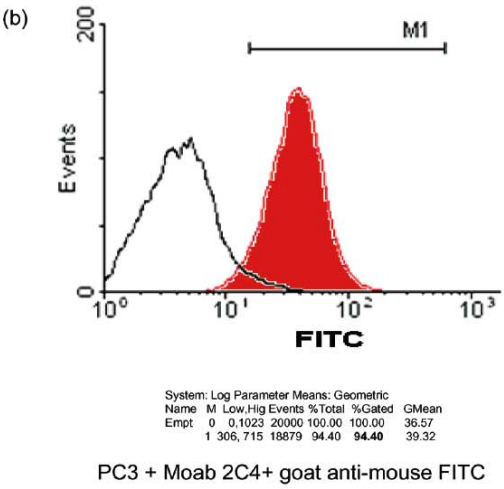
3.3 *Effect of Moab 2C4 on DU145 and PC3*

Moab 2C4 was found to be cytotoxic to both PC3 and DU145 cells in the presence of complement as seen by the MTT assay (figure 2). Complement, at a concentration of up to 12%, exercised minimal toxicity on the cells. However, in the presence of the antibody, cytotoxicity was clearly noticeable, which increased with increasing concentrations of the antibody. At antibody-saturating levels, the percentage of DU145 cells lysed was 83 ± 8.7% (figure 2a) and of PC3 cells it was 80 ± 5.1% (figure 2b). The complement-mediated cytotoxicity exercised by Moab 730 on these cells at antibody-saturating concentrations was of a similar order (Talwar *et al.* 2001). When a combination of Moabs 730 and 2C4 was employed, it caused 100% lysis of both PC3 and DU145 cells (figure 2).

The fact that all tumour cells were killed by the combination of the two Moabs and that only partial killing of the cells occurred after incubation with either one of the Moabs in the presence of complement, was further confirmed by the following experiment. Viable cells of DU145 (figure 3a) and PC3 (figure 3b) were quantified by MTT assay after incubation with Moab 2C4 or Moab 730 in the presence of complement for 12 h. Fresh culture medium supplemented with 10% FCS was added in replicate wells at 12 h and incubation continued for another 24 h. At the end of this period, the viable cells were quantified by MTT assay.

Data from figure 3 show that viable cells were observed in both the tumour cell lines (27–28% in DU145 and 26–29% in PC3 cells) after 12 h of culture with a single Moab in the presence of complement. These numbers increased after culture for a further 24 h with fresh medium and 10%





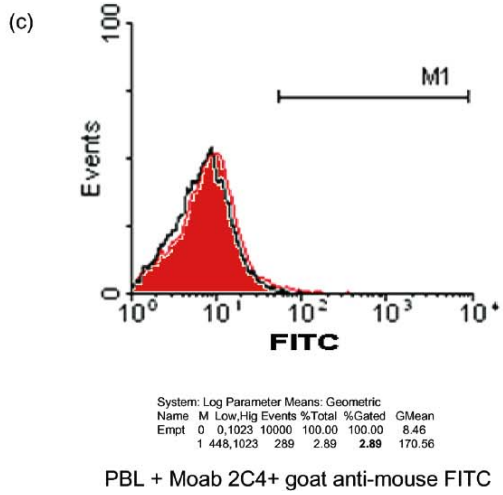
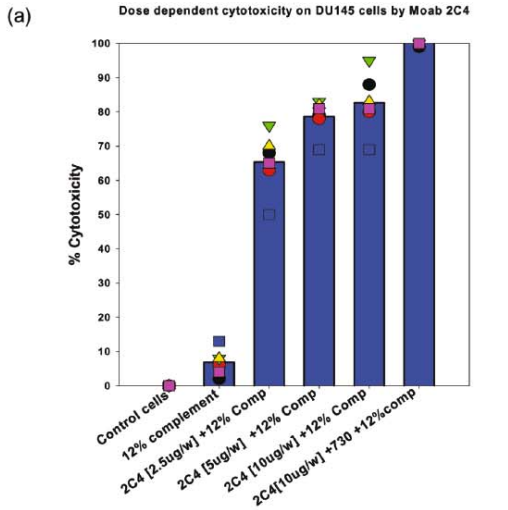
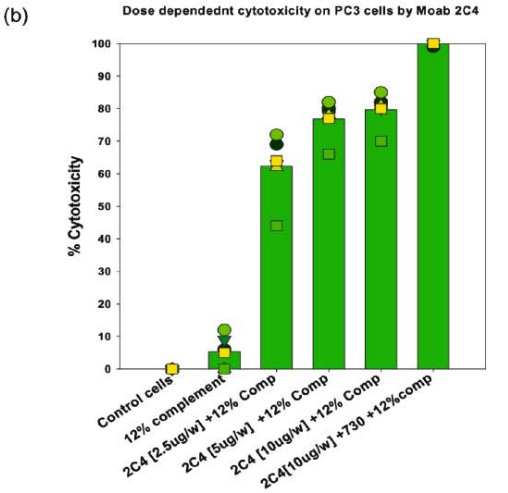


Figure 1. For caption, *see* page No. 913.





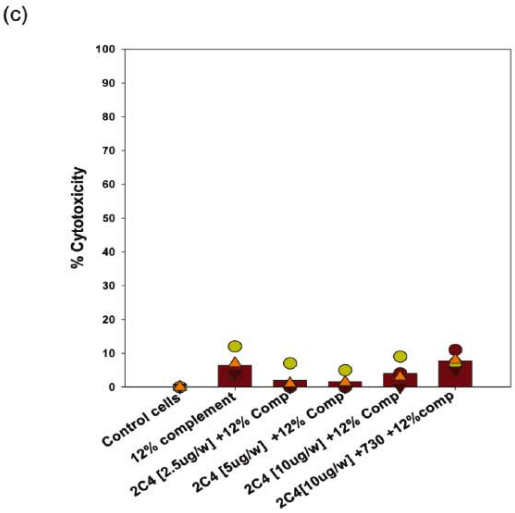


Figure 2. For caption, *see* page No. 913.

FCS. On the other hand, when DU145 and PC3 cells were incubated with a combination of Moab 2C4 and Moab 730 in the presence of complement, no viable cells were measurable at either 12 h or after an additional 24 h of incubation in the culture medium (figure 3).

Killing of androgen-independent prostate carcinoma cells by a combination of Moab 2C4 and Moab 730 was also evident by phase-contrast photomicrographs (figure 4).

It was appropriate to determine whether the two Moabs bound to the same or different surface epitopes on the cells.

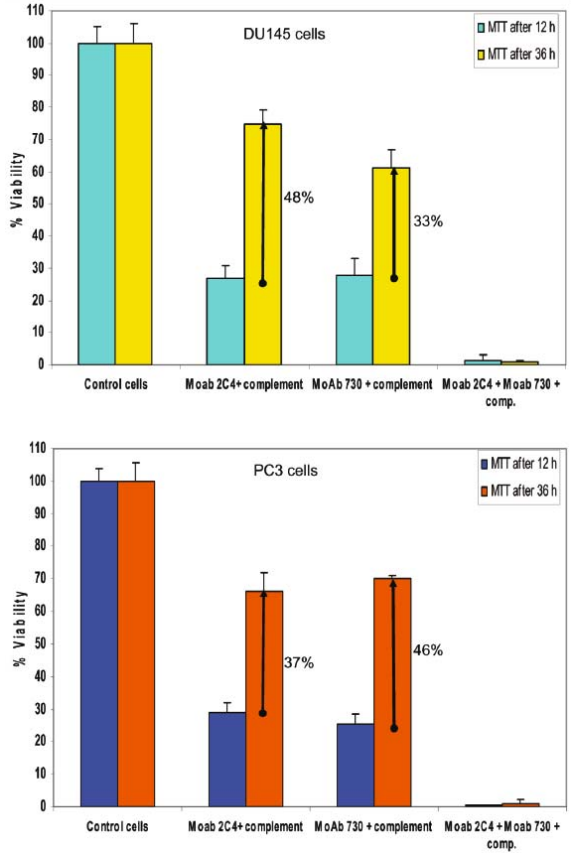


Figure 3. Evidence for partial killing and survival of a fraction of DU145 **(a)** and PC3 cells **(b)** after incubation with either Moab 2C4 or Moab 730 in the presence of complement. After 12 h of incubation with antibodies and complement, the viable cells were quantified by MTT assay. In replicate wells, fresh medium was added at 12 h and culture was continued for another 24 h. No viable cells were observed in the set in which a combination of Moab 2C4 and Moab 730 were employed either at 12 h or at 36 h of culture with fresh culture medium supplemented with 10% FCS.

Figure 1. Binding of monoclonal antibody 2C4 on DU145, PC3 and peripheral mononuclear cells (PBMCs). Moab 2C4 was incubated with viable DU145 **(a)**, PC3 prostate cancer cells **(b)** and peripheral blood leukocytes (PBLs) **(c)** from normal healthy donors. Binding was revealed by using fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody by a fluorescence activated cell sorter (FACS).

Immunofluorescence studies with Moab 730 revealed that it was bound to epitopes dispersed all along the membranes of DU145 and PC3 cells (figure 5). On the other hand, binding of Moab 2C4 was confined and patchy. Another indication that the two Moabs bound to different epitopes is furnished by the western blot carried out on cell lysate of DU145 and PC3 cells (figure 6).

3.4 In vivo *studies in nude mice*

DU145 cells have the ability to form tumours in athymic nude mice. Investigations were carried out to determine whether the combination of the two antibodies prevented the establishment of tumours in nude mice. Figure 7 gives the results. Whereas administration of PBS had no perceptible influence on growth of the tumour, administration of 10 μg each of Moabs 2C4 and 730 thrice weekly prevented formation of the tumour in nude mice. Owing to limitations in the availability of nude mice and lack of maintenance space for these mice, *in vivo* studies could be carried out only on DU145 tumours, even though the *in vitro* cytotoxicity of these antibodies was established in both DU145 and PC3 cells.

4. Discussion

Prostate cancer is among the largest killer of men due to cancer in some countries. According to the Surveillance Epidemiology and End Results fact report of the National Cancer Institute, USA, it is estimated that 192 280 men will be diagnosed with and 27 360 men will die of cancer of the prostate in 2009 in the USA alone (*http://seer.cancer.gov/csr/1975_2006/results_single/ sect_01_table.01.pdf*). Data from other countries are not available, though prostate carcinoma occurs in all countries. Fatalities invariably occur at a stage when the tumour has metastasized, is androgen-independent and refractory to hormones. Currently available drugs are of partial benefit over a limited time span.

The objective of the present study was to develop Moabs that can act selectively on such tumours and bring about their demise. Studies were carried out on cells of two standard androgen-independent prostate carcinoma cell lines obtained from ATCC. A monoclonal antibody developed previously in our laboratory, Moab 730, caused lysis of both DU145 and PC3 cells in the presence of complement. The limitation of this Moab, however, was that it killed only 80% of such cells and the residual viable cells took

Figure 2. Cytotoxicity of Moab 2C4 individually and in combination with Moab 730 on PC3 and DU145 in the presence of complement as determined by MTT assay. **(a)** DU145, **(b)** PC3 cells and **(c)** peripheral blood leukocytes (PBLs) from normal healthy donors. Symbols represent values in different experiments and bars are the mean values.

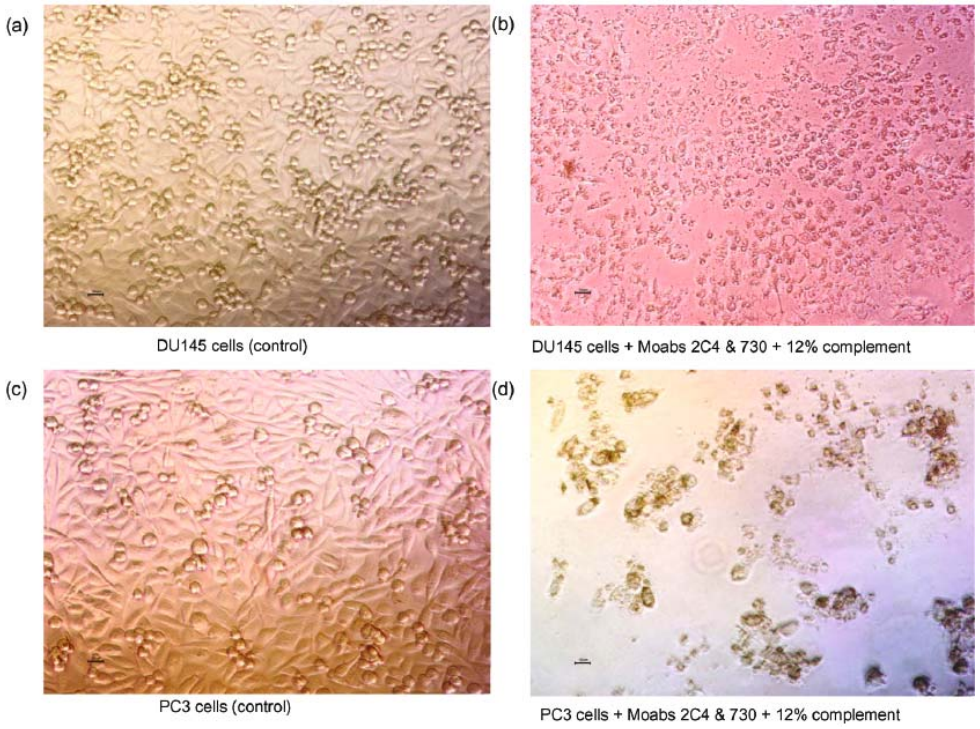


Figure 4. Microphotographs of the prostate carcinoma cells before and after incubation with monoclonal antibodies, in the presence of complement. **(a)** DU145 cells without antibody. **(b)** DU145 cells lysed after co-incubation with Moabs 2C4 and 730 in the presence of 12% complement. **(c)** PC3 cells without antibody and complement, and **(d)** PC3 cells after co-incubation with Moabs 2C4 and 730 in the presence of 12% complement.

over in course of time. The aim of the present work was to develop a complementary antibody that could kill cells resistant to Moab 730. The data presented show that the new Moab 2C4, in combination with Moab 730, can kill 100% of both DU145 and PC3 cells. Killing was gauged not only morphologically but also by MTT assay, which quantitates viable cells by forming formazan crystals.

The binding pattern of the two antibodies differs, as also their isotypes. Moab 730 is IgG1κ and Moab 2C4 is IgG2aκ. The killing of both types of tumours was evident *in vitro* and was confirmed *in vivo* for DU145 cells. The development of tumour in nude mice was effectively prevented by a combination of the two antibodies. The stage is now set to convert the two antibodies into chimeric/humanized recombinant antibodies for therapeutic use.

Several murine/chimeric/humanized antibodies alone, or attached to payloads, have been approved by the US Food and Drug Administration (FDA) and other drug regulatory authorities for the treatment of cancers and other chronic disorders. In the past 12 years, 9 Moabs were approved for clinical use by the US FDA for cancer therapy and about 30 Moabs – murine, chimeric or humanized – are currently undergoing human phase II/III clinical testing as cancer therapy. Out of the 9 FDA-approved Moabs for the treatment of cancers, 6 Moabs act by a mechanism of cytolytic action in the presence of complement, i.e. complement-dependent cytotoxicity (CDC), or by antibody-dependent cell cytotoxicity (ADCC) (Liu *et al.* 2008). The first Moab of this type approved by the US FDA for human use was a murine anti-CD3 monoclonal OKT3, employed for the treatment of organ transplant rejection (Kunq *et al.* 1979). The first Moab approved for the treatment of cancer was rituximab (Rituxan^{TM}), a chimeric anti-human anti-CD20, approved in 1997 for the treatment of non-Hodgkin lymphoma. The use of antibodies such as rituximab (Rituxan^{TM}) (Glennie *et al.* 2007), Campath-1H^{TM} (Bindon *et al.* 1988), ibritumomab (Zevalin^{TM}) (Chinn *et al.* 1999) and others which act by a CDC mechanism provide support for the safety and clinical efficacy of such antibodies.

Although some mouse-derived Moabs such as ibritumomab (Zevalin^{TM}) and tositumomab (Bexxar^{TM}) are approved by drug regulatory authorities for life-threatening conditions, technologies are available to convert mouse-derived antibodies into chimeric or humanized antibodies. We have earlier converted a mouse Moab against human chorionic gonadotrophin (hCG) into a recombinant chimeric antibody which can be expressed in plants at high yield (Kathuria *et al.* 2002a, b). This antibody with mouse variable regions fused to human $IgG_1\kappa$ binds with T-lymphoblastic leukaemia and histiocytic lymphoma cells (Kabeer *et al.* 2005). The

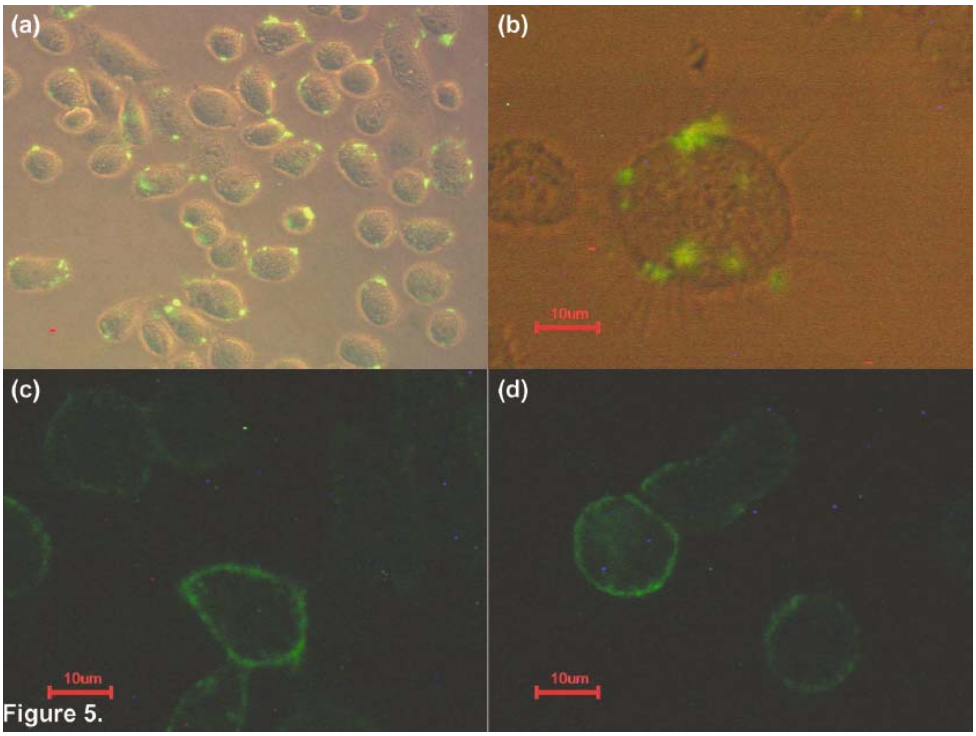


Figure 5. Binding pattern of Moabs 2C4 and 730 on DU145 and PC3 cells. **(a)** Immunofluorescence on PC3 cells with 2C4 under low bright field at 10x magnification. **(b)** 2C4 antibody-binding cell seen at 40x magnification. **(c)** Immunofluorescence due to binding of Moab 730 to DU145 40x, and **(d)** to PC3 cells, 40x as revealed by using fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody.

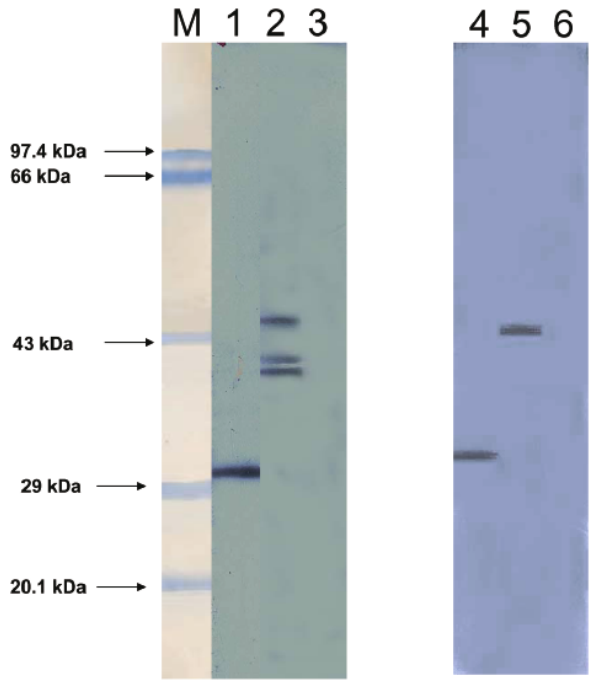


Figure 6. Western blot of Moab 730 and 2C4 on lysate of DU145 and PC3 cells. Lanes 1–3 with lysate of DU145 and lanes 4–6 with lysate of PC3. Lane M, Marker; lanes 3 and 6, control; lanes 2 and 5, Moab 2C4; lanes 1 and 4, Moab 730.

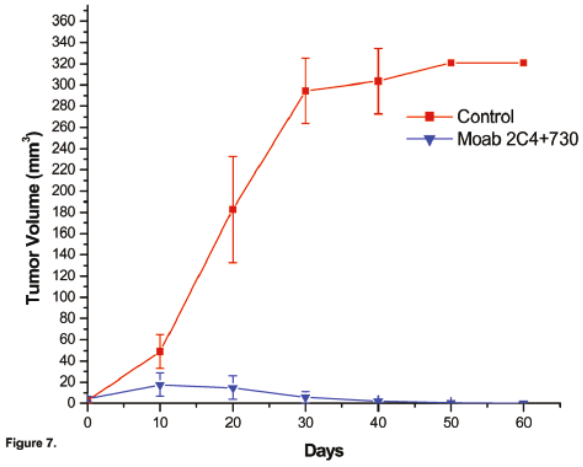


Figure 7. Inhibition of establishment of DU145 prostate tumour in nude mice by a combination of Moabs 2C4 and 730**.** 3 x 10^6 DU145 cells were implanted subcutaneously in the lower flanks of mice. 20 μg each of filter-sterilized Moab 2C4 and Moab 730 dispensed in 100 μl of 10 mM PBS pH 7.2 was injected thrice weekly at the tumour site with controls receiving an equal volume of PBS only. The figure gives the mean tumour size at different time points in six animals in each group with standard deviation.

antibody was used to deliver diferuloylmethane (curcumin) selectively to tumour cells. This immunotoxin was effective in killing 100% of the MOLT-4 T-lymphoblastic leukaemia and U-937 histiocytic lymphoma cells that expressed hCG ectopically (Vyas *et al.* 2009).

Besides Moab 730 (Talwar *et al*. 2001), the only other Moab reported in the literature against prostate carcinoma cells is HuJ591, which is directed against prostate-specific membrane antigen (Bander 2003). Three phase I studies have been carried out with this humanized Moab linked to the *β*-emitting radiometals yttrium 90 and lutetium 177, and the third using a cytotoxin (DM1). The radiolabelled antibody was found useful for imaging of tumours and for delivery of radiation selectively to the tumour cells (Bander *et al*. 2003).

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