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# Inhibition of tumour growth and metastasis of human fibrosarcoma cells HT-1080 by monoclonal antibody BCD-F9

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**Abstract**

BCD-F9 is a murine $IgG_{2a}$ monoclonal antibody (mAb) that recognises a conformational epitope found on the surface of many human tumour cells. The aim of this study was to investigate the ability of BCD-F9 to recognise a variety of neoplastic cell lines and to test BCD-F9 *in vivo* for anticancer activity in subcutaneous (s.c.) and metastatic tumour models. Intravenous (i.v.) administration of BCD-F9 in CD-1 nude mice xenografted s.c. with human HT-1080 cells led to a significant inhibition of tumour growth. We demonstrated that BCD-F9 administrated i.v. significantly prolonged the life-span of CD-1 nude mice inoculated i.v. with the same tumour cell line that induces aggressive lung metastases in the untreated mice. We also investigated the antitumour activity of BCD-F9 *in vitro* in antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent complement-mediated cytotoxicity (ADCMC) assays. The effector cell subpopulations were obtained by macrophage stimulation (thioglycollate) or natural killer (NK) cell enrichment (negative selection). BCD-F9 was found to be effective in mediating tumour cell killing *in vitro* by ADCC and ADCMC mechanisms. These results suggest that the mAb BCD-F9 can have a potential use in immunotherapy for treatment of tumours of different origin. 

*Keywords:* Cancer; Immunotherapy; mAb; *In vivo* animal models

## 1. Introduction

In oncology, tumour-associated antigens are considered good targets for the delivery of anticancer therapy, and mAbs directed at such antigens are increasingly being seen as important immunotherapeutic reagents. There is a consensus that target antigens in general are essentially normal structures that are expressed in a higher density, in an overt fashion, and in an atypical tissue context on tumour cells [1]. A variety of cell surface proteins have been used as targets for mAbs. In the case of cancer cells, the only truly tumour-specific antigens are the clonally expressed receptor idiotypes on B lymphoma cells. Unfortunately, the use of anti-Id mAbs to treat lymphoid cancers is limited by the need to create custom-made mAbs against the tumour from each patient or small group of patients [2]. Thus, most investigators considered other targets such as differentiation antigens, growth factor receptors, oncofetal antigens, unique carbohydrate antigens, or altered normal antigens [3–5]. Such antigens are tumour selective rather than tumour specific, that is, they are present in higher densities on tumour cells when compared with normal cells.

Whatever is their specificity, mAbs in general rarely have the capacity to eradicate large tumour masses. Several factors such as affinity and the number of binding sites on the target cell, antigen expression on the tumour cells versus the normal cells, tumour size and the degree of vascularisation can limit the access of mAbs to the target cells and therefore affect their cytotoxic potential *in vivo* [6]. Moreover, immunotherapy requires injection of relatively high doses of mAb, which leads in most patients to the development of an immune response against the injected rodent mAb with possible side-effects [4,7]. Targeting a highly expressed cell surface antigen, preferentially present on a wide range of neoplastic cells, might improve the efficacy of mAb therapy.

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Most of the unconjugated mAbs displaying anti-neoplastic properties require the contribution of either complement [6,8] or Fcγ receptor (FcγR)-expressing effector cells [9,10] in order to achieve tumour cell killing. The presence in tumours, surgically removed from mAb-treated patients, of infiltrating Natural Killer (NK) cells and macrophages [11,12], as well as of complement deposits [11] are consistent with antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent complement-mediated cytotoxity (ADCMC) playing a role in tumour cell destruction *in vivo*. However, because most tumour cells express large amounts of complement-inhibiting regulators, which protect the cells against lysis by autologous complement [13,14], the main antitumour mechanism of therapeutic antibodies *in vivo* is considered to be ADCC.

The murine mAb BCD-F9 was obtained by immunising BALB/c mice with the human breast carcinoma cell line BT-20 [15]. This mAb recognises an antigen localised on the surface of most neoplastic, and to a lesser extent, normal mammary epithelial cells. In previous studies, BCD-F9 was shown to be a useful tool for the preoperative detection of axillary lymph nodes metastasis in over 30 breast cancer patients with minimal cancer [16]. Moreover, BCD-F9 was shown to be a potential plasmatic marker that correlates with Ki 67 immunoreactivity and tumour aneuploidy [17]. The antigen recognised by BCD-9 has not been identified yet.

In this study, the ability of BCD-F9 to recognise a wide variety of neoplastic cell lines was investigated. The reactivity of the mAb with the human fibrosarcoma HT-1080 enabled us to use this cell line to investigate the antitumour properties of BCD-F9 in an experimental animal model, where either subcutaneous (s.c.) or metastatic tumours can be induced depending on the site of injection [18].

## 2. Materials and methods

### 2.1. Antibodies

The BCD-F9 antibody-producing hybridoma was derived from the fusion of NS-1 myeloma cells with spleen cells from BALB/c mice hyperimmunised with the human breast carcinoma cell line BT-20 [15]. The mAb BCD-F9 is an IgG2a that was purified from ascitic fluid collected from BALB/c mice inoculated intraperitoneally (i.p.) with the hybridoma cell line. A protein-G Sepharose column (Pharmacia, Uppsala, Sweden) was used for that purpose. The mAb BCD-F9 was biotinylated according to a conventional procedure [19]. Fluoroisothiocyanate (FITC)-labelled mouse anti-human CD3 (IgG2a), CD14 (IgG2a), CD15 (IgM), CD40 (IgG1), and all FITC-labelled mAbs used as isotype controls were purchased from Cedarlane (Hornby, Ontario, Canada). The anti-human HLA-ABC class I mAb and the guinea pig complement were also purchased from Cedarlane. Mouse complement was purchased from Rockland (Gilbertville, PA, USA). FITC-conjugated sheep anti-mouse IgG antibody was purchased from Roche Diagnostics (Laval, Quebec, Canada). The anti-PLCγ1 rabbit polyclonal IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal rabbit IgG was purchased from CALTAG Laboratories (Burlingame, CA, USA). The anti-β-Galactosidase mAb (mouse IgG2a, Calbiochem, Cambridge, MA, USA) was used as an isotype control for BCD-F9 in the flow cytometry analysis and immunoprecipitation assays.

### 2.2. Mice and tumour cells

Female athymic CD-1 nude mice were obtained from Charles River, Canada. The mice were maintained in sterilised cages under sterile filter top conditions and were handled in a laminar airflow. They were 6-weeks old when transplanted with the human fibrosarcoma cell line, HT-1080, that was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (v/v). Cells were cultured at 37 °C in a 5% $CO_2$ humidified atmosphere. Other human tumour cell lines, BT-20, MCF-7, MDA/MB-231, SW480, LoVo, Mes-sa, U937, HL-60, Jurkat and Raji used for the cell distribution analysis of the antigen recognised by BCD-F9 were also purchased from the ATCC and were maintained in culture according to the supplier's recommendations.

### 2.3. Flow cytometry analysis

Various tumour cell lines were stained with protein G-purified mAb BCD-F9. Binding of the mAb BCD-F9 was detected with FITC-conjugated sheep anti-mouse IgG antibody. Control samples were incubated with anti-β-Galactosidase mAb (mouse IgG2a) as a primary antibody.

Fresh heparinised blood samples were obtained from healthy donors and processed immediately. Firstly, unwashed blood samples (100 μl) were incubated with biotin-conjugated mAb BCD-F9 (1 μg total) for 20 min at room temperature and washed once in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (w/v). Then, 10 μl of each undiluted FITC-labelled CD3, CD14, CD15 and CD40 antibody were added to the different tubes. Binding of the biotinylated mAb was detected with phycoerythrin (PE)-labelled streptavidin (Beckton Dickinson, Moutain View, CA, USA). For each mAb, control samples were stained with a non-reactive FITC-labelled primary mAb of the same isotype, and the biotinylated anti-β-Galactosidase

IgG2a mAb was used as a negative control for PE-labelled streptavidin staining. Samples were incubated for 20 min at room temperature and washed once in PBS containing 0.1% BSA. Then, 0.5 ml of OptiLyse C solution (Immunotech, Marseille, France) was mixed with the pelleted cells and the samples were incubated for 15 min at room temperature. Finally, 0.5 ml of PBS containing 0.5% formaldehyde (v/v) was added to each sample.

Stained samples were analysed on a Epics XL-MCL flow cytometer (Coulter, Hialeah, FL, USA) equipped with a 488 nm argon laser. Tumour cell populations were gated on the basis of forward and side scatter parameters. T and B lymphocytes were gated on the basis of CD3 and CD40 fluorescence and side-scattering characteristics, whereas monocytes and granulocytes were gated on the basis of CD14 and CD15 fluorescence, respectively. Data analysis based on the collection of 10 000 events per sample was performed using XL software. The degree of reactivity of tumour cells or blood leucocyte populations with the BCD-F9 mAb is expressed as the mean fluorescence intensity (MFI) of the positive cells after substraction of non-specific binding of the anti-β galactosidase isotype control on the same cell population.

### 2.4. Metabolic labelling of HT-1080 cells

For metabolic incorporation of radioactive precursors into proteins, confluent HT-1080 cells were harvested by incubation with PBS containing 0.5 mM ethylene diamine tetra acetic acid (EDTA). Cells were washed twice in a conical tube with 50 ml of methionine-free DMEM by centrifugation. Cells were placed (a) in 20 ml of methionine-free DMEM ($2\times10^8$ cells) containing 5% of complete DMEM and 5% of dialysed FBS, in the presence of [$^{35}$S]-Methionine (80 μCi/ml, ICN Pharmaceuticals, Irvine, CA, USA), for 20 h at 37 °C or (b) in 20 ml of complete DMEM ($2\times10^6$ cells) containing 10% of dialysed FBS, in the presence of [$^3$H]-Glucosamine (12.5 μCi/ml, ICN Pharmaceuticals, Irvin, CA, USA), for 72 h at 37 °C. After metabolic labelling, the cells were harvested and washed twice with PBS by centrifugation. The cell pellets were resuspended by Vortex mixing at room temperature in 1 ml of lysis buffer (25 mM Tris–HCl pH 8.0, 0.15 M NaCl, 5 mM $MgCl_2$, 0.5% Nonidet (N)P-40 (v/v), 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride) and centrifuged at 14 500*g* for 10 min. The supernatants were incubated with 20 μl of a 50% suspension of protein G-Sepharose beads (v/v) and 10 μg of anti-β-Galactosidase (control mAb) for 1 h at 4 °C on a rotary wheel. The beads were removed by centrifugation and kept as a control for non-specific adsorption. Aliquots of the supernatants (250–500 μl) were incubated with 25 μg of mAb BCD-F9 or 10 μl of anti-PLCγ1 polyclonal antiserum as a positive control and 30 μl of protein G-Sepharose beads for 4 h at 4 °C on a rotary wheel. The beads were collected by centrifugation and washed four times with lysis buffer and twice with 50 mM Tris–HCl pH 8.0/0.15 M NaCl. The immunoadsorbed proteins were released from the protein G-Sepharose by boiling for 10 min in 60 μl of 1% $NaDodSO_4$ (w/v)/2% 2-mercaptoethanol (v/v) and analysed by 12% polyacrylamide gel electrophoresis and autoradiography as described in Ref. [20].

### 2.5. In vivo tumour growth

Confluent cultured HT-1080 cells were harvested by incubation with PBS containing 0.5 mM EDTA. Viable cells were counted by trypan blue exclusion. Fractions containing over 95% of viable cells were used in this study. Cells ($1.5\times10^7$ cells/ml) were suspended in PBS, and 0.2 ml of the suspension was inoculated s.c. into the right flanks of 2 groups of 5 CD-1 nude mice on day 0. Normal rabbit IgG (100 μg) or mAb BCD-F9 (100 μg) was administrated intravenously (i.v.) in 0.2 ml of PBS on days 1–4, 7–10 and 13–14. The resulting tumour was measured over the skin in two dimensions using a slide caliper, and the tumour volume was calculated according to the following formula, $(\text{width})^2\times\text{length}/2$. All animals were sacrificed after 30 days, the tumours were completely dissected and then weighed.

### 2.6. Experimental metastasis

For the experimental metastasis model, HT-1080 cells ($2\times10^7$ cells/ml) were harvested in PBS containing 0.5 mM EDTA, centrifuged and resuspended in PBS, and 0.1 ml of the suspension was inoculated i.v. into the tail vein of two groups of five CD-1 nude mice (day 0). Normal rabbit IgG (100 μg) or mAb BCD-F9 (100 μg) was administered i.v. in 0.2 ml of PBS on days 1–2, 5–9, 12–16 and 19–21. Both lungs were recovered from the recently deceased animals or from animals surviving the end of the experiment (day 61 post-injection). Lungs were fixed in 10% buffered formalin (v/v), embedded in parafin, sectioned and stained with haematoxylin and eosin (H&E) for routine histological examination by light microscopy.

### 2.7. Preparation of effector cells

Splenic NK cells from nude mice were enriched by a single filtration through a nylon wool column. Effector cells for macrophage-mediated cytotoxicity were thioglycollate-elicited peritoneal exudate cells harvested from CD-1 nude mice [21].

### 2.8. Antibody-dependent cytotoxicity assays

For ADCC, $3\times10^6$ HT-1080 cells were labelled with 150 μCi of $Na_2^{51}CrO_4$ (ICN Biomedical, Costa Mesa,

CA, USA) for 1 h at 37 °C and washed four times in DMEM. Fifty μl of $^{51}$Cr-radiolabelled HT-1080 cells ($2\times10^5$/ml) were distributed into U-bottomed 96-well microtitre plates and incubated for 1 h at 37 °C with 50 μl of protein G-purified mAb BCD-F9 at a concentration of 80 μg/ml. Then 100 μl of various concentrations of murine effector cells, prepared as described above, were added in quadruplicates to each well. After plates were incubated in a $CO_2$ incubator at 37 °C for 8 h, they were centrifuged for 5 min at 250*g* and supernatants were collected with the Titertek supernatant collection system (Skatron, Oslo, Norway). Radioactivity was measured in a Beckman 7000 gamma counter. The percent specific lysis was calculated using the following formula:

$$\%\ \text{specific lysis} = \frac{\text{counts per minute (cpm) (test)} - \text{cpm (spontaneous)}}{\text{cpm (max)} - \text{cpm (spontaneous)}} \times 100$$

in which spontaneous release (12–17%) was measured by incubating radiolabelled cells in the absence of effector cells and maximum release was obtained by adding 1% Triton X-100 (v/v) to the radiolabelled cells.

For ADCMC, 50 μl of $^{51}$Cr-radiolabelled HT-1080 tumour cells ($2\times10^5$/ml) and 50 μl of various concentrations of protein G-purified mAb BCD-F9 were distributed in triplicates into U-bottomed 96-well microtitre plates. Then 100 μl (1:5 dilution) of Low-Tox guinea pig or mouse complement was added to each well, and the plates were incubated for 45 min at 37 °C in a 5% $CO_2$ humidified atmosphere. Plates were centrifuged for 5 min at 250*g* and culture supernatants were harvested as described above for the ADCC assay. Spontaneous release was measured by incubating radiolabelled cells in the absence of complement and maximum release was obtained by adding 1% Triton X-100 to the radiolabelled cells. The anti-human HLA-ABC class I mAb was used as a positive control in the ADCMC assay.

### 2.9. Statistical analysis

Unpaired two-tailed Student's *t*-test and Cox–Mantel logrank test were performed using INSTAT software. A $P$ value $<0.05$ was considered significant.

## 3. Results

### 3.1. BCD-F9 recognition of neoplastic cell lines and peripheral blood mononuclear cells (PBMC)

Eleven human cell lines harvested when confluent were examined by flow cytometry analysis for the binding of the mAb BCD-F9. As shown in Fig. 1a, BCD-F9 mAb recognises an epitope expressed on the surface of all the cell lines tested. Low reactivity was detected in almost half of them, including the BT-20 cell line used for the BALB/c immunisation. Significant binding of BCD-F9 was observed with MDA/MB-231, SW480, HT-1080 and HL-60. The highest reactivity was found within the LoVo and Raji cells. No obvious correlation was observed between the level of BCD-F9 immunostaining and the tissue origin of the neoplastic cells. No fluorescence was detected when human peripheral blood mononuclear cells (PBMC) were stained with BCD-F9 and the FITC-conjugated anti-mouse IgG antibody (data not shown).

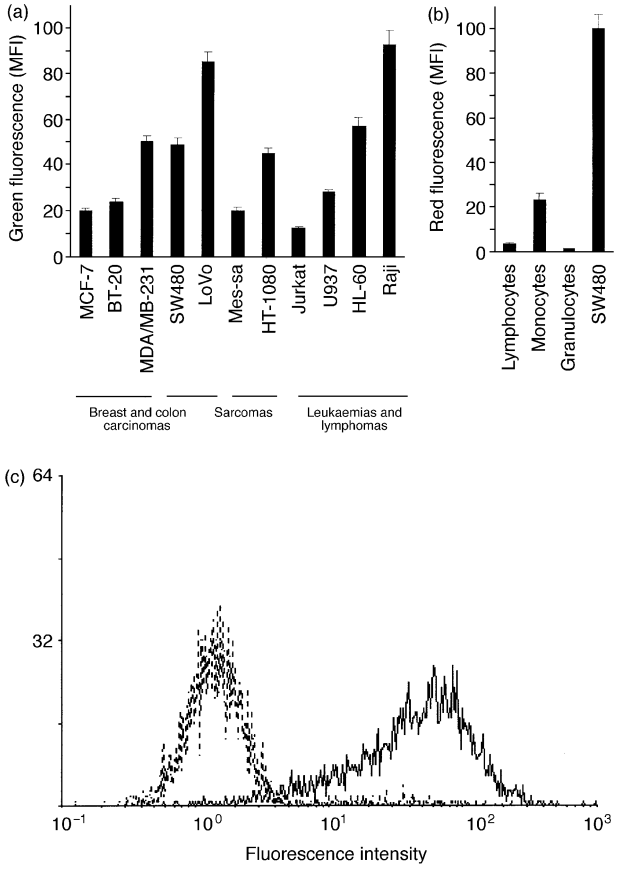


Fig. 1. Reactivity of mAb BCD-F9 with various neoplastic cell lines and peripheral blood mononuclear cells (PBMC). Expression of BCD-F9 epitope on cultured tumour cell lines (a) and on freshly collected PBMC (b) was analysed by flow cytometry. Data correspond to the mean fluorescence intensity (MFI) of positive cells after staining with unconjugated BCD-F9 and FITC-labelled sheep anti-mouse IgG (green fluorescence) or biotinylated-BCD-F9 and PE-labelled streptavidin (red fluorescence). The results illustrated represent mean values±standard deviation (S.D.) of three independent experiments. (c) Histogram fluourescent activated cell sorting analysis (FACS) plot showing the binding of mAb BCD-F9 to the HT-1080 cells. Cells were stained with mAb BCD-F9 (thick line) or anti-β-Galatosidase IgG2a isotype control mAb (dashed line). Bound mAbs were detected with fluoroisothiocyanate (FITC)-conjugated sheep antimouse IgG.

In order to achieve an even greater sensitivity, human PBMC were stained with FITC-conjugated mAbs specific for leucocyte populations and biotinylated mAb BCD-F9 followed by incubation with PE-labelled streptavidin for the detection of BCD-F9 binding. Under these conditions, BCD-F9 positively stained monocytes ($CD14^+$), while weak staining was observed in lymphocytes ($CD3^+$, $CD40^+$) and no staining on granulocytes ($CD15^+$) (Fig. 1b). The human colon carcinoma cell line SW480 expressing the BCD-F9 epitope at an intermediate level was used as a positive control for staining with the biotinylated mAb. The mean fluorescent intensity observed with the tumour cells was more than 4-fold higher than with the monocytes.

Microheterogeneity is recognised in cancer cells and it was hypothesised that every cell in the any cell line may not carry epitope recognised by mAb BCD-F9. Fig. 1c shows over 94% cells of the HT-1080 cells bound with BCD-F9. All other tumour cell lines were found also to express the BCD-F9 antigen at a similar level to the HT-1080 cells (data not shown).

### 3.2. Characterisation of the BCD-F9 reactive antigen

To confirm the data obtained by flow cytometry, lysates from metabolically labelled HT-1080 cells were immunoprecipitated with the mAb BCD-F9. A predominant band with a molecular mass of approximately 57 kDa was detected (Fig. 2, lanes 2). The antigen in the HT-1080 cell lysates is a glycoprotein, since it was labelled by both [$^{35}$S]-Methionine and [$^{3}$H]-Glucosamine. In addition, the mAb BCD-F9 precipitated two secondary proteins with molecular masses of approximately 80 and 25 kDa, respectively (Fig. 2, lanes 2). All other bands were non-specific reactions with the negative mAb control and/or Protein G-Sepharose beads (Fig. 2, lanes 1). PLCγ1, a 145 kDa intracellular signal transduction protein was used as a positive control (Fig. 2, lanes 3). It remains to be determined whether the 80 and 25 kDa coprecipitates are physically associated with the 57 kDa BCD-F9 antigen or if they are cross-reactive with the mAb BCD-F9.

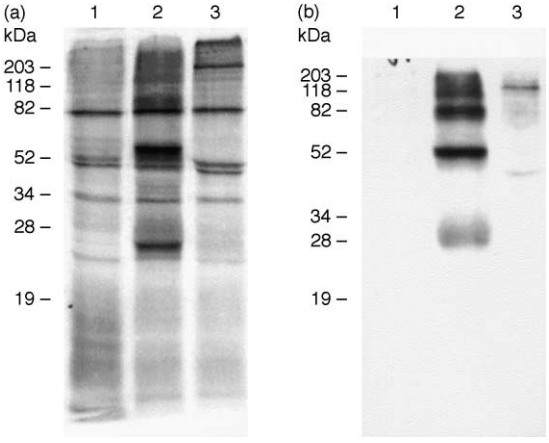


Fig. 2. Characterisation of the BCD-F9-reactive antigen. Aliquots of lysates of HT-1080 cells labelled metabolically with [$^{35}$S]-Methionine (a) or [$^{3}$H]-Glucosamine (b) were immunoprecipitated with anti-β-Galatosidase IgG2a isotype control mAb (lanes 1), mAb BCD-F9 (lanes 2), or anti-PLCγ1 rabbit polyclonal IgG (lanes 3) and analysed by electrophoresis in 12% polyacrylamide slab gels under reducing conditions, followed by autoradiography.

### 3.3. Antitumour activity of BCD-F9

Among all the cell lines stained with the mAb BCD-F9, the fibrosarcoma HT-1080 was selected for the *in vivo* studies mainly because of the rather short time period for tumour growth and lung metastasis development (3 weeks), as well as its ability to produce tumours in all of the injected animals and with all animals with lung metastasis [18]. In order to evaluate the antitumour efficacy of BCD-F9 for *in vivo* tumour cell eradication, CD-1 nude mice were injected s.c. on day 0 with $3\times10^6$ HT-1080 cells into the right flank, followed by 10 i.v. administrations of the mAb BCD-F9 starting at day 1 post-injection. The mean tumour size of all treated mice was monitored from day 9 to 30. All mice were sacrificed at day 30, mainly because of the large tumour volumes and the development of necrotic areas. As depicted in Fig. 3, all injected animals developed palpable tumours within 9 days; however, starting from day 12, a reduced tumour growth rate in the BCD-F9-treated group was observed when compared with the control group. Repeated inoculation of BCD-F9 in mice bearing HT-1080 tumours resulted in significantly lower tumour sizes compared with the control group which had been treated with normal rabbit IgG ($P<0.01$, from

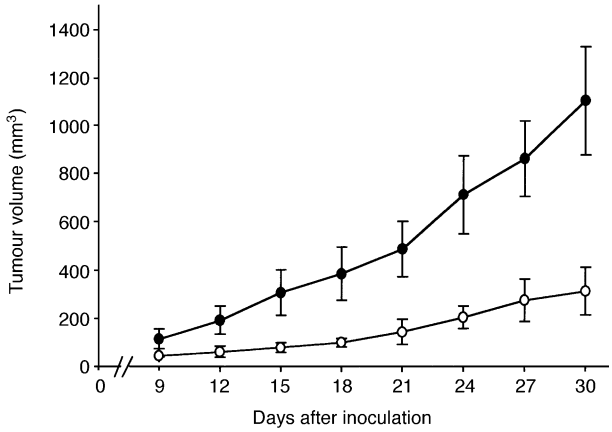


Fig. 3. Antitumour activity of BCD-F9 in HT-1080-xenografted nude mice. Cultured HT-1080 cells were inoculated subcutaneously (s.c.) into CD-1 nude mice on day 0. Normal rabbit IgG (●) or mAb BCD-F9 (○) was administrated intravenously (i.v.) according to a schedule described in the Methods. Results expressed as mean tumour volume±standard deviation (S.D.) were calculated from five growing tumours/group measured at 3-day intervals from 9 to 30 days post-grafting. Statistical significance: $P<0.01$ versus normal IgG, calculated using the Student's *t*- test.

days 15–30 versus control). Starting from day 15, in all of the BCD-F9-treated mice, clear signs of tumour necrosis were visible in the centre of the tumour, surrounded by a rim of viable tumour cell mass. These signs of necrosis were only observed in the BCD-F9-treated mice and not in the control group. Moreover, one tumour in the BCD-F9-treated animals showed clear signs of regression and totally disappeared within 18 days. Tumour weights, as determined at sacrifice in all animals on day 30 post-tumour injection, were compared. In the group given BCD-F9, the mean tumour weight was $0.25 \pm 0.1$ g compared with $1.16 \pm 0.22$ g in the control group, a finding that corresponds to a 78.45% reduction in tumour growth in the BCD-F9-treated group ($P < 0.001$ versus control). These data rule out the possibility that the antitumour activity of BCD-F9 could be due to the non-specific action of the administration of IgG. At no time during the observation period were side-effects, such as loss of body weight, observed in the mice treated with both antibodies.

### 3.4. Effect of BCD-F9 on experimental metastasis

We also examined the antitumour activity of BCD-F9 in an experimental metastasis model. In this case, HT-1080 cells were injected i.v. in CD-1 nude mice for the formation of metastatic foci in the lung. Survival curves show that animals treated with rabbit IgG started to die at 21 days post-injection, apparently due to difficulties in breathing caused by lung metastasis of tumour cells. Mean survival time for the group of mice treated with rabbit IgG was $25 \pm 1$ days (Fig. 4). All animals treated with BCD-F9 survived the experimental period, thus confirming the beneficial antitumour activity of BCD-F9 even when using an aggressive tumour model. Survivors were sacrificed at day 60 post-injection. Histological examination of the lungs of all animals showed diffuse metastases in the case of control animals (Fig. 5a) and no metastatic foci in all of the animals treated with BCD-F9 (Fig. 5b). No side-effects or toxic reactions were observed in mice treated with BCD-F9 throughout the experimental period, thus suggesting that no serious side-effects were caused by the i.v. injection of BCD-F9 in mice.

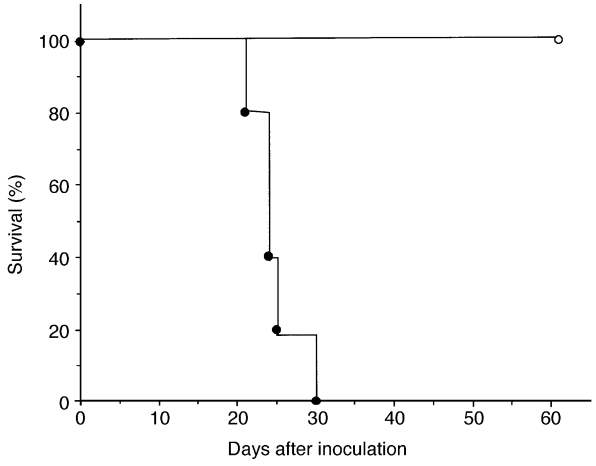


Fig. 4. Prolonged survival time of HT-1080 tumour-bearing mice after treatment with BCD-F9. Cultured HT-1080 cells were inoculated intravenously (i.v.) into two groups of five CD-1 nude mice on day 0. Rabbit IgG (●) or BCD-F9 (○) was administrated i.v. according to a schedule described in the Methods. Statistical significance: $P < 0.05$ versus normal IgG, calculated using the Cox–Mantel test.

### 3.5. BCD-F9 antibody-dependent complement- or cell-mediated cytotoxicity

Two potential effector mechanisms that might contribute to the efficacy of BCD-F9 *in vivo* were investigated *in vitro*. We first examined the ability of the mAb BCD-F9 to kill HT-1080 tumour cells by ADCMC. The assay positive control involved the use of guinea pig complement. With this as the complement source, the

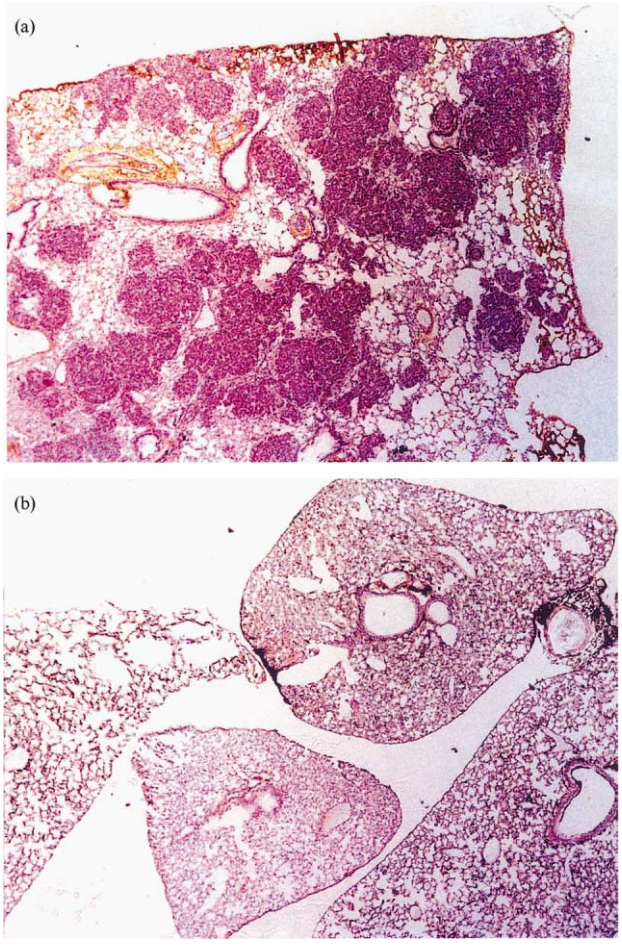


Fig. 5. Tumour histology. Photomicrographs demonstrating histological features of tumours formed in the mouse lung after intravenous (i.v.) injection of human HT-1080 cells. Representative lung colony of the HT-1080 tumour cells in mice treated with normal rabbit IgG (a) or mAb BCD-F9 (b)×100.

mAb BCD-F9 induced substantial cytotoxicity against the HT-1080 cells (Fig. 6). Significant lysis of the tumour cells was also observed in the presence of the mouse complement at an antibody concentration between 3 and 50 μg/ml. ADCMC correlated directly with the mAb concentration and was undetectable at a concentration less than 3 μg/ml (data not shown). These data suggest that a minimal number of antibody molecules had to be bound to its epitope to induce ADCMC. There was no direct cytotoxicity of mAb BCD-F9 for the HT-1080 tumour cells, which were not killed by the mAb at a concentration of 50 μg/ml in the absence of complement. Complement-dependent lysis of HT-1080 tumour cells by anti-human HLA-ABC class I mAb was used as a positive control in the ADCMC assay.

Another mechanism through which antibodies can destroy tumour cells is ADCC. Experiments with splenic NK cells and peritoneal cells from nude mice were performed against BCD-F9 antibody-coated HT-1080 cells at different effector–target ratios. Data generated show that the HT-1080 tumour cell line is not susceptible to spontaneous killing by these effector cells, which however lysed BCD-F9-coated targets in a dose-dependent manner (Fig. 7).

## 4. Discussion

The use of mAbs in immunotherapy was demonstrated previously for various types of tumours [3–5]; however, major improvement is still needed before this therapy reaches its full potential. Selection of the optimal mAb for the treatment of some cancers is still in its early phases and depends on many factors, notably the specificity, affinity and avidity of the antibody for its target antigen [22]. Access to a homogeneously-expressed cell surface antigen as a target is also important for a successful mAb-mediated therapy.

The mAb BCD-F9 raised originally against a breast carcinoma cell line (BT-20) was shown to recognise a cell surface antigen on mammary tumours and, to a lesser extent, on normal breast tissue [23]. The present data demonstrate that the epitope recognised by the mAb BCD-F9 was not restricted to the breast cancer cell lines. It is also present at varying degrees on other neoplastic cell lines from different origins: colon carcinoma, sarcoma, lymphoma and leukaemia. A common reactivity with BCD-F9 implies that these cell lines share a common surface antigen and/or express different molecules with common or cross-reactive epitopes.

The ability of BCD-F9 mAb to recognise the human fibrosarcoma cell line HT-1080 enabled us to use this cell line for an experimental antibody-mediated therapy in a xenograft tumour model. Although BCD-F9 reacted more strongly with the LoVo and Raji cell lines, HT-1080 was preferred for the *in vivo* studies, mainly because of the rapid tumour growth in nude mice transplanted s.c. and the production of lung metastases when the tumour was injected i.v. The antitumour activity of the mAb BCD-F9 is supported by the reduced growth rate of the tumour in BCD-F9-treated mice transplanted s.c., the survival of all mice inoculated i.v. with tumour cells, and the absence of detect-

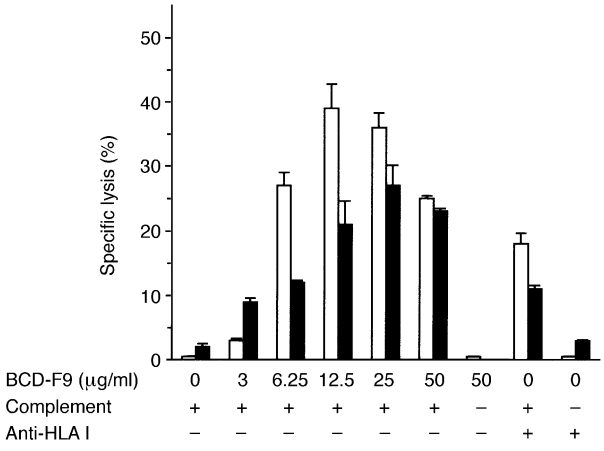


Fig. 6. Capacity of mAb BCD-F9 to kill HT-1080 cells by antibody-dependent complement-mediated cytotoxicity (ADCMC). Lysis of radiolabelled tumour cells was measured in a standard [$^{51}$Cr]-release assay after cells were incubated for 45 min at 37 °C with various concentrations of protein G-purified mAb BCD-F9 and with guinea-pig (open bars) or mouse (solid bars) complement. The values shown are means of triplicate samples±standard deviation (S.D.).

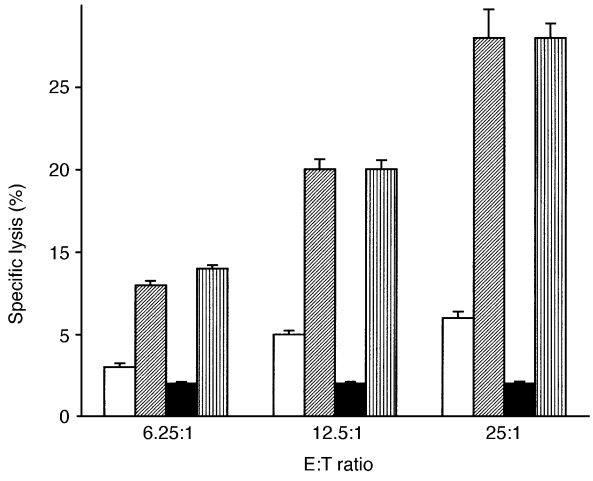


Fig. 7. Capacity of mAb BCD-F9 to kill HT-1080 tumour cells by antibody-dependent cellular cytotoxicity (ADCC). The capacity of protein G-purified mAb BCD-F9 to lyse radiolabelled HT-1080 tumour cells by ADCC was evaluated in a standard [$^{51}$Cr]-release assay using as effector cells Natural Killer (NK)-enriched spleen cells (hatched bars) or thioglycollate-elicited peritoneal cells (lined bars) harvested from CD-1 nude mice. Open bars and solid bars show the resistance of HT-1080 target cells to lysis by splenic NK-enriched cells and peritoneal cells in the absence of antibodies, respectively. The values shown are the mean of quadruplicate samples ±standard deviation (S.D.) containing cells at the indicated Effector:Target (E:T) ratios.

able metastatic foci following histological examination of the lung of sacrificed animals at day 60 post-injection. As *in vitro* analyses showed that BCD-F9 has the ability to kill HT-1080 tumour cells by ADCC or ADCMC, these mechanisms may contribute to the *in vivo* antitumour activity of the mAb. These same mechanisms can explain the presence of necrosis in the centre of s.c. tumours in BCD-F9-treated group. Usually, rapidly growing masses show central necrosis due to insufficient neo-vascularisation [24], while in our study only smaller s.c. tumours showed necrotic foci and only when treatment with BCD-F9 mAb was administered. These data suggest that the BCD-F9 mAb can elicit a specific cytotoxic response that is not observed following the inoculation of non-specific IgG. As no killing was observed after tumour cells were incubated for up to 9 h with BCD-F9 in the absence of effector cells or complement, the mAb alone is unlikely to be efficient in inducing tumour cell apoptosis.

The mechanism of tumour eradication mediated by antibodies *in vivo* is thought to be mainly ADCC, because macrophage and NK cell depletion resulted in 100% increase in tumour take in mAb-treated xenografted nude mice [9]. Although mAb BCD-F9 elicits ADCC by murine effector cells *in vitro*, this finding does not imply that ADCC is the only important mechanism for the inhibition of tumour growth *in vivo*. Another possible mechanism of cytotoxicity in a xenograft model is complement activation. Considering the strong complement-mediated cytotoxicity of BCD-F9 *in vitro*, the participation of the complement-mediated mechanism in the antitumour activity of this antibody *in vivo* cannot be ruled out. Usually, autologous cells will not be killed by complement-mediated mechanisms because they express complement downregulating factors [13,25,26]. However, this tumour evasion mechanism should not occur in nude mice as xenografted human cells lack inhibitors of murine complement. Therefore, although ADCMC may be involved in the control of HT-1080 tumour growth in nude mice, the capacity of BCD-F9 to activate the complement component cascade would probably be of limited interest for a therapeutic application.

The immunoprecipitation of cell lysates after metabolic staining with radiolabelled methionine or glucosamine allowed the identification of a 57-kDa glycoprotein as the major molecule expressing the BCD-F9 epitope. Although less intensively, the mAb reacted with other proteins, for instance with some of 25 and 80 kDa, respectively. Whether the detected proteins coprecipitate with the predominant protein of 57 kDa or share with it a common epitope has to be established. It is conceivable that different band patterns might even be detected after immunoprecipitation of lysates from the other tumour cell lines that reacted with BCD-F9 in the flow cytometry analysis. However, the fact that the mAb binds to several proteins does not limit its interest for immunotherapy. Indeed, the mouse mAb 17-1A, a reagent well known for its therapeutic effect in colorectal cancer patients with minimal residual disease [27], not only detects an epithelial cell adhesion molecule of 33–40 kDa, but also immunoprecipitates other proteins of 50 and 65 kDa from different cell lysates [28]. Whether these secondary antigens play a role in the antitumour activity of mAb 17-1A has not yet been determined.

Taken together, the findings reported in this study suggest that broad recognition of neoplastic cell lines from different origins identifies mAb BCD-F9 as a potentially useful immunotherapeutic reagent. It is worthwhile to study whether mAb BCD-F9 will be capable of suppressing the tumour cell growth and metastasis of other human tumour cells. Should this be the case, the contribution of ADCC to the *in vivo* antitumour activity of the mAb will have to be confirmed and the effector cells involved will have to be identified. The use of NOD/severe combined immunodeficient mice that lack functional B, T and NK cells and are deficient in complement-mediating killing [29] may more directly address the issue of what is really the conventional mechanism of action to explain the efficacy of mAb BCD-F9 *in vivo*. It will then be possible to consider combining treatment with mAb and cytokines to improve the therapeutic effect of BCD-F9. Successes with such a strategy were recently reported for different mAbs, in particular, mAb 14.G2A [30], which reacts with the GD2 ganglioside expressed on several tumours, including neuroblastoma and melanoma, and mAb 17-1A that has already been used for the treatment of minimal residual disease in colon cancer patients [31–33].

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