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ORIGINAL ARTICLE

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Immune responses in advanced colorectal cancer following repeated intradermal vaccination with the anti-CEA murine monoclonal antibody, PR1A3: results of a phase I study

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Abstract *Background and aims:* The aim was to determine the toxicity, clinical and immune responses to the murine monoclonal anti-carcinoembryonic antigen (CEA) antibody, PR1A3, in patients with advanced colorectal cancer. *Materials and methods:* Fifteen patients with advanced colorectal cancer received either 0.5-, 1.0- or 5.0-mg doses of PR1A3 mixed with 10% w/v Alum adjuvant (Superfos Biosector, Denmark) intradermally at 4-week intervals for 3 months. Patient serum was assessed for anti-idiotypic (Ab2), anti-anti-idiotypic (Ab3) and human anti-mouse antibody (HAMA) reactivity. Peripheral blood mononuclear cell (PBMC) proliferation with phytohaemagglutinin (PHA), CEA and PR1A3, stimulated IL-2, IL-4 and IFN-γ levels and PR1A3-stimulated IL-2 receptor expression during immunotherapy were determined. Comparisons were made with 16 age-matched controls without malignant disease. *Results:* Hyperimmune sera from 12 of the 15 patients showed Ab2 reactivity with no detectable Ab3 responses. Strong HAMA reactivity was recorded in 7 of the 15 cases with no adverse clinical effect. Delayed-type hypersensitivity (DTH) responses developed in 12 of the 15 patients. Pre-treatment PBMC proliferation with PHA was subnormal in each patient compared with controls, becoming normal (or supranormal) in all patients during immunisation ($P<0.001$). PBMC proliferation with CEA and PR1A3 increased during immunotherapy ($P<0.001$) along with stimulated production of IL-2, IFN-γ and IL-2 receptor expression. Progressive disease was observed in 14 of the 15 patients with minimal toxicity. *Conclusion:* PR1A3 generated limited idiotypic responses but robust DTH reactivity in most patients. In vitro PBMC proliferation with mitogens and recall antigens is greatly increased during the course of immunisation, with a shift in stimulated cytokine profile.

Keywords Monoclonal antibody therapy · Immunotherapy · Anti-idiotypic antibodies

Introduction

There has been considerable interest in the use of monoclonal antibody-based therapies in colorectal cancer, utilising murine, chimeric and humanised anti-carcinoembryonic antigen (CEA), murine anti-idiotypic and human anti-idiotypic antibodies [1]. Up to 80% of colorectal cancers (CRC) over-express the tumour-associated antigen CEA, with diagnostic and therapeutic monoclonal antibodies (mAb) being well tolerated in patients with advanced disease [2]. Their perioperative adjuvant use in patients with Dukes' C CRC receiving treatment with the murine mAb 17-1A (anti-Ep-CAM) has shown an early 30% improvement in overall survival and an equivalent reduction in metastatic recurrence with durable survival advantage up to 7 years [3, 4], although preliminary phase III evidence using this antibody in stage III colon cancer has not shown improvement either in disease-free or overall survival [5, 6].

Unmodified mAb therapy in solid epithelial malignancy is thought to function by recruiting several antitumour effector systems, including complement-dependent cell death and antibody-dependent cellular cytotoxicity (ADCC) [7, 8], by the induction of tumour cell apoptosis, cell cycle arrest, abrogation of angiogenesis and by the induction of CEA-specific antibody networks. The latter mechanism was originally predicted by Jerne as a cascading generation of antibodies that specifically target the antigenic epitope of CEA through recognition domains in the antigen-binding site [9]. It is postulated that primary administered murine anti-CEA antibodies (Ab_1) will contain components functioning as secondary "epitopes" for recognition by a second line of antibodies referred to as anti-idiotypic antibodies (Ab_2) stereochemically resembling CEA itself. These Ab_2 antibodies may in certain circumstances be sufficiently immunogenic to stimulate a third tier of recognition anti-anti-idiotypic antibodies (Ab_3), which then functionally resemble the administered murine mAb.

Antibody networks, if induced by the primary treatment Ab_1, could regulate cancer development by neutralising circulating CEA as well as by binding to surface immunoglobulin receptors on activated immunocytes resulting in tumour-specific T cell receptor stimulation and cytotoxicity. The use of mAb therapy in patients with advanced colorectal cancer has been shown to induce these antibody networks, although the response is dependent upon whether vaccination is by unmodified murine Ab_1, murine syngeneic Ab_2, or human Ab_2 antibody therapy [10–12].

PR1A3 is an IgG1κ mAb that was produced from mice immunised with normal human colonic epithelium, showing binding to upper crypt columnar epithelium and demonstrating high sensitivity and specificity for colorectal tumours of all grades of differentiation [13]. It has been used in the clinic as a sensitive and specific radioimmunoscintigraphic guide for recurrent CEA-bearing colorectal carcinomas [14, 15], showing no effective binding to soluble circulating CEA or related immunoglobulin superfamily group members such as non-specific cross-reacting antigen and biliary glycoprotein [16]. Unlike other CEA antibodies, PR1A3 has a high affinity for its epitope and its recent mapping to the B3 domain of the protein close to the site of membrane attachment but exclusive of the glycosylphosphatidylinositol (GPI) anchor [17] has resulted in the construction of a recombinant recognition protein for PR1A3 (designated NABA), composed of the B3 domain of CEA and domains from BGP and IgG [18]. This has permitted the development of an ELISA that provides avid PR1A3 binding for assays in cell-free systems.

It is anticipated that PR1A3 will function as a valuable tumour targeting agent for clinical use in advanced disease where there are pre-existing high levels of circulating CEA. In this phase I study we evaluated the use of repeated intradermal PR1A3 vaccination in 15 patients with measurable advanced and/or metastatic CRC, assessing their humoral and cell-mediated responses during immunotherapy.

Materials and methods

Patient selection

The study was approved by the local ethics committee (Imperial College School of Medicine and Technology, Hammersmith Hospital) and all patients provided informed consent for treatment and involvement in the project. The study was conducted according to the guidelines for the use of monoclonal antibodies in phase I clinical trials [19]. All patients had CEA-positive, locally advanced, recurrent and/or metastatic CRC that failed to respond to standard therapy (Table 1). Baseline assessment included complete physical examination, chest radiography, thoracoabdominal computerised axial tomography (CT), serum CEA measurement and routine blood chemistries. Patients were eligible for the study if they had measurable disease, an estimated survival expectancy exceeding 4 months and an adequate performance status (ECOG 0-2) on admission to the study. Exclusion criteria were chemotherapy, radiotherapy or steroid treatment within 3 months of commencement of the study, prior PR1A3 radioimmunoscintigraphy, or a history of anaphylaxis or other serious allergy to xenogeneic proteins.

Monoclonal antibody preparation

The production and purification of murine PR1A3 has been previously described [13, 16]. Quality control procedures for monoclonal antibody production met the guidelines set out by the working party on the clinical use of antibodies, where antibody was formulated and packaged into vials of clinical grade by the Hybridoma Development Unit. PR1A3 was mixed with 10% w/v alum precipitate (Alhydrogel; Superfos Biosector, Frederikssund, Denmark) under laminar flow conditions by our local hospital pharmacy for use

Table 1 Characteristics of patients included in the phase I study. *TTP* time to progression (days), *TTD* time to death (days), *5FU* 5-fluorouracil, *FA* folinic acid, *CisPt* cis-platinum, *RT* radiotherapy

Patient	Age (years)	Dukes' stage	Differentiation	Locale	Prior oncologic therapy	Disease extent	Response	TTP	TTD
1	71	C	Well	Rectum	5FU+FA	Bilobar hepatic metastases	Enlarging hepatic metastases	608	–
2	80	B	Well	Sigmoid	5FU+FA	Pulmonary metastases, abdominal wall recurrence	Enlarging pulmonary metastases	360	440
3	69	C	Moderate	Rectum	5FU+FA	Bilobar hepatic metastases	Enlarging hepatic metastases	420	618
4	52	B	Well	Sigmoid	5FU+FA	Pelvic mass	New pulmonary metastasis	280	–
5	63	C	Moderate	Sigmoid	5FU+FA	Pulmonary/hepatic metastases	Enlarging hepatic metastases, ascites	155	248
6	59	B	Moderate	Sigmoid	Nil	Bilobar hepatic metastases	Enlarging hepatic metastases, ascites	240	325
7	70	C	Moderate[a]	Caecum	5FU+FA	Bilateral pulmonary metastases	New hepatic metastasis	380	600
8	58	C	Poor	Caecum	5FU+FA DXRT	Pelvic mass	Stable pelvic mass	620[b]	–
9	60	C	Poor	Rectum	5FU+FA RT	Pelvic mass, anastomotic recurrence	New cerebral metastasis, new hepatic metastasis	400	–
10	55	C	Poor[a]	Sigmoid	5FU	Bilobar hepatic metastases, anastomotic recurrence	Enlarging hepatic metastases, ascites	260	328
11	53	C	Well	Sigmoid	5FU+FA	Bilobar hepatic metastases	Enlarging hepatic metastases	98	146
12	59	C	Well	Rectum	Infusional 5FU+FA	Bilobar hepatic metastases	Enlarging hepatic metastases	72	118
13	56	C	Poor	Sigmoid	5FU+FA	Bilobar hepatic metastases	Enlarging hepatic metastases	90	140
14	61	C	Moderate	Sigmoid	5FU+FA CisPt Irinotecan	Bilobar hepatic metastases	Enlarging hepatic metastases, jaundice	120	180
15	64	C	Moderate	Sigmoid	5FU + FA	Bilobar hepatic metastases	Enlarging hepatic metastases, jaundice	89	120

[a]Neural/perineural invasion
[b]Follow-up without progression (days)

in three different dosage formulations (0.5, 1 and 5 mg) made up to the same volume (1.4 ml).

Treatment schedule, toxicity and clinical response

The trial design was a dose escalation phase I study with the primary objective of assessing the toxicity of therapy. Fifteen patients (14 men; mean age 62 years; range 52–80 years) were randomly assigned to the three different doses. Comparisons were made with 16 age-matched controls (11 men; mean age 58 years; range 48–78 years) where the absence of neoplastic disease was established by clinical history, physical examination and routine laboratory testing. Patients were immunised intradermally with the PR1A3-alum gel every 4 weeks for 3 months. Dose escalation was only performed after the lower dose group of five patients had completed their three injections and had reached day 28 with no evidence of dose-limiting toxicity evaluated in accordance with National Cancer Institute criteria and review of blood tests. CT assessment and chest X-ray were performed at the beginning and at the end of immunisation. Blood samples prior to and during treatment were analysed for white cell count, absolute neutrophil count, peripheral blood CD4/CD8 ratios, renal and liver function and serum CEA. Sera were also separated and stored at −70°C until required for serological assays. Clinical responses were defined by the UICC (Union Internationale Contre le Cancer) response criteria for measurable lesions.

Delayed-type hypersensitivity reaction

Delayed-type hypersensitivity (DTH) responsiveness was assessed 48 h after each immunisation. No test dosing methodology was used with the responses being examined on the volar aspect of the non-dominant forearm during the immunisation schedule where induration and erythema was measured as mm^2 area using calipers. A full-thickness 2-mm punch biopsy (Stiefel, Offenbach, Germany) of immunisation sites was taken in six patients under local anaesthesia for immunohistochemistry at the end of the vaccination

schedule. Samples were orientated on cork disks for immediate freezing in isopentane and storage at −80°C and for fixation and paraffin embedding. Fixed specimens were stained with haematoxylin and eosin and frozen samples were evaluated for dendritic cells (with the mouse monoclonal antibodies RFD1/Factor XIIIa and CD1a; NA1/34 Clone; DAKO, Glostrup, Denmark) in accordance with previously described techniques [20], CD4, CD8 and CD 22 antibodies (Southern Biotechnology Associates, Birmingham, AL, USA), and with the pan-macrophage marker EBM 11 (anti-CD68; DAKO, Glostrup, Denmark) [21]. Appropriate positive and negative antibody controls were used and comparisons were made with samples of normal skin and purified protein derivative (PPD)-induced DTH-positive controls available from a skin tissue bank (Royal London Hospitals NHS Trust Skin tumour Laboratory).

Human anti-mouse antibodies

Levels of human anti-mouse antibodies (HAMA) were determined in patient sera before and after the vaccination schedule using a commercial HAMA ELISA (Immunomedics, Morris Plains, NJ, USA) following the manufacturer's instructions with values reported from a standard dilution curve as nanograms of precipitable antibody equivalents per ml against a known reference standard of anti-mouse IgG containing 220 ng equivalents/ml [22].

Ab_2 and Ab_3 assays

Anti-idiotypic (Ab_2) antibodies were detected by ELISA using 96-well microtitre plates coated with purified F(ab) fragments of PR1A3 (20 μg/ml). F(ab) fragments were generated by digestion of PR1A3 (4 mg/ml in 0.1 M sodium acetate, pH 6.5, 50 mM dithiothreitol) with papain (0.2 mg/ml) for 4 h at 37°C and purified by Superose and MonoQ chromatography. Serum reactivity was detected using an alkaline phosphatase-conjugated goat anti-human Fc antibody (Sigma, Poole, UK) and developed with *p*-nitrophenyl phosphate substrate (Sigma). Plates were read on a microplate autoreader (Labsystems Multiskan, Basingstoke, UK) at 405 nm. Positive tests were reported if maximal serum concentrations had optical density (OD) values >2 SD beyond a panel of control sera derived from age-matched patients without malignant disease or murine antibody exposure (n=16). Patients were also screened for Ab_3 levels by ELISA where 96-well microtitre plates were coated with a purified recombinant hybrid antigen (NABA) containing the CEA B3 domain, which has previously been shown to contain the PR1A3 epitope [17]. Antibody binding was determined using alkaline phosphatase-conjugated goat anti-human antibody and developed as described above.

Peripheral blood mononuclear cell proliferation assay

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinised whole blood by Ficoll-Hypaque (Nycomed, Asker, Norway) density gradient centrifugation. PBMCs were resuspended in complete RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% pooled normal human AB serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. All proliferation assays were performed using fresh PBMCs. Cells were added to 96-well flat-bottomed plates (Pierce-Warriner, Chester, UK) at 1×10^6 cells/well and stimulated with purified CEA, PR1A3, or control antibody, HMFG1 (0.01–10 μg/ml) or phytohaemagglutinin (PHA; 0.1–10 μg/ml; Sigma). Incubation doses of antibodies and CEA were based on studies of a similar nature during mAb immunotherapy with other murine Ab_1 antibodies (GA 733-2 and 3 H1) directed against CEA [11]. Cells were cultured for 3 days and pulsed with 1 μCi/well of tritiated thymidine (Amersham, Amersham, UK) for 18 h, harvested, and radioactivity was incorporated into DNA estimated by scintillation counting (Wallac, Milton Keynes, UK). The stimulation index (SI) was calculated as mean cpm of stimulated wells/mean cpm of control wells (supplemented medium only).

Immunofluorescent labelling and flow cytometry

Peripheral blood mononuclear cells (1×10^6 cells) were cultured in 96-well plates at 37°C for 7 days in the absence or presence of PR1A3 or control antibody (10 μg/ml) for determination of CD25-expressing CD4+ PBMCs as reported by Kosmas et al. [23]. Cells were harvested and labelled with phycoeryrthritin (PE)-conjugated mouse anti-human-CD4, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human-CD25 (Pharmingen, Oxford, UK) or isotype-matched control antibodies (Caltag, Towcester, UK). Antibody labelling was assessed by flow cytometry with a FACScan (Becton Dickinson, Oxford, UK) where gates were set on viable cells according to forward and side scatter and data were analysed using the Cell Quest software package.

Cytokine assays

Patient PBMCs (1×10^7 cells) were cultured with PHA (10 μg/ml) in 96-well microtitre plates and cell-free supernatant was harvested for IL-2 analysis after 48 h incubation, following preliminary kinetics experiments of control lymphocytes derived from age-matched volunteers without malignant disease (data not shown).

As initial experiments failed to show detectable levels of IL-4 in the supernatants of PHA-stimulated PBMCs, IL-4 production was detected following PBMC stimulation for 12 h using a combination of 10 ng/ml phorbol-12-myristate 13-acetate (PMA; Sigma) and 10 μmol/ml calcium iono-

mycin (Calbiochem, Nottingham, UK) as described previously [24]. IL-2 levels were quantitated by bioassay using the IL-2-dependent mouse cytotoxic lymphocyte cell line CTLL-2 [25]. IL-4 levels were quantified by ELISA using the anti-human IL-4 antibody (426-1A6-10; NIBSC, Potters Bar, UK) at 5 μg/ml for coating and the biotinylated antibody 426-8D4-8 (Pharmingen) for detection as previously described [26] IFN-γ levels were also measured in an ELISA using commercially available antibodies where the sensitivity for both IL-4 and IFN-γ ELISAs was 20 pg/ml. In all cytokine assays, WHO International standards or reference agents available from NIBSC were used.

Statistics

For variables that were measured only once for each subject, unpaired *t* tests or regression analyses were employed. For measurements taken at different immunisation times and under varying doses of mitogen/antigen exposure, random effects regression models were used. The normality of residuals for all data was assessed by the Shapiro–Francia *W′* test. Bartlett's test was employed to ensure equal variances in factors and where these assumptions were not met, data were transformed. Spearman's rank correlation was used to determine the relationship between cytokines produced and IL-2 receptor expression. All *P* values <0.05 are reported.

Results

Clinical response and toxicity

The immunological and clinical responses of the patients are shown in Tables 1 and 2. No serious toxicity was observed in any of the patients with only local reactions (erythema and induration) noted at the injection sites. Three patients (patients 6, 7 and 12) experienced mild fever and flu-like symptoms lasting 48 h after their final injection. None of the patients had observable lymphadenopathy and no haematological (total white blood cell and absolute neutrophil counts), hepatic or renal toxicity was observed, nor was there any observable change in peripheral CD4/CD8 ratios (data not shown). All but one patient (patient 8) eventually developed progressive disease with serial monitoring of serum CEA correlating with disease progression in all cases (data not shown).

DTH response to PR1A3

Positive DTH responsiveness was recorded when at least one dimension of skin induration or observable erythema exceeded 10 mm in diameter (Fig. 1). During the course of PR1A3 vaccination, 12 of the 15 patients developed DTH responses, although 3 patients had reduced responses following their third immunisation compared with their second (patients 1, 4 and 14). The mean area of induration measured after the first immunisation was 86.8±39.07 mm^2 (range 2–459 mm^2), 769±246.9 mm^2 (range 1–3,600 mm^2) after the second immunisation and 618.4±199.8 mm^2 (range 0–2,000 mm^2) after the third immunisation. Haematoxylin and eosin staining of immunisation site biopsies confirmed typical type IV hypersensitivity responses in the six biopsied cases, with extensive perivascular mononuclear cell infiltration of the dermis and dermal oedema. CD1a, RFD1 and EBM11 immunohistochemistry showed diffuse dermal infiltrates of dendritic antigen-presenting cells (Fig. 2) and macrophages, with only scant CD4, CD8 and CD22 infiltration.

HAMA reactivity

When sera was assessed for HAMA responses during the course of PR1A3 immunisations, patients fell into two broad categories of high responders (*n*=7; post-treatment HAMA reactivity 871.3±39.1 ng/ml, range 720.2–1,011.4 ng/ml) and non/low responders (*n*=8; post-treatment HAMA reactivity 88.4±25.7 ng/ml, range 7.6–211.7 ng/ml; Fig. 3). None of the patients showed a positive pre-treatment HAMA reaction.

Ab_2 and Ab_3 production following immunisation with PR1A3

Ab_2 antibodies specific to the F(ab) fragment of PR1A3 progressively developed in 12 of the 15 patients following intradermal immunisation. An example of one of the progressive ELISAs is shown in Fig. 4 (patient 5). Two patients (patients 1 and 6) showed pre-existing low-level Ab_2. Mixed model ANOVA for repeated measurements from each subject failed to show an effect of the PR1A3 immunising dose on Ab_2 production (*P*=0.29). No evidence of Ab_3 antibody production was observed in any of the PR1A3-treated patient sera using the NABA (PR1A3 epitope-bearing) ELISA.

PBMC responses to mitogen, antibody and CEA following vaccination

Regression analyses based on the in vitro dose of mitogen used demonstrated that all patients tested (*n*=12) had subnormal dose-dependent responses to PHA prior to PR1A3 immunisation when compared with age-matched controls without malignant disease (P<0.001). PHA responsiveness was significantly upregulated in all patients during immunisation (*P*<0.001) with no significant differences noted in

Table 2 Immunologic responses of patients treated with PR1A3. *DTH* delayed-type hypersensitivity reaction recorded at some or all points during immunisation, *Ab2* anti-idiotypic antibody, *HAMA* human antimouse antibody (ng/ml equivalents), *Pre* pre-immunisation, *Post* post-immunisation, *PHA* phytohaemagglutinin, *CEA* carcinoembryonic antigen, *HMFG1* human milk fat globulin, *SI* stimulation index (cpm thymidine against mitogen or antigen/cpm against medium), *SEM* standard error of the mean, *1*, *2* first and second immunisations, *ND* not done

Patient	DTH	Ab2	HAMA (ng/ml)		PHA SI			CEA SI			PR1A3 SI			HMFG1 SI		
			Pre	Post	Pre	1	2	Pre	1	2	Pre	1	2	Pre	1	2
1	+	+	−		27.2	28.4	142.1	1.1	18.6	23.1	0.4	4.0	10.0	1.0	1.0	3.0
			92	101												
2	−	+	±		78.0	242.0	37.1	0.8	2.7	0.9	0.8	1.1	0.9	0.6	1.0	0.8
			22	160												
3	+	+	+		40.0	80.0	80.2	3.7	8.8	7.0	3.0	4.0	12.0	3.3	3.1	1.8
			20	900												
4	+	−	−		174.6	201.9	130.0	1.0	4.0	13.7	0.5	16.1	47.1	1.0	3.8	2.7
			10	10												
5	+	+	+		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
			20	780												
6	+	+	+		20.0	38.7	49.9	0.6	4.0	14.0	1.0	4.0	53.9	1.7	1.6	2.0
			40	740												
7	+	+	+		191.1	202.0	237.0	2.8	2.5	6.0	4.0	2.7	2.7	3.0	2.8	2.6
			20	780												
8	+	−	−		30.1	147.1	144.4	4.0	19.9	4.1	3.0	3.0	7.8	3.7	0.6	3.3
			30	20												
9	+	+	−		25.5	54.0	150.0	0.2	1.3	4.0	0.5	2.0	1.6	0.6	0.8	0.7
			60	70												
10	+	−	−		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
			30	20												
11	−	+	+		60.0	149.9	ND	2.0	3.0	7.5	1.4	3.9	8.9	0.6	1.0	ND
			15	1,000												
12	+	+	+		42.2	347.0	120.1	0.2	14.1	15.0	0.1	4.0	18.7	1.0	3.0	1.6
			22	990												
13	+	+	−		45.5	147.7	141.1	0.9	3.0	3.1	1.0	1.8	17.0	2.0	2.7	1.8
			15	60												
14	+	+	+		75.0	100.0	137.9	0.6	6.0	35.1	3.0	13.3	17.6	2.0	0.8	1.1
			22	870												
15	−	+	±		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
			10	180												
Mean ± (SEM) patients					67.4	144.9	124.5	1.49	7.3	11.1	1.55	4.99	16.5	1.71	1.85	1.94
					(16.5)*	(27.0)**	(15.7)	(0.4)	(1.9)**	(2.8)***	0.4	1.3**	4.9****	0.3	0.3	0.2
Controls mean ± (SEM)					99.7			0.8			1.0			0.6		
					(24.2)			(0.05)			(0.05)			(0.03)		

P values for stimulation indices. Comparisons are made in patients for 10 μg/ml with either mitogen (PHA) or antigen (CEA, PR1A3 and HMFG1)
*Controls vs. pre-treatment patients, $P<0.001$
**Pre-treatment vs. first immunisation, $P<0.001$
***First immunisation vs. second immunisation, $P<0.05$
****First immunisation vs. second immunisation, $P<0.001$

responses by the PBMCs of the treated groups between the first or second immunisations ($P=0.17$; Fig. 5; Table 2).

Following immunisation with PR1A3, patient PBMCs proliferated when incubated with both PR1A3 (pre- vs. post-immunisation $P<0.001$; first vs. second immunisation $P<0.001$) and CEA (pre- vs. post-immunisation $P<0.001$; first vs. second immunisation $P=0.04$), with maximal responses occurring after the second injection, although the stimulation indices did not approach the values obtained with PHA. These effects were not PR1A3 dose-dependent

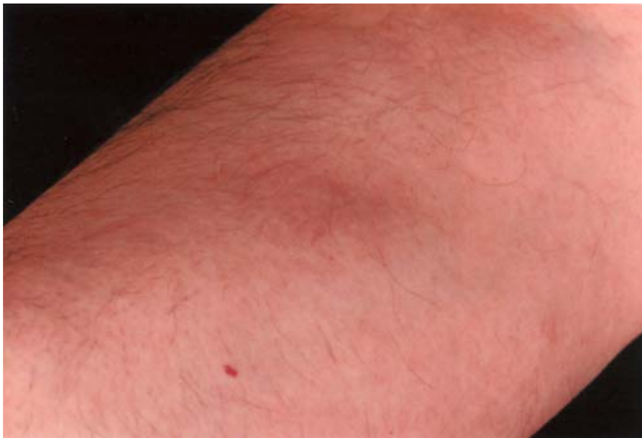

Fig. 1 Delayed-type hypersensitivity (DTH) at 48 h (patient 1) on the non-dominant forearm

on analysis and during immunisation there was minimal proliferation observed in co-culture with the idiotypically irrelevant IgG_1 mAb, HMFG1.

Induction of CD25 expression in vitro

Patient PBMCs incubated with PR1A3 showed a significant increase in the proportion of CD25+/CD4+ cells during the course of immunisation compared with PBMCs incubated with enriched medium only or with HMFG1.

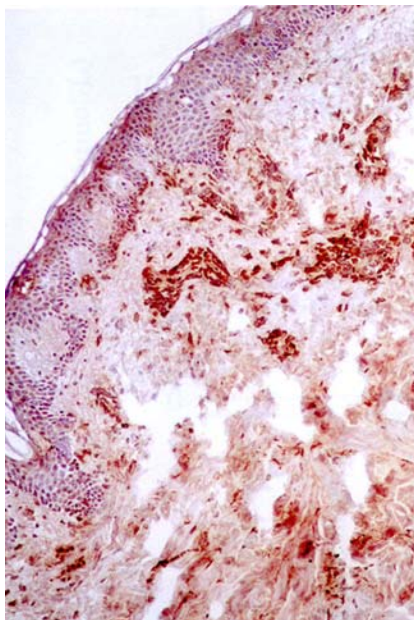

Fig. 2 Immunisation site biopsy immunohistochemistry. A dermal infiltrate of RFD-1-positive (dendritic) cells is shown (magnification ×40)

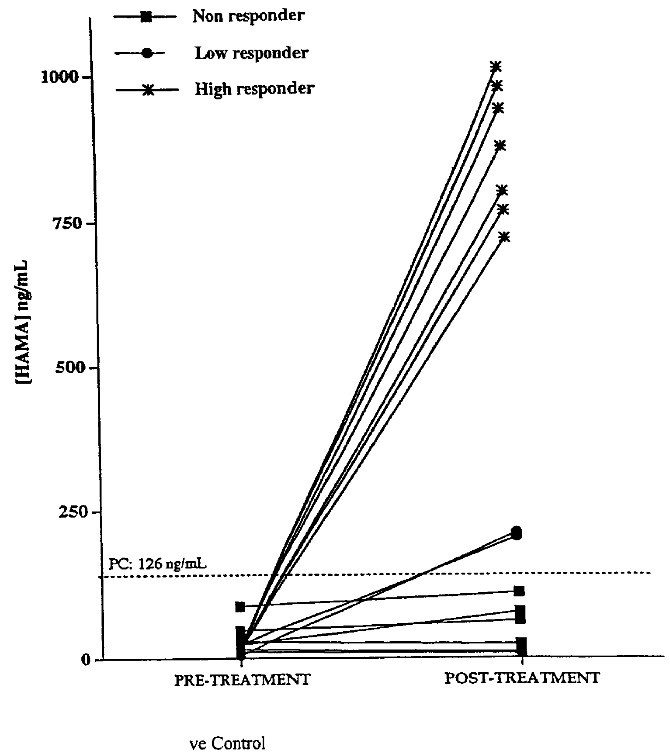

Fig. 3 Human antimouse antibody (*HAMA*) titres during PR1A3 immunotherapy

These values with PR1A3 increased from 4.6±1.1% before immunisation to 10.5±1.8% following the first immunisation, 16.4±3.8% after the second immunisation and 26.2±3.5% by the final vaccination ($P<0.001$). Percentage values for double-staining CD4+ PBMCs on incubation with HMFG-1 were 4.1±0.9% prior to immunisation and 4.4±0.6, 4.5±0.5 and 9.2±2.1% respectively after each PR1A3 immunisation (Fig. 6).

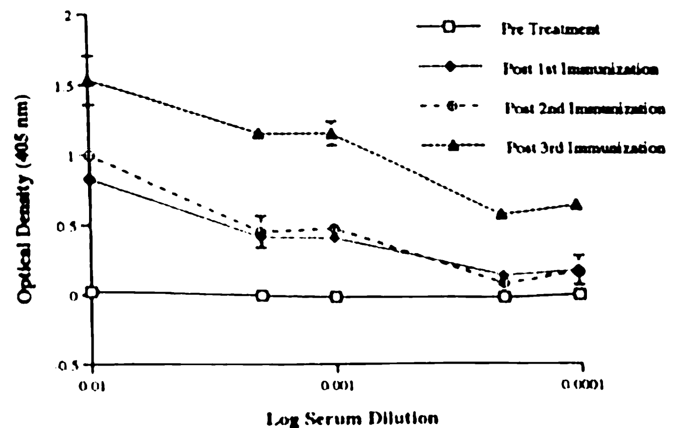

Fig. 4 Ab_2 (anti-idiotypic) responsiveness during PR1A3 immunotherapy (patient 5)

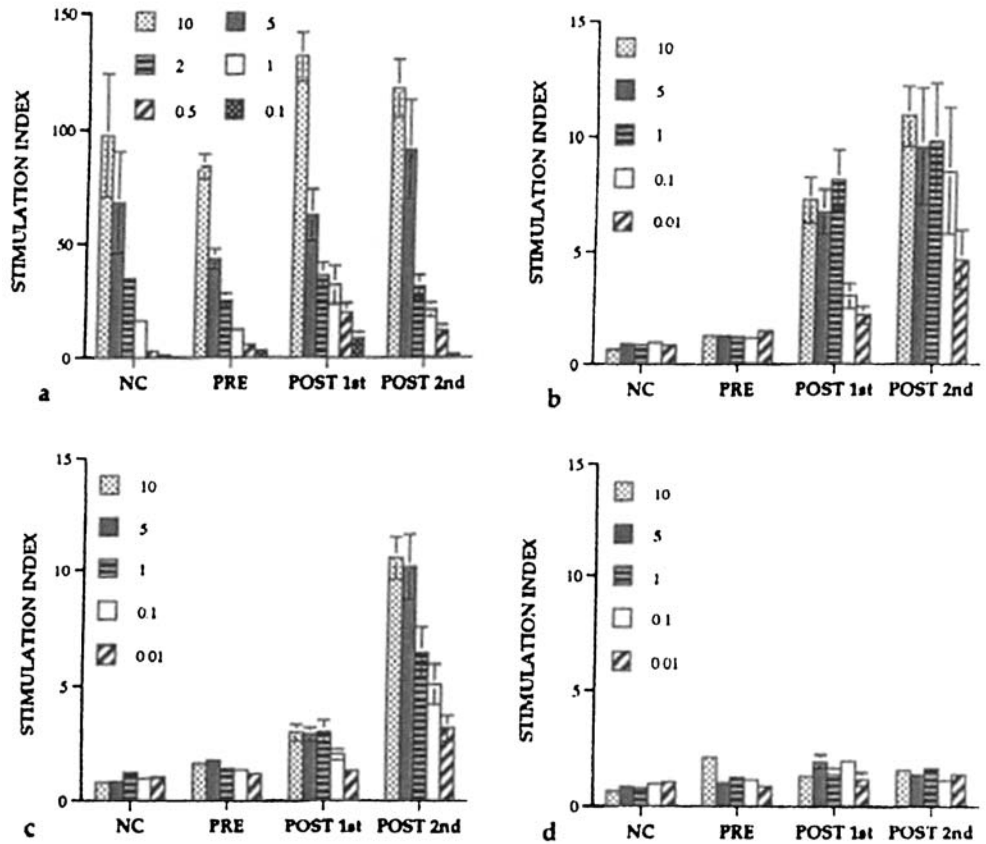

Fig. 5 Stimulation indices for peripheral blood mononuclear cells (PBMCs) in vitro during PR1A3 immunotherapy. **a** Phytohaemagglutinin (PHA), **b** Carcinoembryonic antigen (CEA), **c** PR1A3, **d** HMFG-1. Doses of mitogen/antigens are in μg/ml. *NC* normal controls, *PRE* pre-immunisation, *POST 1st* first immunisation, *POST 2nd* second immunisation. Doses of mitogen/antigen are in μg/ml.

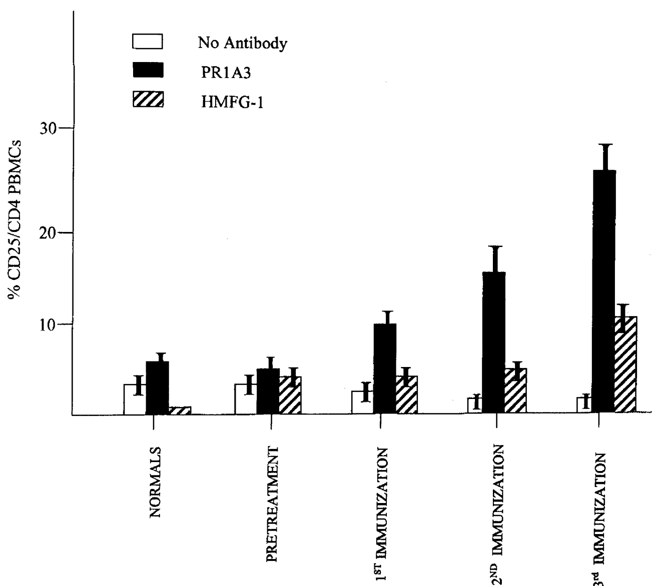

Fig. 6 Percentage of CD25/CD4 double-staining cells by FACS analysis during co-culture of PBMCs with 10 μg/ml of PR1A3 or HMFG-1

Cytokine production during PBMC stimulation in vitro

Prior to PR1A3 immunisation, none of the patients produced IL-2 following PHA stimulation. During PR1A3 vaccination, significant IL-2 upregulation was observed (post first immunisation 3.83±1.6 U/ml; post second immunisation 11.5±3.3 U/ml and post third immunisation 12.8±4.4 U/ml; $P<0.001$). Following completion of PR1A3 treatment, stimulated IL-2 levels from PBMCs exceeded IL-2 production by PBMCs derived from control subjects (control PBMC IL-2=3.83±1.32 U/ml; $P<0.001$; Fig. 7). IFN-γ production in response to PHA stimulation was significantly lower in the pre-treatment patient group compared with control PBMCs (309±78 vs. 4,737.5±845.2 pg/ml respectively; $P<0.001$). Despite IFN-γ levels progressively increasing during PR1A3 immunotherapy ($P<0.001$), IFN-γ production remained subnormal compared with controls during the immunisation schedule (1,800±820 pg/ml after the first immunisation and 2,895±992 pg/ml after the second immunisation; insufficient samples for comparison after the third immunisation). IL-4 levels from pre-PR1A3 treatment patient PBMCs were found to be significantly higher compared with control PBMCs (mean control IL-4=919.4±163.9 pg/ml vs. mean pre-treatment IL-4=1,400.6±265.7; $P<0.001$) and there was a significant overall reduction in IL-4 production during the course of PR1A3

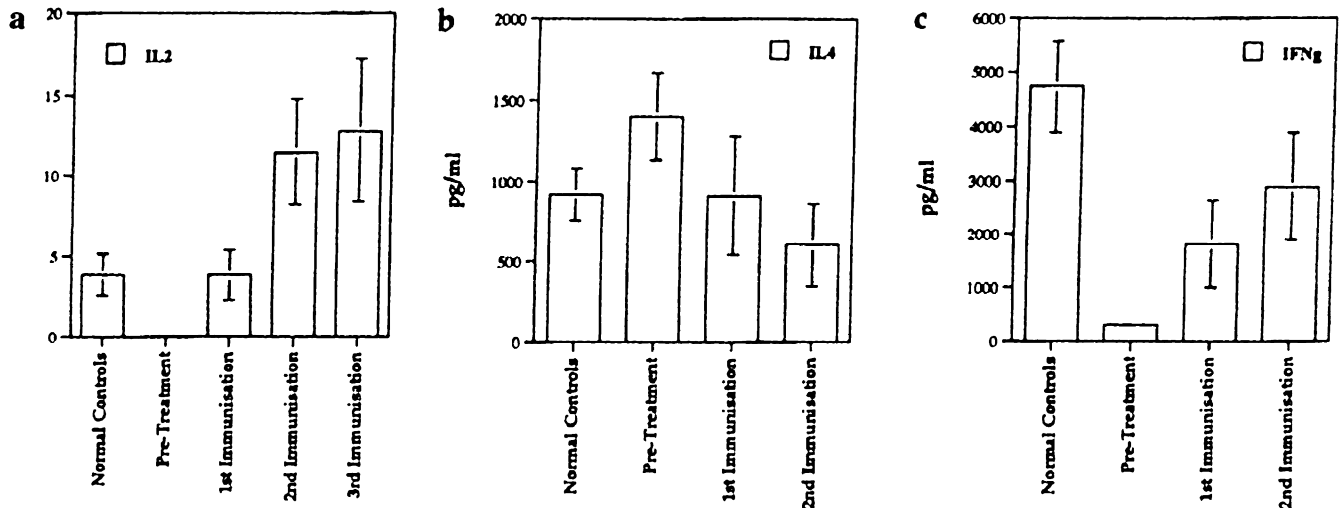

Fig. 7 Supernatant cytokine production by PBMCs in vitro during PR1A3 immunisation (see Materials and methods). **a** IL-2 was measured by bioassay with CTLL-2 cells and **b** IL-4. **c** IFN-γ was measured by ELISA.

immunisation (910.8+ 370.6 pg/ml after the first immunisation and 606.3+258.9 pg/ml after the second immunisation: $P<0.001$; insufficient samples for comparison after the third immunisation).

There was sporadic low level IFN-γ production at variable times in the immunisation schedule in the case of CEA (patient 1, 20 pg/ml after two immunisations; patient 4, 175 pg/ml after three immunisations and patient 14, 150 pg/ml after one immunisation) and in the case of PR1A3 after the second immunisation (patient 4, 175 pg/ml). No correlation was evident between cytokine levels at any time point during immunisation or between stimulated IL-2 levels and the percentage of IL-2 receptor-expressing CD4+ lymphocytes during the course of immunotherapy.

Discussion

The PR1A3 antibody is in theory, an effective reagent for the targeting of CRC as it binds preferentially to cell-bound and not to soluble CEA; a potential advantage in metastatic or locally advanced disease where there are frequently high levels of circulating tumour-associated antigen capable of complexing with other anti-CEA antibodies. In the present study there was no evidence of treatment toxicity, similar to other studies in CRC using different mAbs [3, 5]. Progression of measurable disease was, however, observed in nearly all patients, a finding that has been recently reported in phase II trials of patients with metastatic CRC using the human anti-idiotypic antibody 105AD7 [27]. Immune responses resulted during the treatment schedule in most patients as evidenced by DTH reactivity, immunisation site dendritic cell and macrophage infiltration and by Ab_2 production, although none of the patients showed Ab_3 reactivity. It is accepted that some of this DTH responsiveness may have been due to anti-murine reactivity, although our group felt that it was unethical to subject these patients to contralateral exposure to an isotypically identical but idiotypically irrelevant murine IgG_1 monoclonal. There was high HAMA reactivity detected in half of the treated patients, although without attendant morbidity. Subnormal pre-treatment in vitro PHA responsiveness by PBMCs derived from our patients was normalised in all cases during the course of immunotherapy with an antigen-specific increase in PBMC proliferation in vitro with repeated exposure to CEA and PR1A3. This was accompanied by an increase in PBMC IL-2 and IFN-γ production, a reduction in IL-4 generation and an antigen-specific increase in IL-2 receptor (CD25)-expressing CD4+ PBMCs on co-culture with the immunising antibody.

Only half of the patients developed high HAMA titres during the course of the study. In theory, the presence of HAMA may neutralise mAb directly by immune complex formation, leading to rapid antibody clearance and even serious hypersensitivity, although studies have failed to show any correlation between tumour relapse and HAMA levels in patients with minimal residual CRC treated with adjuvant Ab_1 therapy [28]. Over half the patients in the study developed Ab_2 antibodies with two patients showing pre-existing Ab_2 reactivity; the latter finding having previously been reported in patients with a range of solid tumours undergoing murine Ab_1 therapy [29, 30]. We were unable, however, to show any Ab_3 reactivity, a finding that has been reported before in advanced CRC using human Ab_2 immunotherapy [31]. Other studies, have, however, correlated Ab_3 reactivity with overall cancer-specific outcome during therapy [10], although detection of Ab_3 species may be more dependent upon performance status of the patients undergoing treatment, where only fitter patients living longer may be able to generate extended antibody networks. There is also the potential that Ab_2/Ab_3 complexes may develop during therapy (affecting idiotope rec-

ognition by ELISA) [32], or that metastatic deposits will function as an "antibody sink" for circulating Ab_3, limiting their detection in sera as disease progresses.

There is substantial evidence to show that patients with advanced malignancy have global impairment in their cell-mediated immune responses, which in some cases correlates with disease stage and clinical course. These changes have been manifest as alterations in DTH reactivity to recall antigens, diminished lymphocyte proliferation in vitro to mitogenic stimuli and alterations in T-cell receptor signalling of both PBMCs and tumour-infiltrating lymphocytes [33]. In this study, not only was there upregulation of PHA responsiveness by PBMCs in vitro but also both soluble CEA and the immunising Ab_1 functioned as recall antigens, a finding that mirrors that of serum cytokine changes recently noted in patients with advanced colorectal cancer treated with combination chemoimmunotherapy [34]. The effects on PBMC reactivity in our study were not dose-dependent; in some patients, stimulation indices with very small concentrations of CEA approached pre-treatment responses to PHA. This type of finding has been noted before by Kosmas et al. in patients with advanced ovarian cancer undergoing intraperitoneal mAb therapy with radiolabelled and chelate-conjugated HMFG1 [23], as well as in patients with CRC treated with murine Ab_2 [35], although stimulation indices achieved in our study were much higher than in these other reports. Our findings were accompanied by only poor responsiveness in vitro by PBMCs to an idiotypically irrelevant murine monoclonal IgG1 (HMFG1), supporting the view that antigen-specific immunotherapy has an important bearing on lymphocyte recognition and memory in CRC. In this respect, Lanzavecchia et al. [36] have shown that T cell clones can be raised during mAb administration that are specific for murine immunoglobulin, but which are also capable of killing target tumour cells bound to mAb recognising tumour-associated antigen.

The separation by Mosmann et al. [37] of murine CD4+ PBMCs based on cytokine profiles into Th1 cells capable of producing IL-2, IFN-γ and TNF-β and Th2 cells that secrete IL-4, IL-5, IL-6, IL-10 and IL-13 has implications for the classification of human PBMCs. It is suggested that Th1 dominance is important for the development of DTH responsiveness and activation of cytotoxic lymphocyte function, and that Th2 dominance is more representative of humoral responsiveness. Pellegrini et al. [38] have shown a predominance of Th2 PBMCs from patients with CRC that is stage-dependent and Tsitsilonis and colleagues [39] have recently demonstrated an enhancement of serum Th1 cytokine production during mAb therapy (17-1A) at different stages of CRC. Our study expands on these findings by showing stimulated PBMC conversion in CRC from a Th2-dominant profile to a Th1-dominant phenotype during highly specific antigen-specific immunotherapy. Although both favourable and unfavourable T-helper cell responses can occur alongside progressing tumour burdens [40], more research is needed concerning the importance of the Th1/Th2 paradigm in CRC to define whether it may be predictive either of chemotherapeutic or immunotherapeutic response. Antibody-dependent cellular cytotoxicity (ADCC) appears to be a principal immune effector mechanism for tumour cell killing during unconjugated monoclonal antibody therapy, perhaps stimulating apoptosis and cell cycle arrest as well as inhibiting the process of angiogenesis necessary for metastatic spread [41]. Each is an effect that may be dependent upon the inherent cytokine milieu as well as being enhanced by the concomitant use of colony-stimulating factors [42, 43] The future direction of our work needs to assess the effect this novel antibody has on in vitro ADCC activity.

In conclusion, this antibody proved safe for repeated intradermal use, eliciting antigen-specific humoral and cell-mediated responses in vitro and converting PBMC cytokine production profiles to a pattern more conducive to tumour cytotoxicity. This approach has recently been adopted by Tsitsilonis et al. [39] using the 17-1A monoclonal antibody Edrecolomab, where this group showed enhanced PBMC proliferative capacity and increased lymphokine-activated killer activity against tumour-sensitive targets as well as changes in circulating serum cytokine levels to a more immuno-enhancing pattern. PR1A3 mAb therapy is a treatment of low toxicity and it is likely to be more appropriate for use in patients with minimal residual disease where there is less attendant immunosuppression. The results of this study justify exploration of its clinical use in a minimal residual disease setting.

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