Clin. exp. Immunol. (1989) **75,** 258–264

Humoral immune responses to XMMCO-791-RTA immunotoxin in colorectal cancer patients

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(Accepted for publication 14 October 1988)

SUMMARY

Monoclonal antibody 791 (XMMCO-791) recognizes a colorectal tumour-associated antigen. Antibody 791-ricin A chain immunotoxin (XMMCO-791-RTA) inhibits growth of human tumour xenografts and it is therefore being evaluated for the treatment of colorectal cancer. One of the problems with therapy with mouse monoclonal antibodies is they stimulate humoral responses in patients. However antigens linked to ricin are cytotoxic for B cells and therefore XMMCO-791-RTA may not be immunogenic. The humoral antibody response to murine monoclonal antibody XMMCO-791 (IgG2b) conjugated to the plant toxin, ricin A chain (RTA), was measured in colorectal cancer patients in a phase I clinical trial. All patients produced strong responses to the XMMCO-791 immunoglobulin and to RTA. The predominant response to the antibody was against the idiotypic determinant although anti-subclass and anti-mouse antibodies were also detected. A component of the anti-idiotypic immunoglobulin response in the colorectal cancer patients was directed against the combining site of XMMCO-791. These antibodies inhibited in-vitro binding of XMMCO-791 to target 791 cells and so may be inhibitors of repeated immunotoxin therapy. Immunotoxins do not abrogate the immune response to mouse immunoglobulin *in vivo* but instead are highly immunogenic.

Keywords human anti-mouse antibodies (HAMA) immunotoxin

INTRODUCTION

Monoclonal antibody XMMCO-791 recognizes a 72 kD glycoprotein on tumour cells (Price *et al.*, 1983; Campbell, Price & Baldwin, 1984) and this antibody, labelled with 131-iodine or 111-indium, has been used extensively to gamma camera image primary and metastatic colorectal cancers (Farrands *et al.*, 1982; Armitage *et al.*, 1984; Ballantyne *et al.*, 1986). Flow cytometry analysis of tumour cells derived by collagenase disaggregation of surgically resected colorectal carcinomas also showed that XMMCO-791 antibody reacted with two-thirds of tumours (Durrant *et al.*, 1986). Based upon these studies, XMMCO-791 antibody has been used to construct an immunotoxin by conjugation to ricin A chain (RTA) (Embleton *et al.*, 1986). Immunotoxin XMMCO-791-RTA is specifically cytotoxic *in vitro* for tumour cells expressing the gp72 antigen recognized by the antibody moiety (Embleton *et al.*, 1986), and it specifically and effectively inhibits growth of human tumour xenografts (Byers *et al.*, 1987b). Based upon these studies the immunotoxin is being evaluated for the treatment of colorectal cancer.

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Murine monoclonal antibodies are known to stimulate a human humoral antibody response. Anti-murine antibodies have been detected in patients treated with radiolabelled monoclonal antibody (1 to 5 mg) for tumour imaging (Pimm *et al.*, 1985; Rowe, Pimm & Baldwin, 1985) as well as in patients treated with larger doses (up to 1–2 g) for tumour therapy (Meeker *et al.*, 1985; Schroff *et al.*, 1985; Courtenay-Luck *et al.*, 1986). Furthermore, responses *in vivo* to murine monoclonal antibody OKT3 have been extensively documented in renal allograft patients (Chatenoud, 1986; Chatenoud *et al.*, 1986; Jaffers *et al.*, 1986). This study was therefore designed to analyse the spectrum of antibody responses to murine immunoglobulin in colorectal cancer patients treated with ricin A chain immunotoxin. In addition patients were monitored for antibody responses to the ricin A chain polypeptide component.

The objective of this study was to define the temporal changes in the anti-murine immunoglobulin responses, particularly the anti-idiotypic responses which have been reported to have the most significant influence on biological activity in renal allograft patients (Chatenoud *et al.*, 1986). The generation of anti-RTA responses may also be important in terms of antibody targeting of the immunotoxin. Finally the influence of the cytotoxic moiety in terms of cytotoxicity for antibody-produc-

MATERIALS AND METHODS

Patients

The colorectal cancer patients in this study were entered into a phase I clinical trial of XMMCO-791-RTA immunotoxin. Patients' ages ranged from 30–70 years and all had colorectal cancer metastases either in liver, lung or both organs. Routine clinical tests indicated that patients had good function in all organs at the time of entry into the study.

Immunotoxin Treatment

Immunotoxin XMMCO-791-RTA was prepared by conjugating highly purified ricin A chain to the murine monoclonal antibody XMMCO-791 (IgG2b) via a disulphide linkage (Embleton *et al.*, 1986). After purification it was produced for clinical use in a standard form at a concentration of 1 mg protein/ml in phosphate-buffered saline (PBS), pH 7·5.

XMMCO-791-RTA immunotoxin treatment was given as five daily intravenous infusions diluted in approximately 100 ml normal saline at doses ranging from 0·05 to 0·2 mg/kg/day (total doses 6·8–52·8 mg). Patients were tested for presensitization to mouse immunoglobulin prior to treatment by intradermal skin testing with 200 μg of native XMMCO-791 immunoglobulin. In one patient (EW) treatment was stopped after the first dose.

Detection of human immunoglobulins recognizing XMMCO-791-RTA

The presence of anti XMMCO-791-RTA antibodies was screened in parallel by ELISA assays. ELISA microplates were incubated for 18 h at 4°C with purified XMMCO-791 (5 μg/ml, 250 ng/well in PBS), or RTA (100 μg/ml, 5 μg/well in PBS) or purified myeloma IgG2a (5 μg/ml, 250 ng/well in PBS, Sigma, Poole, UK) prior to washing in PBS-Tween (0·01 M phosphate, 0·005% Tween 20, Sigma, Poole, UK).

The plates were incubated for 1 h at room temperature with serial dilutions (10^{-1}–10^{4}) of patient's serum diluted in 50 mM sodium citrate buffer, pH 4·5, containing 5% BSA. Following extensive washing the plates were incubated for 1 h at RT with a 1 in 1000 dilution of alkaline-phosphatase-conjugated goat anti-human IgG (anti-Fcγ) or anti-human IgM (anti-Fcμ) antiserum (Sigma, Poole, UK). After washing the assay was developed with *p*-nitrophenolphosphate (Sigma, Poole, UK) as the alkaline phosphatase substrate (1 mg/ml diluted in 0·1 M glycine buffer, pH 10·4, containing 0·001 M $MgCl_2$ and 0·001 M $ZnCl_2$). The optical densities of each well were read (Multiscan, Titertek, Flow Labs, Irving, UK) at 405 nM.

All sera were assayed on the same day and a maximum optical density was noted for each ELISA. The serum titration producing 50% of this maximum value was calculated since it was the most sensitive area of the assay where very small increases in antibody concentrations produced large changes in optical density. The 50% serum titre value allows comparison of results between the different ELISA assays. Sera were only considered positive if serial titration produced a significant decrease in optical density.

Detection of anti-combining site antibodies

Serial dilutions (undiluted to 10^{-2}) of serum in PBS containing 1% BSA were incubated for 1 h at RT with 0·1 μg of fluorescein isothiocyanate (FITC)-labelled XMMCO-791 prior to incubation with avidin FITC for 1 h at 4°C and then added to 791T cells (2×10^5). SRL-3 is an IgG2b monoclonal antibody which recognizes B2 microglobulin expressed by 791T cells. As it has a different recombining site to XMMCO-791, human anti-combining site antibodies in patients' serum should not interfere with binding of SRL-3 to 791T cells. However, if anti-subclass or anti-mouse antibodies can prevent antigen-antibody binding both XMMCO-791 and SRL-3 should be equally inhibited. The tests were assayed by flow cytometry (Robins *et al.*, 1986). Fluorescence was excited at 488 nm and collected via a 10 nm band with band pass filter centred at 515 nm after adjustment for standard conditions using fluorochrome-labelled latex beads. Fluorescence intensity expressed as a mean linear fluorescence (MLF) was calculated by multiplying the contents of each channel by its channel number and dividing by the total number of cells in the distribution (Roe *et al.*, 1985).

Anti-combining site antibodies in individual patients at different times were compared by calculating the serum titre which produced a 50% inhibition of XMMCO-791 FITC binding to target cells.

Detection of anti-(anti-combining site) antibodies

791T cells were incubated with patients' sera (50 μl, 10^{-0} dilution) for 1 h at 4°C. The cells were washed in PBS and incubated with FITC-labelled goat anti-human Ig antisera for 1 h at 4°C. Cells were analysed by a FACS IV as described above.

RESULTS

Detection of anti-XMMCO-791-RTA immunotoxin antibodies

All patients produced both IgM and IgG antibodies recognizing XMMCO-791 immunoglobulin (Fig. 1 a,b). The IgM response was first detected between 7–22 days after initiation of immunotoxin therapy, with peak responses in individual patients occurring between 7–32 days. In four patients where sera were analysed up to 40–60 days after treatment, the IgM antibody levels progressively diminished. The IgG antibody response to XMMCO-791 immunoglobulin was initially detected in all but one patient 5–18 days after initiation of therapy. In one patient (RA) IgG antibody did not become detectable until day 28 and coincided with the peak IgM response.

The IgM and IgG antibody responses to ricin A chain are shown in Fig. 2. All but one of the patients produced an IgM antibody response (Fig. 2b); the IgG antibody response was more pronounced will all patients responding (Fig. 2a). The most marked responses were observed in three patients (AF, YBP and IK).

The relative response of patients to XMMCO-791 immunoglobulin and ricin A chain was determined by titrating serial dilutions of serum against both components of the immunotoxin and comparing the dilution producing 50% of the maximum response (Table 1). In patient AF both the IgG and IgM responses to RTA were more pronounced than those

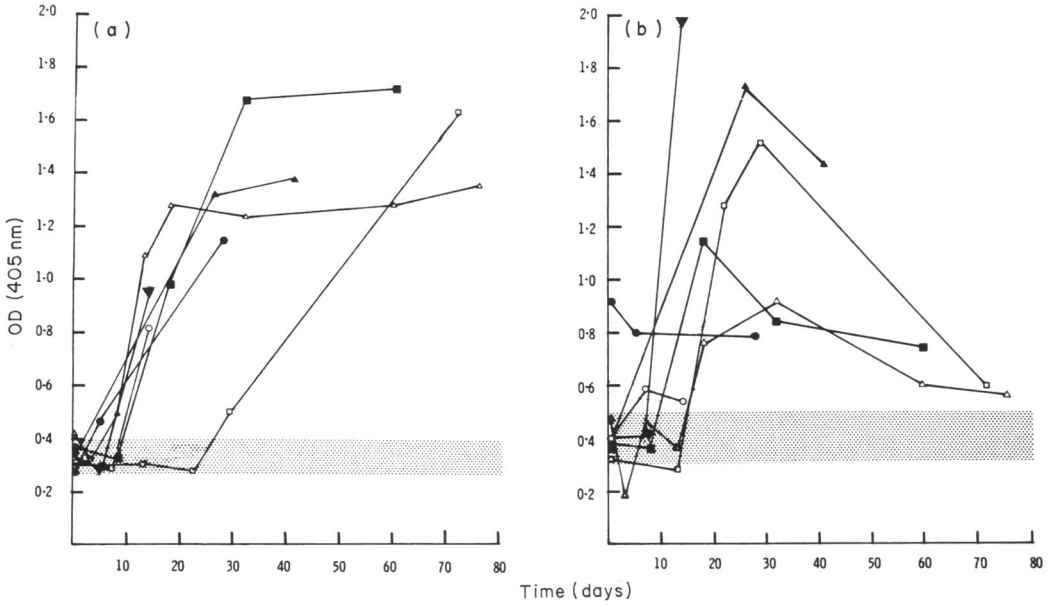

Fig. 1. Anti-murine XMMCO-791 IgG (a) and IgM (b) responses in colorectal cancer patients treated with XMMCO-791-RTA immunotoxin. Sera at a dilution of 10^{-1} in pH 4·5 buffer were screened by ELISA. Patient LF (●); EW (○); AF (■); RA (□); FC (▲), YBP (△); IK (▼). The shaded area denotes background optical intensity minus two standard deviations.

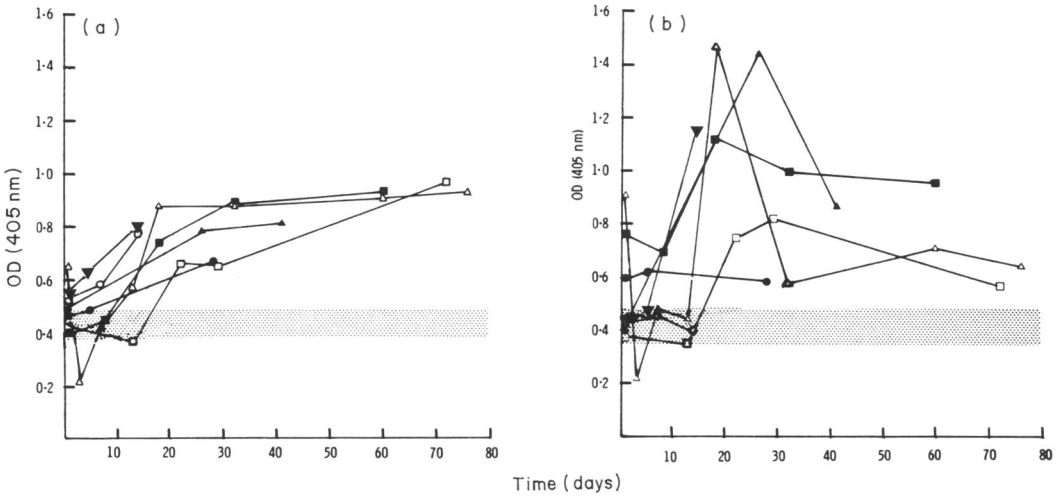

Fig. 2. Anti-ricin A chain antibody responses in colorectal cancer patients. (a) IgG, (b) IgM. Sera were screened at a dilution of 10^{-1} in pH 4·5 buffer. Patient LF (●); EW (○); AF (■); RA (□); FC (▲); YBP (△); IK (▼). The shaded area denotes background optical density minus two deviations.

IgG response to XMMCO-791T was much greater than that to RTA, whereas the IgM response was greater to RTA.

Comparing responses in seven patients (Table 1) few produced higher IgM titres to RTA compared to XMMCO-791; two of these patients produced greater IgG responses to RTA. Only one patient (LF) produced greater IgG and IgM responses to XMMCO-791 when compared to the response to RTA.

Three patients (AF, LF, YP) had pretreatment IgM antibodies which recognized RTA. However these patients did not produce a particularly strong IgM response to RTA following treatment and only one patient produced a strong IgG response. One patient had pretreatment IgG antibodies recognizing RTA and responded strongly following XMMCO-791-RTA treatment.

Five patients (LF, FC, IK, EW, RA) had pretreatment IgG antibodies which recognized XMMCO-791; one (LF) also had a similar IgM response. These patients did not respond, either at an earlier time or with a response following administration of XMMCO-791 stronger than the patients without pretreatment antibodies.

Anti-idiotypic antibodies could not be detected by binding to $F(ab)_2$ or Fab fragments of 791T/36, as this antibody is an IgG2b subclass and fragments produced from this mouse subclass are unstable. Comparative binding assays and inhibition assays were performed therefore.

Anti-mouse, anti-isotype and anti-idiotype responses

Table 1. Anti-idiotypic, anti-isotypic and anti-mouse common determinant antibody responses in colorectal cancer patients treated with immunotoxin XMMCO-791-RTA

Patient	Dose of immunotoxin (mg)*	Serum sample (days)†	Antibody titres‡ against XMMCO-791 IgM	XMMCO-791 IgG	Murine IgG2b IgM	Murine IgG2b IgG	Murine IgG2a IgM	Murine IgG2a IgG	RTA IgM	RTA IgG
LF	0·05/13·0	0	10	50	10	20	10	20	3	0
		5	10	50	10	10	10	10	2	0
		28	20	500	10	70	10	65	0	20
YBP	0·1/25·0	0	0	0	0	0	0	0	140	0
		13	0	10	0	10	0	10	0	25
		18	100	2 500	100	1 780	100	1 700	90	31 600
		32	45	4 000	45	1 500	45	1 400	100	316 000
		60	20	900	20	450	17	420	8	3 160
		76	20	750	15	250	15	250	6	1 250
FC	0·1/37·5	0	0	10	0	4	0	3	0	0
		26	140	2 500	110	1 000	30	35	30	6 310
		41	70	7 000	55	1 000	30	280	250	10 000
IK	0·1/38·0	0	0	10	0	0	0	0	0	45
		14	100	200	100	40	40	0	80	158 500
AF	0·1/42·5	0	0	0	0	0	0	0	16	0
		18	30	20	25	0	15	0	350	1 600
		32	10	1 000	10	210	5	15	200	2 500
		60	10	400	5	100	5	15	80	630
EW	0·1/0·8	0	0	5	0	0	0	0	0	0
		7	5	790	5	180	5	70	0	10
		14	10	1 000	10	250	5	50	1 780	20
RA	0·2/52·8	0	0	10	0	5	0	0	0	0
		22	0	20	0	10	0	0	100	130
		29	50	670	15	280	5	15	180	200
		72	80	7 000	15	1 600	5	1 400	5 000	630

* Daily dose/total dose.

† Day 0 sample taken immediately prior to immunotoxin treatment (day 0 to 5).

‡ Serum samples evaluated by ELISA for IgG and IgM antibodies binding to XMMCO-791 (IgG2b), murine myeloma IgG2b, murine myeloma IgG2a and RTA. Serum titre expressed as dilution giving 50% of maximum response.

immunoglobulin (BALB/c, IgG2b), BALB/c myeloma IgG2b, to detect anti-isotype antibody, and BALB/c myeloma IgG2a to detect antibodies recognizing mouse immunoglobulin common determinants. The relative response to these three immunoglobulins was compared by titrating serial dilutions of serum and determining the dilution producing 50% of the maximum response.

The responses of all seven patients to XMMCO-791, myeloma IgG2b and IgG2a are summarized in Table 1. The most marked response in all patients was the production of IgG antibodies to XMMCO-791, with peak titres ranging from 1/200 to 1/7000. In all patients the anti-XMMCO-791 response was several-fold higher than the response to myeloma IgG2b. For example, with patient FC the maximum titres to XMMCO-791 and normal IgG2b were 1/2500 and 1/1000, respectively.

Antibody reacting with myeloma IgG2a, recognizing mouse common determinants, was detected in all patients. However this response was only substantial in one patient (RA) who

Detection of anti-combining site antibodies

Human antibodies recognizing the combining site of XMMCO-791 were detected by their ability to prevent binding of fluoresceinated XMMCO-791 to 791T cells. Inhibition of binding of XMMCO-791-FITC to its target cell, 791T, by serial dilutions of sera from patient AF is shown in Table 2. Pretreatment and day 8 sera, undiluted, only caused weak inhibition of binding when pre-incubated with XMMC-791. However, by day 18 significant inhibition was produced by undiluted and 1/10 dilution of serum and by day 32 even serum diluted 1/100 produced marked inhibition of binding of XMMCO-791-FITC to 791T cells. The response began to fall by day 60.

Serum samples were titrated and the dilution which produced 50% inhibition of XMMCO-791-FITC binding to 791T cells calculated (Fig. 3). All but one patient (IK) produced anti-combining site antibodies. The patient who failed to respond was only followed for 14 days post therapy. None of the

Table 2. Inhibition of binding of XMMCO-791 antibody or SRL-3 antibody to target cells by serial dilutions of serum from patient AF

Serum Sample (day)*	Mean linear fluorescence		
	Dilution	XMMCO-791	SRL-3
0	10^{-0}	380	181
	10^{-1}	420	283
	10^{-2}	430	269
18	10^{-0}	10	161
	10^{-1}	170	284
	10^{-2}	400	261
32	10^{-0}	20	124
	10^{-1}	20	159
	10^{-2}	220	271
60	10^{-0}	10	134
	10^{-1}	30	261
	10^{-2}	390	232

* Day 0 sample taken immediately prior to immunotoxin treatment.

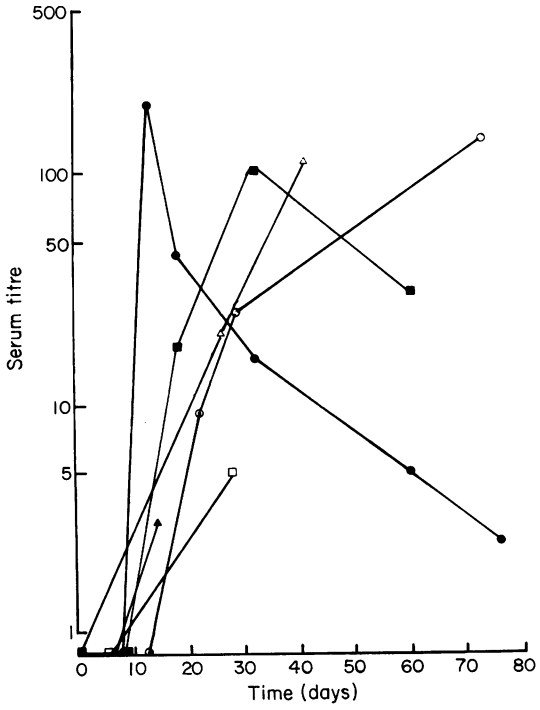

Fig. 3. Serum titre at different times after immunotoxin therapy producing 50% inhibition of binding of XMMCO-791 antibody to its

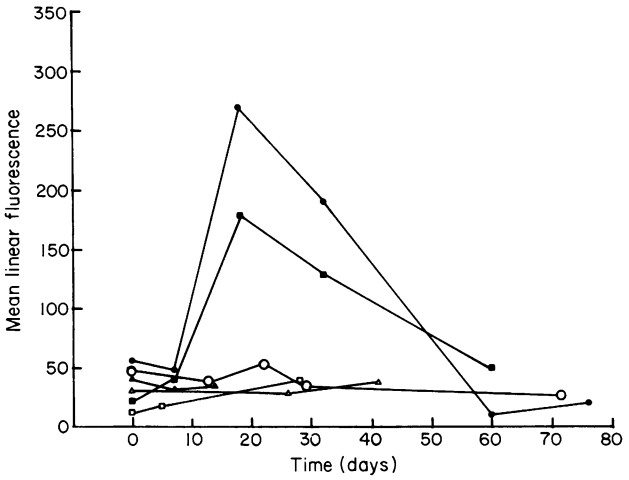

Fig. 4. Flow cytometry analysis of immunofluorescence binding of antibodies from patients serum. Patient LF (□); EW (▲); AF (■); RA (○); FC (△); YBP (●). Serum samples taken during and following treatment with immunotoxin XMMCO-791-RTA.

microglobulin monoclonal antibody SRL-3 to 791T cells. The data for patient AF is shown in Table 2. Furthermore, serum from a patient injected with radiolabelled antibodies for tumour imaging produced human antibodies which reacted equally with XMMCO-791 and myeloma IgG2b in the ELISA assays, but which failed to inhibit binding of XMMCO-791-FITC to 791T cells. Fusion of lymphocytes from this patient with a mouse myeloma resulted in the production of a human anti-IgG2b specific monoclonal antibody. This antibody also failed to inhibit binding of XMMCO-791T-FITC to target cells (Durrant to be published).

Of the four patients who were followed for more than 30 days after therapy, two continued to secrete quantities of anti-combining site antibodies which increased with time post-therapy. The two other patients produced strong responses which peaked at days 13 and 32.

The drop in anti-combining site antibodies could have been due to the formation of anti-(anti-combining site) antibodies. Anti-(anti-combining site) antibodies could also inhibit binding of XMMCO-791 FITC to its target cells and could therefore give a misleading impression of anti-combining site antibodies. However anti-(anti-combining site) antibodies will bind directly to target cells and were detected by their ability to react with 791T cells. Figure 4 shows the presence of human antibodies recognizing 791T cells in the serum from patient YBP and AF. These antibodies could be detected at day 18 and slowly decreased to negligible levels at day 60. None of the sera from other patients bound to 791T cells (Fig. 4).

The peak inhibition of binding of XMMCO-791-FITC to 791T cells was at day 32 in sera from patient AF and at day 13 in sera from patient YBP. However, peak anti-(anti-combining site) antibodies could be detected at day 18 for both patients. If anti-(anti-combining site) antibodies alone were responsible for inhibition of binding of XMMCO-791-FITC to 791T cells the peak responses should have coincided. Furthermore, anti-combining site antibodies fail to bind non-specifically to 791T cells since the majority of patients producing anti-combining

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