

Induction of IgM, IgA and IgE Antibodies in Colorectal Cancer Patients Vaccinated with a Recombinant CEA Protein

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Received: 4 June 2010 / Accepted: 31 January 2012
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Abstract

Purpose Previous clinical studies have indicated that natural IgM antibodies have the ability to induce apoptosis of tumor cells but IgE and IgA may also mediate tumor cell killing (in addition to IgG). The aim of the study was to analyse induction of IgM, IgA and IgE antibodies in patients vaccinated with the tumor associated antigen CEA.

Methods Twenty-four resected CRC patients without macroscopic disease were immunized seven times with CEA± GM-CSF. Four different dose schedules were used over a 12-month period. IgM, IgA and IgE antibody responses

against recombinant CEA were determined by ELISA. Patients were monitored immunologically for 36 months and clinically for 147 months.

Results GM-CSF significantly augmented the anti-CEA response for all three antibody classes. Low dose of CEA tended to induce a higher IgM, IgA or IgE anti-CEA antibody response than higher. Anti-CEA IgA antibodies could lyse CEA positive tumor cells in antibody dependent cellular cytotoxicity (ADCC) as well as in complement dependent cytotoxicity (CDC). A significant correlation between survival and high IgA anti-CEA titers was noted ($p=0.02$) irrespective of GM-CSF treatment.

Conclusions The observation that IgA anti-CEA antibodies were cytotoxic and associated with improved survival might indicate that also these antibodies may exert a clinical anti-tumor effect.

Electronic supplementary material The online version of this article (doi:10.1007/s10875-012-9662-7) contains supplementary material, which is available to authorized users.

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Keywords Colorectal cancer · CEA · GM-CSF ·
Vaccination · IgA · CDC · ADCC

Abbreviations

CRC	Colorectal carcinoma
rCEA	Recombinant carcinoembryonic antigen
GM-CSF	Granulocyte/monocyte colony stimulating factor

Introduction

Class and subclass antibody responses induced by vaccination might be of importance as each immunoglobulin isotype has distinct biological functions. The focus of humoral responses in cancer vaccine trials has mainly been on IgG. However, studies have also suggested that IgM, IgA as well

as IgE antibodies may have a role in eradicating cancer cells [1–6]. Vaccination strategies might have an advantage over treatment with monoclonal antibodies. As opposed to passive administration of antibodies, vaccines are easy to administer and induce a memory response.

The presence of spontaneously induced IgM anti-carcinoembryonic antigen (CEA) antibodies has been associated with improved survival of colorectal cancer (CRC) patients [7]. In melanoma patients vaccinated with a ganglioside, the occurrence of IgM antibodies was related to a prolonged disease-free interval and overall survival [8]. Tumor specific IgM antibodies isolated from cancer patients induced apoptosis of tumor cells [2]. Monoclonal IgM antibodies have been used to treat gastric cancer patients [9]. Also, vaccine induced IgE antibodies have been suggested to have anti-tumor effects [10]. IgE and IgA antibodies have also been proposed to be advantageous in tumor cell killing as compared to IgG [4, 11].

We have previously analyzed the IgG and T cell responses in CRC patients vaccinated with recombinant CEA (rCEA) with or without concomitant GM-CSF administration as an adjuvant cytokine [12, 13]. The capacity of GM-CSF to augment an antitumor response may be attributed to its role of activating antigen-presenting dendritic cells [14]. We chose to use a low dose of GM-CSF since low amounts rather than high have been shown to be effective in stimulating an immune response [15]. GM-CSF had a substantial impact on both the IgG and T cell responses [12, 13, 16]. There are no previous reports on the effects of GM-CSF on the IgM, IgA and IgE antibody outcome.

The aim of the present study was to assess over a 3-year period the IgM, IgA and IgE anti-CEA responses in CRC patients vaccinated with recombinant CEA with or without GM-CSF as an adjuvant cytokine.

Materials and Methods

Patients

Twenty-four patients, 14 males and 10 females, with a median age of 61 years (range 33–80 years) who had been operated on for CRC Dukes' stage A ($n=1$), B ($n=12$) or C ($n=11$) with no remaining macroscopic tumor entered the study (Table 1). Seventeen patients had colon cancer while in 7 patients the primary tumor was located to rectum. The Karnofsky index was $\geq 90\%$ in all patients. Except for surgery only one patient had received adjuvant chemotherapy. The median time from surgery to start of immunization was 5.7 months (range 1–39 months). The study was approved by the Ethic Committee of the Karolinska Institute and informed consent was obtained from each patient.

The first patient was included in Dec. 1994 and the last in April 1996. At that time, adjuvant chemotherapy was not an

accepted standard treatment for colon cancer stage C in Sweden. All patients were followed immunologically for 36 months and clinically for 147 months.

Vaccine Preparation

CEA is a glycoprotein with a molecular mass of about 180 kD (approximately 50% carbohydrate) [17, 18]. The recombinant CEA (rCEA) used in this trial was produced by Protein Sciences Corp. (Meriden, Mass., USA). The gene for CEA was cloned from human colon adenocarcinoma cells (LS174T) and introduced into the baculovirus *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) under the transcriptional control of the AcNPV polyhedrin promoter.

rCEA was engineered to contain all amino acids in the mature CEA. Expression in insect cells and purification has been described previously [16]. The purified rCEA for immunization was formulated in situ with aluminium phosphate (alum) (0.5 mg/ml of aluminium ion as AlPO_4).

Vaccination Protocol

The trial was as an open labelled, dose-ranging study. Six consecutive patients were enrolled at each of four rCEA dose levels: 100 μg , 316 μg , 1,000 μg and 3,000 μg per immunization respectively. rCEA was administered subcutaneously at days 1 and 14 and at months 2, 4, 6, 9 and 12, i.e. the total vaccination period was 1 year. At each dose level of rCEA, half of the patients ($n=3$) were randomized to concomitant administration of recombinant human GM-CSF (Leucomax[®]) (Schering-Plough, Kenilworth, NJ, USA) while the other half received rCEA alone. Eighty $\mu\text{g/day}$ of GM-CSF was given s. c. at each immunization time once daily, days 1–4, at the same site as the rCEA injection.

Clinical Examination and Follow-up

Before immunization a complete case history, physical examination and laboratory tests were done. The patients were followed clinically as scheduled every second month during the first 9 months, then every third month for 24 months and after that every 6 months. Blood were drawn for immunological analyses at pre-immunization and once monthly at 1, 2, 4, 6, 7, 9, 12, 15, 18, 21, 24, 30 and 36 months. Due to the shortage of samples only the following time points were used for the serological assays: pre-immunization, month 1, 2, 4, 6, 9, 12, 15, 18, 24, and 36.

ELISA

The levels of IgM, IgA and IgE antibodies (Ab) against rCEA were assayed by conventional isotype-specific

Table 1 Pre-immunization values and peak concentrations of antibody responses of colorectal carcinoma patients immunized with a recombinant CEA protein $\pm$ GM-CSF as an adjuvant cytokine

Patient no	IgG (AU/mL)		IgM (kAU/mL)		IgA (kAU/mL)		IgE (AU/mL)	
	Pre-value	Peak-value	Pre-value	Peak-value	Pre-value	Peak-value	Pre-value	Peak-value
1	0.16	1.7 (12 m) ^a	26	— ^b	0.20	0.66 (1 m)	<0.20	—
2	0.47	2.4 (12 m)	7.5	25 (1 m)	0.13	0.47 (1 m)	<0.20	3.8 (12 m)
3	0.14	—	9.5	—	1.2	—	<0.20	—
4	0.62	2.4 (12 m)	41	—	0.93	—	<0.20	93 (15 m)
5	0.097	0.68 (18 m)	164	—	1.3	—	<0.20	—
6	0.42	2.7 (12 m)	9.9	63 (1 m)	0.75	4.1 (15 m)	<0.20	70 (15 m)
7	0.50	2.5 (6 m)	29	63 (2 m)	0.47	4.1 (1 m)	<0.20	4.2 (12 m)
8	0.041	0.20 (36 m)	5.6	—	0.18	—	<0.20	—
9	0.12	—	10	207 (2 m)	0.077	0.86 (1 m)	<0.20	6.2 (6 m)
10	0.054	—	3.8	—	<0.050	—	<0.20	—
11	0.23	1.2 (6 m)	17	—	0.36	—	<0.20	—
12	0.086	—	18	590 (1 m)	0.25	5.5 (1 m)	<0.20	—
13	0.79	3.0 (12 m)	5.7	469 (1 m)	<0.050	0.38 (1 m)	<0.20	24 (15 m)
14	0.55	2.1 (18 m)	11	63 (4 m)	0.22	3.4 (1 m)	<0.20	13 (1+2 m)
15	1.3	—	9.8	—	0.65	—	<0.20	—
16	0.076	0.47 (12 m)	4.5	—	0.097	—	<0.20	—
17	0.34	—	9.5	—	0.35	—	0.64	—
18	0.34	2.4 (12 m)	8.6	110 (1 m)	0.86	9.1 (1 m)	<0.20	1.4 (18 m)
19	0.10	—	3.4	—	0.20	0.92 (9 m)	<0.20	—
20	0.15	2.4 (12 m)	3.0	—	0.42	—	<0.20	1.2 (18 m)
21	0.58	—	3.7	23 (1 m)	0.19	—	<0.20	—
22	0.39	2.5 (6 m)	12	—	0.37	—	<0.20	4.8 (15 m)
23	0.32	2.6 (6 m)	5.0	—	<0.050	—	<0.20	—
24	0.49	2.1 (12 m)	9.0	—	1.3	—	<0.20	—

^a month from start of immunization for antibody peak value^b the criteria for an antibody isotype class response were not fulfilled

sandwich ELISA developed and verified in our laboratory. Wells were coated with recombinant human CEA (2 μ g/ml) and serum samples were assayed at 1:500 (IgM), 1:200 (IgA) and 1:10 (IgE) dilutions. Following overnight incubation in the wells antibodies were detected with alkaline phosphatase (ALP)-conjugated rabbit polyclonal isotype-specific F(ab')₂-fragments (IgM and IgA) and biotinylated anti-IgE mAb/ALP-conjugated streptavidin [19] after 2 h incubations, respectively. More details about the ELISA-procedure have been published [20]. IgG anti-CEA ELISA has been described in detail previously [12].

The antibody levels were calculated from standard curves established with chimeric IgM, IgA2 and IgE anti-NIP (5-iodo-4-hydroxy-3-nitro-phenacetyl acid) hapten antibodies [20, 21] using BSA-NIP conjugate (10 μ g/ml) as coating antigen. This procedure permits comparison of results between runs in a semi-quantitative way where 1 AU (arbitrary ELISA unit) approximates to 1 ng of antibody as previously described [20]. The sensitivities of the IgA, IgM and IgE

antibody assays in serum are 0.1 μ g/ml, 2 μ g/ml and 0.4 ng/ml, respectively.

A patient was considered to have developed a significant response if IgM, IgA or IgE antibodies could be detected at at least two different time points and was at least twice that of the pre-immunization value for that patient.

Indirect Immunofluorescence Using Flow Cytometry

SW48 (a human cancer cell line expressing CEA) and A549 (a human lung cancer cell line not expressing CEA) were used for surface staining of a patient serum, pooled sera from normal healthy donors (NHD) and a non-relevant IgA isotype control [22]. Briefly, 10⁶ cells were washed in PBS containing 0.01% sodium azide and incubated with the IgA isotype control, NHD and the patient serum (1:10–1:1000) at 4°C for 1 h followed by 3 washings. A polyclonal rabbit anti human IgA serum (Abcam, Cambridge, USA) (1:100) in washing buffer was then added and incubated at 4°C for

1 h. The cells were finally washed 3 times and fixed by adding 1% paraformaldehyde in PBS. Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, NJ, USA). Flowjo software program (BD Biosciences) was used to determine the frequency of cells.

Complement Dependent Cytotoxicity (CDC)

Stored sera (-70°C) from a patient (no 18) who had developed high IgA anti-CEA antibody titers were used. Sera from two sampling times, 12 and 18 months from start of vaccination with IgA anti-CEA concentrations of 6.6 kAU/ml and 8.2 kAU/ml respectively were pooled to obtain a sufficient amount of serum. IgA from the patient and pooled sera from healthy donors ($n=4$) were isolated using peptide M/Agarose affinity column chromatography yielding highly purified IgA according to manufacturer's instruction (InvivoGen, San Diego, CA, USA). 1.8 mg IgA was obtained from 1.5 ml of the pooled patient serum and 2.5 mg from 3 ml of pooled normal sera.

CDC was performed as previously described [23]. Briefly, SW48 and A549 cells (5×10^4) were plated in V-bottomed microtiter plates (Nunc, Roskilde, Denmark) in 100 μl RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FCS (Invitrogen, Carlsbad, CA, USA) (complete medium). Cells were incubated with 20 $\mu\text{g/ml}$ of purified IgA from the patient and NHD respectively for 30 min at room temperature followed by twice washing with RPMI-1640. After washing, 20% normal human serum (NHS) in complete medium was added to the cells and incubated at 37°C in humidified air with 5% CO_2 for 1 h. Finally, cells were collected, washed twice with 1xPBS and resuspended in 100 μl of 1x binding buffer. Five μl of propidium iodine (BD Biosciences) was added to the cells, vortexed and incubated at room temperature in the dark for 15 min. 100 μl of 1x binding buffer was added to the cells which were analyzed by flow cytometry (FACSCalibur).

Antibody Dependent Cellular Cytotoxicity (ADCC)

ADCC was performed as previously described [24]. In brief, cell lines were labeled with 2.8 MBq sodium ^{51}Cr (PerkinElmer Inc. Wellesley, MA, USA) for 2 h. After 3 washings with DMEM medium (Invitrogen, Carlsbad, CA, USA) 10,000 cells in 100 μl medium were added to each round-bottomed microtiter well (Nunc) and Ficoll-isopaque isolated PBMC of healthy donors ($n=4$) as effector cells to yield target:effector cell ratios of 1:25 and 1:50 to a final volume of 200 μl . The test was run in sixuplicate and the same patient sera as for CDC was used (see above). After 24 h at 37°C the reaction was stopped by centrifugation. ^{51}Cr release was measured. The percentage of target cell lysis was calculated according to the following formula: % specific

lysis = $(\text{experiment cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm}) \times 100$. Maximal ^{51}Cr release was determined by adding RIPA lysis buffer (150 mM NaCl, 25 mM Tris PH=7, 5 mM EDTA, 0.5% SDS and 1% Triton X-100) to target cells and spontaneous release was measured in the absence of antibodies and effector cells.

Statistical Methods

The relationship between survival and anti-CEA Ig-titers were analyzed by Cox-Regression analysis where the through mean values of IgM, IgA and IgE levels respectively for all patients at pre-immunization and at month 1, 2, 4, and 6 were calculated and listed from lowest to highest value. Patients with a through mean value below the median of all patients were labelled value 0. Patients with a mean value above the median of all patients were labelled value 1. This factor was analyzed in an univariate Wilcoxon Gehan exact life table test for relation to survival. As development of antibodies is "time dependant", analysis of the prognostic impact of antibodies was performed after a fixed observation time [25]. As one patient died early, after 7 months, the 6 first months were used as observation time. The observation period and follow-up period were then held apart, avoiding "the pitfall of overlapping exposure and follow-up periods" [26, 27]. Survival in relation to both anti-CEA IgA and anti-CEA IgG titers was also analysed. Patients were grouped according to those having both IgA and IgG anti-CEA antibody levels (through value) above the median ($n=9$) and the remaining patients ($n=15$). Comparison of pre-immunization anti-CEA titers of deceased and alive patients as well as of cytotoxicity between groups were analysed by unpaired t-test. The relationship between anti-CEA IgA titres, GM-CSF and survival were analysed by Cox-regression analyses. The influence on survival by GM-CSF was assessed by univariate Wilcoxon Gehan exact life table test.

Results

Antibody Subclasses Responses

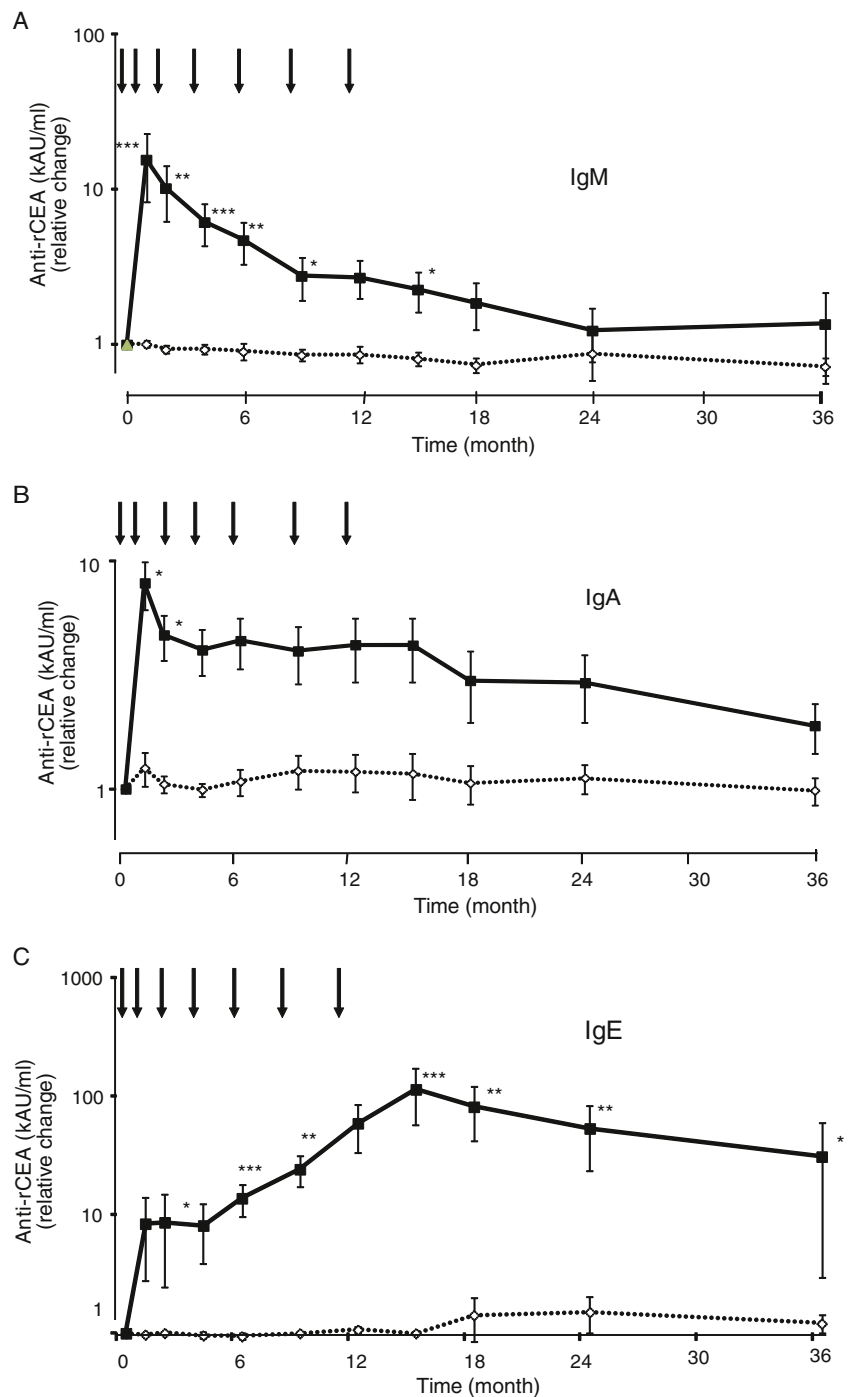
Most patients (83%) had detectable IgM, a majority (54%) IgA and one (4%) patient IgE anti-CEA antibodies before vaccination. Pre-immunization and peak antibody concentrations of individual patients are listed in Table 1. (Part of the IgG responses has previously been published [12] but is included for clarity). A strong IgM response was observed initially during vaccination with maximum titres at 3 months from start of vaccination. A rapid induction of IgA antibodies was noted already after one immunization and then a gradual increase with a peak at 12 months. Repeated

immunizations induced a gradual increase in the IgE anti-CEA antibody levels. Maximum IgE levels were noted at 15 months, i.e. shortly after the last immunization (Fig. 1a–c). The IgM-titers declined as expected much earlier than the IgA and IgE antibody levels. However, IgA and IgE anti-CEA levels clearly above pre-vaccination levels were still detectable 2 years after the last immunization.

Addition of GM-CSF significantly augmented the anti-CEA antibody responses for all antibody classes. Nine

patients (9/12; 75%) in the GM-CSF group developed increasing IgM antibody levels to CEA while none in the CEA alone group. The corresponding figures for IgA and IgE antibodies were 9/12 (75%) and 1/12 (8%) patients respectively. The difference in anti-CEA antibody titers for the various isotypes comparing the GM-CSF and non-GM-CSF group was statistically highly significant ($p < 0.05$ – 0.001) (Fig. 1a–c). There was no statistically significant relation between the dose of CEA and the magnitude of

Fig. 1 IgM (a), IgA (b) and IgE (c) anti-CEA antibody responses (AU/ml) (mean $\pm$ SEM) in colorectal cancer patients vaccinated with CEA together with GM-CSF ($n=12$) (solid line) or without GM-CSF ($n=12$) (dotted line). Arrows indicate immunization times. Asterisk indicate significance levels comparing the GM-CSF group with the non-GM-CSF group at the indicated time points. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The antibody titer curve over time (through values) comparing the two groups were statistically highly significant (ANNOVA): for IgM $p=0.003$; IgA $p=0.006$ and IgE $p=0.007$ respectively



the IgM, IgA and IgE anti-CEA antibody levels but a tendency to a stronger response with a low dose of CEA for all isotypes (Supplementary information Fig. 1S A–C).

Flow-Cytometry Analysis

Isolated IgA from the patient with high IgA anti-CEA titers bound to the CEA expressing cell line (SW48) but not to the CEA negative cell line (A549). The patient serum (1:1000) stained more than 30% of SW48 cells as compared to only 1% of the A549 cells (Fig. 2). Isolated IgA from normal control sera (1:1000) stained less than 1% of CEA positive and negative cell lines. The corresponding figures for the IgA isotype control were less than 2%.

Complement Dependent Cytotoxicity (CDC)

The IgA fraction of a vaccinated patient (no 18) containing anti-CEA antibodies was tested in CDC using SW48 and A549 respectively and could be demonstrated to significantly lysed CEA positive tumor cells in the presence of human complement ($p<0.0001$), but not the IgA fraction alone, complement alone or isolated IgA from healthy donors not containing anti-CEA antibodies (Fig. 3).

Antibody Dependent Cellular Cytotoxicity (ADCC)

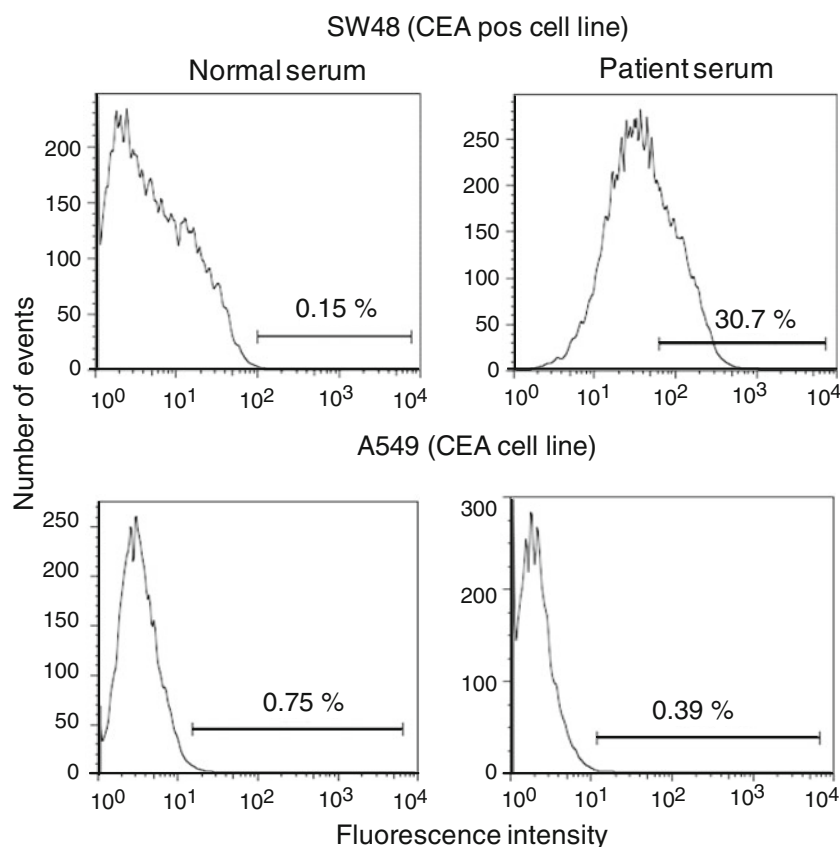
Isolated IgA from a vaccinated colorectal carcinoma patient (no 18) containing high IgA anti-CEA titers was also tested in ADCC using the same cell lines as above. PBMC from healthy donors were used as effector cells. The patient IgA fraction could significantly lyse the CEA positive cell line but not the CEA negative cell line ($p<0.0001$). Isolated IgA from healthy donors did not lyse tumor cells in ADCC (Fig. 4).

Clinical Outcome and Relation to Immune Response

Seven patients in the CEA alone group and six patients in the GM-CSF group relapsed and died during follow up (Table II). Two further patients in the CEA alone group died from other causes than CRC.

Pre-immunization IgA, IgM and IgE anti-CEA titers did not differ significantly comparing deceased and alive patients. There was a statistically significant correlation between IgA anti-CEA antibody levels and survival i.e. those patients with an anti-CEA IgA level above median survived significantly longer than those below ($p=0.02$) (Fig. 5). GM-CSF treatment itself did not significantly contribute to survival ($p=0.55$) (data not shown). Furthermore, irrespective of treatment with GM-CSF, patients with anti-

Fig. 2 Indirect immunofluorescence staining of SW48 (CEA expressing cell line) and A549 (CEA negative cell line) using isolated serum IgA (1:1000) from a colorectal carcinoma patient (no 18) immunized with CEA (containing a high IgA anti-CEA titer) and isolated serum IgA (1:1000) from pooled normal donors ($n=4$)



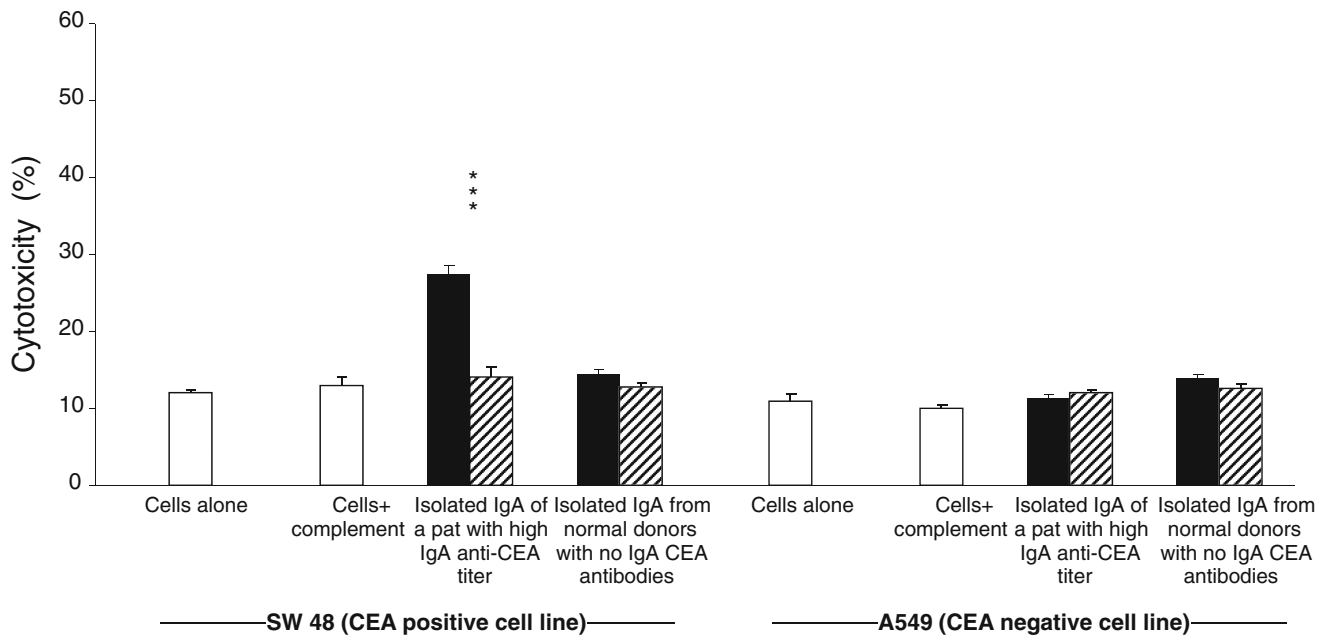


Fig. 3 Lysis of SW48 (CEA expressing cell line) and A549 (CEA negative cell line) in CDC. Isolated serum IgA (containing a high IgA anti-CEA titer) from a colorectal carcinoma patient (no 18) immunized with CEA and isolated IgA from pooled human normal serum ($n=4$) were incubated with the cells and human complement. Results are

presented as percent lysis (mean $\pm$ SEM). (■) cells with isolated IgA and complement (▨) cells with isolated IgA but no complement. Statistical calculations were done comparing cells incubated with IgA and with or without complement, *** $p<0.0001$

CEA IgA levels above the median of all patients had significantly better survival (Fig. 6). No significant relationship was found between the IgM and IgE antibody levels and survival (data not shown). We have previously shown that patients which were alive during the observation period had

a higher IgG titer than those who had deceased ($p<0.05$) [12]. In a univariate regression analyses for survival anti-CEA IgA titers (above and below the median) was significantly related to survival ($p=0.03$) and a clear trend for IgG ($p=0.07$). However, in a multivariate analysis none of these

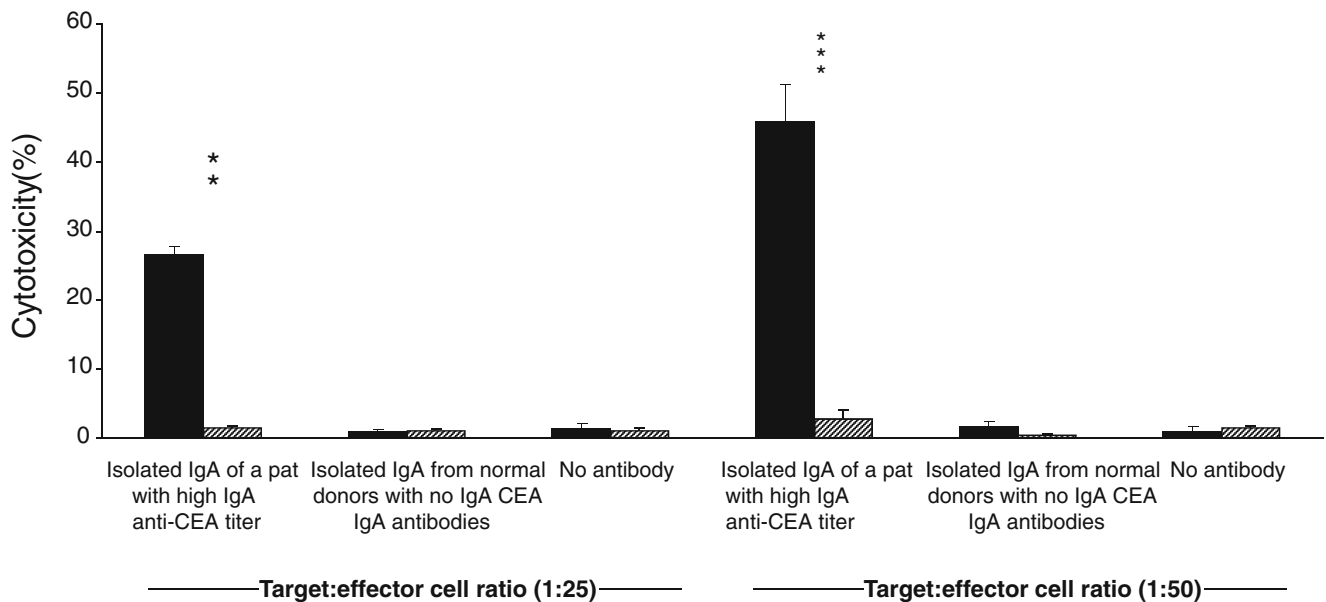


Fig. 4 Antibody dependent cellular cytotoxicity (ADCC) (24 h ^{51}Cr release assay) using SW48 (CEA expressing cell line) (■) and A549 (CEA negative cell lines) (▨) in the presence of human effector cells (PBMC from healthy donors) at different target:effector cell ratios

(1:25–1:50) and isolated IgA (20 $\mu\text{g}/\text{ml}$) from a colorectal carcinoma patient (no 18) immunized with CEA. Data are presented as mean percentage specific lysis $\pm$ SEM of sixplicates. Significance levels ($p<0.0001$) comparing SW48 and A549 are indicated above the columns

Table II Clinical characteristics of colorectal carcinoma patients immunized with a recombinant CEA protein $\pm$ GM-CSF as an adjuvant cytokine

Patient no	Sex	Age	Dose of rCEA per immunization	Adjuvans	Tumor localization	Tumor ^a stage	Relapse	Total survival time (m)
1	F	68	100 μ g	-GM-CSF	Colon	B	+ (9 m)	54
2	M	33		+GM-CSF	Colon	C	—	154+
3	F	54		-GM-CSF	Colon	B	—	153+
4	M	66		+GM-CSF	Colon	C	—	153+
5	M	71		-GM-CSF	Rectum	A	—	59
6	M	43		+GM-CSF	Rectum	C	—	151+
7	M	39	316 μ g	+GM-CSF	Colon	B	—	151+
8	M	75		-GM-CSF	Rectum	B	+ (12 m)	61
9	M	59		+GM-CSF	Colon	C	+ (7 m)	23
10	F	72		-GM-CSF	Colon	B	+ (10 m)	23
11	M	47		-GM-CSF	Colon	C	—	131
12	F	60		+GM-CSF	Colon	B	+ (21 m)	60
13	F	42	1,000 μ g	+GM-CSF	Colon	C	+ (17 m)	55
14	M	74		+GM-CSF	Colon	C	+ (54 m)	54
15	M	68		-GM-CSF	Colon	B	—	147+
16	M	63		-GM-CSF	Colon	B	—	60
17	M	71		-GM-CSF	Colon	C	+ (5 m)	7
18	F	68		+GM-CSF	Colon	B	+ (17 m)	54
19	F	56	3,000 μ g	+GM-CSF	Colon	C	+ (7 m)	16
20	F	72		-GM-CSF	Rectum	B	—	139+
21	F	63		+GM-CSF	Colon	B	—	139+
22	M	55		+GM-CSF	Rectum	C	—	139+
23	F	61		-GM-CSF	Rectum	C	+ (21 m)	28
24	M	80		-GM-CSF	Rectum	B	+ (81 m)	82

^aDukes' staging system

factors was significant for survival. Comparing patients both with an anti-CEA IgA and anti-CEA IgG titers above the

median to the remaining patients showed a significant value for the former group ($p=0.05$) (Fig. 2S).

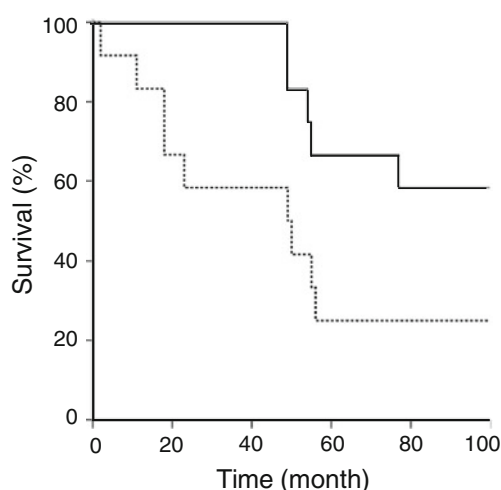


Fig. 5 Overall survival of rCEA vaccinated CRC patients in relation to IgA anti-CEA antibody titers. *Solid line* indicates patients with IgA anti-CEA antibody titers above the median of all patients ($n=12$) and *dotted line* below the median ($n=12$). The difference is statistically significant ($p=0.02$) (univariate Wilcoxon Gehan exact life table test)

Discussion

In the present study, IgM, IgA and IgE antibody responses were analyzed during a 3-year follow-up period of CRC patients immunized for 1 year with rCEA with or without the adjuvant cytokine GM-CSF. A large number of patients had naturally occurring anti-CEA antibodies, mainly in the low range and predominantly of the IgM type.

Addition of the adjuvant cytokine GM-CSF was practically mandatory to elicit a vaccine induced IgM, IgA and IgE anti-CEA response as compared to patients receiving the vaccine alone. This finding corroborates our previous studies where GM-CSF was found to strongly enhance both the T cell and IgG responses [12, 13]. Moreover, the IgM, IgA and IgE responses were not significantly related to the vaccine dose but low doses seemed to be more effective.

We have also reported that anti-CEA IgG antibodies were cytotoxic and that there was a relationship between the IgG antibody response and survival [12], i.e. higher levels

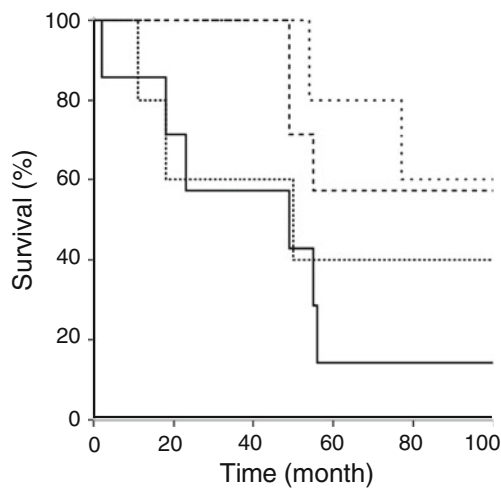


Fig. 6 Overall survival of CRC patients vaccinated with rCEA±GM-CSF in relation to the level of anti-CEA IgA titers. Patients not receiving GM-CSF and with anti-CEA IgA titers above the median (.....) ($n=5$); patients receiving GM-CSF and with anti-CEA IgA titers above the median (---) ($n=7$); patients receiving GM-CSF and with anti-CEA IgA titers below the median (.....) ($n=5$); patients not receiving GM-CSF and with anti-CEA IgA titers below the median (—) ($n=7$). There was a statistically significant difference comparing all patients with anti-CEA IgA titers above the median to those below irrespective of GM-CSF treatment ($p=0.02$) (Log rank Wilcoxon Gehan exact life table test)

seemed to be associated with better survival. In the present trial, we observed a significant correlation between high IgA titers and survival irrespective of GM-CSF treatment, while no such relation was noted for IgM and IgE levels. A survival advantage was also noted for patients with both high IgA and IgG titers. Furthermore, IgA anti-CEA mature antibodies could induce tumor cell killing both in ADCC and CDC. Fc receptors for IgA (Fc α R1) are found on monocytes/macrophages, polymorphonuclear neutrophils and NK-cells, cells participating in ADCC [28]. These findings together may indicate a clinical anti-tumor effect of anti-CEA IgA antibodies and not only of IgG antibodies. Furthermore, the study does not indicate a preference for IgA or IgG antibodies for survival but both might contribute.

In colon cancer patients elevated serum IgA immune complexes have been observed. It has been suggested that the presence of IgA immune complexes could represent a tumor specific antibody response [1]. Moreover, IgG1 and IgA antitumor antibodies were shown to be equally effective in killing tumor cells in ADCC [1]. Furthermore, neutrophils, the most frequent cytotoxic cells in humans, might be more effectively recruited by IgA than by IgG [29]. IgA antibodies may also form dimers with an increased valency, which may augment the capacity to kill tumor cells [11]. It has also been shown that IgA anti-HLA-class II antibodies effectively could trigger killing of lymphoma cells [30], and human IgA may activate the complement system via the mannan-binding lectin [31] or the alternative pathways [32].

There is a lot of evidence to support that also IgM and IgE antibodies have the capability to kill cancer cells. Anti-tumor monoclonal IgM antibodies have been generated from patients with lung, pancreatic, and colon cancer. These antibodies could induce apoptosis of tumor cells [2, 3]. In a neo-adjuvant therapy pilot study, a human monoclonal IgM antibody, specifically inducing apoptosis of signet ring cell gastric cancer, could in some patients be shown to induce a significant regression of the tumor cell mass [9, 33].

In an ovarian cancer xenograft model, a monoclonal IgE antibody against an ovarian-tumor-specific antigen (folate binding protein), induced a better survival than the corresponding IgG1 antibody [4]. IgE antibodies may activate effector cells through the surface IgE receptors [6, 34]. In another animal model, oral immunization with trastuzumab mimotopes induced IgE antibodies against the HER-2 antigen. The antibodies could lyse tumor cells in ADCC [10]. IgE antibodies have also been shown to inhibit the growth of CRC cells in mice [35] and conferred long-term antitumor immunity [36]. Furthermore, mast cells might release TNF- α upon Fc-receptor binding of anti-tumor IgE antibodies and subsequently lyse the tumor cells [37]. IgE antibodies against pancreatic cancer associated antigens have also been detected in sera of pancreatic cancer patients [38].

In a few cancer vaccine trials the induction of other isotype antibodies than IgG has been analysed. A ganglioside vaccine was tested in breast cancer patients and specific IgA antibodies were induced. Two patients with pulmonary metastases had a stable disease for 18 and 40 months [39]. A GD2-KLH vaccine plus the adjuvant QS-21 in melanoma patients induced antibodies against GD2 in the majority of the patients. The IgM response peaked after the third immunization, whereas the IgG response peaked after the fourth or fifth immunization [40]. Adjuvant immunization of breast cancer patients with a sTn—KLH vaccine plus QS-21 induced IgM and IgG responses in all patients. Significant IgM and IgG antibody levels were noted at week 7 and remained elevated for at least 12 months from the first immunization [41]. Four patients with advanced renal cell carcinoma were immunized with dendritic cells loaded with tumor cell lysate. IgM, IgG1, IgG2 and IgG3 as well as IgA antibodies were induced but not IgE [42]. Patients with advanced CRC vaccinated with SART3 peptides developed IgE antibodies [43]. The patterns of vaccine induced isotype antibody responses warrant further studies as the isotype response might be of clinical relevance.

In summary, the present study shows that vaccination with a recombinant CEA protein induces a strong IgM, IgA and IgE antibody response in patients receiving GM-CSF. Previously we have shown induction of IgG antibodies [12, 13]. Although the clinical significance of vaccination induced antibodies of different isotypes is not clear, there

are indications that IgM, IgA and IgE antibodies might have anti-tumor effects in addition to IgG. Induction of IgA anti-CEA antibodies was associated with improved survival and the IgA fraction containing anti-CEA antibodies was cytotoxic.

Acknowledgements We would like to thank Maggy Magnusson for expert technical assistance and Bo Nilsson for excellent statistical support. Recombinant CEA was a kind gift from Protein Sciences Corp. Meriden, CT, USA. This study was supported by grants from the Swedish Cancer Society, the Cancer Society in Stockholm, the King Gustaf V Jubilee Fund, the Cancer and Allergy Foundation, Torsten and Ragnar Söderberg Foundation, Tornspiran, Hesselman, the Goldi and Ludvig Berglund Foundation, the Research Fund of the Department of Oncology, Uppsala University Hospital, Swedish Medical Society and the Karolinska Institute Foundations.

Disclosures The authors declare that they have no conflicts of interest.

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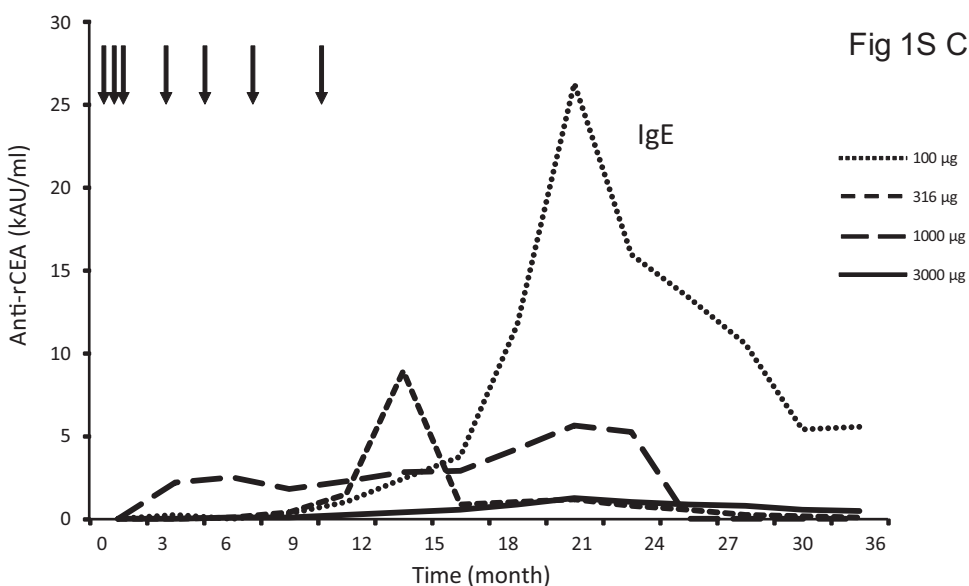
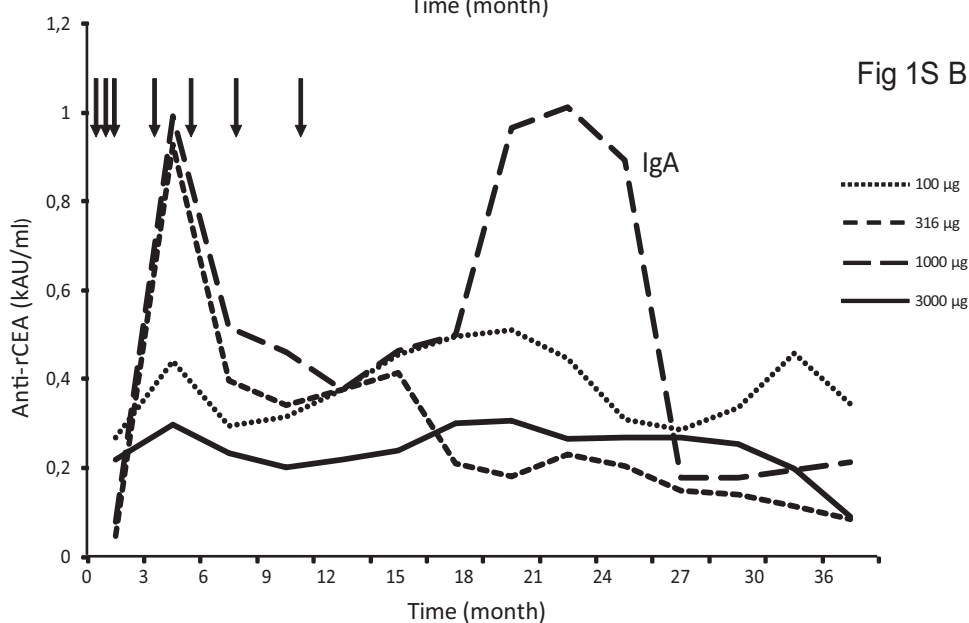
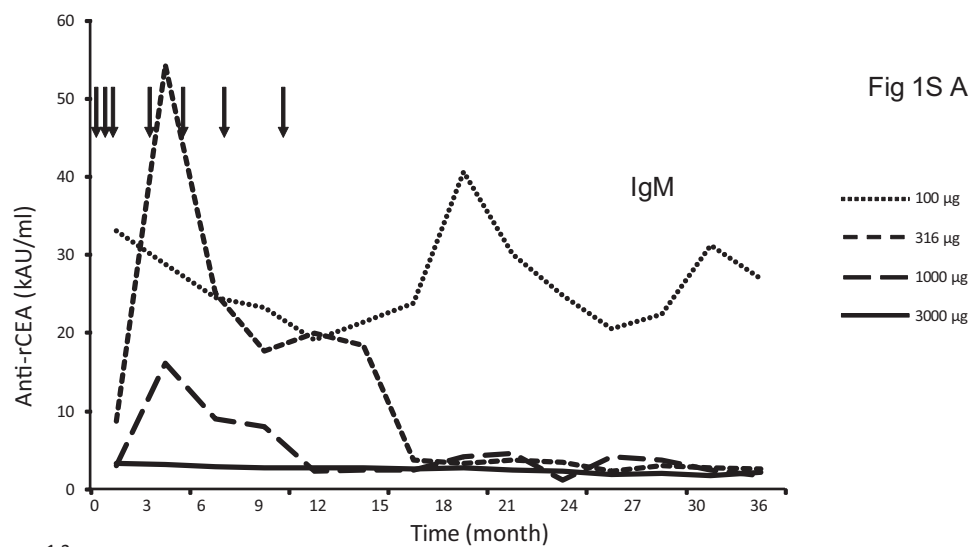


Fig 2S

