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Pharmacological administration of granulocyte/macrophage-colony-stimulating factor is of significant importance for the induction of a strong humoral and cellular response in patients immunized with recombinant carcinoembryonic antigen

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Abstract Eighteen colorectal carcinoma patients without macroscopic disease after surgery were immunized using recombinant (r) human (h) carcinoembryonic antigen (CEA) with ($n = 9$) or without ($n = 9$) the addition of soluble granulocyte/macrophage-colony-stimulating factor (GM-CSF). The dose of rhCEA per immunization was 100 μg ($n = 6$), 316 μg ($n = 6$) or 1000 μg ($n = 6$). rhCEA was given s.c. on day 1 and 80 μg/day of GM-CSF s.c. on days 1–4. The schedule was repeated six times during a period of 9 months. All patients in the GM-CSF group developed a strong rhCEA-dose-dependent IgG antibody response while only one-third of the non-GM-CSF patients mounted a weak antibody response. All patients (9/9) in the GM-CSF group developed a strong rhCEA-specific proliferative T cell response as well as type I T cells (interferon γ secretion). In 45% of the patients also a weak type II T cell response (interleukin-4 secretion) was evoked. Both MHC-class-I- and -II restricted rhCEA-specific T cells were noted. A specific cellular response (proliferation and/or cytokine secretion) against native hCEA could be found in 8/9 patients in the GM-CSF group, although at a significantly lower level than against rhCEA. In the non-GM-CSF group a weak rhCEA-specific T cell response was induced. Three patients had a proliferative response, 4 patients type I T cells and 6 patients type II T cells. No signs of autoimmune reactions were noted. Local pharmacological administration of GM-CSF seemed to be a prerequisite for the induction of a strong immunity against baculovirus-produced hCEA protein. However, the cellular response against native CEA was of a significantly lower magnitude.

Key words rCEA · GM-CSF · Immune response

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Introduction

Many solid tumors relapse after primary surgery, probably because of the presence of micrometastases at diagnosis [23, 31]. Systemic therapy with hormonal or chemotherapeutic drugs is mostly insufficient to control the disease.

The induction of relevant antitumor immune rejection mechanisms by cancer vaccines might be a treatment option to improve the outcome for these patients. There are a number of tumor antigen preparations to be used for immunizations, such as whole tumor cells, tumor-derived proteins, recombinant proteins, rationally designed peptides, proteins expressed in viruses, "naked"-DNA and anti-idiotypic antibodies [25]. Naturally occurring tumor antigens are "self" molecules and poorly immunogenic [9]. There might, however, be several means to break the unresponsiveness and augment an immune response against such antigens.

The external domain of surface tumor-associated protein antigens (TAA) might be used for immunization. Such protein antigens may be processed and presented

to the immune system by antigen-presenting cells (APC). The functional activities of APC (dendritic cells, macrophages/monocytes) might be augmented by granulocyte/macrophage-colony-stimulating factor (GM-CSF) [36, 48].

The overall prognosis for colorectal carcinoma (CRC) patients is poor, not more than 50% being cured by surgery alone. Even in those patients with radically resected tumors, the risk of relapse is substantial. In Dukes' stage C, the cure rate by surgery alone is about 35% [27]. Adjuvant post-surgical chemotherapy seems to increase the survival rate to 50% [28]. CRC patients might, therefore, be good candidates for a vaccination approach. The degree of infiltration of mononuclear cells in CRC tumor lesions seemed to correlate positively to the prognosis [3]. Down-regulation of MHC class I molecules may have a negative impact on the clinical outcome [26]. Auto-antibodies against the TAA CO17-1A have been detected in CRC patients [7] as well as auto-reactive T cells [39] and any means of enhancing those reactivities may assist tumor-directed responses. Passive immunotherapy with monoclonal antibodies induced tumor regression [32, 33] and prolonged survival in the adjuvant setting [34]. Active immunization using whole tumor cells mixed with various adjuvants might induce survival benefits [14, 37, 47].

Most CRC cells express the TAA carcinoembryonic antigen (CEA). CEA has been used for immunization in animals and for colorectal and breast carcinoma patients (2, 12, 18, 45). Mice immunized with recombinant CEA expressed in vaccinia virus were protected against challenge with murine coloncarcinoma cells expressing the human CEA, and regression of established tumors was noted [18]. Patients immunized with vaccinia CEA mounted a CTL response against tumor cells loaded with cytotoxic CEA peptides and against tumor cells exhibiting endogenously produced CEA peptides [45].

In the present trial we have produced the recombinant (r) human (h) CEA protein in baculovirus for vaccination. To augment antigen presentation, the patients received simultaneously locally administered GM-CSF in an aqueous formulation. The main purpose of this first report is to describe the augmenting effects on the humoral and cellular immune response against rhCEA by the simple procedure of delivering GM-CSF. All patients have been followed for 9 months. GM-CSF seemed to be mandatory for the induction of a specific humoral and cellular rhCEA immune response.

Materials and methods

Patients

Eighteen patients, 12 male and 6 female, with a median age of 60 years (range 30–75 years), who had been operated on for Dukes' class A ($n = 1$), B ($n = 8$) or C ($n = 9$) CRC with no remaining tumor, entered the study; 15 patients had colon cancer while in 3 patients the primary tumor was located in the rectum. Except for surgery, only 1 patient had received adjuvant chemotherapy. The median time from surgery to the start of immunization was 7 months (range 1–39 months). The study was approved by the local Ethics Committee. Informed consent was obtained from each patient.

Clinical examination and follow-up

Before immunization a complete case history, physical examination and laboratory tests [hemoglobin concentration, white blood cells with a differential count, platelet count, blood chemistry including blood urea nitrogen, creatinine, electrolytes, albumin, total protein, liver function test and serum tumor markers (CEA, CA19-9, CA50), standard urine analysis] were done. The patients were monitored as above at least every second month during the first 9 months. Other examinations were performed when clinically needed.

Carcinoembryonic antigen

Native (n) human CEA is a glycoprotein with a molecular mass of about 180 kDa (approximately 50% carbohydrate) [29, 38]. The gene for CEA was cloned from human colon adenocarcinoma cells (LS174T) and then introduced into the baculovirus *Autographa callfornica* nuclear polyhedrosis virus (AcNPV) under the transcriptional control of the AcNPV polyhedrin promoter. The rhCEA was engineered to contain all of the amino acids found in the mature hCEA. In addition, rhCEA was expressed in such a manner as to prevent the addition of glycosylglycerophosphoinositol at the C terminus to facilitate secretion of the rhCEA in insect cells. The rhCEA is glycosylated with *N*-linked glycans, and has a molecular mass of 120kDa, which is smaller than the 180-kDa nhCEA because it lacks the complex carbohydrate structures of glycoproteins made in insect cells. rhCEA was purified by a series of hydrophobic interactions and anion-exchange chromatography under non-denaturating conditions to at least 99% purity. Contaminants may be baculovirus and insect cell proteins. rhCEA was tested for purity by quantitative scanning densitometry and for safety in mice and guinea-pigs as well as for sterility and pyrogenecity. The purified rhCEA for immunization was formulated in situ with aluminium phosphate (0.5 mg/ml aluminium ion as $AlPO_4$).

A baculovirus control protein (BCP) was produced for in vitro tests. Purification of proteins from insect cells infected with a control non-CEA-producing baculovirus expression vector by using the rhCEA purification protocol resulted, however, in the recovery of non-measurable protein. Therefore, a control preparation was made that contained a mixture of insect cell and baculovirus proteins from cells infected with a control baculovirus expression vector. The procedure employed was to extract essentially all of the insect cell and baculovirus proteins in infected cells, bind them to a single anion-exchange column, and recover the control protein preparation in a phosphate-buffered solution. This procedure gave a BCP preparation that contained all the major insect cell and baculovirus proteins, which should represent any of the low-level contaminants found in purified rhCEA.

nhCEA was purified from CRC liver metastasis as described [13, 19] and was a kind gift from Prof. S. Hammarström, Department of Immunology, Umeå University, Umeå, Sweden.

As a control for nhCEA, three different proteins were used during the study period: biliary glycoprotein (Sigma-Aldrich, Stockholm, Sweden), nonspecific cross-reacting antigens (a gift from Prof. B. Wahren, MTC, Karolinska Institute, Stockholm) and glycoprotein-α1 (Sigma-Aldrich). These proteins are members of the CEA immunoglobulin superfamily and highly homologous cross-reacting proteins to hCEA [43]. There were no statistically significant differences between these three control proteins stimulated cellular responses in vitro and therefore the data were grouped together. The comprehensive term, $nhCEA_{cp}$ (native human CEA control protein) was used.

Immunization protocol

The trial was an open-label, randomized, dose-ranging study. Six consecutive patients were enrolled at each of three rhCEA dose levels: 100 μg, 316 μg or 1000 μg for each immunization. rhCEA was administered subcutaneously on days 1 and 14 and during months 2, 4, 6 and 9. All patients received rhCEA formulated with alum. At each dose level of rhCEA, half of the patients ($n = 3$) were randomized to concomitant administration of recombinant human GM-CSF (Leucomax; Schering-Plough, Kenilworth, N.J., USA). A dose of 80 μg/day GM-CSF was given s.c. at each immunization once a day on days 1–4 at the same site as that used for rhCEA injection.

Enzyme-linked immunosorbant assay

Antibodies against rhCEA and BCP were assayed using 96-well microtiter plates (Nunc, Odense, Denmark). The plates were coated with 5 μg/ml proteins in 0.05 M sodium carbonate buffer (pH 9.5) at room temperature overnight. After washing, sera diluted 1:100 in phosphate-buffered saline with 0.5% bovine serum albumin, 0.05% Tween 20 and 0.01% merthiolate were added. The plates were incubated for 2 h at 37 °C. Alkaline-phosphatase-conjugated goat anti-(human IgG) (Sigma, St. Louis, Mo., USA) was added and incubated for 2 h at 37 °C. As a substrate *p*-nitrophenyl phosphate (Sigma) was used. The reaction was stopped with 1 M NaOH after 30 min at 37 °C. Results are expressed as the mean absorbance (at 405 nm) of duplicate wells after subtraction of background values.

The absorbance values of the 18 patients before immunization were 0.250 ± 0.199 (mean ± SD) for rhCEA and 0.279 ± 0.126 for BCP. In healthy control donors ($n = 10$), the absorbance values for rhCEA and BCP were 0.364 ± 0.256 and 0.385 ± 0.182 respectively. A post-immunization value of more than 0.876 and more than 0.795 for rhCEA and BCP respectively was considered positive (above the mean + 2 SD of the healthy controls).

Isolation of blood cells

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of heparinized venous blood on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient (density: 1.077 g/ml). The cells were resuspended in HEPES-buffered RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with antibiotics (100 IU penicillin and 100 μg streptomycin/ml), L-glutamine (2 mM) and 10% heat-inactivated normal human AB^+ serum. Adherent cells were removed by incubation for 30 min at 37 °C in tissue-culture flasks (Nunc). Non-adherent cells, peripheral blood lymphocytes (PBL), were collected and resuspended in complete medium. Adherent cells were removed from the flasks using a rubber policeman and used as APC.

Proliferation assay (DNA synthesis)

PBL were supplemented with 10% autologous adherent cells. The cells (10^5/well) were cultured in 96-well round-bottomed microtiter plates (Nunc) at 37 °C in humidified air with 5% CO_2 for 6 days. rhCEA, nhCEA and the control proteins were added at concentrations of 0.1 pg/ml–100 ng/ml respectively. Concanavalin A (80 μg/ml), phytohemagglutinin (10 μg/ml), purified protein derivative of tuberculin (PPD; 2.5 μg/ml) and tetanus toxoid (TT; 50 ng/ml) were used as positive controls. During the last 18 h of incubation, 1 μCi/well [^{3}H]thymidine (specific activity 5 Ci/mmol; Amersham, Life Sciences, UK) was added.

The cells were harvested by an automatic cell harvester (Skatron, Lier, Norway). Radioactivity was measured in a liquid scintillation counter (LKB 1212, Rackbeta, Pharmacia). The results are expressed as the mean of triplicates. A stimulation index (SI) was calculated for each set of three results by dividing the mean radioactivity (cpm) of stimulated cells by that of unstimulated cells. The highest SI value induced by the different concentrations in each test was used [6].

Before the start of immunization there were no statistically significant differences between patients allocated to immunization with or without GM-CSF with regard to background values (unstimulated cells) or response to mitogens (data not shown) or recall antigens (see Results). SI values (mean + 2 SD) of 23 operated non-immunized CRC patients were 1.64 + 1.22 for rhCEA, 1.63 + 1.18 for BCP, 1.63 + 1.12 for nhCEA and 1.70 + 1.50 for $nhCEA_{cp}$. The proliferative response of healthy control donors ($n = 14$) against nhCEA was 1.94 + 1.26. On the basis of these results an SI above 3.2 was considered a positive value. Moreover, to be regarded a positive test against rhCEA and nhCEA respectively, the value had to be twice that of the control protein. Furthermore, an SI value above 3.2 but less than 4.0 was considered a weak positive response, (+), and values of 4.0 and higher a significant positive response, +.

Reverse enzyme-linked immunospot (ELISPOT) assay

An assay to identify cells secreting interferon γ (IFNγ) and interleukin-4 (IL-4) was used as described earlier [49]. Briefly, PBMC (10^5/well) were incubated with rhCEA, BCP or nhCEA (0.1 pg/ml–1 μg/ml) for 48 h in humified air with 5% CO_2 at 37 °C. Cells incubated with medium alone, PPD (2.5 μg/ml) or concanavalin A (10 μg/ml) were used as the control. Spots corresponding to cytokine-secreting cells were counted blind under a dissection microscope. All samples were done in duplicate. The coefficient of variation between duplicates was 8.5%. The results are expressed as the numbers of cells secreting IFNγ or IL-4/10^5 PBMC. The number of spots in response to stimulation (the total number of spots minus the number of spots without stimulation) is shown.

The number of spots following rhCEA stimulation spots for non-immunized CRC patients who had undergone operation ($n = 12$) was 7.38 ± 4.19 for IFNγ (mean ± SD; range 2–13) and 7.33 ± 3.70 for IL-4 (range 3–13). The corresponding values for BCP were 3.43 ± 1.87 (range 0–7) for IFNγ and 3.46 ± 1.90 (range 1–7) for IL-4. The numbers of spots obtained when nhCEA was used were 6.44 ± 3.54 (range 3–12) for IFNγ and 5.78 ± 4.49 (range 1–12) for IL-4. In healthy control donors ($n = 5$) the IFNγ response to rhCEA, BCP and nhCEA was 3.90 ± 3.42, 2.10 ± 0.65 and 2.30 ± 0.84 respectively (range for all antigens 1–9). The IL-4 response to rhCEA, BCP and nhCEA was 3.80 ± 2.14, 3.60 ± 3.34 and 1.60 ± 0.82 respectively (range for all antigens 1–9). On the basis of these results, at least 15 spots following stimulation was considered a positive value for all antigens assessing IFNγ as well as IL-4-secreting cells. Moreover, a value of at least 15 to less than 20 was considered a weak positive response, (+), and values of 20 or more a significant positive response, +.

Major histocompatibility complex (MHC) blocking experiments

To study MHC restriction of the proliferative response, mouse monoclonal antibodies (mAb) against human MHC class II molecules (HLA-DR, (IgG2B; Immunotech, Marseille, France), or class I molecules (HLA-ABC, (IgG2B; Chemicon International Inc., Temecula, Calif., USA) were used. A non-relevant mouse mAb (IgG2B) was used as a control (Immunotech). Antibodies (1 μg/ml) were added at the start of culture together with the antigen. The results are expressed as the percentage inhibition of cell proliferation according to the formula: inhibition (%) $= 100 \times (A - B)/A$, where A is the radioactivity (cpm) measured in cells stimulated with the antigen alone and B is the radioactivity (cpm) measured in cells incubated with both antigen and antibody. The unspecific inhibition of the control mouse mAb IgG2B was 2.8 ± 7.2% (mean ± SD, $n = 9$). More than 40% inhibition was required to be regarded a positive response.

Statistical analyses

To test changes in SI and ELISPOT, a *t*-test on the ratios between stimulated and unstimulated cells was used. To estimate differences

between means of different groups, an independent *t*-test was carried out.

Results

Antibody response

All patients (9/9) in the GM-CSF group developed IgG antibodies against rhCEA, which titers gradually increased by repeated immunizations (Fig. 1). The IgG titers seemed to increase with higher doses of rhCEA (Fig. 2). In contrast, only 3 patients in the non-GM-CSF group showed the induction of a weak anti-rhCEA IgG antibody response (Fig. 1). One of these patients had IgG antibodies reacting with rhCEA prior to immunization, which titer was boosted. A weak antibody response against BCP was noted in 3 patients (2 in the GM-CSF group and 1 in the non-GM-CSF group).

Proliferative T cell response

The kinetics of the T cell response is shown in Fig. 3. All patients (9/9) in the GM-CSF group mounted a strong response against rhCEA and a modest T cell response was seen in only 3 of the 9 patients in the non-GM-CSF group ($P < 0.005$) (Fig. 3, Tables 1, 2 and 4). The response induced in the non-GM-CSF group was slower than that in the GM-CSF group. A proliferative response against nhCEA was noted in 5 patients (55%) in the GM-CSF group. In the non-GM-CSF group the nhCEA proliferative response was not analyzed on a regular basis. Occasional responses were noted. The T cell response induced by nhCEA occurred later than the response against rhCEA (Figs. 3, 4). All patients in the GM-CSF group developed a weak proliferative T cell response against BCP in time but this was much lower than that against rhCEA ($P < 0.005$) (Table 2). No significant response against $nhCEA_{cp}$ over time was noted (Fig. 4). The highest rhCEA/BCP ratio of immunized patients was 10.3 ± 4.9 (means ± SEM) in the GM-CSF group. In the non-GM-CSF group all patients also developed a BCP T cell response against BCP in time (SI > 3.2) but the rhCEA/BCP ratio in this group was 1.0 ± 0.3. Thus, a clear rhCEA-specific proliferative T cell response was only noted in the GM-CSF group.

The proliferative T cell response induced against rhCEA was compared to that against the recall antigen PPD (Fig. 5). The PPD response was unchanged during the observation period and of the same magnitude in patients receiving GM-GSF as in those not receiving GM-CSF. In the GM-CSF group at the end of the observation period, the rhCEA response reached the same level as that for PPD. The response against the recall antigen TT was lower than that with PPD. The TT response did not change significantly over time and there was no difference between the GM-CSF and non-GM-

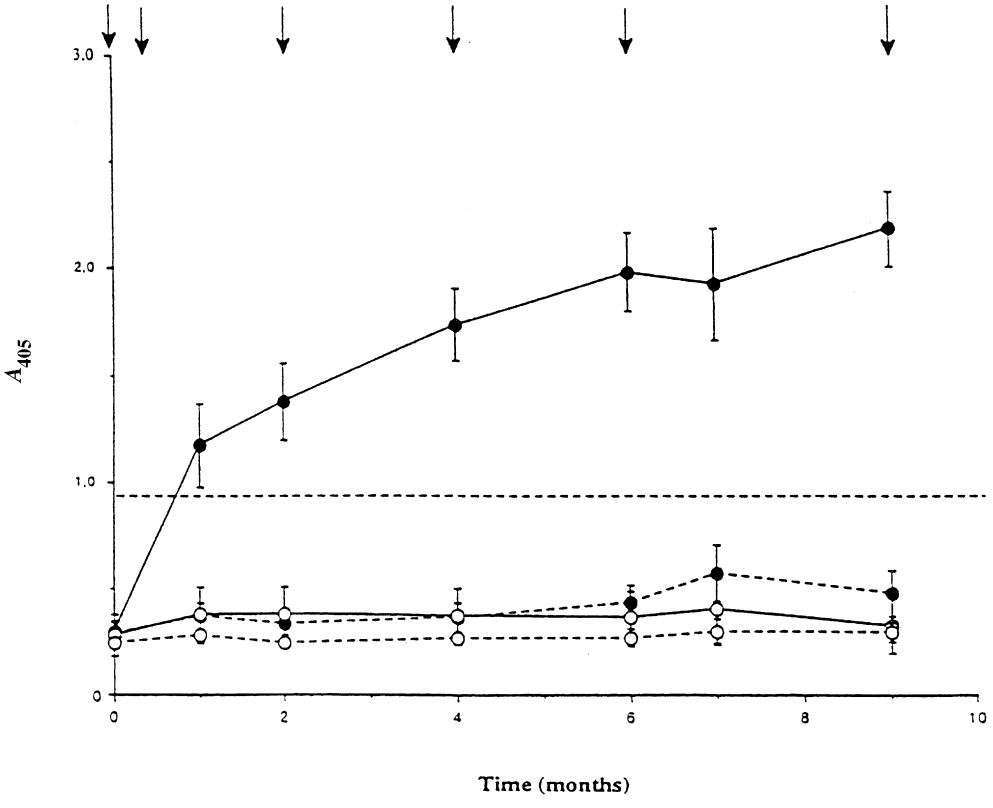

Fig. 1 Kinetics of anti-(recombinant human carcinoembryonic antigen) (anti-rhCEA) IgG antibodies (mean ± SEM; serum dilution: 1:100) in colorectal carcinoma patients immunized with rhCEA in combination with granulocyte/macrophage-colony-stimulating factor (GM-CSF: —; $n = 9$) or without GM-CSF (- - -; $n = 9$). Antibodies against rhCEA (●) and against baculovirus control protein (○). *Arrows* immunization times. *Dashed line* cut-off level (mean +2 SD of results from healthy controls)

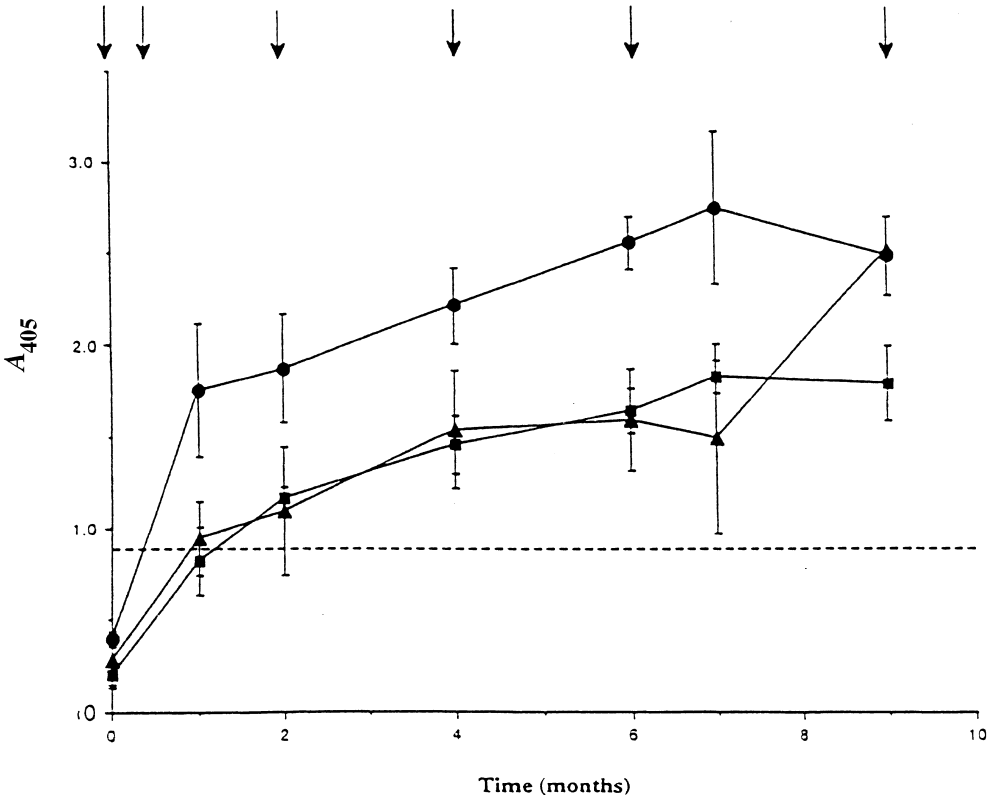

Fig. 2 Kinetics of anti-rhCEA IgG antibodies (mean ± SEM; serum dilution: 1:100) in relation to the rhCEA immunization dose in colorectal carcinoma patients immunized with rhCEA in combination with GM-CSF. ■ 100 μg rhCEA/immunization ($n = 3$); ▲ 316 μg rhCEA/immunization ($n = 3$); ● 1000 μg rhCEA/ immunization ($n = 3$). *Arrows* immunization time. *Dashed line* cut-off level (mean +2 SD of results from healthy controls)

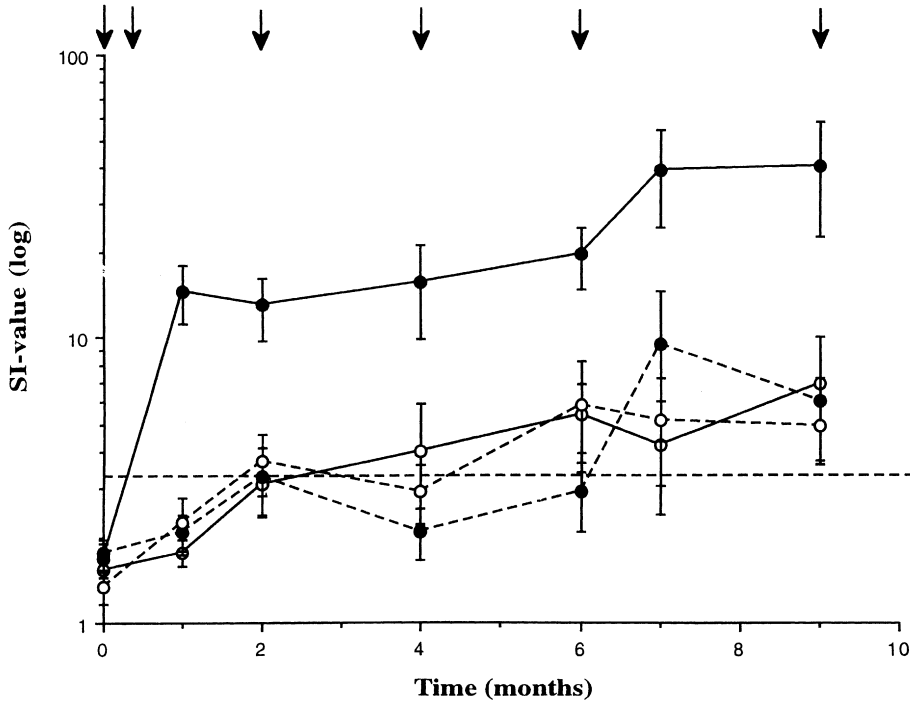

Fig. 3 DNA synthesis (stimulation index, *SI*; mean ± SEM) of lymphocytes stimulated by rhCEA (●) and baculovirus control protein (○) in vitro, from colorectal carcinoma patients immunized with rhCEA in combination with (—; $n = 9$) or without (- - -; $n = 9$) GM-CSF. *Arrows* immunization times. *Dashed line* cut-off level

CSF group. At the end of the observation period, the nhCEA response had reached the same level as that of TT (data not shown).

Cytokine-secreting T cells (ELISPOT)

T cell cytokines were secreted upon antigen-specific activation. In the GM-CSF group all patients (9/9) mounted an IFNγ response (type I cells) against

rhCEA. In 7/9 a type I T cell response was also induced against nhCEA. The response was, however, at a lower level than that against the recombinant protein and, in 4 cases, the response was weak, although specific (Fig. 6; Tables 3, 4). rhCEA-specific IFNγ- secreting cells were induced in 4 out of 9 patients in the non-GM-CSF group; in 2 of them the response was weak. The nhCEA IFNγ response was not analyzed (Fig. 6; Tables 3, 4). Low numbers of IFNγ secreting cells were also induced against BCP (in 1 patient in the GM-CSF group and 2 patients in the non-GM-CSF group). The maximum numbers of IFNγ-secreting T cells were, however, statistically highly significantly different when those induced by rhCEA and nhCEA respectively were compared to those induced by the control protein ($P < 0.001$ and $P < 0.005$ respectively). Thus, a strong type I T cell response was induced in all patients in the GM-CSF group against rhCEA and in the majority also against nhCEA but at a lower level. In the non-GM-CSF group, a weaker type I response was induced against rhCEA in a few patients.

A type II cellular response (IL-4 secretion) was mounted against rhCEA in 4/9 patients in the GM-CSF group, although the response magnitude was significantly lower than that of the type I response ($P < 0.001$) (Fig. 6; Tables 3, 4). In 1 patient a weak response (17 spots) against nhCEA was evoked. In the non-GM-CSF group a weak rhCEA IL-4 response was induced in 6 patients. Only in 1 patient in the GM-CSF group was

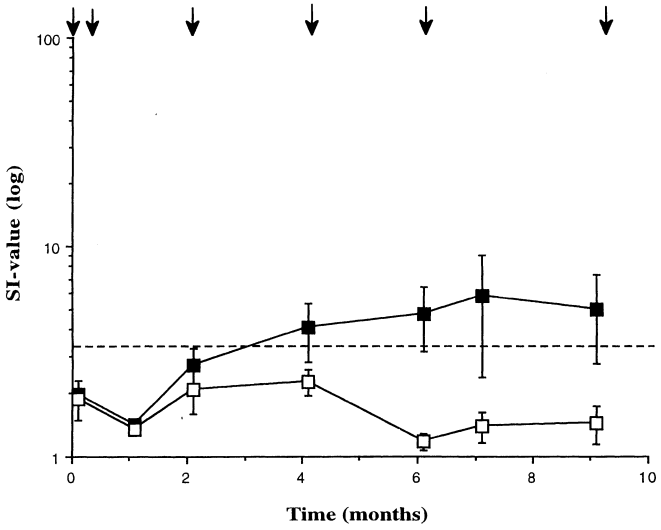

Fig. 4 DNA synthesis (stimulation index, SI; mean ± SEM) of lymphocytes stimulated by nhCEA (■) and $nhCEA_{cp}$ (□) in vitro, from colorectal carcinoma patients immunized with rhCEA combined with GM-CSF ($n = 9$). *Arrows* immunization times. *Dashed line* cut-off level

Table 1 Individual data of the proliferative response (DNA synthesis; stimulation index) against recombinant human carcinoembryonic antigen (*rhCEA*) and natural (*n*) nhCEA (and the respective control proteins) in colorectal carcinoma patients immunized with rhCEA with or without granulocyte/macrophage-colony-stimulating factor (*GM-CSF*). Pre-immunization and maximum values against rhCEA and nhCEA respectively during the observation period are shown. *BCP* baculovirus control protein. The values "At rhCEA max" correspond to the point for the maximum value against rhCEA and nhCEA respectively. Bold type indicates a positive value. Numbers in parentheses indicate the month at which the maximum value was recorded for that particular patient. *ND* not done

Patient no.	Stimulation index							
	rhCEA		BCP		nhCEA		$nhCEA_{cp}$	
	Pre	Max	Pre	At rhCEA max	Pre	Max	Pre	At nhCEA max
With GM-CSF								
2	2.7	**26.0** (9)	2.3	7.1	1.3	**17.4** (9)	1.3	1.4
4	1.0	**46.4** (4)	2.1	1.4	2.0	2.4 (2)	1.0	1.3
6	1.8	**91.9** (9)	1.3	7.2	1.6	4.7 (4)	1.5	3.1
7	1.1	**40.5** (6)	2.3	4.5	2.0	5.8 (6)	4.0	4.0
9	2.7	**53.7** (7)	1.3	2.8	2.1	**24.1** (7)	1.7	2.3
12	1.9	**26.7** (1)	2.8	3.6	2.4	**14.8** (6)	1.3	2.2
13	1.5	**120.1** (9)	1.8	11.1	1.6	**10.0** (4)	1.2	1.4
14	0.8	**19.2** (1)	0.9	1.9	1.8	1.6 (2)	1.8	1.5
18	1.7	**28.5** (2)	1.1	9.0	1.4	**4.1** (2)	1.6	1.5
Without GM-CSF								
1	1.3	2.9 (4)	1.2	2.4	2.7	2.7 (1)	2.3	2.3
3	1.9	**18.4** (4)	1.6	5.9	1.1	**8.7** (4)	1.1	1.7
5	1.1	5.5 (2)	0.8	9.5	1.4	1.9 (2)	2.6	2.3
8	1.5	**6.6** (6)	1.7	1.4	1.5	ND	1.6	ND
10	1.8	1.9 (9)	1.6	3.0	2.3	ND	2.4	ND
11	2.6	2.4 (6)	1.9	2.3	2.7	ND	2.4	ND
15	2.3	7.8 (9)	2.2	5.7	1.7	**3.7** (9)	1.9	0.7
16	1.5	**25.2** (9)	1.6	9.2	ND	ND	ND	ND
17	0.7	4.9 (4)	0.4	6.9	1.2	ND	1.9	ND

Table 2 Maximum stimulation index (DNA synthesis) of colorectal carcinoma patients immunized with rhCEA ± GM-CSF. A stimulation index greater than 3.2 was considered a positive value. Results are means ± SEM. The *P* value on the row between the pre- and post-immunization values refers to comparison between these two variables. *Post-immunization* (*max*) is the highest value for each patient noted during the 9 months of follow-up from the start of immunization. *NS* not significant, *NA* not applicable. Statistical comparison: post-immune rhCEA GM-CSF$^+$ versus post-immune rhCEA GM-CSF$^-$ $P < 0.005$

Patient groups		Stimulation index			
		rhCEA	BCP	nhCEA	$nhCEA_{cp}$
rhCEA with GM-CSF ($n = 9$)	Pre-immunization	1.7 ± 0.2	1.8 ± 0.2	1.9 ± 0.1	1.7 ± 0.3
		$P < 0.01$	$P < 0.05$	$P < 0.02$	NS
	Post-immunization (max)	50.3 ± 11.4	9.5 ± 3.2	9.4 ± 2.6	3.3 ± 0.8
		< 0.005		< 0.05	
rhCEA without GM-CSF ($n = 9$)	Pre-immunization	1.6 ± 0.2	1.4 ± 0.2	1.8 ± 0.2	2.0 ± 0.2
		$P < 0.02$	$P < 0.01$		
	Post-immunization (max)	8.5 ± 2.7	9.1 ± 2.1	NA	NA

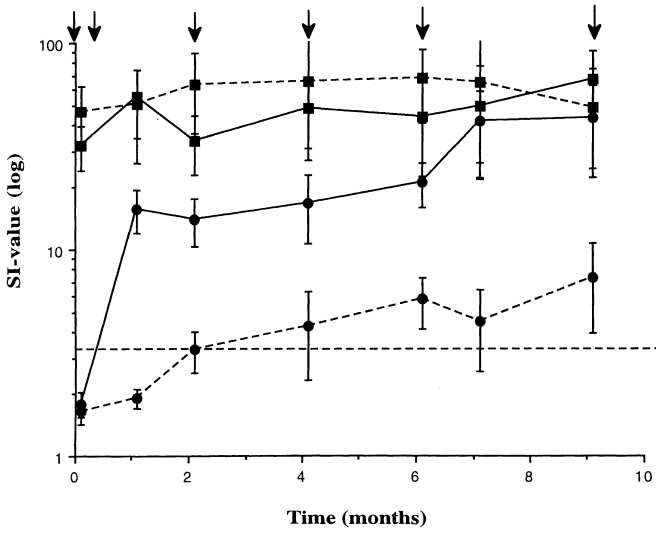

Fig. 5 DNA synthesis (stimulation index, *SI*; mean ± SEM) of lymphocytes stimulated with rhCEA (●) and purified protein derivative (■) in vitro, from colorectal carcinoma patients immunized with rhCEA in combination with (—; $n = 9$) or without (- - -; $n = 9$) GM-CSF. *Arrows* immunization times. *Dashed line* cut-off level

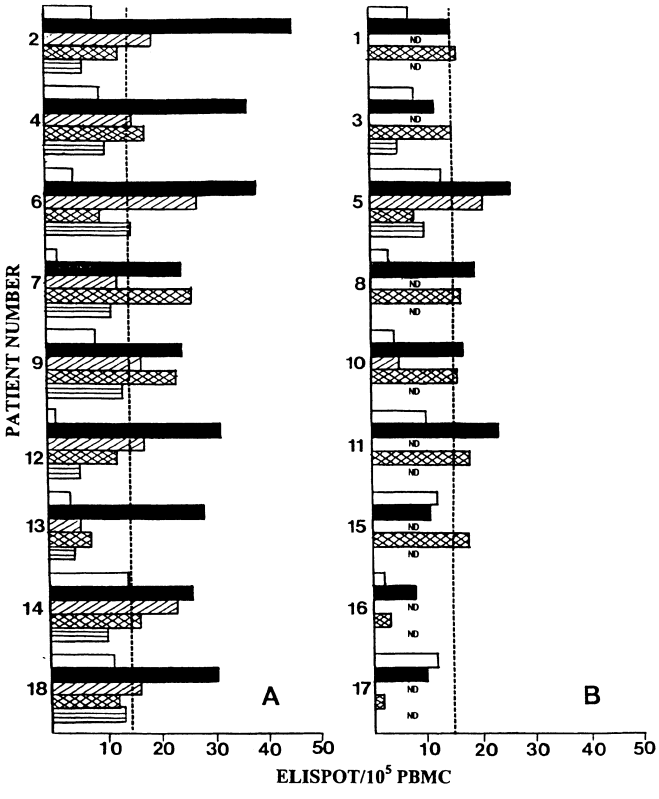

Fig. 6A, B Maximum number of cytokine-secreting cells (reverse enzyme-linked immunospot assay, *ELISPOT*) of individual patients. **A** Patients who received GM-CSF. **B** Patients who did not receive GM-CSF. ■ rhCEA-induced IFNγ-secreting cells; ▨ nhCEA-induced IFNγ secreting cells; ▩ rhCEA-induced IL-4-secreting cells; ▤ nhCEA-induced IL-4-secreting cells; □ highest number of cytokine-secreting cells (IFNγ or IL-4) at testing time induced by the control protein

an IL-4 response against the control protein noted. Thus, a weak type II cellular response was induced in about half of the patients in both groups.

Blocking of the rhCEA-specific cellular response by MHC class I and II monoclonal antibodies

For an indication whether the rhCEA-specific cell-mediated response was confined to MHC-class-II- and/or MHC-class-I-restricted T cells, 6 patients in the GM-CSF group were analyzed (Fig. 7). PBMC were stimulated with rhCEA in the absence or presence of anti-MHC class I and II antibodies respectively. In 3 patients (patients 4, 13, 14) an MHC-class-II-restricted T cell response was noted, while in 3 other patients (patients 6, 7, 18) MHC-class-II- as well as MHC-class-I-restricted rhCEA-specific T cells seemed to be present. In all 6 patients, a strong type I T cell response (IFNγ secretion) was found (mean value: 31 ELISPOTS/10^5

Table 3 Maximum number of induced spots (cytokine-secreting cells) of colorectal carcinoma patients immunized with rhCEA ± GM-CSE. A reverse enzyme-linked immunospot (*ELISPOT*) value of at least 15 spots/10^5 peripheral blood mononuclear cells (*PBMC*) was considered positive. Results are means ± SEM. The *P* value on the row between the pre- and post-immunization values refers to comparison between these two variables. Post-immunization (max) is the highest value for each patient noted during the 9 months of follow-up from the start of immunization. *ND* not done, *NS* not significant, *IFN* interferon, *IL* interleukin. There were no statistically significant differences in ELISPOT values prior to immunization when the two patient groups were compared. Statistical comparison: post-immune IFNγ rhCEA GM-CSF$^+$ versus post-immune IFNγ rhCEA GM-CSF$^-$ $P < 0.001$; post-immune IFNγ rhCEA GM-CSF$^+$ versus post-immune IL-4 rhCEA GM-CSF$^+$ $P < 0.001$

Patients		IFNγ ELISPOTS/10^5 PBMC			IL-4 ELISPOTS/10^5 PBMC		
		rhCEA	BCP	nhCEA	rhCEA	BCP	nhCEA
rhCEA with GM-CSF ($n = 9$)	Pre-immunization	9.6 ± 1.9	3.7 ± 0.9	8.0 ± 0.9	7.0 ± 1.3	3.9 ± 0.7	3.9 ± 1.1
		$P < 0.001$	$P < 0.005$	$P < 0.005$	$P < 0.05$	$P < 0.05$	$P < 0.005$
	Post-immunization (max)	32.3 ± 2.4	8.6 ± 1.1	17.9 ± 2.3	15.9 ± 2.1	8.6 ± 1.8	10.0 ± 1.5
		$P < 0.001$	$P < 0.005$		$P < 0.02$		
rhCEA without GM-CSF ($n = 9$)	Pre-immunization	7.7 ± 1.4	3.9 ± 0.4	ND	7.3 ± 1.2	5.9 ± 1.3	ND
		$P < 0.01$	NS		NS	NS	
	Post-immunization	17.2 ± 2.2	7.1 ± 2.4	ND	12.6 ± 2.1	4.7 ± 1.2	ND
		$P < 0.01$			$P < 0.01$		

PBMC, range 25–39), while a type II T cell response (IL-4 secretion) was noted in only 3 of the patients (numbers 4, 7, 14) and at a lower level (ELISPOT values: 18, 27 and 17/10^5 PBMC respectively).

Clinical side-effects

At the local injection site, especially in the GM-CSF group, redness, swelling and tenderness were noted. The intensity of the local reaction increased by each immunization. In no patient had treatment to be withdrawn or medication given. No patient had gastrointestinal symptoms. Blood cell counts remained unchanged during follow-up as did liver function tests and kidney function analyses.

Discussion

Nine colorectal carcinoma patients without macroscopic disease were immunized with recombinant human CEA in combination with GM-CSF. A further 9 patients received an identical immunization schedule but without GM-CSF. All patients in the GM-CSF group developed high titers of rhCEA-specific IgG antibodies. The limited data suggest that the titers induced by rhCEA may have been dose-dependent. A summary of the individual cellular responses is shown in Table 4. All patients in the GM-CSF group developed an rhCEA-specific T cell response (DNA synthesis). The specific T cells seemed to be of type I (IFNγ secretion) but a minority of the patients also had type II (IL-4 secretion) cells. Both MHC-class-I- and -class-II-restricted rhCEA-specific T cells were induced. In the GM-CSF group, where a T cell response against rhCEA was noted in all patients (proliferation as well as IFNγ secretion) the response against nhCEA varied. Five patients had a proliferative response and 7 an IFNγ response. Only 1 patient (patient 7) did not recognize nhCEA. The varying responses to the native antigen might be explained by the observation that different T cell subsets and T cells of varying maturity may have a varying response profile [17]. In particular, antigen-specific cell proliferation may not correlate to antigen-specific cytokine production, suggesting that a proliferation test only may not be sufficient to reveal an antigen-specific T cell population [40]. In accordance with the present study, a recent report on idiotype immunization in multiple myeloma showed that both proliferation and cytokine secretion tests were necessary to detect an antigen-specific T cell response [49].

Furthermore, the response against nhCEA was at a significantly lower level than that against rhCEA. The reason for this is not clear, but it may be due to differences in glycosylation as the amino acid sequence of the protein backbone is exactly the same for the two proteins. Preliminary data indicate, however, that T cells recognized a restricted number of overlapping peptides

Table 4 Summary of recombinant human CEA (*r*) and native human CEA (*n*) specific cellular responses in patients immunized with rhCEA ± GM-CSF. ELISPOT results: + a significant positive specific response; (+) a weak but specific positive response; − no specific positive response. *ND* not done

Patient no.	DNA synthesis		IFNγ produced		IL-4 produced	
	r	n	r	n	r	n
With GM-CSF						
2	+	+	+	+	−	−
4	+	−	+	(+)	(+)	−
6	+	−	+	+	−	(+)
7	+	−	+	−	+	−
9	+	+	+	(+)	+	−
12	+	+	+	(+)	−	−
13	+	+	+	−	−	−
14	+	−	+	+	(+)	−
18	+	+	+	(+)	−	−
Without GM-CSF						
1	−	−	−	ND	(+)	ND
3	+	+	−	ND	(+)	−
5	−	−	+	+	−	−
8	+	ND	(+)	ND	(+)	ND
10	−	ND	(+)	ND	(+)	ND
11	−	ND	+	ND	(+)	ND
15	−	(+)	−	ND	(+)	ND
16	+	ND	−	ND	−	ND
17	−	ND	−	ND	−	ND

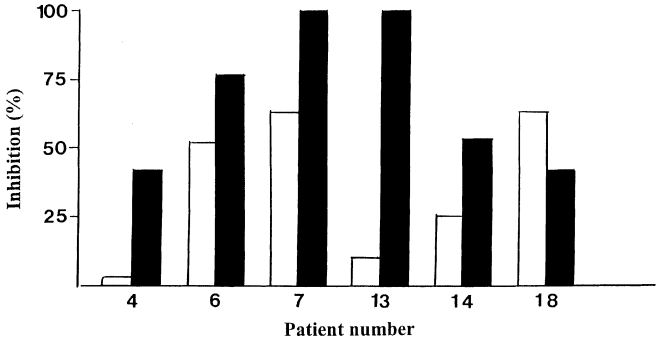

Fig. 7 Inhibition of rhCEA-specific T cell response by MHC class I (*open column*) and MHC class II (*filled column*) monoclonal antibodies. Peripheral blood mononuclear cells were stimulated by rhCEA in the presence of anti-(MHC class I), anti-(MHC class II) or a control mAb. DNA synthesis was measured. Values are given as percentage inhibition of cultures with the control antibodies

of the external domain of the human native CEA molecule, indicating that a cellular response was induced against T cell epitopes of the naturally occurring human CEA molecule [8]. (A detailed analysis of the humoral and cellular responses against nhCEA will be presented separately.) In the non-GM-CSF group only one-third developed IgG antibodies at a low level. A weak rhCEA-specific T cell response (proliferation and/or cytokine secretion) was noted in most of the patients. However, owing to the weak response against the recombinant protein, the response against the native protein was not analyzed in detail in the non-GM-CSF group. Thus, GM-CSF seemed to be mandatory for the regular induction at a significant level of a humoral and cellular immune response against the baculovirus-produced recombinant CEA protein. However, immunity against the natural human CEA was of a significantly lower magnitude. This observation should be kept in mind when developing vaccine strategies using recombinant proteins as immunogens.

Local administration of soluble GM-CSF may elicit similar effects to those of the cytokine secreted by transduced tumor cells [5, 21, 46]. Such a therapeutic approach should be simpler, more reproducable and more cost-effective. However, a single dose of GM-CSF given together with the tumor cells was not effective in inducing an antitumor immunity in an animal model, but administration of GM-CSF encapsulated in microspheres, together with irradiated tumor cells, induced an antitumor immunity comparable to that of GM-CSF-gene-transduced tumor cells [10]. Vaccination of rhesus monkeys with an anti-idiotypic antibody mimiking the Lewis Y antigen induced a strong humoral response against the nominal antigen if the anti-idiotype was administered together with GM-CSF for 4 consecutive days, starting at the day of vaccination, but not if GM-CSF was given for only 1 day or if the anti-idiotype was given alone [24]. Local administration of GM-CSF for 4 days together with the autologous idiotype in an animal lymphoma model also induced a strong protective cell-mediated immunity [20]. Moreover, soluble GM-CSF contributed, in an animal model, to the induction of a cellular response against a self oncoprotein [neu (c-erbB-2) protein] [4]. In melanoma patients, addition of soluble GM-CSF was necessary to induce a clinical response when they were immunized with melanoma-associated peptides [16].

Immunization of mice with naked plasmid DNA encoding the regulatory HIV-1 Nef protein and an expression vector encoding GM-CSF also resulted in a

marked humoral and mainly Th1 response. The immunostimulatory activity of GM-CSF was locally restricted [41]. Thus, our data and those of others clearly indicate that soluble GM-CSF is an important adjuvant cytokine to combine with tumor-associated antigens for vaccination, but prolonged local administration seems to be required.

Low doses of GM-CSF, not inducing a systemic effect, evaluated as no rise in white blood cell counts, seemed to be sufficient. The low-dose efficacy is in line with an observation showing that high doses of GM-CSF produced by tumor cells resulted in a suboptimal antitumor immunity compared to low-dose producers [1]. Furthermore, a lower dose of GM-CSF together with the idiotype induced a better protective cellular immunity than did a higher dose in an animal lymphoma model [20].

CEA has been used in other studies to induce a tumor-specific immune response. In a mouse model, recombinant vaccinia CEA (rV-CEA) induced a strong CTL response while native CEA was less effective [18]. Immune mice were protected against challenge with murine coloncarcinoma cells expressing human CEA and regression of established tumors was noted. A proliferative T cell response to CEA was observed when patients with minimal residual disease were immunized with rV-CEA [2] while this was not the case in advanced disease [12]. Precursor cytotoxic T lymphocytes were generated at low frequency, which lysed autologous Epstein-Barr-virus-transformed B cells expressing CEA peptides or HLA-class-I-matched CEA-expressing colorectal carcinoma cell lines [45].

A strong humoral immune response was induced, as indicated by high IgG antibody titers. Such antibodies might have therapeutic effects by mediating antibody-dependent cellular cytotoxicity or complement lysis. Moreover, as CEA belongs to the group of epithelial cell adhesion molecules modulating cell-to-cell contact [15], anti-CEA antibodies might induce contact inhibition contributing to inhibition of tumor growth.

The major part of the T cells recognizing rhCEA seemed to be type I T cells. MHC-class-II-restricted but also MHC-class-I-restricted cells seemed to have been induced. The functional significance of these T cells is not established. Classical CD8 CTL recognizing endogenously presented antigens have been considered mandatory for the rejection of malignant cells. Exogenous antigens may also use the endogenous antigen-presentation pathway [35] and induce specific CD8 T cells recognizing MHC-I-restricted peptides. GM-CSF may facilitate such a presentation [30]. Furthermore, CD4 as well as CD8 T cells have been shown to be of importance for tumor rejection in various systems using exogenous protein antigens, cell-based vaccines or viral constructs as immunogens [11, 21, 22, 44]. In the present study, however, we have no proof whether functional cytolytic T cells were induced or not. Studies are in progress to analyze the presence of cytotoxic T cells.

In conclusion, pharmacological administration of GM-CSF together with the recombinant tumor antigen CEA induced an immune response against rhCEA. However, the response against native CEA was of a significantly lower magnitude, which finding might be crucial and warrants further studies in man to find an optimal way to present the CEA antigen for vaccination. No signs and symptoms were noted that indicated auto-immune reactions. Moreover, pharmacological administration of GM-CSF might be an effective general principle for the induction of a strong immune response against tumor-associated antigens, anti-idotypes and microbial antigens [10, 24, 42]. Clinical trials are warranted to explore this simple and inexpensive therapeutic principle for the induction of a clinically effective immunity in malignant as well as non-malignant diseases.

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