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Induction of IgG Subclass Responses in Colorectal Carcinoma Patients Vaccinated with Recombinant Carcinoembryonic Antigen¹

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ABSTRACT

There is scanty information on the IgG subclass response after vaccination against cancer antigens. The induction and development of the IgG subclass responses in 18 colorectal carcinoma patients vaccinated s.c. seven times with recombinant human carcinoembryonic antigen (rhCEA) over a 12-month period were analyzed by ELISA. The patients were followed for 3 years. Four rhCEA doses were used, and half of the patients also received granulocyte macrophage-colony stimulating factor (GM-CSF) as an adjuvant. Anti-rhCEA-specific IgG1 and IgG4 responses and, to a lesser degree, IgG2 responses were markedly enhanced by concomitant GM-CSF administration, whereas the antigen dose was of minor importance. Almost no IgG3 response was observed. A significant antibody response was noted within the first weeks for IgG1 and IgG2 but noted several months later for IgG4. The responses gradually increased by repeated immunizations and peaked around 12 months for IgG1 and a few months later for IgG2 and IgG4. A sustained but reduced response was noted for these three subclasses at 24 and 36 months. Interestingly, there was a gradual shift from a predominant IgG1 response at 6 months to an IgG4 response at 15 months. No significant change in total concentrations of the four IgG subclasses was observed comparing prevaccination concentrations with concentrations at 12 months, indicating an antigen-specific effect of GM-CSF administration on the anti-rhCEA response. The clinical significance of the individual IgG subclass antibodies for tumor response is not clear and requires additional studies.

INTRODUCTION

Despite tremendous therapeutic efforts, the prognosis for CRC³ is still poor, and new treatment modalities are needed. Specific immunotherapy involving cancer vaccines and monoclonal antibodies may induce tumor regression (1, 2) and survival benefits in the adjuvant setting (3, 4).

CEA, a surface-expressed tumor-associated antigen (5), is a well-characterized glycoprotein present at a high density on most malignant tumors of the gastrointestinal tract. Vaccination against colon cancer with anti-idiotypic antibodies mimicking CEA (6), rCEA (7), and CEA expressed in virus (8, 9) has been shown to induce both cellular and humoral immune responses.

CEA is therefore being explored in cancer patients as a therapeutic vaccine, but unresponsiveness to self-antigens like CEA might occur. In such instances, it may be necessary to administer strong adjuvants and perform repeated immunizations. We have recently shown an enhancing effect of the adjuvant cytokine GM-CSF on both the

humoral and cellular anti-CEA response in CRC patients after CEA vaccination (7). The capacity of GM-CSF to augment an antitumor response is attributed to its role in activating antigen-presenting dendritic cells (10–12). Although the induction of antigen-specific IgG antibodies has previously been shown, the distribution of individual IgG subclasses has only partly been analyzed (6, 9, 13).

There are four subclasses of human IgG (IgG1, IgG2, IgG3, and IgG4). In serum of healthy adults, the total concentrations of these various subclasses represent 65%, 25%, 6%, and 4%, respectively (14). The distribution between the IgG subclasses of the antibody response varies depending on the nature of antigen, dose, route of entry, and also the host genotype. Complement activation and opsonization are two main effector functions of IgG antibodies. These functions are mediated by the Fc part after interaction of the antibody with antigen. Cellular effector functions mediated by antibodies are complex, depend on the type of cell and isoform of FcγR involved (15), and include ADCC, enhanced antigen presentation, and regulation of antibody response. In general, the effector capacity among the IgG subclasses may be ranked as IgG1 ≈ IgG3 ≫ IgG2 ≈ IgG4 both with regard to complement activation via C1q (complement-dependent cytotoxicity) and FcγR binding (14).

Vaccination with tumor antigens elicits both a humoral and cellular response, the respective importance of which may differ for various malignancies. The humoral response may be of greater significance than previously anticipated, and its clinical significance warrants further evaluation. There are several monoclonal antibodies in use for treatment of hematological as well as solid tumors.

The aim of the present study was to assess over a 3-year period the IgG subclass anti-CEA-specific response in CRC patients vaccinated with rCEA. We also investigated the effects of the antigen dose, the addition of GM-CSF, and total serum IgG subclass levels on the IgG subclass responses.

MATERIALS AND METHODS

Antigen. rhCEA (Protein Sciences Corp., Meriden, CT) was produced in baculovirus-infected insect cells as described previously (7). Briefly, the gene for CEA was cloned from human colon adenocarcinoma cells (LS174T) and engineered to contain all of the amino acids found in native human CEA. The rhCEA is glycosylated with N-linked glycans and has a molecular mass of 120 kDa, which is smaller than the anticipated 180-kDa molecular mass of native human CEA. The difference is caused by the lack of complex carbohydrate structures in rhCEA. rhCEA was purified under nondenaturing conditions and tested for purity (>99%), safety, and sterility (7). The purified rhCEA was formulated *in situ* with alum (0.5 mg/ml aluminum ion as Al₃PO₄; Swedish Institute for Infectious Disease Control, Stockholm, Sweden) before immunization.

Baculovirus and insect cell proteins may contaminate purified rhCEA. Therefore, a BCP was prepared that contained a mixture of insect cells and baculovirus proteins from cells infected with a control baculovirus expression vector as described previously (7).

Patients. Eighteen patients (12 males and 6 females) who had been operated on for Dukes' class A (*n* = 1), B (*n* = 10), and C (*n* = 7) CRC with no remaining tumor entered the study. The median age was 64 years (range, 33–80 years). Consecutive patients were enrolled at each of four dose levels of rhCEA (100, 316, 1000, and 3000 μg/immunization), which was administered

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³The abbreviations used are: CRC, colorectal carcinoma; rhCEA, recombinant human carcinoembryonic antigen; CEA, carcinoembryonic antigen; rCEA, recombinant carcinoembryonic antigen; GM-CSF, granulocyte macrophage-colony stimulating factor; FcγR, Fcγ receptor; ADCC, antibody-dependent cellular cytotoxicity; BCP, baculovirus control protein; AU, arbitrary unit(s).

s.c. at weeks 0 and 2 and months 2, 4, 6, 9, and 12. Six patients receiving 100 μg of rhCEA and three patients receiving 316 μg of rhCEA constituted the low-dose group. Five patients receiving 1000 μg of rhCEA and four patients receiving 3000 μg of rhCEA formed the high-dose group. In a randomized fashion, four patients in the low-dose group and five patients in the high-dose group were given GM-CSF (80 $\mu\text{g}/\text{day}$; Leucomax, Schering-Plough/Novartis, Kenilworth, NJ) s.c. at each immunization once a day, days 1–4, at the same site as the rhCEA injection. The IgG subclass response was analyzed on all available sera taken at regular intervals (0–36 months). The study was approved by the Ethic Committees of the Karolinska Institute, and informed consent was obtained from each patient.

IgG Subclass Assays. The total serum concentrations of the four IgG subclasses were analyzed by standard methods and compared with the age-related reference population (95% confidence intervals) used at the Department of Clinical Immunology, Karolinska Hospital.

Antigen-specific IgG1, IgG2, IgG3, and IgG4 antibodies were determined by sandwich ELISA using monoclonal antibodies essentially as described previously (16). Briefly, microtiter plate wells were coated with purified rhCEA (2 $\mu\text{g}/\text{ml}$). All serum samples were initially assayed at a final 40-fold dilution after preincubation with rhCEA (10 $\mu\text{g}/\text{ml}$), BCP (10 $\mu\text{g}/\text{ml}$), or diluent alone. High-titered sera were assayed at higher dilutions. The net results of absorbance at 405 nm (difference between diluent alone and antigen as inhibitor) were used to interpolate the concentrations of anti-rhCEA and anti-BCP antibodies from standard curves of chimeric IgG1, IgG2, IgG3, and IgG4 anti-NIP (5-iodo-4-hydroxy-3-nitro-phenacetyl acid) hapten antibodies run in parallel using BSA-NIP conjugate (10 $\mu\text{g}/\text{ml}$) as coating antigen (16). The anti-rhCEA IgG concentrations were calculated after subtraction of the anti-BCP results, which, in general, were very low. Taking the serum dilution into account and considering a net absorbance of 0.020 as cutoff, the serum sensitivity in the assays was 6.0 (IgG1 and IgG2) and 4.0 (IgG3 and IgG4) AU/ml. This ELISA procedure permits semiquantitative evaluation where 1 AU roughly corresponds to 1 ng of antibody.

Statistical Methods. Differences in frequency between patient groups were compared by Fisher's exact test. Medians of continuous parameters were compared for two groups by the Mann-Whitney *U* test and by the Kruskal-Wallis test after further stratification. Wilcoxon's rank-sum test was used to analyze paired samples. Correlations between continuous variables were performed using Spearman rank analysis.

RESULTS

Patterns and Kinetics of the IgG Subclass Response to rhCEA.

The antigen-specific IgG subclass antibody response in CRC patients vaccinated with rhCEA at four doses in the presence or absence of GM-CSF was evaluated over a 3-year period. The kinetics of the individual anti-rhCEA IgG subclass responses showed that most patients mounted a significant IgG1, IgG2, and IgG4 anti-rhCEA response but a weak IgG3 response (Fig. 1). The order of the response with regard to absolute antibody concentration was $\text{IgG1} \approx \text{IgG4} > \text{IgG2} \gg \text{IgG3}$. Eleven (61%) patients had a >10 -fold increase in antibody concentration at one or more sampling times for at least two IgG subclasses in comparison with levels before treatment. For most patients, the antibody levels gradually increased after each immunization. With a few exceptions, induction of an IgG1 and IgG2 antibody response was noted within the first few weeks of treatment, followed by a surge of the IgG4 subclass at 6 months (Fig. 1).

The last vaccination with rhCEA was given at month 12, and for most patients, the antibody concentrations of IgG1, IgG2, and IgG4 gradually declined after a maximum level was reached (this occurred at 12 months for IgG1 and at 15 months for IgG2 and IgG4; Fig. 1). High anti-rhCEA antibody concentrations (arbitrarily defined as 10 times the pretreatment levels) for at least one subclass were still present in 10 of 16 patients (63%) at 24 months and in 4 of 12 patients (33%) at 36 months (Fig. 1).

A few patients had detectable anti-rhCEA antibody levels before

treatment, and their antibody response patterns were similar to those of other patients (Fig. 1).

Five patients relapsed during the study, but no particular pattern of the anti-rhCEA IgG subclass response could be found (data not shown).

Effects of the rhCEA Dose and GM-CSF on the Antibody Response. The IgG subclass levels against rhCEA were assessed in relation to the dose of rhCEA given and to concomitant administration of GM-CSF used as an adjuvant. Because of the limited number of patients, patients were grouped into two categories with regard to the CEA dose: (a) the high-dose group (1000/3000 μg); and (b) the low-dose group (100/316 μg ; Fig. 2).

Irrespective of GM-CSF administration, there was no dose response for IgG1, whereas for IgG2, there was a tendency to higher antibody concentrations in the high-dose group (Fig. 2). The IgG3 response was too low for a meaningful comparison. The minor dose-dependent response for IgG4 observed among patients not receiving GM-CSF could probably be attributed to patient 20 (Fig. 1). Considering the dose alone, statistically significant higher responses (at 12 and 15 months) in the high-dose group were noted for IgG2 only (Fig. 3).

In comparison with the weak effect of the rhCEA dose, GM-CSF induced a marked effect on the anti-rhCEA IgG response (Fig. 2). To more closely examine the effect of GM-CSF on the pattern of IgG subclass responses, the patients were divided into two groups based on whether they had received GM-CSF or not, irrespective of antigen dose (Fig. 4). Significant differences in the anti-rhCEA IgG1 response were already observed after 2 months of treatment and remained during follow-up of 3 years (Fig. 4). For IgG2, statistically significant higher levels were noted in the GM-CSF group only at 1 month and at the 3-year time point, but at all other time points during the evaluation period, the IgG2 antibody concentrations were notably higher in the GM-CSF group but not statistically significant. The IgG4 concentrations were significantly higher in the GM-CSF group after 6 months and during 18 months of follow-up in comparison with the group of patients receiving rhCEA alone (Fig. 4).

GM-CSF treatment not only affected the magnitude but also induced an earlier appearance of the antibody responses. In comparison with baseline, significantly higher IgG1 and IgG2 response levels were already noted at 1 month and 2 months of therapy in the GM-CSF group as compared with 9 and 12 months for the CEA only group. For IgG4, a significant antibody response was initially seen in both groups at 6 months of therapy, but higher levels were noted in the GM-CSF group during that time period.

All nine patients treated with GM-CSF developed strong antibody responses to rhCEA (>10 times the pretreatment level) in at least two IgG subclasses, mainly IgG1/IgG4. In contrast, only two patients (patients 1 and 20) in the CEA only group mounted such responses, which were IgG1/IgG4 and IgG2/IgG4, respectively. The difference between the two groups was statistically significant ($P < 0.003$).

The median increase in the anti-rhCEA antibody concentrations at 12 months in comparison with pretreatment concentrations was calculated (Fig. 5). The increase in anti-rhCEA IgG1 was 57-fold for the GM-CSF group as compared with 2.8-fold for the group not receiving GM-CSF ($P < 0.001$). The corresponding figures for anti-rhCEA IgG2 were 9.8-fold *versus* 2.0-fold ($P = 0.07$), and the corresponding figures for IgG4 were 72-fold *versus* 1.4-fold ($P < 0.01$), respectively.

Anti-BCP Response. Only minor IgG subclass responses against the BCP were detected. Although the median increases were higher in the GM-CSF group, they were not significantly different from those of the group that did not receive GM-CSF (Fig. 5).

Relation between IgG Subclass Responses. In 13 patients with a strong antibody response (>10 times the pretreatment level at any

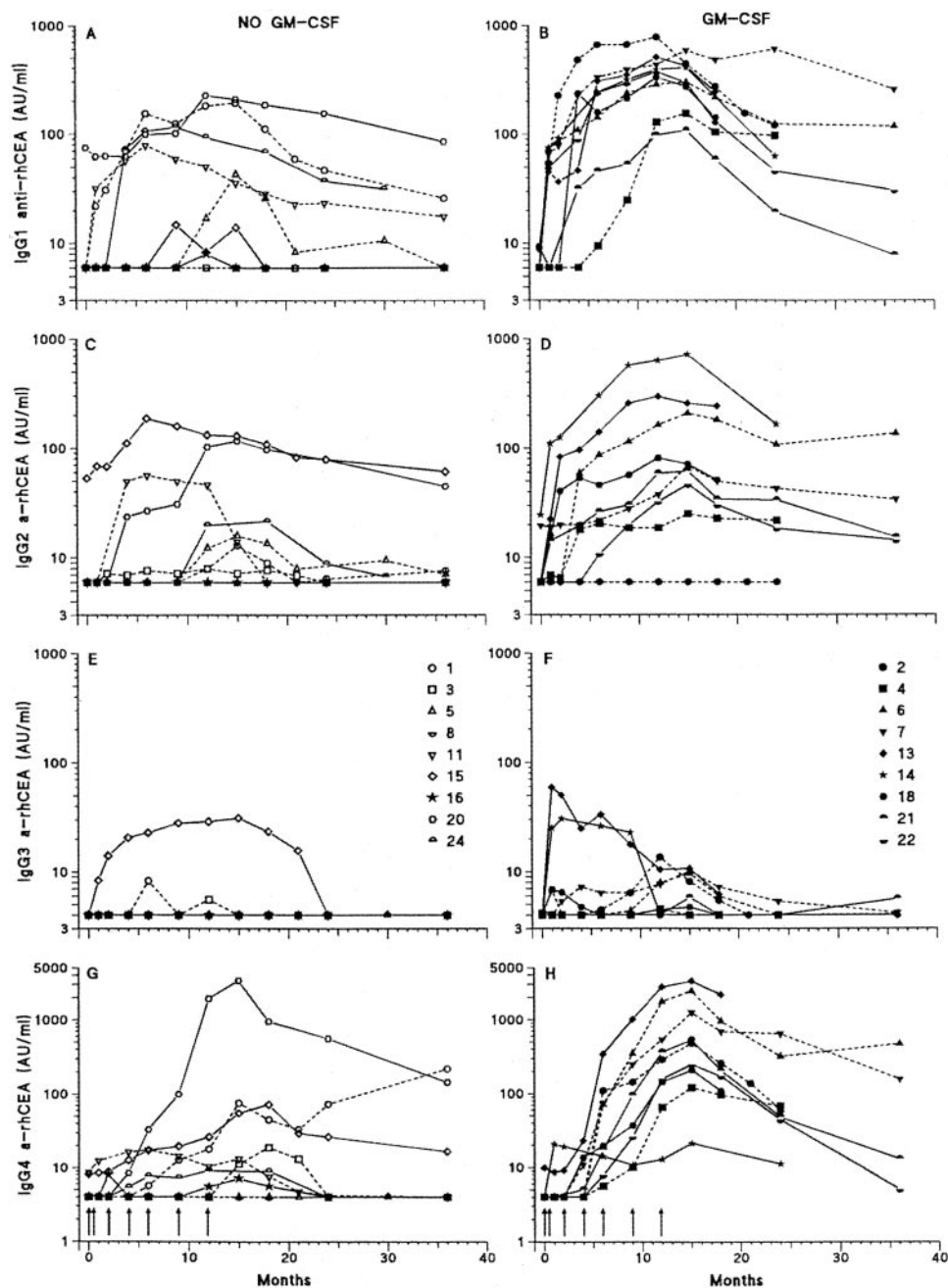


Fig. 1. Kinetics of individual serum IgG1, IgG2, IgG3, and IgG4 anti-rhCEA antibody responses (AU/ml) as analyzed by ELISA in CRC patients ($n = 18$) vaccinated with rhCEA. Arrows (at the bottom) indicate immunization times. Nine patients were allocated to a low-dose group (100 or 316 μ g rhCEA/immunization; ---), and nine patients were allocated to a high-dose group (1000 or 3000 μ g rhCEA/immunization; —). Left panels represent patients who did not receive GM-CSF (open symbols), and right panels represent those who received GM-CSF (filled symbols). The different symbols indicate individual patients. For additional details, see "Materials and Methods."

sampling time), an IgG1 response was the most common ($n = 12$), followed by IgG4 ($n = 10$), IgG2 ($n = 6$), and IgG3 ($n = 1$). In nine patients, a simultaneous IgG1/IgG4 response dominated, which also included IgG2 in four patients.

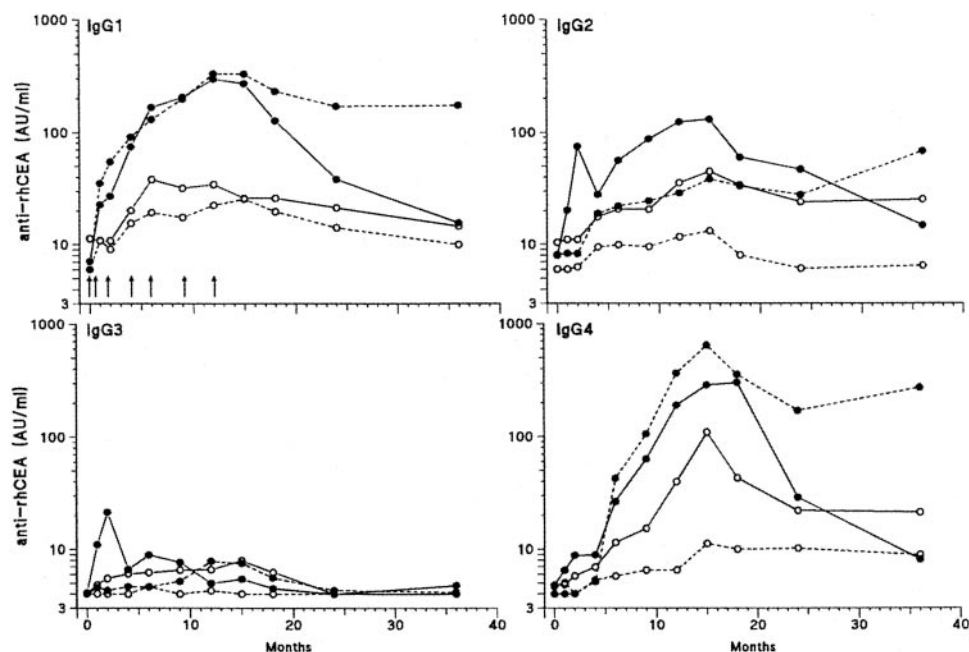
Analyses at 12 months showed a significant correlation between the IgG1 and IgG4 anti-rhCEA concentrations ($r_s = 0.76$; $P < 0.001$; $n = 18$) as well as between the IgG2 and IgG4 concentrations ($r_s = 0.57$; $P < 0.05$; $n = 18$).

Because our ELISA permits semiquantitative comparisons, the distribution of the anti-rhCEA IgG subclass responses from the 6th to 15th month was calculated in the group of patients receiving rhCEA together with GM-CSF (Fig. 6). At 6 months, the response was predominantly IgG1 (58%), followed by IgG2 (23%), IgG4 (16%), and IgG3 (3%). Thereafter, however, the IgG1, IgG2, and IgG3 levels gradually declined, whereas the IgG4 levels increased. At 15 months, the IgG4 subclass was predominant (53%), followed by IgG1 (33%),

IgG2 (13%), and IgG3 (1%). Because the levels of anti-rhCEA IgG subclass antibodies among the nine patients who did not receive GM-CSF were often undetectable, comparisons could only be performed in a few patients, for whom a similar shift from IgG1 to IgG4 by time was demonstrated (data not shown). This suggests that the shift is related more to the immunization protocol than to GM-CSF administration.

Total IgG Subclasses. Total serum concentrations of the four IgG subclasses were analyzed before vaccination and at 12 months. The levels were outside the normal range (95% confidence intervals) in five patients. There were no statistically significant differences in total IgG subclass concentrations at prevaccination or at 12 months with respect to the dose of rhCEA or to treatment with or without GM-CSF. Furthermore, no significant changes were observed in the IgG subclass concentrations when comparing paired samples taken before vaccination and at 12 months.

Fig. 2. Serum IgG1, IgG2, IgG3, and IgG4 anti-rhCEA antibody concentrations (AU/ml; geometric mean) in relation to dose of rhCEA and GM-CSF therapy. $\circ$ - $\circ$, low-dose group without GM-CSF ($n = 5$); $\circ$ - $\circ$, high-dose group without GM-CSF ($n = 4$); $\bullet$ - $\bullet$, low-dose group with GM-CSF ($n = 4$); $\bullet$ - $\bullet$, high-dose group without GM-CSF ($n = 5$). Arrows indicate immunization times. No significant differences were observed for any subclass at any time point by statistical analysis with the Kruskal-Wallis test. For additional details, see the Fig. 1 legend and "Materials and Methods."



To assess whether there was a relation between total IgG subclass levels and the ability of the patients to mount an anti-rhCEA antibody response, the levels at 12 months were compared. No significant correlations were observed when analyzing all 18 patients with regard to the rhCEA dose or treatment with or without GM-CSF. A low total IgG subclass level did not exclude a substantial anti-rhCEA response, as illustrated by patient 14, who, despite a low serum concentration of IgG4 (25 mg/liter), mounted a relatively high anti-rhCEA IgG4 response (Fig. 1).

DISCUSSION

In this study, IgG subclass antibody responses were analyzed during a 3-year period in CRC patients immunized for 1 year (seven times) with rhCEA with or without concomitant GM-CSF therapy. The rhCEA vaccination protocol elicited a strong IgG1 and IgG4 response, a moderate IgG2 response, and a weak anti-rhCEA IgG3 response. The predominant anti-rhCEA IgG subclass response shifted from IgG1 to IgG4 during vaccination. GM-CSF had a marked influence on the induction of the antibody response, whereas the antigen dose seemed to be of minor importance. All nine patients receiving

GM-CSF as an adjuvant, irrespective of the rhCEA dose, developed a strong antibody response in at least two subclasses compared with only two of nine patients who received CEA alone. For most of the 18 patients in this study, the IgG1 and IgG2 responses appeared within a few weeks, whereas the IgG4 response was delayed a few months. The titers reached a plateau at 12–15 months from start of immunization. The last vaccination was done at month 12. Sustained responses could be seen in several patients for up to 2 years after immunization.

GM-CSF is known to have a substantial impact on both humoral and cellular responses in rodents (17) and humans (7). However, there has been no study demonstrating the effect of GM-CSF on individual IgG subclasses. Our results demonstrate that GM-CSF strongly enhanced the rhCEA vaccination-induced IgG1 and IgG4 responses and moderately affected IgG2 levels but did not affect IgG3 levels at all.

rhCEA is a heavily glycosylated protein. It might be anticipated that IgG1 and IgG4 responses are elicited against the protein backbone, whereas IgG2 antibodies react with the carbohydrate structures (14). The weak effect on IgG3 is unclear and suggests additional mechanisms for switching to this isotype. The distribution pattern of the IgG subclass responses indicates that both Th1 (IgG1) and Th2 (IgG4) T-cell responses are induced, which is supported by detection of rhCEA-specific IFN- γ and interleukin-4-secreting T cells (7).

We did not note any significant effect of the doses of rhCEA on the anti-rhCEA responses, except for a weak effect on IgG2 concentrations despite a 30-fold dose difference in rhCEA. This may be attributed to the limited number of patients or to a minor dose effect of rhCEA that is overshadowed by the profound effect of GM-CSF. Thus, from a therapeutic point of view, a strong and long-lasting IgG1/IgG4 response can be obtained with a low dose of antigen if it is given together with GM-CSF as an adjuvant.

The kinetics of the antibody response was similar for most patients, irrespective of the antigen dose or GM-CSF therapy. Concomitant administration of GM-CSF substantially enhanced the amplitude, giving a rapid appearance and longer duration of the IgG1, IgG2, and IgG4 antibody responses. Whereas IgG1 and IgG2 appeared after a few weeks, there was a delay in the IgG4 response. A similar antibody response pattern has been described after immunization of atopic and

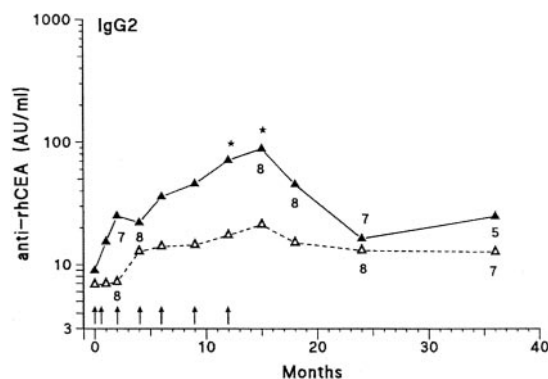


Fig. 3. Serum IgG2 anti-rhCEA antibody response (AU/ml; geometric mean) in relation to rhCEA dose. Nine patients received a low dose (100 or 316 μ g rhCEA/immunization; $\triangle$ - $\triangle$), and nine patients received a high dose (1000 or 3000 μ g rhCEA/immunization; $\blacktriangle$ - $\blacktriangle$). *, $P < 0.05$.

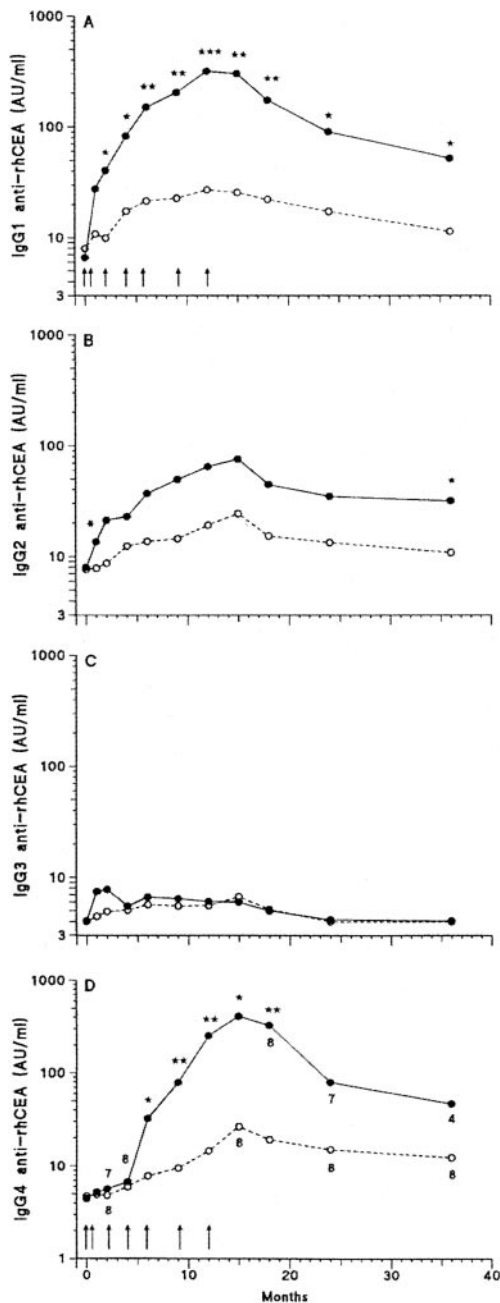


Fig. 4. Influence of adjuvant GM-CSF therapy on serum IgG1, IgG2, IgG3, and IgG4 antibody response (AU/ml; geometric mean) in CRC patients vaccinated with rhCEA. Nine patients received GM-CSF (●—●), and nine patients did not receive GM-CSF (○—○). The numbers next to the curves in the IgG4 panel (bottom) indicate the actual number of patients analyzed at the indicated time points for each IgG subclass. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

nonatopic subjects with various exogenous antigens *e.g.*, keyhole limpet hemocyanin and allergens (18, 19). In agreement with these studies, it seems that repetitive immunizations are required to evoke a strong IgG4 response as in chronic exposure (18). Interestingly, as the number of immunizations increased, there was a gradual shift in the anti-rhCEA response from a predominant IgG1 at 6 months to IgG4 after 12 months. This shift is probably not a consequence of GM-CSF treatment but rather caused by the repetitive immunization protocol.

IgG antibodies exert their physiological effects mainly through complement activation and interaction with FcγRs, which are present on various effector cells of the immune system. IgG1 and IgG3 are more powerful in mediating phagocytosis, ADCC, and complement-

dependent cytotoxicity than the other two subclasses. Therefore, humanized monoclonal IgG1 antibodies against tumor-associated antigens are being used for treatment of lymphoma and breast cancer (1, 2). The strong IgG1 anti-rhCEA response we observed may cause tumor cell destruction. Although total IgG4 represents the lowest serum concentration of the IgG subclasses, the magnitude of the IgG4 antibody response to rhCEA was as high as that for IgG1. IgG4 and IgG2 probably have weak antitumor effector functions, but they may inhibit cell-cell contact and interfere with the metastatic process because CEA belongs to the group of epithelial cell adhesion molecules (20). However, these subclasses might also down-regulate effector functions of IgG1 by interfering at the level of immune complex formation. This may attenuate complement activation and FcγR interactions (18). If these effects are of importance, vaccination protocols should be aimed to favor mainly an IgG1 antibody response. It might also be of interest to try other adjuvants to induce IgG3 antibodies, which are powerful effectors, as illustrated by preclinical studies using chimeric antitumor antigen antibodies of different IgG subclasses (21, 22).

There are few studies on the induction of IgG subclass responses using cancer antigens as vaccines. Immunization with a monoclonal anti-idiotypic antibody generated high-titer humoral responses against CEA in colon cancer patients with a dominance for IgG1, IgG2, and IgG4 responses (6), which is consistent with our findings. Vaccinia-CEA immunization induced significant IgG anti-CEA responses in patients with CEA-expressing adenocarcinomas. These patients were

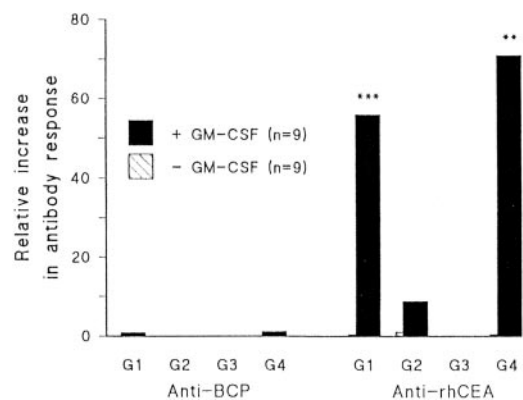


Fig. 5. Effect of GM-CSF on IgG anti-rhCEA and anti-BCP (control antigen) subclass responses (median) comparing pretreatment and 12 month values in CRC patients immunized with rhCEA. Nine patients received GM-CSF (■), and nine patients did not receive GM-CSF (▨). **, $P < 0.01$; ***, $P < 0.001$.

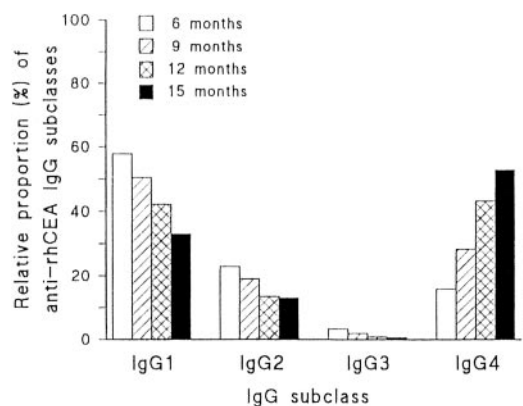


Fig. 6. Relative proportions (median) of anti-rhCEA IgG subclass antibody levels in relation to time after immunization with rhCEA and adjuvant GM-CSF therapy in nine CRC patients.

only tested once at 5–8 weeks postimmunization, and a primarily IgG1 response was noted (9). In another study, a strong IgG1 and IgG3 response against MUC1 was noted in breast cancer patients, whereas the IgG2 response was modest (13). This discrepancy may be due to the nature of the antigen used, vaccine formulation, use of different adjuvants, immunization schedule, and different tumor types and patients.

In summary, vaccination with the recombinant protein CEA in combination with GM-CSF as an adjuvant induced a strong IgG1 and IgG4 response and a moderate IgG2 response but practically no IgG3 anti-CEA response. In the absence of GM-CSF, the response was weak and delayed. Although the clinical significance of vaccination-induced anti-CEA antibodies is unclear, IgG1 mediates ADCC and complement-dependent cytotoxicity, which may cause tumor cell destruction. The biological significance of IgG4 is not well characterized and is warranted because an IgG4 response may be of importance in a cancer vaccination approach.

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