Original article

Annals of Oncology 14: 461–466, 2003
DOI: 10.1093/annonc/mdg102

Epidermal growth factor-based cancer vaccine for non-small-cell lung cancer therapy

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Received 26 April 2002; revised 16 September 2002; accepted 14 October 2002

Background: The role that growth factors and their receptors play in human cancer growth and progression makes them interesting targets for novel treatment modalities. Our approach consisted of active immunotherapy with the epidermal growth factor (EGF). Two pilot clinical trials were conducted to examine the safety and immunogenicity of a five-dose immunization protocol and to compare different adjuvants and treatment designs.

Patients and methods: Forty patients with advanced non-small-cell lung cancer were enrolled in both trials. They were randomized to be treated with aluminum hydroxide or montanide ISA 51 as adjuvants in the EGF vaccine preparation. The use of cyclophosphamide prevaccination treatment was evaluated in the second trial.

Results: Pooled data from both trials showed that the use of montanide as adjuvant increased the percentage of good antibody responders (GAR). Cyclophosphamide prevaccination treatment did not provoke improvements in antibody response. GAR had a significant increase in survival as compared with poor antibody responders. Response duration was also related to a significant improvement in survival rates.

Conclusions: Vaccination with five doses of EGF vaccine is safe and immunogenic. Montanide ISA 51 increased the percentage of GAR. There is a direct relationship between anti-EGF antibody titers and immune response duration with survival time.

Key words: cancer vaccine, epidermal growth factor, non-small-cell lung cancer

Introduction

During the 1990s, the epidermal growth factor receptor (EGF-R) has become one of the most attractive targets for the design of new anticancer drugs. In many cancer cells, growth factors and/or their receptors are overexpressed. In 1984, our research found that EGF-Rs were overexpressed in ~60% of human breast tumors and that this related to a poor prognosis [1, 2]. This relationship has also been demonstrated in other tumors [3–7]; specifically in non-small-cell lung cancer (NSCLC), EGF-R expression correlated with a high rate of metastasis, tumor invasiveness, poor prognosis and worse overall survival [8–10].

In the last few years, different therapeutic approaches targeting the EGF-R have been evaluated and chimeric and humanized monoclonal antibodies against the EGF-R have been used successfully in clinical trials [11, 12]. We undertook an alternative approach which consisted of vaccination with one of the main EGF-R ligands, the epidermal growth factor (EGF), coupled to a carrier protein, in an attempt to induce a specific anti-EGF antibody response that would block ligand–receptor binding.

In previous reports, we have shown that immunization with autologous EGF in mice and monkeys provoked an antibody response [13]. We also showed that mice with antibody titers against self-EGF had better survivals when transplanted with an EGF-R expressing tumor [13, 14]. Moreover, we demonstrated that there is a direct relationship between anti-EGF antibody titers and survival [14]. More recently, we reported that vaccination with autologous EGF in patients with epithelial tumors is immunogenic and well tolerated. An anti-EGF antibody response was obtained in 60% of patients immunized with a two-dose protocol [15].

In the present paper, pooled data from two pilot clinical trials of EGF vaccination are presented. The results allowed us to compare the effect of different adjuvants on patients' antibody response. The effect of a prevaccination treatment with low-dose cyclophosphamide was also studied, as was the relationship between survival and anti-EGF antibody titers in vaccinated patients.

Patients and methods

Eligibility criteria

Patients with histologically proven NSCLC at advanced stage (IIIb or IV) and not amenable to any other modality of oncospecific therapy were eligible.

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They were included in this protocol 4 weeks after finishing their last oncospecific treatment. Other eligibility criteria were age between 18 and 80 years, WHO performance status 0–2, normal liver, kidney and bone marrow functions, no pregnancy or lactation, no severe uncontrolled comorbidity, no second malignancies and no previous history of hypersensitivity to foreign proteins.

Study design and patient evaluation

Pooled data from two pilot clinical trials were evaluated. Both trials were open-label randomized studies, intended to compare safety and immunogenicity of vaccination with an EGF-based vaccine when two different adjuvants were used [aluminum hydroxide (alum) or montanide ISA 51 (Seppic, Paris, France)]. Data from both trials were taken in order to evaluate the effect of the pretreatment with cyclophosphamide. The simple randomization method was used for creation of the randomization list.

In the first study, 20 patients were randomized to vaccination with EGF-P64K adsorbed to alum (10 patients) or emulsified in montanide ISA 51 (10 patients). The vaccine was administered intramuscularly on days 0, 7, 14, 21 and 51. In the second study, an additional 20 patients were randomized and vaccinated similarly but all received a single dose of cyclophosphamide 200 mg/m^2 3 days prior to the first vaccination.

In both trials, serum was collected on days 0, 14, 28 and 60, and then monthly for antibody titer determination. Patients were revaccinated when antibody titers decreased to at least 50% of their peak titer at the induction phase. Patients underwent a complete blood count prior to inclusion, on days 0, 14, 28, 45 and 60, and then monthly during the follow-up period. Tumor response was evaluated on the first month after inclusion and then every 3 months by chest X-ray, abdominal ultrasound, and thoracic and abdominal computed tomography (CT) scan. Objective responses were classified according to WHO criteria [16].

The protocol was approved by Institutional Review Boards from the hospitals and by the State Center of Drug Quality Control, the national regulatory agency. All patients signed the informed consent prior to inclusion.

Immunogens

The vaccine was composed of human recombinant EGF conjugated to a carrier protein, the P64K *Neisseria meningitides* recombinant protein [17, 18], as previously described [15]. EGF and P64K were purchased from the Center of Genetic Engineering and Biotechnology of Havana, Cuba.

For preparations in which alum was used as adjuvant, conjugates were mixed after filtration with 2 mg/dose of alum: adsorption was achieved by constant stirring at room temperature for 1 h under sterile conditions. All procedures were performed according to good manufacturing practices.

When montanide ISA 51 was used as the adjuvant, the conjugate was mixed with an equal volume of the adjuvant until emulsification immediately before injection. One dose of vaccine is equivalent to 50 μg of EGF.

Measurement of antibody titers

Antibody titers against hu-EGF were measured through an enzyme-linked immunosorbent assay (ELISA) as previously described [15]. Antibody titer was defined as the maximum sera dilution with an absorbance measurement higher than the blank signal plus 3× the standard deviation. Specificity of antibodies against EGF has been demonstrated previously [15]. Antibody titers were more than 80% suppressed if EGF (0.1 μg/ml) was added to serum samples.

Antibody response was considered positive (seroconversion) when antibody titers were at least twice their pre-immunization values. Patients were additionally classified as good antibody responders (GAR) if antibody response reached titers equal or higher than 1:4000 and at least 8× the pre-immunization values and as poor antibody responders (PAR) if not. For immunoglobulin subclass testing, the same ELISA procedure was performed up to patient sera incubation and washing steps; plates were then incubated with 1:5000 diluted biotin conjugated mouse anti-human IgG1, IgG2, IgG3 or IgG4 monoclonal antibodies. After washing, alkaline phosphatase-conjugated streptavidin (1:1000 diluted) was added, color developed with ortophenilendiamine and the absorbance at 405 nm measured.

The capacity of patients' sera to inhibit the binding of EGF to its receptor was assessed through a radio receptor assay as previously described [15].

Statistical analysis

Peak antibody titers were compared through an ANOVA using the logarithmic transformation. The proportions of seroconversion, GAR and PAR in treatment groups were compared using Fisher's exact test and χ^2 tests [19]. These tests were also used to compare the capacity of sera to inhibit EGF/EGF-R binding. The duration of the immune response was compared with the ANOVA [19]. Estimations of mean survival times were done with Kaplan–Meier curve estimates, and survival comparisons were evaluated with the log-rank test [20].

Results

Forty patients with histologically confirmed NSCLC were included in two independent trials. Their mean age was 62 years (range 35–80). Seventeen patients had received prior surgery, chemotherapy and/or radiotherapy. Twenty-three subjects were consider ineligible for any oncospecific therapy at diagnosis. Table 1 summarizes their main characteristics (sex, performance status, stage and previous therapy).

Higher antibody responses were obtained when montanide ISA 51 was used as the adjuvant and pretreatment with low-dose

Table 1. Patient characteristics

Characteristic	No. of patients	Percentage
Entered	40	100
Sex		
Male	32	80
Female	8	20
Performance status		
0	5	12.5
1	26	65
2	9	22.5
Stage		
III	16	40
IV	24	60
Previous therapy		
Surgery + RTP + CTP	3	7.5
RTP + CTP	6	15
Surgery + RTP	3	7.5
CTP	3	7.5
RTP	1	2.5
Surgery	1	2.5

CTP, chemotherapy; RTP, radiotherapy.

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Table 2. Geometric means (ranges) of anti-EGF antibody titers in different treatment groups

	Alum group	Montanide group	All treated patients
Trial 1: EGF vaccine	1100 (100–8000)	3020 (100–32 000)	2691 (100–32 000)
Trial 2: CPM (200 mg/m^2) + EGF vaccine	2238 (100–160 000)	10 592 (4000–400 000)	5000 (100–400 000)

EGF, epidermal growth factor; CPM, cyclophosphamide.

Table 3. Percentage of seroconversion, GAR and PAR in different treatment groups

	Alum group (%)	Montanide ISA 51 group (%)	All treated patients (%)
Trial 1: EGF vaccine			
Seroconversion	78	100	90
GAR	22	73	50
PAR	78	27	50
Trial 2: CPM (200 mg/m^2) + EGF vaccine			
Seroconversion	90	100	95
GAR	30	70	50
PAR	70	30	50

EGF, epidermal growth factor; CPM, cyclophosphamide; GAR, good antibody responders; PAR, poor antibody responders.

cyclophosphamide was given before vaccination, as shown in Table 2. However, the observed differences were not statistically significant.

Percentages of GAR were significantly higher for montanide treatment groups in both trials (73% and 70%) compared with alum groups (22% and 30%), as shown in Table 3.

More than 90% of all vaccinated patients were seroconverted and GAR was achieved by 50% of all vaccinated patients. Cyclophosphamide pretreatment did not improve the percentage of seroconversion or the percentage of GAR.

Ninety-five percent of GAR sera inhibited the binding of EGF to its receptor. However, only 30% of PAR sera showed EGF/EGF-R binding inhibition capacity, and this difference was statistically significant.

Figure 1 shows the kinetics of anti-EGF antibody titers in two immunized patients. Re-immunizations were performed when antibody titers decreased. Even when re-immunizations provoked an increase in antibody titers, this was only up to the same maximal levels reached before and did not give permanent long-lasting responses. For maintaining antibody titers, maintained re-immunizations were required. The same behavior was observed in all re-immunized patients.

The duration of antibody response was evaluated. In the follow-up period, 45% of patients showed maintained antibody titers of at least twice their original value for ≥60 days, and 27.5% of patients showed maintained antibody titers of at least 1:2000 and 4× original levels for ≥60 days. The duration of antibody titers was not related to the kind of adjuvant or to cyclophosphamide pretreatment.

Anti-EGF antibodies were IgG. No specific IgM was detected. The antibody isotype was mainly IgG3 for all treatment groups.

No evidence of severe clinical toxicity was observed. Secondary reactions were mild or moderate, limited to 14 patients, seven of whom developed moderate (grade 2) adverse symptoms requiring standard medication. Those reactions consisted of chills, fever, vomiting, nausea, hypertension, cephalea, dizziness, flushing, pain at the site of injection, bone pain, mouth dryness or hot flashes that disappeared after medication. Hematological data and blood chemistry remained within the normal ranges during the immunization and follow-up period. No local cutaneous reactions at the site of injection were observed in any treated group.

During the 6-month evaluation, 12 patients (30%) showed clinical and radiological stable disease. Twelve months after the first vaccine dose, two patients (5%) continued with stable

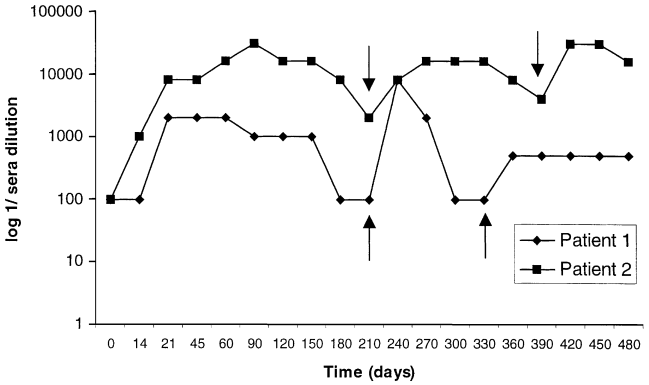

Figure 1. Kinetics of anti-EGF antibody responses in two vaccinated patients. Abscissa is the time after first immunization. Ordinate is antibody titer, log (1/sera dilution). Patients were re-immunized when antibody titers decreased; arrows indicate re-immunization dates.

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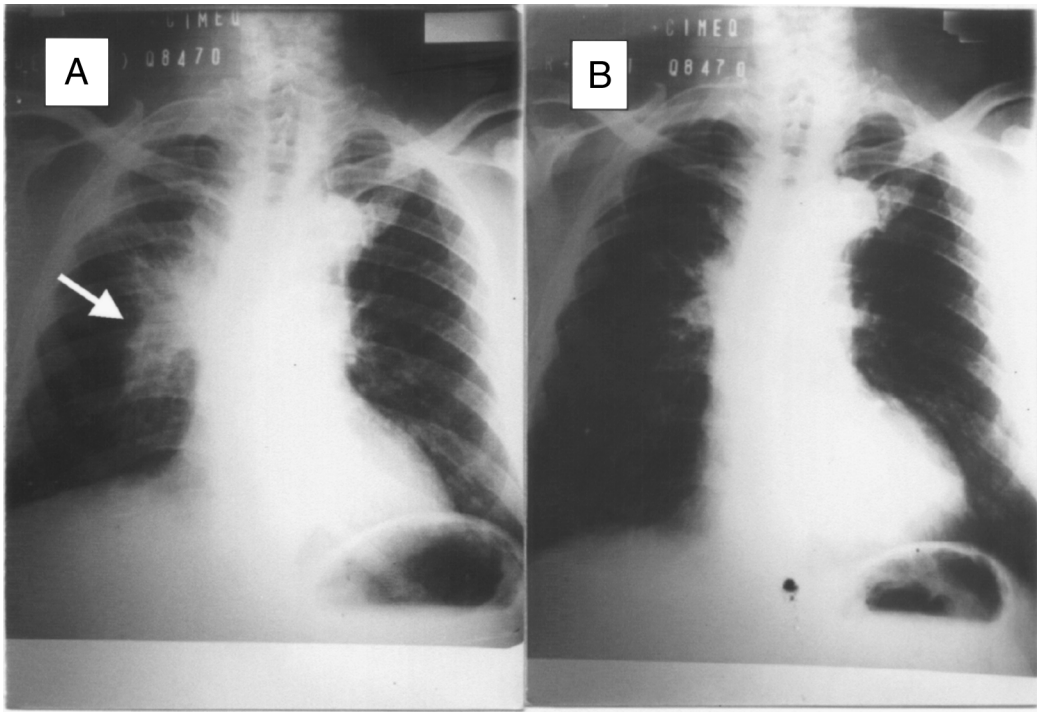

Figure 2. Chest X-ray of a stage IV non-small-cell lung cancer patient: (**A**) before EGF vaccination; (**B**) 12 months after EGF vaccination.

disease. Disease stabilization during the follow-up period was observed mainly in GAR. One of the patients who reached a maintained high anti-EGF antibody response showed a tumor regression 12 months after first vaccination, this was evaluated with radiology (Figure 2).

Survival times were calculated from patient randomization until death. Treated patients were divided into GAR ($n = 19$) and PAR ($n = 21$) for survival comparison between groups, as shown in Figure 3. The median survival in the pooled GAR group was 9.1 months (mean 12.41) and 4.5 months in the pooled PAR group (mean 5.47 months). The median survival of all vaccinated patients (GAR + PAR) was 8.17 months (mean 9.64).

Patients with ≥60 days response duration, either in 2× or 4×, 1:2000 antibody titer levels, showed a significant increase in survival times compared with the corresponding groups with response duration <60 days (Table 4). There was no relationship between survival and the type of adjuvant used (not shown).

Discussion

Our previous reports of preclinical studies demonstrated the immunogenicity and antitumoral activity of immunization with self-EGF in mice [13, 14]. Although EGF is not the only known ligand of EGF-R, these preclinical data showed that, at least in some tumor models, EGF-vaccination is enough to elicit an antitumor response. In the clinical setting, vaccination with two doses of human EGF coupled to a carrier protein and administered in an adequate adjuvant was immunogenic and safe in advanced cancer patients [15].

The two pilot clinical trials reported in this paper were performed to examine the safety and immunogenicity of vaccination with five doses of EGF coupled to P64K as the carrier protein. Two different adjuvants were compared and pretreatment with low-dose cyclophosphamide before vaccination was evaluated. Results from those trials demonstrated that when a more intense immunization protocol was used, either with alum or montanide ISA 51 as adjuvants, the EGF vaccination was again immunogenic and safe.

Our results show that montanide ISA 51, used as adjuvant for EGF vaccination, provoked a better antibody response than alum in terms of the percentage of GAR. This agrees with our previous preclinical results and, taken together with the absence of local toxicity of the adjuvant administered by intramuscular route, suggests the selection of this novel oil-based adjuvant.

Previous reports showed that cyclophosphamide pretreatment before vaccination increased antibody responses [21, 22], which was explained by its effect on suppressor T cells. In our study, no significant increase was observed in antibody titer levels, percentage of seroconversion or GAR when low-dose cyclophosphamide was administered before vaccination. Although a trend to increased antibody titers was observed, the high variability prevented statistical significance being achieved. New preclinical studies should be undertaken, changing the dose and time of cyclophosphamide administration, for an optimization of this pretreatment effect.

Sera from GAR showed higher EGF/EGF-R binding inhibition capacity than sera from PAR. This agrees with our working hypothesis of ligand-receptor binding inhibition through specific anti-ligand antibodies. It was also observed that re-immunization with EGF when antibody titers decreased did not provoke a characteristic booster effect (stronger and maintained antibody responses). Even when antibody titers increased after re-immunization, they

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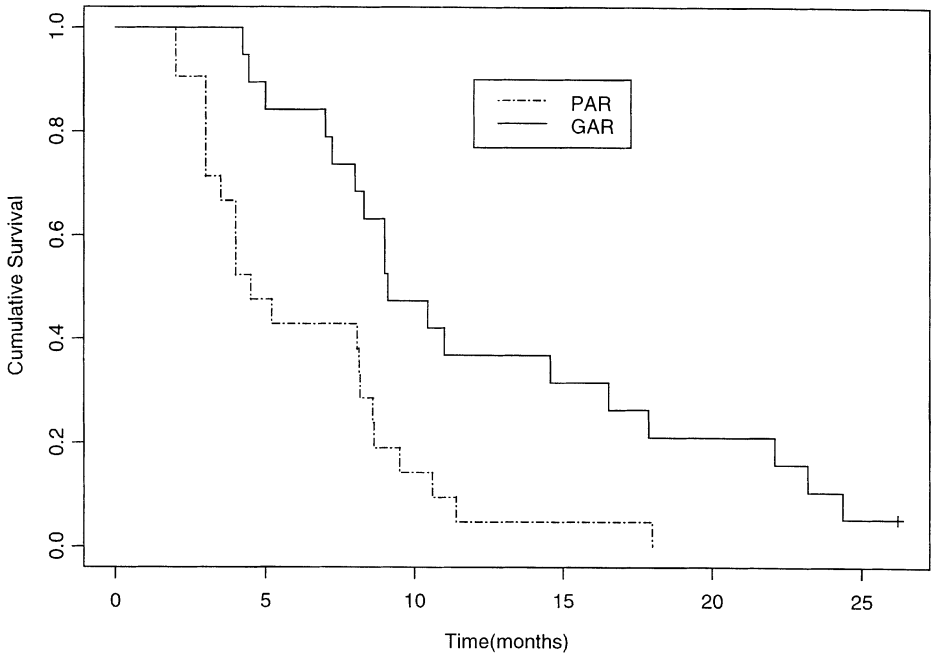

Figure 3. Survival functions for good antibody responders (GAR) and poor antibody responders (PAR). Median survival for GAR was 9.1 months (mean 12.41 months). Median survival for PAR was 4.5 months (mean 5.47 months). The difference in survival was statistically significant (P <0.05). Six-month survival was achieved by 84% of GAR and 38% of PAR. Twelve-month survival was achieved by 37% of GAR and 4% of PAR.

Table 4. Relationship between response duration and survival times

Group of patients	Response duration <60 days (months)	Response duration ≥60 days (months)	Statistical significance
Ab titers ≥2× original levels	Median SV: 4.23	Median SV: 10.43	P = 0.0001
	SD: 0.8	SD: 2.02	
Ab titers ≥4× original levels and at least 1:2000	Median SV: 8.07	Median SV: 10.43	P = 0.0469
	SD: 2.63	SD: 1.65	

Ab, antibody; SV, survival; SD, standard deviation.

only reached the same levels as previous maximal values, and decreased again in a short time. To maintain antibody titers, continuous re-immunizations were necessary. This fact could be related to the 'self' characteristic of the immunogen [23].

Because the EGF is a self growth factor, EGF deprivation could be expected to provoke toxicity. However, the lack of toxicity of EGF immunization in adult animals has been previously reported. EGF deprivation in rats provoked adverse effects on the fetus but not on normal adult tissues [24, 25]. Previous clinical results demonstrated no toxicity when cancer patients were treated with a less intensive immunization protocol [15]. Our present results show that there is no evidence of severe toxicity, even using a five dose immunization schedule with re-immunization when antibody titers decreased.

All of these results suggest that this growth factor plays a key role in fetal development and tumor growth, but not in normal adult tissue physiology. If this is the case, EGF blockade through inducing anti-EGF antibodies could affect mainly tumor development, without undesirable side-effects.

Although the evaluation of antitumor activity was not the main goal of these trials and no objective remissions were expected with any procedure at such advanced stages, a tumor regression was documented in one treated patient. This is a single case which should be examined carefully, but it should be noted that this was one of the patients who developed higher and maintained anti-EGF antibody titers after vaccination.

Additionally, better survival times were observed in the GAR compared with PAR, although this relation between anti-EGF antibody titers and survival only demonstrates correlation between these parameters and not causality. Previously, we reported a direct relationship between anti-EGF antibody levels and survival in tumor challenged mice [14]. Our results indicate that a similar association occurs in humans.

There was a significant increase in survival for patients with maintained antibody responses. Inside the GAR patient subgroup, the duration of the antibody titers showed an additional correlation with survival.

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These results are consistent with our hypothesis that anti-EGF antibodies block the binding between EGF and its receptor, slowing down tumor cell proliferation. However, confirmation of this EGF vaccination effect on survival requires a randomized phase II trial, in which vaccinated patients could be compared with a concurrent best supportive care treatment arm. This trial is already ongoing.

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