A pilot study of monoclonal antibody cG250 and low dose subcutaneous IL-2 in patients with advanced renal cell carcinoma

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The chimeric monoclonal antibody cG250 recognizes the CAIX/MN antigen. cG250 induces antibody-dependent cellular cytotoxicity (ADCC) responses in vitro that can be enhanced by IL-2. We studied the effects of adding daily low-dose subcutaneous IL-2 to cG250 for treatment of clear cell renal cell carcinoma (RCC). The primary endpoints of the trial were toxicity and immunological effects (human anti-chimeric antibodies [HACA], ADCC, natural killer [NK] and lymphokine-activated killer cell [LAK] activity); secondary endpoints were cG250 biodistribution and pharmacokinetics (PK) and tumour response rates. Eligible patients had unresectable metastatic or locally advanced clear cell RCC with measurable or evaluable disease. Nine patients were treated with six doses of cG250 (10 mg/m²/week, first and fifth doses trace-labelled with 131I), and 1.25 x 10⁹ IU/m²/day IL-2 for six weeks. Treatment was generally well tolerated with no adverse events attributable to cG250. Two patients required a 50% dose reduction of IL-2 due to toxicity. No HACA was detected. 131I-labeled cG250 showed excellent targeting of tumour deposits. 131I cG250 PK: T½α 20.16 ± 6.59 h, T½β 126.21 ± 34.04 h, CL 39.67 ± 23.06 mL/h, Cmax 5.12 ± 0.86 µg/mL, V₁ 3.88 ± 1.05 L. IL-2 did not affect cG250 PK. A trend for increased percentage of circulating CD3+/CD16+/CD56+ NK cells was observed. Some patients showed enhanced ADCC or LAK activity. No antitumour responses were observed. In conclusion, weekly cG250 with daily low-dose subcutaneous IL-2 is well tolerated. IL-2 does not influence cG250 biodistribution or increase HACA.

Keywords: clinical trial, renal cell carcinoma, human CA9 protein, cG250, chimeric antibody, IL-2

Introduction

In 2007, over 51,000 people in the USA are expected to develop renal cell carcinoma (RCC) or cancer of the renal pelvis, with more than 12,000 deaths (1). Patients with localized disease have 5-year survival rates of more than 90% (2); patients with metastatic disease have 5-year survival rates of around 30% (2) and the median survival is 10 months (3). RCC is therefore curable only if it is resectable.

To date chemotherapy has not demonstrated sufficient anti-tumour activity to prolong the survival of patients with metastatic RCC (4). Single agent or multiple agent chemotherapy has response rates less than 15-20%. Recent data indicate that inhibition of receptor tyrosine kinases (5, 6) or of mTOR leads to clinical benefit (7); however, these options are not available or suitable for many patients with RCC. The combination of these less than satisfactory responses to chemotherapy and surgery, together with indirect evidence that host immune mechanisms play a significant role in the natural history of RCC, supports continued exploration of the role of immunotherapy in this disease.

A recent Cochrane review concluded that interferon-α (IFN-α) provided a modest survival advantage in comparison to other therapies with an odds ratio for death at one year of 0.56 (95% confidence intervals 0.40 to 0.77) (8). In a recent phase III study, the response rate to high-dose interleukin-2 (IL-2) was 23.2% (22/95 patients), which was superior to low dose IL-2 plus interferon-α2b (9.9%, 9/91 patients) (9). Some long term remissions were described. In another meta-analysis involving 670 patients in 24 trials, patients receiving any cytokine therapy were compared against chemotherapy (10). Cytokine therapy was associated with an improved median survival for all levels of risk, with the benefit mainly seen in favourable and intermediate risk groups. However, only 4.5% of patients survived more than 5 years, although some long term survivors were rendered disease-free with surgery. These data suggest that cytokine-based immunotherapy may still have a role for carefully selected patients.

The G250 antigen (CAIX / MN) is a heat-sensitive transmembrane cell surface antigen homologous to carbonic anhydrase IX (11). G250 is present on more than 85% of RCCs, almost exclusively in the clear cell subtype, but expression in normal tissues is restricted to the gastric epithelium, biliary ducts, and some pancreatic acini (12, 13). Expression of this antigen is most common in clear cell RCC (14). Low CAIX staining is correlated with a worse prognosis (14-16). After treatment with high dose IL-2, survival of RCC patients is prolonged in the group in which G250/CAIX expression is observed, and prolonged survival beyond five years is only seen in the context of G250/CAIX expression (17). This correlation is independent of tumour grade and stage (17).

The murine monoclonal IgG1 antibody G250 recognizes the G250 antigen and has been used in clinical trials (13, 18, 19). The development of human anti-mouse antibody (HAMA)
cG250 induces ADCC in vitro against G250 positive RCC lines (20), which is enhanced significantly by IL-2 at doses achievable in vivo (21). When IL-2 was added to the culture in concentrations above 10 IU/mL, significant enhancement of ADCC occurred and was maintained for seven days, with associated lymphokine-activated killer (LAK) cell activity observed after three days. Activity was seen at concentrations as low as 1 IU/mL (21).

cG250 has been studied in clinical trials as a single agent, either as cold antibody or labelled with various radioisotopes (19, 22-28). Treatment was well tolerated and human anti-chimeric antibody (HACA) responses were infrequent. cG250 targets clear cell RCC efficiently (22) and does not bind significantly to biliary tract cancers (29). In terms of clinical outcomes, some patients had stable disease and two partial remissions and one complete remission have been reported (23, 27). A phase I multiple dose study has been completed at our site in conjunction with the Mayo clinic (trial LUD 98-011) (30, 31). This trial showed that cG250 is safe, has a long half-life, targets RCC effectively and has some biologic activity (30).

The combination of cG250 with IL-2 has been previously studied. In a phase II trial of 35 patients with progressive RCC, a partial response was demonstrated at week 16 in one patient and 11 patients had stable disease. Clinical benefit, defined as the sum of patients with partial or complete responses and patients with stable disease of at least 24 weeks, was observed in 7 out of 30 evaluable patients (23%). The median survival was 22 months and the 2-year survival 45% (27). The effects of IL-2 on the biodistribution, tumour uptake or pharmacokinetics of cG250 were not assessed in this trial.

In another trial reported to date only in abstract form (32) and recently updated (27), 31 patients with metastatic RCC were treated with cG250 (20 mg weekly for three months) combined with interferon-α2a (3 MIU three times per week subcutaneously). Of the 31 patients, no grade 3 or 4 toxicities or HACA responses were observed. At week 16, 2/26 (8%) evaluable patients had a partial response and 14/26 (54%) had stable disease. Clinical benefit was observed in 11/26 patients (42%) (32). In this study the median survival was 30 months and the 2-year survival 57%.

We hypothesized that a combination of cG250 and low dose IL-2 would be safe, that IL-2 would have no effect on the pharmacokinetics or biodistribution of cG250, and that immunological effects of the combination would be observed.

Results

Patients

Patient characteristics are shown in Table 1. Nine patients (seven males, two females) participated and all were evaluable. The median age was 55 (range 39-71). Four patients had prior immunotherapy; no patient had prior chemotherapy. Three patients had not had prior nephrectomy. For those who had undergone nephrectomy, this had occurred between six weeks to four years prior to the first infusion of cG250. Median Karnofsky performance status was 90% (range 80-100%).

In a retrospective analysis, the following prognostic indicators were determined for each patient where available: presence or absence of prior nephrectomy; time since diagnosis or nephrectomy; baseline haemoglobin; baseline lactate dehydrogenase (LDH). No correlations of these parameters with study outcome were found. The two patients who received an additional cycle of treatment did not have more favourable prognostic indicators than other patients.

Toxicity

Toxicities are summarized in Table 2. All patients completed the study. Weekly cG250 with daily low-dose subcutaneous IL-2 was well tolerated and most adverse events were attributable to the underlying cancer. No adverse events were thought related to cG250. Adverse events attributable to IL-2 were as expected. IL-2 toxicities were mild to moderate in severity: one patient (patient 3) required two dose reductions over two cycles due to IL-2-related dyspnea. This patient successfully completed the
Table 2
Summary of toxicities related to IL-2.

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>N</th>
<th>Grade 1-2</th>
<th>Grade 3-4</th>
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<tbody>
<tr>
<td>Fatigue</td>
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<tr>
<td>Injection site reaction</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Chills</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Sweating</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Flu-like symptoms</td>
<td>3</td>
<td>3</td>
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<td>Anorexia</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cough</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Peripheral edema</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Anxiety/agitation</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Depression</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Light-headedness</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pleural effusion increased</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>45</td>
<td>3</td>
</tr>
</tbody>
</table>

* Toxicities at least possibly related to IL-2 are listed in descending order of frequency. No cG250 related toxicities were observed.

second cycle of treatment after the dose reduction. Two serious adverse events (SAEs) occurred, both in patient 4 (hospitalization for bony metastatic disease); both events were unrelated to the study drugs. This patient was found to have previously undiagnosed metastatic disease in the right femur, which was discovered on gamma camera imaging after the first infusion of $^{131}$I-cG250 (Figure 1). The patient was admitted for prophylactic orthopedic fixation. The second hospitalization for this patient was for cancer-related back pain occurring one week after the final cG250 infusion.

Haemoglobin fell slightly in all patients but this was not clinically significant. Mild eosinophilia was observed as expected for IL-2. No significant changes in patient blood chemistry were observed. Thyrotropin (TSH) fell to below normal in 3/9 patients, but there was no clinical or other biochemical evidence to suggest IL-2-induced hyperthyroidism. Subsequent assessment of thyroid function in these patients after the conclusion of the study showed normal TSH levels in two patients; no follow-up sample was available for the remaining patient.

Figure 1

**Biodistribution of $^{131}$I-cG250 in patient 4.** (A) Anterior and (B) posterior whole body gamma camera image eight days after first infusion. Tumour uptake of $^{131}$I-cG250 (arrows) is seen in the right kidney (primary) and in the left scapula and L2 vertebra, as well as a previously undiagnosed right femur metastasis. (C) Single photon emission computed tomography (SPECT) 3D anterior coronal image of the abdomen and chest, showing uptake in tumour (arrows) in the right kidney, right upper rib and left scapula. (D) CT scan showing right kidney tumour (arrow). (E) Anteroposterior X-ray image of right femur showing lytic metastasis. (F) Whole body bone scan (posterior view) showing the previously undiagnosed right femur metastasis.
Biodistribution of cG250

No differences in biodistribution and tumour uptake between the first and final $^{131}$I-cG250 infusion gamma camera images were observed in any patient. Targeting of tumour was excellent (Figure 1). The initial pattern of $^{131}$I-cG250 biodistribution in all patients was consistent with blood pool activity that cleared gradually with time (data not shown). Minor activity in the gut was also observed, similar to that seen in past cG250 protocols. Some visualization of thyroid and stomach was observed, related to dehalogenation of $^{131}$I-cG250 in vivo (Figure 1). Distinct uptake of $^{131}$I-cG250 in sites of primary and/or metastatic RCC where lesions were larger than 1.5 cm was evident in all patients, often as early as day 2 post-infusion. One occult metastatic lesion in the left femur of patient 4 was also detected (Figure 1). No other uptake of $^{131}$I-cG250 was evident in normal tissues.

Pharmacokinetics of cG250

Summarized data for infusion 1 are presented in Table 3. By measurement of blood $^{131}$I radioactivity, the initial ($T\frac{1}{2\alpha}$) and terminal ($T\frac{1}{2\beta}$) half-lives of $^{131}$I-cG250 were 20.16 ± 6.59 h and 126.21 ± 34.04 h respectively (mean ± standard deviation). There was no significant difference in pharmacokinetic parameters of $^{131}$I-cG250 between infusions 1 and 5 (data not shown). Cmax and Cmin values measured by ELISA showed increasing levels with consecutive infusions. The Cmin value for infusion 1 as determined by ELISA was 1.41 ± 0.75 µg/mL. This indicates that the 10 mg/m² dose of cG250 produced serum values greater than 1 µg/mL in most patients at one week post-infusion of cG250, a concentration that reproducibly produces in vitro ADCC of G250 target cells (21).

**Table 3**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$^{131}$I-cG250</th>
<th>Serum cG250 ELISA</th>
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<tr>
<td>Mean</td>
<td>SD</td>
<td>Fitted value</td>
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<tr>
<td>$T\frac{1}{2\alpha}$ (h)</td>
<td>20.16</td>
<td>6.59</td>
</tr>
<tr>
<td>$T\frac{1}{2\alpha}$ (h)</td>
<td>126.21</td>
<td>34.04</td>
</tr>
<tr>
<td>AUC (µg/mL)</td>
<td>568.39</td>
<td>180.49</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>5.12</td>
<td>0.86</td>
</tr>
<tr>
<td>Cmin (µg/mL)</td>
<td>3880</td>
<td>1050</td>
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</table>

Abbreviations: AUC, area under the concentration curve extrapolated to infinite time; Cmax, maximum serum concentration; CL, total serum clearance; SD, standard deviation; $T\frac{1}{2\alpha}$ and $T\frac{1}{2}$, half-lives of the initial and termination phases of disposition, respectively; V., volume of central compartment.

Immunological assays

FACS analysis demonstrated a trend for an increase in the percentage of circulating CD3-/CD16+CD56+ NK cells during the study (Table 4). Small increases or decreases in total white blood cell counts and total lymphocyte counts, as well as CD3+ and CD4+ and CD3+/CD8+ lymphocyte subsets, were observed in all patients during the study (data not shown).

The level of ADCC at the start of the study was patient dependent and did not correlate with clinical outcome. Specific ADCC of 5-15% was observed in 3/9 patients (patients 1, 5 and 8) and a low specific ADCC of <5% was observed in the other 6/9 patients.

The observed level of ADCC and NK cell (LAK) activity varied greatly between individual patients and no consistent pattern was observed. Patient 3 demonstrated increasing ADCC activity throughout the study (Figure 2); however, no change to cG250 specific ADCC was observed for 7/9 patients on study, and patient 1 demonstrated a drop in activity over the course of treatment.

A marked increase in NK cell (LAK) activity was observed for patients 1, 2, 3 and 5 (Figure 2) while patients 4 and 8 demonstrated increased NK cell activity at week 3. The increased LAK activity correlated with increased NK cell (LAK) numbers for patients 1 and 5. In contrast, patients 2 and 3 demonstrated a decrease in NK cell numbers over the 2 cycles of therapy (Table 4). No change was observed in the NK cell (LAK) activity of the other patients, despite increased NK cell numbers being observed for these patients over the study period.

**Table 4**

Effects of IL-2 on CD3-/CD16+CD56+ NK cells during study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cycle</th>
<th>Week</th>
<th>% CD3+/CD16+/CD56+ NK Cells</th>
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<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6.47</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>11.50</td>
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<td>3</td>
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<td>20.20</td>
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<td>4</td>
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<td>1</td>
<td>33.30</td>
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<td>5</td>
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<td>1</td>
<td>30.80</td>
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<td>6</td>
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</tr>
<tr>
<td>9</td>
<td>1</td>
<td>1</td>
<td>24.80</td>
</tr>
</tbody>
</table>

**% Change Over Study**

| 1     | 1.93 |
| 2     | -5.95|
| 3     | -11.60|
| 4     | 16.70|
| 5     | 6.50 |
| 6     | 18.20|
| 7     | 13.40|
| 8     | 1.10 |
| 9     | 6.60 |

**% Change (Mean ± SD)**

| 7.11 ± 10.92 |

Abbreviations: ND, not determined.

Human anti-chimeric antibody (HACA) assay

All sera tested were HACA negative. High background interference with the ELISA analysis was apparent in all samples from patient 4. However, a positive immune response was not observed and clinical manifestations of HACA were absent.

Clinical outcomes

No antitumour effects were observed. Seven patients had progressive disease at the end of cycle 1. At completion of cycle 1, two patients (patients 2 and 3) had stable disease. These patients were offered and successfully completed a second cycle of treatment. At restaging at the end of that cycle, one had continued stable disease and one had progressive disease. Neither patient was eligible for further treatment.

Statistics

The primary objectives of this pilot study were to assess the safety and immunogenicity of cG250 and IL-2 in patients with advanced renal cell carcinoma. Nine patients were planned to be accrued. For the safety component, if two or fewer of the planned nine patients experienced a dose-limiting toxicity, the study had approximately 90% power to show that the probability of dose-limiting toxicity was less than 0.49. With respect to immunological efficacy, the error rates of missing a promising agent or selecting a non-promising agent were set to be at most 10% and were computed employing an empirical Bayes formulation, using a large set of exploratory vaccine trials.
Figure 2

ADCC and/or NK cell (LAK) activity of PBMCs isolated from patients 1 and 3. Week 1 (filled circles), week 3 (open circles) and week 7 (filled triangles). (A) LAK activity of patient 1, showing increasing LAK activity during the study. (B) ADCC activity of patient 3 (cycle 2 data). An increase in ADCC activity (E:T ratio 25:1) at week 3 was observed, which decreased to baseline level by week 7.

performed at Memorial Sloan-Kettering Cancer Center (33). Clinical responses and pharmacokinetic parameters were not primary endpoints and were to be reported descriptively.

Discussion

This trial confirms that treatment with weekly infusions of cG250 for six doses is safe. The only significant adverse events during the study were due either to the underlying disease or to concurrent IL-2, with no adverse events attributable to cG250. However, the addition of low-dose IL-2 to weekly cG250 was generally well tolerated.

The half-life of cG250 was found to be long, and blood trough levels of cG250 were easily detectable at the time of the subsequent dose. The calculated pharmacokinetic results (Table 3) showed no significant difference to the values determined for our previous study LUD98-011 (31). These values are longer that those observed for murine G250 (13, 18) and are highly comparable with values observed for other chimeric antibodies. Results obtained for clearance, volume of distribution and Cmax were also comparable to the reported human cG250 trials. It can be concluded that IL-2 as used in this study did not affect cG250 pharmacokinetics.

Targeting of cG250 to tumour was excellent. In at least one patient, a clinically significant but previously undiagnosed metastasis was detected by gamma camera imaging following the first infusion of $^{131}$I-cG250. Most sites of disease, particularly if greater than 1.5 cm in size, were easily detected by scanning after $^{131}$I-cG250 infusion. The pattern of biodistribution of $^{131}$I-cG250 was similar to our previous cG250 protocol LUD 98-011 (31) and an earlier cG250 study (17) and was consistent in all nine patients.

IL-2 did not increase the incidence of HACA; indeed, HACA was not detected during the study. Variable effects were observed with respect to white blood cell count, lymphocyte count, and lymphocyte subset (CD3+/CD4+ and CD3+/CD8+) analysis. A trend for increase in CD3-/CD16+CD56+ NK cell numbers was also observed. The increase in CD3-/CD16+CD56+ NK cell numbers in this study due to IL-2 treatment is consistent with previous reported trials (34, 35). Some patients showed enhanced ADCC or NK cell (LAK) activity, but this was not a consistent finding. Prior trials have also shown only modest increases in ADCC and NK cell (LAK) activity following cytokine treatment in cancer patients (34); this is most likely explained by the suppression of immune responses in patients with advanced or metastatic cancer.

Overall, the results from this study are comparable to those from our previous phase I study using cG250 alone (31). In that study, cG250 was well tolerated. Clinical effects were observed in that trial but not in the current trial; this discrepancy may be due to the small number of patients treated.

Another trial involved 35 patients with progressive clear cell RCC after nephrectomy (36). Patients received cG250 20 mg weekly (week 2 to 12) combined with daily low dose IL-2 (1.8 MIU daily), with a pulse of 5.4 MIU on days 1 and 3 of weeks 3, 5, 7, 9 and 11 for 12 weeks. In week 16, a partial response was demonstrated in one patient and stable disease in 11 patients. Seventeen patients with clinical benefit at week 16 received further treatment. The percentage of peripheral blood NK cells increased but lytic activity remained stable. The combination therapy was safe and well tolerated, although HACA was observed in 2 of 28 evaluable patients without apparent clinical sequelae. In agreement with our observations, an increase in NK cell populations and ADCC / NK cell activity following IL-2 treatment with cG250 were also reported. This trial, however, did not evaluate the biodistribution of cG250 following IL-2 treatment, nor the impact of IL-2 on cG250 pharmacokinetics, as was performed in our study.

Monoclonal antibody cG250 treatment has a potential role in the management of metastatic RCC; however, particular patient subgroups that may benefit from this treatment need to be delineated. Other investigators have used combinations of cG250 with cytotoxic drugs such as vinblastine (37). A large international phase III trial is underway to study the effects of
adjuvant therapy with cG250 after resection of RCC (38). Future work will address other methods for enhancing the anti-tumour effect of cG250/CAIX-targeted therapies, such as radioimmunotherapy approaches with 131I-cG250 (39, 40), 90Y-cG250 and 177Lu-cG250, or combining cG250 with other therapeutics that have activity in RCC, such as sunitinib.

Abbreviations
RCC, renal cell carcinoma

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We gratefully acknowledge the staff of the Austin Hospital Department of Nuclear Medicine and of the Cancer Clinical Trials Centre. This study was sponsored by the Ludwig Institute for Cancer Research. Part of this study has been presented at the 2004 annual meeting of the American Society of Clinical Oncology (ASCO) in New Orleans, LA.

References


Materials and methods

Trial design

Trial LUD00-010 was an open-label pilot study to investigate the effects of combining weekly infusions of cG250 with daily subcutaneous injections of low-dose IL-2 (Proleukin, Chiron). The primary objectives were (a) safety and (b) immunological effects of the combination. Safety was assessed by toxicity as defined by the National Cancer Institute Common Toxicity Criteria (CTC) Scale Version 2.0 (41). Immunological endpoints were serum HACA activity, peripheral blood natural killer cell (NK) phenotype as determined by flow cytometry, and peripheral blood ADCC activity. Secondary objectives of the study were to determine the blood pharmacokinetics, organ distribution and tumour distribution of cG250 given weekly in combination with daily low dose IL-2, and to evaluate the clinical efficacy of the combination.

Inclusion criteria were as follows: unresectable locally advanced or metastatic, histologically proven, clear cell carcinoma of the kidney; progressive disease prior to study entry, defined as an increase in the size of disease on two clinical or radiological assessments at least one month apart; measurable or evaluable disease; at least four weeks after chemotherapy, radiotherapy or immunotherapy; no other effective therapy or evaluable disease; at least four weeks after chemotherapy, radiotherapy or immunotherapy; no other effective therapy available or appropriate; full recovery from surgery; expected survival of at least three months; Karnofsky performance status of at least 70%; adequate bone marrow, renal and hepatic function; age at least 18 and able to give written informed consent. Exclusion criteria: prior exposure to monoclonal antibody unless there was no evidence of circulating human anti-mouse, anti-chimeric, or anti-human antibody; active central nervous system metastases (unless adequately treated and no evidence of progression for at least three months); positive HIV test; other clinically significant medical illness; other coexisting or previous malignancy within five years (except treated cervical carcinoma in situ or treated non-melanoma skin cancer); concomitant treatment with systemic glucocorticoids, anti-histaminic or non-steroidal anti-inflammatory drugs (unless used in low doses for prevention of an acute cardiovascular event); participation in a clinical trial involving another investigational agent within 4 weeks; pregnancy or lactation; women of childbearing potential not using medically acceptable means of contraception; psychiatric or addictive disorders that may compromise the ability to give informed consent; lack of availability of a patient for immunological and clinical follow-up assessment. All patients provided written informed consent prior to any study-specific procedures. The trial protocol was approved by the Human Research Ethics Committee of Austin Hospital and was performed under the Australian Therapeutic Goods Administration Clinical Trials Notification (CTN) scheme and FDA IND BB-8023. All patients were treated at the Austin Hospital in Heidelberg, Melbourne, Australia.

The treatment regimen was as follows. A total of 6 doses of cG250 (Biological Production Facility, Ludwig Institute for Cancer Research, Melbourne, Australia) were given at 10 mg/m²/dose, infused over 30 minutes once weekly; the first and fifth weekly doses were trace-radiolabeled with 8-10 mCi (296-370 MBq) iodine-131 (¹³¹I) to facilitate the evaluation of cG250 biodistribution and pharmacokinetics. IL-2 (1.25 x 10⁶ IU/m²/day) was administered by subcutaneous injection, commencing on the day of the first cG250 infusion. Patients received potassium iodide solution, 10 drops orally twice a day for seven days, beginning on the morning of the ¹³¹I-cG250 infusions (day 1 and 29). Dose modifications of cG250 (reduction in infusion rate or cessation of infusion) were permitted for adverse events occurring during the infusion. If dose-limiting toxicity attributable to IL-2 occurred, IL-2 treatment was to be withheld until the toxicity had resolved to grade 2 or less. Subsequent doses were to be reduced by 50%, with a further 50% reduction (to 25% of the initial dose) permitted if necessary. If further dose reductions were required then the patient was to be removed from the study.

Patients with stable or responding disease were permitted to receive a second six-week cycle of treatment, to commence between four and eight weeks after completion of the first cycle. Only patients with responding disease and with no evidence of HACA were eligible to receive a third or subsequent treatment. Patients who demonstrated an increased clearance of ¹³¹I-cG250 during week 5 of cycle 1 were not eligible to receive further treatment cycles. Increased clearance was defined as more than 50% decrease in the half-life of whole body clearance of ¹³¹I-cG250, in conjunction with reduced tumour uptake compared to that observed for week 1 imaging.

Blood samples were collected before treatment and at various time points during the study as described below. Tumour evaluations were performed prior to treatment and two weeks after the sixth dose.

¹³¹I-cG250 biodistribution

Infusions of cG250 trace-labelled with 8-10 mCi (296-370 MBq) of ¹³¹I were administered in weeks 1 and 5 for determination of tumour targeting, normal organ biodistribution, and pharmacokinetics. Gamma camera imaging with anterior and posterior whole body scans using conjugate view methodology were performed on five occasions after each trace-labelled infusion: 1-4 hours after ¹³¹I-cG250 infusion (day 1) and on day 2, days 3 or 4, 5 or 6 and 7 or 8. A standard was included in the field of view at each imaging time point. Single-photon emission computed tomographic (SPECT) imaging of relevant areas of disease were performed on at least one occasion following ¹³¹I-cG250 infusion.

Pharmacokinetic studies

Pharmacokinetics of cG250 were determined by measurement of radioactivity in blood samples after ¹³¹I-cG250 infusion (weeks 1 and 5), and by measurement of cG250 protein in blood by ELISA. Blood samples for these assays were drawn in weeks 1 and 5 on the day of infusion (immediately prior to the infusion, 10 minutes, 1 hour, and 4 hours after completion of the infusion), day 2, days 3 or 4, 5 or 6 and 7 or 8, and were stored at -70°C to -80°C for later ELISA analysis. Blood samples (10 mL) were collected for cG250 trough and peak serum levels immediately prior to each cG250 infusion and one hour after the completion of the infusion at weeks 2, 3, 4 and 6. Serum obtained after infusion of ¹³¹I-cG250 was counted in a gamma counter (Packard Instruments, Canberra, Australia). Duplicate standards prepared from the injected material were counted at each time point with serum samples to enable calculations to be corrected for the isotope's physical decay. The results were expressed as percent injected dose per liter serum.
(%ID/L) and converted to µg/mL for pharmacokinetic calculations.

Chimeric G250 antibody levels in serum samples, standards or relevant control antibody samples were assessed by a sandwich ELISA. Briefly, wells of microtiter plates were coated with anti-G250 idiotype (NUH-82) monoclonal antibody (42). Biotinylated NUH-82 was used as the tracer antibody. Biotinylation of NUH-82 was performed according to the manufacturer’s instructions (Pierce, Australia). The range of measurement was 3.15 ng/mL to 10.0 µg/mL after half-log serial dilutions of serum. All samples were assayed in triplicate and were diluted by a factor of at least 1:2.

Pharmacokinetic parameters were estimated from individual patient 131I and pooled ELISA serum clearance data using a curve fitting program (WinNonlin, Pharsight Co., Mountain View, CA). A two-compartment model with macro-parameters, no lag and a bolus i.v. was used to calculate pharmacokinetic parameters of Cmax (maximum serum concentration); AUC (area under the serum concentration curve extrapolated to infinite time); CL (total serum clearance); T½½ and T½β (half lives of the initial and terminal phases of disposition); and V1 (volume of central compartment).

Immunological assays

Flow cytometry

Flow cytometry was performed on peripheral blood mononuclear cells (PBMCs) to determine lymphocyte numbers, T cell subsets, NK numbers and phenotype. The following markers were examined in the following combinations: CD3+/CD4+ (CD4+ T cells), CD3+/CD8+ (CD8+ T cells), CD3-/CD16+/CD56+ (NK cells), CD20+ (B cells), and CD14+ (monocytes). Blood samples (10 mL) for flow cytometry were collected prior to the infusion on day 1, prior to the third infusion, and at day 43 after completion of the treatment cycle. Direct immunofluorescence was performed on the day of sample collection using standard techniques on a Coulter Epics Elite ESP flow cytometer (Coulter Corporation, Miami, Florida, USA). Lymphocyte population subsets in the peripheral blood of patients were expressed as the percentage of lymphocytes and total number in 1 mL blood. CD14+ macrophages were expressed as a percentage of all white blood cells and the total number in 1 mL blood. Total number of each subclass of lymphocytes from 1 mL of fresh blood was calculated according to the FACS and blood count data.

ADCC and NK activity

Patient blood samples were collected before treatment (week 1) and at weeks 3 and 7 for assessment of immune function and white blood cell profiles. Freshly isolated PBMCs were tested in triplicate with G250-positive SK-RC-52 target cells at an effector:target (E:T) ratio range of 50:1 to 0.39:1 in the presence of a predetermined optimal concentration of cG250 (1 µg/mL) (21) or IgG1 isotype control (huA33; Ludwig Institute for Cancer Research, Melbourne, Australia) to evaluate ADCC (24). To evaluate the nonspecific cellular lytic activity of patient PBMCs, K562 cells were used as targets at E:T ratios of 50:1 to 0.39:1, in the absence of cG250 antibody. After incubation at 37°C for four hours, cells were pelleted by centrifugation and 50 µL of supernatant harvested for quantification of 51Cr release. Controls included in the assay corrected for spontaneous release (medium alone) and total release (10% Tween20/PBS). The percentage cell lysis (cytotoxicity) was calculated according to the following formula: Percentage cytotoxicity = [(sample counts - spontaneous release) x 100] / (total release - spontaneous release). Specific cytotoxicity mediated by cG250 was determined by subtracting any activity observed with isotype control antibody at each of the E:T ratios examined. Cytotoxicity assessments were performed in triplicate and the mean values reported with standard deviations. The data were expressed as specific cG250 cytotoxic activity (ADCC) on target SK-RC-52 cells, and NK cell (or LAK) activity on target K562 cells at different E:T ratios.

Human anti-chimeric antibody assays

Patient serum samples for HACA assessment were collected prior to each infusion, at day 43 and, where possible, three months after patients completed treatment. The assay of anti-cG250 antibodies in human serum was performed by ELISA based upon previously published methods (22). Serum samples (450 µL) were diluted 1:2 and then serially diluted for analysis in duplicate. The cG250 anti-idiotype monoclonal antibody NUH-82 was included as a positive control. The limit of quantitation (in samples at minimum dilution) was 16 ng/mL for the NUH-82 anti-idiotype Ab and 150 ng/mL for anti-IgG Abs. Samples with values greater than the anti-idiotype NUH-82 limit of quantitation (16 ng/mL) were considered HACA positive. Results were reported as ‘negative’ (<16 ng/mL) or ‘positive’ (with the titer of anti-cG250 antibodies provided in ng/mL).

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