Superantigen-targeted Therapy: Phase I Escalating Repeat Dose Trial of the Fusion Protein PNU-214565 in Patients with Advanced Gastrointestinal Malignancies

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ABSTRACT

Antibody-directed, superantigen-induced cytotoxicity has been shown to have potent in vitro and in vivo antitumor effects in preclinical models. In the present study, PNU-214565, a recombinant fusion protein consisting of the Fab of the monoclonal antibody C242 and staphylococcal enterotoxin A (SEA), was used in an escalating repeat dose Phase 1 clinical trial in patients with advanced gastrointestinal malignancies. A prior single-dose Phase I clinical trial had demonstrated safety at doses of 1.5 ng/kg with toxicities of fever and hypotension that were not dose related. Twenty-seven patients (age range, 36–75 years; median, 62; 14 males and 13 females; 23 colorectal and 4 pancreatic) were treated in the present study with one cycle of four consecutive daily 3-h infusions of PNU-214565 at doses of 0.15 ng/kg (n = 3); 0.5 ng/kg (n = 3), 1.5 ng/kg (n = 4), 2.75 ng/kg (n = 12), and 3.5 ng/kg (n = 5). All patients had a good performance status (Eastern Cooperative Oncology Group: PS = 0 (n = 15), PS = 1 (n = 12)). As in the single-dose trial, fever and hypotension were the most common toxicities. Dose-limiting toxicity (DLT), consisting of transient hypotension responsive to dopamine, was experienced by one patient treated at the 2.75 ng/kg dose level. One patient with pancreatic cancer metastatic to the liver experienced a partial response of hepatic metastases with stable pancreatic head abnormalities by computed tomography scan. Further dose escalation was suspended when two patients treated in a companion repeat dose Phase I study experienced DLT at the 4 ng/kg dose level. Multiparameter analyses on all patients treated in the two companion single-dose and two-repeated-dose Phase I trials revealed that the levels of patients' pretreatment anti-SEA antibodies protected against toxicity at a given drug dose. By jointly considering weight and the baseline anti-SEA concentration in a patient, it is possible to assign a PNU-214565 dose that will induce systemic cytokine release (a surrogate test to assess for the presence of uncomplexed drug and its ability to induce systemic cellular activation) without DLT. This pharmacodynamically based dosing scheme will be tested in future repeated-dose clinical trials and will define maximally tolerated doses of this powerful new immunotherapy approach.

INTRODUCTION

Biological response-modifying agents designed to augment immune responsiveness have been the standard in preventative medicine since the turn of the century in the form of vaccines to enhance adaptive immune responses to infectious agents. More recently, therapeutic strategies incorporating biological response modifiers have aimed to enhance cell-mediated TH1 responses for the treatment of human diseases. Both TH1 and TH2 responses require antigen processing and presentation, are MHC restricted, and are dependent on the initial antigen-specific activation of T-cell subsets through the TCR. Antigen-specific T-cell frequency has been estimated at one in 10^4-10^6 cells (1). In attempts to increase both the frequency and activation of tumor antigen-specific cells, treatment modalities incorporating cytokines and growth factors have been used to up-regulate effector cell function (2), alone or in concert with adoptive immunotherapy and the infusion of ex vivo expanded tumor infiltrating lymphocytes or lymphokine activated killer cells (3, 4). Immunotherapy with monoclonal and bispecific antibodies has been used to activate and target T-cell and natural killer-cell populations, both systemically and at tumor site (5–7).

SAgs harness potent immunomodulatory capabilities. The activation potential of SEA is exhibited when the SEA molecule has been used to activate and target T-cell and natural killer-cell populations, both systemically and at tumor site (5–7).

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proliferation. SEA contains two independent but cooperative binding sites for MHC class II (8–12) and, in addition, a binding site for specific TCR Vβ subsets (1, 13, 14). SEA interacts with APCs in a MHC class II-dependent but not MHC-restricted manner, with MHC class II binding occurring outside the peptide binding groove. SEA activity does not require APC internalization or processing before the simultaneous SEA-T cell interaction (1, 15–19). Optimal SEA activity is induced when both SEA-MHC class II sites are engaged. Engagement can occur between two SEA molecules on one APC, one SEA with two APCs, or by cross-linking two MHC class II molecules on the same APC, resulting not only in a stable alignment for SEA-T cell interactions but at the same time activating the APC (8). The number and nature of the SAg-MHC class II interactions may determine the outcome of the SAg-T cell encounter, which could induce activation, proliferation, depletion, or energy of T-cell Vβ subsets (20). SAg have the potential to activate more than 10–25% of the T-cell population (1, 12–14).

SEA targets T-cell cytotoxicity in a MHC class II-dependent manner, making MHC class II-expressing tumor cells susceptible to SEA-induced effects. MHC class II-dependent cytotoxicity has been termed SDCC (21–25). Treatment modalities exploiting the therapeutic potential of SAg require SEA modification to decrease the systemic activation resulting from SEA-MHC class II interaction with monocytes and B cells and to localize the cytotoxic capabilities of SAg-activated T cells at tumor sites. Genetic fusion of SEA to the Fab of a tumor-targeting monoclonal antibody has resulted in molecules that show a 10-fold reduction in SEA-MHC class II binding and contain an antibody portion with a 100-fold stronger tumor antigen affinity than the SEA-MHC class II affinity (26–32). Such fusion proteins support superantigen-mediated antibody-dependent cell cytotoxicity, with lessened MHC class II dependence, and endow tumor cells with superantigenicity capable of activating tumor infiltrating T cells exhibiting selective SEA-Vβ specificities (27).

Phase I clinical trials using a single dose of the superantigen-based agent PNU-214565, a recombinant fusion protein of SEA and the Fab of the tumor targeting monoclonal antibody C242, have been conducted at this institution (33) and at Rigshospitalet in Copenhagen, Denmark. The single dose trials demonstrated the activation potency of this novel reagent, identified DLTs, and outlined a safe starting dose for the repeated dose trial presented here. The present trial has revealed a relationship of the clinical toxicities to pretreatment anti-SEA sera antibody concentrations and administered drug doses and has led to the development of a pharmacodynamically based dosing strategy.

### Table 1. Dose escalation schema

<table>
<thead>
<tr>
<th>Dose (ng/kg)*</th>
<th>No. of patients</th>
<th>Cumulative dose range (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>3</td>
<td>32.6-68.9</td>
</tr>
<tr>
<td>0.5</td>
<td>3</td>
<td>88.0-209</td>
</tr>
<tr>
<td>1.5</td>
<td>4</td>
<td>340.2-498</td>
</tr>
<tr>
<td>2.75</td>
<td>12</td>
<td>258-1112.6</td>
</tr>
<tr>
<td>3.5</td>
<td>5</td>
<td>888-1263.6</td>
</tr>
</tbody>
</table>

* Administered by 3-h infusion daily for 4 consecutive days.

### Table 2. Patient demographics

<table>
<thead>
<tr>
<th>Total entered</th>
<th>27</th>
</tr>
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<tbody>
<tr>
<td>Female</td>
<td>13</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>62 (36–75)</td>
</tr>
<tr>
<td>Baseline performance status (ECOG*')</td>
<td>0 15</td>
</tr>
<tr>
<td>1 12</td>
<td></td>
</tr>
<tr>
<td>Primary tumor site</td>
<td>Rectal 5</td>
</tr>
<tr>
<td>Colon 18</td>
<td></td>
</tr>
<tr>
<td>Pancreatic 4</td>
<td></td>
</tr>
<tr>
<td>Prior treatment</td>
<td>Surgery 24</td>
</tr>
<tr>
<td>Chemotherapy 26</td>
<td></td>
</tr>
<tr>
<td>Radiation 7</td>
<td></td>
</tr>
</tbody>
</table>

* ECOG, Eastern Cooperative Oncology Group.

**PATIENTS, MATERIALS, AND METHODS**

**Patient Population.** The inclusion criteria for the present repeated-dose study were consistent with those outlined in the previously described single-dose clinical trial (33). Eligible patients were required to have a histological diagnosis of incurable advanced pancreatic or colorectal adenocarcinoma. All patients were 18 years of age or older with an Eastern Cooperative Oncology Group performance status of 0 or 1. Patients had received no radiotherapy, chemotherapy, immunotherapy, or biological therapy within 4 weeks of entry into this study (6 weeks for mitomycin C or nitrosoureas). All patients had adequate bone marrow (WBC ≥ 3,000/mm³, absolute neutrophil count ≥ 2,000/mm³, and platelets ≥ 150,000/mm³) and adequate hepatic and renal function (total bilirubin ≤ 1.5 times upper limit of normal; aspartate aminotransferase and alanine aminotransferase less than or equal to twice the upper limit of normal unless liver metastases, then ≤ 4 times the upper limit of normal; and serum creatinine ≤ 1.5 mg/dl). The use of β-blockers, either for hypertension or glaucoma, had to be discontinued a minimum of 5 days before treatment. Patients with pulmonary disease requiring active therapy, New York Heart Association classes II–IV heart disease, or uncontrolled hypertension were excluded from therapy. Written informed consent was obtained in accordance with federal, state, and institutional guidelines. Prior to treatment initiation, a medical history, physical examination, and clinical laboratory evaluations, including hematology, coagulation, and chemistry profiles, were performed, and samples were obtained for the baseline laboratory investigative studies. Laboratory evaluations included flow cytometry analyses, lymphocyte proliferation assays, sera cytokines, soluble CA242 antigen, anti-SEA and HAMA levels, and plasma drug levels. Serial blood samples were obtained throughout the treatment course. Tumor assessment was performed at baseline and day 28 after treatment using standard response criteria (34).

**Treatment Plan.** Patients were admitted for the duration of PNU-214565 treatment. The patients received a daily 3-h i.v. infusion on 4 consecutive days. Treatment was administered using a CADD-micro pump (Pharmacia-Deltec, St. Paul, MN). The starting dose of PNU-214565 was 0.15 ng/kg/day with escalation as described in Table 1. Three patients were accrued to each dose level. Patients received only one 4-day course of treatment.
The 2.75 ng/kg dose level was expanded after DLTs were observed in a concurrently running companion trial. Dose escalation was terminated at 3.5 ng/kg, when the observed relationship between clinical toxicity and the contribution of the baseline sera anti-SEA concentrations relative to administered drug dose became apparent.

Expression and Purification of PNU-214565. The fusion protein of C242 Fab-SEA (PNU-214565) was prepared and provided by Pharmacia/Upjohn (Lund, Sweden) as described previously (33).

Cytokine Determinations. Immunoenzymetric assays were performed using Medgenix Diagnostics kits (Fleurus, Belgium) following manufacturer’s procedures for the quantitative measurements of IL-2 and TNF-α. Serial samples were analyzed daily at preinfusion, 3, 5, 11, and 24 h after the final drug infusion.

Flow Cytometry. Flow cytometric analysis was performed on peripheral blood mononuclear cell preparations obtained and processed using Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation preinfusion days 1 and 2, 24 h after the last PNU-214565 infusion, and on day 11. Cells were dually labeled (FITC/PE) with CD4/CD45RO, CD8/CD45RO, CD14/CD16, CD4/HLADR, CD8/HLADR, and CD19/HLADR. The fluorescein (FITC)- and PE-conjugated monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA). Analysis was performed on a FACScan flow cytometer (Becton Dickinson) equipped with Cell Quest software BDIS. Gates were selected to include the CD45 positive lymphocyte/monocyte populations for analyses. Bridging assays were performed by pre-incubating the patient’s lymphocytes, suspended in either PBS or autologous plasma, in the presence and absence of PNU-214565 for 30 min at 37°C followed by dual labeling with CD3-FITC and CD14-PE.

PNU-214565 Plasma Levels. Blood samples were drawn, and plasma was obtained at baseline and at hour 3 on days 1 and 4. Plasma was stored at −70°C for batch analyses. Infusion (final dilution) solutions were analyzed as a quality control measure. Analyses were performed by Pharmacia/Upjohn (Helsingborg, Sweden) as described previously (33). Analysis of the infusion drug doses indicated that all patients received the intended dose. Circulating PNU-214565 levels were undetectable at the doses administered in this study. The range of the cumulative doses administered in this study was 32.6 to 1263.6 ng.

Humoral Immune Responses: Human Anti-Immune Antibody (HAMA), Anti-SEA, and Soluble CA242 Antigen Levels. Blood samples were drawn, and plasma was obtained at baseline and days 11 and 28 after initiation of treatment. Plasma was stored at −20°C for batch analyses. HAMA was analyzed using Immun STRIP HAMA fragment (Immunomedics, Morris Plains, NJ) following the manufacturer’s procedure. Anti-SEA was determined as described previously (33). CA242 circulating antigens levels were performed using the EIA CanAg CA242 kit (CanAg Diagnostics, Gothenburg, Sweden) following the manufacturer’s instructions.

Lymphocyte Proliferation Assays. PNU-214565-induced lymphocyte proliferation assays were performed on Ficoll-prepared lymphocytes from blood samples collected at baseline, prior to the day 2 infusion, 24 h after the last PNU-214565 infusion, and on day 11 as described previously (33). Additional proliferation assays using a standard lymphocyte donor and patient autologous plasma at baseline were performed to assess the contribution of autologous plasma factors. All assays used serial dilutions of PNU-214565. PHA was used as a positive control stimulator. Assays were incubated 72 h at 37°C with [3H]thymidine added during the last 4 h of incubation. Selected patient plasma samples were affinity chromatography absorbed to remove the anti-SEA. Repeat lymphocyte
proliferation assays were performed to assess the contribution of the anti-SEA to the overall plasma inhibition factor.

**HLA-DR Analyses.** Selected patients were HLA-DR typed as described previously (33).

**Toxicity Model Derivation.** The equation below used to calculate the predicted dose for a 50% probability of cytokine induction was derived retrospectively following analysis of data obtained from patients treated with a single dose of PNU-214565 (33), omitting patient 2, and those described in this present trial to assess the association of the pretreatment baseline anti-SEA concentration and the administered dose to the observed treatment-induced toxicities.

Predicted dose $= \exp \left[ \ln \left( \frac{\text{probability}}{100} - \text{probability} \right) \right] - 5.83 + 1.770 \times \ln \left( \frac{\text{pmol/ml anti-SEA}}{0.824} \right)$

The predicted dose in ng/kg required to elicit a systemic cytokine response at a predetermined probability rate is calculated using this formula. Any desired probability (e.g., 10%, 50%, or 90%) that a systemic cytokine induction will be elicited is inserted, as is the patient’s baseline pretreatment anti-SEA concentration (expressed in pmol/ml) to derive the dose to be used.

**Statistics.** Statistics were made using the standard Student’s $t$ test.

**RESULTS**

Twenty-seven patients were treated on this study. Characteristics of the patients are listed in Table 2. The age range was 36–75, with a median of 62; 14 were males, and 13 were females. Of the 27 patients treated, 23 had colorectal cancer and the remaining 4 had pancreatic cancer. Prior to treatment, 24 patients had undergone surgery, 26 had received chemotherapy, and 7 had received radiation therapy. The performance status was either 0 ($n = 15$) or 1 ($n = 12$).

**Clinical Assessment.** No DLTs were observed in patients treated at the 0.15-, 0.5-, or 1.5-ng/kg dose levels. The 2.75-ng/kg dose was expanded to include 12 patients after DLT (grade 4 hypotension) was experienced by one patient, as described below. Two additional grade 4 toxicities were observed at the 4-ng/kg dose level in a companion clinical trial performed at Righospitalet. Four additional patients were treated at 3.5 ng/kg with no DLTs. The study was closed at this dose level after the observation of a relationship of the pretreatment anti-SEA sera concentration relative to the administered drug dose and the probability of clinical toxicities.

Twenty-five of 27 patients received the full 4-day cycle of PNU-214565. Treatment was terminated after one dose of drug in patient 20 (2.75 ng/kg), who developed a transient grade 4 hypotension responsive to i.v. fluid and low-dose dopamine. Day 4 treatment was not administered in patient 23 after the development of a grade 2 skin rash on day 3 that did not resolve. As in the single-dose trial, fever and hypotension were the most common nonhematological toxicities experienced after infusion of PNU-214565 (1). Grade 1 fever occurred in 41% (11 of 27) of the patients, and grade 2 fever occurred in 26% (7 of 27). Fever was not dose-limiting. Hypotension was encountered in 89% (24 of 27) of the treated patients: 11 events (41%) were grade 1; 12 (44%) were grade 2; and 1 patient (4%) exhibited a dose-limiting grade 4 toxicity (see above). Other toxicities experienced included diaphoresis, nausea, vomiting, rigors, headache, diarrhea, anxiety, and edema. Except for one episode of grade 2 diaphoresis and one grade 2 edema of the feet, none of the additional toxicities advanced further than a grade 1. In general, toxicities were most prominent on day 1. Toxicities were low grade, transient, and subsided within 24 h of the conclusion of treatment. All treated patients experienced transient losses in WBC.

**Table 3  Toxicity correlates**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Toxicity</th>
<th>Percentage of loss from baseline at nadir</th>
<th>Mean peak cytokine induction (pg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type WBC PMN</td>
<td>Lympocytes</td>
<td>Monocytes</td>
</tr>
<tr>
<td>≤1</td>
<td>(n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Hypotension</td>
<td>18.4</td>
<td>27.9</td>
</tr>
<tr>
<td>2</td>
<td>Fever</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fever plus hypotension</td>
<td>38</td>
<td>32.7</td>
</tr>
</tbody>
</table>

* Significant levels for IL-2 exceed 94.3 pg/ml; significant TNF-α levels exceed 40 pg/ml.

subsets. The degree of loss correlated with toxicity and cytokine induction. Fig. 1 illustrates the correlation of the hematological subset fluctuations and cytokine induction for patient 12. Table 3 shows the correlation of lymphocyte subsets decrease, toxicity grade, toxicity type, and peak cytokine induction. For toxicities less than or equal to grade 2, no significant difference was observed in the loss of WBCs and polymorphonuclear lymphocytes. The development of fever, alone or in combination with hypotension, correlated with lymphocyte loss and cytokine induction. The IL-2 induction (grade 2 fever plus hypotension) had greater impact on the lymphocyte and monocyte decreases than TNF-α induction at equivalent toxicity grade. The one patient with grade 4 toxicity had significant cytokine induction, and all white cell subsets were drastically reduced. However, platelet decreases were not significant in any of the patients.

**Clinical Outcomes.** An overall response lasting 2.5 months was experienced by patient 12, who had been diagnosed with pancreatic cancer metastatic to the liver. This patient was treated at 2.75 ng/kg. At 28 days, a partial response of the hepatic metastases with stable pancreatic head abnormalities was demonstrated by computed tomography scan (Fig. 2).

**Laboratory Assessment: PNU-214565-associated Effects.** Samples from 16 of 27 patients (59%) showed a 1.1-3-fold increase in lymphocyte counts on day 11 when compared with baseline values. Flow cytometry analysis showed some evidence of activation during and after the course of treatment in the majority of patients. Activation was indicated by increases in the total percentage of dually labeled CD14+/CD16+ monocytes, CD8+ lymphocyte populations, or CD16+ natural killer populations, accompanied by mean fluorescent index increases for HLA-DR and CD45RO both on CD4+ and CD8+ cells (data not shown).

Humoral immune responses were assessed to determine the response to the Fab C242 mouse-derived portion of the fusion protein. All patients were followed a minimum of 1 month after treatment. As predicted by the low protein level administered and the Fab nature of the mouse component, no patient showed a HAMA response. One patient (patient 22) demonstrated 39.5 ng/ml HAMA to Fab mouse fragment pretreatment with a drop to 4.1 ng/ml at the 1 month interval. The pretreatment value was unexplained. The patient’s medical history gave no indication of prior murine monoclonal antibody exposure.

Anti-SEA concentrations were evaluated at baseline, day 11, and 1 month after treatment to investigate immune responses to the SEA portion of the fusion protein. Baseline anti-SEA concentrations ranged from 15.4 to 2091 pmol/ml. No patients at the 0.15-ng/kg dose level showed increases over baseline in their anti-SEA concentrations. Two patients treated with 0.5 ng/kg dose level showed a 2-fold increase at 1 month over baseline. Fifteen patients treated at the 1.5-ng/kg and higher dose levels had increases in their anti-SEA concentrations of 2-381-fold at 1 month after treatment (mean, 39.5-fold; median, 3.3-fold). The greatest increase was observed in a patient treated at 3.5 ng/kg with a baseline concentration of 24 pmol/ml. This patient’s anti-SEA concentration increased to 9152 pmol/ml at 1 month after treatment. The “threshold” for anti-SEA induction

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**Table 4 HLA-DR typing**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>HLA-DR allotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>4/701, 53</td>
</tr>
<tr>
<td>17</td>
<td>4, 53</td>
</tr>
<tr>
<td>18</td>
<td>3/701, 52/53</td>
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<td>15/14, 51/52</td>
</tr>
<tr>
<td>28&quot;</td>
<td>1/2, 51</td>
</tr>
</tbody>
</table>

* Patients with grade ≥2 toxicity.
ranged from undetectable to 10,087 units/ml at baseline. No patient in this study had greater than a 4.7-fold increase at 1 month, 12 patients had 1.5-fold or less, 10 patients demonstrated a 1.6–3.0-fold increase, and five patients had a 3.3–4.7-fold increase. There were no noted correlations of circulating CA242 antigen levels and disease status.

Table 4 gives the HLA-DR allotypes for the patients evaluated. A number of the allotyped patients expressed the DR53 allele. The DR53 allele has an amino acid alteration at position 81, changing the histidine to tyrosine. This histidine at position 81 is responsible for the SEA-DR1 moderate affinity binding (35–37). Although investigations have not shown SEA-MHC class II binding to be affected significantly by the HLA-DR allotype (37, 38), augmentation of this moderate affinity MHC class II-SEA binding could contribute to the outcome resulting from the subsequent SAg-TCR Vβ interaction. However, no associations between allotypes and toxicities have been observed.

**Pretreatment Anti-SEA Concentration and Association with Toxicity.** Evaluations following the single-dose trial (1) and the initial part of the present repeated-dose trial indicated that the observed clinical toxicities were not solely dependent on the weight-based dose assignment. When baseline anti-SEA concentrations in relation to administered dose were considered, an association with toxicity was revealed. Fig. 3 shows data for the 84 patients treated in the single- and repeated-dose trials at this facility and Rigshospitalet. The highest grade of clinical toxicity experienced by each patient during the course of treatment is plotted against the ratio of the baseline anti-SEA concentration (pmol/ml) to the daily administered dose (ng/kg). Patients with grade 3 toxicities all had ratios less than 30:1, with all three grade 4 toxicities occurring in patients having a ratio of 6:1. Likewise, significant IL-2 and TNF-α induction was detected in those patients with ratios less than 10:1.

Calculations were derived (see “Materials and Methods”) to define a dose with 50% probability of inducing systemic cytokines, which is used as an indication of the presence of uncomplexed drug and its ability to induce systemic cellular activation, or DLT. As the calculation indicates, the relationship between the predicted dose and the anti-SEA antibody concentration is not linear. Using a 50% probability of systemic cytokine induction (IL-2 and TNF-α), Fig. 4 (upper panel) illustrates the ratio of actual to predicted administered dose for the treated patients in this study and indicates that 7 of 27 patients received a ratio greater than 1. Fig. 4 (lower panel) gives the peak IL-2 (□) and TNF-α (●) levels for those patients. Three of the 7 (or 50%, as predicted by the calculation) did in fact exhibit a cytokine induction. Patient 20, who experienced a grade 4 hypotension with significant levels of circulating cytokines, received 9.2 times the dose predictive for cytokine induction (Table 5). Cytokine induction was detected in Patient 12 (grade 2 fever) and patient 23 (grade 2 fever and hypotension). These patients received 3 and 1.3 times, respectively, the predicted dose. Although there was sufficient uncomplexed drug to induce systemic cytokine, the degree of cytokine induction in these patients did not induce DLT. However, patient 28 (4.5 times predicted dose and grade 2 hypotension), patient 17 (3.0 times predicted dose, no toxicity), patient 19 (2.3 times predicted dose, grade 2 fever and hypotension), and patient 14 (1.3 times
predicted dose, no toxicity) had only minimal detectable levels of cytokines. These data illustrate a relative association between the anti-SEA concentration and the administered drug dose with respect to toxicity. However, components of the total anti-SEA concentration may play a more toxicity "protective" role as shown by the outcome of patients 12 and 23 compared with patients 28, 17, 19, and 14.

Lymphocyte proliferation assays provide a functional means to assess the impact of inhibitory anti-SEA components in patient plasma. Fig. 5 compares the PNU-214565 molar concentrations required for half-maximal stimulation plotted against the baseline anti-SEA concentrations (pmol/ml) for each treated patient. These assays were performed using a single healthy donor's lymphocytes to assess the impact of patients' circulating anti-SEA concentration. Donor lymphocytes assayed in FBS were half-maximally stimulated at a PNU-214565 concentration of $9 \times 10^{-13}$ M. Thus, half-maximal PNU-214565 concentrations approaching $9 \times 10^{-13}$ M would indicate a lower inhibitory/neutralizing component in a given patient's plasma.

Table 5 shows that the plasma of six patients (patients 2, 4, 12, 17, 20, and 23) had lowered half-maximal PNU-214565 concentration requirements for lymphocyte proliferation. Con-
centrations in the $10^{-11}$ M range indicate minimal inhibition. Although patients 2 and 4 had half-maximal drug concentrations in this range of $10^{-11}$ M, their lack of severe clinical toxicity and a lack of cytokine induction was most likely related to the low drug doses these patients received. In contrast, patients 12, 20, and 23 also had half-maximal drug concentration requirements in this range, but all experienced clinical toxicity and significant cytokine induction (Fig. 5, ↓). All of these patients received greater than the predicted drug dose based on their total baseline anti-SEA concentration (Fig. 4). Half-maximal concentrations and anti-SEA/drug dose ratios would have predicted that patient 17 would experience severe toxicity and cytokine induction. However, this was not observed. Five patients demonstrated half-maximal PNU-214565 concentration requirements of $10^{-10}$ M (patients 3, 6, 15, 19, and 28). Toxicity would not have been predicted for patients 3 and 6. These two patients demonstrated an intermediate level of inhibition and received low drug doses. The lack of toxicity experienced by patients 15, 19, and 28, who received 0.3, 2.3, and 4.4 times the predicted dose, respectively, demonstrates that this intermediate level of inhibition is sufficient to protect against severe toxicity at the drug doses received. All remaining patients contained a sufficient level of neutralizing anti-SEA to protect against toxicity at the doses administered.

Affinity chromatographic absorption was performed on selected patient plasma samples to remove the anti-SEA to definitively assess its contribution to the half-maximal drug concentrations required for proliferative stimulation. Table 6 indicates that lowered drug concentrations are capable of half-maximally stimulating donor lymphocytes after anti-SEA absorption. Patients 8 and 12 had similar preabsorption anti-SEA concentrations. The plasma from patient 12 exhibited little inhibition of PNU-214565-induced lymphocyte proliferation. This patient exhibited grade 2 fever and cytokine induction after treatment with $2.75 \operatorname{ng/kg}$ PNU-214565. In comparison, lymphocyte proliferation was inhibited by plasma from patient 8, who exhibited only grade 2 hypotension and had no detectable cytokine induction. Patients 16 and 24 had neither clinical toxicity nor cytokine induction, suggesting the presence of adequate PNU-214565-neutralizing anti-SEA for the drug dose administered.

Fig. 6 depicts the SEA-induced cell-cell interaction that can occur systemically. It illustrates the variable capacity of the plasma of two patients with comparable "low" anti-SEA concentrations, and who received the predicted drug dose, to block the PNU-214565-induced bridging of monocytes/B cells and T cells resulting from MHC class II-SEA and TCR Vβ-SEA interactions. Fig. 6A shows that PNU-214565-induced bridging was reduced from 5.61% in PBS to 1.25% in baseline plasma for patient 19. This patient had a baseline anti-SEA of 30 pmol/ml
Fig. 5 Relationship of anti-SEA to PNU-214565 concentrations required for half-maximal stimulation. The assay was performed with donor cells and 10% patient plasma. $10^{-8}$ M PNU-214565 was the highest concentration used in the assay. Toxicity grade for each patient: ●, ≤grade 1; ○, grade 2 hypotension alone; □, grade 2 fever alone or in combination with hypotension; ◻, grade 4 hypotension. ↓, patients showing significant cytokine induction.

Table 6 Lymphocyte proliferation after affinity chromatography absorption

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Preabsorption</th>
<th>Postabsorption</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Anti-SEA (pmol/ml)</td>
<td>Half-maximal stimulation (molar drug)</td>
</tr>
<tr>
<td>8</td>
<td>66</td>
<td>$5.5 \times 10^{-9}$</td>
</tr>
<tr>
<td>12</td>
<td>65</td>
<td>$4 \times 10^{-11}$</td>
</tr>
<tr>
<td>16</td>
<td>203</td>
<td>$5 \times 10^{-10}$</td>
</tr>
<tr>
<td>24</td>
<td>1504</td>
<td>$6 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

* Half-maximal stimulation for healthy donor cells in FBS was $4.5 \times 10^{-13}$ M.

DISCUSSION

The present clinical trial is the first report of repeated dosing with an SEA-based fusion protein and shows that PNU-214565 can be administered safely in a repeated-dose regimen. No cumulative dose-related toxicities were observed. The toxicities encountered were manageable and of short duration. Laboratory and clinical findings showed no detectable HAMA to the C242 Fab portion at the doses administered, but there were variable responses to the SEA portion of the fusion protein. Toxicity correlated with systemic cytokine induction and the extravasation of WBC subsets. The required PNU-214565 concentration for half-maximal lymphocyte proliferation stimulation correlated with treatment-induced cytokine release, the type and grade of toxicity, and the ratio of baseline circulating anti-SEA concentration to administered drug dose.

T-cell activation using immunoconjugates of SAg has advantages over other monoclonal antibody-based strategies (39). SAg activates a limited, although significant, fraction of T lymphocytes, and in contrast to pan T-cell activators, can be expected to leave the majority of the T-cell repertoire intact, thereby avoiding general immune suppression. In addition, whereas monoclonal antibodies penetrate tumors poorly, T-lymphocytes are not limited by such factors as interstitial pressure and, depending on the relevant chemotactic signal, can move freely throughout large tumor masses.

Cytokine induction was the most reliable indicator of a systemic immunological response and was used as a surrogate measure of uncomplexed circulating drug and SEA-induced cell-cell activation mediated by the SEA portion of the agent in this study. Such responses indicate that circulating drug levels are sufficient to exceed the circulating inhibitory anti-SEA plasma...
component, allowing drug to intravascularly stimulate patient cells via SDCC. Also, systemic IL-2 induction would suggest the presence of the appropriate SEA-TCR Vβ subsets. Circulating drug plasma levels at the doses administered in this trial are estimated to be in the attomolar range ($10^{-16}$ to $10^{-18}$ M), and the observed toxicities underscore the remarkable activation potency of this agent. In vitro testing shows SEA-induced lymphocyte proliferation to be half-maximal at drug concentrations in the pico-femtomolar range when assays are performed in FBS and in the nanomolar range in the presence of plasma.

The relative ratio of the baseline circulating anti-SEA concentration to drug dose, although not linear, has been the best predictor of toxicity and showed patients having ratios $<$10–15 at the most risk of toxicity. Drug doses must be balanced to achieve circulating levels in excess of anti-SEA with acceptable toxicities. In this trial, this balanced excess of drug was accomplished in only 7 of 27 patients (patients 12, 14, 17, 19, 20, 23, and 28; Table 5; Fig. 4). In the present clinical trials that build on these results, the assigned dose is based on weight and uses the baseline anti-SEA concentration as a covariate predicting for probability of cytokine induction/DLT. The dose escalation between patients uses a Bayesian dose escalation scheme. The dose for each patient will be determined so that, on the basis of all available data, the probability of DLT is 0.1 and the probability that the dose exceeds the MTD is equal to $\alpha$, which gradually increases from the current level of 0.25 to 0.50.

In patients demonstrating relative anti-SEA/drug ratios below 30, the anti-SEA impact on toxicity has been variable. In vitro lymphocyte proliferation assays are capable of revealing the level of the inhibitory component in plasma with a given total anti-SEA concentration. This functional assessment of the total anti-SEA concentration was predictive in 26 of the 27 patients treated in this study (Fig. 5) and could serve as an associated predictive factor for toxicity in future clinical trials, where drug dose is calculated to be in excess of the total anti-SEA in circulation. The data presented here do not exclude the possibility that factors other than anti-SEA antibodies modulate the SEA-induced immune responses observed systemically and potentially at the tumor site. However, determining the contribution of the neutralizing anti-SEA component relative to the total anti-SEA concentration is likely to enable accurate dosing of this agent.

The clinical toxicity and cytokine induction are reflective of the systemic intravascular cellular activation resulting from the SEA-induced cell-cell interaction. However, an individual’s innate cellular capability must be considered when assessing the overall effectiveness of such biological response-modifying agents. Additional factors could affect PNU-214565-induced immune responses at tumor sites. Of the 27 patients entered into study, 18 patients (67%) demonstrated an adequate lymphocyte mitogenic stimulatory (PHA) capacity when assayed in FBS (data not shown). Ten of these 18 patients maintained that capacity when assayed in autologous plasma. No patient had a greater mitogenic stimulation in plasma compared with FBS-containing assay systems. These results suggest that autologous plasma contains as yet unidentified inhibitory factors that could modify the proliferation/activation potential at tumor site. When SEA-induced mitogenic stimulation was examined, it was found that 16 of 27 patients (59%) had significant SEA-induced lymphocyte proliferation in FBS-containing assay systems (data not shown). Plasma anti-SEA concentrations account for a majority of the inhibition of SEA-induced proliferation in assays performed in plasma (Table 6). Diminished PHA mitogenic re-

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sponses, as revealed in FBS-containing assay systems, could indicate an innate decreased lymphocyte proliferative capacity. The level of immunoglobulin and other potential inhibitory factors at the tumor site milieu is unknown. Factors affecting the SEA-induced proliferation, in addition to immune suppressive factors, could include variability in the expression of TCR VB-SEA reactive subsets, improper presentation of SEA, MHC class II polymorphisms, or a lack of accessory molecule engagement. Any of these factors could, in combination with the inhibitory/blocking anti-SEA, modulate lymphocyte responsiveness to SEA (19, 35, 36).

Systemic activation resulting from MHC class II-dependent SDCC most likely will be dose-limiting for this agent, and the anti-SEA/fusion protein complex formation could potentially limit the delivery of therapeutic drug to tumor sites. Preclinical studies are presently in progress with mutant SEA molecules that demonstrate decreased MHC class II binding (9, 12, 40, 41). Strategies that minimize SDCC and maximize staphylococcal enterotoxin antibody-dependent cell-mediated cytotoxicity could greatly enhance the potential for therapeutic effect of this potent and novel immunoregulatory treatment modality.

This trial has led to the development of a novel pharmacodynamic dosing model encompassing both weight-based dosing and a dosing schema based on the relationship of the pretreatment anti-SEA concentrations to a dose sufficient to provide the needed balance between the plasma anti-SEA inhibitory component and the agent's SEA treatment doses that provide the needed balance between the plasma anti-SEA inhibitory component and the agent's SEA immunostimulatory capability. It is anticipated that by adjusting the doses administered to account for the anti-SEA concentration, other factors contributing to toxicity can be identified and more fully explored.

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