Abstract

Purpose: To assess the tolerability and effectiveness of 105AD7 vaccination in colorectal cancer patients. 105AD7 is a human anti-idiotypic antibody mimicking CD55, a glycoprotein, which is more than expressed on colorectal cancer cells and protects them from attack by complement.

Experimental Design: Colorectal cancer patients (n = 67) eligible for primary surgery were randomized to receive the anti-idiotypic antibody 105AD7 + Bacillus Calmette-Guerin/alum or to no treatment (control group). The immunizations were given i.d./i.m. before surgery and continued for a period of 2 years. The patients were monitored in enzyme-linked immunospot (ELISPOT; γ-IFN), proliferation assay, and Luminex cytokine assays.

Results: No serious adverse events were recorded. Of the 32 investigated immunized patients, 14 (44%) were considered to be responders in the ELISPOT assay. Induced proliferative responses were noted in 17 of 40 (43%) monitored patients. There was no correlation between the ELISPOT and proliferation assays. Luminex analyses revealed tumor necrosis factor-α and granulocyte macrophage colony-stimulating factor responses not only to the vaccine but also toward the native antigen CD55 in 9 of 13 (69%) patients.

Conclusions: Immune responses to vaccination were induced in a majority of monitored patients measured by ELISPOT and proliferation assay. The lack of correlation between the ELISPOT and proliferation assays may reflect the fact that the two methods measure different T-cell responses and highlights the importance of multiple readouts in evaluating a potential cancer vaccine. Responses to both the anti-idiotype and the CD55 antigen were measurable, adding support to the use of CD55 as a target in cancer treatment.

105AD7 is a human monoclonal anti-idiotypic antibody, which shows both amino acid and structural homology with CD55 (decay accelerating factor), a glycosylphosphatidylinositol-anchored integral membrane protein of the membrane complement regulatory protein family (1), which protects cells from complement. Low level expression of CD55 occurs in all cells exposed to complement; however, increased expression of CD55 has been shown in multiple tumor types, including up to 80% of colorectal cancers, making it an attractive target for specific immunotherapy (2). Immunization with 105AD7 can stimulate both antibody and T-cell responses (via Fc receptor–mediated targeting of antigen-presenting cells; ref. 3) resulting in an immune response directed against tumor cells bearing CD55. Phase I studies in 13 patients with advanced colorectal cancer showed that 105AD7 was nontoxic, with immunized patients showing evidence of T-cell blastogenesis in response to CD55-expressing cells and increased interleukin (IL)-2 production (4). Subsequently, a double-blind randomized phase II trial of 105AD7 on alum versus placebo involving 162 patients with advanced disease failed to show any significant survival benefit (5). Work has since focused on the use of 105AD7 in the neoadjuvant/adjuvant setting where patients in a phase I trial undergoing potentially curative surgery receiving preoperative immunizations showed enhanced infiltration of CD4 and CD8 lymphocytes and natural killer cells in their resected tumors when compared with matched controls (6). In a recent sarcoma trial, the potency of this vaccine concept was underlined by two long lasting clinical responses (7).

Here, we report the results of the largest adjuvant trial thus far with 105AD7. In this randomized trial, we show enhanced anti-idiotype specific responses to 105AD7 in vaccinated patients when compared with unimmunized controls and thus provide further evidence of the ability of 105AD7 to elicit a specific functionally active response in vivo.
Material and Methods

Patients. All patients had a WHO performance status 0 to 2, normal hematologic variables, and normal renal and liver function (<25% deviation from normal values). Exclusion criteria were as follows: any autoimmune or chronic hematologic condition, concomitant other anticancer treatment, or preoperative radiotherapy in patients with rectal cancer.

Patients assigned to the treatment arms were immunized preoperatively on the day of recruitment, with a further immunization after 2 weeks if still awaiting surgery. Postoperatively, immunizations were continued at 3, 6, and 12 weeks and then at 3 monthly intervals up to a maximum of 24 months. Patients who underwent postoperative chemotherapy as clinically indicated (n = 28) were suspended from the trial during this period, resuming immunizations after chemotherapy was completed. When the trial was conceived, there was concern for the potential immunosuppressing effect from chemotherapy. Recent studies now suggest that these concerns are unfounded. Blood for immunologic assays was collected at recruitment, at surgery, and at the time of the 3, 6, and 12 weeks postoperative immunizations. Samples were then acquired 1 month after each subsequent immunization (Fig. 1). Approval of the local Ethical Committee was obtained, and all patients gave written informed consent for their participation. The study was carried out in accordance with the Declaration of Helsinki.

The first patient was included in June 2000 and the last in May 2002. The mean follow-up time is 39 months (range, 18-56 months). Sixty-seven patients, 38 males and 29 females, with primary colorectal cancer scheduled to undergo surgical resection of their primary tumor were randomized to receive neoadjuvant 100 μg 105AD7 with alum (aluminum hydroxide) only i.m. (n = 28) or 100 μg 105AD7 with Bacillus Calmette-Guerin (BCG; Oncotice) i.d. at the first immunization with subsequent vaccinations with alum as an adjuvant (n = 17) or no treatment (control group; n = 22; Fig. 1). The vaccine dose was selected from previous studies, indicating that a dose of 100 μg is optimal in inducing T-cell responses (8). Mean age at study entry was 66 years (range, 43-85 years). The last 15 included patients were randomized between control and alum groups only due to local reactions from BCG. Twenty-eight patients had colon cancer, whereas in 39 patients, the primary tumor was located to rectum. Twelve had stage Duke’s A, 20 had Duke’s B, 29 had Duke’s C, and 5 had Duke’s D. One patient refused surgery and the correct stage could therefore not be assessed.

Vaccine formulation. Clinical grade 105AD7 human antibody was produced as described previously (9) using the guidelines of the Cancer Research Campaign (presently Cancer Research-UK), United Kingdom (10). Antibody was prepared as 100 μg doses/mL in sterile saline. Alum was prepared as aluminium hydroxide gel (alhydrogel 85, Superphos Biosector, Vedbaek, Denmark).

Collection of peripheral blood mononuclear cell. Samples of peripheral venous blood were separated on Ficoll-Hypaque density gradient separation, washed with PBS, counted in trypan blue, and suspended in RPMI 1640 containing 20% FCS and 10% DMSO. Cells were stored at −196°C in liquid N2 in cryovials at 5 × 10^6 to 10 × 10^6 viable cells per vial.

T-cell stimulation. The cryopreserved peripheral blood mononuclear cells (PBMC) were quickly thawed, washed, and resuspended in 10% FCS in RPMI 1640. The enzyme-linked immunospot (ELISPOT) assays were done after overnight resting of the cells in a humidified incubator of 5% CO2 at 37°C. All samples from each patient were stimulated with the optimal concentration of purified protein derivative (Statens Seruminstitut, Copenhagen, Denmark), 105AD7, CD55-lg, and human IgG1 (10 μg/mL) for 36 to 40 hours and then analyzed simultaneously using a modified ELISPOT assay for IFN-γ secretion to determine the frequency of antigen-specific T cells and for cytokine secretion by Luminex assay. Having previously established using healthy volunteers the optimal incubation time to allow internalization, processing, and presentation of the protein antigens (data not shown). As previous studies have shown that CD55 with a glycosylphosphatidylinositol linker is inhibitory to T-cell responses (11), we used CD55 human Fc-y1 fusion protein in these assays. The human IgG1 control is therefore a good control for both 105AD7 and CD55-lg and clearly shows that the response is not to Fc-y1.

For ELISPOT assays, plates were coated with capture antibody against human IFN-γ (R&D Systems, Abingdon, United Kingdom). Cells were then removed after 36 hours and developed with biotinylated human anti-IFN-γ detection antibody (R&D Systems) and streptavidin-alkaline phosphatase/5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate using standard protocols.

A responder is defined as having a positive response at least one time point after the first immunization. To be considered a positive time point, the duplicate values of 105AD7 must be greater than the highest value of the IgG duplicates for that time point as well as greater than the highest 105AD7 value at preimmunization and the mean value of 105AD7 must exceed the mean + 2 SD of the corresponding value at preimmunization. After subtracting the background (hIgG), the number of spots must exceed 50 per million PBMCs. We selected our level as positive due to no responses in our immunonuclear controls.

Cytokine assay. Supernatants were analyzed for cytokines using Luminex assays. Briefly, supernatant was incubated with detection antibody (R&D Systems) and developed with streptavidin-phycocerythrin. The plates were read in the Luminex machine (Bio-Rad, Hemel Hempstead, United Kingdom).

A responder is defined as having a positive response at least one time point after the first immunization. To be considered a positive response, the cytokine concentration [granulocyte macrophage colony-stimulating factor (GM-CSF) or tumor necrosis factor-α (TNF-α)] for 105AD7 or CD55 must be at least twice as high as hIgG for that time point and at least twice as high as the corresponding value at preimmunization.

Proliferation assay. A complete set of cryopreserved (−85°C) PBMC for each patient was defrosted for simultaneous analysis. The PBMCs were stimulated with the optimal concentration of 105AD7 (10 μg/mL), positive control purified protein derivative (10 μg/mL), or negative control human IgG antibody (10 μg/mL) in RPMI 1640 containing 5% autologous human plasma with 1 mL/well (10^5 cells/mL) in 24-well plates (Corning, Inc., Corning, NY) as described previously (7). Soluble CD55 has been shown to inhibit proliferative responses and therefore was not included in these assays.

A responder is defined as having a positive response at least one time point after the first immunization. To be considered a positive response, the stimulation index must be >2 for 105AD7/hIgG and the cpm for 105AD7 at that time point must be at least twice as high compared with
preimmunization. One-way ANOVA showed that all samples that responded with a 2-fold increase in response to 105AD7 compared with human IgG were significant.

Results

Patients. Patients’ characteristics are shown in Table 1. The mean number of immunizations received was 8 (range, 2-13 immunizations). Except for low-moderate local reactions from the immunizations (especially BCG), no adverse events, including autoimmune reactions, were recorded.

Proliferative T-cell response. Patients (17 of 40) showed a significant proliferation response to 105AD7 but not to control human IgG (Table 2). Figure 2A shows these results for patient OX14. This patient showed a strong proliferation response that peaked following the initial immunizations dipped postsurgery and was then reboosted with postsurgical immunizations. Although there was a high degree of variation among patients in their kinetic response to 105AD7, this was the most common pattern as shown by the mean stimulation indices for all responding patients (Fig. 2B). The induction of a proliferative anti-105AD7 response was noted already after the first immunization with a mean of 6.9 in stimulation index (range, 0.3-53; Fig. 2B). In general, the response decreased after surgery but a second peak was seen at 5 months, after the fourth immunization with a mean of 6.7 in stimulation index (range, 0.4-45). After the second peak, the response gradually returned to preimmune levels. All controls (n = 22) were assessed in proliferation assay (Fig. 2C) and two were defined as responders according to our criteria (see above) but only based

Table 1. Clinical characteristics of colorectal carcinoma patients

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Site (n)</th>
<th>Stage, Duke’s (n)</th>
<th>Gender (n)</th>
<th>Mean age* (n)</th>
<th>Chemotherapy† (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated with 105AD7 + alum or with 105AD7 + BCG and alum (45)</td>
<td>60% rectum (27), 40% colon (18)</td>
<td>20% A (9), 31% B (14), 36% C (16), 11% D (5), 2% unknown (1)</td>
<td>42% female (19), 58% male (26)</td>
<td>65.9</td>
<td>40% (18)</td>
</tr>
<tr>
<td>Controls (22)</td>
<td>55% rectum (12), 45% colon (10)</td>
<td>13% A (3), 27% B (6), 59% C (13)</td>
<td>45% female (10), 55% male (12)</td>
<td>65.7</td>
<td>45% (10)</td>
</tr>
</tbody>
</table>

*At study entry.  †Adjuvant.  ‡No surgery.

Table 2. Immune responses in colorectal carcinoma patients vaccinated with 105AD7 plus alum or with 105AD7 plus alum and BCG

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Vaccine group</th>
<th>Adjuvant chemotherapy</th>
<th>T-cell assays</th>
<th>Luminox</th>
<th>ELISPOT (γ-IFN)</th>
<th>Proliferation assay</th>
<th>TNF-α</th>
<th>GM-CSF</th>
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<tr>
<td>14</td>
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<td>18</td>
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<td>33</td>
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Abbreviations: R, responder; NR, nonresponder.
of the 17 patients who showed a proliferative response to 105AD7, 6 showed a strong response (Fig. 2D), 5 showed a moderate response (Fig. 2E), and 6 showed a weak response (Fig. 2F). There was no difference in the number of responders who were primed with BCG compared with patients just receiving 105AD7 on alum. The induction of a proliferative anti-105AD7 response was noted already after the first immunization with a mean of 6.9 in stimulation index (range, 0.3-53.1; Fig. 2A). In general, the response decreased after surgery but a second peak was seen at 5 months, after the fourth immunization with a mean of 6.7 in stimulation index (range, 0.4-44.7). After the second peak, the response gradually returned to preimmune levels. Induced proliferative responses against 105AD7 were noted in 17 of 40 vaccinated monitored patients (Table 2). All controls (n = 22) were assessed in proliferation assay (Fig. 2B) and two were defined as responders according to our criteria (see above) but only based on one time point and only in this assay.

Cytokine response (IFN-γ; ELISPOT). Of the 32 investigated immunized patients, 14 (44%) were considered to be anti-vaccine responders in the ELISPOT assay (Table 2). A classic response is shown in Fig. 3A with peak ELISPOT responses to 105AD7 of 1 per 10⁴ PBMC cells and no response to control human IgG (Fig. 3A). When the mean ELISPOT response for all
responding patients were plotted, there was again considerable patient variation in their kinetic responses but for several patients two peaks were noted, one after the first immunization before surgery and one after 1 year after seven to eight vaccinations (Fig. 3B). All assessed controls (n = 12) were recorded as negative (Fig. 3C). The difference between the number of responders in the vaccinated group versus the control group was highly significant in \( \chi^2 \) statistics (\( P = 1.5 \times 10^{-6} \)). Four patients made very strong ELISPOT responses to 105AD7 with peak frequencies of 1 per 5,000 PBMCs (Fig. 3D). Two of these patients were primed with 105AD7/BCG and two with 105AD7/alum. Six patients made a moderate ELISPOT response to 105AD7 with peak frequencies of 1 per 10,000 PBMCs (Fig. 3E) and four patients made weak responses to 105AD7 with peak responses of <1 per 10,000 PBMC (Fig. 3F).

Multiple cytokine (Luminex) assay. In the first assay, supernatants from proliferation assay and ELISPOT assays were included for evaluation of IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IFN-\( \gamma \) (data not shown), GM-CSF, and TNF-\( \alpha \) responses. These results indicated that responses toward CD55 had been induced but only for GM-CSF and TNF-\( \alpha \). Therefore, a second and third experiment was carried out with the focus to assess GM-CSF and TNF-\( \alpha \) responses only, in supernatants from the ELISPOT assay. In these latter experiments, all patients who were responders in proliferation and/or ELISPOT assay (with available supernatants) were assessed. Of the 14 assessed patients, 8 were anti-vaccine responders for TNF-\( \alpha \) and 6 for GM-CSF (Table 2). Of the 13 assessed, an induced TNF-\( \alpha \) response against the native antigen, CD55, was recorded for 7 patients and an anti-CD55 GM-CSF response for the same number of patients (Table 2). The kinetics of the induced

![Graphs](https://via.placeholder.com/150)
responses are depicted for patient 32 (TNF-α; Fig. 4A) and patient 37 (GM-CSF; Fig. 4B). The response against the vaccine, 105AD7, is accompanied by a less pronounced anti-CD55 response. Several patients received adjuvant chemotherapy but the number of immune responders in this group is similar to the group not receiving chemotherapy.

**T-cell response and relation to clinical outcome.** This trial was not designed to study a possible effect of the vaccinations on survival. Nineteen of the patients died during follow-up. Two patients died from lung cancer and two from myocardial infarction. Fifteen patients died from colorectal cancer. Seven of these patients had advanced disease at the time point of primary surgery and an additional patient with Duke’s C disease was incompletely resected. Ten patients relapsed during the follow-up period. Two (14%) responders in ELISPOT (IFN-γ) relapsed compared with 5 (28%) in the nonresponder group. The numbers are too small to show any effect of vaccination on survival.

**Discussion**

Sixty-seven colorectal cancer patients were randomized before primary surgery to immunization with an anti-idiotypic antibody, 105AD7, mimicking CD55 and no treatment. This is the first report on the thus far largest adjuvant 105AD7 vaccination trial.

Several anti-idiotypic antibodies mimicking tumor-associated antigens have been used in clinical trials and have been shown to induce antibodies that recognize both the anti-idotype and antigen (12–15). No antibody responses were recorded in the present trial (data not shown), which is consistent with previous clinical 105AD7 vaccine studies (4–6). The lack of humoral responses was probably due to the low alum dose because 105AD7 combined with a high dose of alum induced antibody responses in mice (16). Some of these anti-idiotypes also induce anti-tumor T-cell responses (13, 17). However, the advantage of 105AD7 is that it is a human monoclonal antibody that can target Fc receptors on dendritic cells giving more efficient presentation of T-cell epitopes and a high frequency of response (1 per 5,000 PBMCs are specific for 105AD7). This study shows that the induced T cells also recognize and respond to the target antigen CD55.

Proliferation assay was applied as the primary readout system for vaccine induced responses in this study. In addition, IFN-γ responses (ELISPOT) were monitored in the vast majority of the patients. To reduce the variability, all samples for an individual patient were always assessed in the same experiment. The proliferation and ELISPOT (IFN-γ) assays may not overlap, as antigen-specific cells may respond to antigen stimulation by proliferation and/or cytokine production dependent on involved T-cell subsets and stages of maturation (18, 19). The combination of assays is needed to thoroughly determine the response (20, 21).

For the majority of the immunized patients, a T-cell response against the vaccine was recorded in proliferation and/or ELISPOT (IFN-γ) assay. Further characterization of the T-cell response with regard to which T-cell subpopulation is responsive would have been desirable. However, because of the limited number of PBMCs, there were not enough cells to carry out these assays. As previously published, the peptides in 105AD7, which mimic CD55, are discontinuous and do not stimulate T-cell responses. This suggests that there is limited homology between CD55 and 105AD7 T-cell epitopes.

Unfortunately, there were insufficient numbers of PBMCs to screen all the potential peptides from 105AD7 and CD55. Recently, it has been suggested that T-cell epitopes in 105AD7 are in NH2-terminal region. We have used these peptides in our ELISPOT assays but no responses were observed, suggesting that these are not T-cell epitopes (22). We have also assessed potential 105AD7 and CD55 T-cell epitopes in patients with HLA-A1, HLA-A13, and HLA-A124 and HLA/DR1, HLA/DR3, and HLA/DR7 phenotypes in a previous study (23). Of the 14 responders in ELISPOT (IFN-γ), only 50% were responders in proliferation assay, indicating no correlation at all between these two assays. This may be due to proliferation assays, which predominantly measure CD4 responses, whereas the ELISPOT measures both CD4 and CD8 cells secreting IFN-γ. A tendency for two peaks in the response was noted in both T-cell assays. The first peak follows the start of the immunization schedule and reflects an induction of the response. This first peak is followed by a decline after surgery, which may reflect a negative effect by surgery on the immune system (24). The second peak is observed some months later, after further immunizations.

To our knowledge, this is the first cancer vaccination trial, in which GM-CSF and TNF-α responses have been assessed. There seems to be a positive correlation between IFN-γ (ELISPOT) and TNF-α (Luminex) secretion with a concordance in 10 of 14 (71%) with 105AD7 stimulation. Most notably, an induced anti-CD55 response was observed for 9 of 13 (69%) of assessed patients in the Luminex assay (GM-CSF and TNF-α). The concordance for GM-CSF and TNF-α anti-CD55 responses was...
9 of 13 (71%). TNF-α and GM-CSF are known to have potent antitumoral effects (25, 26). TNF-α induces apoptosis in cancer cells (25). Some efficacy has been seen with TNF-α treatment in pancreatic cancer patients (27) and colorectal cancer patients (28). High-dose TNF-α plus chemotherapy produced between 70% and 80% complete remission in cases of in transit melanoma metastases and between 25% and 36% complete remission in cases of inoperable soft-tissue sarcomas (29). Vaccination of mice with a tumor vaccine induced secretion of TNF-α (30). GM-CSF is vital for antigen presentation (31, 32) and has successfully been used as an adjuvant in colorectal cancer vaccine trials (20, 21). Vaccination of mice with a tumor cell vaccine indicated that GM-CSF production is a determining factor for treatment efficacy (26).

TNF-α is produced by T cells (33), monocytes (34), and natural killer cells (35). GM-CSF could be produced by a wide range of cells (36), including T cells and monocytes (37).

It could be assumed that a prerequisite for an anti-CD55 response would be an induced response against the vaccine. However, in a few patients (n = 3), an anti-CD55 response in the Luminex assay was observed without a corresponding increase in the anti-105AD7 levels. This might be explained by enhanced antigen presentation of CD55 derived from tumor cells. The same mechanism could explain the reason for the proliferative responses noted in two control patients.

There were fewer patients relapsing in the T-cell responder groups. Although this is an interesting finding, the material is too heterogeneous (rectal and colon cancer patients, different stages, etc.) and limited to draw any conclusions from this observation. Importantly, despite CD55 being a self-antigen, no adverse effects, except for local reactions, including autoimmune reactions, were observed.

In summary, vaccination with 105AD7 induced T-cell responses in the majority of the patients. T-cell and cytokine responses to both the anti-idiotypic and the CD55 antigen were measurable, adding support to the use of CD55 as a target in cancer treatment. The data presented from the two T-cell assays and the Luminex assay used highlight the importance of monitoring responses in cancer vaccine trials with different assays to accurately assess the response.

Acknowledgments

We thank Cancer Research-UK, Onyxav, and all the patients, and R. Moss for technical assistance.

References

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