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Advances in Brief

Vaccine-induced Apoptosis: A Novel Clinical Trial End Point?[1]

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Abstract

The functional end point of immunotherapy is to induce tumor regression. Because immune effector mechanisms usually result in apoptosis, the aim of this study was to determine whether measurement of tumor apoptosis *ex vivo* is a good end point to evaluate the efficacy of cancer vaccines. A prototype vaccine, 105AD7, was administered to colorectal cancer patients before resection of their primary tumors. There was a significant increase in apoptosis of tumor cells within immunized patients compared with control patients as assessed by immunohistochemistry ($P = 0.005$; $n = 16$) or by flow cytometry ($P = 0.003$; $n = 34$). Preoperative immunization and measurement of tumor cell apoptosis may be a valuable clinical end point for evaluation of new vaccine and other biological approaches.

Introduction

A better understanding of the molecular basis for immune recognition has led to the development of many new and exciting approaches to cancer immunotherapy (1). There is an increasing need to adopt a rational trial design to assess the antitumor effects of these approaches. Phase I toxicity trials are necessary to verify the safety of any new agent. However, trials designed to assess maximum tolerated doses are not applicable for immunotherapeutics because increasing the dose does not necessarily increase the effectiveness of the agent (2). It may be possible to optimize immunotherapy by monitoring immune responses induced in patients. However, in several recent trials in melanoma, tumor regression was observed in the absence of peripheral blood immune responses (3, 4). Because immune cells only transit in the blood *en route* to target tissues, the most effective antitumor immunization would result in immune responses within the tumor; however, this may be difficult to assess in advanced solid tumors. A further complication is that very few immunotherapeutics induce objective regression of advanced cancer. This may be because the immune response stimulated is insufficient to have a significant impact on bulky disease, although other factors associated with advanced disease such as immunosuppressive effects and evolution of escape mechanisms by the tumor may also be involved. Immunotherapy may be more successful when administered to patients with minimal residual disease (5). For example, the mAb[3] 17-1A was able to reduce recurrence and enhance survival when administered to Dukes' C cancer patients (6). Unfortunately, this trial required 189 patients and took 5 years to evaluate. Thus, large numbers of patients would be involved in studies lasting several years, making it unfeasible to assess numerous new approaches simultaneously. There is therefore a need to design rapid trials in patients with early-stage tumors to allow selection of the most promising agents. New therapies that have been shown to be nontoxic in Phase I clinical trials could be used to immunize patients at diagnosis before surgical resection. This would allow direct monitoring of immune responses on the resected tumor.

105AD7 is a human anti-idiotypic antibody that mimics the CD55 antigen (7). In a Phase I clinical trial in advanced colorectal cancer patients, both helper and cytotoxic immune responses were induced in the peripheral blood (8). T cells that proliferated to either 105AD7 or to tumor cells expressing CD55 antigen were measured (9). Enhanced plasma interleukin 2 was detected, and an accumulation of CD4/CD45RO and CD8/CD45RO cells was seen with successive immunizations. However, although three patients showed prolonged periods of stable disease, no regression of any liver metastases was observed. We therefore assessed immune infiltration in metastatic colon cancer by evaluating hepatic metastases; however, insufficient tissue was obtained from most patients to give a representative view of the lesion. A new trial was therefore designed whereby patients were immunized at diagnosis and before resection of their primary tumor. The resection specimen could then be analyzed for immune effector cells. Enhanced infiltration of CD4 and NK cells was observed in 105AD7-immunized tumors as compared with grade- and stage-matched tumors from control patients (10). Furthermore, the infiltrating cells within the immunized tumors expressed significantly more CD25 than control tumors (11). This suggested that the immune cells did reach the tumor and remain active. However, the real goal was to show that these activated cells could induce tumor cell death. Immune effector cells cause apoptosis of their target cells either directly or by cytokine release. We therefore investigated whether measuring tumor cell apoptosis could be a sensitive method to assess the antitumor effectiveness of the 105AD7 vaccine and whether this may represent a rational approach for evaluation of other immunotherapies.

Materials and Methods

Patients. This trial was run under the auspices of the CRC, United Kingdom, Phase I Targeting Trial Committee. Local ethical approval was obtained from the recruiting hospital. Patients with histopathologically proven colorectal adenocarcinoma who were scheduled for elective surgery were recruited. Patients had to have a WHO performance status of 0–2, a hemoglobin of >10 g/dl, a WBC count of $>2 \times 10^9$/liter, and platelets $> 50 \times 10^9$/liter. All patients had normal renal and liver function (no more than 25% deviation from normal values). All patients gave written informed consent and were registered with the CRC data center. Patients with any acute intercurrent illness, with autoimmune or chronic hematological disorders, or receiving other concomitant anticancer therapy were excluded. No women of child-bearing age or receiving planned preoperative radiotherapy to primary rectal tumors were included. Patients could receive postoperative chemotherapy if indicated. However, 3 months after the completion of chemotherapy, they completed their postoperative 105AD7 course.

Human mAb. Clinical grade human mAb was produced as described previously (9) using the guidelines of the CRC (12). Samples of the seed lots passed testing for sterility and viral contamination. Antibody for clinical use was prepared as either 10 μg of antibody in sterile saline for skin test doses or as an aluminum hydroxide gel (alHydrogel 85; Superphos Biosector, Vebaek, Denmark) precipitated i.m. doses of 100 μg antibody/ml. The antibody can be

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[3] The abbreviations used are: mAb, monoclonal antibody; NK, natural killer; CRC, Cancer Research Campaign.

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stored at 4°C. Stability studies have shown that the antibody can be stored at 4°C for a minimum of 5 years with no loss in binding activity.

Clinical Protocol. Twenty-one of the total 50 patients recruited were immunized with the human mAb 105AD7. The clinical protocol initially stated that patients should receive an intradermal skin test of 105AD7 (10 μg), and if there was no adverse reaction after 24 h, they could then receive the i.m. dose of 105AD7 precipitated on alum (50 or 100 μg). Patients received an initial dose of 105AD7 at diagnosis of their colorectal cancer and then were boosted at 6-weeks, 12-weeks, and then at 3-month intervals after their surgical resection. The remaining patients were not immunized and were treated as a control group.

Immunohistochemistry. A pathologist took samples from two edges of the tumor where possible. Tumor tissue from both immunized and nonimmunized (age- and gender-matched) patients was stored in liquid nitrogen. Tumor blocks from both the edges of an immunized tumor and a stage-, grade-, and site-matched control tumor selected from the tumor bank were selected. Sections (5 μm) were fixed in acetone for 10 min and labeled with mAb APO 2.7 (Ref. 13; Beckman Coulter, Luton, United Kingdom) or normal mouse IgG1 (Sigma, Dorset, United Kingdom), using an indirect avidin-biotin complex technique (Dako, Ely, United Kingdom).

Image Analysis. Sections were viewed under ×125 magnification, and the image was digitized and transferred by camera to an Apple Macintosh Quadra 660AV computer. Using the NIH image program, it was possible to quantify the degree of staining and express it as a pixel count. Sections were analyzed from two edges of the tumor. Infiltration was quantified on five randomly selected areas for each section. Immunized and nonimmunized tumors were coded before staining and analysis to ensure that there was no observer bias. Pixel counts on immunized and matched nonimmunized tumors were decoded and analyzed for significance by a two-tailed paired Wilcoxon signed rank test.

Flow Cytometry. Fresh samples were obtained from four growing edges of the tumor and finely minced. They were then dissociated in 0.05% collagenase (Boehringer Mannheim, Lewes, United Kingdom) and 0.1% DNase (Boehringer Mannheim). Cells for Apo2.7 staining were fixed immediately in 0.5% formaldehyde, and an aliquot of unfixed cells was stained with FITC-labeled annexin V (Biowhittaker, Workingham, Berks, United Kingdom) and propidium iodide according to the manufacturer's protocol. Saponin (0.1%; Sigma Chemicals, Poole, United Kingdom)-permeabilized cells were stained with Apo2.7-PE-conjugated mAb (Beckman Coulter, Luton, United Kingdom) or PE-conjugated mouse IgG in control tubes, together with the epithelial marker BerEp4 FITC-conjugated mAb (Dako) or the leukocyte marker CD45 FITC-conjugated mAb (Dako). BerEp4 binds to both normal and malignant epithelial cells. Analysis was performed on a Becton Dickinson FACScan, and 5,000–10,000 events were collected; Apo2.7 staining was measured in epithelial cells and leukocytes gating on the BerEp4-positive cells and CD45-positive cells, respectively.

The percentage of apoptotic cells within immunized tumors was compared with stage-, grade-, and site-matched tumors from nonimmunized patients and analyzed for significance by a two-tailed paired Wilcoxon signed rank test. Nonpaired data were analyzed for significance using a nonparametric Wilcoxon signed rank test.

Results

The cancer vaccine 105AD7 had been shown to induce enhanced infiltration of T cells and NK cells within the tumors of immunized patients as compared with control patients matched for age, gender, and tumor location, grade, and stage. These tumors were therefore analyzed for apoptosis. Tumor sections were cut and stained by indirect immunoperoxidase staining with mAb Apo2.7. Three sections were analyzed from each block, and five fields were quantified by image analysis on each section (Table 1). There was a significant increase in apoptosis in the 105AD7-immunized tumors compared with control tumors (median, 3.12 pixel count *versus* 1.78 pixel count; $P < 0.005$) when analyzed by a Wilcoxon two-tailed test paired comparison for immunized *versus* control tumors for each region. Tumors from immunized and control patients were also stained with an IgG1 control antibody. Staining was low, and there was no significant difference between immunized and control tumors. The pixel counts for Apo2.7 for each tumor were then summated, and Fig. 1 shows the cumulative pixel count for each immunized tumor as compared with its matched control. Six of eight immunized patients had higher levels of apoptosis than matched controls. To attempt to exclude sampling error as a possible explanation for this, a new prospective study was designed whereby all tumor tissue not necessary for pathological evaluation was disaggregated, stained, and analyzed by flow cytometry.

Cell death within resected tumors was initially measured by Apo2.7, which measures the mitochondrial antigen 7A6 that is ex-

Table 1 *Apo2.7 immunohistochemical staining and image analysis of tumor from patients immunized with 105AD7 or matched control tumors*

Patient	Age[a] (yr)	Gender	Operation[b]	Site[c]	Stage[d]	Pixel counts[e]
Immunized						
1	79	F	14	Sigmoid	B	395, 249, and 368
						351, 412, and 565
2	76	M	14	Sigmoid	A	161, 192, and 265
						349, 342, and 324
3	75	M	26	Rectum	A	175, 241, and 286
4	56	M	9	Rectum	B	766, 693, and 566
5	70	M	13	Rectum	B	64, 87, and 60
6	62	F	20	Rectum	C	206, 191, and 182
						90, 196, and 82
7	87	M	20	Cecum	B	632, 523, and 451
8	82	M	11	Cecum	C	292, 289, and 222
Controls						
1	75	F		Sigmoid	B	217, 173, and 59
						167, 188, and 177
2	79	M		Sigmoid	A	262, 155, and 170
						182, 210, and 163
3	70	M		Rectum	A	152, 96, and 156
4	60	M		Rectum	B	214, 262, and 222
5	73	M		Rectum	B	53, 62, and 81
6	65	F		Rectum	C	299, 221, and 300
						363, 356, and 204
7	81	M		Cecum	B	165, 177, and 176
8	75	M		Cecum	C	87, 94, and 104

[a] Age of patient at trial entry.
[b] Time in days between immunization and tumor resection.
[c] Site of primary tumor.
[d] Dukes' stage.
[e] Mean pixel count for five areas of a tumor section stained with Apo2.7. The same regions of immunized and control tumors were paired and analyzed for significance by a two-tailed Wilcoxon signed rank test. Significantly ($P < 0.005$) more infiltration was seen in immunized tumors than in control tumors.

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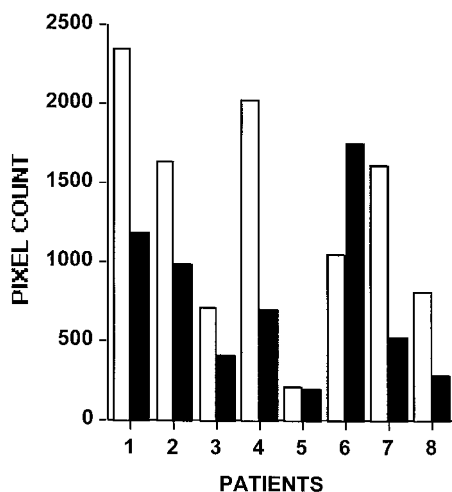

Fig. 1. Tumor sections from patients immunized with 105AD7 (□) or nonimmunized controls (■) were stained by indirect immunoperoxidase staining with the antiapoptotic mAb Apo2.7. Cumulative pixel counts for each tumor are presented.

posed as an early event of apoptosis. Tumor cells were disaggregated by a 1-h incubation with collagenase and then fixed before Apo2.7 staining, thus minimizing the time and processing steps to which the unfixed cells were subjected. Apo2.7 staining also had the advantage that dual staining with cell type-specific antibodies could be used to identify the cells undergoing apoptosis. Thirteen patients were immunized with 105AD7 preoperatively (median, 14 days between immunization and surgery). There were eight males and five females; six patients had rectal tumors, and seven patients had colon cancers. All tumors were of moderate grade. One patient had Dukes' A cancer, five patients had Dukes' B cancer, and seven patients had Dukes' C cancer. Twenty-one tumors from nonimmunized patients were also processed. There were 12 males and 9 females; 8 patients had rectal tumors, and 13 patients had colon tumors. All tumors were of moderate grade. One patient had Dukes' A cancer, 7 patients had Dukes' B cancer, 12 patients had Dukes' C cancer, and 1 patient had Dukes' D cancer. Similar proportions of epithelial cells (53% *versus* 56%) and leukocytes (12% *versus* 10%) were released by collagenase from the immunized and control tumors. Fig. 2 shows a scatter plot of the proportion of apoptotic cells from immunized and control tumors. There is an increase in apoptosis in the tumor cells of the immunized patients as compared with nonimmunized tumor cells (22% *versus* 7.8%; $P = 0.0023$). However, there was no difference in apoptosis of leukocytes from either immunized or control tumors (7.2% *versus* 5.5%; $P = 0.55$). Interestingly, the level of apoptosis in the tumors of nonimmunized patients was similar to the level of apoptosis of the intratumor leukocytes (7.8% *versus* 5.5%; $P = 0.50$) from these tumors, suggesting that both cell types had a similar turnover.

Thirteen nonimmunized patients were selected as age-, gender-, site-, stage-, and grade-matched controls for the vaccine-immunized patients (Fig. 3). Apoptosis was significantly elevated in the tumors of immunized patients compared with control tumors (22% *versus* 7.0%; $P = 0.003$). However, there was no difference in the proportion of apoptotic leukocytes from immunized tumors as compared with control tumors.

Discussion

Although early trials with nonspecific immunotherapy including interleukin 2 therapy, adoptive transfer of tumor-infiltrating lymphocytes, and whole cell vaccines have shown some encouraging results (14), these results were frequently seen on only a small subset of cancers and usually in melanoma patients. In the last decade, a much better understanding of the molecular basis for immune recognition and activated has been elucidated. This has led to the identification of new antigens recognized by T cells and to many new approaches to specifically activate antitumor immunity (1, 14). However, it is still unclear how these new agents should be evaluated in the clinic because few Phase I trials have resulted in regression of advanced disease. This may be a reflection of a numerical balance between tumor cells and immune effector cells that may be irrevocably tipped in favor of tumor in patients with bulky disease and/or the tumors acquiring resistance to immune attack.

An effective cancer vaccine must stimulate all arms of the immune response including helper T cells that aid both antibody and cytotoxic T-cell production but also release cytotoxic cytokines (IFN-γ and tumor necrosis factor β) and recruit nonspecific effector cells such as NK cells and tumoricidal macrophages. All of these immune mediators may induce apoptosis in their target tumor cells. Measurement of tumor cell apoptosis may therefore prove to be a good method of assessing the overall efficacy of a vaccine. In colorectal cancer patients, there is a window of opportunity of 2–3 weeks between diagnosis and resection of the primary tumor to allow for such an evaluation to be made. This study shows that if patients are immunized with the prototype vaccine 105AD7 immediately after diagnosis of their tumor, it is possible to measure antitumor effects of the

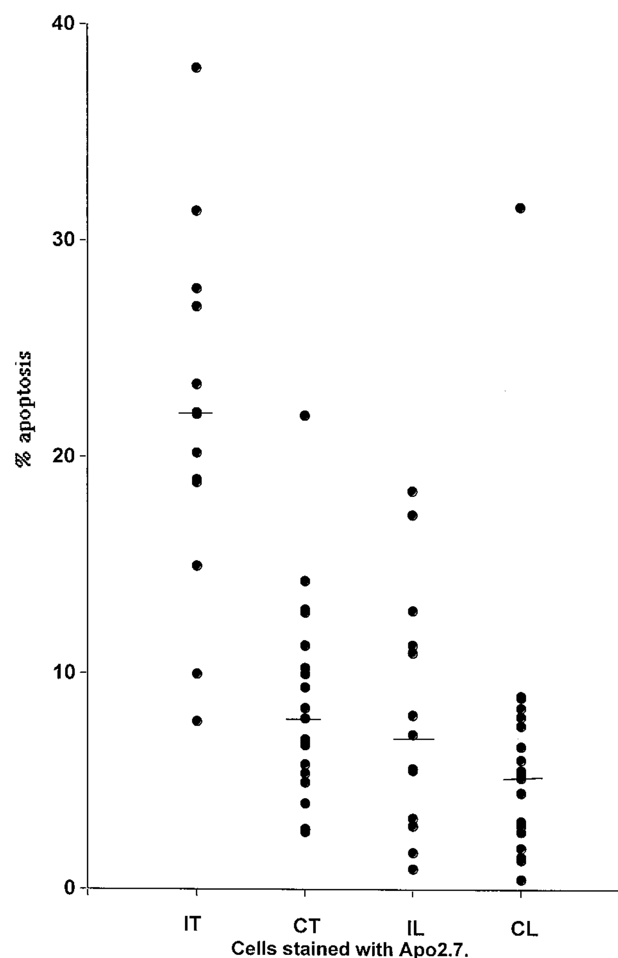

Fig. 2. Tumors from 105AD7-immunized (*I*) or nonimmunized control patients (*C*) were disaggregated with collagenase/DNase. Tumor cells stained with the antiepithelial antibody BerEp4 (*T*) or leukocytes stained with the antileukocyte antibody CD45 (*L*) were gated and analyzed for costaining with the antiapoptotic mAb Apo2.7.

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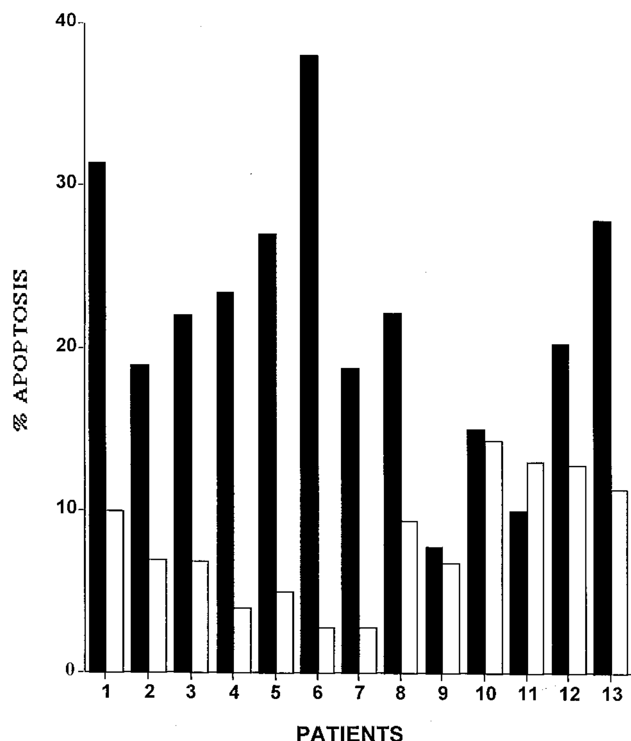

Fig. 3. Tumors from 105AD7-immunized (■) or nonimmunized control patients (□) were disaggregated with collagenase/DNase. Tumor cells stained with the antiepithelial antibody BerEp4 were gated and analyzed for staining with the antiapoptotic mAb Apo2.7.

vaccine by measuring immune cell infiltration and by enumerating the degree of apoptosis of tumor cells in the resected cancer.

The 105AD7-immunized tumors exhibited enhanced apoptosis compared with matched control tumors as assessed by immunohistochemistry with mAb Apo2.7 (13). This suggested that 105AD7 stimulated immune cells that infiltrated the primary tumor and caused extensive cell death. However, quantitative image analysis is difficult because infiltration can vary greatly in different regions of the tumors, and slight variations in tissue thickness can also have dramatic effects on results. To avoid these problems, and also because it was unclear whether the enhanced apoptosis was a result of the immune effector cell or tumor cell death, a prospective study of 13 patients immunized with 105AD7 and 21 nonimmunized control patients was therefore performed to address these issues. Immunized patients were injected with 105AD7 at diagnosis of their colorectal tumor, and control patients were not immunized. Apoptosis in this study was measured on disaggregated tumor samples collected at resection.

Apoptosis was initially measured by annexin V staining, but the processing time and the necessity of staining, washing, and analyzing unfixed cells resulted in increased tumor cell death. Apo2.7 staining was possible on cells processed and fixed much more quickly, and the percentage of apoptotic tumor cells in control tumors using this protocol more closely correlated with levels reported by terminal deoxynucleotidyl transferase-mediated nick end labeling staining of colorectal tumor tissue sections (3.6–11%; Ref. 15). The 105AD7 cancer vaccine induced a 3-fold increase in the proportion of apoptotic tumor cells in 13 immunized patients compared with stage-, grade-, and site-matched nonimmunized controls, and the degree of apoptosis in control tumors was similar in all Dukes' stages. Earlier studies have shown that apoptosis decreases in the transition from normal mucosa to polyp to carcinoma, but that subsequent tumor progression has little effect (16). It was of interest that the proportion of apoptotic tumor cells was similar to the proportion of apoptotic leukocytes in control tumors, suggesting that both populations have a similar turnover. Also there was no difference in the number of apoptotic leukocytes between immunized and control tumors, suggesting that there was no evidence for dramatic killing of infiltrating leukocytes in the tumor environment. In contrast, immunization with 105AD7 selectively results in tumor cell apoptosis.

There has been much debate as to whether tumor vaccines targeting overexpressed self-antigens can stimulate antitumor immune responses. However, there is now a plethora of data to support this. There has also been a lack of direct evidence that the immune response observed in the blood of immunized patients would be mirrored in the tumor environment. In fact, there is evidence that the tumor environment is particularly hostile to cell-mediated immunity, with reports of tumors expressing transforming growth factor β and Fas ligand, although recent reports have suggested that Fas ligand is more likely to stimulate rather than suppress an inflammatory response (17–19). In this study, it has been possible to show, at least in primary colorectal cancer, that a vaccine mimicking CD55 antigen can induce immune responses that result in a significant increase in tumor cell apoptosis. We would suggest that this trial design (using an apoptosis index) could represent a valid end point for Phase II trials assessing immunotherapy. It would also be possible to assess tumor infiltration by immune effector cells. 105AD7 induced infiltration of NK cells and T cells that expressed the activation marker CD25 as assessed by quantitative immunohistochemistry (11). However, recent advances in immunology may allow a more accurate quantification of vaccine-specific T cells within both the blood and the tumor of immunized patients. MHC tetramers (20) or MHC Fc chimeric molecules (21) can be folded around specific T-cell epitopes and then used to stained for epitope-specific T cells. Similarly, short-term *in vitro* culture with antigen and measurement of intracellular cytokines can give accurate frequencies of antigen specific T cells (22).

The neoadjuvant approach can measure immune infiltration of tumors and can be used to assess the ability of the induced immune response to kill tumor cells within primary lesions. This in itself will justify for each individual patient a rationale for continuing immunization. However, it may not predict survival. We do not know the effects of this therapy on micrometastases that may be left after surgery. The effect on overall survival will need to be assessed in classical Phase III trials with tumor recurrence and patient survival as the primary end points. Likewise, to achieve long-term survival, it may be necessary to induce a sustained immune response over several years to induce a continuous response to what is a self-antigen. Therefore, although measuring tumor cell apoptosis in response to therapy may not necessarily predict outcome, it may be sufficient to justify large Phase III randomized trials to evaluate new immune therapies that result in increased apoptosis at the primary tumor site. These trials can be performed rapidly because response is evaluable within 3–4 weeks at tumor resection. Measuring tumor apoptosis with Apo2.7 is sensitive, accurate, and simple, and in this trial, using colorectal cancer vaccine 105AD7, Apo2.7 shows significant differences between treated and control patients. Additional studies are needed to test this approach in the evaluation of other immunotherapies.

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