Intratumoral Vaccination and Diversified Subcutaneous/ Intratumoral Vaccination with Recombinant Poxviruses Encoding a Tumor Antigen and Multiple Costimulatory Molecules

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ABSTRACT

Purpose: **Intratumoral (i.t.) vaccination represents a potential modality for the therapy of tumors. Previous i.t. vaccination studies have focused on the efficacy of i.t. vaccination alone. There are no reports that clearly compared i.t. vaccination with systemic vaccination achieved by s.c., intradermal, or i.m. injection, or combining both modalities of systemic and i.t. vaccination. Here, we compared the antitumor effects induced by a systemic vaccination regimen (s.c.) and i.t. vaccination, and a sequential s.c/i.t. vaccination regimen. In this study, we used a recombinant vaccinia virus containing the transgenes for carcinoembryonic antigen (CEA) and a triad of T-cell costimulatory molecules (B7–1, ICAM-1, and LFA-3; designated rV-CEA/TRICOM) for s.c. priming and a replication defective avipox (fowlpox) virus containing the same four transgenes (designated rF-CEA/TRICOM) for i.t. vaccination or s.c. booster vaccinations.**

Experimental Design: **Vaccination was started on day 8 after s.c. implantation with CEA-positive tumors. We compared the antitumor activity induced by these vaccines when administered via the i.t. route *versus* the s.c. route. Subsequent therapy studies examined the sequential combination of these routes, s.c. priming with rV-CEA/TRICOM followed by i.t. boosting with rF-CEA/TRICOM. Initial studies were conducted in conventional mice to define optimal vaccine regimens and then in CEA-transgenic mice that expressed CEA as a "self" antigen in a manner similar to that of an advanced colorectal cancer patient.**

Results: **The results demonstrate that the antitumor activity induced by i.t. vaccination is superior to that induced by s.c. vaccination. For more advanced tumors, a s.c. priming vaccination, followed by i.t. boosting vaccinations was superior to either s.c. or i.t. vaccination alone. Both of these phenomena were observed in tumor models where the tumor-associated antigen is a foreign antigen and in a CEA-transgenic tumor model where the tumor-associated antigen is a self-antigen. The cytokine, granulocyte macrophage colony-stimulating factor admixed in vaccines, was shown to be essential in inducing the antitumor activity.**

Conclusions: **These studies demonstrate that the diversified vaccine regimens that consisted of s.c. prime and i.t. boosts with CEA/TRICOM vectors could induce antitumor therapy superior to that seen by either route alone.**

INTRODUCTION

Cancer vaccines are being investigated for the therapy of tumors. Many tumor-associated antigens (TAAs) have been identified, and a variety of tumor-specific vaccines have been generated and developed (1–4). However, many antigens expressed on tumors have not been identified. Therefore, investigators have been examining whole tumor cells as potential vaccines, because these are thought to contain the entire antigenic repertoire of the tumor. Tumor cells, either autologous (5, 6) or allogeneic (7), have been manipulated *ex vivo* with cytokines (8–10) or costimulatory molecules (5) before being reintroduced to the patient. An alternative approach would be the direct introduction of immunostimulatory molecules into the tumor milieu, thereby exploiting the presence of multiple tumor antigens, known and unknown, to induce and potentiate tumor-specific immune responses.

Intratumoral (i.t.) approaches can be divided into two types based on the proposed antitumor mechanism: (*a*) direct eradication of tumor cells after the introduction of genetic materials, *e.g.,* a replacement or inactivation of defective genes such as *p53* in tumor cells, leading to tumor cell apoptosis (11–13); or (*b*) indirect approaches mediated by immune cells, *e.g.,* the introduction of immunostimulatory molecules or vectors encoding immunostimulatory transgenes to influence the immune milieu of the tumor, culminating in the introduction of antitumor immune responses. For this purpose, biological response modifiers such as *Bacillus* Calmette-Guérin were originally used intratumorally not only in animal models (14–17) but also in clinical trials (18, 19). Recently, particular cytokines [*e.g.,* interleukin 2, tumor necrosis factor α, granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin 12] have been noted to elicit antitumor immunity effectively, and viral vectors (*e.g.,* vaccinia virus and herpes simplex virus) encoding a cytokine have been injected intratumorally in animal models (4, 20–26) and used in clinical trials (27, 28). Also, adenovirus vectors encoding individual costimulatory molecules have been introduced intratumorally to enhance tumor-antigen-specific T-

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cell responses (4, 29–32). However, efficacy of i.t. vaccination has not been compared with conventional vaccination or as part of a diversified route of a vaccination regimen (s.c./i.t.) to additionally potentiate antitumor immunity. Moreover, no previous studies have used vectors intratumorally to amplify the expression of a given tumor antigen, and none have used vectors containing more than one costimulatory molecule.

We have described previously poxvirus vaccine vectors that contain the carcinoembryonic antigen (*CEA*) transgene, transgenes for a triad of T-cell costimulatory molecules (*B7–1*, *ICAM-1*, and *LFA-3*; designated TRICOM), and transgenes for CEA in combination with TRICOM. Two types of poxvirus vectors were used, replication-competent recombinant vaccinia (rV) and replication-defective recombinant fowlpox (rF; Refs. 33–36). In a peripancreatic metastasis tumor model, it has been shown previously that: (*a*) rV-CEA/TRICOM or rF-CEA/TRICOM was more effective than rV-CEA and rF-CEA; and (*b*) a diversified prime and boost regimen where rV-CEA/TRICOM was used as a prime and rF-CEA/TRICOM as a boost was more efficacious than s.c. priming and s.c. boosting vaccinations with rF-CEA/TRICOM in the induction of CEA-specific T-cell responses and antitumor activity (34, 36). In this study, we sought to compare the magnitude of antitumor activity induced by systemic *versus* i.t. vaccination in the hope that i.t. vaccination would not only induce immunity to the endogenous antigen, but also additionally increase its immunogenicity by hyperexpression of the endogenous antigen. Moreover, we examined the diversified vaccination by i.t. boosting after s.c. priming.

Finally, studies were conducted in mice in which CEA is a self-antigen in an attempt to understand the potential clinical relevancy of i.t. vaccine therapy. These CEA-transgenic mice express CEA in normal gastrointestinal tissue and fetal tissue in a manner similar to that expressed in humans (37, 38). Additionally, CEA-transgenic mice have CEA protein in sera at levels similar to those found in patients with advanced colorectal carcinoma (5–100 ng/ml). We demonstrate here for the first time: (*a*) the superiority of i.t. vaccination *versus* conventional s.c. vaccination in inducing antitumor effects; (*b*) the superiority of the antitumor activity induced by s.c. vaccination followed by i.t. boosting to that induced by s.c. prime and boost vaccinations; (*c*) the importance of having i.t. vectors also containing a transgene for a TAA (CEA) in mediating antitumor activity; and (*d*) the importance of priming (s.c.) with a recombinant vaccinia virus *versus* priming (s.c.) with recombinant fowlpox virus when using a diversified s.c./i.t. vaccination regimen.

MATERIALS AND METHODS

Animals and Tumor Cells. Female C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). C57BL/6 mice transgenic for human CEA were obtained from a breeding pair provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). The generation and characterization of the CEA-transgenic mouse has been described previously (37, 38). Mice were housed and maintained under pathogen-free conditions in microisolator cages until used for experiments at 6–8 weeks of age.

Parental murine colon adenocarcinoma MC38 cells and MC38 expressing human CEA (designated MC38-CEA$^+$) were used (39). Before implantation to mice, these adherent cells were trypsinized and washed in HBSS.

Recombinant Poxviruses. The recombinant vaccinia and fowlpox viruses containing the murine *B7–1*, *ICAM-1*, and *LFA-3* genes (designated rV-TRICOM and rF-TRICOM, respectively) or in combination with the human *CEA* gene (designated rV-CEA/TRICOM and rF-CEA/TRICOM, respectively) have been described (33, 40). The recombinant fowlpox virus containing the gene for murine GM-CSF (designated rF-GM-CSF) has been described (41). Nonrecombinant vaccinia virus (Wyeth strain) was designated V-WT and nonrecombinant wild-type fowlpox virus was designated FP-WT. Therion Biologics Corp. (Cambridge, MA) kindly provided all of the orthopox viruses.

I.t. Vaccination Studies. Mice were s.c. implanted with MC38-CEA$^+$ tumor cells (3×10^5 cells/mouse) into the right flank. In protocols not using priming with recombinant vaccinia viruses, mice were vaccinated i.t. with rF-CEA/TRICOM on days 8, 15, 22, and 29 after tumor implantation. In protocols with priming, mice were vaccinated s.c. with rV-CEA/TRICOM on day 8, and i.t. with rF-CEA/TRICOM on days 15, 22, and 29. In indicated experiments, mice received s.c. vaccination in place of i.t. vaccination. Each virus was injected at 10^8 plaque-forming units/mouse admixed with 10^7 plaque-forming units/mouse of rF-GM-CSF except where indicated otherwise. Tumor size was measured using calipers 1–2 times a week. Tumor volumes were calculated as follows: Tumor volume (mm^3) = $0.5 \times$ Length $\times$ Width^2. In all of the experiments, mice were sacrificed when either size (length or width) of tumors exceeded 20 mm.

Statistical Analysis. Statistical significance for differences was evaluated using ANOVA with repeated measures using Statview 4.1 (Abacus Concepts Inc., Berkeley, CA). For graphical representation of data, *Y*-axis error bars indicate the SD of the data for each point on the graph.

RESULTS

Efficacy of i.t. Vaccination with TRICOM Vectors. Previous studies have shown that s.c. vaccination using CEA/TRICOM vectors could eliminate established tumors when vaccination was begun 3 or 4 days after s.c. tumor transplant at a distal site from vaccination. In the studies presented here, vaccination was administered 8 days after s.c. tumor transplant. We first compared the antitumor effects elicited via i.t. vaccination and s.c. vaccination on day 8-established MC38-CEA$^+$ tumor models. The mean tumor volume of a day 8-established tumor was 40 $mm^3 \pm 6$ mm^3. As a control, mice were administered PBS i.t. on days 8, 15, 22, and 29 after tumor implantation (Fig. 1*A*). Tumors grew in this group at the same rate as unmanipulated tumors, indicating that mechanical damage by i.t. injection was not effective in suppressing tumor growth. When mice were vaccinated i.t. with control fowlpox vector FP-WT four times at weekly intervals, tumor growth was suppressed in some mice, and 1 of 10 tumors disappeared (Fig. 1*B*); this antitumor effect was significant to that in the PBS-treated group ($P = 0.0006$). This is most likely due to the introduction of foreign gene products of fowlpox into the tumor and/or the fact that the

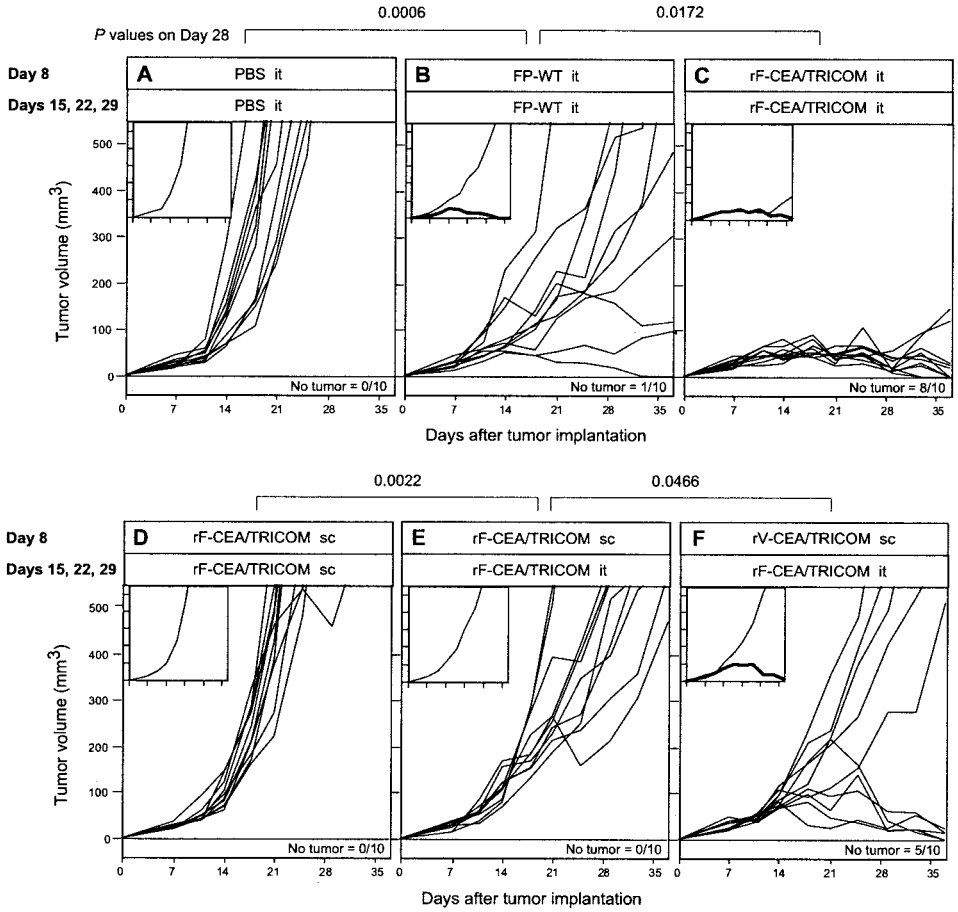

Fig. 1 Efficacy of intratumoral (i.t.) vaccination with recombinant fowlpox (rF)-carcinoembryonic antigen (CEA) and a triad of T-cell costimulatory molecules (TRICOM) on day 8 tumors. C57BL/6 mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. The mean tumor volume of a day 8 established tumor was 40 mm^3 ± 6 mm^3. *A*, control mice were administered PBS i.t. on days 8, 15, 22, and 29. *B*, mice were vaccinated i.t. with wild-type fowlpox virus (FP-WT) on days 8, 15, 22, and 29. *C*, mice were vaccinated i.t. with rF-CEA/TRICOM on days 8, 15, 22, and 29. Efficacy of i.t. vaccination with rF-CEA/TRICOM on day 15 tumors. C57BL/6 mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. The mean tumor volume on day 15 was 105 mm^3 ± 12 mm^3. *D*, mice were vaccinated s.c. with rF-CEA/TRICOM on days 8, 15, 22, and 29. *E*, mice were vaccinated with rF-CEA/TRICOM s.c. on day 8, and i.t. on days 15, 22, and 29. *F*, mice were vaccinated s.c. with recombinant vaccinia (rV)-CEA/TRICOM on day 8, then boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. Each virus was admixed with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored 1–2 times a week. *Inset*, mean tumor volumes of mice responding to (*heavy line*) or failing (*thin line*) vaccine therapy.

replication defective fowlpox is cytocidal for the cells it infects on the needle track. When mice received i.t. vaccination with rF-CEA/TRICOM at 4 weekly intervals, however, tumor growth was significantly suppressed in all of the mice ($P = 0.0172$ as compared with FP-WT), and 8 of 10 mice were completely cured (Fig. 1*C*). In contrast, when mice received rF-CEA/TRICOM vaccination (prime and boost) via the s.c. route (at a site distal from the tumor), tumor growth was not retarded (Fig. 1*D*; $P = 0.2544$ *versus* controls). Previous studies using other experimental models (2, 34, 36) have shown that s.c. vaccination with a recombinant vaccinia virus followed by boosting with a recombinant avipox virus was superior to the continued use of one vector. We thus asked if priming with rV-CEA/TRICOM (s.c.) is superior to priming with rF-CEA/TRICOM (s.c.) when boosted vaccinations of rF-CEA/TRICOM are given intratumorally. When mice were primed s.c. with rV-CEA/TRICOM on day 8, the mean tumor volume on day 15 was 105 mm^3 ± 12 mm^3, *i.e.*, similar to tumor volumes in the untreated group. This is in contrast to the tumor size (40 mm^3 ± 6 mm^3) of mice treated on day 8 shown in Fig. 1. When these mice were boosted weekly by i.t. vaccination with rF-CEA/TRICOM, however, tumor regression was observed, and 5 of 10 mice were cured (Fig. 1*F*). This antitumor activity was significantly higher than that in mice vaccinated s.c. with rF-CEA/TRICOM on day 8 followed by rF-CEA/TRICOM i.t. boosted vaccinations ($P = 0.0466$ to Fig. 1*E*). These data demonstrated that the efficacy of i.t. vaccination was superior to that of s.c. vaccination. However, when the treatment of tumors via i.t. vaccine administration was held until day 15 after tumor transplant, systemic priming with rV-CEA/TRICOM was required to more effectively induce antitumor activity of i.t. boosting with rF-CEA/TRICOM in the case of late-phase therapy of tumors (Fig. 1*F*).

Importance of the Tumor Antigen Transgene (CEA) in the Primary Vaccine Preparation. Next, we sought to determine whether the TAA, CEA, coexpressed as a transgene in the recombinant TRICOM vectors, contributed to the antitumor activity elicited by the combination of s.c. prime and i.t. boosts. Control mice were administered PBS s.c. on day 8, and i.t. on days 15, 22, and 29 (Fig. 2*A*). When mice were primed s.c. with rV-TRICOM and boosted i.t. with rF-TRICOM three times at weekly intervals (no CEA in vaccinations), tumor growth was significantly inhibited (Fig. 2*B; P* = 0.0001 as compared with the control group), and 2 of 15 mice were cured. When mice received weekly i.t. boosts with rF-CEA/TRICOM after priming with rV-TRICOM (no CEA in vector at priming), there were no cured mice, although the antitumor effect was significant as compared with that in the control group (Fig. 2*C; P* = 0.0276). In contrast, when mice were primed s.c. with rV-CEA/TRICOM followed by i.t. boosts with rF-TRICOM, tumor growth was more strongly inhibited than that seen in mice primed with rV-TRICOM followed by i.t. boosts with rF-TRICOM (*P* = 0.0123 to Fig. 2*B*), and 3 of 15 mice were cured (Fig. 2*D*). Furthermore, when mice were primed with rV-CEA/TRICOM followed by i.t. boosts with rF-CEA/TRICOM, 6 of 15 mice were cured (Fig. 2*E*). This inhibitory effect was significantly higher than that seen in mice primed with rV-TRICOM followed by i.t. boosts with rF-CEA/TRICOM (*P* = 0.0008 to Fig. 2*C*). In either group primed with rV-CEA/TRICOM (Fig. 2, *D* and *E*), antitumor effect was significant compared with that in the control group (*P* = 0.0001), and there were no significant differences between them (*P* = 0.6989). These data demonstrate that the presence of the CEA tumor antigen in the primary vaccination is a significant factor in the combination vaccine regimen (s.c. prime/i.t. boost) for tumor therapy.

Vaccine Regimen Efficacy on CEA-Negative Tumors. To examine the hypothesis that multiple costimulatory molecules, when introduced into the tumor microenvironment, could potentially induce T-cell responses to endogenous tumor antigens, we evaluated the vaccine regimen using another tumor model that was negative for CEA expression, parental MC38. Control mice were administered PBS i.t. on days 8, 15, 22, and 29 after tumor implantation (Fig. 3*A*). When mice were vaccinated i.t. with rF-CEA/TRICOM four times at weekly intervals, the tumor growth was suppressed in 2 of 5 mice, and significant differences were seen compared with that in the control group (Fig. 3*B; P* = 0.0032 on day 21). When taking into consideration the impact of i.t. vaccination with fowlpox viruses on day-8 tumors (Fig. 1*B*), it is speculated that viruses injected i.t. caused growth suppression of MC38 tumors. When i.t. vaccination began on day 15 after priming on day 8, mice vaccinated with the alternate regimens, rV-TRICOM and rF-TRICOM (Fig. 3*C*), rV-TRICOM and rF-CEA/TRICOM (Fig. 3*D*), or rV-CEA/TRICOM and rF-TRICOM (Fig. 3*E*), did not show remarkable retardation or regression (*P* > 0.1 to control group). As shown in Fig. 3*F*, when mice were primed with rV-CEA/TRICOM and boosted i.t. with rF-CEA/TRICOM, tumor growth was slightly

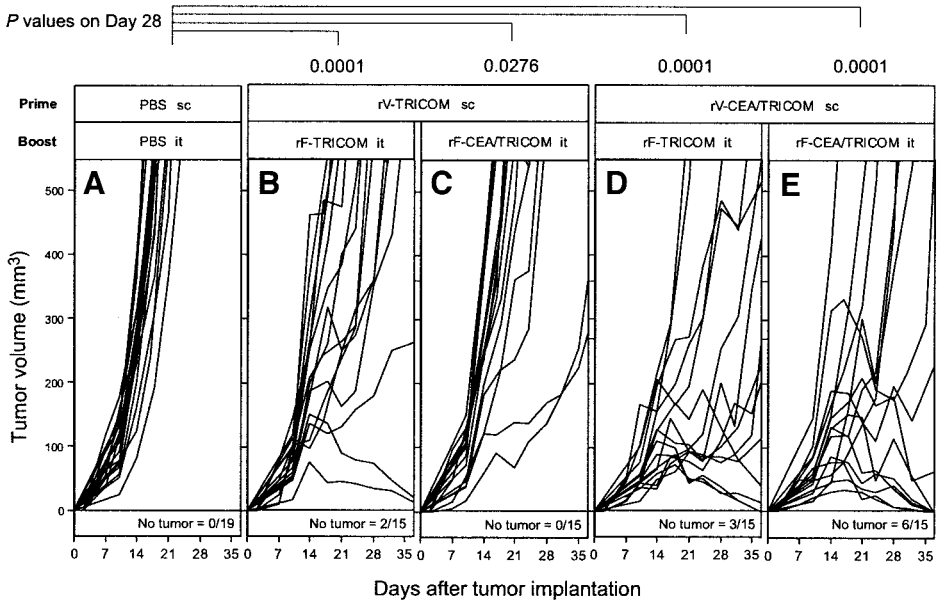

Fig. 2 Contribution of vaccine-encoded tumor antigen to the antitumor activity induced by intratumoral (i.t.) boosting after s.c. priming with recombinant vaccinia (rV)-carcinoembryonic antigen (CEA) and a triad of T-cell costimulatory molecules (TRICOM). C57BL/6 mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. *A*, control mice were administered PBS s.c. on day 8, and intratumorally on days 15, 22, and 29. *B*, mice were vaccinated s.c. with rV-TRICOM on day 8. Then mice were boosted i.t. with recombinant fowlpox (rF)-TRICOM on days 15, 22, and 29. *C*, mice were vaccinated s.c. with rV-TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. *D*, mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-TRICOM on days 15, 22, and 29. *E*, mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. Each virus was admixed with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored 1–2 times a week. These data are the compilation of three separate experiments.

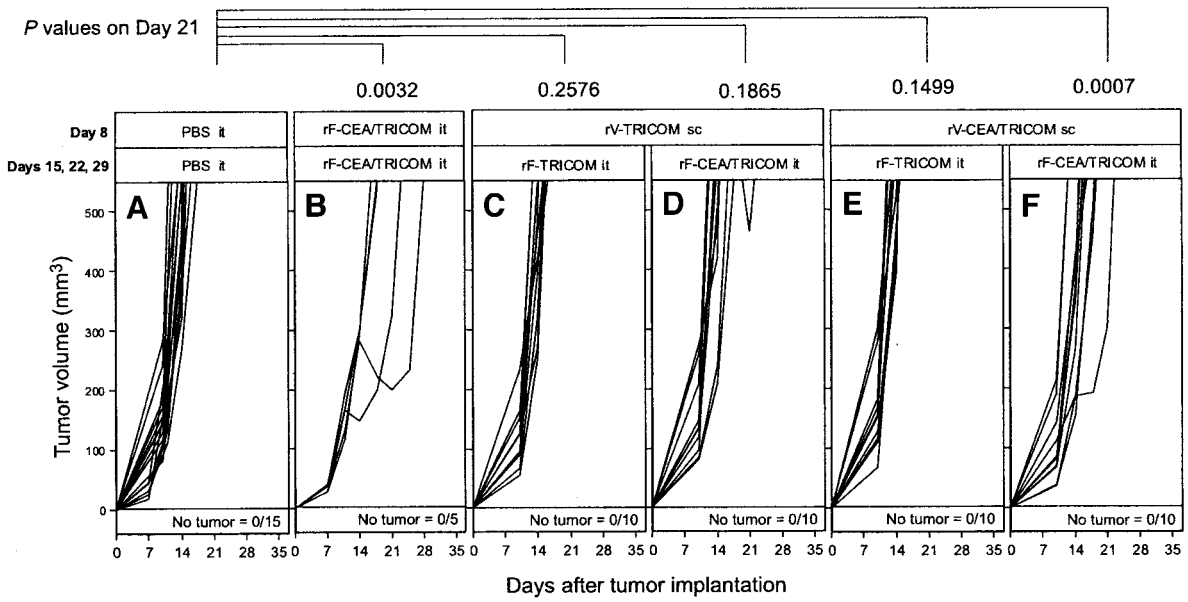

Fig. 3 Intratumoral (i.t.) vaccination of carcinoembryonic antigen (CEA)-negative MC38 tumors. C57BL/6 mice were implanted s.c. with parental MC38 tumors on day 0. *A,* control mice were administered PBS i.t. on days 8, 15, 22, and 29. *B,* mice were vaccinated intratumorally with recombinant fowlpox (rF)-CEA and a triad of T-cell costimulatory molecules (TRICOM) on days 8, 15, 22, and 29. *C,* mice were vaccinated s.c. with recombinant vaccinia (rV)-TRICOM on day 8. Then, mice were boosted i.t. with rF-TRICOM on days 15, 22, and 29. *D,* mice were vaccinated s.c. with rV-TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. *E,* mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-TRICOM on days 15, 22, and 29. *F,* mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. Each virus was admixed with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored 1–2 times a week. These data are the compilation of two separate experiments.

suppressed, and the antitumor effect was statistically significant to that in the control group ($P = 0.0007$ on day 21). However, there were no cured mice and the degree of inhibition was not noted as compared with that seen in MC38-CEA$^+$ tumor models (Fig. 1*F;* Fig. 2*E*). These results demonstrate the specificity of i.t. vaccination therapy with CEA/TRICOM vectors for CEA-positive tumors.

I.t. Vaccination Studies Using a Self-Antigen System: rF-GM-CSF Requirement for i.t. Vaccination with CEA/TRICOM Vectors. In the above studies, conventional C57BL/6 mice were used to define optimal parameters of vaccine regimens because of the limited availability of CEA-transgenic mice. CEA-transgenic mice express CEA in normal gastrointestinal tissues and fetal tissues in a manner similar to that expressed in humans, and these mice have CEA proteins in sera similar to the level (5–100 ng/ml) found in patients with CEA-expressing tumors (37, 38). For subsequent *in vivo* studies, CEA-transgenic mice in which CEA is a self-antigen were used to verify the antitumor activity of the vaccine regimen optimized in conventional mice. In the vaccination regimens described above using conventional mice, rF-GM-CSF was admixed with all of the vaccines. Using CEA transgenic mice, we examined whether rF-GM-CSF was required for antitumor activity induced by the vaccine regimen that consisted of heterogeneous (s.c./i.t.) administration routes. When CEA-transgenic mice were primed s.c. with rV-CEA/TRICOM admixed with rF-GM-CSF on day 8 and then boosted i.t. with rF-CEA/TRICOM admixed with rF-GM-CSF on days 15, 22, and 29, 3 of 5 mice were cured of tumor (Fig. 4*A*). The antitumor effect was significant as compared with that in mice vaccinated with rF-GM-CSF alone (Fig. 4*E; P* = 0.0475). These data indicated that the vaccine regimen with CEA/TRICOM vectors optimized in conventional mice was also effective in inhibiting tumor growth in a CEA self-antigen system. In contrast, when mice were primed s.c. with rV-CEA/TRICOM without the addition of rF-GM-CSF, the therapeutic effect was decreased significantly as compared with that seen when admixed with rF-GM-CSF (Fig. 4*B; P* = 0.0103). Significant reduction of antitumor effects was also seen when mice received i.t. boosts with no rF-GM-CSF (Fig. 4*C; P* = 0.0076). There were no cured mice in both groups. As seen in Fig. 4*D,* when mice were vaccinated without the addition of rF-GM-CSF at both s.c. prime and i.t. boosts, the tumor of one mouse was cured, but significant differences were not found when compared with mice vaccinated with rF-GM-CSF alone ($P = 0.4454$ to Fig. 4*E*) or mice treated with PBS ($P = 0.8909$). These data demonstrated that rF-GM-CSF was necessary to induce potent antitumor activity induced by the CEA/TRICOM vectors.

Efficacy of i.t. Vaccination in a Self-Antigen System. On the basis of the data described above, tumor therapy studies were carried out to additionally verify the antitumor activity induced by i.t. vaccination compared with s.c. vaccination using vectors admixed with rF-GM-CSF. As a control, CEA-transgenic mice were administered PBS i.t. on days 8, 15, 22, and 29 after tumor implantation (Fig. 5*A*). When mice were vaccinated with rF-CEA/TRICOM via the s.c. route only, there were no

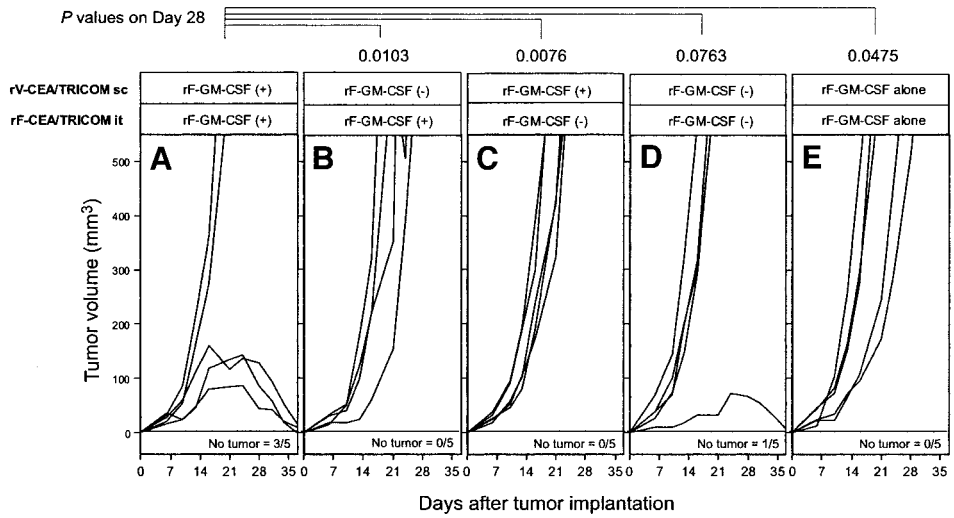

Fig. 4 Requirement of recombinant fowlpox (rF)-granulocyte macrophage colony-stimulating factor (GM-CSF) for the antitumor activity induced by intratumoral (i.t.) boosting with carcinoembryonic antigen (CEA) and a triad of T-cell costimulatory molecules (TRICOM) vectors. CEA-transgenic mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. *A*, mice were vaccinated s.c. with recombinant vaccinia (rV) -CEA/TRICOM + rF-GM-CSF on day 8. Then, mice were boosted intratumorally with rF-CEA/TRICOM + rF-GM-CSF on days 15, 22, and 29. *B*, mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM + rF-GM-CSF on days 15, 22, and 29. *C*, mice were vaccinated s.c. with rV-CEA/TRICOM + rF-GM-CSF on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. *D*, mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. *E*, control mice were vaccinated with rF-GM-CSF s.c. on day 8, and rF-GM-CSF i.t. on days 15, 22, and 29. Tumor volume was monitored 1–2 times a week.

cured mice, and significant differences were not seen as compared with the control group (Fig. 5*B; P* = 0.5696). However, i.t. vaccination with rF-CEA/TRICOM was greatly effective in inhibiting tumor growth (*P* = 0.0002 to Fig. 5*A*), and 12 of 18 mice were completely cured of tumors (Fig. 5*C*).

When i.t. vaccination with rF-CEA/TRICOM was started on day 15, tumor growth was not significantly inhibited as compared with that in the PBS-treated group (*P* = 0.9884 to Fig. 6*A*), and no mice were cured (Fig. 6*B*). However, when rV-CEA/TRICOM was used as a prime on day 8 before i.t. vaccination with rF-CEA/TRICOM on day 15, tumor development was significantly inhibited as compared with that seen when mice were vaccinated i.t. on day 15 without priming (*P* = 0.0001 to Fig. 6*B*), and 14 of 20 mice were cured (Fig. 6*C*). In

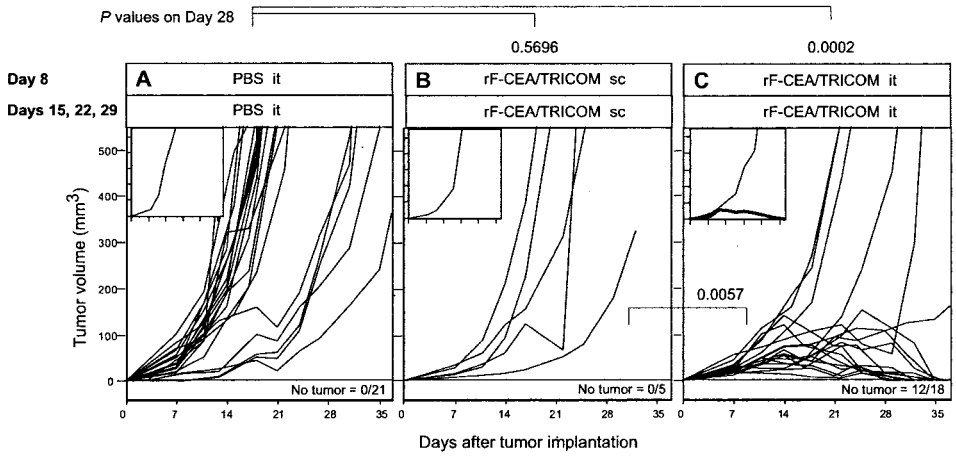

Fig. 5 Efficacy of intratumoral (i.t.) vaccination in a self-antigen system. Carcinoembryonic antigen (CEA)-transgenic mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. *A*, control mice were administered i.t. PBS on days 8, 15, 22, and 29. *B*, mice were vaccinated s.c. with recombinant fowlpox (rF)-CEA and a triad of T-cell costimulatory molecules (TRICOM) on days 8, 15, 22, and 29. *C*, mice were vaccinated i.t. with rF-CEA/TRICOM on days 8, 15, 22, and 29. Each virus was admixed with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored 1–2 times a week. These data are the compilation of four separate experiments. *Inset*, mean tumor volumes of mice responding to (*heavy line*) or failing (*thin line*) vaccine therapy.

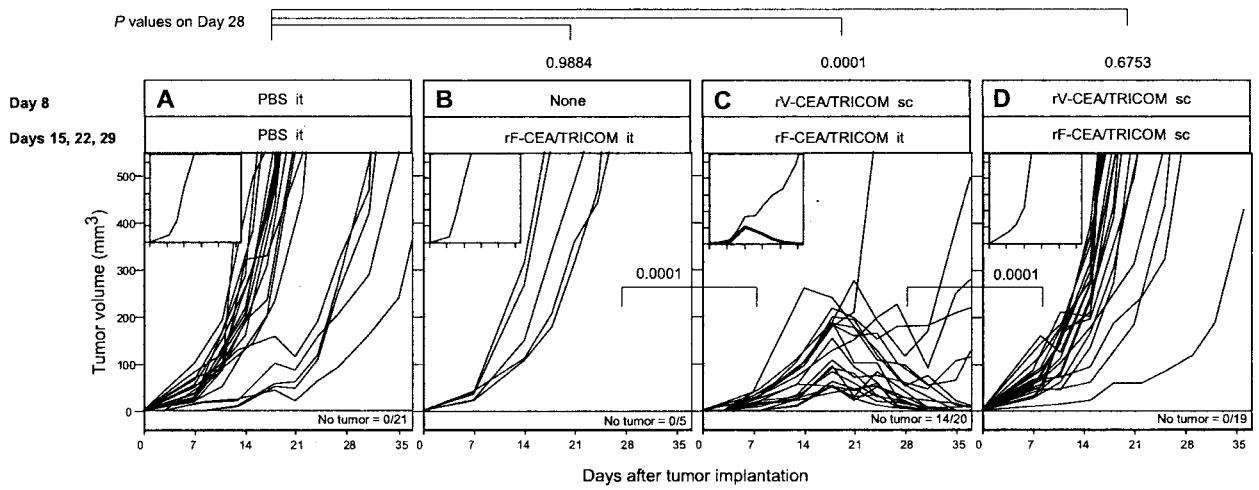

Fig. 6 Efficacy of intratumoral (i.t.) vaccination after s.c. priming with recombinant vaccinia (rV)-carcinoembryonic antigen (CEA) and a triad of T-cell costimulatory molecules (TRICOM) on advanced tumors in a self-antigen system. CEA-transgenic mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. *A,* control mice were administered i.t. PBS on days 8, 15, 22, and 29. *B,* mice were vaccinated i.t. with recombinant fowlpox (rF)-CEA/TRICOM on days 15, 22, and 29. *C,* mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22, and 29. *D,* mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted s.c. with rF-CEA/TRICOM on days 15, 22, and 29. Each virus was admixed with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored 1–2 times a week. These data are the compilation of four separate experiments. *Inset,* mean tumor volumes of mice responding to (*heavy line*) or failing (*thin line*) vaccine therapy.

contrast, when mice were boosted s.c. with rF-CEA/TRICOM after priming with rV-CEA/TRICOM, tumor growth was not strongly inhibited, and the antitumor activity was significantly lower than that seen in i.t. boosted mice after priming (Fig. 6*D;* $P = 0.0001$ to Fig. 6*C*). Taken together, the data using CEA-transgenic mice, a self-antigen system, reinforced the data obtained with conventional mice in that i.t. vaccination with rF-CEA/TRICOM was superior to s.c. vaccination. Moreover, i.t. booster vaccination was more effective in inhibiting growth of larger tumors (day 15 after implantation) when combined with rV-CEA/TRICOM as a prime.

To determine whether long-term immunological memory existed as a result of the heterologous s.c./i.t. vaccine regimen, CEA-transgenic mice cured with rV-CEA/TRICOM (s.c.) and rF-CEA/TRICOM (i.t.) in Fig. 4*A* (3 mice) and Fig. 6*C* (8 mice) were rechallenged with MC38-CEA$^+$ tumors 146 days after the first MC38-CEA$^+$ tumor implantation (Table 1). The rechallenged MC38-CEA$^+$ tumors were rejected in 73% (8 of 11) of those mice. One month after rechallenge of MC38-CEA$^+$ tumors, these mice were challenged with parental (CEA-negative) MC38 tumors, and 75% (6 of 8) of mice rejected them (Table 1). In addition, these mice were challenged with B16 melanoma cells 2 months after MC38 challenge. The B16 tumor growth was significantly suppressed in all of the mice as compared with that in control mice ($P = 0.0072$), and 1 of 6 mice (17%) rejected them completely. As a control, MC38-CEA$^+$, parental MC38, or B16 tumors were injected into normal C57BL/6 mice, and these tumors grew normally.

DISCUSSION

Previous i.t. vaccination studies have focused on the efficacy of i.t. vaccination alone (20–26). There are no reports that systematically compared i.t. vaccination with systemic vaccination achieved by s.c., intradermal, or i.m. injection, or combining both systemic and i.t. modalities. Here, we compared the antitumor effects induced by a systemic vaccination regimen and an i.t. vaccination regimen, and demonstrated the superiority of i.t. vaccination after s.c. priming to s.c. priming and s.c. boosting vaccinations in both conventional tumor models where the TAA is a foreign antigen (Fig. 1) and in a CEA-transgenic

Table 1 Long-term protection from tumor challenge in mice cured by intratumoral vaccination

	Percentage of tumor-free mice after challenge with		
Groups	1st MC38-CEA$^+$	2nd MC38-CEA$^+$	Parental MC38
Vaccinated[a]	73% (11/15)[b]	73% (8/11)[c]	75% (6/8)[d]
Control[e]	0% (0/10)	0% (0/5)	0% (0/5)

[a] Carcinoembryonic antigen (CEA)-transgenic mice were implanted (s.c.) with MC38-CEA$^+$ tumor cells on day 0. Mice were vaccinated with recombinant vaccinia-CEA and a triad of T-cell costimulatory molecules (TRICOM) (s.c.) on day 8 and with recombinant fowlpox (rF)-CEA/TRICOM (i.t.) on days 15, 22, and 29. All vaccines were coadministered with rF-granulocyte macrophage colony-stimulating factor.

[b] Tumor-free mice following vaccine regimen.

[c] Mice cured by vaccination ($n = 11$) were rechallenged on day 146 with MC38-CEA$^+$ tumor cells. As a control, naive mice were implanted with MC38-CEA$^+$ tumor cells at the same time.

[d] Mice that rejected rechallenge of MC38-CEA$^+$ tumor cells ($n = 8$) were rechallenged on day 176 with parental (CEA-negative) MC38 tumor cells. As a control, naive mice were implanted with MC38 tumor cells at the same time.

[e] Control mice were administered PBS s.c. on day 8 and i.t. on days 15, 22, and 29.

tumor model where the TAA is a self-antigen, thus simulating cancer patients with CEA-positive tumors (Fig. 6). Moreover, we have examined antitumor effects of i.t. vaccination without s.c. priming on smaller tumors (vaccination on days 8, 15, and 22 after MC38-CEA$^+$ tumor implantation). Tumor growth was strongly inhibited in 17 of 20 mice, and 70% of mice were cured (Fig. 6*C*). In contrast, no mice were cured by s.c. priming and boosting (Fig. 6*D*). In the clinical setting, there are some issues, such as safety and accessibility, concerning i.t. injection. For this reason, in the studies reported here, only the replication-defective fowlpox virus recombinants were used for i.t. vaccination. However, although internal cancers differ from accessible tumors such as cutaneous melanoma or cutaneous breast carcinoma lesions, it could be feasible to inject visceral tumors at surgery.

Kaufman *et al.* (29, 31) have administered a viral vector encoding costimulatory molecules directly into melanoma lesions in clinical trials, suggesting that potential TAAs can be recognized without initial identification of melanoma antigens and that the immune response may be directed to multiple melanoma antigens. Our data support these observations; i.t. administration of rF-TRICOM significantly reduced the growth rate of tumors (Fig. 2). However, we suggest that this antitumor activity can be additionally enhanced by the addition of a tumor antigen in the viral vectors. Here, i.t. vaccination of more advanced tumors with rF-CEA/TRICOM was most effective when combined with s.c. priming with rV-CEA/TRICOM (Fig. 1*F;* Fig. 6*C*). When mice were primed with rV-TRICOM, the antitumor activity induced by subsequent i.t. boosts with rF-TRICOM or rF-CEA/TRICOM was significantly lower (Fig. 2). These results indicate that systemic priming with rV-CEA/TRICOM resulted in an important contribution to vaccination. Previously, it was shown that diversified priming with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM was more efficacious than s.c. priming and s.c. boosting vaccinations with rF-CEA/TRICOM in the induction of CEA-specific T-cell responses and antitumor activity using peripancreatic metastasis models (34, 36). However, we demonstrate here for the first time that the introduction of a specific tumor antigen, CEA, in vaccines used as priming is advantageous in enhancing antitumor activity induced by subsequent i.t. boosts. The i.t. boosts with rF-CEA/TRICOM would not only induce immunity to the endogenous antigen (in this case, CEA), but also make the endogenous antigen more immunogenic by hyperexpression in the tumor.

The s.c. priming and i.t. boosting regimen described here used the admixture of rF-GM-CSF. Kass *et al.* (41) have shown previously that rF-GM-CSF enhances T-cell responses and antitumor activity when given s.c. as an admixture with CEA/TRICOM vectors (34, 36). In this study, the omission of rF-GM-CSF in either the s.c. prime or the i.t. boost dramatically reduced the antitumor effects induced by this vaccine regimen (Fig. 6). These demonstrate that vaccination plays a critical role in i.t. vaccination as well as that observed previously for s.c. vaccination by numerous groups.

The vaccine regimen of s.c. priming followed by i.t. boosting with CEA/TRICOM vectors was not effective on CEA-negative MC38 tumors, highlighting the requirement of CEA on the tumor for optimal vaccine therapy (Fig. 3). However, mice cured of tumors via this vaccine regimen rejected secondary tumor challenge, not only of MC38-CEA$^+$ tumors but also of CEA-negative parental MC38 tumors and B16 tumors (Table 1). These results indicate that cured mice could acquire long-term antitumor immunity, which would be significant to protect cancer patients from recurrence and metastasis.

Tumor therapy was accompanied by the induction of immune responses to other antigens in addition to CEA, such as p53 and gp70 expressed on MC38-CEA$^+$ tumors (data not shown), demonstrating that the diversified regimen of s.c. prime and i.t. boosts could induce potent antitumor activity specific not only for a particular antigen contained in vaccines but also for other antigens contributed by the tumor. Considering this, it can be inferred that the addition of endogenous tumor antigens is a key step in the induction and propagation of antigenic cascades. Markiewicz *et al.* (42) have shown that cured mice vaccinated with a P815 tumor peptide rejected a P815-derived cell line that did not express the vaccine peptide, indicating that antigen-negative tumors were rejected by the presentation of additional epitopes and broadened CTL responses. It has also been shown that >50% of mice vaccinated with DNA encoding Her-2/*neu* rejected Her-2/*neu*-negative tumors, and this effect was abolished by CD4$^+$ T-cell depletion before tumor challenge (43).

Taken together, these data suggest that i.t. vaccination with an avipox vector containing a tumor antigen and multiple costimulatory molecules combined with systemic priming is an effective modality in the therapy of tumors. This vaccine regimen holds promise not only for the therapy of s.c. tumors but also for other tumors accessible by surgery.

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