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J Immunol 2007; 178:3260-3264; ;
doi: 10.4049/jimmunol.178.5.3260
<http://www.jimmunol.org/content/178/5/3260>

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Immunotherapy of Tumors with Neuroimmune Ligand Capsaicin

Jacqueline Beltran, Amiya K. Ghosh, and Sreyashi Basu¹

Red chili pepper (*Capsicum frutescens*) is a highly consumed spice throughout the world. Its principal pungent ingredient is the phenol capsaicin (8-methyl-*N*-vanillyl-6-nonenamide). Capsaicin causes neurogenic inflammation and has analgesic and anti-inflammatory activities. We have observed previously that dendritic cells, a key cell type in immune responses, have the receptor for capsaicin, and engagement of this receptor has powerful immune consequences. In this study, we demonstrate that intratumoral administration of capsaicin into a preexisting tumor results in retarded progression of the injected tumor regardless of whether the tumor is at its early or late stage. Furthermore, it leads to significant inhibition of growth of other, uninjected tumors in the same animal. Capsaicin-elicited immunity is shown to be T cell-mediated and tumor-specific. These results reflect the immunological potency of a neurological ligand in modulating immune response against an established tumor. *The Journal of Immunology*, 2007, 177: 3260–3264.

Red chili peppers (*Capsicum frutescens*) have been used for several thousand years as food additives and for a broad variety of medical applications in Indian, Native American, African, and Chinese medical traditions (1, 2). Red peppers have been claimed to: enhance immune response (and thereby prevent infections), act in an anti-inflammatory manner (and thereby prevent atherosclerosis and reduce risk of heart disease), lower blood pressure, reduce excessive blood clotting, reduce blood sugar levels, but no formal examination of these claims has been published (3, 4). Capsaicin is the pungent component of red peppers. Chemically, it is a derivative of vanillyl amide (8-methyl-*N*-vanillyl-6-nonenamide) and has a m.w. of 305.42. With respect to potency, target, and mechanism of action, capsaicin is known to have two kinds of effects: a brief stimulation of primary afferent nerve fibers followed by desensitization to capsaicin and a cell nonselective effect. This nonselective effect does not have any long-lasting consequence on the responding cells (5). The capsaicin receptor, vanilloid receptor 1 (VR1),² has been shown to be highly expressed by nociceptive neurons in dorsal root and trigeminal ganglia (6–8). Capsaicin binds to VR1 on sensory neurons to convey the sensation of pain. Apart from its neurological functions, capsaicin has also been shown to be active immunologically in generating more Ab-producing cells compared with untreated controls (9). Dietary capsaicin in BALB/c mice has been shown to enhance lymphocyte proliferation and serum Ig levels (9). Inhalation of capsaicin has been shown to interfere with neural responses involved in inflammation in the lungs of Lewis rats, and such interference modulates immunity to inhaled Ags (10).

Recently we identified that immune cells, e.g., dendritic cells (DCs), express VR1. Engagement of VR1 on immature DCs by treatment with capsaicin leads to maturation of DCs as measured by up-regulation of Ag-presenting and costimulatory molecules. This effect is seen in DCs of VR1^{+/+} but not VR1^{-/-} mice. In the VR1^{+/+} mice, this effect is inhibited by VR1 antagonist capsazepine. Furthermore, intradermal administration of capsaicin leads to migration of DCs to the draining lymph nodes in VR1^{+/+} but not VR1^{-/-} mice (11). This adjuvant ability of capsaicin led us to test its use in tumor therapy.

We report here the immunomodulatory activity of capsaicin in treatment of pre-existing tumors. Our studies show that intratumoral administration of capsaicin leads to regression of the injected tumor and significant inhibition of growth of antigenically alike but not antigenically distinct, uninjected tumors in the same animal. This treatment causes regression of early as well as advanced pre-existing tumors. This antitumor activity of capsaicin is T cell-dependent as well as tumor-specific.

Materials and Methods

Mice, reagents, and cells

BALB/cJ and BALB/cJ *nu/nu* mice were obtained from The Jackson Laboratory. Tween 80 and ethanol were purchased from Sigma-Aldrich. Capsaicin was purchased from BioMolecules. Methylcholanthrene (Meth A)-induced fibrosarcomas and CMS5 (BALB/c) have been previously described (12). Meth A was maintained in ascites formed in BALB/cJ mice by weekly i.p. passage of cells. CMS5, CT26, and RAW 264.7 cells were maintained in vitro and cultured in DMEM with 10% FBS.

Intratumoral therapy of tumors

Six-week-old BALB/cJ mice were injected with live tumor cells (100 × 10³) on day 0. Tumors were treated intratumoral on day 5 and day 10 or day 10 and day 15 after tumor challenge with PBS, vehicle (10% Tween 80, 10% ethanol in PBS), 100 or 200 μg of capsaicin in same vehicle. Tumor diameter was monitored every 2–3 days. The significance of differences in tumor size was determined by two-tailed Student's *t* test.

Apoptosis detection by TUNEL method

The apoptotic cell death was identified by labeling their DNA 3'-OH nick ends with TUNEL staining. Staining for cell death was conducted according to the manufacturer's protocols (APO-DIRECT kit; BD Biosciences). In brief, cells were harvested, washed in PBS, and fixed with 1% (w/v) paraformaldehyde for 30 min on ice. After washing, cells were suspended in wash buffer and washed twice by centrifugation at 300 × *g* for 5 min.

Center for Immunotherapy of Cancer and Infectious Diseases, University of Connecticut School of Medicine, Farmington, CT 06030-1601

Received for publication August 18, 2006. Accepted for publication December 12, 2006.

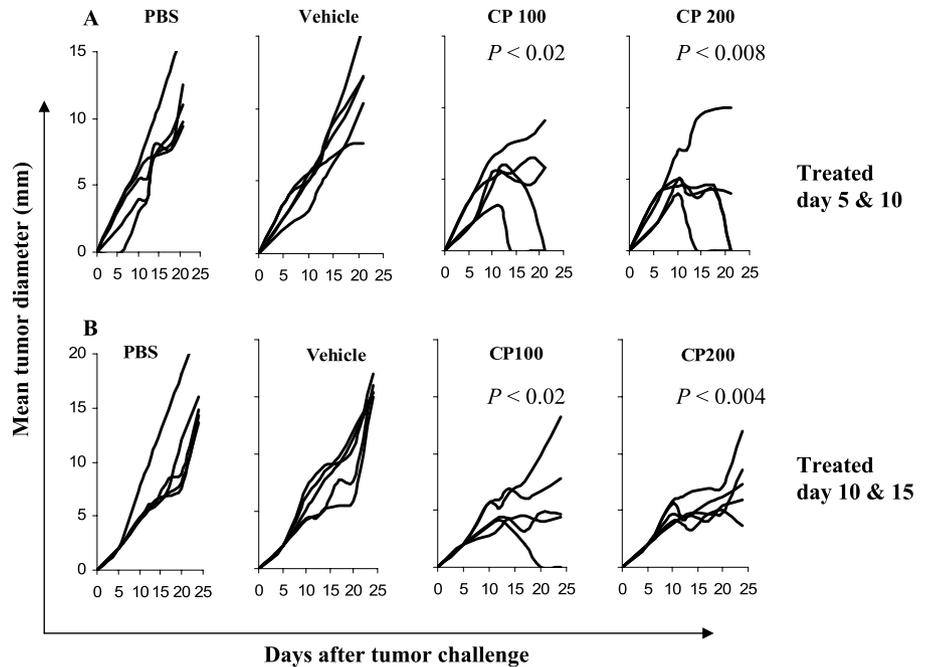
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¹ Address correspondence and reprint requests to Dr. Sreyashi Basu, Center for Immunotherapy of Cancer and Infectious Diseases, Mail Code 1601, University of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06030-1601. E-mail address: basu@up.uhc.edu

² Abbreviations used in this paper: VR1, vanilloid receptor 1; DC, dendritic cell.

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FIGURE 1. Effect of intratumoral administration of capsaicin on growth of Meth A. BALB/c mice were intradermally challenged on day 0 with 1×10^5 live Meth A cells. On day 5 after Meth A challenge (A) or day 10 after Meth A challenge (B) tumors were treated with PBS, vehicle, or two doses of capsaicin (CP) as indicated (100 and 200 μ g). Tumors were again treated similarly 5 days after the last treatment and tumor growth was monitored twice a week. Each line represents an individual mouse. Student's *t* test for CP100 ($p < 0.02$) and CP200 ($p < 0.008$) in A, and for CP100 ($p < 0.02$) and CP200 ($p < 0.004$) in B are against respective vehicle controls.



Cells were then resuspended in 50 μ l of staining solution (reaction buffer, TdT enzyme, FITC-dUTP, distilled water) and incubated overnight in the dark at room temperature. At the end of incubation, cells were resuspended in rinsing buffer and centrifuged at $300 \times g$. Supernatant was discarded. The cell pellet was resuspended in 0.5 ml of PI/RNase Staining buffer and incubated in the dark for 30 min at room temperature. Cells were acquired in PI/RNase solution by flow cytometry.

Induction of CTLs

Six-week-old BALB/cJ mice were injected with live CT26 tumor cells (500×10^3) on day 0. After 5 days tumors were treated either once on day 5 or twice (on day 5 and day 10) by intratumoral injection of vehicle (10% Tween 80, 10% ethanol in PBS) or 200 μ g of capsaicin in same vehicle. Splenocytes were harvested on either day 10 or day 14 of tumor challenge and cultured with 1 μ M AH1 (SPSYVYHQF) synthetic peptide for 5 days. Splenocytes were then tested for cytotoxicity in a chromium release assay using RAW 264.7 cells alone or RAW 264.7 cells pulsed with AH1 peptide as targets.

Results

Immunotherapy with capsaicin is effective in therapy of Meth A fibrosarcoma

A widely used and extremely aggressive fibrosarcoma Meth A was examined in a therapy model. By 5 days postchallenge, Meth A tumor becomes visible or palpable and by 10 days it measures between 5 and 8 mm in diameter. We treated the mice starting at day 5 or day 10 after tumor challenge. Mice were again treated 5 days after initial treatment, and tumor diameter was measured every 2–3 days. Tumors grew progressively in PBS as well as in vehicle-treated mice, but treatment with capsaicin either slowed the growth of the primary tumor or resulted in complete regression of the primary tumor regardless of the size of the tumor at the time it received initial treatment (Fig. 1).

Capsaicin induces apoptosis in tumor cells but not normal cells

In an attempt to examine the mechanism by which tumor growth retardation is induced by capsaicin, we tested the effect of capsaicin on tumor cells and compared it with normal cells. Tumor cells, e.g., Meth A and CMS5 fibrosarcoma, and normal cells, e.g., mouse embryonic fibroblasts, were cultured in vitro with vehicle or capsaicin for 72 h and apoptosis was measured by TUNEL assay.

Compared with vehicle, treatment with capsaicin induced significant apoptosis in the fibrosarcoma lines but not in the normal mouse embryonic fibroblasts (Fig. 2). This phenomenon is dose- and time-dependent (data not shown).

Capsaicin elicits T cells against the primary tumor

Because capsaicin activates DCs in vitro and in vivo (11), we sought to determine whether capsaicin could augment the capacity

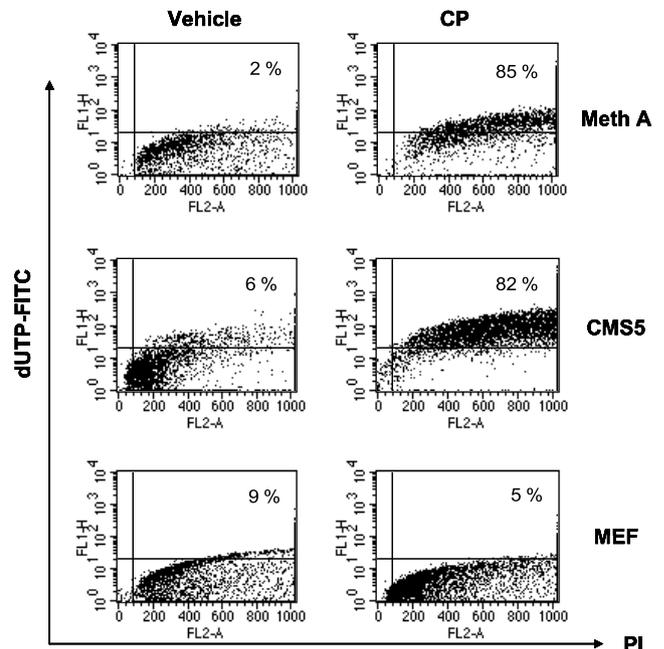
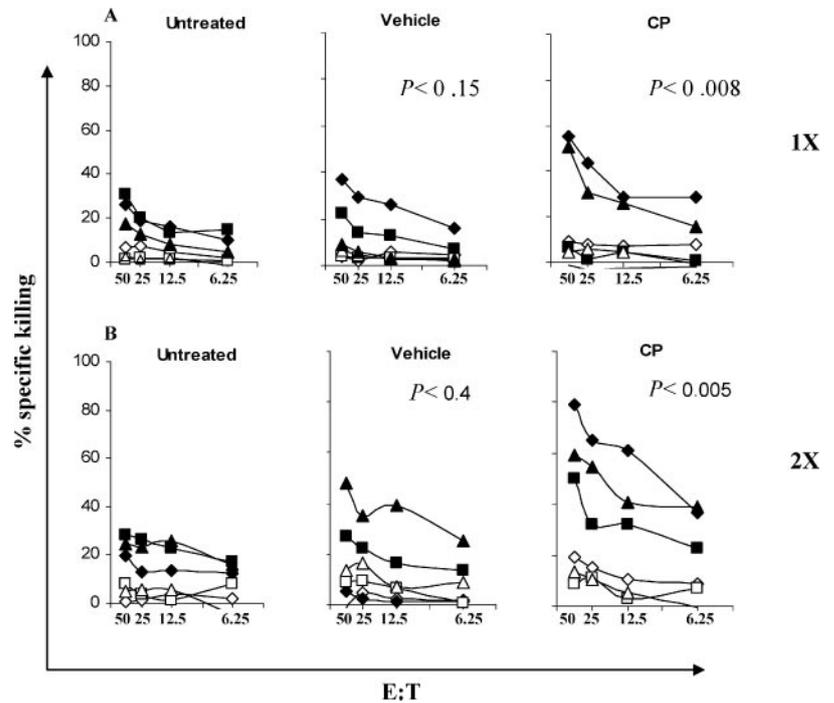


FIGURE 2. Fibrosarcomas but not normal fibroblasts undergo apoptosis in response to capsaicin in vitro. Fibrosarcomas Meth A and CMS5 or normal fibroblasts were treated in culture (1×10^6) with vehicle or capsaicin (100 μ M) for 72 h and tested for apoptosis by TUNEL assay. Cells were analyzed by flow cytometry using FACScan (BD Biosciences). The percentage in each panel represents apoptotic cells (FITC-dUTP staining) in the total number of cells (PI staining).

FIGURE 3. Intratumoral injection of capsaicin induces tumor-specific T cells. BALB/c mice were challenged intradermally with 500×10^3 live CT26 cells on day 0. **A**, On day 5 of tumor challenge, mice were treated once (1X) with either 50 μ l of vehicle or 200 μ g of capsaicin in 50- μ l volume intratumoral. Splenocytes were harvested on day 10 and cultured with 1 μ M AH1 peptide for 5 days. Splenocytes were then tested for cytotoxicity. **B**, Mice were treated twice (2X) first on day 5 and then on day 10 of tumor challenge with either 50 μ l of vehicle or 200 μ g of capsaicin in 50- μ l volume intratumoral. Splenocytes were harvested on day 14 of tumor challenge and cultured with 1 μ M AH1 peptide for 5 days. Splenocytes were then tested for cytotoxicity. Cytotoxicity testing was done using RAW 264.7 cells unpulsed (open symbols) or pulsed (closed symbols) with AH1 peptide as targets by chromium release assay. Each symbol represents an individual mouse. Student's *t* test for vehicle-treated mice ($p < 0.15$ for once treated, $p < 0.4$ for twice treated) and capsaicin-treated mice ($p < 0.008$ for once treated, $p < 0.005$ for twice treated) are against untreated controls and for capsaicin-treated ($p < 0.02$ for once treated and $p < 0.003$ for twice treated) when compared with vehicle control.



of tumor-infiltrating DCs to prime antitumor T cell immunity. CT26 (H2^d), colon carcinoma line was chosen for this study; the CD8 T cell epitope AH1 (SPSYVYHQF) recognized by T cells against CT26 tumor is well defined (13). We challenged BALB/c mice with live 5×10^5 CT26 cells. On day 5 after tumor injection, CT26 tumor was palpable. Mice were treated with capsaicin either once on day 5 (Fig. 3A) or twice (on day 5 and day 10) (Fig. 3B). Splenocytes from the treated or untreated mice were tested for antitumor CTL activity after in vitro culture as described in *Materials and Methods*. CT26 tumor-bearing mice that were left untreated showed some antitumor CTL activity. But splenocytes from capsaicin-treated mice had significantly higher CTL activity

than vehicle-treated controls. The difference in CTL activity was also evident in mice that received only one treatment.

Antitumor activity of capsaicin is T cell-dependent

Capsaicin is lethal to tumor cells and is effective in enhancing antitumor T cell response in vivo. Thus we examined whether the antitumor activity of capsaicin is solely dependent on its cytotoxic effects. For this we compared immunotherapy of pre-existing Meth A tumors in wild-type BALB/c (+/+) and BALB/c *nu/nu* mice (lacking functional T cells). Palpable Meth A tumors were treated as described in Fig. 1 in wild-type BALB/cJ (+/+) and BALB/c *nu/nu* (T cell-deficient) mice (Fig. 4). Treatment with capsaicin

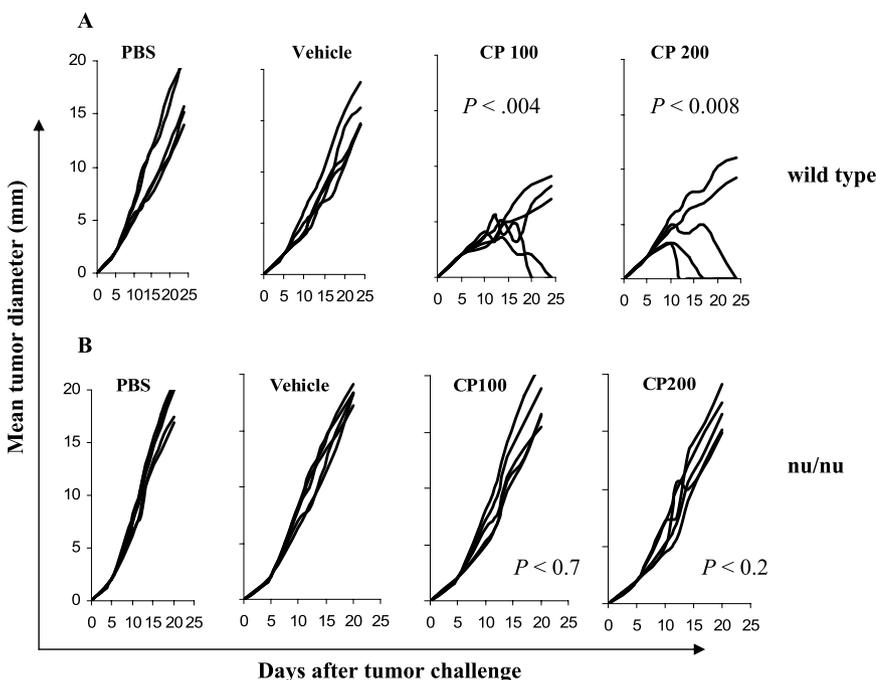
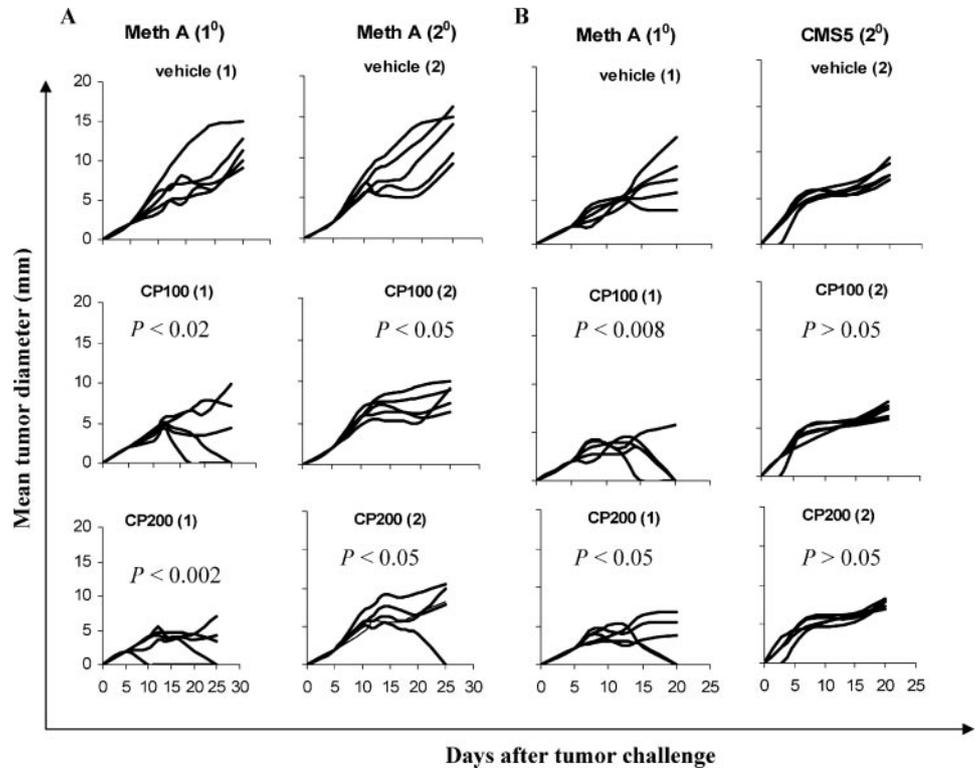


FIGURE 4. Tumor growth retardation is T cell-dependent. BALB/c mice (A) or BALB/c *nu/nu* mice (B) were intradermally challenged on day 0 with 1×10^5 live Meth A cells 5 days after Meth A challenge, tumors were treated with PBS, vehicle, or two doses of capsaicin (CP) as indicated. Tumors were treated similarly for the second time on day 10 and tumor growth was monitored twice a week. Each line represents an individual mouse. Treatment in wild-type mice ($p < 0.004$) of 100 μ g and ($p < 0.008$) of 200 μ g and treatment in *nu/nu* mice ($p < 0.7$) of 100 μ g and ($p < 0.2$) of 200 μ g are compared with respective vehicle controls by Student's *t* test.

FIGURE 5. Immunotherapy of secondary tumors is specific to the primary treated tumor. BALB/c mice were challenged on the right flank with (1×10^5) Meth A (A) or with (1×10^5) CMS5 (B) and on the left flank with (1×10^5) Meth A (A) and (B) by intradermal administration on day 0. On day 5 after tumor challenge left tumors (tumor 1) were treated with vehicle or two doses of capsaicin (CP) as indicated and right tumor (tumor 2) was left untreated. Tumor 1 was again treated for the second time similarly on day 10 and growth of both the tumors (1 and 2) were monitored twice a week. Each line represents an individual mouse. Treatment using 100 μg ($p < 0.02$) and 200 μg ($p < 0.002$) in Meth A left flank; 100 μg ($p < 0.05$) and 200 μg ($p < 0.05$) in Meth A right flank; 100 μg ($p < 0.008$) and 200 μg ($p < 0.05$) in Meth A left flank; and 100 μg ($p > 0.05$) and 200 μg ($p > 0.05$) in CMS5 right flank are compared against vehicle control in each group using Student's *t* test.



slowed the growth of the primary Meth A tumor, in wild-type BALB/c (+/+) mice, whereas treatment in BALB/c (*nu/nu*) mice had no effect in therapy. This effect was observed in mice bearing 10-day-old tumors as well (data not shown). The lack of antitumor activity of capsaicin in BALB/c (*nu/nu*) mice suggests that T cell functions are involved in capsaicin-mediated immunotherapy.

Immunotherapy of tumors with capsaicin is specific to the primary tumor

To determine whether capsaicin-mediated tumor regression induces a systemic as well as tumor-specific immune response we used two antigenically distinct sarcomas both of BALB/c origin, Meth A and CMS5. BALB/cJ mice were either challenged with live Meth A cells on each flank designated as Meth A-Meth A mice or Meth A on one flank and CMS5 on opposite flank designated as Meth A-CMS5 mice. When both the tumors were visible and palpable, the Meth A tumors on one flank were treated with vehicle or capsaicin following a previously mentioned administration schedule. Tumor growth was monitored for both tumors every 2–3 days. In both Meth A-Meth A and Meth A-CMS5 mice, capsaicin-treated Meth A tumors (tumor 1) either completely regressed or their growth was significantly retarded as previously observed (Fig. 1). In capsaicin-treated Meth A-Meth A mice (Fig. 5A), the growth of the untreated Meth A tumor (tumor 2) was also retarded significantly as compared with vehicle-treated controls. Interestingly in capsaicin-treated Meth A-CMS5 mice (Fig. 5B), the untreated CMS5 tumor (tumor 2) grew progressively similar to the vehicle-treated mice.

Discussion

The role of capsaicin in treatment of cancer has been implicated for many years but has never been formally tested in vivo (3, 4). Our study shows for the first time that capsaicin can be used as an immunological adjuvant in treatment of established cancers. All animals with pre-existing tumors when treated with capsaicin had significant reduction in tumor growth compared with vehicle treat-

ment. This treatment is effective, irrespective of the size of the tumor. Capsaicin causes apoptosis of tumor cells but a direct cytotoxic effect of capsaicin responsible for tumor growth retardation seems unlikely. Treatment with capsaicin eliciting specific antitumor T cells is shown by the following criteria: 1) enhanced priming of antitumor T cell immunity occurs in tumor-bearing (+/+) mice when treated with capsaicin. Compared with vehicle-treated or untreated tumor-bearing mice, capsaicin-treated mice elicited a robust T cell response with effector function specific to the treated tumor (Fig. 3). 2) Treatment with capsaicin slowed the growth of the primary Meth A tumor in wild-type BALB/c (+/+) mice, whereas treatment in BALB/c (*nu/nu*) mice (lacking functional T cells) had no effect in therapy. This lack of antitumor activity of capsaicin in BALB/c (*nu/nu*) mice suggests that T cell functions are essential in capsaicin-mediated immunotherapy (Fig. 4). 3) Ag specificity of the antitumor immune response is clearly revealed in tumor protection studies using antigenically distinct tumor models. T cells generated by treatment of a primary tumor (Meth A) with capsaicin can only protect mice from the growth of another Meth A tumor growing at a distant site but not from an antigenically distinct tumor such as CMS5 as shown in Fig. 5. Ag specificity is solely possible through the adaptive immune response such as T cell response.

These observations can be explained by at least two mechanisms that are not mutually exclusive: 1) increased apoptosis of tumor cells by capsaicin and subsequent initiation of an enhanced antitumor immune response by apoptotic cells. Although the phenomenon of apoptosis is considered to be immunologically quiescent, we suggest that capsaicin-induced apoptosis of tumors in vivo is immunologically active. This idea is in accordance with recent reports suggesting that tumors when treated with certain apoptotic agents such as local gamma radiation (14), intratumoral injection of doxorubicin (15), or i.p. injection of gemcitabine (16) can elicit antitumor T cell response in tumor-bearing animals. It is also reported that apoptotic agents vary in their ability to promote the delivery of suitable Ag to DCs (17) such that the products of early

apoptosis cannot efficiently stimulate MHC class I-restricted anti-tumor T cells even in the presence of DC maturation factors. On the contrary, secondary necrosis was found associated with robust T cell response. Our data implies that capsaicin-induced apoptosis of tumor cells might be providing a maturation signal to DCs (18–20), affecting Ag expression and/or the production of immunomodulating substances, which may significantly influence the generation of a more efficient tumor-specific immune response; 2) DC activation by capsaicin can lead to better priming of antitumor T cell response. We have previously shown that capsaicin injection significantly increases the number as well as the activation status of skin-derived DCs in the draining lymph nodes (11). These activated DCs acquire endotoxin-free Ag from the site of injection (skin) of capsaicin and process and present it to activate Ag-specific T cells in the draining lymph nodes. It would be of interest to perform therapy of tumors in VR1^{-/-} mice where the DCs would be unresponsive to capsaicin.

It is our belief that both of these mechanisms are involved in capsaicin-mediated immunotherapy. Tumor as a source of Ag when undergoing apoptosis by capsaicin can lead to efficient/enhanced loading of tumor-associated Ags into neighboring capsaicin-responsive DCs, which in turn leads to efficient priming of tumor-specific T cells.

We are currently investigating the effectiveness of capsaicin in tumor therapy through routes other than intratumoral. Capsaicin is a common component of foods of many cultures. The question of effect of enteral capsaicin on immune system is worthy of investigation. Regardless, the potency of capsaicin as an immuno-active ligand along with its cytotoxic effects suggests that immunotherapy by capsaicin can elicit a specific immune response that can be tumor protective in a therapeutic setting. This strategy widens the potential therapeutic application for capsaicin and may be a promising new approach in cancer immunotherapy.

Acknowledgments

We acknowledge Pramod Srivastava for critical comments on the manuscript. J. Beltran performed research and analyzed data, A. K. Ghosh designed research, performed research, and analyzed data, and S. Basu designed research, performed research, analyzed data, and wrote the paper.

Disclosures

The authors have no financial conflict of interest.

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