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Antitumour effects of genetically engineered *Salmonella* in combination with radiation

J. Platt [a], S. Sodi [a], M. Kelley [b], S. Rockwell [b], D. Bermudes [c], K.B. Low [b], J. Pawelek [a,*]

[a] *Department of Dermatology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA*
[b] *Department of Therapeutic Radiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA*
[c] *Vion Pharmaceuticals, Four Science Park, New Haven, CT 06511, USA*

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Abstract

The antitumour efficacy of lipid A mutant *Salmonella* was evaluated alone and in combination with X-rays in mice bearing B16F10 or Cloudman S91 melanomas. Each treatment alone slowed tumour growth and prolonged survival, and the combined treatments produced supra-additive antitumour effects. That is, in dose–response studies with single doses of *Salmonella* and increasing doses of radiation, the two agents together caused suppression of tumour growth that was greater than that calculated for additivity. The results suggest that the combination of these genetically engineered *Salmonella* with radiotherapy could be a new and beneficial treatment for solid tumours.

Keywords: *Salmonella*; X-rays; Tumour targeting; Supra-additivity; Melanoma

1. Introduction

This study investigated whether *Salmonella* might be useful when combined with X-ray therapy for melanomas and other solid tumours. Whereas earlier reports suggested that melanomas are relatively radioresistant compared with other tumours, a number of more recent studies have shown that X-ray treatment can indeed elicit complete responses and even local control in a significant percentage of patients [1]. It was recently reported that genetically engineered *Salmonella* have many of the desirable properties of an antitumour vector, including targeting of tumours from a distant inoculation site, selective replication within tumours, tumour growth suppression, and the ability to express effector genes such as the herpes simplex thymidine kinase [2]. Lipid A-modified (*msbB*$^-$) *Salmonella* auxotrophs (*pur*$^-$) were developed that were attenuated in toxicity in mice and swine. These mutants showed significantly reduced host tumour necrosis factor (TNF)α induction, yet retained the abilities for tumour-targeting, amplification and growth suppression, achieving tumour accumulations of 10^9 colony forming units (cfu)/g of tumour with ratios in excess of 1000:1 over normal tissues in mice [3–5]. A candidate *msbB*$^-$, *purI*$^-$ strain, VNP20009 [6], is currently in phase I clinical trials (Vion Pharmaceuticals, New Haven, CT, USA). Thus, we were interested to test the effectiveness of combined treatments of *Salmonella* + X-rays against melanomas and other solid tumours.

2. Materials and methods

2.1. Bacteria and cancer cells

Two independent lipid A-modified (*msbB*$^-$), auxotrophic (*purI*$^-$) strains of *Salmonella*, YS1456 and YS1646, were used. Both of these strains exhibited anticancer phenotypes in terms of targeting, amplification and tumour growth suppression. YS1456 was more virulent than YS1646, and antibiotics were used in mice injected with this strain, as described below. YS1646, also designated 'VNP20009' [6] was derived from strain

* Corresponding author. Tel.: +1-203-785-4411; fax: +1-203-785-7637.
E-mail address: john.pawelek@yale.edu (J. Pawelek).

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YS72 [2,3]. Both YS72 and YS1456 were derived directly from the wild-type strain 14028 (American Type Culture Collection, CDC6516-60) [2]; data not shown). B16F10 mouse melanoma cells were kindly provided by I. Fidler, (M.D. Anderson Cancer Center, Houston, TX, USA). Cell line 94-H48 is a highly metastatic derivative of the Cloudman S91 melanoma [7]. Melanoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium with 10% horse or bovine sera and antibiotics by routine culture methods.

2.2. Tumour implantation and bacterial injection

Tumours were implanted on the mid-right side of the mice. C57B6 or DBA/2J female mice 6–8 weeks old were implanted subcutaneously (s.c.) with 5×10^5 B16F10 or 94-H48 cells respectively. As indicated, mice were further inoculated intraperitoneally (i.p.) or intravenously (i.v.) with *Salmonella*, 2×10^5 cfu/mouse unless otherwise stated, in 0.1–0.2 ml 0.9% saline, at periods from 7 to 14 days post-tumour implantation.

2.3. *Antibiotics* in vivo

In experiments with *Salmonella* strain YS1456, 2 weeks following injection of bacteria, mice were given Baytril™ (Bayer, Inc.) diluted 1:100 in the drinking water for 1 week every other week for up to 7 weeks post-tumour implantation. No antibiotics were used with *Salmonella* strain YS1646.

2.4. X-irradiation

X-rays were administered using a Siemens Stabilipan 250 kV 15 mA X-ray machine with 2 mm aluminum equivalent filtration. Mice were anaesthetised and placed in a lead-shielded mouse tumour localiser. The region of the tumour implantation site was drawn through a 15 mm aperture for irradiation, with the remaining portion of the mouse, including vital organs, shielded. The dose rate was 1.109 Gy/min, as determined by the radiological physics group of the Therapeutic Radiology Department, Yale University, CT, USA, using standard radiation therapy dosimetry techniques.

2.5. Tumour growth

Mice were tagged and followed individually over time for tumour growth. Tumour measurements were collected with Mitutoyo Digimatic Calipers connected to an Apple PowerBook® computer. Volume was calculated and data were rearranged with the Scriptable Text Editor and AppleScript™ software from Apple Computer, Inc. The formula volume $= L \times W \times H \times 0.52$ was used for volume calculations. Effects of treatments on tumour growth were expressed either as mean tumour volumes versus time, the time (days) to 1 g tumours, or as the per cent suppression of tumour growth, expressed as $1 - T/C \times 100$ where T = treated volume and C = control tumour volume. Thus, if treated tumours were 20% of the volume of control tumours on a given day, tumour suppression in the treated group was 80% (Table 1).

2.6. Determination of resistance to Salmonella

B16F10 tumours that had begun growing in mice in the presence of *Salmonella* were dissociated into single cell suspensions, grown for 1–2 weeks in culture with antibiotics to kill residual *Salmonella*, implanted into fresh mice and again challenged with *Salmonella*, with the procedures repeated five times.

2.7. Statistics

Means and standard errors were calculated according to standard formulas with AppleScript™ software. Curve fitting was done with Cricket Graph III® from Computer Associates, Inc. Other statistics, including *P*-value calculations, were done with StatView® from Abacus Concepts, Inc.

2.8. Animal welfare and euthanasia

All experimental protocols were approved by the Yale University Animal Care and Use Committee. Methoxyflurane and CO_2 were each used for mouse euthanasia, consistent with the recommendations of the Panel on

Table 1
Salmonella dose and tumour growth suppression in C57B6 mice bearing B16F10 melanomas

Strain	cfu[a] Injected	% Tumour suppression
YS1646	None	0
	2×10^5	78
	2×10^6	80
YS1456	None	0
	2×10^5	84
	2×10^6	88

C57B6 mice were implanted subcutaneously (s.c.), in the flank, with 5×10^5 B16F10 melanoma cells. *Salmonella* were injected intraperitoneally (i.p.) 7 days later at the cfu indicated, when tumours were 10–50 mg. Tumour growth was assessed after 20 days, when all mice were alive and control tumours were still <2 g. Untreated tumour-bearing control mice were euthanised between days 21–26 post-tumour implantation due to their tumours reaching >2 g. The per cent suppression of tumour growth is calculated using the formula $1-T/C\times100$ where T = treated volume and C = control volume. Results are means for 10/10 mice per group.

[a] Colony forming units.

Euthanasia of the AVMA. Animals were euthanised when tumours reached 2–4 g, or sooner if they showed signs of distress (cessation of eating and drinking, weight loss, listlessness). Yale University is registered as a research facility with the United States Department of Agriculture. The School of Medicine is fully accredited by the American Association for Accreditation of Laboratory Animal Care. An Animal Welfare Assurance is on file with OPRR-NIH.

3. Results

3.1. Salmonella *and tumour growth suppression*

Salmonella suppressed the growth of B16F10 melanomas (Table 1). The number of bacteria inoculated had only a small effect on tumour suppression: i.p. injections of 2×10^5 and 2×10^6 cfu inhibited tumour growth by 78 and 80%, respectively, 20 days post-tumour implantation (Table 1). In a similar experiment with i.v. injections at 7 days and tumour measurements at 24 days post tumour implantation, bacterial inoculi were increased in increments over a 100-fold range, while tumour suppression increased from 80% at 10^4 cfu/mouse to 94% at 10^6 cfu/mouse (data not shown). This lack of strong dependence of tumour suppression on the number of inoculated bacteria presumably results from amplification of the *Salmonella* within tumours [2,3]. For example, when *Salmonella* were injected i.p. into B16F10 melanoma-bearing mice in doses ranging from 10^2 to 10^5 cfu/mouse, tumour bacterial counts consistently reached levels of 10^9 cfu/g by 6 days (data not shown). Therefore, with these *Salmonella*, the final numbers of bacteria eventually plateaued at similar levels within the tumours, independent of the original bacterial inoculum. Thus, in the experiments described below, single doses of *Salmonella*/mouse were used throughout, in combination with either single or multiple doses of X-rays.

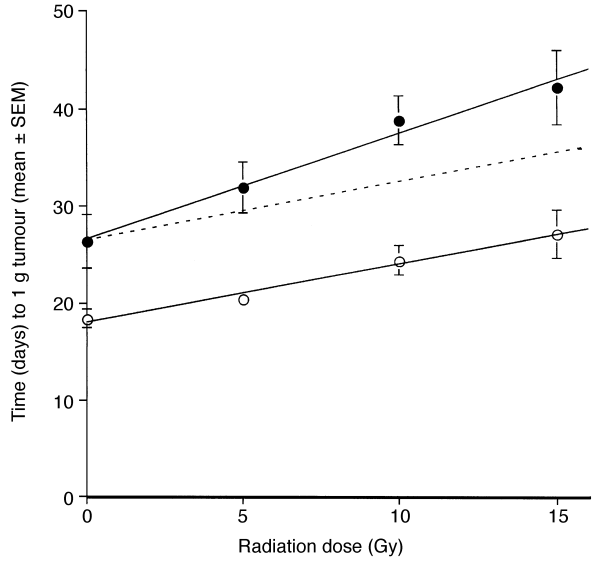

Fig. 1. Time (days) to 1 g tumour after subcutaneous (s.c.) implantation of 5×10^5 B16F10 mouse melanoma cells into C57B6 mice ($n=8$–10/group), followed 7 days later, when tumours were not yet palpable, by i.v. injection of *Salmonella* YS1646. Four days after *Salmonella* injection, mice were irradiated with X-rays at doses of 5–15 Gy. Controls with or without *Salmonella* were sham-irradiated for the time equivalent to 15 Gy. Points represent the mean±SEM (standard error of the mean). The dashed line denotes the antitumour effects expected from additive interactions between the X-ray and *Salmonella* treatments [8]. All treatment categories differed significantly from the sham-irradiated controls, with P values ranging from <0.0001 to 0.03. *Salmonella*+X-rays differed from X-rays alone at each X-ray dose, with P values ranging from <0.0001 to 0.002. The experiments were performed three times with similar results (Table 2). ○, No *Salmonella*; ●, with *Salmonella*.

3.2. Salmonella *and X-rays*

The effects of single X-ray doses ranging from 5 to 15 Gy, with and without i.v. injected *Salmonella*, on B16F10 growth suppression were investigated (Fig. 1). Antitumour activity was assessed by determining the number of days post tumour implantation needed to form 1 g tumours. *Salmonella* alone prolonged the time to 1 g from the control value of 18±1 days (mean±SEM) to a value of 26±3 days. X-rays alone also prolonged the time to 1 g. The dose–response curve relating radiation dose to tumour growth delay was linear over the dose range studied in these experiments. The combination of *Salmonella*+X-rays showed supra-additive antitumour effects, with the slope of the dose–response curve being greater than expected for additivity (Fig. 1). Supra-additivity was indicated in all three of the three

Table 2
X-ray dose–response ±*Salmonella* strain YS1646 versus tumour growth

Treatment	Experiment no.	Slope + y intercept[a]	r^2
X-rays only	177	$y=0.605x+18.0$	0.99
(0–15 Gy)	172	$y=0.490x+17.9$	0.60
	185	$y=0.524x+16.4$	0.99
	Pooled data	$y=0.514x+17.6$	0.83
Salmonella+X-rays	177	$y=1.089x+26.6$	0.99
(0–15 Gy)	172	$y=1.350x+21.2$	0.99
	185	$y=0.912x+21.5$	0.43
	Pooled data	$y=1.075x+23.3$	0.61
Expected for additivity	177	$y=0.605x+26.7$	n.a.
	172	$y=0.490x+21.2$	n.a.
	185	$y=0.524x+21.5$	n.a.
	Pooled data	$y=0.514x+23.3$	n.a.

r^2, Correlation coefficient. na, Not available.

[a] y Intercept for X-rays only = mean number of days for tumours to reach 1 g in sham X-rayed control mice (compare with Fig. 1); y intercept for *Salmonella* plus X-rays = mean days for tumours to reach 1 g in control mice treated with *Salmonella* only plus sham radiation.

X-ray dose–response experiments in mice using the B16F10 melanoma, as shown by comparing the actual slopes of the dose–response curves obtained to those slopes expected for simple additivity (Table 2) [8].

Tumour growth curves from the experiment in Fig. 1 are shown in Fig. 2, where it is seen that the combination of *Salmonella* and a single dose of 15 Gy X-rays markedly slowed B16F10 melanoma growth and prolonged mouse survival compared with the other treatments. Similar results with a single dose of 15 Gy X-rays in combination with *Salmonella* were obtained with the Cloudman S91 melanoma line 94-H48 implanted s.c. in DBA/2J mice (Fig. 3).

Higher cumulative X-ray doses of 25 Gy (Fig. 4) and 50 Gy (Fig. 5) were achieved by delivery of smaller weekly increments (arrows). When the total X-ray doses from all of our experiments were compared (range: 5–50 Gy), the results consistently demonstrated that tumour growth suppression with and without *Salmonella* increased with increasing X-ray dosage, and that at all X-ray doses measured, the combined effects of *Salmonella* + X-rays were greater than expected for additivity. In the experiments with X-ray doses of 25–50 Gy (Figs. 4 and 5) we could not formally assess whether the combination of radiation + *Salmonella* produced supra-additive effects, because this would have required full fractional dose–response curves which were not performed. None the less, the strongest tumour suppression and longest survival of mice were achieved at the highest cumulative dose (50 Gy) of X-rays + *Salmonella* (Fig. 5). For this combination, B16F10 melanomas reached 1 g an average of 100 days post tumour implantation, a time 6 times longer than for the sham irradiated controls, 5 times longer than with *Salmonella* alone, and approximately 50% longer than with X-rays alone.

Although regressions were transient (data not shown), '*Salmonella*-cycled' tumours and the clones derived from them did not appear to be resistant to *Salmonella* and showed a similar sensitivity as control B16F10 melanomas when rechallenged with *Salmonella* (data not shown).

4. Discussion

The above results demonstrated in two melanoma tumour models that combined administration of *Salmonella* and X-rays produced significantly greater anti-tumour effects than either of the treatments alone. With both tumour models, when treatment was initiated after tumours were palpable, numerous cases of tumour regression were documented in individual experimental animals, most frequently with the combined treatments of *Salmonella* + X-rays. While in these cases tumour growth was markedly retarded in comparison with controls, the regressions were transient and tumours eventually recurred (data not shown). The mechanisms for this tumour escape are not understood; however, one possibility, an enrichment of *Salmonella*-resistant

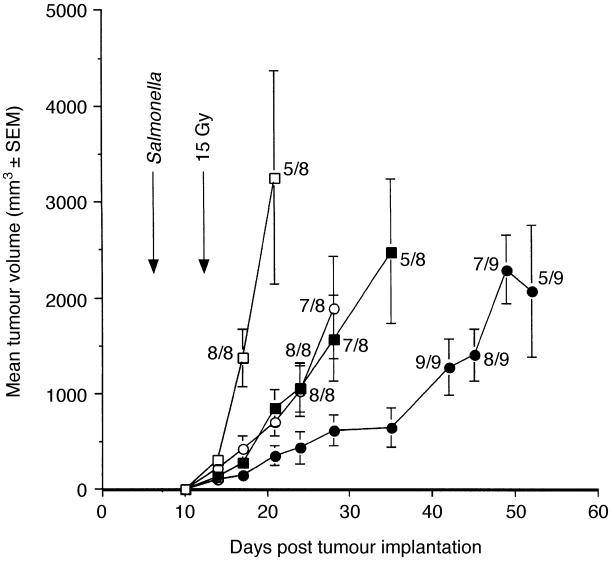

Fig. 2. Effects of single X-ray doses (15 Gy), with and without *Salmonella* strain YS1646, on the growth of B16F10 mouse melanomas after subcutaneous (s.c.) implantation of 5×10^5 B16F10 cells in C57B6 mice. Data are from the same experiment shown in Fig. 1. □, sham-irradiated controls; ○, *Salmonella* only; ■, X-rays only; ●, *Salmonella* plus X-rays. Experiments were performed three times with similar results. The fractions on the graphs are the *n* living mice/*n* mice initially in the treatment group, shown in this and Figs. 3–5 below. SEM, standard error of the mean.

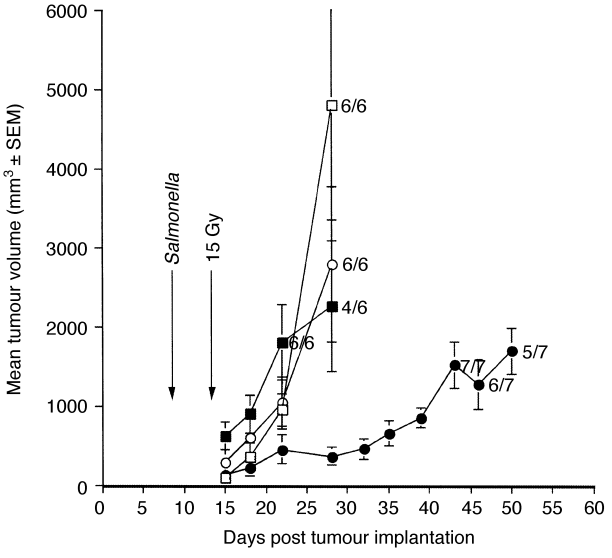

Fig. 3. Effects of single X-ray doses (15 Gy), with and without *Salmonella* strain YS1646, injected intravenously (i.v.), on the growth of Cloudman S91 mouse melanoma 94-H48 after subcutaneous (s.c.) implantation of 5×10^5 94-H48 cells in DBA/2J mice. Experiments were performed twice with similar results. □, Sham-irradiated controls; ○, *Salmonella* only; ■, X-rays only; ●, *Salmonella* plus X-rays. SEM, standard error of the mean.

tumour cells, did not seem to be the case. In resistance experiments in all cases, the '*Salmonella*-cycled' tumours, and clones derived from them, were still suppressed by *Salmonella* when re-implanted in mice, similar to control B16F10 melanomas that had received no previous *Salmonella* exposure. Thus, there was no evidence of acquired melanoma cell resistance to *Salmonella*, and the phenomenon of tumour escape from *Salmonella* suppression could not be explained by this mechanism.

Although the mechanisms are not yet understood, the antitumour effects of combined treatment with X-rays and *Salmonella* are likely to be multifaceted, and we plan to try to elucidate mechanism(s) with the following considerations. *Salmonella* could secrete molecules which increase cellular radiosensitivity, e.g. by inhibiting repair of radiation damage. X-ray treatment, though not lethal to all tumour cells at the doses used here, might render some of the tumour cells more vulnerable to *Salmonella* infection and/or to *Salmonella* toxins [9–13]. X-rays might alter the tumour environment rendering it more accessible to *Salmonella* infection. *Salmonella* might recruit cells of the immune system to tumours (e.g. lymphocytes, macrophages, neutrophils) and X-rays might render tumour cells more vulnerable to immune attack. Another possibility, that X-rays somehow induce an increase in the number of *Salmonella* in the tumours, was not borne out. That is, *Salmonella*/g tumour were quantitated over 5 days post X-ray treatment (15 Gy) and no effect of X-rays on *Salmonella* number in tumours was found (data not shown).

In experiments not shown, we found that exposure of the *Salmonella* cultures *in vitro* to 20 Gy X-rays resulted in >90% survival of the bacteria. Thus, the X-ray treatments used in the tumour experiments (highest single dose 15 Gy) would have had very little effect on the survival of the *Salmonella* in the tumours. The resistance of the *Salmonella* to 20 Gy X-rays was consistent with that reported for the response of wild-type *Escherichia coli* to irradiation [14].

We used the term 'supra-additivity' as discussed by Steel and Peckham [8] for combined radiotherapy and chemotherapy in which the effect of a combination appears to be greater than would be expected for the two agents on the basis of additivity. Since *Salmonella* amplify within tumours, the effects on bacterial density within tumours, as well as on tumour suppression, are largely independent of the initial inoculum, and therefore there is no dose–response curve for the effects of

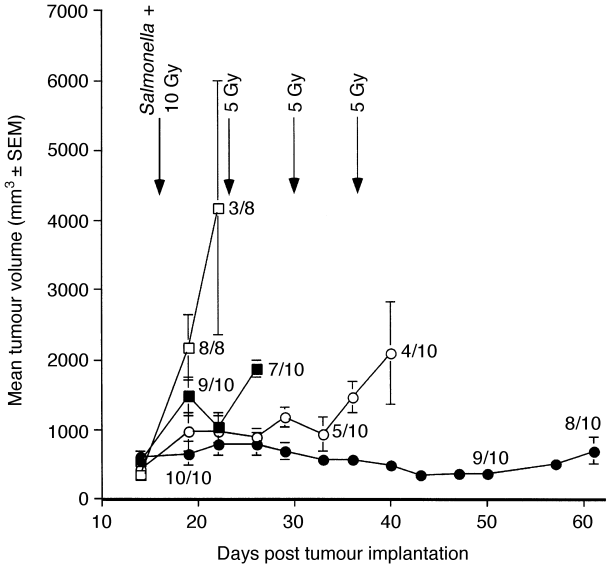

Fig. 4. Effects of multiple X-ray treatments, to a cumulative dose of 25 Gy, with and without *Salmonella* strain YS1456, on the growth of B16F10 mouse melanomas. C57B6 mice were implanted subcutaneously (s.c.) with 5×10^5 B16F10 mouse melanoma cells. Tumours were irradiated locally with 10 Gy 14 days post tumour cell inoculation, when tumours were approximately 0.5 g. *Salmonella* was injected intraperitoneally (i.p.) later on the same day. Sham-irradiations or 5 Gy of X-rays were then given weekly for the next 3 weeks. Schedules for irradiations and *Salmonella* injection are indicated by arrows. Antibiotic treatment with Baytril was terminated 32 days post tumour implantation. Experiments were performed twice with similar results. □, Sham-irradiated controls; ○, *Salmonella* only; ■, X-rays only; ●, *Salmonella* plus X-rays. SEM, standard error of the mean.

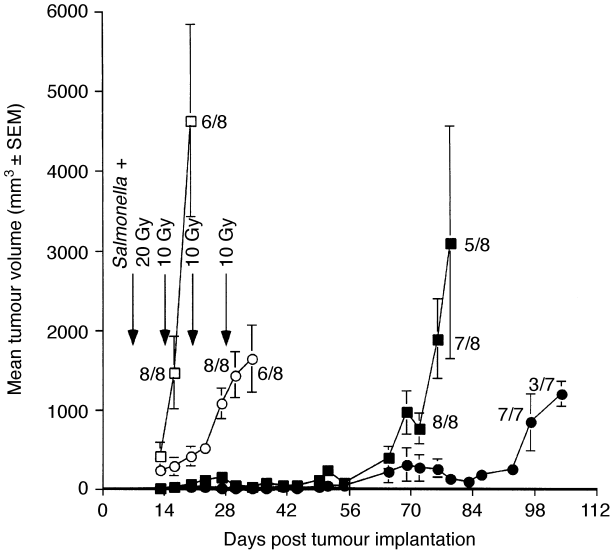

Fig. 5. Effects of multiple X-ray treatments, to a cumulative dose of 50 Gy, with and without *Salmonella* strain YS1456, on growth of B16F10 mouse melanomas. C57B6 mice were implanted subcutaneously (s.c.) with 5×10^5 B16F10 mouse melanoma cells. On day 9 post implantation, when tumours were not yet palpable, the regions in which the tumours had been implanted were irradiated with 20 Gy of X-rays. *Salmonella* strain YS1456 was injected intraperitoneally (i.p.) later on the same day. The same groups received additional treatment with 10 Gy X-rays at 14, 21 and 28 days post-tumour implantation. Schedules for irradiations and *Salmonella* injection are indicated by arrows. Antibiotic treatment with Baytril was terminated 49 days post-tumour implantation. Experiments were performed twice, with similar results. □, Sham-irradiated controls; ○, *Salmonella* only; ■, X-rays only; ●, *Salmonella* plus X-rays. SEM, standard error of the mean.

Salmonella alone, using tumour suppression as an endpoint. The term *synergism* was not used because the term implies that the two agents were working together and, as stated above, the mechanisms underlying the supra-additive antitumour effects of *Salmonella* and X-rays are unknown. Whatever the mechanisms, these results suggest that the combined use of *Salmonella* and radiation would be of therapeutic value in cases where X-irradiation is indicated for the therapy of solid tumours.

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