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Tumour burden and interleukin-2 dose affect the interaction between low-dose total body irradiation and interleukin 2

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Abstract

Low-dose total body irradiation (LTBI) has a synergistic immune-mediated antitumour effect when used in combination with interleukin 2 (IL-2) in a murine metastatic malignant melanoma model. To optimise the use of this combination treatment this study was performed to test the effect of tumour burden and dose of both LTBI and IL-2 on the therapeutic potential of this treatment strategy. Ten-week-old female C57BL/6 mice were inoculated intravenously (day 0) with 1 million B16F1 malignant melanoma cells. Groups of mice received no treatment, a single fraction of LTBI alone, IL-2 treatment alone, or a combination of LTBI and IL-2. Two doses of LTBI and IL-2 were tested. LTBI was given on day +10 and IL-2 treatment started on day +11. On day +18 the mice were killed. The lungs were removed and analysed for tumour burden. Lung sections were also tested for infiltrating leucocytes using immunohistochemical staining. In one experiment, mice were treated at day +7 with low-dose IL-2 with and without LTBI. LTBI (in the two tested doses) showed no independent therapeutic effects. An IL-2 dose of 300,000 Cetus units (CU) that was effective and showed synergism with LTBI when mice were treated on day +7 failed to show a therapeutic effect when mice were treated on day +10, at which time the initial tumour burden had doubled. High-dose IL-2 (600,000 CU), in contrast, led to a significant reduction in metastatic burden compared to the control group. Combining high-dose IL-2 with LTBI led to a further significant reduction in tumour burden. Moreover, this combination was associated with a less severe vascular leakage syndrome compared to IL-2 alone. IL-2 and combination treatment was associated with an increase in the number of tumour-infiltrating immune cells, but only the number of tumour-infiltrating natural killer cells reflected therapeutic efficacy. It was concluded that tumour burden at the time of treatment and IL-2 dose are two crucial factors affecting the synergism between LTBI and IL-2. The combination may not only be more effective than IL-2 alone but also less toxic.

Keywords: Low-dose total body irradiation; Interleukin 2; B16 melanoma; Therapy effect

1. Introduction

We have previously shown that low-dose total body irradiation (LTBI) in a single fraction of 0.75 Gy has a synergistic, immune-mediated antitumour effect when used in combination with interleukin 2 (IL-2) in a murine metastatic malignant melanoma model [1]. The data pointed to natural killer (NK) cells and macrophages as probable major effectors of the synergistic outcome of the combined treatment.

The immune modulatory effects of LTBI are known to occur within a narrow window of radiation dose [2]. Exploiting the immunomodulatory effects of TBI necessitates, therefore, a careful choice of radiation dose. Hosoi and colleagues [3] have shown that a reduction of metastasis seen in experimental animals with a LTBI dose of 0.2 Gy was lost (or even reversed) when the dose was raised to 0.5 Gy. A study on non-tumour-bearing mice showed that a single LTBI fraction of only 0.075 Gy increased the expression of IL-2 receptors on

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concanavalin A-stimulated thymocytes 24 h after irradiation, and increased the reactivity of splenic cells to exogenous IL-2 [4]. The investigators state that the most prominent immune stimulation after low-dose irradiation occurred in the T lymphocytes and that this potentiation occurred in a dose range lower that 0.25 Gy. These observations warranted the testing of other lower TBI doses in this model as it raised the possibility that a lower LTBI dose might stimulate a stronger/additional immune response with possibly greater synergism.

Initial studies with IL-2 in a similar model showed that the time at which treatment is initiated affects the optimal IL-2 dose needed to achieve a therapeutic outcome [5]. We treated mice on day 7+ post-inoculation and found that an IL-2 dose of 300,000 Cetus units (CU) given over 5 days could reduce the tumour burden appreciably and synergise with LTBI at a dose of 0.75 Gy. By day 7+, 2.7% (±0.5%) of the lungs had been invaded by melanoma cells, while by day 10+, the tumour burden had almost doubled, with metastases constituting an average of 5% (±1.5%) of the total lung volume (unpublished data). The question then arose whether treating mice on day 10+ would have an influence on treatment outcome.

With the aim of optimising the use of this treatment strategy, the following experiments were performed to test how tumour burden at the time of treatment initiation, LTBI and IL-2 dose would affect both the therapeutic efficacy and the toxicity of this potentially useful combination.

2. Materials and methods

2.1. Animals

Female C57bL/6J mice were purchased from M&A A/S (Ry, Denmark). The mice were kept under specific pathogen-free conditions at the Department of Experimental Clinical Oncology, Aarhus University Hospital, and given food and water *ad libitum*. They were first acclimatised for a minimum of 1 week and then used when 10–12 weeks of age.

2.2. Tumour cells

B16F1PVI, a murine malignant melanoma subline syngeneic to C57BL/6J mice, was maintained *in vitro* at 37 °C in a humidified atmosphere containing 5% CO_2 and cultured in RPMI-1640 (Gibco-BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Harlan Sera Lab Ltd., Belton, UK), 2 mM L-glutamine, 20 mM Hepes buffer, penicillin 100 units/ml and streptomycin 100 μg/ml. Adherent cells were detached with 0.02% EDTA for 10 min, and washed twice in RPMI-1640 medium. Before injection the cells were resuspended in RPMI supplemented with 2% FCS. Cell viability was always >95% before injection.

2.3. Interleukin 2

Human recombinant (r)IL-2 (Proleukin®) was kindly provided by Chiron (Amsterdam, NL). Each ampoule (containing 1.1 mg/ml = 18×10^6 IU/ml) was rehydrated in 1 ml ultrafiltered water (UF) and 5 ml phosphate-buffered saline (PBS). Mice were injected intraperitoneally three times daily for five consecutive days. Two doses of IL-2 were tested: 60,000 CU /day to a total of 300,000 CU and 120,000 CU/day to a total of 600,000 CU.

2.4. Irradiation

Irradiated mice were restrained in a Plexiglas box and the dose was delivered to the whole body from a Philips 250 kV X-ray therapy machine with a dose rate of 0.05 Gy/min. Two doses were tested: 0.075 Gy and 0.75. Non-irradiated groups were restrained in the Plexiglas box and sham treated.

2.5. Experimental plan and treatment schedule

The mice were inoculated in the tail vein with 1×10^6 B16F1 cells on day 0. They were then randomised into nine groups receiving no treatment (control group), LTBI alone (two dose levels), IL-2 treatment alone (two dose levels), or a combination of LTBI (×2 dose levels) and IL-2 (×2 dose levels). The experiment was repeated three times and the final number of mice included in each experimental group ranged from 12 to 18. LTBI was given on day +10 after tumour inoculation and IL-2 treatment started on day +11. The mice were killed on day +18 when whole blood was collected by heart puncture. The chest wall was then opened and 0.5 ml of Tissue-tek (OCT compound 4585; Miles Inc., Elkhart, USA) injected into the trachea before the lungs were extracted, submerged in Tissue-tek and snap frozen in −70 °C Hexan. The frozen lungs were later sectioned and stained for estimation of tumour burden as previously described [1,6]. The abdomen was then opened, the internal organs inspected for metastasis, and the liver and spleens dissected and weighed. In one experiment, mice were treated at day +7 with low-dose IL-2 with and without LTBI.

2.6. Tumour-infiltrating leucocytes (TIL)

The lung sections were stained by a standard two-layer immunoperoxidase technique. After fixation in acetone for 10 min, the slides were washed in Coon's phosphate buffer (NaCl, Na_2HPO_4) containing 5% bovine serum albumin (BSA) followed by the addition of polyclonal rabbit antimouse asialoGM_1 antibody (Waco Chemical Industries, Japan) in a 1:600 dilution,

rat anti-CD8, IgG2b (ATCC) in a 1:200 dilution, rat anti-CD4, IgG2b (ATCC) in a 1:200 dilution, or rat antisialoadhesin (Serotec) in a 1:100 dilution. Sections were incubated at room temperature in a humidified chamber for 1 h and washed four times in Coon's buffer supplemented with 5% BSA. Peroxidase-conjugated goat antirabbit IgG2b (Zymed, code 65-6120) or peroxidase-conjugated goat antirat IgG2b (Zymed, code 7063-7221) supplemented with a blocking murine serum at 1:1 to prevent background activity were used as the second-layer antibody in a 1:25 dilution. As controls, normal rabbit serum or irrelevant rat IgG2b isotype monoclonals were used. After 30 min of incubation the sections were washed four times in Tris buffer 0.05 M, pH 7.6. 3,3-diaminobenzidine tetrahydrochloride was used as chromogen in all experiments. Sections were counterstained with Meyer's haematoxylin for 10 min. The number of positively stained cells was determined using the 400× objective of an Olympus microscope in an arbitrarily chosen, fixed tumour area of 300,000 μm^2.

2.7. Statistical methods

The data from all experiments were then pooled and reported as mean ± SEM. A two-tailed independent samples *t* test for equality of the means was used to determine whether two sample means were significantly different. The two-tailed Spearman correlation was used to test correlation between TIL and tumour burden. The analysis was done using the statistical program *SPSS 9.0 for Windows*.

3. Results

3.1. Therapeutic efficacy of LTBI and IL-2

The data in Fig. 1 show the percentage of lung area infiltrated with metastases in each of the experimental groups. The results showed that LTBI alone (in the two tested doses) does not manifest any significant therapeutic effect in this model. The therapeutic efficacy of IL-2 was dose dependent; whereas 300,000 CU was 'non-therapeutic', a dose of 600,000 CU led to a significant reduction in tumour burden from 29 ± 4% to 13 ± 3%. Combining LTBI (in either of the tested doses) and the lower non-therapeutic dose of IL-2 added no significant therapeutic advantage, but combining LTBI with the higher dose of IL-2 produced a further significant reduction of metastatic burden to 5.7% (±0.9%) in the 0.075 Gy group and 7.0% (±1.3%) in the 0.75 Gy group. The difference between high-dose IL-2 alone and each of the combination treatment groups was statistically significant, while that between the two combination groups (with two different LTBI doses) was not.

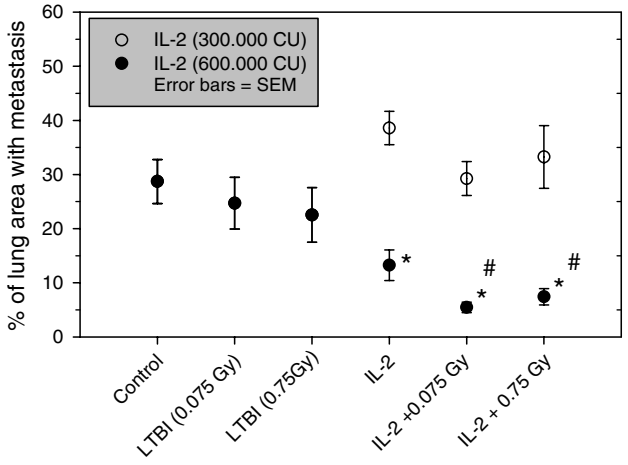

Fig. 1. Tumour burden in the various experimental groups. The burden of metastatic B16 malignant melanoma cells in the lungs of C57bl/6 mice in the different treatment groups. Treatment began on day +10 after tumour implantation and mice were killed on day +18. Tumour burden is represented as percentage of lung area occupied by metastases. There were 12–18 mice in each experimental group. Error bars are SEM. The symbol marked with (*) is significantly different ($P < 0.05$) from the control group; that marked with (#) is significantly different ($P < 0.05$) from the corresponding interleukin 2 (IL-2) group. LTBI, low-dose total body irradiation.

3.2. Tumour burden at the time of treatment

Fig. 2 shows how the significant 57% (±8%) reduction in tumour burden achieved by treating mice on day 7 with LTBI (0.75 Gy) and 300,000 CU IL-2 (Fig. 2a) was lost when mice were treated with the same combination but on day 10 instead (Fig. 2(b)). Treating mice on day 10 with double the IL-2 dose (Fig. 2(c)) led to a restoration of the therapeutic effect of IL-2 alone (32 ± 10% reduction in tumour burden) and restitution of the synergistic effect of the combination treatment (50 ± 12% reduction in tumour burden).

3.3. Tumour-infiltrating leucocytes

Analysis of TIL is shown in Table 1. The most important findings can be summarised as follows:

- LTBI alone led to a significant, dose-independent increase in the number of tumour-infiltrating macrophages.
- Treatment with IL-2 alone led to dose-dependent increase in the number of all the tumour-infiltrating cells tested apart from macrophages after the lower dose of IL-2.
- Compared to high-dose IL-2 alone, the addition of 0.75 Gy of TBI led to a further significant increase in the number of tumour-infiltrating NK cells.
- LTBI seems to limit (in a dose-dependent manner) the IL-2-induced increase in the number of tumour-infiltrating CD4+ cells though this only reached a statistical significant level in the group treated with low-dose IL-2 and 0.75 Gy TBI.

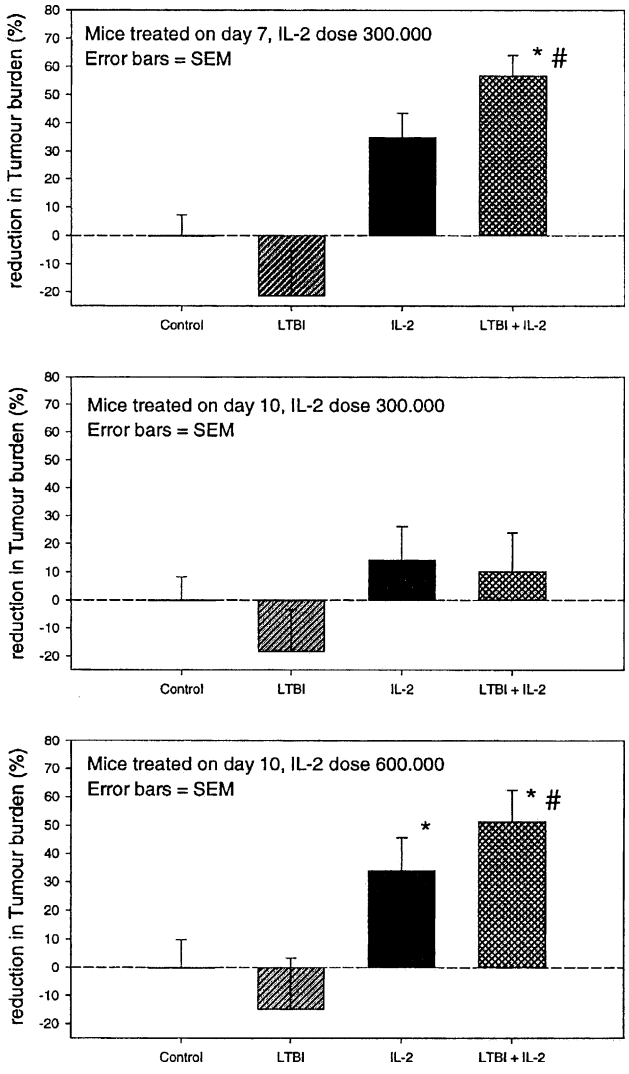

Fig. 2. Effect of time of treatment start and interleukin 2 (IL-2) dose on therapeutic efficacy, expressed as percentage reduction of tumour burden. The time of treatment start is specified on the figures. Each bar chart represents data from a minimum of 11 mice. Low-dose total body irradiation (LTBI) dose was 0.75 Gy = Error bars, SEM.

The Spearman correlation between percentage reduction in tumour burden and tumour-infiltrating cells was only significant for NK cells ($P = 0.047$).

3.4. Vascular leakage syndrome (VLS)

Fig. 3 shows how liver and spleen weights were affected by the administered treatment with a similar pattern in both organs. LTBI alone had no effect on liver and spleen weight. IL-2, conversely, led to a significant and dose-dependent increase in the weight of both organs due to VLS and also, in the case of the spleen, an increase in the number of cells (data not shown). Combining LTBI and IL-2 led to a dose-dependent reduction of the organ weight compared with IL-2 alone, the difference in organ weight between IL-2 and combination treatment being seen most significantly when high-dose IL-2 was combined with 0.75 Gy of TBI ($P = 0.055$ and 0.03 for liver and spleen, respectively).

4. Discussion

The results of this study confirm our previously reported data on the synergistic effect of combining LTBI with IL-2 in this tumour model [1].

The demonstration of a dose-response relation between IL-2 and tumour burden is in agreement with earlier reports using IL-2 in a similar model [5]. As in our previous work we determined tumour burden histologically, expressed as the percentage of the total lung area (our chosen endpoint). Counting superficial metastasis, apart from being inherently inaccurate, would have not been feasible, especially in the control and the LTBI-alone groups where more than 25% of lung area was occupied with confluent metastasis that precluded accurate counting. The merits of the chosen endpoint have been discussed elsewhere [6]. Our ability to detect a dose–response relation for a standard treatment such as IL-2 with this endpoint is an additional argument for its validity.

Loss of the therapeutic effect of 3×10^5 CU IL-2 when treatment is delayed from day +7 to day +10 is in agreement with other experimental and clinical reports showing that the potency of IL-2 [7] and other forms of immunotherapy depends on tumour burden at the time of the start of treatment [8].

There was no obvious difference in the therapeutic effects of the two TBI doses either when given alone or in combination with IL-2, although the therapeutic effects of the lower dose had a higher statistical significance, which may suggest a greater synergism at the lower doses. This would be in agreement with other data reporting an 'inverted U' phenomenon or a reversal of the dose–response relation for many biological-response modifiers and LTBI [2].

The increase in the number of macrophages infiltrating the metastases following LTBI is in line with data from Ibuki and Goto [9,10], who have shown that LTBI activates the proliferation of macrophages. The lack of association between this increase and a reduction in tumour burden casts some doubt on the part that these cells play in the therapeutic synergism between LTBI and IL-2. Therapeutic efficacy presented as the percentage reduction in tumour burden was significantly correlated only with tumour-infiltrating NK cells. This confirms our previous observation and suggestion that NK cells may be a major player in the synergistic interaction between IL-2 and LTBI. This notion is further supported by a recent study showing that LTBI increases the functional activity of NK cells in the spleens of low-dose irradiated mice [11].

Table 1
The statistical significance of change in the average number (±SEM) of immune cells infiltrating 300,000 μm^2 of lung metastasis in the various experimental groups

Experimental group (No. of mice)	NK cells	*P*	Mø** cells	*P*	CD4+ cells	*P*	CD8+ cells	*P*
Control	8		5		9		3	
(12)	(0.8)		(0.7)		(1.3)		(0.6)	
LTBI (0.075 Gy)	7	*N.S.	8	*0.01	6	*N.S.	2	*N.S.
(12)	(1.3)		(0.9)		(0.9)		(0.7)	
LTBI (0.75 Gy)	10	*N.S.	9	*0.04	7	*N.S.	2	*N.S.
(12)	(2.0)		(2.1)		(1.1)		(0.6)	
IL-2 (3×10^5 CU)	11	*0.03	6	*N.S.	16	*0.00	7	*0.00
(12)	(1.0)		(1.1)		(1.9)		(1.2)	
IL-2 (3×10^5 CU) + 0.075 Gy	14	*0.00	9	*0.00	16	*0.00	9	*0.00
(12)	(1.0)	***N.S.	(1.5)	***N.S.	(2.3)	***N.S.	(1.9)	***N.S.
IL-2 (3×10^5 CU) + 0.75 Gy	12	*0.05	11	*0.00	9	*N.S.	12	*0.00
(12)	(1.6)	***N.S.	(2.6)	***N.S.	(1.6)	***0.01–	(2.8)	***N.S.
IL-2 (6×10^5 CU)	20	* 0.01	10	*0.00	25	*0.00	18	*0.00
(12)	(3.7)		(1.8)		(4.3)		(3.5)	
IL-2 (6×10^5 CU) + 0.075 Gy	32	*0.05	11	*0.00	37	*0.00	22	*0.00
(12)	(9.8)	***N.S.	(1.7)	***N.S.	(7.1)	***N.S.	(3.5)	***N.S.
IL-2 (6×10^5 CU) + 0.75 Gy	38	*0.02	15	*0.00	18	*0.00	22	*0.00
(12)	(9.0)	***0.05	(2.3)	***N.S.	(7.8)	***N.S.	(6.3)	***N.S.

LTBI, low-dose total body irradiation; IL-2, interleukin 2; N.S., nonsignificant.
* Comparison with control.
** Macrophages.
*** Comparison with corresponding dose of IL-2 alone.

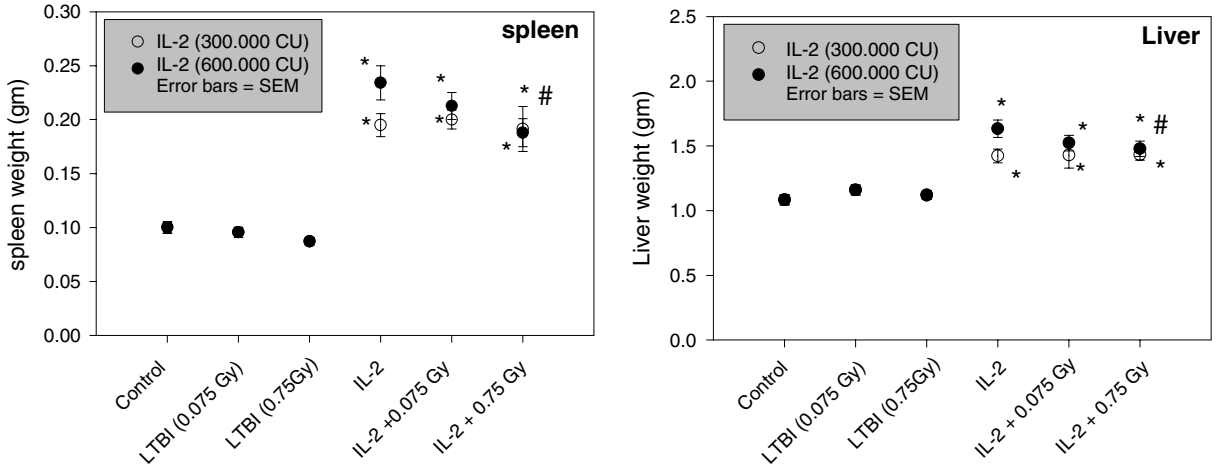

Fig. 3. Effect of low-dose total body irradiation (LTBI) and interleukin 2 (IL-2) dose on average liver and spleen weight in the various treatment groups. Each bar chart represents data from 12 to 18 mice. The symbol marked with (*) is significantly different ($P < 0.05$) from the control group; that marked with (#) is significantly different ($P < 0.05$) from IL-2 alone. Error bars = SEM.

Hashimoto and colleagues have shown that a single TBI dose of 0.2 Gy has a suppressive effect on lung and lymph node metastasis in rats harbouring the allogeneic hepatoma (KDH-8) tumour. This was associated with an increase in tumour-infiltrating lymphocytes, of which 75% were CD8+ cells [12]. In the current study, the percentage of CD8+ tumour-infiltrating cells did not differ significantly between groups and ranged from 7% to 12% in the control and LTBI-treated groups to 15–25% in the groups receiving IL-2 either alone or in combination with LTBI. Infiltrating NK cells were commonly seen, with consistent representation in the successfully treated groups of around 35%. The difference between these results and those of Hashimoto might be related to differences in the animal/tumour model, the time of testing for tumour-infiltrating cells, the use of IL-2 or the difference in the dose of LTBI.

Our finding of a protective effect of LTBI against IL-2-induced VLS is in perfect agreement with the study of Fourquet and colleagues [13]. In contrast to that study, in which the mice were killed immediately after the last IL-2 dose, our animals were killed 3 days after

the last IL-2 dose. However, the protective effect of LTBI could still be detected. Though Fourquet and colleagues did not test this combination in tumour-bearing mice, the therapeutic efficacy of our combination treatment is also in agreement with their data showing that the addition of the LTBI to IL-2 did not affect the cytotoxic activity of NK cells.

5. Conclusion

We conclude that tumour burden at the time of treatment and IL-2 dose are two crucial factors affecting the synergy between LTBI and IL-2. The combination may not only be more effective but also less toxic than IL-2 alone. Within the range of radiation dose tested, TBI dose does not seem to influence the antitumour effect of the combination nor the NK response. Further studies are needed to investigate the mechanisms behind the synergism, in particular the part played by NK cells and possibly of the NKT subset.

Acknowledgements

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