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Inhibition of spontaneous pulmonary metastases of Lewis lung carcinoma by oral treatment with Respivax and Broncho-Vaxom

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Summary. The antimetastatic activity of orally administered polybacterial vaccines, Broncho-Vaxom (BV) and Respivax (RV) was examined in C57BL/6 mice, bearing implants of Lewis lung carcinoma (3LL) in the footpad. The oral administration of BV or RV for 10 consecutive days before or after surgery caused significant reduction of the number and volume of lung metastases. In addition, the therapeutic potential of BV and RV was examined in combination with chemotherapy to determine if there is additive activity. In animals bearing pulmonary micrometastases, treatment with a combination of cyclophosphamide at 50–150 mg/kg with BV or RV was found to be more effective than each of these treatments alone. In immune function studies it was established that the oral administration of BV and RV induced an increase in the number of cells, recovered by broncho-alveolar lavage, and alveolar macrophages were dominant in these cell populations. Furthermore, oral treatment of mice with these vaccines rendered their alveolar macrophages tumoricidal for syngeneic metastatic 3LL cells in vitro. These results show that pulmonary macrophages induced by oral administration of BV and RV played a key role in the inhibition of metastasis in 3LL-bearing mice.

Key words: Metastases – Macrophages – Chemo-immunotherapy

Introduction

The limited success to date of conventional cancer therapy in the treatment of disseminated malignancies has prompted many attempts to develop immunotherapeutic regiments that aim to activate host defences in situ. As the lung is frequently a site of development of primary and/or metastatic tumours, many efforts have been made to activate cells found in this organ that have potential antitumour activity, such as natural killer (NK) cells [26, 28, 29] or macrophages [9, 12, 22, 23].

Broncho-Vaxom (BV) is a polyvalent bacterial lysate used in the treatment of respiratory tract infections, especially acute and chronic bronchitis [1, 13, 14, 19]. Several in vitro experiments indicate that BV can affect various immunological mechanisms including stimulation of the metabolic and functional activities of macrophages [15], activation of NK cells [30] and enhancement of interleukin-1 (IL-1) [5], IL-2, tumour necrosis factor (TNF) α and IFNγ production [30]. Treatment of mice per os increased the levels of IgA in gut and lung secretions [3]. In animals immunosuppressed with cyclophosphamide, BV restored serum immunoglobulin levels as well as the cell proliferation in the thymus and the number of anti-(sheep erythrocyte) plaque-forming cells in the spleen [4]. Respivax (RV) is a similar preparation intended for oral immunotherapy and immunoprophylaxis of non-specific respiratory diseases with a good therapeutic effect in children [11] and adults [7]. In the lungs of mice, orally treated with RV a strong hyperplasia of the peribronchial lymphoid structures, thickened alveolar walls and dense mononuclear infiltrates were observed [6].

Successful host defence against invading microorganisms and cancer depends on the migration, accumulation and activation of effector cells at the relevant sites. Alveolar macrophages (AM) are recognized as the major cellular immune mechanism contributing to the protection of the lower respiratory tract against microbial invasion [27]. AM also play an important role in pulmonary defences against developing neoplasms in lung parenchyma or against the establishment of metastatic foci [8, 10, 21].

The clinical and experimental evidence suggesting that BV and RV may increase non-specific defence mechanisms in the lung, the most frequent target organ for haematogenous metastasis, encouraged us to explore the possibility that these vaccines might inhibit the formation and the growth of pulmonary metastases. It seemed quite possible that prolonged maintenance of high levels of immune

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activation in the lung, induced by continuous oral treatment with BV and RV, could represent a barrier against circulating tumour cells reaching this target organ and also might influence the growth of metastatic foci located in lung parenchyma. To test this hypothesis we designed a study, in which by using an experimental transplantable tumour model in syngeneic mice we investigated the antimetastatic effect of RV and BV either alone, or in combination with chemotherapy. The number and the cytolytic activity of AM induced by oral administration of BV and RV in C57BL/6 mice were also determined.

Materials and methods

Animals. Specific-pathogen-free female C57BL/6 mice, 8 weeks of age, were obtained from Šumice Experimental Animal Production Area (Praha, Czechoslovakia).

Tumours. In these studies we used the Lewis lung carcinoma (3LL), which is syngeneic to the C57BL/6 mice. 3LL was maintained by serial biweekly i.m. passage in inbred female C57BL/6 mice. A local tumour grown in the thigh was removed aseptically and minced in Hanks' balanced salt solution (HBSS). Single-cell suspensions were made by filtering mechanically chopped tumour pieces through four-fold gauze. In experiments investigating AM-mediated cytotoxicity we used fresh explants of 3LL metastatic tumours in short-term cultures as the source of tumour cell targets. Metastases were isolated aseptically from the lungs of control C57BL/6 mice, and a cell suspension in RPMI-1640 medium (Flow Laboratories Inc., Irvine, Scotland) was made using crossed scalpels and filtering the chopped material through four-fold gauze. The recovered cells were adapted to growth in culture and used in cytotoxicity assays after two or three passages. The primary cultures of 3LL metastatic cells were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and 10% heat-inactivated fetal bovine serum (Flow Laboratories Inc., Irvine, Scotland).

Spontaneous metastasis model. To produce spontaneous metastases, 5×10^5 viable 3LL tumour cells in 0.05 ml RPMI-1640 medium were injected s.c. into one hind footpad of each mouse. After 11 days, when spontaneous lung micrometastases had been established, the foot with the growing tumour was amputated. This time period was determined in a previous experiment and corresponded to the minimal postimplantation interval when the surgery could not prevent the appearance of lung metastases (i.e. save the mice). The local tumour was removed under sodium pentobarbital anaesthesia by amputating the tumour-bearing leg at midfemur to include the popliteal lymph node. Surgical procedures were done aseptically with sterile instruments. After amputation the animals were injected s.c. with 0.5 ml sterile 0.9% saline to prevent dehydration during the postanaesthetic recovery. Mice were autopsied 22 or 25 days after tumour implantation, and their lungs were fixed overnight in Bouin's fixative. The metastases were counted at the lung surface and measured using a stereomicroscope (magnification, ×10) with a graticule. The volume of individual metastases was calculated from the diameter, assuming the foci to be spherical in shape [18]. The total volume of pulmonary metastases in each lung was estimated by summing (computer programme) the volumes of all metastases in the corresponding lung.

Agents. Broncho-Vaxom (BV; Laboratories OM, Meyrin, Geneva, Switzerland) is a lyophilized alkaline lysate of eight strains of bacteria usually involved in the respiratory tract infections (*Diplococcus pneumoniae, Neisseria catarrhalis, Streptococcus pyogenes, Streptococcus viridans, Haemophilus influenzae, Staphylococcus aureus, Klebsiella pneumoniae, Klebsiella ozaenae*). Respivax (RV; Scientific-Productional Enterprise of Pharmacy, Sofia, Bulgaria) contains freeze-dried lysate and the dead bacterial bodies of the same microbial species present in BV except for *Klebsiella ozaenae* and *Streptococcus viridans*. RV was a gift from Professor B. Petrunov (Research Institute of Infectious Diseases, Sofia, Bulgaria). Cyclophosphamide (Endoxan; ASTA Pharma AG) was dissolved in 0.9% NaCl solution before use and injected i.p. in a volume of 0.01 ml/g body weight.

Treatment of animals. BV and RV were dissolved in saline and given orally in a daily dose of 50 mg/kg for 10 consecutive days before or after surgery. Cyclophosphamide at a single dose of 50, 100 or 150 mg/kg was injected i.p. 2 days after tumour amputation. Oral treatment with RV or BV was initiated 48 h following cyclophosphamide injection.

Preparation of AM cultures. AM were collected by broncho-alveolar lavage as described by Talmadge et al. [25]. Briefly, C57BL/6 mice were anaesthetized with an i.p. injection of pentobarbital sodium, and exsanguinated by severing one or both renal arteries. After opening the chest cavity to produce pneumothorax, the trachea was canulated with a 20-gauge needle without bevel. The lungs were lavaged with 1 – 1.5 ml Ca^{2+}- and Mg^{2+}-free HBSS prewarmed at 37° C. The process was repeated five times for each mouse and the alveolar cells of ten mice per group were pooled. The total number of cells collected was determined in white-blood-cell counting solution using a haemocytometer. The viability of nucleated cells was checked by Trypan blue dye exclusion and was >95%. The macrophage number was estimated by counting the neutral-red-positive cells. The alveolar cell population was also determined microscopically by staining with May-Grünwald's and Giemsa's solutions. The lavage fluid was centrifuged and resuspended in serum-free RPMI medium at 1×10^6AM/ml. The macrophage suspensions were plated (0.2 ml/well) in 96-well flat-bottomed microtest plates (Flow Laboratories Inc. Irvine, Scotland) and were allowed to adhere for 2 h at 37° C in a 5% CO_2 humidified atmosphere. Then each well was washed twice with warm medium to remove nonadherent cells and the resultant AM monolayers were used for the in vitro cytotoxicity assays.

Assay of AM-mediated cytotoxicity. The AM-mediated cytotoxicity assay was performed using the ^{51}Cr-release method, as described by Nagao et al. [16]. Briefly, 3LL target cells (1×10^6), suspended in 0.2 ml complete medium were labelled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham, UK) at 37° C for 1 h in a water bath. After incubation, the cells were washed three times with warm medium to remove unincorporated radiolabel and adjusted to a concentration of 5×10^4/ml. After washing the AM monolayers, prepared as described above, the medium was aspirated and 1×10^4 ^{51}Cr-labelled 3LL cells were added (0.2 ml/well) to each well to obtain an AM: target cell ratio of 20:1. The macrophage target cell cultures were then incubated for 20 h at 37° C in a humidified atmosphere containing 5% CO_2. After incubation the plates were centrifuged for 5 min at 250 *g*. ^{51}Cr experimental release (ER) was determined by harvesting 0.1 ml supernatant from each well and counting the radioactivity in a gamma counter. Total release (TR) was determined by the addition of detergent (2% sodium dodecyl sulphate). Spontaneous release (SR), obtained by the addition of medium only was always less than 20% of the total. Percentage cytotoxicity was calculated by the formula:

$$^{51}Cr \text{ release } (\%) = [(ER-SR)/(TR-SR)] \times 100$$

Statistical analysis. The statistical significance of differences between the groups was determined using Student's *t*-test.

Results

Antimetastatic activity of RV and BV

In the initial set of experiments we found that oral therapy with RV inhibited spontaneous development of lung metastases in mice implanted with different numbers of 3LL cells (Table 1). As shown in Table 1 (Experiment I), oral administration of RV for 10 consecutive days caused significant reduction, compared to the surgery alone, of the

Table 1. Inhibition of spontaneous lung metastases by Respivax in mice implanted with different numbers of 3LL cells[a]

Agent	Treatment schedule (days)[b]	Lung metastases			Incidence[c]	Survival time (days)
		Number	Nodule volume (mm^3)	Total volume (mm^3)		
Experiment I						
Untreated	–	75.7 ± 27.6[d]	1.26 ± 0.14[d]	95.3 ± 34.6[d]	10/10	26.6 ± 4.2[e]
Respivax	−10 to −1	20.1 ± 6.2***	0.31 ± 0.06***	6.2 ± 3.6*** (7%)[f]	10/10	28.0 ± 3.6
	1 to 10	22.0 ± 4.5***	0.21 ± 0.02***	4.6 ± 2.1*** (5%)	10/10	31.1 ± 4.4
	12 to 21	40.7 ± 14.1**	0.94 ± 0.11***	48.2 ± 19.2** (51%)	10/10	33.2 ± 5.3*
Experiment II						
Untreated	–	5.8 ± 3.3[d]	0.31 ± 0.07[d]	1.80 ± 1.05[d]	10/10	ND
Respivax	−10 to −1	2.0 ± 1.0**	0.09 ± 0.04***	0.18 ± 0.11*** (10%)[f]	6/10	ND
	1 to 10	1.3 ± 0.5**	0.05 ± 0.03***	0.07 ± 0.04*** (4%)	3/10	ND
	12 to 21	3.2 ± 1.7*	0.20 ± 0.06**	0.64 ± 0.24** (36%)	8/10	ND

[a] 5×10^5 (experiment I) or 1×10^5 (experiment II) tumour cells were inoculated into the footpad on day 0
[b] Time from tumour implantation. For details, see Materials and methods
[c] Number of mice with pulmonary metastases / number of mice examined
[d] Mean ± SD of ten mice per group
[e] Mean ± SD of eight mice per group
[f] Numbers in parentheses, percentage of control value
*, **, *** significantly different from untreated control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 2. Inhibition of spontaneous pulmonary metastases by Broncho-Vaxom and Respivax

Agent	Treatment schedule (days)[a]	Lung metastases		
		Number	Nodule volume (mm^3)	Total volume (mm^3)
Untreated	–	38.3 ± 14.0[b]	1.38 ± 0.17[b]	52.9 ± 18.6[b]
Respivax	1 to 10	9.8 ± 3.7***	0.22 ± 0.02***	2.2 ± 1.0*** (4%)[c]
	12 to 21	19.4 ± 9.1**	1.24 ± 0.11*	24.1 ± 8.8*** (46%)
Broncho-Vaxom	1 to 10	8.0 ± 3.2***	0.21 ± 0.03***	1.7 ± 0.8*** (3%)
	12 to 21	16.8 ± 7.9***	1.04 ± 0.09***	17.5 ± 6.2*** (33%)

[a] Time from tumour implantation. For details, see Materials and methods
[b] Mean ± SD of ten mice per group
[c] Numbers in parentheses, percentage of control value
*, **, *** Significantly different from untreated control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

number and volume of lung metastases. Some extension of life-span of the treated animals was also observed. The therapeutic efficacy of RV, as estimated by the decreased number and volume of lung metastases, was greater when the treatment was initiated prior to or immediately after implantation of the tumour. However, only the postsurgical immunotherapeutic schedule prolonged the survival significantly ($P < 0.05$), suggesting that the strong inhibitory effect of RV on lung micrometastases decreased after the end of the treatment. The therapeutic efficacy of RV depended on the degree of dissemination of 3LL cells. As shown in Table 1 (Experiment II), oral administration of RV was significantly more effective when used for the treatment of a comparatively minor secondary tumour burden. In this study 20%–70% of the animals treated with RV were without visible lung metastases on day 22 after tumour implantation. Although impressive, this effect of immunotherapy might be attributed mainly to the relatively minor tumour burden, which does not accurately represent the clinical state of patients with more advanced disease.

Next, we decided to investigate whether BV, another oral polyvalent vaccine, will exhibit the same activity as RV in the treatment of spontaneous pulmonary metastases. As shown in Table 2, a similar therapeutic effect was observed following oral administration of the two agents for 10 days before or after surgery. The greatest reduction of the number and volume of lung metastases was achieved when the treatment was initiated in the early phases of tumour dissemination. Administering BV or RV after surgical resection of the primary tumour also produced a significant therapeutic response in animals with established spontaneous metastases. In several consecutive experiments we observed that BV was more effective than RV in inhibiting the volume (i.e. the growth) of preexisting pulmonary metastases, but the differences were not always significant.

Collectively these results demonstrated that RV and BV were effective against lung metastases when administered orally and that maximal therapeutic activity was limited by the metastatic tumour burden. Therefore, the therapeutic potential of BV and RV was examined in combination with chemotherapy to determine if there is additive activity for the treatment of well established metastatic lesions.

Chemoimmunotherapy with cyclophosphamide and BV or RV

The effects of combined therapy with cyclophosphamide and RV or BV are summarised in Table 3. Development of

Table 3. Combination therapy of spontaneous lung metastases with cyclophosphamide (CY) and Respivax (RV) or Broncho-Vaxom[a] (BV)[a]

CY (mg/kg)	Oral vaccine	Lung metastases		Incidence[b]	Survival time (days)
		Number	Total volume (mm^3)		
–	–	46.1 ± 13.6[c]	67.6 ± 18.2[c]	10/10	28.2 ± 5.1[c]
50	–	19.3 ± 7.1	23.2 ± 9.1	10/10	35.8 ± 3.6
100	–	9.5 ± 4.2	5.6 ± 2.0	10/10	43.9 ± 5.6
150	–	4.0 ± 2.2	2.1 ± 1.0	6/10	ND
–	RV	30.3 ± 8.8	38.5 ± 11.4	10/10	33.1 ± 4.2
50	RV	9.4 ± 3.4***	10.8 ± 2.8***	10/10	43.0 ± 3.8***
100	RV	4.7 ± 1.2**	4.2 ± 2.1	9/10	47.7 ± 6.3
150	RV	1.8 ± 0.7**	1.9 ± 0.6**	3/10	ND
–	BV	28.1 ± 6.9	30.2 ± 11.0	10/10	34.4 ± 5.1
50	BV	7.2 ± 2.4***	8.0 ± 3.2***	10/10	45.2 ± 5.9***
100	BV	5.5 ± 1.3**	3.1 ± 1.3**	8/10	48.2 ± 5.3
150	BV	2.0 ± 1.0*	1.4 ± 0.2*	2/10	ND

[a] Mice (n = 10/group) were treated as described in Materials and methods
[b] Number of mice with pulmonary metastases / number of mice examined
[c] Mean ± SD values
*, **, *** Significantly different from cyclophosphamide alone: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4. Activation of alveolar macrophages (AM) by Broncho-Vaxom and Respivax in normal and tumour-bearing mice

Treatment[a]	Tumour[b]	Number of AM /mouse ($\times 10^4$)	AM-mediated cytotoxicity (%)	Number of pulmonary metastases
Saline	–	3.2 ± 0.1[c]	4.4 ± 2.1[c]	–
Respivax	–	25.5 ± 3.1[d]	17.5 ± 4.0[d]	–
Broncho-Vaxom	–	21.8 ± 2.3[d]	21.8 ± 3.5[d]	–
Saline	+	5.5 ± 0.5[d]	2.8 ± 0.9	61.0 ± 14.2[c]
Respivax	+	18.5 ± 3.2[dc]	13.2 ± 2.3[de]	40.2 ± 8.5[e]
Broncho-Vaxom	+	20.0 ± 2.8[de]	16.7 ± 3.3[de]	35.3 ± 6.0[e]

[a] Broncho-Vaxom or Respivax was administered p.o. for 10 consecutive days. The study was terminated 3–4 h following the last administration
[b] 5×10^5 3LL cells were inoculated i.m. on day 0. Therapy was initiated on day 11
[c] Mean ± SD values of ten mice per group
[d] Significantly different from saline-treated normal mice ($P < 0.001$)
[e] Significantly different from tumour-bearing control mice ($P < 0.001$)

pulmonary metastases was inhibited by each of these treatments alone. Cyclophosphamide exhibited a strong inhibitory effect in a dose-dependent manner against lung metastases and the dose of 150 mg/kg cured 40% of the animals. Oral immunotherapy with BV or RV had comparatively minor therapeutic activity against the large tumour burden of preexisting pulmonary metastases. The combination of cyclophosphamide at each dose tested with BV or RV had additive inhibitory effects on lung metastases. The mice receiving a single dose of cyclophosphamide at 50 mg/kg in combination with RV or BV showed a significantly reduced number and volume of lung metastases, as compared to animals injected with cyclophosphamide only. This combined therapy also significantly prolonged survival as compared to chemotherapy alone. In contrast, the addition of BV or RV to the treatment protocol did not significantly prolong survival of mice injected with 100 mg/kg cyclophosphamide. However, in this case a significant reduction of the number and volume of lung metastases was also observed. Four out of ten mice receiving a single dose of cyclophosphamide at 150 mg/kg were free of lung metastases, while no metastatic nodules could be seen in six or seven of ten mice treated with cyclophosphamide plus RV or BV respectively. In summary, these results demonstrated that the therapeutic effect of cyclophosphamide on preexisting pulmonary metastases was enhanced by subsequent oral treatment with RV or BV.

Accumulation and activation of alveolar macrophages

As shown in Table 4, the cytolytic activity of AM recovered after an oral therapy with BV or RV for 10 days was significantly higher than that of the control macrophages (from both normal or 3LL-bearing mice). In mice with i.m. implants of 3LL cells, there was a significant correlation between the increase of AM tumoricidal activity and the inhibition of metastatic tumour growth in their lungs, demonstrating that macrophage activation was implicated in eradication of lung metastases. Another interesting observation was the difference in the numbers of AM harvested. To confirm and expand on this observation in the final set of experiments, we determined the effect of oral treatment with BV and RV on the number of AM in normal and tumour-bearing mice.

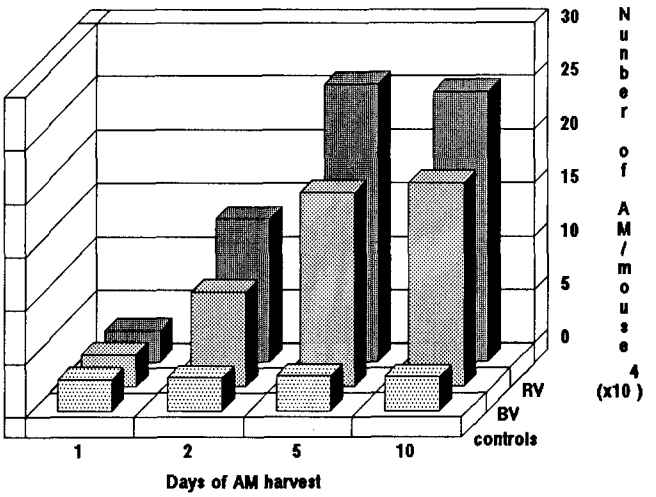

Fig. 1. Effect of oral treatment with Broncho-Vaxom (*BV*) or Respivax (*RV*) on the number of alveolar macrophages (*AM*) in normal C57BL/6 mice. BV and RV at 50 mg/kg were administered orally for 10 days and the AM were collected on days 1, 2, 5 and 10

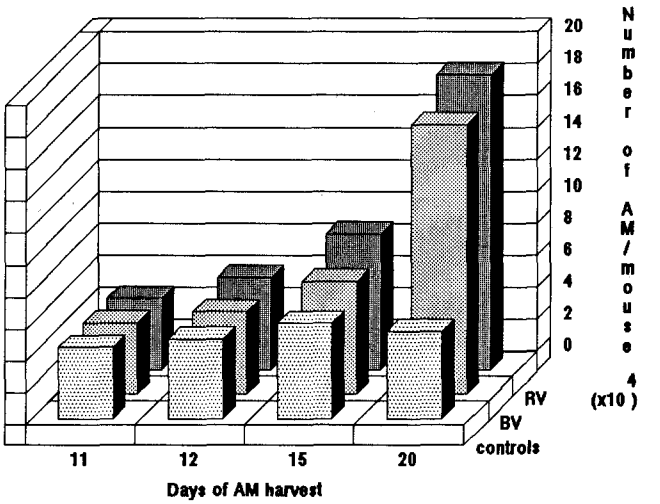

Fig. 2. Effect of oral treatment with Broncho-Vaxom (*BV*) or Respivax (*RV*) on the number of alveolar macrophages (*AM*) in 3LL-bearing C57BL/6 mice. Samples of 5×10^5 3LL cells were inoculated i. m. on day 0. BV and RV at 50 mg/kg were administered for 10 days, beginning on day 11. The AM were collected on days 11, 12, 15 and 20

In normal C57BL/6 mice, 2–4 h following the first administration the number of AM recovered was unchanged but 24 h later (on day 2) there was a significant increase (Fig. 1). On the 5th and 10th days this increase was even more pronounced. Ten days after the end of the treatment there was still a significant difference between the numbers of AM harvested from the lungs of the treated and control animals (data not shown). The presence of progressively growing lesions in the lungs of 3LL-bearing control mice resulted in a slight increase in the number of AM harvested (Fig. 2). In tumour-bearing mice, oral administration of BV and RV caused an increase in the number of AM, which was statistically non-significant at the beginning of the treatment but at the end was nearly as large as that in normal animals. These results indicate that oral therapy with BV and RV was able to induce a long-lasting influx of tumoricidal macrophages in the lungs of the treated animals.

Discussion

In the present study we demonstrated that oral administration of two polyvalent bacterial vaccines, BV and RV, inhibited spontaneous pulmonary metastases in mice through accumulation and activation of pulmonary macrophages in vivo. Thus, we have provided new information regarding their therapeutic potential and some insight into the mechanism of their protective action.

Clinically, metastasis has already occurred at the time of diagnosis, which is why, in the preclinical evaluation of biological response modifiers, much attention has been directed to the effect of immunotherapy on spontaneously disseminated tumour cells in syngeneic hosts after surgical resection of their primary tumour [24]. Our experimental model aimed to simulate the clinical situation as closely as possible. A subcutaneous inoculum of syngeneic tumour cells was allowed to grow and to produce spontaneous pulmonary micrometastases; it was then removed by surgery, thus simulating the clinical state of patients with a minimal residual disease.

In this study, oral administration of BV and RV was highly effective in preventing pulmonary metastases in syngeneic mice, suggesting that these vaccines had potent antimetastatic activity at the stage of dissemination and micrometastasis in the lung (Table 2). An inhibition of metastatic foci in the lung was also observed when therapy was begun after surgical resection of the primary tumour. The results were less spectacular but nevertheless significant. The demonstration of oral activity against preexisting metastatic disease is of particular interest, as in the case of postsurgical immunotherapy the parallel between the experimental model and the clinical situation is conserved. The oral route is very attractive for chronic administration but unfortunately the majority of biological response modifiers, including bacillus Calmette-Guérin (BCG) [2] and *Corinebacterium parvum* [20], are without any effect on organ metastases when applied orally. Therefore, the identification of orally active, immunotherapeutic agents with a proven clinically safe administration to children and adults is of particular interest.

The results presented here also indicate that combination therapy with cyclophosphamide and BV or RV is more effective than cyclophosphamide alone both in eradication of spontaneous pulmonary metastases of 3LL cells and in prolonging the survival of the mice with metastases (Table 3). This effect of combination therapy appears to result from the reduction in metastatic tumour burden through cytotoxic therapy to a level where the immunotherapeutic properties of BV and RV might be active. In particular, combination of cyclophosphamide at a suboptimal dose (50 mg/kg) with BV or RV showed significant therapeutic activity compared to chemotherapy alone. In this case the effect of the combined treatment was comparable to that observed following a single injection of twice as large a dose (100 mg/kg) of cyclophosphamide

alone. Therefore, this combination therapy could also avoid the severe side-effects associated with high doses of cyclophosphamide. This suggestion is supported by the previous finding that oral treatment with BV was able to restore the lowered immune responses in mice immunosuppressed with cyclophosphamide (200 mg/kg i.p.) [4]. Collectively our results suggested that the use of BV and RV could be beneficial in the therapy of a relatively small secondary tumour burden – pulmonary micrometastases or residual metastatic foci after most of the tumour cells have been removed by other means such as chemotherapy.

The success of immunotherapy of tumour metastases depends on the ability of the treatment protocol to activate immune mechanisms selectively within the target organ, which contains metastatic tumour foci or, to mobilize systemically activated effector cells to the site of metastatic tumour growth. In order to investigate the possible correlation between effector cell activation and antimetastatic activities of BV and RV in a second set of experiments we assessed the activity of alveolar macrophages (AM), a population closely associated with the target organ for BV and RV stimulation. We first looked at the tumoricidal activity of AM elicited by oral administration of BV and RV and found them cytotoxic against syngeneic 3LL metastatic cells (Table 4). According to North et al. [17] the length of time in culture influences the susceptibility of tumour cells to macrophages and the most suitable cells for the assessment of the functional status of macrophages in vivo are their natural target cells isolated directly from local or metastatic tumour growing in situ. Therefore, in cytotoxicity assays we decided to use 3LL metastatic cells in short-term culture as a source of tumour cell targets. We also determined the effect of oral treatment with BV and RV on the number of AM in normal (Fig. 1) and tumour-bearing mice (Fig. 2). The results obtained enabled us to establish a relationship between the ability of BV and RV to induce an accumulation of tumoricidal AM in vivo and the acquisition of antimetastatic properties. The increase in the number of AM may be attributed to an influx of monocytes through migration of these cells to the lungs of the treated animals [21]. A local multiplication of macrophages could also be implicated, as shown for the BCG vaccine [27].

Recently Mauel et al. reported that BV strongly potentiated metabolic and functional activities of mouse peritoneal and bone-marrow-derived macrophages [15]. Such in-vitro-activated macrophages from C57BL/6 mice were lytic towards P815 tumour target cells. In the present study, we demonstrated that BV can activate a new category of macrophages in vivo, namely mouse alveolar macrophages. This fact is of particular interest as the accumulation of non-specifically activated AM might explain the protective effect afforded by the oral administration of BV and RV in patients with chronic respiratory tract infections.

As an important factors in the immunotherapy of pulmonary micrometastases Sone [21] suggested the rate of recruitment of new macrophages into the lungs and the tumoricidal capacity of the recruited macrophages. Our experimental data indicate that by oral treatment with BV and RV it is possible to provoke an influx of tumoricidal AM in the lungs of the treated animals. It is therefore reasonable to conclude that pulmonary macrophage activation was one of the mechanisms involved in the antimetastatic effects observed in vivo. However, it is impossible to exclude the participation in these processes of additional cells such as NK cells, neutrophils, or of humoral factors such as natural antibodies, IFNγ or cytokines. Indeed, we have recently found that RV induce the secretion of IL-1 and IFNγ production (results to be published), as shown for BV [5, 30]. In contrast, the NK activity in the spleen, evaluated by a 4-h ^{51}Cr-release assay against YAC-1 target cells, never increased and even decreased slightly after an oral treatment with BV or RV (data not shown). This observation may have been associated with the inappropriate source of effector cells for these assays. Some biological response modifiers are capable of augmenting NK activity in the lungs and livers to a greater degree than in the blood and spleen and thus increase the resistance to metastasis formation [28, 29]. Therefore, we are now trying to elucidate the involvement of lung NK cells in the antimetastatic activity exhibited by BV and RV.

In conclusion, the results of the experiments reported here add to the known properties of BV and RV that of inhibition of lung metastases through induction of an influx of tumoricidal AM in vivo. Our experimental data might provide the rationale for future clinical trials using these oral vaccines in the treatment of cancer patients. This approach is especially attractive for the prevention and/or eradication of postoperative tumour micrometastases in the lung, the first site encountered by most tumour cells after they have been shed into the venous circulation.

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