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Superiority of *Micrococcus lysodeikticus* to BCG in Chemo-immunotherapy of Advanced L1210 Leukaemia*

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Abstract—*When CDF*$_1$ *mice initially inoculated with* 10^5 *leukaemic cells were treated once by* 12 mg/kg *of nitroso-urea BCNU on* day 6.5 *(when the tumour burden reaches* $> 10^8$ *cells) and by* 1 mg *Micrococcus injections on* days 8, 11, 14, 17 *and* 20 *or on* days 8, 9, 10, 11 *and* 12, 30% *of cured mice were recorded. Immunotherapy with* 1 mg *injections of BCG,* B. subtilis *or* Micrococcus lysodeikticus *given on* days 8, 11, 14, 17 *and* 20 *after BCNU chemotherapy (one* 12 mg/kg *injection on* day 6.5) *respectively cured* 5, 10 *and* 50% *of leukaemic mice after a graft of* 4000 L1210 *cells on* day 0 *and* 0, 10 *and* 50% *of leukaemic mice after a graft of* 25,000 *cells. Using this chemo-immunotherapy combination, Micrococcus immunotherapy proved to be significantly effective* (χ^2 *test,* $P < 0.005$) *against* $> 10^8$ L1210 *cells whereas* B. subtilis *or BCG immunotherapy was not statistically significant. Mice cured by chemo-immunotherapy (BCNU + Micrococcus) rejected new local as well as systemic grafts of* 10^4 L1210 *cells (on* day 120).

INTRODUCTION

PREVIOUS studies by Skipper *et al.* clearly demonstrated that chemotherapy of neoplasms (such as murine L1210 leukaemia) obeys first-order kinetics [1], indicating that after cytotoxic chemotherapy, an additional therapy like immunotherapy may be required to eliminate the last tumour cell. Although effective chemotherapy may induce temporary tumour regression, it may also lead to the *in vivo* selection of drug resistant neoplastic cell clones that kill the host after a new proliferative phase. In this perspective, it is quite understandable that immunotherapy provides an attractive antitumour weapon since it offers the possibility to cure.

Numerous reports describing the protecting effect of Bacillus Calmette–Guérin (BCG) and anaerobic Corynebacteria against many syngeneic mouse tumours have been published (for a review see [2–4]). Although the BCG strain of *Mycobacterium bovis* has become a popular anticancer immunotherapeutic agent, the general use of BCG is make difficult because of many disadvantages: BCG can cause ulceration, pyrexia, liver function subnormalities, tuberculosis and, in a few reported cases, death or even enhancement of tumour growth [5–7].

Micrococcus lysodeikticus is a non-pathogenic and easy-to-eliminate (substrate of lysozyme) gram-positive bacterium that elicits the production of large amounts of antibodies of restricted heterogeneity and clonal dominance in rabbits [8] and mice [9]. Antimicrococcus antibodies are directed against carbohydrates and bind to certain lymphocytes [10, 11] and to several tumour cell types (L1210 lymphoid, Ehrlich carcinoma cells) [12] but not to erythrocytes [13]. The interaction of antimicrococcus antibodies with receptors on neoplastic cells was confirmed by others and a cell cycle stage dependency was established [14].

Although adjuvants such as BCG exert various biological activities like, for instance, T-cell adjuvanticity [15], induction of natural killer cells [16], increased lymphocyte trapping [17] and exoctyosis into tumour cells [18], it was

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clearly demonstrated that antitumour activity was inhibited by antimacrophage agents [19]. Complement activation is a very important step in inflammation that switches on multiple biological processes such as B-cell proliferation, macrophage activation [20] and attraction of polynucleated cells to the inflammation site [21]. The phenomena occur after binding of the activated components on specific membrane receptors (C3d) or (C3b) and are non-lytic to the affected cell [22]. Previously, we have demonstrated that *Micrococcus lysodeikticus* is able to activate complement by the alternative pathway [23].

Although one single injection of Micrococcus suspended in incomplete Freund adjuvant does not display an adjuvant effect for protein antigens [24], we have demonstrated that multiple Micrococcus and live BCG (Brussels GL2 strain) 1 mg injections elicit a comparable and five-fold increased number of plaque-forming cells to sheep erythrocytes, whereas Micrococcus treated mice exhibited much less toxicity than BCG treated animals [25]. In an attempt to maximize the chemotherapeutic response to antitumour agents by immunotherapy, this study was undertaken to compare the therapeutic values of Micrococcus with the activity generated by *B. subtilis*, another Gram-positive bacterium, and BCG. Since L1210 leukaemia kills untreated mice early and with low standard deviations, we have chosen this often solicited and reliable animal tumour model that allows activity prediction against human leukaemia [26].

ANIMALS, MATERIALS AND METHODS

Animals

Female [female BALB/c (H_2^d) × male DBA/2 (H_2^d)]F_1 hybrid mice called CDF1 were purchased from Charles River Breeding Laboratories, Calco, Italy. These mice were stored for 3 weeks in an isolation room and were used before they were 10 weeks old. Animals weighing 19–23 g were used. Ten to twenty mice per test group and 20 control mice were used.

Tumours

The L1210 leukaemia obtained from Dr. A. Bogden (Mason Research Institute, Worcester, Mass., U.S.A.) was maintained in ascitic form by weekly transfer in DBA/2 mice. Tumour transplants were performed as earlier described [12]. Mean survival time (M.S.T.) of treated and control mice as well as the doses and timing of each experiment are specified in the tables and figures. Mice being tumour-free on the 90th day after tumour grafting were considered as long-term survivor and they were eliminated from evaluation of M.S.T. Relapses after day 90 were not observed.

Bacteria

Micrococcus lysodeikticus. Bacterial suspensions were made as reported before [11].

Bacillus subtilis. Exponentially growing cultures offered by Professor N. Glansdorff were washed 3 times with 0.15 M NaCl and centrifuged at 300 *g* for 20 min. Bacteria were frozen at −28°C and lyophilized. Dried bacteria were stored at −28°C, weighed and resuspended in 0.15 M NaCl at appropriate times, kept at 4°C and used within 12 hr.

Bacillus Calmette–Guérin. 80 mg/ml vials of "fresh" Bacillus Calmette–Guérin were weekly obtained from the Pasteur Institute, Brussels. The suspension was constantly kept in the dark at 4°C and used within 1 week. The Brussels strain of BCG (GL_2) was studied for adjuvanticity, suppression of lectin stimulation and induction of immune interferon by Huygen *et al.* Both the Brussels GL2 strain and the Paris strain were shown to display a considerable adjuvant capacity whereas the Montreal strain was ineffective [27, 28]. Whereas the Paris strain yielded higher lectin stimulation values, the adjuvant activity of the Brussels GL2 was more effective in adjuvant activity tests. This BCG preparation is actually used in the clinical immunotherapy trials of the E.O.R.T.C. and contains approximately $3–9.10^6$ colony forming units per mg of bacteria (mean = 5.10^6) (K.Huygen, personal communication).

Drugs

One hundred milligram containing vials of 1,3 bis (2-chloro-ethyl)-1-nitroso-urea, named BCNU (NSC-409962), melphalan (NSC 8806), 5-fluoro-uracil (NSC 19893) and cyclophosphamide (NSC 26271) were a gift of the National Cancer Institute to the NCI Liaison Office, Brussels.

RESULTS

1. *Immunotherapy trial*

First, the immunotherapeutic effectiveness of Micrococcus, "fresh" BCG and *B. subtilis* were studied after grafting i.p. 5×10^4 viable L1210 cells to CDF1 mice. All further chemo-immunotherapy regimens were performed with this number of initially grafted cells (5×10^4–10^5 L1210 cells). Immunotherapy was performed by repeated 1 mg injections of Micrococcus, BCG or *B. subtilis* on days 1, 2, 3, 4 and 5 (schedule A) or by intermittent injections on days 1, 4 and 7 (schedule B) in the mouse

peritoneum. Mathé *et al.* have shown previously that 1 mg/mouse is the optimal immunostimulating dose of BCG [29]. As seen in Table 1, neither BCG, *B. subtilis* nor *Micrococcus lysodeikticus* immunotherapy alone was able to affect the proliferation of L1210 leukaemia significantly (Student–Fisher *t*-test).

2. *Investigation of different chemo-immunotherapeutic protocols for activity against L1210 mouse leukaemia*

As seen in Table 2, different doses of potent anticancer drugs and different treatment schedules were assayed in order to obtain a 60–90% increase in M.S.T. of i.p. treated mice as compared to untreated controls after the i.p. graft of 10^5 L1210 cells on day 0. The administration of 2.5 mg/kg of melphalan on days 1, 3 and 5 or 5 mg/kg on days 1 and 5 leads respectively to a 63 and 57% increase in M.S.T. with 17% of mice surviving on day 90 in both groups. L1210 chemotherapy with either 100 mg/kg of 5-fluoro-uracil or 80 or 60 mg/kg of cyclophosphamide on day 1 or with 60 and 40 mg/kg of 5-fluoro-uracil on days 1 and 5 resulted in a substantial increase in lifespan of treated mice but no cures were recorded.

Next, we compared the immunotherapeutic values of Micrococcus after non-curative chemotherapy and no longer used melphalan in

Table 1. *Immunotherapy of L1210 leukaemia by* Micrococcus lysodeikticus, *BCG or* Bacillus subtilis

Bacterium	Schedule of treatment	% of cured mice†	M.S.T. (days) ± S.D.‡	T/C%§
Micrococcus l.	A	0	9.46 ± 0.5	104*
Micrococcus l.	B	0	9.08 ± 0.3	100*
"Fresh" BCG	A	0	10.30 ± 1.8	113*
"Fresh" BCG	B	0	9.50 ± 0.5	104*
B. subtilis	A	0	9.40 ± 0.5	103*
B. subtilis	B	0	9.33 ± 0.5	103*
Untreated controls	—	0	9.10 ± 3.0	100

*Not significant (Student–Fisher *t*-test)

On day 0, intact CDF_1 mice were challenged intraperitoneally with 5×10^4 L1210 cells. Mice were treated intraperitoneally with 1 mg injections of bacteria either on days 1, 2, 3, 4, 5 (schedule A) or on days 1, 4, 7 (schedule B) after tumour grafting.

†Percentage of mice surviving day 90.

‡Mean survival time ± the standard deviation of mice dying before day 90.

§Mean survival time of treated but tumour bearing mice as compared to untreated mice, expressed in percentage.

Table 2. *Evaluation of different chemotherapeutic regimens on mouse L1210 leukaemia*

Drug	Dose (mg/kg)	Treatment (days)	% of cured mice§	M.S.T. (days) ± S.D.‖	T/C%¶
Melphalan	5.0	1.5	17	14.6 ± 2.1	157*
(NSC 8806)	2.5	1, 3, 5	17	15.2 ± 2.8	163‡
5-fluoro-uracil	100	1	0	14.6 ± 0.5	157‡
(NSC 19893)	60	1, 5	0	13.0 ± 2.4	140†
	40	1, 5	0	13.8 ± 1.3	149‡
Cyclophosphamide	80	1	0	13.8 ± 0.9	149‡
(NSC 26271)	60	1	0	10.7 ± 4.4	115
	40	1, 5	0	16.3 ± 1.1	175‡
Untreated controls	—	—	0	9.3 ± 0.5	100

Student–Fisher *t*-test:* $0.01 < P < 0.02$, †$P < 0.01$, ‡$P < 0.001$.

On day 0, 10^5 leukaemia L1210 cells were grafted intraperitoneally to CDF_1 mice.

Mice were treated intraperitoneally.

§% of the animals surviving day 90.

‖Mean survival time ± the standard deviation of treated mice dying before day 90.

¶Mean survival time of treated but tumour bearing mice as compared with untreated mice, expressed in percentage.

our tests. Mice were treated by chemotherapy only or by chemotherapy plus 1 mg injections of Micrococcus administered on days 8, 9, 10, 11 and 12 (schedule A) or on days 8, 11, 14, 17 and 20 (shcedule B) after grafting 10^5 L1210 cells i.p. on day 0. The latter schedule was constantly used to allow an evaluation of immunotherapy after different but comparably effective chemotherapeutic regimens. We observed that the combination of Micrococcus immunotherapy with 5-fluoro-uracil or cyclophosphamide, administered at doses earlier mentioned, was not more effective than cyclophosphamide or 5-fluoro-uracil chemotherapy alone. By contrast, when L1210 tumour bearing mice were treated once i.p. with 12 mg/kg of BCNU on day 6.5, 30% of mice could be cured by repeated 1 mg injections of Micrococcus following schedules A and B described in Table 3. This chemo-immunotherapy protocol was originally described by Cantrell *et al.* [30]. Since our experiments were conducted with congenic CDF1 (H_2^d, H_2^d) mice, one may consider that grafting 10^5 L1210 cells on day 0 to CDF1 mice exceeds 10^8 leukaemic cells on day 6.5 (which was the estimated tumour burden for 10^4 L1210 cells grafted to BDF1 mice (H_2^k, H_2^d) on day 0 [30]).

3. *Effect of repeated administration of Micrococcus, BCG or* Bacillus subtilis *in chemo-immunotherapy of advanced L*1210 *leukaemia*

We then compared the immunotherapeutic acitivity of BCG and *B. subtilis* with Micrococcus. Intact CDF1 mice were inoculated i.p. either with 25,000 or 4000 cells on day 0. Leukaemic mice were treated once i.p. by a 12 mg/kg injection of BCNU on day 6.5. Mice were treated by immunotherapy by 1 mg injections of bacteria administered on days 8, 11, 14 and 17 after tumour grafting. As seen in Fig. 1, we were able to cure 50, 10 and 5% of mice challenged with 4000 L1210 cells (day 0) with Micrococcus, *B. subtilis* and BCG chemo-immunotherapy respectively. When mice challenged with 25,000 L1210 cells were treated by chemo-immunotherapy with Micrococcus, *B. subtilis* or BCG, 50, 10 and 0% of long-term survivors were respectively recorded (Fig. 2). In these protocols, no long-term survivors could be obtained by BCNU chemotherapy alone.

We further investigated whether chemo-immunotherapy yielded a temporary enhanced non-specific immune resistance or whether a specific immune response was built up by chemo-immunotherapy. Therefore, long-term survivors were rechallenged with 10^4 L1210 cells

*Table 3. Screening of different chemo-immunotherapeutic protocols for activity against L*1210 *mouse leukaemia*

Drug	Chemotherapy Dose (mg/kg)	Chemotherapy Treatment (days)	Immunotherapy schedule	% of cured mice*	M.S.T. (days) ± S.D.†	T/C‡
5-fluoro-uracil	120	1	—	0	15.2 ± 0.3	165§
(NSC 19893)	120	1	*A*	0	14.2 ± 2.9	155§
	120	1	*B*	0	15.5 ± 0.8	168§
5-fluoro-uracil	50	1,4	—	0	16.4 ± 2.8	179§
(NSC 19893)	50	1,4	*A*	0	14.9 ± 3.9	162§
	50	1,4	*B*	0	16.0 ± 2.2	174§
Cyclophosphamide	100	1	—	0	14.8 ± 0.6	161§
(NSC 26271)	100	1	*A*	0	14.4 ± 1.4	156§
	100	1	*B*	0	16.0 ± 1.6	174§
Cyclophosphamide	40	1,5	—	0	15.0 ± 0.0	163§
(NSC 26271)	40	1,5	*A*	0	15.3 ± 0.9	166§
	40	1,5	*B*	0	15.2 ± 0.6	165§
BCNU	12	6.5	—	0	17.7 ± 5.4	192§
(NSC 409962)	12	6.5	*A*	30	15.7 ± 0.7	171§
	12	6.5	*B*	30	20.4 ± 5.6	222§
Untreated controls	—	—	—	0	9.2 ± 3.1	100

On day 0, intact CFF_1 mice were challenged intraperitoneally with 10^5 L1210 cells. Mice were treated intraperitoneally by chemotherapy and immunotherapy. Immunotherapy was performed by 1 mg intraperitoneal injections of *Micrococcus lysodeikticus* either on days 8, 9, 10, 12, 12 (schedule A) or on days 8, 11, 14, 17 and 20 after tumour grafting (schedule B).

*% of mice surviving day 90.

†Mean survival time ± standard deviation of mice dying before day 90.

‡Mean survival time of treated but tumour bearing mice as compared with untreated mice, expressed in percentage.

§Student–Fisher *t*-test: $P < 0.001$.

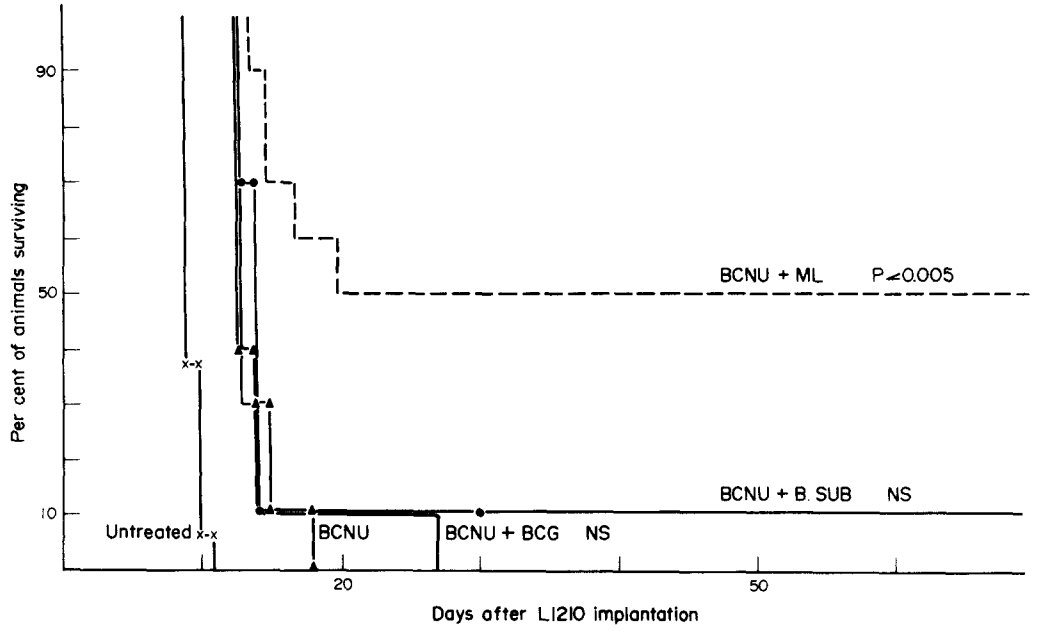

Fig. 1. Effect of repeated administration of Micrococcus, BCG or Bacillus subtilis *in chemo-immunotherapy of advanced L1210 leukaemia.*

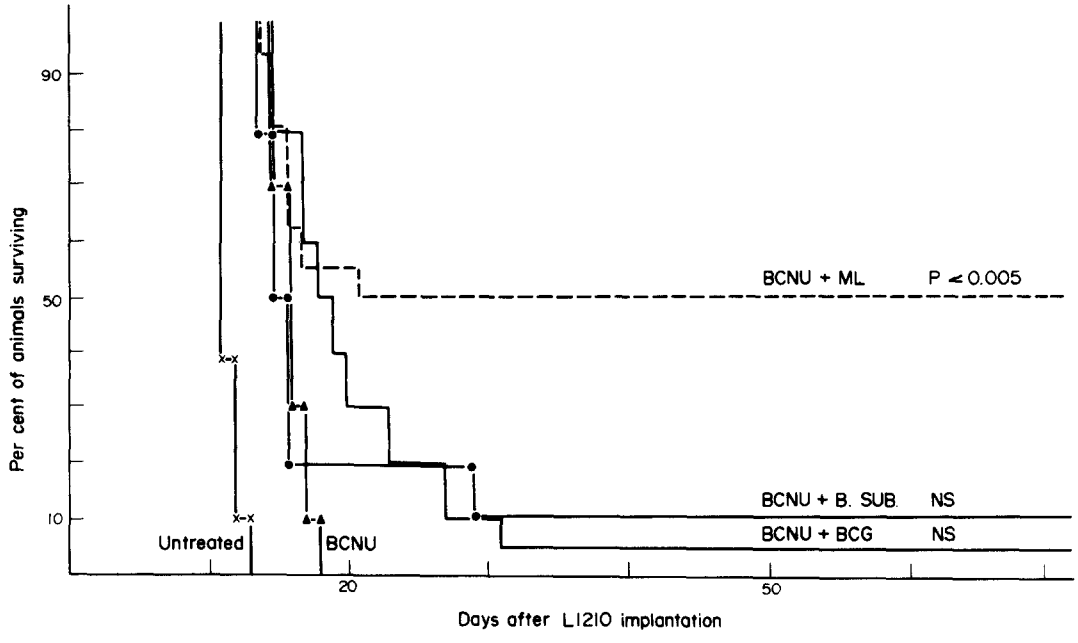

Fig. 2. Effect of repeated administration of Micrococcus, BCG or Bacillus subtillis *in chemo-immunotherapy of advanced L1210 leukaemia.*

Notes for Figs. 1 *and* 2. *Intact* CDF_1 *mice received a transplant either of* 2.5×10^4 *leukaemic L1210 cells (Fig.* 1) *or* 4×10^5 *cells (Fig.* 2) *Intraperitoneally on* day 0. *Mice were treated by a single intraperitoneal injection of BCNU* (12 *mg/kg*) *on* day 6.5. *Mice were treated by immunotherapy and received an intraperitoneal injection of* 1 *mg of bacteria on* days 8, 11, 14 *and* 17 *after tumour grafting. The % of surviving untreated* (-x-x-), *BCNU treated* (-▲-▲-), *BCNU + BCG* (—), *BCNU + B. subtilis* (-●-●-), *BCNU + ML*(----) *mice is plotted.*

Statistical evaluations were determined by χ^2 *distribution for difference with mice given BCNU only. NS = Not significant.*

on day 121. Although all untreated control mice died after 10.33 ± 0.5 days, mice previously cured by chemo-immunotherapy rejected this tumour graft (surviving on day 90), indicating a specific and durable immune resistance. Similarly, all mice previously cured by chemo-immunotherapy rejected an intramuscular graft of 10^4 L1210 cells whereas all intact control mice died (M.S.T. $= 11.0 \pm 0.4$) with proliferative solid L1210 tumours.

DISCUSSION

Immunotherapy alone with either Micrococcus, *B.subtilis* or BCG did not result in a significant lengthening of survival of mice grafted with 5×10^4 L1210 cells. This could be explained by the fact that immunotherapy is only active against minimal residual disease and is ineffective in mice against grafts of $\geq 10^4$ cells [31]. Although chemotherapeutic doses and treatment schedules were selected so as to induce a mean lifespan of 60–90% over controls, the combination of Micrococcus immunotherapy with 5-fluoro-uracil chemotherapy or with cyclophosphamide was unsuccessful after the graft of 10^5 L1210 cells. We assume that this ineffectiveness can be attributed to one or all of three factors:

(a) the initial inoculum level was too high (5×10^4 L1210 cells): the curative effect of BCG administered after cyclophosphamide has been demonstrated in the case of an initial inoculation of 10^3 leukaemic cells [32];

(b) since the same immunotherapeutic treatment was combined with the different chemotherapeutic protocols in order to demonstrate the effectiveness of immunotherapy, it can be assumed that the optimal schedule of treatment with the combination therapy was not reached. Immunotherapy may have indeed been started at a time when immunosuppression induced by chemotherapy reached a peak and when mice were unresponsive. Therefore, the kinetics of immunosuppression and the responsiveness of tumour-bearing mice should be better investigated for each chemotherapeutic drug;

(c) immunotherapy may have affected the inadequate immune cell subpopulations. During the last decade, it became clear that the overall immune response is the outcome of action of particular cell clones (i.e., T-helper, T-killer cells) which in turn are affected by antagonists (i.e., T-suppressor cells) but which differ in kinetics of cell proliferation. We cannot emphasize strongly enough the importance of immune cell kinetic studies combined with pharmaco-dynamic activity studies both in humans and in animals. Possibly, there is hope in the future for considerable enhancement of the antitumour immune responsiveness through elimination of the inhibitory cell populations by administration of cytotoxic drugs at the time when those inhibitory cells display the highest activity. This may eventually offer a new argument in favour of immunotherapy. At present there is no clear-cut evidence explaining the failure of immunotherapy after cyclophosphamide or 5–fluoro-uracil chemotherapy. By contrast, Micrococcus immunotherapy combined with BCNU chemotherapy started on day 6.5 after an initial inoculation of 100,000, 25,000 or 4000 L1210 cells cured respectively 30, 50 and 50% of tumour-bearing mice. The results with "fresh" BCG and *B. subtilis* were strictly inferior to those obtained with BCG-SP (33). Also, working with "fresh" Paris BCG, Mathé *et al.* found that 1 mg injections (7×10^6 viable units per mg) is the immunostimulating dose which yields statistically significant enhanced adjuvanticity on day 2 after intraperitoneal administration in mice [29]. We found that multiple 1 mg injections of Micrococcus or BCG (5×10^6 viable micro-organisms per mg) were shown to yield more effective adjuvanticity than one single injection [25, 33]. However, since injections of 10^9 bacilli were less effective than 10^7 in regressing line 10 tumour-bearing guinea pigs [34] and high doses of BCG were immunosuppressive [35], we decided to use intermittent multiple 1 mg injections rather than one single high dose of bacilli. Even more important, no schedule dependency could be demonstrated when mice were treated with Micrococcus immunotherapy after BCNU chemotherapy.

The regular therapeutic response pattern obtained by multiple 1 mg *Micrococcus lysodeikticus* injections, the high cure rate and the non-pathogenicity and non-toxicity of Micrococcus promote this easy-to-eliminate bacterium (substrate of lysosyme) as an attractive new candidate for new clinical antitumour strategies.

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