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Comparison Between the Effects of *Micrococcus Lysodeikticus*, Bacterial Cell Wall and Related Polysaccharides in the Non-Specific Tumour Immunotherapy of Ehrlich Ascites Tumour Growth*

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Abstract—*By analyzing, at different times after grafting* 300,000 *Ehrlich ascites cells to BALB/c mice, the primary immune response to* 2.10^8 *sheep erythrocytes, the carcinoma monitored immunosuppression was outlined. The fact that murine ascites fluid contains less immunoglobulins and less pre-albumin migrating components than serum of tumour bearing mice suggests that the tumour site may be the consuming focus. When different treatment schedules were assayed, we found that the intraperitoneal route (intratumoral) was better than other treatment routes: for* 30,000 *initially grafted tumour cells, i.p. treatment with* 0.08 *and* 0.4 mg *heat-killed Micrococcus on* day 1, 3, 5, 7 *and* 9 *resulted respectively in a* 120 *and* 148% *increase in mean life span over control mice and* 50 *and* 20% *of long-term survivors were recorded. However, the administration of* 1 mg *Micrococcus on* days 1, 5, 9 *and* 12 *after grafting* 300,000 *Ehrlich carcinoma cells considerably enhanced tumour growth. The administration of* 1 mg *of Micrococcus, cell wall, cell wall conjugated chitin, chitin and zymosan A on* days 1, 2, 3, 4 *and* 5 *resulted in a* 71, 45, 29.5, 68 *and* 82% *increase in mean life span over control mice and* 30, 20 *and* 30% *of long-term survivors were recorded for chitin, cell wall conjugated chitin and zymosan A respectively.*

INTRODUCTION

EARLIER studies by Skipper *et al.* clearly demonstrated that chemotherapy of neoplasms obeys first-order kinetics [1], indicating that, after reduction of the tumour cell mass (particularly in human patients), an additional therapy such as immunotherapy is required to eliminate the last tumour cell.

Although the administration of a single drug or a combined drug therapy may induce temporary tumour regression, they also may enable the *in vivo* selection of drug resistant malignant cell clones that kill the host after their proliferative phase. In this perspective, it is quite understandable that, if adequately monitored, immunotherapy provides an attractive anti-tumour weapon since it offers the possibility to cure.

Immune responses are of limited strength but, for instance, they were shown to be effective in mice when the tumour burden is inferior to 10^5 cells [2]. Chemically induced [3] or virally transformed [4] tumour cells express tumour specific antigenic epitopes but it was shown that the evoked immune responses were feeble as a result of the weak antigenicity of those cells [5].

Although the humoral anti-tumour immunity (antibodies) collaborates with Fc receptor [6] bearing lymphoid cells in an effec-

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tor mechanism (antibody dependent cellular cytotoxicity [7]) that was shown to be operationally effective, it is worthy of note that a minimal amount of tumour cell coating immunoglobulin is required and that a sufficient number of effector cells must be recruited. In accordance with the immunostimulation theory of Prehn [8], it was observed that a vigorous immune response inhibits tumour growth whereas a small response facilitates neoplastic cell proliferation [9].

Immunostimulating compounds such as levamisole [10], Bacillus Calmette-Guérin [2] and *Corynebacterium parvum* [11] were successfully used to reinforce the immunologic potential of transplantable tumour bearing mice.

In this study, we have investigated the parameters that influence the non-specific immune resistance generated by inoculation of heat-killed and non-pathogenic *Micrococcus lysodeikticus* cells (American Type Culture Collection 4698) on the growth of Ehrlich ascites carcinoma. This well defined tumour grows in BALB/c mice (lethal dose for 100% of the animals = 1000 cells) but also in rats and other mouse strains [12] presumably as a result of the absence of membrane histocompatibility antigenic markers [13].

Ehrlich ascites tumour growth is facilitated by a tumour monitored generalized immunosuppression: the cell free ascites tumour fluid contains an immunosuppressive factor that, upon inoculation in intact mice, reduces the number of plaque forming anti-sheep-erythrocyte cells [14] and considerably prolongs mean life span of mouse skin grafts [15].

Furthermore, it was demonstrated that this factor inhibits the maturation of precursor T cells [16] and finally leads to a defective immune machinery. However, it must be stressed that immunocompetent cells of immunosuppressed (tumour bearing) animals were able to build nearly normal immune responses when transferred into normal mice with a X-rays inactivated immune system, demonstrating the reversibility of the suppression [17].

A similar situation is encountered in clinical cancer. Human patients often show impaired immune responses as reflected in phytohaemagglutinin stimulated cultures of T cells [18]. Circulating tumour antigen [19], antigen–antibody complexes [20], macromolecular immunosuppressive globulins [21] and dialysable peptides [22] were held responsible for the immunosuppressive status of the host. These factors were claimed to cause the inhibition of an effective antitumour response by repulsing the macrophages [23] and to decrease the expression of a committed immune cell proliferation by interference with normal inhibitory feed-back mechanisms.

The antineoplastic immune reactivity is mediated by activated macrophages and phagocytes [24] that express membrane receptors for the third component of complement (C_3) [25].

Since those particular cell types are known to be activated through the serum complement [26] system, we have compared the therapeutic values of the complement activating bacterium *Micrococcus lysodeikticus* [27] with the activity generated by related complement triggering polysaccharides.

MATERIALS AND METHODS

Micrococcus lysodeikticus

A suspension was made of lyophilized *Micrococcus lysodeikticus* (ML) (Worthington Biochem. Corporation) after repeated washings and centrifugations at 3000 ***g*** for 15 min at 4°C in phosphate buffered saline (0.15 M NaCl–0.01 M potassium phosphate; pH = 7.3) = PBS buffer.

Micrococcus cells were heat-killed by incubation of the suspension at 60°C for 30 min and used within one week.

Chitin, Micrococcus wall and cell wall conjugated chitin

They were prepared as earlier described [28].

Zymosan A

Cell walls of the yeast *Saccharomyces cerevisiae* (Z4250) were obtained from the Sigma Company (lot No. 34C-2650). The insoluble cell walls were resuspended in PBS, boiled for 1 hr, washed thrice with PBS and centrifuged at 3000 ***g*** for 20 min.

Erythrocytes of sheep

Ten milliliter samples of sterile, heparinized blood of sheep were purchased from the Pasteur Institute, Brussels, and stored upon delivery in Alsever solution at 4°C.

The sheep red blood cells (SRBC) were washed five times with PBS buffer and centrifuged at 3000 ***g*** for 15 min. They were diluted in PBS buffer and counted in a Thoma cell haemocytometer.

Quantitative agglutination analyses

These were carried out in U shaped wells of Linbro agglutination plates. 0.05 ml of the antiserum was mixed with 0.05 ml of phosphate buffered saline and consecutive twofold dilutions were made. After a preincubation of

30 min at 37°C, 0.05 ml of the SRBC (10^8 cells/ml) or the micrococcus suspension (2 mg/ml) were added and the agglutination was scored after 2 hr at 37°C and expressed in $\log_2$ units.

Electrophoretic procedures

Cellulose acetate electrophoreses were carried out with a Beckman microzone cell model R200.

Electrophoresis was carried out in barbital buffer (I = 0.075, pH = 8.6) for 20 min, at 250 V, at 4°C. After staining with Ponceau S, the strips were stained with 5% acetic acid and scanned with a Vitatron Modular photometer (absorbance at 546 nm) with a scanning accessory.

Mice and tumour

BALB/c mice were originally obtained from the T.N.O., Rijswijk, The Netherlands, fed and watered *ad libitum*. Either male mice, weighing minimum 19 g, or female mice weighing at least 18 g were used.

An hyperdiploid line of the Ehrlich ascites tumour was obtained from Dr. Maisin, Belgium. This tumour has been transplanted for years by Mr. A. Cordier in inbred BALB/c mice, by weekly passage of 10^7 tumour cells to intact mice.

Ehrlich cells were aspirated from BALB/c mice and diluted in phosphate buffered saline. 0.1 ml of the suspension was injected intraperitoneally (i.p.) to obtain the ascitic form of the carcinoma. Animals were randomized into test groups and control groups. For each experiment, 10–20 mice were used. The mean survival time ± the standard deviation of the treated mice and the control mice (M.S.T. ± S.D) as well as the dosage and timing of each experiment are specified in the tables. Survivors on day 90 after tumour grafting were considered as long-term survivors and did not contribute to the final evaluation of the mean survival time.

RESULTS

1. *Effect of Ehrlich ascites tumour growth on murine physiology*

The present study aims at investigating whether a non-specific antitumour immune resistance could be generated in Ehrlich ascites tumour bearing mice by the administration of *Micrococcus lysodeikticus*. Therefore, we have tried, in a first approach, to outline some changes in murine physiology that accompany the growth of the ascites tumour.

For 200,000 tumour cells grafted on day 0 in the mouse peritoneum, we found that inoculation of 2.10^8 sheep erythrocytes injected intravenously on days 0, 4, 9 and 12 after tumour grafting resulted in a gradually decreasing primary immune anti-SRBC response, as measured by the serum haemagglutination titers of mice, bled 4 days after priming.

Whereas normal mice respond to this cellular antigen with closely resembling (antibody) haemagglutination titers of high magnitude ($\log_2 = 9.56 \pm 1.15$), immune responsiveness of Ehrlich tumour bearing mice decreased 5 days after tumour transplant. BALB/c mice were completely unresponsive to new antigenic challenges 13 days after tumour grafting, as indicated in Fig. 1 (lower diagram).

Ascites tumour cells are known to proliferate exponentially according to a

$$V = V_0 \exp\left[\frac{A}{\alpha(1-e^{at})}\right],$$

Gompertz function [29] and most remain

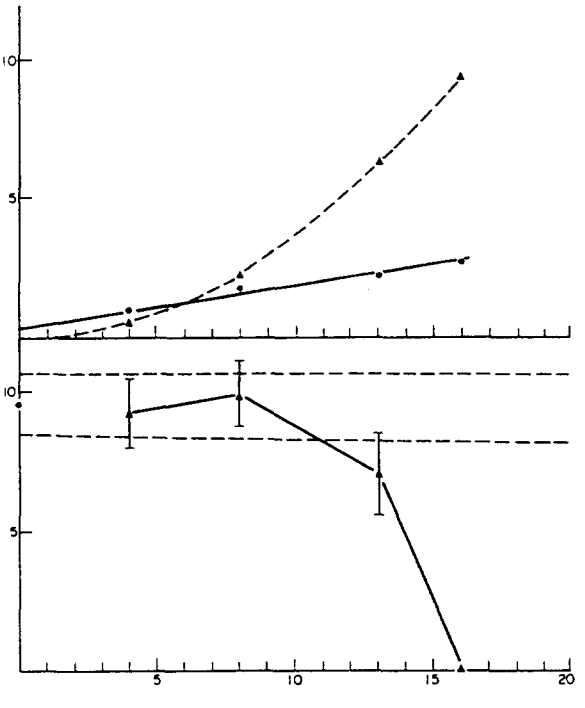

Fig. 1. Influence of Ehrlich ascites tumour growth on murine physiology. BALB/c mice were challenged i.p. with 200,000 *Ehrlich carcinoma cells on* day 0. *Changes of body weight and primary anti-sheep erythrocytes immune responses of normal* (●) *and tumour bearing mice* (▲) *are shown. Mean values are presented for* 5–10 *animals.*
Upper diagram: abscis: tumour age (days). *ordinate: absolute increase in body weight* (g).
Lower diagram: abscis: tumour age (days). *ordinate: haemagglutination titer* ($\log_2$ *units*).

Table 1. Occurrence of serum components in the tumour fluid (EAF) of Ehrlich carcinoma-bearing mice

Percentage of decrease of concentration of alpha$_1$ EAF/α_1 serum vs albumin standard	Percentage of decrease of concentration of gammaglobulin EAF/gamma serum vs albumin standard	Percentage of decrease of concentration of prealbumin components EAF/prealbumin components serums vs albumin standard	Percentage of decrease of concentration of gamma EAF/gamma normal serum vs albumin standard	Percentage of decrease of concentration of gamma EAF serum/gamma normal serum vs albumin standard
20.42	49.76	69.36	80	23.1
17.27	41.96	68.49	70.73	65.33
29.39	46.5	41.55	69.54	43.07
26.85	15.56	65.7	57.32	26.27
29.87	73.86	62.1	59.68	19.75
Mean values				
24.76	=45.53	=61.44	=67.43	=35.5

Intact syngeneic BALB/c mice were inoculated i.p. with 2.10^5 Ehrlich ascites tumour cells on day 0.
On day 16, Ehrlich ascitic fluid and serum of the individual mice were collected after exsanguination of the animal. After microzone electrophoresis, the electrophoretic patterns were scanned by a Vitatron scanner and the individual concentrations were calculated by automated peak integration.
No trace of hemorraghia was observed.

inside the peritoneal cavity. Cellular multiplication goes hand in hand with development of massive ascites (up to 10 ml fluid for Ehrlich carcinoma) and leads to an exponential gain in body weight, as seen in Fig. 1 (upper diagram).

Microzone electrophoresis patterns of the ascites fluid revealed the presence of all components of normal mouse serum. After densitometric scanning with a Vitatron Modular Photometer, the individual component amounts were quantitatively determined by automated peak integration. Peak concentrations were compared on an albumin standard peak basis. As illustrated in Table 1, we found a 45.5% decrease in gammaglobulin (IgG), 24.8% decrease in α1 and 61.4% decrease in pre-albumin migrating components in the cell free ascites fluid as compared to the serum amounts of the same tumour bearing mice. Serum of sick animals contained 35% IgG less than normal mice.

2. *Effect of the inoculation of* Micrococcus lysodeikticus *on Ehrlich tumour growth*

BALB/c mice received intraperitoneally a transplant of 300,000 Ehrlich ascites cells on day 0.

We found that neither 0.01, 0.05, 0.25, 0.75 nor 1.5 mg of heat-killed *Micrococcus lysodeikticus* given 14 days before the graft considerably protected mice against tumour challenge (Table 2, A).

Similarly, we also observed that 1 mg dosages administered 24 hr before tumour grafting or 1 day after the graft did not affect mouse survival.

On the contrary, we found that inoculation

Table 2. Effect of the inoculation of Micrococcus lysodeikticus *on the growth of murine Ehrlich carcinoma*

Schedule	Treatment by Micrococcus dosage + route + day	M.S.T. (days) ± S.D.	%T/C	% of mice cured (day 90)
A	0.01 mg, i.p., −14	22.89 ± 2.2	105	0
	0.05 mg, i.p., −14	22.67 ± 2.2	104	0
	0.25 mg, i.p., −14	23.20 ± 2.0	106	0
	0.75 mg, i.p., −14	24.17 ± 3.49	110	22
	1.50 mg, i.p., −14	22.33 ± 3.0	102	0
	none (control mice)	21.83 ± 2.3	100	0
B	1 mg, i.p., −1	21.71 ± 1.6	104	0
	1 mg, i.p., +1	21.57 ± 3.2	103	0
	1 mg, i.p., +1, 5, 9 and 12	14.14 ± 1.2	67	0
	none (control mice)	21.00 ± 0.7	100	0

BALB/c mice were challenged with 300,000 Ehrlich ascites cells intraperitoneally, on day 0.
Mean survival time (M.S.T.) and the standard deviation (S.D.) of mice (T), treated either 14 days before tumour grafting or after tumour challenge is shown and the ratio with control mice (C) is expressed in percentage (T/C per cent).

of 1 mg of heat-killed Micrococcus on days 1, 5, 9 and 12 to tumour bearing animals resulted in an enhanced mortality: treated mice died with a mean survival time of 14.14 days ± 1.2 and control mice after 21.00 ± 0.7 days (Table 2, B).

3. *Determination of the parameters influencing the immunotherapeutical trials of* Micrococcus lysodeikticus *on Ehrlich ascites tumour growth*

Since inoculation of heat-killed Micrococcus leads to a growth enhancement, when administered to tumour bearing mice during the period of decreasing immunoresponsiveness, we decided to treat mice before the 9th day of the tumour graft.

We looked for an immunotherapeutical effect of the bacterium on the proliferation of 30,000 (C) and 300,000 (D) initially grafted cells (day 0).

As demonstrated in Table 3C, intravenous inoculation of 0.08 and 0.4 mg on days 1, 3, 5, 7 and 9 after tumour grafting resulted respectively in 40 and 11% of long-term survivors, whereas the 1 mg dose given on day +1 or day +5 yielded 11 and 0% of long-term surviving mice.

When the bacterium was subcutaneously injected, the lower dosage (80 μg) had no effect but after the administration of 1 mg (day 1) or 0.4 mg (days 1, 3, 5, 7 and 9), we respectively found 30 and 11% of mice cured on day 90.

Intraperitoneal treatment proved to be superior to the other treatment routes and yielded 50 and 20% of long-term survivors after the repeated administration (days 1, 3, 5, 7 and 9) of 0.08 and 0.4 mg. A 1 mg injection on day 5 was ineffective but injection on day 1 gave 25% of cures. It is remarkable that systemic inoculations lead to an all or none reaction that cures some animals but does not considerably prolong the mean survival time of the other treated mice over control mice.

Intratumoral treatment (intraperitoneal) yields both an increased mean survival time and a percentage of cured mice.

It is also worthy of note that there was no direct correlation between the serum anti-Micrococcus antibody titer (as measured on day 12 after tumour challenge mean survival time and the percentage of cured animals.

For 300,000 grafted Ehrlich cells (Table 3D), treatment by the intravenous route was ineffective irrespective of the treatment schedule.

Intraperitoneal administration of 0.05 and 0.75 mg of heat-killed bacteria on days 1, 3, 5, 7 and 9 succeeded in 22 and 20% of animals cured on day 20.

4. *Comparison of the immunotherapeutical effects of Micrococcus with the activity generated by related polysaccharides*

In a last set of experiments, we have examined and compared the immunotherapeutical effects of *Micrococcus lysodeikticus*, the bacterial cell wall, chitin (the naturally occurring polymer of *N*-acetyl-D-glucosamine isolated from the shell of crabs), cell wall conjugated chitin and zymosan A. We have previously demonstrated that both the bacterium and the polysaccharides are complement activating substances which trigger complement under conditions where only the alternative pathway may proceed. Since preceding results indicate that treatment after the fifth tumour transplant day is ineffective, we have administered 1 mg doses on days 1, 2, 3, 4 and 5 after challenging 300,000 tumour cells in the mouse peritoneum (day 0). We found that the administration of Micrococcus, cell wall, chitin, cell wall conjugated chitin and zymosan A yielded an increase in mean life span of respectively 71, 45, 68, 29.5 and 82% over control mice, as illustrated in Table 4.

Treatment by chitin, cell wall conjugated chitin and zymosan A resulted respectively in 30, 20 and 30% of mice cured on day 90 (long-term survivors).

DISCUSSION

In this paper, we provide evidence that the bacterium *Micrococcus lysodeikticus* induces a beneficial immune resistance against Ehrlich ascites carcinoma growth in BALB/c mice.

We have previously demonstrated that this Gram-positive but non-pathogenic bacterium is able to activate complement under conditions that allow only the alternative pathway to proceed [27].

It is established now that complement activation is a very important step in inflammation that switches on multiple biological processes such as B cell proliferation, macrophage activation [26] and attraction of polynucleated cells to the inflammation site [30].

Those phenomena are brought about after binding of the activated compounds on specific membrane receptors (C3b or C3d) and are non-lytic to the affected cell.

Especially for the macrophage, the complement induced signal leads to an activation followed by extrusion of the lysosomal content in the surrounding medium [31]. Therefore, it

Table 3. Determination of the parameters that influence the immunotherapeutical trials of Micrococcus lysodeikticus *on Ehrlich ascites tumour growth*

Schedule	Treatment by micrococcus dosage + route + days	Number of Ehrlich cells grafted (day 0)	M.S.T. (days) ± S.D.	%T/C	% of mice cured on day 90	Mean $\log_2$ micrococcus agglutination titer
C	0.08 mg i.v. 1, 3, 5, 7 and 9	30,000	25.66 ± 1.8	110	40	3.11 ± 3.06
	0.4 mg i.v. 1, 3, 5, 7 and 9	30,000	26.62 ± 1.9	114	11	3.50 ± 2.40
	1 mg i.v. 1	30,000	24.22 ± 1.7	104	11	2.67 ± 2.59
	1 mg i.v. 5	30,000	22.75 ± 1.7	98	0	0.0 ± 0.0
	none (control mice)	30,000	23.29 ± 2.7	100	0	0.0 ± 0.0
	0.08 mg sub. 1, 3, 5, 7 and 9	30,000	26.30 ± 1.6	105	0	0.75 ± 0.54
	0.4 mg sub. 1, 3, 5, 7 and 9	30,000	30.50 ± 12.3	121	11	0.33 ± 0.56
	1 mg sub. 1	30,000	25.71 ± 1.5	102	30	0.45 ± 0.96
	1 mg sub. 5	30,000	24.77 ± 2.7	98	0	0.22 ± 0.44
	none (control mice)	30,000	25.12 ± 1.1	100	0	0.0 ± 0.0
	0.08 mg i.p. 1, 3, 5, 7 and 9	30,000	54.80 ± 16.3	220	50	0.1 ± 0.32
	0.4 mg i.p. 1, 3, 5, 7 and 9	30,000	61.80 ± 26.9	248	20	2.65 ± 1.45
	1 mg i.p. 1	30,000	36.67 ± 12.5	147	25	0.92 ± 1.62
	1 mg i.p. 5	30,000	25.40 ± 13.4	102	0	0.0 ± 0.0
	none (control mice)	30,000	24.90 ± 1.8	100	0	0.0 ± 0.0
D	0.01 mg i.v. 1, 3, 5, 7 and 9	300,000	22.44 ± 3.8	106	0	
	0.05 mg i.v. idem	300,000	20.20 ± 2.2	95	0	
	0.25 mg i.v. idem	300,000	21.44 ± 3.8	101	0	
	0.75 mg i.v. idem	300,000	22.60 ± 1.6	106	0	
	none (control mice)	300,000	21.25 ± 5.2	100	0	
	0.01 mg i.p. 1, 3, 5, 7 and 9	300,000	21.75 ± 3.0	109	0	
	0.05 mg i.p. idem	300,000	19.28 ± 5.0	96	22	
	0.25 mg i.p. idem	300,000	21.50 ± 4.2	107	0	
	0.75 mg i.p. idem	300,000	20.00 ± 2.3	100	20	
	none (control mice)	300,000	20.00 ± 1.7	100	0	

Ehrlich carcinoma tumour bearing BALB/c mice were treated intravenously (i.v.), intraperitoneally (i.p.) or subcutaneously (sub.) with heat-killed *Micrococcus lysodeikticus* and the mean survival time (M.S.T.) and the standard deviation (S.D.) of treated mice (T) and control mice (C) of each schedule is listed and their ratio expressed in percentage (T/C%). On day 12, after tumour grafting, mice were bled in the retro-orbital plexus and their micrococcus serum agglutination titer was scored.

Table 4. Comparison of the effect of Micrococcus lysodeikticus, *the bacterial cell wall and related polysaccharides in the immunotherapy of Ehrlich ascites tumour growth*

Immunotherapy by	M.S.T. ± S.D. (days)	%T/C	% of mice cured (day 90)
Micrococcus l.	36.60 ± 21.3	171	0
Micrococcus cell wall	31.10 ± 14.5	145	0
Chitin	36.00 ± 15.4	168	30
Cell wall conjugated-chitin	27.71 ± 11.3	129.5	20
Zymosan A	39.00 ± 24.9	182	30
None (control mice)	21.43 ± 2.3	100	0

BALB/c mice received a tumour graft of 300,000 Ehrlich carcinoma cells in the peritoneum on day 0.
Some were treated by immunotherapy: on days 1, 2, 3, 4 and 5 after tumour grafting, they received 1 mg injections intraperitoneally.
Mean survival time (M.S.T.) and the standard deviation (S.D.) of the treated group (T) and the control group (C) are presented and their ratio expressed in percentage (T/C per cent).

was not surprising that the intratumoral route (i.p.) was the most effective.

In a first experiment, we have tried to circumline the described tumour induced immunosuppression for a given number of i.p. transplanted tumour cells. We found that the expression of a primary anti-SRBC immune response rapidly declines 9 days after tumour transplant, leading to complete abrogation of immunoresponsiveness after the 13th day, as seen in Fig. 1. We also noticed that tumour growth is accompanied by an exponential net increase in body weight, which is a direct consequence of the Gompertz-like development of massive intraperitoneal amounts of tumour fluid (up to 10 ml ascitic fluid). This ascitic fluid was analyzed by microzone electrophoresis and the components quantitated by automated peak concentration integrations and finally compared to the serum components of the same mouse. On an albumin peak standard concentration, we found a 45.5% decrease in IgG, 24.8% decrease in $\alpha 1$ and 61.4% decrease in prealbumin components in the cell free fluid, as illustrated in Table 1. Since the immunoglobulin content of the fluid $= IgG_f < IgG_{serum} < IgG_{normal\,serum}$ we assume that the tumour site is the IgG consuming focus.

A similar order of decrease was found for the pre-albumin components. Since those components are known to contain predominantly proteolytic inhibitors and since it was described that Ehrlich tumour contains a plasminogen activator as well as a kinin-forming proteolytic system, we hypothesize that the reduced concentration is due to consumption by the tumour cells.

Neither 0.01, 0.05, 0.25, 0.75 nor 1.5 mg of heat-killed bacteria inoculated fourteen days before tumour challenge were able to protect mice (Table 2A).

One milligram injected 24 hr before or after tumour challenge was also ineffective whereas inoculation of 1 mg on days 1, 5, 9 and 12 resulted in an enhanced mortality (Table 2B).

Inoculation of 0.08 and 0.4 mg on days 1, 3, 5, 7 and 9 after grafting 30,000 cells resulted in 40 and 11% of long-term survivors for the intravenous route, 0 and 11% for the subcutaneous route and 50 and 20% for the intraperitoneal treatment.

A 1 mg injection on day 5 was always ineffective but a 1 mg injection on day 1 yielded 11% (intravenous route), 30% (subcutaneous) and 25% (i.p.) of mice cured on day 90.

Although anti-Micrococcus antibodies were shown to agglutinate Ehrlich ascites cells [32], we found that the described treatment schedules yielded very low antibody titers (as measured on day 12) and that there was no direct correlation with the outcome of the experiment.

For 300,000 grafted Ehrlich cells, we found that only the administration of 0.05 and 0.75 mg bacterium on days 1, 3, 5, 7 and 9 resulted in 22 and 20% of long-term survivors, as seen in Table 3.

In a comparative trial against 300,000 Ehrlich carcinoma cells, we found that the administration of 1 mg of Micrococcus, cell wall, cell-wall conjugated chitin, chitin and zymosan A on days 1, 2, 3, 4 and 5 resulted in a 71.45, 29.5, 68 and 82% increase in mean life span over control mice and 30, 20 and 30% of long-term survivors were re-

corded for chitin, cell-wall conjugated chitin and zymosan (Table 4).

Our data indicate that an anti-tumour immune resistance can be generated by Micrococcus and related complement triggering polysaccharides if those substances are administered in the early proliferative stages of the carcinoma, when the murine immune capacity is maintained.

Administration of the bacterium at 1 mg dosages in mice with an ageing tumour (after the 9th tumour transplant day) leads to an enhanced mortality. Since this murine carcinoma develops a para-symptology, frequently encountered in clinical cancer, we stress the point that non-specific immunotherapeutical trials should be applied only in individuals who show a good immune prognosis. Further experiments may reveal whether the immunosuppressive status, which is reversible in murine Ehrlich carcinoma, can be altered in human patients.

Further studies may indicate if Micrococcus or complement triggering polysaccharides can be successfully used in specific immunotherapy of mice or in immunotherapeutical trials, after a tumour cell reducing chemotherapy.

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