Europ. J. Cancer Vol. 16, pp. 1199–1204

0014-2964/80/0901-1199 $02.00/0

The Influence of Alkyl-Lysophospholipids and Lysophospholipid-Activated Macrophages on the Development of Metastasis of 3-Lewis Lung Carcinoma*

WOLFGANG E. BERDEL,† WALTER R. BAUSERT, HANS U. WELTZIEN, MANUEL L. MODOLELL, K. H. WIDMANN and PAUL G. MUNDER‡§

Max-Planck-Institut für Immunbiologie, D-7800 Freiburg im Breisgau, Federal Republic of Germany

Abstract—*Alkyl-lysophospholipids are synthetic analogs of natural occurring 2-lysophosphatidylcholine. They inhibit the development of metastasis of 3-Lewis lung tumor in the lung of C57Bl/6 mice if given i.v., i.c. or even orally as demonstrated by the increase of the median survival time and the number of surviving animals. Furthermore, i.v. injections of lysophospholipid-activated bone marrow macrophages increase the number of surviving animals and cause also a prolongation of the median survival time.*

INTRODUCTION

AMONG the many models in which tumor-host relationship can be studied *in vivo* the Lewis lung tumor (3-LL) is of particular relevance as it metastasizes regularly to the lung and is highly malignant probably due to its low antigenicity [1].

In an extensive study on the immunemodulating effect of various lysophospholipids we noted also a remarkable anti-tumor effect of some of these compounds [2]. Growth of several tumors growing i.p. or i.c. were either greatly retarded or completely inhibited when mice were treated with various alkyl-lysophospholipids. In order to investigate the effect of alkyl-lysophospholipids on the development of metastasis we have used the 3-LL carcinoma in syngeneic C57Bl/6 mice. Several other different immunemodulating substances like pyran or BCG have been tested in this system for their possible anti-tumor effect [3–8].

As macrophages can be activated by alkyl-lysophospholipids *in vivo* and *in vitro* [2, 9] the effects of i.v. injected activated macrophages were also tested.

Alkyl-lysophospholipids as well as lysophospholipid activated macrophages seem to inhibit metastatic growth in the lung as indicated by the number of surviving animals and the prolongation of the median survival time.

Accepted 15 February 1980.

*Work performed in partial fulfilment of the requirements for the M.D. degree, University of Freiburg, Federal Republic of Germany.

†Present address: I. Medical Clinic, Technical University, Munich, Federal Republic of Germany.

‡Supported by the Deutsche Forschungsgemeinschaft grants Mu 226/7 and 226/8 and the Volkswagenstiftung 1/34-75.

§To whom requests for reprints should be addressed.

Abbreviations: CSF, colony stimulating factor: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 3-LL, 3-Lewis lung carcinoma; 2-LPC, 2-lysophosphatidylcholine; LPA, lysophospholipid analogs; PBS, phosphate buffered saline; SPF, specific pathogen free; i.c., intracutaneously.

MATERIALS AND METHODS

Inbred female 2–3 month old C57Bl/6 mice were obtained from the SPF unit of the breeding center of the institute.

Lysophospholipids

2-Lysophosphatidylcholine (2-LPC) was prepared from egg lecithin as described [10] or purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. The lysophospholipid analogs racemic 1-octadecyl-glycero-3-

phosphocholine (ET-18-OH) and racemic 1-octadecyl-2-methyl-glycero-3-phosphocholine (ET-18-OCH_3) were synthesized as described previously [11]. The commercially available 2-LPC and the synthetic compounds were pure on thin-layer chromatography. The 2-LPC, prepared from egg 3-*sn*-phosphatidylcholine contained traces (<1%) of sphingomyelin.

Tumor

The 3-LL cell line was kept *in vivo* by injecting 10^6 tumor cells into the axilla. Fifteen to 20 days later, the tumor was removed and brought into single cell suspensions. In brief, the following procedure was used [12]. The tumor was cut into small pieces (<1 mm^3) which were then suspended into 50–100 ml cold PBS (pH 7.2) under continuous slight stirring for 10 min. Subsequently the supernatant was sucked off and the sediment trypsinized (10 min, 20°C) with 100 ml of a 0.25% solution of trypsin (lyophilized *ca.* 33 U/mg, Fa. Boehringer Mannheim GmbH F.R.G.). After spontaneous sedimentation the supernatant was discarded and the remaining tissue fragments trypsinized again (30 min, 37°C). Trypsination was stopped by adding 10 ml FCS. The cell suspension was filtrated through a stainless steel sieve, then centrifuged at 300 ***g*** after being underlayered with 3–5 ml FCS and then again washed twice in FCS-free DMEM. Viability was determined by the trypan blue exclusion test, and was usually between 80–90%.

Tumor growth and surgical removal

One million viable tumor cells in 0.05 ml DMEM + 10 mM HEPES were injected into the left hind foot-pad. Within 6–7 days the injected foot reached a diameter of 0.5–0.6 cm. The tumors were surgically removed by amputating under phenobarbital anaesthesia the left leg just above the knee joint [12]. The mice were randomized either 1 day after the amputation or in some experiments shortly before amputation. The mice began to die from metastasis 21 days after the primary tumor transplantation. Surviving animals were observed up to 120 days and then considered as survivors.

Bone marrow macrophages

Bone marrow macrophages were collected from the femurs essentially as described elsewhere [13]. Seven days-old supernatant of L929 fibroblasts was used as source of CSF and added in a final concentration of 30% to the DMEM + 10% FCS. The bone marrow cells were kept in liquid culture in Petri dishes having a highly hydrophobic gas permeable membrane for cellular support (Petriperm, Fa. W.C. Heraeus GmbH, D-6450 Hanau, F.R.G.). The mature macrophages attach only slightly to this membrane. They detach spontaneously when the dishes are placed for 10 min at room temperature on a tumbler. One hundred per cent of macrophages can be recovered, only <5% being usually trypan blue positive.

Bone marrow macrophages were activated for 48 hr by adding 5 μg/ml of ET-18-OCH_3 or ET-18-OH to the culture medium.

RESULTS

Inhibition of metastasis by alkyl-lysophospholipids

In this communication we confine ourselves to those experiments where treatment was begun 1 day after surgical removal of the

Table 1. *The effect of alkyl-lysophospholipids on the development of lung metastasis of* 3-*Lewis lung tumor*

Route	LPA (10 μg/mouse)†	Survivors*/total LPA groups‡	Control groups§	χ^2	*P* ¶	MST‖ LPA groups	Control groups
i.c.	ET-18-OH	6/10	2/10	3.33	0.035	34 (28–41)	22 (20–25)
	ET-18-OCH_3	14/30	6/30	4.80	0.014	44 (37–53)	27 (24–30)
i.v.	ET-18-OH	6/15	2/15	2.73	0.05	35 (29–42)	31 (27–35)
	ET-18-OCH_3	11/30	4/30	4.36	0.018	39 (34–45)	31 (29–33)
s.c.	ET-18-OH	1/10	0/14	1.46	0.110	n. calc.	n. calc.
	ET-18-OCH_3	9/18	8/19	0.23	0.327	n. calc.	n. calc.

*Number of survivors were determined 120 days after primary tumor transplantation.
†Daily treatment for 21 days beginning 1 day after surgical removal of the primary tumor.
‡Significance level for the therapeutic effect for all treated groups with significant higher number of survivors was $P = 0.002$ (t-distribution).
§Mice were injected daily with 0.2 ml PBS.
‖Median survival time in days (confidence limits for 19/20 probability).
¶Exact significance level in 2 × 2 contingency analysis, one tailed test.

Table 2. The influence of oral treatment with ET-18-OCH_3 on the development of metastasis of 3-Lewis lung carcinoma

Experiment No.	ET-18-OCH_3 (μg/mouse)†	Survivors*/total Treated groups	Control groups‡	χ^2	P§	MST‖ Treated groups	Control groups
1	10	4/10	0/10	5.00	0.013	36 (26–43)	30 (27–34)
	100	4/10	0/10	5.00	0.013	35 (23–56)	30 (27–34)
2	10	8/15	4/15	2.22	0.069	n. calc.	n. calc.
	100	6/15	4/15	0.60	0.219	n. calc.	n. calc.
3	10	6/14	1/15	5.18	0.011	37 (31–43)	29 (26–33)
4	10	7/14	1/15	6.81	0.004	47 (36–61)	31 (28–34)
5	10	5/14	0/15	6.47	0.005	37 (29–47)	31 (29–35)
	10 (total)	30/67	6/70	23.16	<0.001		
	100 (total)	10/25	4/25	3.57	0.030‖		

*Number of survivors were determined 120 days after primary tumor transplantation.
†Daily treatment for 21 days beginning 1 day after surgical removal of the primary tumor.
‡0.2 ml PBS.
§Exact significance level in 2 × 2 contingency analysis, one tailed test.
‖See Table 1.

primary tumor, although the start of treatment was extended up to 3 weeks after tumor transplantation. Different routes of injection and different doses of alkyl-lysophospholipids were used. Table 1 summarizes a series of experiments showing the anti-tumor effect of 2 alkyl-lysophospholipids, given at different routes. Whereas daily application of 10 μg ET-18-OCH_3 or ET-18-OH i.v. or i.c. had a protective effect against the development of lung metastasis, the s.c. and i.p. route was ineffective so far.

Treatment starting later than 21 days after transplantation of the tumor was completely ineffective (data not shown).

Surprisingly, oral application of the alkyl-lysophospholipids was as protective as i.v. or i.c. injections (Table 2 and Fig. 1). In these experiments ET-18-OH was as effective as ET-18-OCH_3.

There is not only a retardation of death due to metastasis but also a significant difference in the number of survivors. The surviving animals were observed up to day 120 after tumor transplantation and then checked for lung metastasis. No lesions in the lungs were found.

Inhibition of metastasis by lysophospholipid activated macrophages

LPA-activated macrophages kill tumor cells very effectively *in vitro* [9]. As activated macrophages have been reported to inhibit the development of lung metastasis *in vivo* [14] we also studied the influence of LPA-induced and activated peritoneal cells on the metastatic spread of 3-LL. In five different experiments the injection of $5–15 \times 10^6$ LPA induced peritoneal cells caused a significant prolongation of the median survival time as well as an increase in the absolute number of surviving animals (data not shown). These experiments, however, have to be repeated with normal purified peritoneal macrophages which already have been shown to be ineffective *in vitro* and *in vivo* [9, 15]. Recovery of purified peritoneal macrophages *in vitro* is, however, rather difficult.

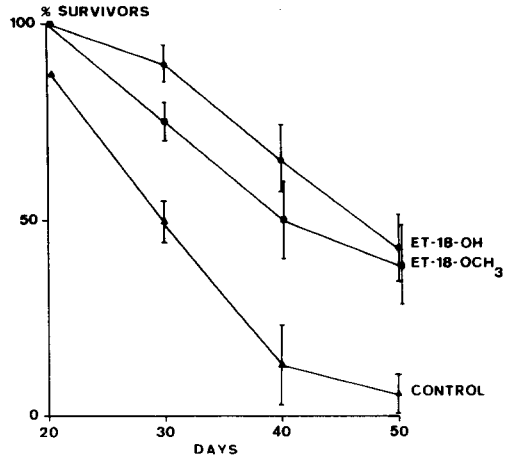

Fig. 1. Oral application of alkyl-lysophospholipids and the development of metastasis of 3-Lewis lung tumor. Racemic 1-octadecyl-glycero-3-phosphocholine (ET-18-OH) and racemic 1-octadecyl-2-methyl-glycero-3-phosphocholine (ET-18-OCH_3) were given orally in a concentration of 10 μg/mouse/day for 30 days starting 1 day after surgical removal of the tumor. The curves of the LPA-treated mice were computed as the means of three different experiments (14 mice/group, 15 mice/control group). In the 2 × 2 contingency analysis, one tailed test, all P values of the treated groups were <0.001 at days 30, 40 and 50. Day 20 was not significant. Median survival time of mice treated with (a) ET-18-OCH_3:40 (36–44); (b) ET-18-OH:44 (39–49); (c) PBS:32 (30–34).

As peritoneal cells contain about 20–30% of small and medium sized lymphocytes these experiments were repeated with pure 12–14 day-old syngeneic bone marrow derived macrophages. As shown in Fig. 2 normal bone marrow macrophages have already some protective effect when given twice a week i.v. ($P<0.05$). This effect was significantly better, when macrophages had been preincubated for 48 hr with the alkyl-lysophospholipid ET-18-OH (5 μg/ml) before their injection.

DISCUSSION

The presented data indicate that alkyl-lysophospholipids as well as lysophospholipid-activated macrophages are able to inhibit either the spread of 3-LL cells from the primary transplantation area or its local development into a metastatic lesion in the lungs. Application of alkyl-lysophosopholipids immediately after amputation of the primary tumor as well as oral treatment starting as late as 20 days after the primary tumor implant proved to be effective. At this time, tumor cells have already been found in the lung [16]. Thus, inhibition of local metastatic growth in the lung seems possible.

Alkyl-lysophospholipids might act in two ways:

1. We have shown that alkyl-lysophospholipids interfere with phospholipid metabolism of the tumor cell selectively destroying neoplastic cells *in vitro* [9]. In the present study, this mechanism seems unlikely as the LPA concentrations used were rather low (1, 10, 100 μg/mouse) to be cytolytically effective *in vivo*. However, accumulation of alkyl-lysophospholipids in the tumor cell after prolonged application cannot be excluded as many tumor cells have been shown to lack an 1-*O*-alkyl-cleavage enzyme [17], which could prevent such an accumulation. If these compounds accumulate, alkyl-lysophospholipids like ET-18-OCH_3 or ET-18-OH might then act as specific antimetabolites in the synthesis of 3-*sn*-phosphatidylcholine in tumor cells [18].
2. Application of alkyl-lysophospholipids could activate macrophages *in vivo* as these compounds increase the tumoricidal activity of normal peritoneal cells and bone marrow derived macrophages *in vitro* [9]. Although a variety of chemical and microbial substances, dead or living bacteria and lymphocyte derived factors have been described of being able to activate macrophages (for review see [19]) alkyl-lysophospholipids have the advantage of being synthesized and clearly defined chemical substances. Their biological activity depend on certain chemical structures in the molecule [9]. This should offer an approach for understanding the elicitation of tumorcidal macrophages in biochemical terms. Direct evidence for the involvement of macrophages in mediating the anti tumor effect of alkyl-lysophospholipids comes from our experiments with bone marrow macrophages (Fig. 2). The fact that pure bone marrow macrophages acquire a tumorcidal capacity in the absence of lymphocytes seems to argue against a role for lymphocytes in mediating the anti tumor effect of alkyl-lysophospholipids.

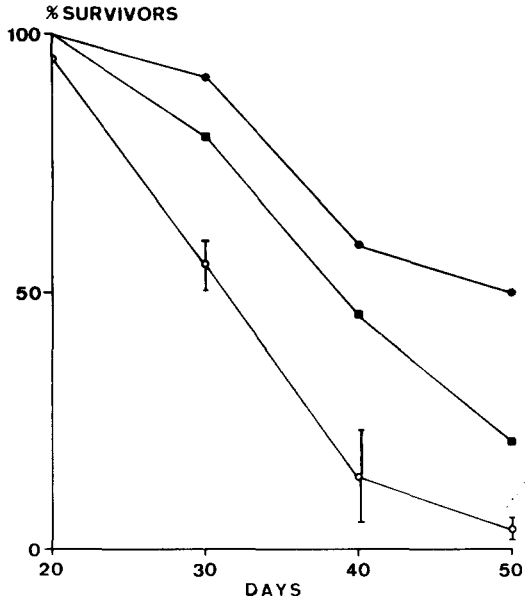

Fig. 2. The influence of activated bone marrow macrophages on the development of metastasis of 3-Lewis lung tumor. (●—●) *Racemic 1-octadecyl-glycero-3-phosphocholine (ET-18-OH)-activated bone marrow macrophages were injected i.v. twice a week in a concentration of* 1×10^6 *cells/mouse* (15 *mice/group*) *for* 3 weeks. (■—■) *Normal bone marrow macrophages were injected i.v. twice a week in a concentration of* 1×10^6 *cells/mouse* (15 *mice/group*) *for* 3 weeks. (○—○) *The control consisted of two different groups* (15 *mice/group*) *injected i.v. with* 0.5 ml *PBS. In the* 2×2 *contingency analysis, one tailed test, the groups treated with activated macrophages had* P *values of* 0.01 (day 30), 0.007 (day 40) *and* 0.0085 (day 50). *Normal macrophages had* P *values of* 0.02 (day 30), 0.05 (day 40), n.s. (day 50). *Median survival time of mice treated with* (*a*) *activated macrophages:* 45 (36–56), (*b*) *normal macrophages :*36 (30–44), (*c*) *PBS:* 31 (29–33).

The tumorcidal capacity of normal bone marrow macrophages cultured in the presence of FCS might be due to minute amounts of endotoxin often present in the serum. It

has been shown [20, 21] that 1–10 pg/ml of lipopolysaccharides increase the tumorcidal capacity of macrophages *in vitro*. Nevertheless these macrophages, however, can further be activated by incubation with alkyl-lysophospholipids as shown in Fig. 2.

Finally, it should be mentioned that no signs of morbidity have been observed during the prolonged treatment with any of the alkyl-lysophospholipids used. The same holds true for the repeated injections of bone marrow derived macrophages. In four different species the LD_{50} of LPA was about 50–60 mg/kg when given i.v. Thus, the therapeutic dose is about 100-fold lower than the LD_{50}.

In conclusion, we believe that alkyl-lysophospholipids inhibit the development of metastatic lesions primarily by activating macrophages which then attack more efficiently neoplastic cells. A direct specific cytotoxic effect of LPA on neoplastic cells *in vivo* seems possible [18] and might act synergistically.

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