Effects of the Immunomodulator LS 2616 on Growth and Metastasis of the Murine B16-F10 Melanoma

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ABSTRACT

The carboxamide-quinoline LS 2616 is a novel immunomodulator augmenting natural killer (NK) cell activity and T-lymphocyte related effector functions. To investigate the possible usefulness of LS 2616 in immunotherapy of tumors, the effect of the substance on growth and metastasis of the B16-F10 melanoma in syngeneic C57BL/6 mice was investigated. Treatment with LS 2616 from the time of s.c. inoculation of B16-F10 cells significantly reduced tumor take. Continuous treatment of mice with LS 2616 initiated 4 days prior to i.v. injection of tumor cells reduced the number of pulmonary metastases by 85%. When treatment with LS 2616 was started 4 days after i.v. injection of tumor cells, a time when established tumor foci were readily detectable in the lungs, a significant reduction in the number of pulmonary metastases resulted. LS 2616 significantly reduced the number of spontaneous pulmonary metastases developing from a B16-F10 tumor growing in the footpad. When treatment with LS 2616 was initiated after the establishment of grossly visible spontaneous pulmonary metastases, no significant effect on the number of metastases was found after 2 weeks of treatment. However, combined treatment with a dose of cyclophosphamide which in itself was ineffective resulted in a statistically significant 70% reduction in the number of remaining pulmonary metastases.

Injection of antibodies to asialomonoganglioside which strongly reduce NK cell activity in various organs was used as a probe for the involvement of NK cells in the effects of LS 2616 on the B16-F10 tumor. The therapeutic efficiency of LS 2616 on tumor take when given from the time of s.c. inoculation, on the number of i.v. induced pulmonary metastases when treatment was started before tumor cell injection, as well as the spontaneous development of pulmonary metastases during exposure to the substance was abrogated by simultaneous injection with antibodies to asialomonoganglioside. In contrast, the beneficial effects of LS 2616 on already established i.v. produced or spontaneous pulmonary metastases were unaltered in mice made NK cell deficient by injection of anti-asialomonoganglioside antibodies. In conclusion, LS 2616 has potent antitumor activities mediated by NK cells as well as non-NK cell related defense mechanisms.

INTRODUCTION

The growth of tumors and the development of metastases are highly complex processes, the outcome of which is dependent on an interplay between host factors and intrinsic characteristics of the tumor (1, 2). Recently, much effort has been spent to understand the relationship of the host immune status to progressive tumor development (for review, see Ref. 3). Immunosuppression commonly accompanies the tumor bearing state, and the detection of several biological response modifiers with potent immunoenhancing capabilities has added immunological intervention as a mean of studying the antitumor potential of the immune system as well as a potentially useful clinical approach to cancer (4, 5).

We have recently described a new chemically defined immunomodulator, LS 2616, a quinoline-3-carboxamide, with characteristics of possible value in tumor therapy (6). LS 2616 increased the delayed hypersensitivity response to bacterial antigens (7) and enhanced the lymphoproliferative response to T-cell mitogens and alloantigens (8). Moreover, the substance profoundly increased NK7 cell activity by a mechanism apparently not related to interferon production (6).

In this study, we demonstrate that LS 2616 affects growth and metastasis of the B16-F10 melanoma in syngeneic mice by NK cell dependent as well as NK cell independent mechanisms and show promising effects either alone or combined with chemotherapeutics in an experimental therapeutic model.

MATERIALS AND METHODS

Animals. All animals were inbred C57BL/6 mice obtained from Gamle Bomholtgård, Ry, Denmark, and entered the experiments at the age of 6–8 weeks.

Drug Treatment. LS 2616 (AB Leo, Helsingborg, Sweden) (Fig. 1) was made up as an isotonic solution in water and was administered to the mice in their drinking water corresponding to doses and according to schedules indicated below. These concentrations did not affect total daily water intake of the mice. Control animals were given ordinary drinking water only.

Tumor Cells. The B16-F10 cell line originally developed by Fidler (9) was kindly provided by Dr. Lennart Lögberg, Laboratory of Immunology, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH, Bethesda, MD. The cells were cultured in RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (Flow Laboratories), penicillin (100 units/ml), streptomycin (100 µg/ml), 25 mM N-(2-hydroxyethyl)piperezine-N-2-ethanesulfonic acid (Flow Laboratories) and 2 mM L-glutamine (complete medium). The cells were harvested from subconfluent cultures for use in the experiments. The harvesting procedure consisted of a 2–3-min incubation at 37°C in 0.25% trypsin (Flow Laboratories) in PBS, pH 7.4, supplemented with 0.02% (w/v) EDTA (Flow Laboratories). Viability of the recovered cells was always exceeded 95% as determined by trypan blue exclusion. YAC-1 cells to be used as target cells in the NK cell assay were kept as continuous suspension cultures in complete medium.

Local Tumor Growth. The mice were inoculated s.c. in the flank with 105 B16-F10 cells in 0.1 ml PBS and followed daily by palpation for the occurrence of tumors. Experimental animals were given LS 2616 in the drinking water corresponding to a daily intake of 160 mg/kg from the day of tumor transplantation; control animals were given ordinary drinking water.

The principal design of the metastasis experiments is outlined in Fig. 2.

Artificial Pulmonary Metastases. The mice were given i.v. injections, into the tail vein of 103 B16-F10 cells in 0.1 ml PBS. Preliminary studies indicated that pulmonary metastases were detectable microscopically at day 3 after i.v. injection and were easily evaluable macroscopically 14 days after injection. Thus the animals were killed at day 14, the lungs were removed and fixed in Bouin’s fluid, and lung colonies were counted in a dissection microscope. Various concentrations of LS 2616 was administered continuously in the drinking water starting either 4 days before or 4 days after injection of tumor cells (Fig. 2, A and B).

Spontaneous Pulmonary Metastases. The development of sponta-
neous metastases was studied in mice inoculated with $5 \times 10^5$ B16-F10 tumor cells in 0.05 ml PBS in the footpad. Initial experiments revealed that a suitable number of small to medium-sized pulmonary metastases was detectable 20–30 days after tumor transplantation when the primary tumor reached a diameter of 0.7–1.0 cm. The prophylactic effect of LS 2616 on spontaneous pulmonary metastases was studied in mice inoculated with tumor cells in the footpad and given LS 2616 (160 mg/kg/day) or ordinary drinking water from the day of tumor inoculation. The number of lung colonies was determined as described above at day 24 (Fig. 2C). To study the effect of LS 2616 on established pulmonary metastases, the tumor-bearing leg was resected at midfemur to include the popliteal lymph node at day 24, and treatment with LS 2616 (160 mg/kg/day) or vehicle was started the same day and continued to the end of the experiment. The number of lung colonies was determined as described above at day 38 (Fig. 2D).

In a similar experimental setup, the effect on established pulmonary metastases of cyclophosphamide (Sendoxan; Pharmacia, Uppsala, Sweden; 50 mg/kg in 0.3 ml PBS i.p.) on days 24, 28, 32, and 36, alone or combined with continuous treatment with LS 2616 (160 mg/kg/day) from day 24, was investigated.

Elimination of NK Activity in Vivo with Antibodies to aGMI. Lyophilized rabbit antiserum to the glycosphingolipid aGMI [Wako Pure Chemical Industries, Osaka, Japan; anti-aGMI (10)] was reconstituted with PBS and kept at −20°C. For in vivo experiments, 200 μl antiserum diluted 1:10 were injected i.p. every fourth day. The B16-F10 tumor was found not to express aGMI, as determined by indirect immunofluorescence.

![Fig. 1. Structure of LS 2616.](image)

**Fig. 1.** Structure of LS 2616.

**Fig. 2.** Experimental design of studies on prophylactic and therapeutic effects of LS 2616 on i.v. induced or spontaneous pulmonary metastases from the B16-F10 tumor. For details, see "Materials and Methods."

**Fig. 3.** Percentage of palpable tumors in C57BL/6 mice inoculated s.c. with $10^5$ B16-F10 cells. Animals were given LS 2616 (160 mg/kg/day) in the drinking water or ordinary drinking water (C) continuously from the time of tumor inoculation. Both experimental groups consisted of 16 mice.

Natural Killer Cell Assay. Natural killer cell assay was as described in detail earlier (11). Briefly, splenocytes were obtained by teasing the spleen through a stainless steel mesh and RBC were removed by hypotonic shock treatment and tested for NK cell activity against 51Cr labeled YAC-1 target cells in a 4-h isotope release assay. All assays were run at effector:target ratios of 100:1, 50:1, and 25:1 but because similar results were obtained with different target:effector ratios, only the former are shown. The data are presented as percentage of specific cytotoxicity which was determined by

\[
\text{Test cpm} - \text{spontaneous cpm} \\
\times 100 \\
\text{Total cpm} - \text{spontaneous cpm}
\]

Lymphoproliferative Response to Mitogens. Spleen cells were obtained as described above and cultured at a cell density of $10^5$ cells/ml in 200 μl complete medium for 72 h at 37°C in an atmosphere of 5% CO₂. The cells were stimulated with Con A (1, 5, or 10 μg/ml; Sigma Chemical Co.), and cellular proliferation was measured by incorporation of [³H]thymidine. For details of the procedures, see Ref. 12.

Statistical Analysis. Tumor frequencies after s.c. transplantation were compared using the χ² test. The incidence of metastases does not correspond to a normal distribution and was therefore analyzed with the Mann-Whitney U test.

RESULTS

Effect of LS 2616 on Local Tumor Growth. When LS 2616 was given continuously from the time of s.c. injection of B16-F10 cells, the frequency of growing tumors was reduced from 80% in the control group to 40% (Fig. 3). The difference was statistically significant ($P < 0.01$). Since all animals developing palpable tumors finally succumbed, these data also reflect survival.

Prophylactic and Therapeutic Effect of LS 2616 on i.v.-induced Pulmonary Metastases. Based on earlier studies on the kinetics of induction of NK cell activity by LS 2616 (6), treatment of the mice was started 4 days prior to the inoculation with B16-F10 tumor cells (Fig. 2A). The results are summarized in Fig. 4 showing that LS 2616 exerted a strong and dose dependent inhibition of the number of lung colonies detected 14 days later. The mean number of lung colonies was reduced from 74 in the control group to 11 in animals given LS 2616 (160 mg/kg daily) ($P < 0.01$). A representative sample of lungs of LS 2616 and control treated animals is shown in Fig. 5. To investigate the possible relation of the increased resistance to the B16-F10 tumor and NK cell activity, some animals were in addition pretreated with aGMI antibodies. There was a close correlation between NK cell activity and the ability to resist i.v. induced lung metastases (Fig. 6). Moreover, the protective effect of LS
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2616 was completely eliminated after injection of anti-aGMI. LS 2616 treatment increased the lymphoproliferative response to Con A while the amount of anti-aGMI used did not affect this response.

To evaluate the effect of LS 2616 on established lung metastases, treatment was started 4 days after i.v. injection of tumor cells (Fig. 2B). Also in this experimental situation, LS 2616 treatment was able to significantly reduce the number of detectable lung colonies after 14 days of treatment with a reduction in the mean number of detectable pulmonary metastases from 62 to 29 (Table 1). However, when aGMI was given simultaneously with the start of exposure to LS 2616 and later every fourth day, NK cell activity was almost completely abrogated while no effect on the therapeutic efficacy of LS 2616 was seen.

Prophylactic and Therapeutic Effect of LS 2616 on Spontaneous Pulmonary Metastases. As an alternative model for the study of the effects of LS 2616 on pulmonary metastases, spontaneous seeding of metastases from a tumor in the footpad was used. When the animals were exposed to LS 2616 from the time of tumor inoculation (Fig. 2C), a significantly reduced number of lung metastases in animals developing tumors was found (Table 2). Elimination of NK cell activity by aGMI antibodies indicated that the metastatic process was highly dependent on NK cells. Moreover, the prophylactic effect of LS 2616 on spontaneous metastases was abrogated by anti-aGMI treatment.

The therapeutic potential of LS 2616 was tested by allowing the tumor to grow for 24 days when the primary tumor was resected, and established metastases were treated continuously until the termination of the experiment at day 38 (Fig. 2D). A reduction in the mean number of metastases per animal from 27 in the control group to 14 in the LS 2616 treated group was found (Table 3). However, this difference was not statistically significant.

It was noted that not all animals in the control group developed metastases and that the size of the spontaneously developed pulmonary metastases was much more heterogeneous than those produced i.v.

Combined Treatment of Established Spontaneous Metastases with LS 2616 and Cyclophosphamide. Based on the tendency of LS 2616 treatment to reduce the number of established spontaneously developed lung metastases by almost 50%, the possible benefit of combination therapy with cyclophosphamide was explored. Cyclophosphamide or LS 2616 alone both reduced the number of lung colonies about 50%, a reduction that was not statistically significant (Table 3). However, combined treatment with cyclophosphamide and LS 2616 resulted in a consistent and statistically significant reduction of the number of lung metastases at day 38 ($P < 0.05$). Cyclophosphamide strongly reduced the activity of NK cells, an inhibition which was only partially overcome by simultaneous treatment with LS 2616 (Table 3). Moreover, injection of aGMI antibodies did not influence the therapeutic efficiency of the combined cyclophosphamide-LS 2616 treatment.

DISCUSSION

Based on the documented effects of LS 2616 on NK cell activity and T-cell mediated immunity (6–8) two major questions were approached in the present study. (a) Can the immunomodulator LS 2616 be useful in experimental tumor therapy? (b) What is the role of NK cells relative to other effector mechanisms in the effects of the substance on experimental tumors?

Using the syngeneic melanoma B16-F10 which metastasizes to the lungs with high frequency (9), we were able to show that treatment with LS 2616 inhibited growth and metastasis of this tumor in various experimental situations. Treatment of mice with LS 2616 reduced the number of growing tumors after s.c. transplantation and clearly reduced the frequency of spontaneous pulmonary metastases originating from the growing tumor. Moreover, exposure to LS 2616 was highly effective in preventing the establishment of i.v. produced pulmonary metastases when treatment was started before tumor cell injection.

Fig. 4. Number of pulmonary metastases per animal given various doses of LS 2616 in the drinking water from 4 days before i.v. injection of 10⁶ B16-F10 cells until termination of the experiment 14 days after tumor cell injection. □, mean number of metastases per animal; ○, single animals. Results from 2 separate experiments with 13—15 animals per group are shown.

Fig. 5. Representative sample of lungs taken from animals given ordinary drinking water (top) or LS 2616 (160 mg/kg/day (bottom)) continuously from 4 days before i.v. injection of 10⁶ B16-F10 cells until termination of the experiment 14 days after tumor cell injection.
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Fig. 6. Relation between NK cell activity, lymphoproliferative response to Con A, and the number of pulmonary metastases per animal continuously treated with LS 2616 (160 mg/kg/day) from day 4 before i.v. injection of 10⁵ B16-F10 cells to termination of the experiment 14 days after tumor cell injection. Some animals were also given an i.p. injection of antibodies to aGMI at the start of LS 2616 treatment and later every fourth day. Results from a single experiment with 10 mice/group are shown.

Table 1 Therapeutic effect of LS 2616 on established i.v. induced pulmonary metastases

C57BL/6 mice were given LS 2616 (160 mg/kg/day) in the drinking water or ordinary drinking water only (control) from day 4 after i.v. injection of 10⁵ B16-F10 cells. In one experiment the animals were also given an i.p. injection of aGMI at day 4 after tumor cell injection and later every fourth day. The number of lung colonies was determined at day 14.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of metastases/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27</td>
</tr>
<tr>
<td>LS 2616</td>
<td>26</td>
</tr>
<tr>
<td>Control + aGMI</td>
<td>15</td>
</tr>
<tr>
<td>LS 2616 + aGMI</td>
<td>15</td>
</tr>
</tbody>
</table>

No. Mean Range
19-150 15-70 34-6 6-72

* Statistically different from control animals, at \( P < 0.05 \).

Table 2 Prophylactic effect of LS 2616 on spontaneous pulmonary metastases

C57BL/6 mice were given LS 2616 (160 mg/kg/day) in the drinking water or ordinary drinking water only (control) from the time of inoculation of 5 x 10⁵ B16-F10 cells in the footpad. Some animals were also given an i.p. injection of antibodies to aGMI at the time of tumor inoculation and later every fourth day. The number of lung colonies was determined at day 24.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of metastases/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
</tr>
<tr>
<td>LS 2616</td>
<td>14</td>
</tr>
<tr>
<td>aGMI</td>
<td>13</td>
</tr>
<tr>
<td>LS 2616 + aGMI</td>
<td>13</td>
</tr>
</tbody>
</table>

No. Mean Range
4-6 0-23 2-73

* Statistically different from control animals, at \( P < 0.05 \).

Table 3 Therapeutic effect of LS 2616 and cyclophosphamide on established pulmonary metastases

C57BL/6 mice were inoculated in the footpad with 5 x 10⁵ B16-F10 cells which were allowed to grow for 24 days. The primary tumor was then resected, and treatment of the established metastases was started. Animals were given LS 2616 (160 mg/kg/day) in the drinking water continuously from day 24 until the termination of the experiment at day 38, cyclophosphamide (50 mg/kg every fourth day) i.p., antibodies to aGMI every fourth day, or combinations of these treatments. Control animals were given vehicles according to similar schedules. The number of lung colonies as well as NK activity (effectoctumor cell ratio, 100:1) was determined at day 38.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of metastases/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
</tr>
<tr>
<td>LS 2616</td>
<td>26</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>16</td>
</tr>
<tr>
<td>Control + aGMI</td>
<td>15</td>
</tr>
<tr>
<td>LS 2616 + aGMI</td>
<td>15</td>
</tr>
<tr>
<td>Control + cyclophosphamide + aGMI</td>
<td>11</td>
</tr>
<tr>
<td>LS 2616 + cyclophosphamide + aGMI</td>
<td>10</td>
</tr>
</tbody>
</table>

No. Mean Range
0-51 0-32 0-29 1-54 0-35 2-25 0-18 0-20

* Statistically different from control animals, at \( P < 0.05 \).

** Treatment with antibodies to aGMI which almost completely eliminated NK cell activity in vivo, indicated that NK cells were of major importance for the development of pulmonary metastases but that their role was mainly limited to the period before extravasal establishment of the tumor cells. The importance of NK cells for the development of pulmonary metastases from NK cell sensitive tumors evident from this study is in accordance with recent reports applying similar depletion techniques or adoptive transfer of NK cell containing cell populations (13–15). Removal of aGMI bearing cells has been found to be one of the most efficient ways of removal of NK cell activity both in vitro and in various organs in vivo (10, 16). Of particular importance for studies on the growth of pulmonary metastases is the observation that alveolar NK cells are also inactivated by in vivo administered aGMI antibodies (17). While precursors of cytotoxic T-lymphocytes as well as some macrophages express a low amount of membrane aGMI, NK cells can apparently be quantitatively removed without affecting these cell populations (Ref. 16; this paper). Our data indicate that NK cells are able to effectively counteract metastatic development before the tumor cells invade the tissue only. Since high NK cell activity normally is present in the interstitial tissue of the lungs (18), local depression of NK cell activity by tumor cells is the most likely explanation of the inability of NK cells to inhibit tumor growth in the tissue. Such depression of NK cell activity in tumor infiltrating lymphocytes has been described for human breast and pulmonary cancers as well as for murine tumors (19–21).

Talmadge et al. (17) recently found that organ specific enhancement of NK cells by muramyl tripeptide-phosphatidyethanolamine incorporated in liposomes was able to affect tumor cells after their extravasation. The enhancement of the antitumor effects of the treatment was sensitive to treatment with antibodies to aGMI. Thus host defense mechanisms rendered inactive by the tumor may nonetheless be important targets for immunoenhancing therapy.

LS 2616 apparently affected growth and metastasis of the B16-F10 tumor in a complex manner. No evidence has been found that LS 2616 acts directly on tumor cells as judged by testing of several tumors, including B16-F10 in vitro.² Abolishment of the effect of LS 2616 by treatment with antibodies to aGMI clearly indicated that the antitumor effects of the substance were related to its immunomodulating properties. Evidence has been presented that LS 2616 in contrast to most earlier reported agents that increase NK cell activity acts in an interferon independent manner (6). Such a mechanism may be of vital importance for the therapeutic efficacy of LS 2616 mediated by NK cells since it does not evoke the well docu-
mented protective effect of interferon on NK target cells (22). However, the therapeutic effects of LS 2616 toward already established pulmonary metastases which are resistant to treatment with antibodies to αGM1 indicate immunomodulating effects in addition to enhancement of NK cell activity. The immunological effects of LS 2616 are only partially characterized. The substance increased delayed hypersensitivity responses and augmented the lymphoproliferative response to T-cell mitogens and alloantigens (8, 9). Recently, we have been able to show that LS 2616 enhanced the production of IL-2 of rat spleen lymphocytes stimulated with Con A. Moreover, the enhanced proliferative response of spleen cells from LS 2616 treated compared to control mice to Con A was equaled by addition of excess exogenous IL-2 to the cultures. These observations suggest that LS 2616 in addition to stimulation of T-lymphocytes (23) in support of this assumption is the observation that injection of rabbit anti-thymocyte globulin eliminated the therapeutic effect of LS 2616 towards dimethylbenzanthracene induced rat tumors. The most relevant model for testing of the possible clinical value of LS 2616 is the effect on established spontaneous metastases after resection of the primary tumor. In this situation, neither LS 2616 nor cyclophosphamide in the doses tested was able to significantly reduce the number of pulmonary metastases. However, a 50% reduction in the number of detectable lung colonies was found in independent experiments. A basic technical problem in these experiments was that not all animals developed metastases even in the control group, thereby reducing the sensitivity of the method. However, combined treatment with cyclophosphamide and LS 2616 significantly reduced the number of lung colonies after 2 weeks of treatment. In addition to an obvious direct cytoreductive effect on tumors, cyclophosphamide has a preferential effect on suppressor T-cells (24). Several studies have shown that cyclophosphamide may abrogate T-cell suppressor mechanisms thereby allowing expression of antitumor immunity by adoptively transferred T-lymphocytes or in conjunction with immunization with tumor associated transplantation antigens (25–27). Thus while cyclophosphamide either alone or combined with LS 2616 strongly reduced NK cell activity, T-cell mediated responses induced by LS 2616 could presumably be promoted by a simultaneous elimination of suppressor T-cells otherwise down regulating the response. Direct studies on the role of T-cell mediated immunity in LS 2616 treated tumor bearing mice are presently being pursued in our laboratory.

The mechanism of action of LS 2616 on the growth and metastasis of the B16-F10 tumor remains only partially understood. An effective host defense to metastasizing tumor cells in the intravasal stage evoked by LS 2616 appears to be related to NK cells, while the immune response to established tumors are dependent on non-NK cell effector mechanisms. It can be concluded, however, that LS 2616 has been shown to be a promising immunomodulator for use in experimental tumor therapy with significant effects on various aspects of tumor development deserving further studies.

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ACKNOWLEDGMENTS

The excellent technical assistance of Ingegerd Andersson is gratefully acknowledged.

T. Stålhandes, personal communication.

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