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# GENERATION AND CONTROL OF METASTASIS IN EXPERIMENTAL TUMOR SYSTEMS; INHIBITION OF EXPERIMENTAL METASTASES BY A TILORONE ANALOGUE

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**The role of the chemical compound RMI 10,874DA (3,6-bis[2-(dimethylamino)-ethoxyl]-9H-xanthene-9-one dihydrochloride) in the abrogation of the metastatic spread of tumor cells was studied. Pre-treatment of BALB/c mice with the RMI 10,874DA compound (referred to below as tilorone analogue) completely eliminated lung colonization of an H-2-negative (GR9.B9) MCA-induced fibrosarcoma clone in an experimental metastasis assay. Other murine tumors, including H-2-positive and H-2-negative chemically induced fibrosarcoma clones and B16 melanoma, were also sensitive to the treatment; orally administered tilorone analogue given one day before the i.v. injection of tumor cells markedly inhibited lung colonization. The effect was not due to direct toxicity of tilorone analogue on tumor cells, but instead it was dependent on NK cells; this was suggested by the finding that anti-asialo $GM_1$ treatment of mice abrogated the effect of tilorone analogue. Kinetic studies of splenic NK activity in tilorone-treated mice showed a rapid boosting of NK-cell activity, the greatest stimulation occurring the day before removal of splenocytes for $^{51}Cr$-release assay against YAC-1 target cells. These kinetics correlated with the inhibition of *in vivo* lung colonization after tilorone analogue treatment. Inhibition of experimental tumor metastasis was dose-dependent and was observed when animals were treated the day before or the day after tumor-cell injection. Furthermore, repeated treatment of mice with this tilorone analogue significantly reduced lung colonization.**


Metastasis formation is a highly selective multi-step process in which tumor-cell dissemination is dependent on immunological as well as non-immunological factors. To establish metastases, tumor cells must complete all steps in the metastatic process: invasion, embolization, survival in the circulation, arrest in a distant capillary bed, extravasation into organ parenchyma, and multiplication and development into secondary foci (Schirrmacher, 1985; Poste and Fidler, 1980). Little is known about the exact mechanism that leads to the destruction of most circulating tumor cells, but interruption of the sequence at any of these stages may prevent the production of clinical, visible metastasis.

The development of therapeutic modalities leading to either prevention or elimination of tumor metastasis is currently one of the principal challenges facing oncologists. A large body of work in experimental models supports the importance of the following parameters in the outcome of immunological intervention: (i) immunogenicity of the tumor, a factor influenced by the expression of major histocompatibility complex (MHC) antigens on the tumor cells (Ruiz-Cabello *et al.,* 1991; Garrido and Klein, 1991); (ii) the size and location of the tumor (Urban *et al.,* 1982); (iii) heterogeneity of the tumor-cell population (Fidler and Kripke, 1977); and (iv) the immunocompetent status of the host, *i.e.* its ability to mount cell-mediated responses (Kedar and Klein, 1993). The administration of various naturally occurring or synthetic biological response modifiers (BRMs) has been reported to enhance host immune activity and, in combination with systemic transfer of immune cell populations, to elicit anti-tumor activity.

Quite early in NK-cell research it became clear that the injection of several different agents into mice, including bacterial and viral products, mitogens and tumor cells, rapidly boosted NK-cell activity. Individual mouse strains vary with regard to NK activity, some being strong and some being weak reactors (Herberman *et al.,* 1975; Kiessling *et al.,* 1975). Several of the agents mentioned above are also known to increase the resistance of mice to transplantable tumors (Gorelik *et al.,* 1982; Wiltrout *et al.,* 1985; Barlozzari *et al.,* 1985; Riccardi *et al.,* 1986).

Tilorone hydrochloride has been shown to enhance NK activity in different animal models (Gidlund *et al.,* 1978). This was first discovered by Krueger and Mayer, who found in the late 1960s that this orally active anti-viral agent was capable of modulating the immune system, and also possessed a potent anti-inflammatory activity (Krueger and Mayer, 1970; Mayer and Krueger, 1970, 1980). Anti-viral activity in mice and NK activity has been ascribed to interferon induction (Welsh, 1984; Megel and Gibson, 1984). More than 800 compounds were synthesized in the tilorone series during the search for orally active antiviral and immunoregulatory compounds. Anti-tumor effectiveness of tilorone and several analogues has been tested in rodents against spontaneous, chemically induced and virally induced tumors as measured by survival time and tumor growth (Mayer and Krueger, 1980; Albrecht, 1977; Munson *et al.,* 1972; Wampler and Regelson, 1977). Gidlund *et al.* (1978) described several tilorone analogues with different capacities to boost NK activity in mice. However, the effect of this compound on the inhibition of metastasis has not been studied.

We investigated the biological effect of the RMI 10,874DA compound (3,6-bis[2-(dimethylamino)-ethoxyl]-9H-xanthene-9-one dihydrochloride) on the prevention of metastases in experimental tumor systems. The model systems analyzed have been described in relation to clonal heterogeneity, H-2 antigen expression and the biological behavior of individual tumor clones (Garrido *et al.,* 1986; Pérez *et al.,* 1985, 1990; Algarra *et al.,* 1989, 1991; Gaforio *et al.,* 1991).

## MATERIAL AND METHODS

### *Animals*

Four-week-old BALB/c mice were obtained from the Animal Center of our Institution. C57Bl/6 mice aged 4–6 weeks were supplied by the Animal Center of the Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden. The average weight of the mice used was 20 g.

### *Cell lines*

Tumor cells included GR9 ($H\text{-}2^d$), a chemically induced fibrosarcoma produced and characterized in our laboratory (Garrido *et al.,* 1986; Pérez *et al.,* 1985), and the following GR9 tumor clones: GR9.B9 ($K^d$, $D^d$, $L^d$-negative) (Garrido *et al.,*

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1986; Pérez *et al.,* 1985; Algarra *et al.,* 1989; Gaforio *et al.,* 1991), B7.1.B5 ($K^d$, $D^d$, $L^d$-negative); B7.1.B4 and B7.2.38 ($K^d$, $D^d$, $L^d$-positive) (Algarra *et al.,* 1991). B16 ($K^b$, $D^b$ low) melanoma was also used. For *in vitro* NK experiments, YAC-1 lymphoma cells were used as the target.

In vivo *treatment with tilorone and NK-cell depletion with anti-asialo $GM_1$*

Different tilorone analogues [named according to Richardson-Merrell international numbers (Albrecht, 1977)] were used: RMI 10,874DA. Cat. T-8014; R11,513DA. Cat. T-7639; R11,877DA. Cat. T-7264 and R11,567DA. Cat. T-7514, Sigma, St. Louis, MO. Unless noted otherwise mice were treated orally by cannula with RMI 10,874DA compound (100 mg/kg) dissolved in 200 μl water. This compound will be referred to below as tilorone. Control mice received water without tilorone. Treatment with Asialo $GM_1$ anti-serum (Wako, Osaka, Japan) was performed with an i.p. injection on day −1 with 200 μl of a 1/50 dilution. For kinetics experiments, the mice were treated on days −6, −3, −1, 0, +3, +6 and +10. In another set of experiments, treatment starting on day +6 was repeated on day +9 or at days +9, +12 and +15.

In vitro *NK cytotoxicity assays*

Spleen cells from tilorone-treated BALB/c mice were used as effector cells. A standard 4- to 6-hr $^{51}Cr$-release assay was used for *in vitro* NK experiments against YAC-1 lymphoma cells (for details see Algarra *et al.,* 1989). Mice were treated with tilorone on days −21, −14, −7, −3 and −1 before the *in vitro* cytotoxicity assays. To obtain repeated peaks of NK activity, treatments were repeated on day −1 in all cases.

*Lung colonization of radiolabelled tumor cells*

$^{51}Cr$-radiolabelled B9 cells ($0.3 \times 10^6$) were inoculated into the tail vein of normal BALB/c mice which had been treated with either asialo $GM_1$ antiserum or tilorone the previous day. A rabbit anti-mouse Ig was used as a negative control with no effect (Mule *et al.,* 1987). The lungs were excised 20 hr after injection and radioactivity was determined in an LKB gamma counter.

*Experimental metastasis assays*

Tumor cells were injected i.v. into the tail vein of C57Bl/6 and BALB/c mice. The animals were killed between days 15 and 30, depending on the tumor-cell line inoculated, and lung colonies were counted macroscopically after fixation of the tissue in Bouin solution (for details see Pérez *et al.,* 1990).

## RESULTS

*Effect of a tilorone analogue (RMI 10,874DA) and anti-asialo GM1 treatment on lung colonization of different tumor cells*

The effect of tilorone was initially detected when the tilorone analogue (RMI 10,874DA) was administered orally 1 day before the injection of tumor cells. Table I shows the number of lung colonies obtained in control animals and in animals treated with tilorone (100 mg/kg) on day −1. The tumor cells used were GR9, a chemically induced fibrosarcoma, and GR9.B9, a previously characterized H-2 negative clone of GR9. Three independent clones derived from chemically induced sarcomas were also tested; B7.1.B5 (H-2-negative), B7.1.B4 (H-2-positive) and B7.2.38 (H-2-positive); in addition, we assayed the well-characterized B16 melanoma. Control animals usually contained large numbers of lung colonies, depending on the tumor and the number of cells inoculated. In contrast, mice treated with the tilorone analogue had few colonies or none at all, depending on the number of cells injected in the same experimental model. Anti-asialo $GM_1$ treatment of mice increased the number of lung colonies, in some cases more than 4-fold, compared with control animals (Table I).

*Dose response of the tilorone analogues on i.v. metastasis of the GR9.B9 clone*

Figure 1 shows the results obtained for lung colonization of clone GR9.B9 after treatment of BALB/c mice with different doses of RMI 10,874DA compound. The minimum dose of tilorone that totally inhibited lung colonization was 100 mg/kg/mouse, with $5 \times 10^5$ GR9.B9 cells. A dose of 50 mg/kg was also effective in preventing lung colonization, although some colonies were found in individual mice. Lower doses were not effective in controlling metastases. Other tilorone analogues (R11,513DA; R11,567DA and R11,877DA) were used. Nevertheless, the results obtained were not efficient for prevention of metastasis (data not shown).

*Kinetics of tilorone treatment on i.v. lung colonization of GR9.B9 clone*

To examine whether the effect of treatment was persistent, groups of BALB/c mice were given the tilorone analogue (RMI 10,874DA compound) (100 mg/kg) from day −6 to day +10 (Fig. 2). It was evident that the effect on lung colonization was transient, since mice treated on day −6 before i.v. inoculation of tumor cells, or on day +10 after i.v. inoculation, developed similar numbers of colonies to those observed in the control animals. However, when mice were given tilorone on day −1, day 0, or day +1, virtually no colonies were observed when the GR9.B9 fibrosarcoma clone was used.

Animals treated with a single dose of tilorone on days −1, 0 or +1 (Fig. 2) survived without evidence of disease.

*Activity of tilorone analogue on established lung colonization*

Table II shows that a single dose of RMI 10,874DA compound on day +6 significantly reduced lung metastases in BALB/c mice after the injection of GR9.B9 tumor cells, in comparison to control mice. Repeated doses on day +9 or days +9, +12 and +15 had a clear curative effect in terms of number of lung colonies.

*Time-course of splenic NK activity in tilorone-treated mice*

Tilorone analogues have previously been used to boost NK activity (Gidlund *et al.,* 1978). In the present study, the animals also expressed enhanced NK activity in an *in vitro* $^{51}Cr$ release assay using YAC-1 lymphoma as the target cell (Fig. 3). Furthermore, the time-course of this enhancement paralleled the *in vivo* effects on reduction of lung colonization. Those animals treated with a single dose of tilorone analogue (100 mg/kg) on days −3 or −1 showed the strongest NK activity. A second dose of tilorone on day −1 maintained high NK activity, suggesting that this effect could be used to prevent lung colonization for longer periods (Fig. 3, Table II).

*Lung clearance of radiolabelled GR9.B9 cells from control, tilorone-treated and anti-asialo $GM_1$-treated mice*

To investigate the role of NK cells, in relation to our observations, we tested the effect of asialo $GM_1$ antiserum treatment in mice treated with RMI 10,874DA compound. A 1/50 dilution of asialo $GM_1$ anti-serum was injected intraperitonealy (200 μl) into BALB/c mice. Anti-asialo $GM_1$ inhibited the clearance of $^{51}Cr$-labelled tumor cells from the lung. The combination of tilorone + anti-asialo $GM_1$ treatment abrogated the protective effect of tilorone (Fig. 4).

*Effect of NK depletion on tilorone activity*

Figure 5 shows that the anti-metastatic properties of tilorone were not due to the direct cytotoxicity of tilorone on tumor cells. NK-cell depletion of BALB/c mice, using a single i.p. injection of asialo $GM_1$ before (day −2) or after (day −1) tilorone treatment, led to higher numbers of lung colonies than in untreated control mice. Day −2 and day −1 refer to the injections of tumour cells on day 0.

**TABLE I** - EFFECT OF TILORONE TREATMENT IN CONTROLLING EXPERIMENTAL METASTASIS FORMATION

| Tumor | Cell dose | Treatment of hosts | Number of mice with metastasis out of total injected | Arithmetic mean (±SD) values for number of colonies | Number of pulmonary colonies in each mouse |
|---|---|---|---|---|---|
| GR9 (wild-type) | $10^5$ | Control | 2/4 | 1 ± 1 | (2, 2, 0, 0) |
| | | Tilorone-1 | 0/4 | 0 | (0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 4/4 | 9 ± 2 | (10, 5, 9, 12) |
| | $2.5 \times 10^5$ | Control | 4/4 | 3 ± 1 | (1, 6, 2, 4) |
| | | Tilorone-1 | 0/4 | 0 | (0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 4/4 | 40 ± 12 | (20, 40, 50, 53) |
| | $5 \times 10^5$ | Control | 5/5 | 34 ± 6 | (15, 25, 8, 50, 75) |
| | | Tilorone-1 | 2/4 | 2 ± 2 | (3, 0, 0, 5) |
| | | Anti-asialo $GM_1$ | 6/6 | 136 ± 10 | (130, 160, 150, 170, 110, 100) |
| GR9.B9 (H-2-) | $10^4$ | Control | 1/8 | 1 ± 1 | (0, 2, 0, 0, 0, 0, 0, 0) |
| | | Tilorone-1 | 0/8 | 0 | (0, 0, 0, 0, 0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 6/8 | 3 ± 2 | (0, 2, 2, 3, 6, 8, 7, 0) |
| | $10^5$ | Control | 8/8 | 19 ± 9 | (10, 10, 20, 35, 9, 11, 22, 18) |
| | | Tilorone-1 | 0/6 | 0 | (0, 0, 0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 4/4 | 89 ± 39 | (45, 90, 120, 100) |
| | $2.5 \times 10^5$ | Control | 6/6 | 38 ± 13 | (55, 41, 30, 25, 20, 22) |
| | | Tilorone-1 | 0/6 | 0 | (0, 0, 0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 4/4 | 100 ± 9 | (110, 105, 96, 89) |
| | $5 \times 10^5$ | Control | 6/6 | 105 ± 8 | (125, 98, 105, 98, 100, 105) |
| | | Tilorone-1 | 0/8 | 0 | (0, 0, 0, 0, 0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 6/6 | 130 ± 19 | (115, 105, 140, 160, 100, 100) |
| | $10^6$ | Control | 4/4 | > 150 | (161, 165, 145, 170) |
| | | Tilorone-1 | 0/6 | 0 | (0, 0, 0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 4/4 | > 200 | (160, 210, 200, 245) |
| B7.1.B5 (H-2-) | $10^5$ | Control | 4/4 | 4 ± 2 | (2, 3, 5, 7) |
| | | Tilorone-1 | 2/5 | 1 ± 1 | (1, 3, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 5/5 | 152 ± 14 | (150, 160, 170, 130, 150) |
| | $5 \times 10^5$ | Control | 5/5 | 190 ± 63 | (220, 250, 230, 100, 150) |
| | | Tilorone-1 | 5/5 | 6 ± 2 | (6, 9, 4, 4, 7) |
| | | Anti-asialo $GM_1$ | 5/5 | > 200 | (240, 250, 255, 280, 243) |
| | $10^6$ | Control | 6/6 | > 150 | (130, 140, 160, 168, 150, 150) |
| | | Tilorone-1 | 5/5 | 10 ± 3 | (2, 15, 3, 15, 16) |
| | | Anti-asialo $GM_1$ | 4/4 | 145 ± 3 | (150, 150, 150, 130) |
| B7.1.B4 (H-2+) | $10^5$ | Control | 5/5 | 23 ± 6 | (30, 20, 30, 16, 18) |
| | | Tilorone-1 | 4/4 | 5 ± 2 | (8, 6, 4, 3) |
| | | Anti-asialo $GM_1$ | 4/4 | 145 ± 46 | (140, 210, 130, 100) |
| B7.2.38 (H-2+) | $10^5$ | Control | 4/5 | 18 ± 23 | (1, 0, 3, 38, 50) |
| | | Tilorone-1 | 0/5 | 0 | (0, 0, 0, 0, 0) |
| | | Anti-asialo $GM_1$ | 4/4 | 100 ± 72 | (190, 70, 20, 120) |
| | $5 \times 10^5$ | Control | 5/5 | > 200 | (180, 210, 220, 200, 230) |
| | | Tilorone-1 | 3/5 | 1 ± 1 | (2, 0, 1, 0, 2) |
| | | Anti-asialo $GM_1$ | 6/6 | > 200 | (200, 180, 250, 250, 210, 150) |
| | $10^6$ | Control | 4/4 | > 200 | (200, 210, 200, 200) |
| | | Tilorone-1 | 4/4 | 38 ± 20 | (60, 84, 7, 4) |
| | | Anti-asialo $GM_1$ | 4/4 | > 200 | (200, 200, 200, 200) |
| B16 (wild-type) | $10^5$ | Control | 4/4 | 57 ± 13 | (70, 56, 40, 63) |
| | | Tilorone-1 | 4/4 | 13 ± 5 | (20, 8, 15, 9) |
| | | Anti-asialo $GM_1$ | 4/4 | > 200 | (200, 180, 250, 250, 210, 150) |

Tumor cells $10^4$, $10^5$, $2.5 \times 10^5$, $5 \times 10^5$ and $10^6$ were injected i.v. in syngeneic BALB/c or C57BL/6 mice in 500 µl of PBS. On the previous day mice were treated orally with 100 mg/kg (0.2 ml water) of tilorone analogue (RMI 10,874DA compound). Control mice received water without tilorone. Tumor cells included GR9 wild-type ($H-2^d$), a chemically induced fibrosarcoma, and the following tumor clones: GR9.B9 ($K^d$, $D^d$, $L^d$-negative), B7.1.B5 ($K^d$, $D^d$, $L^d$-negative); B7.1.B4 and B7.2.38 ($K^d$, $D^d$, $L^d$-positive). B16 ($K^b$, $D^b$ low) melanoma was also used. Asialo $GM_1$ anti-serum treatment was performed with an i.p. injection on day −1 of 200 µl of a 1/50 dilution. A rabbit anti-mouse Ig was used as negative control with no effect. Mice were killed between days 15 and 30, depending on the tumor and cell dose used, and lung colonies were counted macroscopically after fixation in Bouin solution. Duplicate experiments were performed in the different control and treated animals, with similar results.

## DISCUSSION

Cancer metastasis is one of the major clinical problems facing oncologists and modern medicine (Fidler and Kripke, 1977; Nicolson, 1987). Many attempts have been made to prevent and treat metastatic disease by enhancing the immune system with a variety of mechanisms (Gresser *et al.*, 1988; Goldstein and Laszlo, 1986; Shu and Rosenberg, 1985; Ettinghausen and Rosenberg, 1986). However, therapeutic approaches are complicated, and acceptable results have been achieved only in some tumor systems (Kedar and Klein, 1993).

Several recently published reviews of various aspects of cancer immunology and immunotherapy (Schirrmacher, 1985; Kedar and Klein, 1993) conclude that an immune response against the tumor is a pre-requisite for achieving a therapeutic

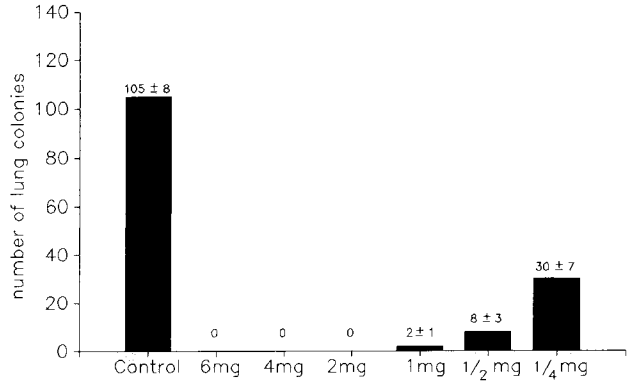


**FIGURE 1** – Dose response to tilorone treatment after i.v. injection of GR9.B9 tumor cells. BALB/c mice (5 animals per group) were treated on day −1 with a single dose per mouse of either 6 mg, 4 mg, 2 mg, 1 mg, 0.5 mg or 0.25 mg tilorone analogue (RMI 10,874DA) and lung colonies were counted macroscopically 30 days after the injection of $5 \times 10^5$ GR9.B9 cells. Mean and SD of 2 experiments is shown. $IC_{50}$: 0.140 mg.

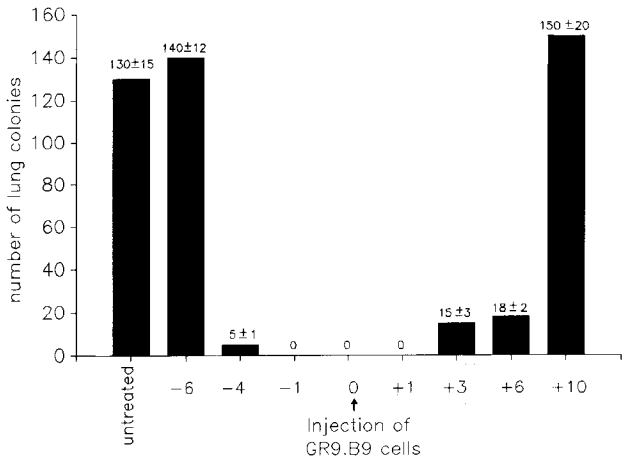


**FIGURE 2** – Kinetics of tilorone treatment on GR9.B9 i.v. metastasis. BALB/c mice (4 animals per group) were treated with a single dose of tilorone (RMI 10,874DA compound) (100 mg/kg/200 µl water) at different time points (days −6, −4, −1, 0, +1, +3, +6, +10), and $5 \times 10^5$ GR9.B9 fibrosarcoma tumor cells were injected i.v. into each animal on day 0. Control mice were injected with the same number of cells, but received no tilorone treatment. Mice were killed after 30 days, the lungs were fixed in Bouin solution, and lung colonies were counted macroscopically. Triplicate experiments were done, with similar results.

effect, and that many biological response modifiers (BRMs) act by improving a non-adoptive immunotherapeutic activity.

We have now shown that the oral administration of a single dose of a tilorone analogue prevents lung colonization and metastasis formation after the i.v. injection of tumor cells into mice. The effect was observed in a panel of tumor cells of different origins and MHC class-I expression in 2 strains of mice. At the dose used (100 mg/kg), the tilorone analogue was able to abolish the formation of experimental lung metastases by GR9.B9 tumor cells, resulting in no clinical evidence of disease in the mice. In the case of B16 melanoma, the inhibition of experimental metastasis formation was even more efficient in other organs than in the lungs of C57Bl/6 mice, a finding that will be investigated further.

**TABLE II** – INHIBITION OF ESTABLISHED METASTASIS AFTER TILORONE TREATMENT

| Treatment[1] of mice | Number of metastases[2] mean ± SD |
|---|---|
| Untreated | 103 ± 12 |
| Tilorone (−1) | 0 |
| Tilorone (+6) | 23 ± 5 |
| Tilorone (+6, +9) | 2 ± 1 |
| Tilorone (+6, +9, +12, +15) | 1 ± 1 |

[1]BALB/c mice were treated with a single dose of tilorone analogue at the indicated days.–[2]Number of lung colonies. BALB/c mice (5 animals per group) received a single dose (100 mg/kg) of tilorone analogue (RMI 10,874DA compound) on day −1 and on day +6, and were injected with $5 \times 10^5$ GR9.B9 tumor cells. Repeated treatment was given in 2 independent groups of animals on days +6 and +9 and on days +6, +9, +12 and +15. Thirty days later lung metastases were counted macroscopically. Results from 3 independent experiments are shown.

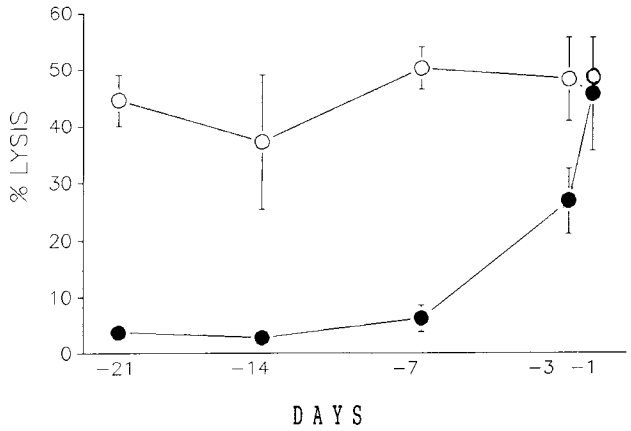


**FIGURE 3** – Tilorone enhancement of NK activity. Mice were treated with a single dose of tilorone analogue (100 mg/kg/200 µl water) on days −21, −14, −7, −3, and −1 (●-●) and this treatment was repeated in all cases on day −1 (○-○) before the spleen cells were tested for NK activity in an *in vitro* 4- to 6-hr $^{51}$Cr-release assay against YAC-1 lymphoma cells. Enhancement of NK activity was observed when a single treatment was performed between days −3 and −1 (●-●). When the treatment was repeated on day −1 (○-○), NK activity reach the highest levels, as with treatment on day −1 alone. E/T ratio was 100:1. Mean ± SD of 2 independent experiments.

The prevention of metastases was not due to a toxic effect of the agent as such on the tumor cells (Megel and Gibson, 1984); anti-asialo $GM_1$ treatment of the animals prior to tilorone administration led to higher numbers of lung colonies than in untreated control animals (Fig. 5). Lung clearance of GR9.B9 cells was much more efficient in tilorone-treated than in anti-asialo $GM_1$-treated mice, whereas a combination of both treatments had no effect on GR9.B9 clearance from the lung.

The greatest effect of tilorone treatment was seen between days −1 and +1. A significant reduction in metastasis was also seen when treatment was given 3 days after tumor-cell injection. Our findings show that continuous treatment with 2 or 4 doses of tilorone, starting on day +6, induced a significant reduction in macroscopically visible metastases (Table II).

Since NK cells have been implicated as an anti-tumor defense mechanism (Gorelik *et al.*, 1982; Wiltrout *et al.*, 1985; Barlozzari *et al.*, 1985; Hanna, 1982) and tilorone was reported to be a potent modulator of *in vitro* NK activity (Gidlund *et al.*, 1978), we examined whether the immunomodulation induced

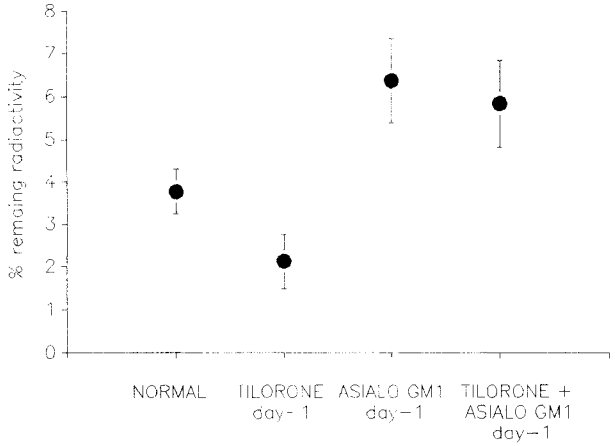


**FIGURE 4** – Depletion of NK cells abolished the effect of tilorone. The lung clearance of $3 \times 10^5$ $^{51}$Cr-labelled GR9.B9 cells from i.v. injected BALB/c mice was studied. Five mice per group were used, and radioactivity remaining in the lung was determined 20 hr after tumor-cell injection. Animals were treated as indicated on the abscissa. Injection of a rabbit anti-mouse Ig was used as a negative control with no effect. Mean ± SD from 3 independent experiments.

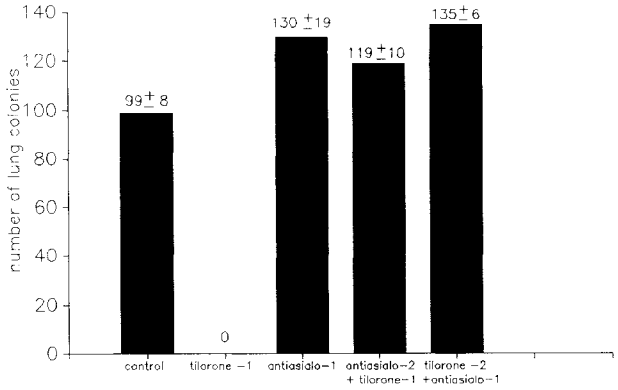


**FIGURE 5** – Effect of NK-cell depletion on tilorone activity. Different groups of BALB/c mice were treated as follows: (I) tilorone (100 mg/kg) on day −1; (II) anti-asialo $GM_1$ on day −1 (200 µl i.p. 1/50 dilution); (III) anti-asialo $GM_1$ on day −2 + tilorone on day −1; (IV) tilorone on day −2 + anti-asialo $GM_1$ on day −1. In the last 2 cases tilorone and antiasialo $GM_1$ concentrations were the same as in the first 2. GR9.B9 cells ($5 \times 10^5$) were injected i.v. and lung colonies counted macroscopically after 30 days. Means ± SD of 2 independent experiments.

by the RMI 10,874DA compound in mice results in any effect on experimentally induced pulmonary metastases in different murine model systems.

Few products have been reported to be capable of reducing the number of metastatic colonies or of partially preventing lung colonization (Humphries *et al.*, 1988; Nakatsuka *et al.*, 1991; Matsuzaki and Yokokura, 1987; Talmadge *et al.*, 1987). *Corynebacterium parvum* and an indolizidine alkaloid (Swainsonine) have been shown to affect experimental metastasis (Humphries *et al.*, 1988; Nakatsuka *et al.*, 1991).

The anti-tumoral effectiveness of several tilorone analogues, as measured by survival time and tumor growth (Megel and Gibson, 1984; Albrecht, 1977; Munson *et al.*, 1972; Wampler and Regelson, 1977; Roye *et al.*, 1971; Adamson, 1971; Pearson *et al.*, 1974; Wampler *et al.*, 1972), has been tested against spontaneous, chemically induced, and some virally induced tumors in rodents. We now report the successful abrogation of experimental metastasis formation.

In summary, the oral administration of a single dose of tilorone analogue is able to inhibit experimental metastases in tumoral systems (Algarra *et al.*, 1992). Preliminary data in a spontaneous metastasis assay model show that tilorone is also able to completely prevent the formation of metastases from different organs when wild-type GR9 tumor cells are injected (data not shown). It remains to be unequivocally established whether tilorone analogues or similar substances are also effective in preventing tumor metastases in other mammals, including humans.

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