

AN EXPERIMENTAL STUDY ON THE LYMPHATIC DISSEMINATION OF THE SOLID EHRlich TUMOR IN MICE

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DAGLI, M.L.Z.; GUERRA, J.L.; SALDIVA, P.H.N. An experimental study on the lymphatic dissemination of the solid Ehrlich tumor in mice. *Braz. J. vet. Res. anim. Sci.*, São Paulo, v.29, n.1, p.97-103, 1992.

SUMMARY: Metastasis to regional lymph nodes is an important step in the dissemination of cancer. The mechanisms of this dissemination are poorly understood, probably due to the paucity of authentic experimental models. The aim of the present study was to characterize the Ehrlich tumor as a model of lymphatic metastasis in mice. Animals were inoculated into the footpad and the popliteal lymph nodes were collected at several times post-inoculation in order to evaluate their weight, histopathological aspects, and mast cells quantitation, since these cells are reported to be implicated in host's response to tumor. The migration of tumor cells was detected by a biological assay as early as 1 hour post-inoculation. The solid tumor growth in the footpad was measured until day 30 post-inoculation and the histological alterations during this period were also studied. Ehrlich tumor was considered as a suitable model for the study of lymphatic metastasis.

UNITERMS: Lymphatic metastasis; Carcinoma, Ehrlich tumor; Lymph nodes; Mast cells; Mouse

INTRODUCTION

In many human and animal cancers, metastasis to regional lymph nodes is an important step in the dissemination of the disease. Yet, its mechanisms are

not thoroughly understood. A major cause of this has been the lack of suitable experimental animal models²⁶.

Metastases, and particularly lymphatic metastasis, are reported to occur infrequently with transplanted animal tumors^{8,25,27}. Many models have been used, but most of them were performed in rats^{8,17,23,25,27,28} or rabbits¹⁶ and few of them in mice^{12,13}.

The Ehrlich tumor, that grows in mice as an ascitic tumor²⁰, presents a solid phase when inoculated subcutaneously¹. Lymphatic and hematogenous metastases of this tumor are reported^{18,24}, but no experimental studies on this subject have been published.

The aim of this study was to characterize the Ehrlich tumor as a model of lymphatic metastasis in mice. The timing and patterns of growth of this tumor in footpad and in regional popliteal lymph nodes are described.

MATERIAL AND METHOD

Animals: Male Swiss mice weighing 25-35 g, fed with balanced diet and water "ad libitum", were used.

Transplantable tumor: The Ehrlich tumor is kept in our laboratory by serial intraperitoneal transplantation in mice, performed at 10 days intervals. Approximately 3 ml of ascitic fluid were collected from a donor mouse and centrifuged at 1000 g for 3 minutes. The cells in the pellet were washed three times in phosphate buffered saline (PBS), resuspended in the same solution and counted in order to standardize a concentration of 5×10^7 viable tumor cells/ml (by using Trypan blue viability test). To obtain a solid tumor, 0.05 ml of this suspension, containing 2.5×10^6 cells were carefully inoculated into the left footpad of mice.

Experimental protocols: To the following experiments, 45 mice were inoculated into the footpad as described above. Eighteen mice received the same amount of PBS solution into the footpad as controls.

a) **Quantitation of footpad tumor and popliteal lymph nodes growth.** The thickness of the footpad was measured daily with a Moore and Wright micrometer until the 30th post-inoculation day. Five popliteal lymph nodes were excised after sacrifice of mice, and weighed in analytical balance at 1 hour and on days 7,15 and 30 post-inoculation.

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- b) *Histology*. Five mice that received tumor cells and two controls were sacrificed by prolonged exposition to ether at 1, 4, 6, 12, 24 hours and on days 2, 7, 15 and 30 post-inoculation. Their left footpad and the ipsilateral popliteal lymph nodes were collected, fixed in 10% buffered formalin for at least 24 hours, and conventionally embedded in paraffin. The five micrometers sections were stained in Hematoxilin and Eosin (H.E.) for histological examination.
- c) *Mast cells counting in lymph nodes*. Five micrometers semi-serial sections were obtained from the whole lymph nodes collected at 1 hour and on days 7, 15 and 30 post-inoculation. These were treated with the Gomori's method¹⁴ that stains the mast cells granules in red. Those cells were localized and all of them were counted by scanning the whole lymph nodes sections.
- d) *Detection of tumor cells in lymph nodes*. The presence of tumor cells in lymph nodes histological preparations could not be surely evidenced until day 7 post-inoculation into the footpad. So a bioassay was performed, in which mice were inoculated into the footpad as described earlier: at 1, 6, 12, 24 hours and on days 2, 3, 4, 5, 6 and 7 post-inoculation their popliteal lymph nodes were collected and implanted subcutaneously into the dorsal region of receptor mice. After 15 days, these were sacrificed and examined grossly and histologically for the presence of a solid Ehrlich tumor in the site of the implants.
- e) *Statistics*. Student's T test was employed for comparisons between tumor and control groups ($p < 0.05$).

RESULTS

All the mice inoculated into the footpad, except controls, developed solid tumors.

- a) *Quantitation of the footpads and popliteal lymph nodes* - The thickness of the footpads until day 30 post-inoculation are shown in Fig. 1. Significant differences ($p < 0.05$) from controls were obtained from the 3rd day post-inoculation until the end of the experiment. The growth curve presented an exponential behaviour since that time.

The weights of popliteal lymph nodes are presented in Fig. 2. The Student's T test revealed significant differences ($p < 0.05$) between tumor and

control groups in all the times studied. On day 30, popliteal lymph nodes of tumor inoculated mice were 19.5 times heavier than controls.

- b) *Histological examination of footpads* - An inflammatory infiltrate composed mostly by polymorphonuclear neutrophils was observed until 12 hours post-inoculation, when some mononuclear cells could also be seen. The rate of polymorphonuclear/mononuclear cells was comparable at 24 hours and at days 2, 7 and 15 post-inoculation. An inflammatory infiltrate was observed around and within the growing dermic and subcutaneous tumor mass. At day 30, tumor cells invaded intensely the superficial dermis and, in some sections, ulcerations could be seen. The cells were extremely pleomorphic, highly anaplastic, with many mitotic figures, some of them being atypical. Necrosis occurred in extensive areas, and the survivor cells were localized mostly around blood vessels. The inflammatory infiltrate was composed mainly by polymorphonuclear cells.
- c) *Histological examination of lymph nodes* - No histological alterations were found in control lymph nodes. All of them showed typical lymphoid follicles with germinal centers.

In tumor-inoculated animals, lymph nodes alterations began at 6 hours post-inoculation, with the development of germinal centers in lymphoid follicles and a hypercellularity in both subcapsular and paracortical areas.

Tumor cells in this period were not surely seen within lymph nodes. At 12, 24 hours and on day 2 post-inoculation an exacerbation of the above picture could be seen. On day 7 post-inoculation, the lymphoid follicles were greatly hyperplastic. Germinal centers presented various mitotic figures and many tingible body macrophages. Hyperplasia of the paracortical and inter-follicular regions was also observed. Many cells resembling neoplastic ones, sometimes presenting mitotic figures, could be seen in subcapsular and in inter-follicular regions. In the medullary region the medullary cords presented hyperplasia. On day 15 post-inoculation, the above alterations persisted; some cells resembling plasma cells were found in paracortical and in inter-follicular areas. In the medullary sinuses, a hypercellularity was also observed. In two lymph nodes tumor cells foci were detected in the subcapsular region. On day 30 post-inoculation, there was an exacerbation of the above picture. Tumor cells could be seen in the subcapsular region, single or in clumps, and many of them were multinucleated. Many small neoplastic foci were observed as if growing from the subcapsular space, and

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an exuberant tumor mass distorting completely its architecture was seen in one among five lymph nodes.

d) *Mast cells counts in lymph nodes* - Mast cells counts in lymph nodes are presented in Tab. 1. No statistical differences were obtained in total number of mast cells between control and tumor-inoculated groups. In some lymph nodes no mast cells were found, while in others there were plenty of them.

e) *Detection of tumor cells in transplanted lymph nodes* - The results of the bioassay to detect the presence of tumor cells in lymph nodes at different times after inoculation are showed in Tab. 2. From one hour until day 7 post-inoculation tumor cells were detected in lymph nodes.

DISCUSSION

The Ehrlich tumor is widely used for experimentation and may be considered a suitable model for the study of lymphatic metastasis, according to the criteria defined by VAN DE VELDE and CARR²⁶ (1977): it is reproducible, tumor dose is measurable, and its natural spreading avoids the direct intralymphatic inoculation. Its growth characteristics in both ascitic and solid forms represent an advantage for the use of this tumor.

The footpad's tumor growth curve is exponential until day 28 post-inoculation, with a tendency to behave thereafter as a plateau until day 30, corresponding to various naturally occurring or experimental tumors^{19,21}.

Tumor cells evoked an inflammatory response in the footpad that was similar to control groups until 48 hours post-inoculation, suggesting an influence of mechanical trauma (inoculation). The persistence of an inflammatory response after this period may be due to necrosis. These results are similar to those obtained by HARTVEIT and HALLERAKER¹⁵ (1971) with the same tumor.

Tumor cells were detected in popliteal lymph nodes since the first hour post-inoculation in the footpad. At the beginning, this migration could be due to the opening of the lymphatic junctions promoted by oedema²⁶. Other authors detected tumor cells in lymph nodes at more advanced times in experimental models of lymphatic metastasis performed in rats^{4,5,6}. The bioassay to detect tumor cells in regional lymph nodes revealed to be an useful method, and despite this, it is rarely employed in the study of metastasis. It can be used with many transplantable tumors, to detect tumor cells in tissues supposed to contain them.

Popliteal lymph nodes enlarged progressively until day 30 post-inoculation; this was mostly due to the hyperplastic response histologically evidenced as earlier as 6 hours post-inoculation. The follicular hyperplasia, with the development of germinal centers, is associated with a B lymphocyte response⁷. The paracortical and interfollicular (T-dependent areas) hyperplasia may be related to the development of a delayed hypersensitivity²². These histological findings are compatible with other studies using various experimental tumors^{4,5,9}, but the observable alterations in those experiments began at different times. The cells that participate of the immune response to these tumor cells should be typified with the aid of immunohistochemical methods.

Regional lymph nodes are related to be the organs that initially recognize antigens from the primary tumor²⁹. The immunologic role of this response in the tumor growth is, however, controversial. Lymph nodes are not considered true barriers for tumor cells¹², and these can be found migrating by lymphatic⁸ or hematogenous routes²⁷ after leaving these lymphoid organs. Even a direct communication between lymphatic and blood vessels was described¹¹.

In our experiments, Ehrlich tumor cells determined few metastatic foci in lymph nodes. The causes of this are unknown, but a preference of some tumor cells for migrating by lymphatics and establish metastatic foci in lymph nodes is reported in the literature^{10,30}.

Mast cells have been shown to participate in the host response to tumor cells³. They were found in greater numbers in draining lymph nodes of women with mammary cancer that experimented a better prognosis². In our study, no conclusive findings were obtained. The great standard deviations observed in the groups may be related to the heterogeneity of these cells in lymph nodes, even in normal animals. It should be noticed that in the above mentioned clinical studies, based on the evaluation of single (and not serial) histological sections, high standard deviations were also obtained.

It can be concluded that Ehrlich tumor may be used as a model for studying lymphatic dissemination in neoplasia, and further studies should be encouraged in order to answer some intriguing questions on this subject.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Ferruccio Betti for technical informations about mast cells quantitation and to Claudio Arroyo and Paulo Sergio de Souza for preparing the histological materials.

DAGLI, M.L.Z.; GUERRA, J.L.; SALDIVA, P.H.N. Estudo experimental sobre a disseminação linfática do tumor de Ehrlich na forma sólida em camundongos. *Braz. J. vet. Res. anim. Sci.*, São Paulo, v.29, n.1, p.97-103, 1992.

RESUMO: As metástases para linfonodos regionais representam fase importante na disseminação neoplásica. Os mecanismos que concorrem para este processo são pouco conhecidos, devido provavelmente à escassez de modelos experimentais adequados. O objetivo do presente estudo é o de caracterizar o tumor de Ehrlich como modelo para o estudo da disseminação linfática em camundongos. Para tanto, animais foram inoculados com células tumorais no coxim plantar e seus linfonodos poplíteos foram colhidos vários tempos após a inoculação, com a finalidade de avaliar seu peso, aspectos histopatológicos e quantificar os mastócitos, já que estas células parecem estar implicadas na resposta do hospedeiro ao tumor. A migração das células tumorais para os linfonodos poplíteos foi detectada a partir de 1 hora após a inoculação. O crescimento do tumor sólido no coxim plantar foi acompanhado até 30 dias após a inoculação, e as alterações histopatológicas foram estudadas durante esse período. O tumor de Ehrlich foi considerado um modelo experimental adequado para o estudo da disseminação linfática.

UNITERMOS: Metástase linfática; Carcinoma de Ehrlich; Linfonodos; Mastócitos; Camundongos

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Recebido para publicação em 24/04/91

Aprovado para publicação em 16/01/92

TABLE 1 - Mast cells counts in lymph nodes excised at several times after the inoculation of Ehrlich tumor cells (TUMOR) or buffered saline (CONTROL) into the footpad of mice. Results are expressed as mean \pm s.d. São Paulo, 1989.

TIMES	TUMOR	CONTROL
1 hour	228 \pm 69	189 \pm 27
7 days	129 \pm 69	169 \pm 94
15 days	673 \pm 889	527 \pm 328
30 days	205 \pm 57	174 \pm 42

TABLE 2 - Biological assay to detect tumor cells in lymph nodes. Gross and histological evaluation of the subcutaneous formations 15 days after the lymph nodes implants. São Paulo, 1989.

Lymph nodes (LN) excision Times	number of implanted LN	NUMBER OF gross observation	OF TUMORS histological observation
1 hour	8	7	7
6 hours	8	6	3
12 hours	8	6	4
24 hours	8	6	5
2 days	8	6	5
3 days	8	6	3
4 days	8	7	5
5 days	8	6	5
6 days	8	3	1
7 days	8	5	3

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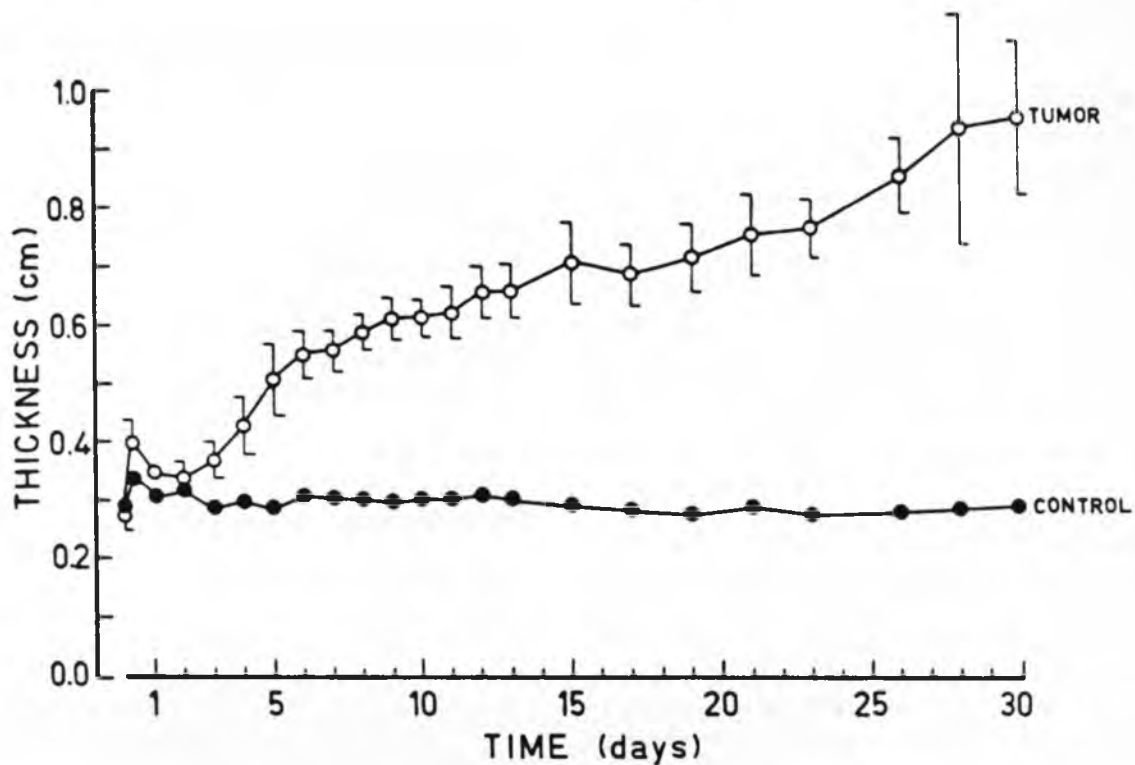


FIGURE 1— Thickness of mice footpads (cm) various times after the inoculation of tumor cells (TUMOR) and buffered saline (CONTROL).

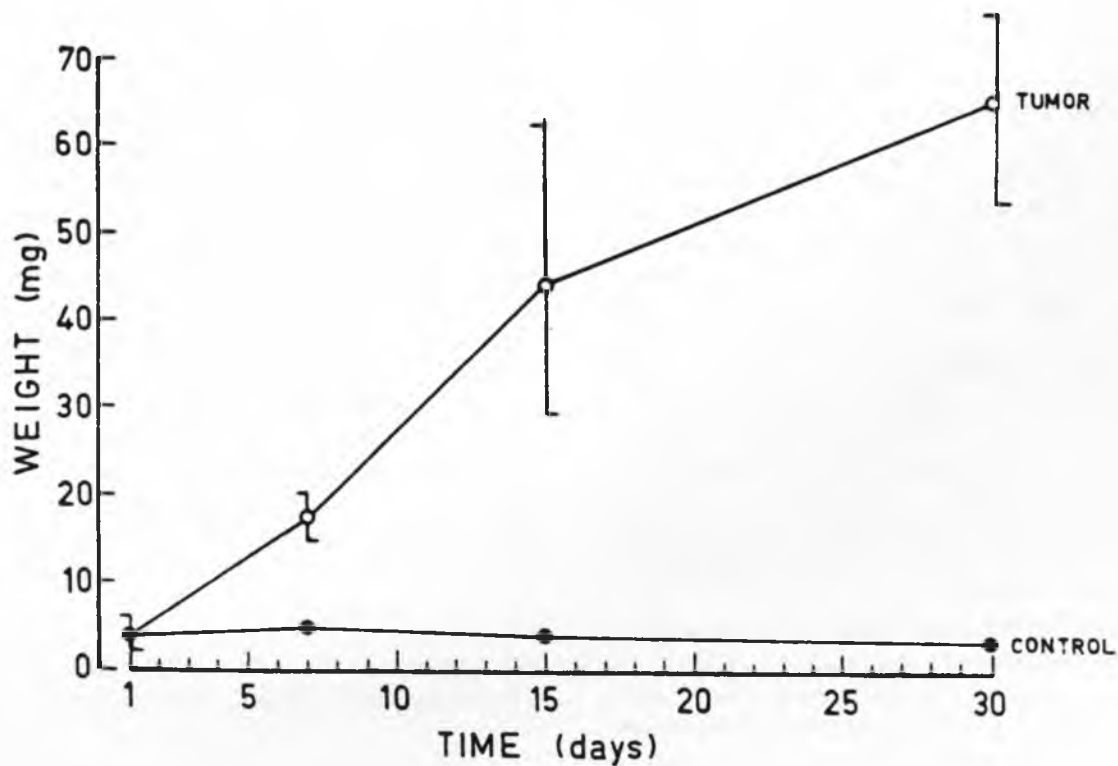


FIGURE 2 — Weights of mice popliteal lymph nodes (g) various times after the inoculation of tumor cells (TUMOR) and buffered saline (CONTROL) into the footpad.