# Inhibition of Ehrlich ascites carcinoma (EAC) growth by carbonyl iron[1,2]

A. Pessina, P. Brambilla and Daniela Balzarin[3]

*Department of Cellular Pathology of C.N.R., University of Milan, 31 Mangiagalli, I-20133 Milano (Italy), 10 April 1978*

*Summary.* Treatment of Ehrlich ascites carcinoma (EAC) cells with carbonyl iron (20 mg/ml) produces a significant decrease in growth rate of tumor inoculum both in Swiss and in C57BL/6 mice. Possible interaction of the carbonyl iron or $Fe^{+++}$ ions with cell surface is suggested.

The treatment of spleen cells with carbonyl iron powder has been suggested and used by various authors as a simple and rapid method to obtain a population of nonadherent cells. The phagocytic cells ingest the iron particles and could be removed by a strong magnet placed to the flat bottom of the vessel[4]. The iron concentration used by various authors was variable and included values of 0.15 mg/ml[5]; 0.5 mg/ml[6]; 10 mg/ml[7]; and 40-80 mg/ml[4,8]. Cellular suspensions of $20\times10^6$ cells/ml were generally used.

In attempt to eliminate adherent cells present in cellular suspension of Ehrlich ascites carcinoma (EAC) by using this method, we observed inhibitory effects on subsequent in vivo growth when iron was added to the tumor cell suspension at highest concentration, prior to inoculation.

*Material and methods.* Animals: female mice of Swiss and C57BL/6 strains 8-10 weeks old from Charles River Italy. Weight: C57BL/6 = 20±0.3 g; Swiss = 28±0.4 g.

Tumor: a half-diploid and half-tetraploid strain of EAC was used. It was maintained for several years by serial transfers in Swiss albino mice[9].

Carbonyl iron: the batch of carbonyl iron SF-grade (General Aniline and Film Corp., New York) was kindly provided by Netherland Cancer Institute of Amsterdam, Holland.

Treatment of tumor suspension: EAC cells were suspended in MEM to final concentration of $20\times10^6$ cells/ml. Cell viability, as estimate by trypan blue exclusion test, was above 95%. Tumor cells were incubated: a) at 37 °C for 40 min together with carbonyl iron at concentration of 1 mg/ml and 20 mg/ml; b) at 37 °C for 40 min in absence of carbonyl iron. At the end of incubation, iron was removed by a magnet.

EAC inoculum and check of tumor growth: cellular suspension of EAC obtained as above-mentioned was washed twice in PBS and than injected i.p. into mice (Swiss and C57BL/6) of experimental groups ($3\times10^6$ cells of EAC suspended in PBS 0.25 ml). In all groups, viability of tumor cells determined by trypan blue exclusion test, was over 85%. Growth of tumor was expressed as measured weight minus initial weight of each mouse, in g[10].

Statistical analysis: the homogeneity of variances was examined by Cochran's test, and the averages of weight increase at the various days were compared by Student's t-test.

*Results.* Mice given an inoculum of tumor cells, pretreated at 37 °C for 40 min with carbonyl iron 20 mg/ml, showed a very slow inoculum growth rate in comparison to mice injected with tumor cells preincubated at 37 °C for 40 min. The kinetics of tumor growth in mice which received tumor cells pretreated with carbonyl iron 1 mg/ml, did not differ from that of the mice injected with EAC cells pretreated without carbonyl iron ($p<0.05$) (figure 1).

The statistical analysis with Student's t-test shows highly significant differences between the means of weight increase taken on carbonyl iron-treated group-20 mg/ml and control group (without carbonyl iron) of both Swiss and C57BL/6 strain, from day 7 ($p<0.05$). The levels of mortality support the above-mentioned effect. The mortality is reduced only in the groups of mice which received tumor cells pretreated with carbonyl iron 20 mg/ml. At day 24, the mortality in Swiss was reduced by 25% and in C57BL/6 by 50% (figure 2).

*Discussion.* The data, both for the Swiss strain, habitually used for serial transplantation of EAC, and for C57BL/6, a strain more resistant to EAC taking, show clearly that the growth rate of EAC inoculum, pretreated with carbonyl iron (20 mg/ml) is strongly reduced (figure 1). This effect appears to be related to carbonyl iron concentration in suspension, since treatment with carbonyl iron (1 mg/ml)

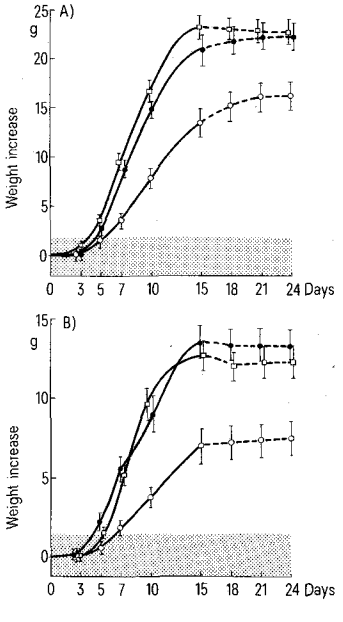


Fig. 1. Tumor growth on various days after the inoculum of $3\times10^6$ Ehrlich ascites carcinoma cells in Swiss mice (A) and C57BL/6 mice (B). Tumor growth is expressed as weight increase of mice above their initial weight. Each point represents the mean of weight increases of 30 mice±SE. Untreated EAC cells (□——□); EAC cells treated with 1 mg/ml carbonyl iron (●——●); EAC cells treated with 20 mg/ml carbonyl iron (○——○); range of physiological variations of weight (screen field).

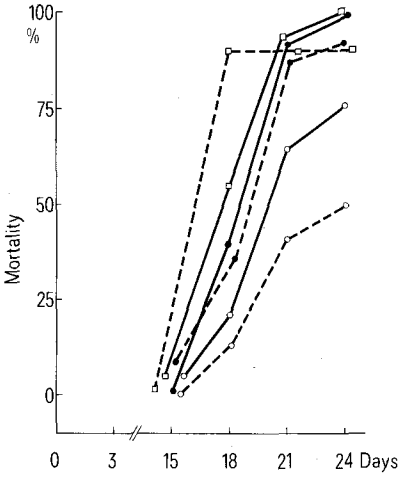


Fig. 2. Mortality percent in Swiss and C57BL/6 mice at various days after the inoculum of Ehrlich ascites carcinoma cells ($3\times10^6$). Swiss (——); C57BL/6 (- - -). Untreated EAC cells (□); EAC cells treated with 1 mg/ml carbonyl iron (●); EAC cells treated with 20 mg/ml carbonyl iron (○).

has no significant effect on subsequent tumor growth. About 35% of the EAC cells are lost during removal of carbonyl iron at the end of incubation. This loss, however, does not affect the size of inoculum because it was performed by injecting in all animals the same number of EAC cells at viability over 85%.

One hypothesis would be that carbonyl iron treatment exerts a delayed cytotoxic effect on the cells. However, it would be necessary to suppose a very drastic decrease in cell number following inoculation, because we have found that the tumor growth rate is independent of inoculum size, at least over the range $1 \times 10^6$ to $6 \times 10^6$ EAC cells injected (unpublished data). This result indicates that simple reduction in cell survival after inoculation does not explain the observed alteration in the growth curve, so that more specific effects of carbonyl iron on the cells may be involved. One such possible effect would be interaction of the carbonyl iron with the cell surface, resulting in altered antigenic properties and increased immunogenicity of the inoculated cells. The greater reduction in mortality in C57BL/6 mice than in Swiss mice correlates well with the observation that the EAC tumor is more immunogenic in C57BL/6 than in Swiss mice[11]. This increased immunogenicity may be caused by high concentration of $Fe^{+++}$ ions released in the EAC suspension during incubation. In fact, about 1300 µg/100 ml of $Fe^{+++}$ions are detected in the supernatant of carbonyl iron-treated group-20 mg/ml; while very low amounts are detected in the supernatant of carbonyl iron-treated group 1 mg/ml (82 µg/100 ml) and in supernatant of control group without carbonyl iron (17 µg/100 ml). If carbonyl iron (20 mg/ml) is mixed with EAC cells and immediately removed, the tumor growth does not differ from the EAC growth of the control group. After removal $Fe^{+++}$ions detected were 97 µg/100 ml. (Determinations performed by: tripyridyltriazine (TPTZ)-test, set-64331, Harleco, Philadelphia, USA.)

The presence of $Fe^{+++}$ions in the supernatant after incubation of EAC cells with carbonyl iron, could also inhibit the cells replication. This hypothesis is supported by observations from various authors who found that multivalent cations interact with membrane functions of EAC cells[12]. Whatever the mechanism, high concentrations of carbonyl iron are by no means biologically inert, and this fact must be considered when using this method to obtain populations of nonadherent cells[4,8].

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2 The abbreviations used are: EAC (Ehrlich ascites carcinoma); PBS (Dulbecco's phosphate buffered saline); MEM (Eagle's minimum essential medium).
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## Autoradiographic localization of the uptake of $^3$H-β-alanine in rat nervous tissue cultures

Elisabeth Hösli and L. Hösli

*Department of Physiology, University of Basel, Vesalgasse 1, 4051 Basel (Switzerland), 28 June 1978*

*Summary.* Autoradiographic studies on the uptake of $^3$H-β-alanine have shown that, in spinal cord and brain stem cultures, both neurones and glial cells have accumulated the amino acid. In contrast, in cultures of cerebellum and dorsal root ganglia, $^3$H-β-alanine was only taken up by glial elements.

It has been proposed that β-alanine may function as an inhibitory transmitter substance in various regions of the central nervous system (CNS)[1]. Although the concentration of β-alanine in the mammalian CNS is quite low, the regional distribution of the amino acid varies considerably, being similar to that of GABA[2]. Microelectrophoretically administered β-alanine has a depressant action on neurones of the mammalian CNS which is blocked by strychnine[3-6]. Furthermore, β-alanine was found to be taken up by high and low affinity transport mechanisms in the frog spinal cord[7] and in brain slices of the rat[8,9]. Autoradiographic studies of the uptake of β-alanine in isolated rat sensory ganglia, and in small slices of cortex and cerebellum, have shown that the amino acid was exclusively accumulated by glial cells[10,11], whereas in the retina both amacrine neurones and glial cells were labelled[12]. The present study is concerned with the cellular localization of the uptake of $^3$H-β-alanine in cultures of spinal cord, brain stem, cerebellum and dorsal root ganglia (DRG) of the rat using autoradiography.

The cultures were prepared from the spinal cord, medulla oblongata-pons, cerebellum and dorsal root ganglia of fetal and newborn rats and grown in the Maximov double coverslip assemblies for 8-35 days[13]. For the autoradiographic studies, the cultures were incubated at 37 °C in Hank's solution containing $^3$H-β-alanine (NEN, specific activity 31.5 Ci/mM) in a concentration of $5 \times 10^{-7}$ and $10^{-6}$ M for 1-15 min. Some experiments were performed at 0 °C or in $Na^+$-free solution[13]. After the incubation, the cultures were rinsed in Tyrode solution, fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, dehydrated and air-dried. Ilford L4 emulsion was placed over the cultures by the loop technique[14]. The autoradiograms were developed after 2 weeks with a Kodak D 19 developer.

After incubation of spinal cord and brain stem cultures with $^3$H-β-alanine, it was observed that both neurones and glial cells have accumulated the amino acid. There was a difference in the number of labelled neurones between spinal cord and brain stem. In cultured brain stem, a relatively great number of neurones have taken up the