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*Tumor and Stem Cell Biology*

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# Tyrosine Isomers Mediate the Classical Phenomenon of Concomitant Tumor Resistance

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## Abstract

Concomitant tumor resistance (CR) is a phenomenon originally described in 1906 in which a tumor-bearing host is resistant to the growth of secondary tumor implants and metastasis. Although recent studies have indicated that T-cell–dependent processes mediate CR in hosts bearing immunogenic small tumors, manifestations of CR induced by immunogenic and nonimmunogenic large tumors have been associated with an elusive serum factor. In this study, we identify this serum factor as tyrosine in its meta and ortho isoforms. In three different murine models of cancer that generate CR, both meta-tyrosine and ortho-tyrosine inhibited tumor growth. In addition, we showed that both isoforms of tyrosine blocked metastasis in a fourth model that does not generate CR but is sensitive to CR induced by other tumors. Mechanistic studies showed that the antitumor effects of the tyrosine isoforms were mediated, in part, by early inhibition of mitogen-activated protein/extracellular signal-regulated kinase pathway and inactivation of STAT3, potentially driving tumor cells into a state of dormancy. By revealing a molecular basis for the classical phenomenon of CR, our findings may stimulate new generalized approaches to limit the development of metastases that arise after resection of primary tumors, an issue of pivotal importance to oncologists and their patients. *Cancer Res; 71(22); 7113–24. ©2011 AACR.*

## Introduction

Concomitant tumor resistance (CR) is the phenomenon according to which a tumor-bearing host inhibits or retards the growth of secondary tumor implants. It was first described by Ehrlich in 1906 (1) but, apart from a few isolated papers (2, 3), this phenomenon remained virtually forgotten for about 60 years (4). Even after its renascence, CR has not received much attention as compared with other areas of cancer research despite the fact that it has been detected in association with human cancer and despite its relevance to the mechanisms of metastases control. In this regard, it has been observed that the removal of human and murine tumors may be followed by an abrupt increase in metastatic growth (5–11), suggesting that, upon certain circumstances, a primary tumor exerts a controlling action on its metastases which could be considered as natural secondary tumor implants.

CR has received different explanations. According to the immunologic hypothesis, originally proposed by Bashford (2), the growth of a tumor generates a specific antitumor immune response which even though not strong enough to inhibit the primary tumor growth, is still capable of preventing the development of relatively small secondary tumor implants. This interpretation, known as "concomitant immunity," is supported by solid evidence mainly based on experiments with strongly immunogenic murine tumors induced by chemicals or viruses (12, 13). However, it does not explain why CR can also be induced by spontaneous murine tumors of nondetectable immunogenicity (14, 15).

As for nonimmunologic explanations, basically 2 hypotheses have been formulated. Ehrlich (1) and Tyzzer (16) believed that nutrients essential for tumor growth are consumed by the primary tumor, making it difficult for a second implant to develop (atrepsis theory). Others (15, 17, 18) postulated that, during primary tumor growth, antiproliferative nonspecific substances or antiangiogenic molecules are produced that limit the replication of the secondary tumor implant. These nonimmunologic hypotheses offer a putative explanation for the CR induced by nonimmunogenic tumors but not for the specific inhibition of secondary tumor implants observed during the growth of immunogenic tumors.

For the last 25 years, we have studied the phenomenon of CR associated with the growth of 17 murine tumors with widely different degrees of immunogenicity, in an attempt to integrate the different hypotheses into a coherent picture.

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Our results (13, 15, 19, 20; Supplementary Table S1) describing 2 temporally separate peaks of CR during primary tumor growth may explain many apparently contradictory results reported by different authors (3, 12, 13, 17) which were probably related to the different stages of tumor growth at which each of these authors looked for CR and to the different characteristics of both peaks. In effect, the first peak was only induced by small (<500 $mm^3$) immunogenic tumors; it was tumor specific and associated with a typical T cell–dependent antitumor immune reaction. On the contrary, the second peak was not related to any conventional immune reaction and was induced by most immunogenic and nonimmunogenic large tumors (≥2,000 $mm^3$); it was tumor nonspecific, thymus independent, and correlated with the activity of a serum factor(s), different from antibodies or complement, that inhibited the *in vitro* and *in vivo* proliferation of tumor cells. When this serum inhibitory activity was absent—the only 2 cases were mice bearing the highly metastatic C7HI and MM3 mammary adenocarcinomas—the second peak did not appear. These results suggested a direct correlation among the second peak of CR, the capacity to restrain metastatic growth and the titer of serum growth inhibitory activity. Furthermore, lung metastases produced by C7HI and MM3 tumors were significantly inhibited by both, the concomitant presence of unrelated tumors that induced CR and by the daily administration of serum from mice bearing these unrelated tumors, which displayed a high titer of growth inhibitory activity (8, 9).

Partial characterization of this inhibitory activity was previously carried out in our laboratory, rendering a heat-, acid-, and alkali-resistant factor of low molecular weight apparently unrelated to other well characterized growth-inhibitory molecules such as interferons, TNF-α, TGF-β, angiostatin, endostatin, etc., taking into account the larger molecular weight of the latter and other physical and biological properties (9, 15, 20).

However, despite these efforts, the origin and chemical nature of that factor remained elusive for years, as well as the paradoxical question about why such a factor could inhibit the proliferation of a secondary tumor but not of a large primary one composed of the same type of cells.

Herein, starting from mice bearing a nonimmunogenic lymphoma (called LB), that produces the strongest second peak of CR among all our tumor models, we report the origin, isolation, and identification of the serum factor(s) associated with the phenomenon of CR. We also report its biological antitumor activity and the putative mechanisms of tumor inhibition.

## Materials and Methods

### Animals

BALB/c mice of both sexes, 3- to 5-month old, raised in our own colony, were used. Nude mice were obtained from the Comisión Nacional de Energía Atómica, Argentina. Care of animals was according to the NIH Guide and Use of Laboratory Animals.

### Tumors

LB is a T-lymphoma spontaneously arisen in a BALB/c male and maintained by serial subcutaneous passages in syngeneic mice. More detailed description of this tumor is given elsewhere (21).

The following tumors were also used: MC-C (fibrosarcoma), CEI (epidermoid carcinoma), and the highly metastatic C7HI (mammary adenocarcinoma), that were described previously (9, 22, 23).

Tumor volume = 0.4 ($a \times b^2$), where $a$ and $b$ represent the larger and smaller tumor diameters, respectively (13).

### Histopathologic studies

Samples were fixed as previously reported (22). A measure of cell proliferation was obtained by immunostaining for Ki-67 protein with goat polyclonal antibodies to Ki-67 (M19; Santa Cruz Biotechnology). Macroscopic (diameter ≥0.1 mm) and microscopic (diameter <0.1 mm) lung metastases were counted as described (9).

### Serum and medium

Serum was prepared as described (15, 21). Medium was RPMI-1640 (Gibco), with penicillin G sodium (10 μg/mL), streptomycin sulphate (25 μg/mL), and amphotericin B as fungizone (25 μg/mL). When necessary, the medium was supplemented with 10% fetal calf serum.

### Drugs, agents, amino acids, and treatments

All the drugs, agents, and amino acids (Sigma) were diluted in saline. Removal of spleen and adrenals and depletion of macrophages by intravenous treatment with silica and liposome-encapsulated clodronate were carried out as previously reported (14, 24).

### Flow cytometry

Cell-cycle analysis was conducted as described (20). The fluorescence of individual cells (proportional to DNA content) was measured in a flow cytometer (Becton Dickinson), and the data were analyzed with CellQuest and ModFit softwares, both of Becton Dickinson.

### [$^3$H]-thymidine uptake assay

A total of $3 \times 10^5$ tumor cells in 0.1 mL of medium were cultured with 0.1 mL of several 2-fold dilutions of serum, serum fractions, or sponge fluids or several concentrations of different defined molecules and 1 μCi/mL of [$^3$H]-thymidine (Dupont NEN Research Products) as described (15). After 18 hours (in selected experiments after 4 and 8 hours), radioactivity incorporated into the cells was determined in a β counter (Beckman). The titer of growth inhibitory activity was defined as the reciprocal of the sample dilution or the concentration of a defined molecule, producing 50% inhibition of [$^3$H]-thymidine uptake by tumor cells as compared with controls ($GIU_{50}$/mL).

### Chromatography on Sephadex G-25 and G-15

Samples were applied successively to an 84 × 0.7 cm column of Sephadex G-25 and to a 66 × 0.7 cm column of Sephadex G-15; in both, elution was carried out with water with 0.45 mL/min flow rate.

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### High-performance liquid chromatography

Samples were applied to a high-performance liquid chromatography (HPLC) column C18, 22 × 0.21 cm with a chromatograph model 140 from Applied Biosystems with a diode array detector; elution was carried out with water using a linear gradient of 100% solvent A [trifluoroacetic acid (TFA), 0.1% in water] that was changed to 60% solvent B (acetonitrile 80% in solvent A) over 60 minutes with 0.15 mL/min flow rate.

Samples were also applied to a second HPLC column C18, 25 × 0.4 cm, using a linear gradient of 100% solvent A' (methanol/TFA/water, 5:1:94 by volume) that was changed to 100% solvent B' (methanol/TFA/water, 20:1:79 by volume) over 20 minutes with 1 mL/min flow rate. Solvents were removed from HPLC fractions before testing on *in vitro* tumor cell proliferation.

### Amino acid analysis and sequencing

Samples were applied to an Amino Acid Analyzer 420 from Applied Biosystems with the method of derivatization with phenyl isothiocyanate and separation of the phenylthiocarbamil amino acid with liquid chromatography. Amino acid sequencing was carried out by automated Edman degradation on 477A protein sequencer from Applied Biosystems with a Premix gradient.

### Mass spectrometry and tandem mass spectrometry

Samples were analyzed in an LCQ DUO mass spectrometer, ESI-IT (Thermo Fisher) with high resolution ion-trap electrospray mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Samples were loaded to the mass spectrometer through a 5-μL loop, in the middle of a 40 μL/min flow (methanol:water:acetic acid). The pump is a Surveyor (Applied Biosystems). Data were acquired in full scan mode between 0 and 2,000 *m/z*. MS/MS data were obtained between 50 and 600 *m/z* scale, with 35 *m/z* as a normalized collision energy.

### Western blotting

Western blotting was carried out with standard techniques as described (23) and analyzed by ImageQuant software. The following antibodies were used: anti–p-Erk 1/2, anti-ERK 1/2, anti–p-STAT3, anti-STAT3 (Santa Cruz), and anti–β-actin (Cell Signaling Technology).

### Statistical analysis

The Student *t* test and Mann Whitney *U* test were used. Values were expressed as mean ± SE. Differences were considered significant when $P \leq 0.05$.

## Results

### Origin of the factor associated with CR

Conditioned medium of cultures of LB tumor cells did not exhibit any inhibitory activity on *in vitro* proliferation of LB tumor cells. Nude, splenectomized, adrenalectomized, and macrophage-depleted tumor-bearing mice exhibited a similar level of serum inhibitory activity as compared with control tumor-bearing mice (not shown). This suggested that neither tumor cells per se, thymus, spleen, adrenals, nor macrophages play a main role in the generation of the factor. On the contrary, because LB tumor growth is accompanied by overt manifestations of systemic inflammation—evidenced by a significant increase of circulating proinflammatory cytokines (TNF-α and interleukins 1β and 6), phase acute proteins (SAA protein), neutrophils, and myeloid-derived suppressor cells (MDSC; not shown), we investigated the relationship between these manifestations and the serum antitumor activity. We observed a significant reduction of that serum activity upon treatment of LB tumor–bearing mice with different steroidal and nonsteroidal anti-inflammatory agents [(dexamethasone, indomethacin, promethazine, chlorpromazine, phenidone, and nordihydroguaiaretic acid (NDGA)], with gemcitabine [that in the concentration used by us and others(25) sharply depleted the number of MDSCs without lowering the number of T cells, B cells, and macrophages] and catalase (that prevents oxidative damage). L-NAME, that prevents peroxynitrite generation, and losartan, which exhibits a weak anti-inflammatory effect (26), did not reduce the serum inhibitory activity. The strongest effects were obtained with gemcitabine and catalase (Table 1). Furthermore, we observed that MDSCs isolated from blood from LB tumor–bearing mice exhibiting the second peak of CR—but not from normal mice or from mice bearing a small LB tumor that did not yet induce CR—spontaneously produced a brightly fluorescent FL-1 product of dihydrorhodamine 123 indicating a high production of reactive oxygen species (ROS) by MDSCs, which, in turn, could oxidize phenylalanine to produce meta- and ortho-tyrosine. These data suggested that ROS released by MDSCs are involved in the generation of the serum antitumor factor.

### Isolation and difficult characterization of the serum factor associated with CR

Fractionation of serum from mice bearing an subcutaneous LB tumor (size ≥2,000 $mm^3$) was carried out through several steps of purification (Supplementary Fig. S1). Serum from normal mice was similarly fractioned as control. At all stages of the purification, the presence of the inhibitory factor was monitored by the [$^3$H]-thymidine uptake assay as a measure of LB tumor cell proliferation. First, serum was decomplemented and subjected to dialysis. The inhibitory factor was recovered only in the dialyzable fraction (MW < 12,500 Da) and was then concentrated by lyophilization and applied successively to Sephadex G-25 and G-15 chromatographic columns, where activity was recovered at fractions corresponding to a molecular weight below 1,000 Da. These fractions were lyophilized and further purified with an HPLC column C18 in a gradient of acetonitrile in TFA (first HPLC). In 12 similar and independent experiments, growth inhibitory activity was systematically recovered in only 1 fraction eluted at near 20% acetonitrile (Fig. 1). At first approach, characterization of this active fraction only revealed the presence of tyrosine not incorporated into a peptide (as evaluated by amino acid analysis and sequencing and by MS and MS/MS spectrometry) but not of putative inhibitory factors of low molecular weight sometimes present in biological fluids such as fatty acids, polyamines, creatinin, uric acid, urea, and prostaglandins E2, A1, A2, and J (see Supplementary information). This result was puzzling because tyrosine is neither inhibitory on tumor cell

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**Table 1.** Effect of gemcitabine, antireactive oxygen, anti-inflammatory, and antireactive nitrogen species drugs on the inhibitory activity present in serum from LB tumor–bearing mice

| Serum from | $GIU_{50}$/mL ($X \pm SE$)[a] | $n$[b] |
|---|---|---|
| Normal mice | 37 ± 7 | 7 |
| LB tumor–bearing mice | 303 ± 30 | 7 |
| LB tumor–bearing mice treated with | | |
| Gemcitabine[c] | 40 ± 6 | 2 |
| Catalase[d] | 49 ± 5 | 3 |
| Dexamethasone[e] | 70 ± 18 | 3 |
| Promethazine[f] | 80 ± 9 | 3 |
| INDO + NDGA[g] | 81 ± 16 | 5 |
| Chlorpromazine[h] | 84 ± 19 | 3 |
| Phenidone[i] | 103 ± 19 | 2 |
| NDGA[j] | 116 ± 14 | 5 |
| INDO[k] | 142 ± 16 | 6 |
| L-NAME[l] | 266 ± 34 | 3 |
| Losartan[m] | 300 ± 30 | 3 |

NOTE: Recovery of the titer of serum inhibitory activity occurred 72 hours after the last dose of the different agents suggesting that this serum activity is continuously produced in tumor-bearing mice. No effect was observed in serum from normal mice that received the same schedule of treatment.

[a]GIUso/mL, titer of growth-inhibitory activity was defined as the reciprocal of the serum dilution producing 50% inhibitory of [$^3$H] thymidine uptake by LB tumor cells as compared with medium only and expressed by milliliter of serum.

[b]*n*, Number of independent experiments.

[c–m]Mice bearing an s.c. LB tumor (volume > 2,000 mm$^3$) received indomethacin (0.5 mg/kg), dexamethasone (0.75 mg/kg), losartan (1 mg/kg), promethazine (1 mg/kg), chlorpromazine (1.2 mg/kg), NDGA (5 mg/kg), phenidone (5 mg/kg), a mixture of indomethacin and NDGA, catalase (350,000 units/kg), L-NAME (25 mg/kg), or gemcitabine (120 mg/kg) by the intraperitoneal route, 48 and 24 hours before testing the titer of serum growth inhibitory activity.

[c]*P*: not significant versus normal serum, $P < 0.01$ versus LB serum.

[d]*P*: not significant versus normal serum, $P < 0.001$ versus LB serum.

[e]*P*: not significant versus normal serum, $P < 0.002$ versus LB serum.

[f]$P < 0.01$ versus normal serum, $P < 0.002$ versus LB serum.

[g]$P < 0.02$ versus normal serum, $P < 0.001$ versus LB serum.

[h]$P < 0.02$ versus normal serum, $P < 0.002$ versus LB serum.

[i]$P < 0.01$ versus normal serum, $P < 0.02$ versus LB serum.

[j]$P < 0.001$ versus normal serum, $P < 0.001$ versus LB serum.

[k]$P < 0.001$ versus normal serum, $P < 0.001$ versus LB serum.

[l]$P < 0.001$ versus normal serum, *P*: not significant versus LB serum.

[m]$P < 0.001$ versus normal serum, *P*: not significant versus LB serum.

proliferation nor a common product of MDSC, or the result of an oxidative damage as the serum factor seemed to be. The elucidation of this puzzle began when this active fraction was applied to a second HPLC, in which the gradient was methanol in TFA, and yielded 3 peaks—instead of only 1 obtained in the first HPLC (Fig. 2A). The first and more conspicuous peak was characterized as tyrosine by comparing its retention time in the gradient with that of commercial tyrosine and by MS and MS/MS spectrometry. The second and the third peaks were characterized as 3-hydroxyphenylalanine (commonly known as meta-tyrosine or m-tyrosine) and 2-hydroxyphenylalanine (ortho-tyrosine or o-tyrosine), respectively, 2 isomers of tyrosine that it is thought to be absent from normal proteins. It is worth noting that tyrosine and its isomers share the same MS spectrum (a major signal at *m/z* 182, consistent with a protonated molecule) but they can be distinguished by the relative abundance of the ions resulting from the fragmentation of the protonated molecule by the MS/MS analysis (Fig. 2B). To further confirm the identity of these isomers, graded concentrations of m-tyrosine and o-tyrosine were added to the biological sample, resulting in an increase of the intensity of the peaks 2 and 3, respectively, in a dose-dependent manner (Supplementary Fig. S2). That is, the active fraction was actually a mixture composed by tyrosine:m-tyrosine:o-tyrosine in a proportion near 19:1.4:1 (tyrosine = 600 ± 134 μg/mL, m-tyrosine = 44 ± 11 μg/mL, and o-tyrosine 31 ± 4 μg/mL; mean of 3 experiments) as calculated by comparing the absorption at 274 nm of the 3 real peaks against calibration curves obtained from known concentrations of commercial tyrosine, m-tyrosine, and o-tyrosine. In addition, we could show that when the

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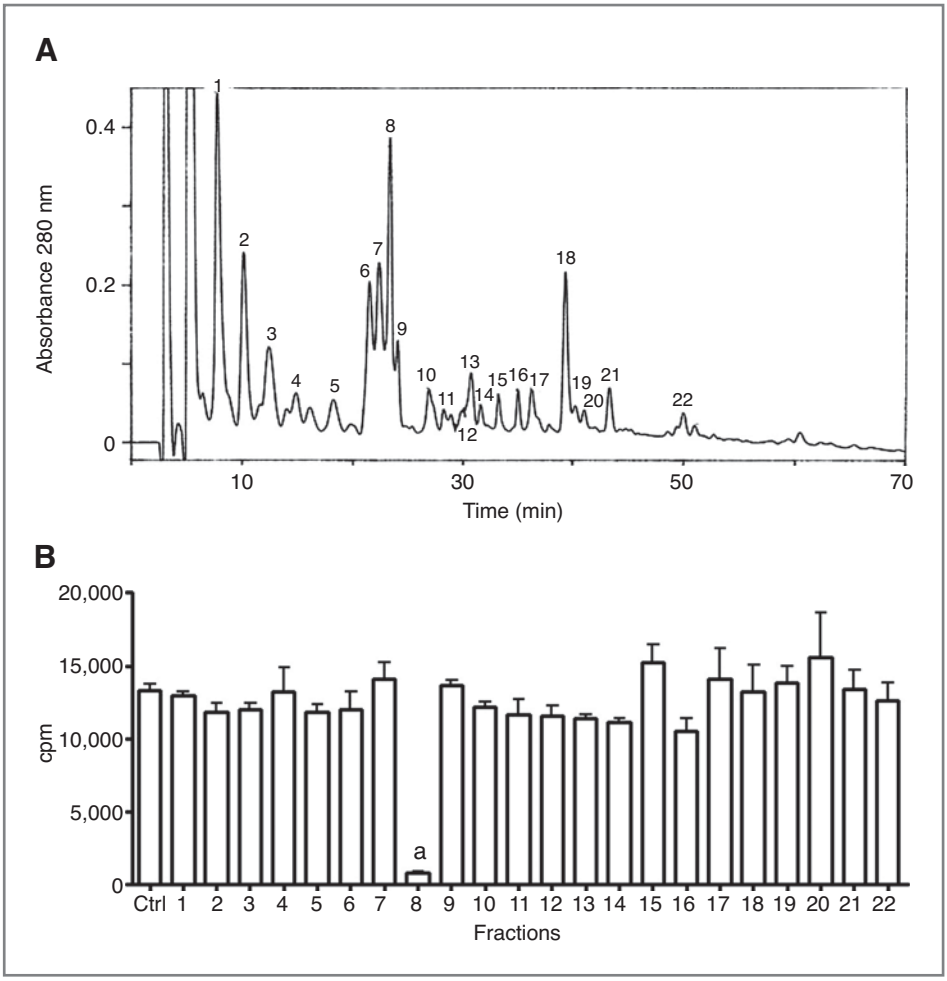


**Figure 1.** A, representative experiment showing the HPLC elution profile of the growth-inhibitory activity present in serum from LB tumor–bearing mice, using a gradient of TFA and acetonitrile (first HPLC). B, effect of each fraction on *in vitro* LB tumor cell proliferation ([$^3$H]-thymidine uptake). Number of assays ($n$) = 3 for each fraction and $n = 6$ for control. a, $P < 0.001$ versus control and the other fractions. Serum from normal mice did not exhibit any fraction with growth-inhibitory activity.

ratio between the amount of tyrosine and that of the sum of its isomers was 7.5 or higher, such as it occurred in the mixture, MS/MS spectrometry was indistinguishable from that of tyrosine alone (Fig. 2C), explaining why the active fraction from the first HPLC seemed to contain tyrosine only.

**Biological assays**

**In vitro *experiments.*** By using an array of equivalent concentrations of the serum purified peaks 2 and 3 from the second HPLC, and commercial m- and o-tyrosine, very similar dose–response curves on the *in vitro* LB tumor cell proliferation were obtained, indicating that m- and o-tyrosine could account for most of the growth-inhibitory activity present in the serum. The inhibitory effect produced by m-tyrosine was about 10 times more robust than that produced by o-tyrosine ($GIU_{50}$/mL for m-tyrosine = 4.5 ± 0.9 μg/mL; for o-tyrosine = 46.7 ± 5.2 μg/mL, $P < 0.002$, $n = 3$ experiments; Fig. 3A). This figure also shows that the peak 1 from the second HPLC and commercial tyrosine were innocuous for LB cells even with high concentrations of both.

Even an excess of commercial tyrosine in relation to m- and o-tyrosine—such as present in the active serum fraction—did not reduce the inhibitory activity of the latter. In contrast, phenylalanine and, to a lesser degree, glutamic acid, aspartic acid, and glutamine, counteracted the inhibitory effect produced by both m- and o-tyrosine in a dose-dependent manner whereas histidine only counteracted the inhibitory effect produced by m-tyrosine. No counteracting effect was observed with the remaining protein amino acids (Fig. 3B and C).

The inhibitory effect produced by m- and o-tyrosine was reversible: after 18 hours of culture, LB tumor cells could reassume their normal growth by replacing the old medium (containing m- or o-tyrosine) by fresh medium (not shown).

The inhibitory effect of m- and o-tyrosine was not restricted to LB tumor cells: *in vitro* proliferation of MC-C, CEI, and C7HI tumor cells was also inhibited by m- and o-tyrosine in a dose-dependent manner (not shown).

**In vivo *experiments.*** When phenylalanine was periodically inoculated at the site of a secondary LB tumor implant—otherwise inhibited by CR (14, 15, 21)—this secondary implant grew similarly to controls. On the contrary, when m-tyrosine was inoculated at the site of a primary tumor implant or systemically, this implant did not grow (Fig. 4A). o-Tyrosine was also inhibitory on LB tumor implants although its effect was weaker than that of m-tyrosine (not shown). In control tumors and in secondary tumor implants treated with

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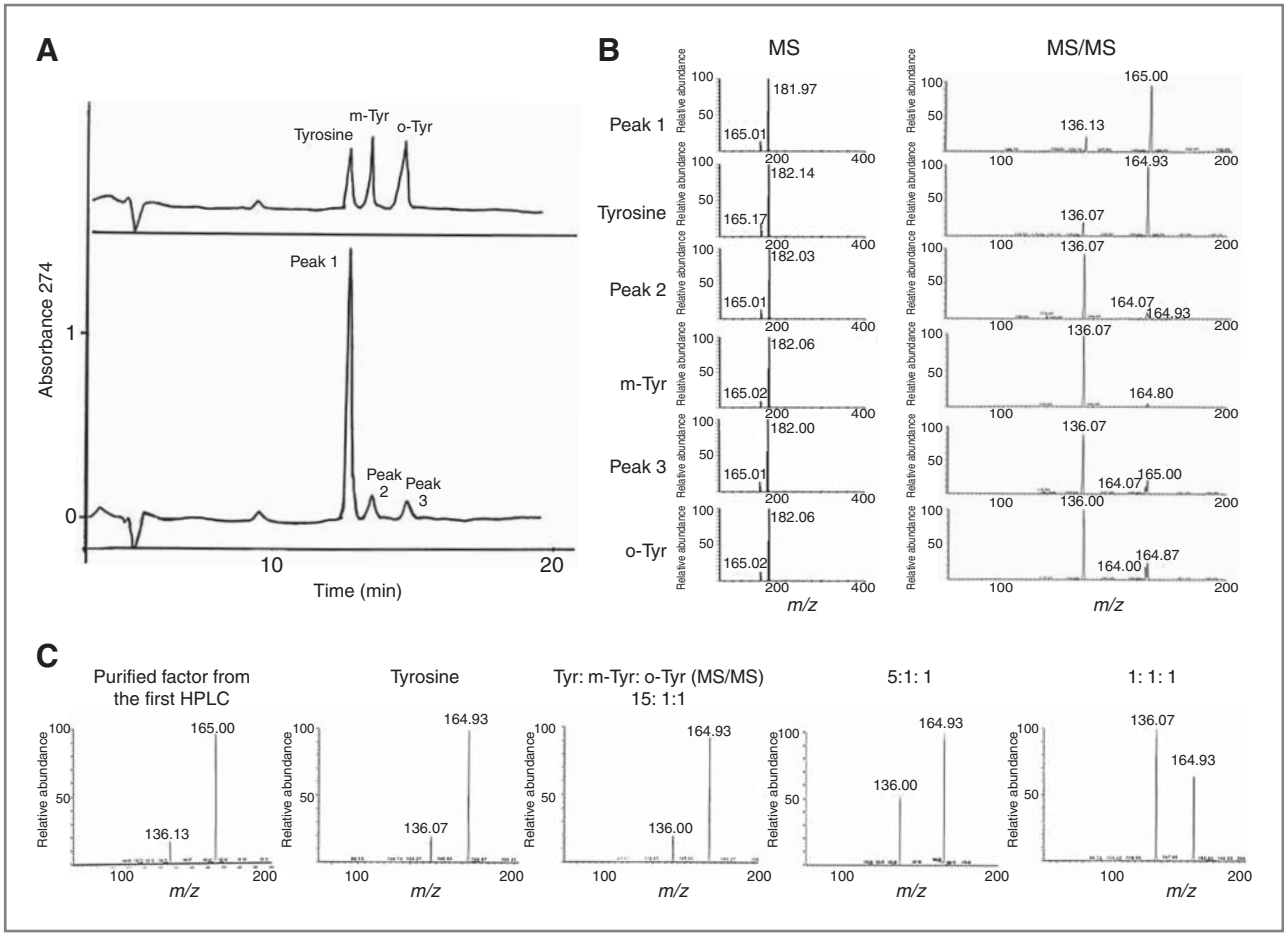


Figure 2. A, representative experiment showing the second HPLC elution profile of the active fraction obtained from the first HPLC, with a gradient of TFA and methanol. Commercial tyrosine, m-tyrosine (m-Tyr), and o-tyrosine (o-Tyr; 60 μg/mL) were comparatively analyzed. B, high resolution ion-electrospray MS and MS/MS of the 3 peaks obtained from the second HPLC and of commercial tyrosine, m- tyrosine, and o-tyrosine. C, comparative analysis by MS/MS of the active fraction from the first HPLC and of a mixture composed by commercial tyrosine, m-tyrosine, and o-tyrosine at different relative concentrations.

phenylalanine, abundant tumor cells, displaying high expression of the cell proliferation marker Ki-67 protein (present in $G_1$–M phases but not in $G_0$), were observed (Fig. 4B). Reciprocally, the inhibition produced by exogenous injection of m-tyrosine mimicked the secondary tumor inhibition produced by CR: in both cases, tumor inhibition was associated with the presence of few tumor cells exhibiting low expression of Ki-67—meaning that most inhibited tumor cells were in $G_0$ (Fig. 4B), a decrease in $G_2$–M phases and an increase of the S phase population—considered the consequence of an S phase arrest (Fig. 4C).

In addition, both a secondary tumor inhibited by CR and a tumor inhibited by exogenous injection of m-tyrosine, could reassume their growth when transplanted in a normal mouse or when treatment with m-tyrosine was interrupted, respectively.

m-Tyrosine not only proved to be inhibitory on tumor implants but also on established s.c. LB tumors (Fig. 4A) and on ascitic LB tumor cells (not shown). Identical inhibition of tumor cells by m- and o-tyrosine was obtained in euthymic and in nude mice indicating that their inhibitory effects were not T-cell mediated. More compelling evidence supporting the contention that m- and o-tyrosine cause (at least in part) the second peak of CR was provided by the following experiment: LB primary tumor–bearing mice bearing a secondary LB tumor implant—inhibited by CR—were treated with gemcitabine plus catalase for 4 consecutive days. As expected by the results shown in Table 1, the titer of serum antitumor activity dropped to control values 2 days after the first inoculation while the previously arrested secondary tumor began to grow. HPLC analysis did not reveal the presence of m- and o-tyrosine in the serum lacking antitumor activity; in contrast, in nontreated tumor-bearing mice, where the secondary tumor was permanently inhibited, the serum displayed both a high titer of antitumor activity and the presence of m- and o-tyrosine (for more details see Supplementary information). Furthermore, m-tyrosine also showed sharp inhibitory effects—without exhibiting toxic side effects—on the growth of MC-C fibrosarcoma and CEI epidermoid carcinoma—2 tumors that induce CR (Supplementary Information)—and on the growth of established spontaneous lung metastases generated by the highly metastatic C7HI mammary adenocarcinoma that does not

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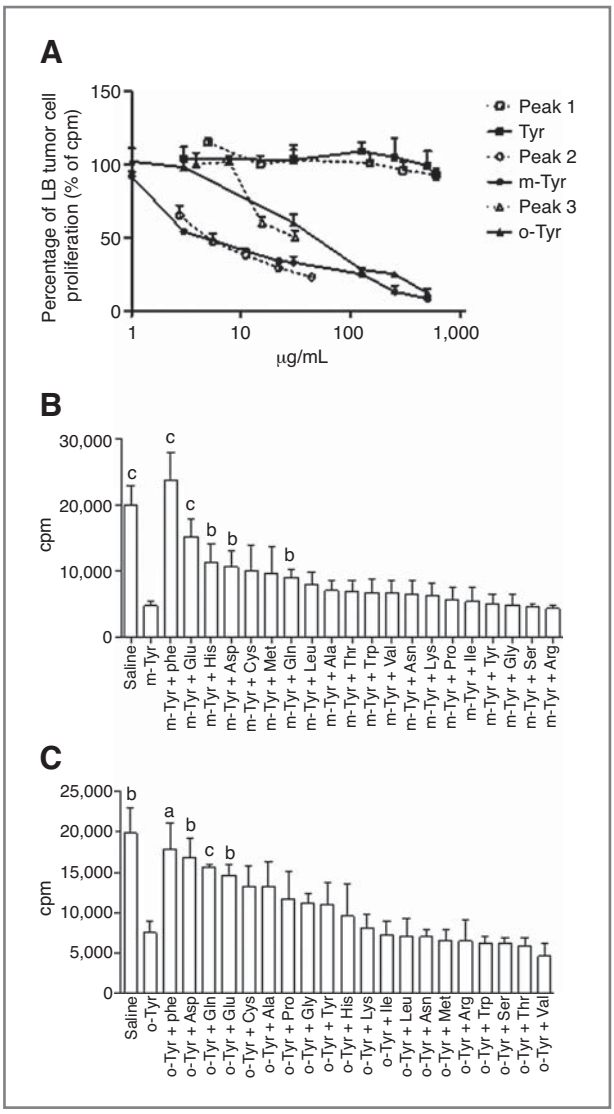


**Figure 3.** A, comparative effect of the 3 peaks obtained from the second HPLC and commercial tyrosine, m-tyrosine (m-Tyr), and o-tyrosine (o-Tyr) on *in vitro* LB tumor cell proliferation. Each point (percentage of cpm incorporated by LB cells as compared with the control) represents the mean ± SE of 3 experiments. Significance versus control, m-tyrosine and peak 2: $P < 0.001$ at >2.75 μg/mL; peak 2: $P < 0.05$ at 2.75 μg/mL. o-Tyrosine: $P < 0.001$ at 500 to 125 μg/mL; $P < 0.002$ at 30 μg/mL. Peak 3: $P < 0.002$ and $P < 0.01$, at 31 and 15.5 μg/mL, respectively. Rescue of *in vitro* LB tumor inhibition mediated by m-tyrosine (150 μg/mL) (B) or o-tyrosine (150 μg/mL) (C) by coculturing with 150 μg/mL of individuals protein amino acids. Each value represents the mean ± SE of 8 to 18 determinations for (B) and 4 to 9 determinations for (C). a, $P < 0.02$; b, $P < 0.01$; c, $P < 0.001$ versus m- or o-tyrosine. Data with saline was similar to data obtained with each amino acid alone.

induce CR but is sensitive to the CR induced by other tumors (Fig. 5).

### On the central paradox of concomitant resistance

In an attempt to understand why a secondary tumor is inhibited while the primary one continues to grow, mice bearing an s.c. LB-growing tumor, received an implant of a sterile polystyrene sponge fragment (about 0.5 $cm^3$) near the site of tumor growth and a similar sponge fragment at the contralateral flank, at the site of a putative secondary tumor implant. A sponge implanted in the flank of normal mice, served as control. Seven days later, the cell-free fluids collected from the sponges were dialyzed and amino acid content of the dialyzable fraction was evaluated. The fluid from the sponges placed near the primary tumor (size ≥2,000 $mm^3$) showed a significantly higher concentration of 16 (out of 20) amino acids than that observed in the sponges implanted at the contralateral flank, including the 5 amino acids (phenylalanine, glutamic acid, aspartic acid, glutamine, and histidine) that counteracted the inhibitory effect of m- and/or o-tyrosine (Supplementary Table S2). This could protect, at least in part, the primary tumor against the antitumor effects mediated by m- and o-tyrosine. In fact, the fluid collected from the sponges placed near the primary tumor exhibited an inhibitory effect on *in vitro* tumor cell proliferation (170 ± 25 $GIU_{50}$/mL, $n = 4$ experiments) more than twice lower than the fluid collected from the sponges placed at the contralateral flank (349 ± 67 $GIU_{50}$/mL; $n = 4$; $P < 0.05$) and than the serum from LB tumor–bearing mice (380 ± 25 $GIU_{50}$/mL; $n = 4$; $P < 0.002$). The basal titer of both the fluid collected from control sponges and normal serum—without detectable amounts of m- and o-tyrosine—were 65 ± 25 $GIU_{50}$/mL ($n = 4$) and 47 ± 10 $GIU_{50}$/mL ($n = 4$), respectively.

To test directly whether a "cocktail" of amino acids similar to that detected close to the "primary site" was more counteracting of the antitumor effects produced by m- and o-tyrosine than a "secondary site cocktail," different concentrations of a mixture of m-tyrosine + o-tyrosine (ratio 1:1) were assayed on LB tumor cell proliferation, alone or together with a primary or a secondary site cocktail of amino acids. The tumor growth inhibitory effect produced by the mixture ($GIU_{50}$/mL = 18 ± 3 μg/mL) was counteracted by both the primary and the secondary sites cocktails. However, the counteracting effect of the primary site cocktail was more than twice greater than that of the secondary site cocktail ($GIU_{50}$/mL of m-tyrosine + o-tyrosine in the presence of the primary site cocktail was = 170 ± 8 μg/mL vs. 70 ± 4 μg/mL in the presence of the secondary site cocktail, $P < 0.01$, mean of 2 experiments) indicating that the inhibitory effect generated by m- and o-tyrosine could be tempered near the primary tumor as compared with the secondary tumor site.

### Molecular analysis

LB tumor cells cultured with m-tyrosine displayed significant changes in the pattern of protein phosphorylation in a dose-dependent manner (not shown). On this basis, we analyzed, first, the effect of m-tyrosine on ERK1 and ERK2 as 2 examples of protein kinases that are activated by the mitogen-activated protein/extracellular signal-regulated kinase (MAP/ERK) signal transduction pathway that normally couples intracellular responses associated with cell proliferation, to the binding of growth factors to cell surface receptors (27). Both, ERK1 and ERK2 are constitutively activated in LB tumor cells, but when these cells were cultured with m-tyrosine, that activation was significantly reduced as early as 3 minutes after

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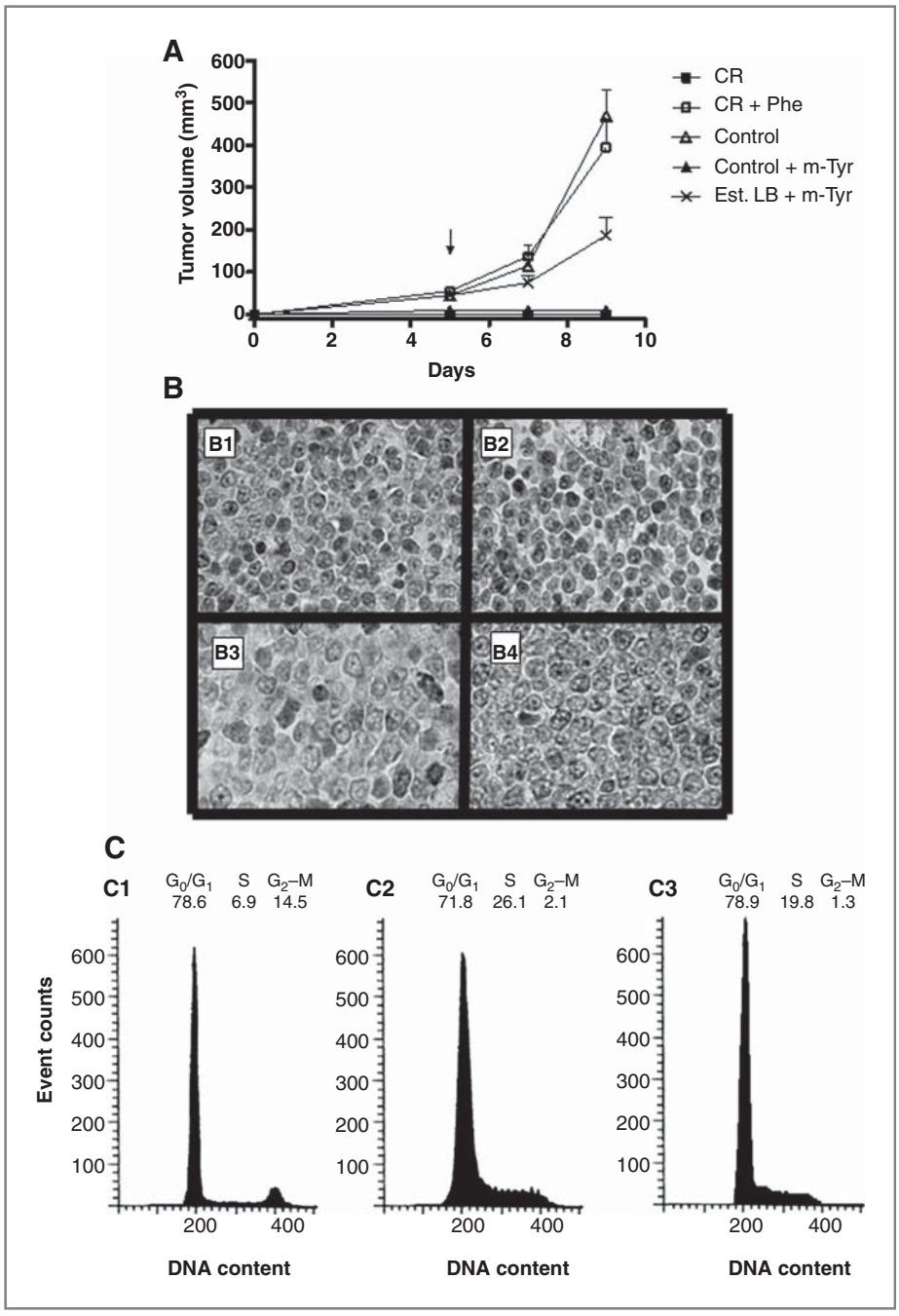


**Figure 4.** A, counteracting effect of phenylalanine on the *in vivo* tumor growth inhibition induced by CR and inhibitory effect of m-tyrosine (m-Tyr) on *in vivo* tumor growth mimicking the inhibition induced by CR. Twenty mice received an s.c. implant of $1 \times 10^6$ LB cells in the right flank, and, 7 days later, an s.c. secondary implant of $1 \times 10^5$ LB tumor cells in the left flank. Ten mice did not receive any additional treatment and the secondary implant did not grow [CR: (-■-)]. The remaining 10 mice received 0.2 mL of phenylalanine (500 μg/mL) at the site of the secondary implant, daily, for 8 days, starting 1 hour after the secondary inoculum [CR + Phe (-□-)]. Controls were 10 mice receiving $1 \times 10^5$ LB tumor cells in the left flank only [control (-Δ-)]. A fourth group received the tumor inoculum in the left flank but also m-tyrosine at the site of tumor implant (0.2 mL of 500 μg/mL, $n = 10$) or by the intraperitoneal route (0.5 mL of 1,000 μg/mL, $n = 6$), daily, for 8 days, starting 1 hour after tumor inoculum [control + m-Tyr: (-▲-)]. A fifth group received the tumor inoculum in the left flank and also m-tyrosine (0.2 mL of 500 μg/mL, $n = 5$) at the site of tumor growth, daily for 5 days, starting (see arrow) when LB was an established tumor measuring 60 to 70 $mm^3$ [est. LB + m-Tyr: (-**x**-)]. Abcissa, days after tumor implant in the left flank. Ordinate, volume of the tumors implanted in the left flank. CR and control + m-Tyr versus control: $P < 0.001$ at day 9 and $P < 0.002$ at days 5 and 7; CR and control + m-Tyr versus CR + Phe: $P < 0.001$ at days 5, 7, and 9; est. LB + m-Tyr versus control and CR + Phe: $P < 0.02$ at day 9. Control + tyrosine or saline, control; CR + tyrosine or saline, CR. B, microscopic view showing many tumor cells expressing Ki-67 protein in both CR + phenylalanine (B1) and control (B2) groups and few tumor cells expressing Ki-67 in both CR (B3) and control + m-tyrosine (B4) groups (H × 400). C, representative experiment showing the frequency distribution histogram of viable LB cells from a control tumor (C1), from a secondary tumor inhibited by CR (C2), and from a tumor inhibited by periodic intratumoral injection of m-tyrosine (C3). Samples of tumor cells were collected 9 days after the s.c. inoculation of $1 \times 10^5$ LB tumor cells.

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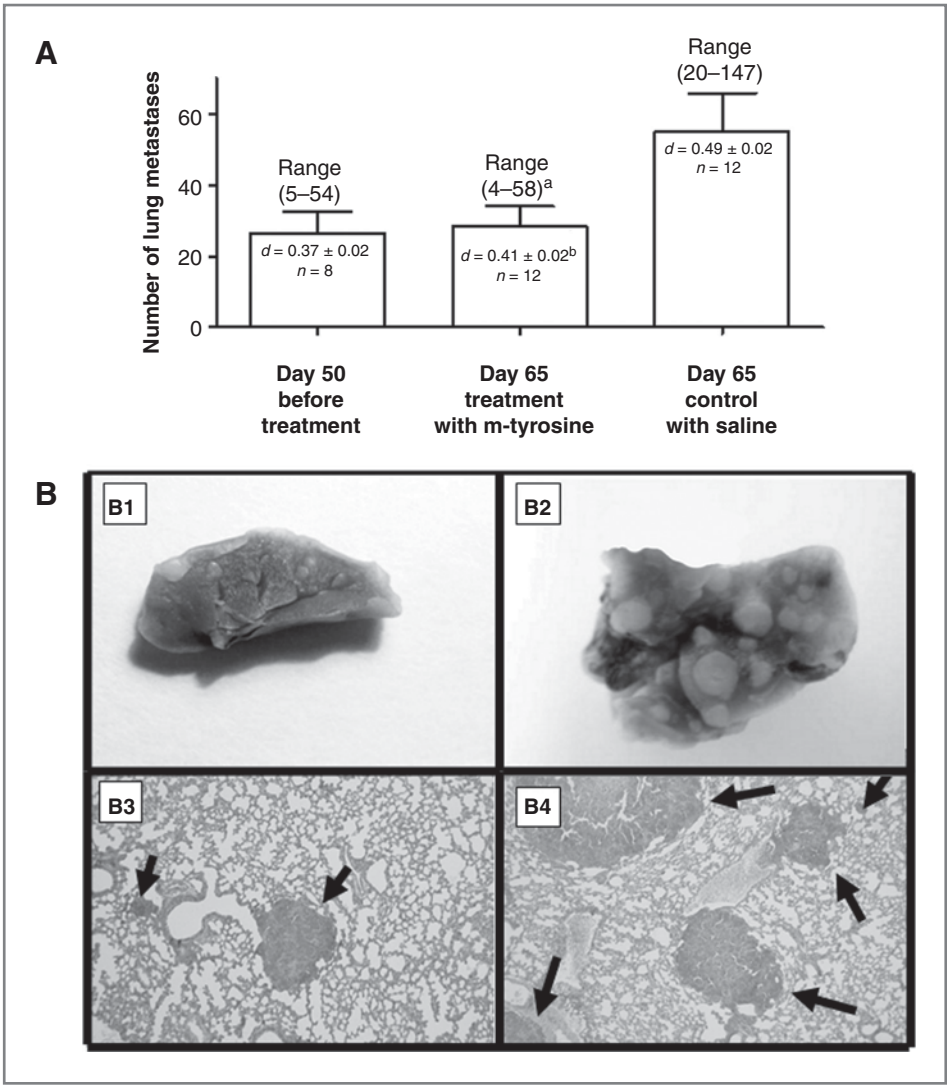


Figure 5. Inhibition of spontaneous C7HI-lung metastases by m-tyrosine. A, mice bearing a C7HI tumor for 50 days received, between days 50 and 64, a daily intravenous injection of m-tyrosine (0.3 mL of 1,000 μg/mL) or saline. Mice were sacrificed at day 50 ($n = 8$, before treatment, primary tumor volume 241 ± 16 $mm^3$) or at day 65 from treated ($n = 12$, primary tumor volume 1,080 ± 101 $mm^3$) or control ($n = 12$, primary tumor volume 1,091 ± 88 $mm^3$) mice and number and size of macroscopic metastases were determined; $d$ = diameter of metastases in mm; a, $P < 0.05$ (Mann–Whitney $U$ test and Student $t$ test) and b, $P < 0.02$ (Student $t$ test) versus control. The difference between the size of the primary tumors at day 65, in experimental and controls was not significant. B, macroscopic (B1; 10×) and microscopic (B3; H&E 100×) view of a lung from a m-tyrosine–treated mouse showing less and smaller metastatic foci (arrows) as compared with a control (B2 and B4). H&E, hematoxylin and eosin.

the onset of the culture, whereas the addition of phenylalanine reversed that effect (Fig. 6A and B). Second, we analyzed the effect of m-tyrosine on STAT3, that is activated (among others) by MAP/ERK cascade and in turn activates several genes involved in cell-cycle progression (28, 29). STAT3 is also constitutively activated in LB tumor cells, but when these cells were cultured with m-tyrosine, that activation was significantly reduced 8 hours after the onset of the culture, whereas the addition of phenylalanine reversed that effect (Fig. 6C and D). The low expression of p-STAT3 was temporally correlated to a low expression of Ki-67 protein, to a cell-cycle distribution identical to that observed in tumor cells inhibited by CR or by m-tyrosine *in vivo* and to the onset of the inhibition of [$^3$H]-thymidine uptake by LB tumor cells (not shown). Furthermore, with different concentrations of m-tyrosine, we could show that the inhibitory effect produced by m-tyrosine on LB tumor cell proliferation was proportional to the reduction of the expression of p-ERK 1/2, p-STAT3, and Ki-67 protein. In consequence, the above experiments suggest that the partial inactivation of ERK1/2 and STAT3 mediated by m-tyrosine could be involved—at least in part—in a relatively rapid mechanism that may drive tumor cells into a state of dormancy.

## Discussion

Clinical and experimental evidence accumulated during the last century indicate that the removal of human and murine tumors may be followed by an accelerated growth of metastases (30, 31). This fact suggests that a primary tumor can exert an inhibitory effect on its metastases which may be considered a particular case of CR. On the contrary, the phenomenon of concomitant enhancement, by which the presence of a primary tumor can stimulate the growth of its metastases has also been observed (32). However, in our experience (21), the magnitude of this stimulatory effect whenever it is present proved to be rather modest as compared with the magnitude of the inhibitory effect produced by CR.

In previous papers, we suggested that 2 temporally separate peaks of CR could be detected throughout primary tumor growth: the first, only observed with small immunogenic

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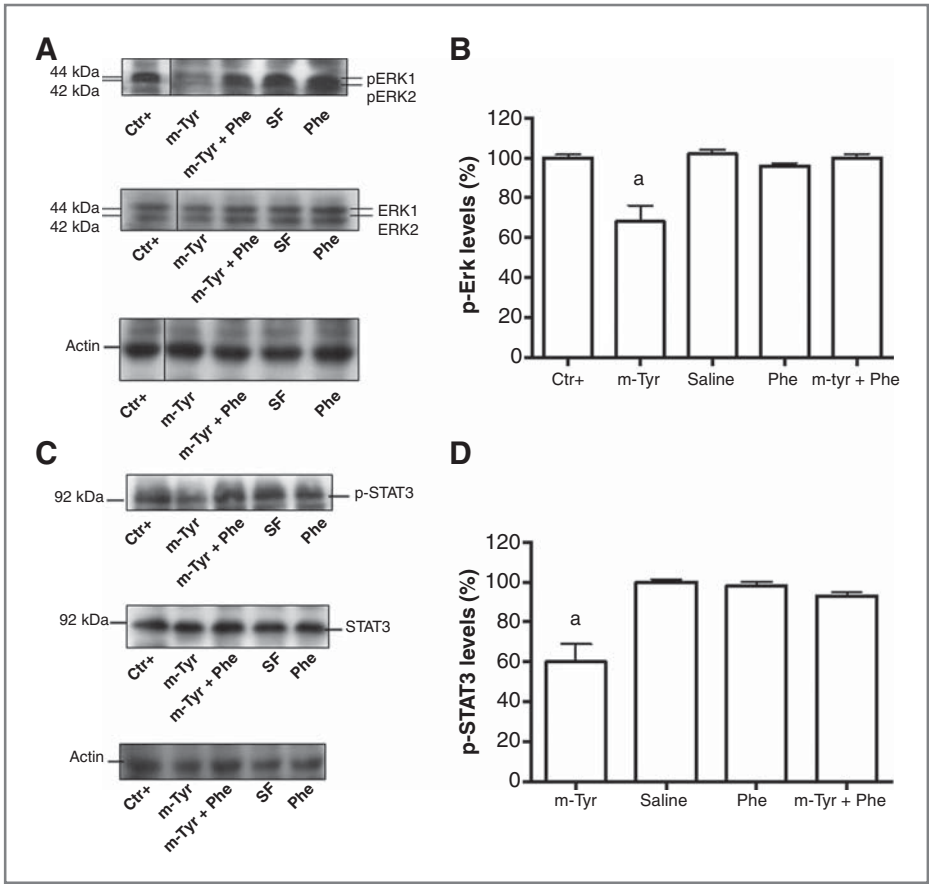


**Figure 6.** Effect of m-tyrosine on the expression of phosphorylated (p)-Erk 1/2 and p-STAT3 in LB tumor cells using Western blot: $3 \times 10^5$ LB tumor cells in 0.1 mL of medium were cultured in the presence of 0.1 mL of m-tyrosine (m-Tyr; 250 μg/mL), phenylalanine (Phe; 250 μg/mL), m-tyrosine (250 μg/mL) + phenylalanine (250 μg/mL), or saline for 3 minutes (to measure p-ERK1/2) or for 8 hours (to measure p-STAT3). Positive control was obtained by treating LB cells with pervanadate. Controls with actin, total ERK 1/2, and total STAT3 were also added. A, representative experiment showing p-Erk1/2 expression (full blot available in Supplementary Fig. S3). B, histogram showing the mean of 5 experiments; a, $P < 0.01$ versus saline and versus m-tyrosine + phenylalanine; $P < 0.02$ versus phenylalanine. C, representative experiment showing p-STAT3 expression (full blot available in Supplementary Fig. S3). D, histogram showing the mean of 6 experiments; a, $P < 0.01$ versus saline and versus phenylalanine; $P < 0.02$ versus m-tyrosine + phenylalanine.

tumors and the second associated with large immunogenic and nonimmunogenic tumors (13, 20). Some years ago, an intermediate peak of CR was reported to be associated with a particular type of mild-sized tumors that restrain secondary tumors by limiting tumor neovascularization (18). Although the mechanisms associated with the first and intermediate peaks of CR have been elucidated as T cell- and angiostatin-dependent, respectively, the molecular basis of the most universal manifestation of CR, that is, the second peak, has remained an enigma for many years.

In former studies, we showed that the second peak of CR could be attributed to a tumor-inhibitory factor(s) of low molecular weight, present in the serum of tumor-bearing mice, and unrelated to previously characterized growth inhibitors and immunologic mediators (9, 13, 15). In this article, we report the characterization of this factor(s) using as tumor model the LB lymphoma that produces the strongest second peak of CR among all the tumors studied in our laboratory. This task was long and difficult due to the overwhelming amount of tyrosine present in the purified antitumor serum fraction, which masked the existence of other molecules and considerably retarded the process of characterization. The elucidation of this puzzle was achieved when minimal amounts of m- and o-tyrosine, 2 isomers of tyrosine that it is thought to be absent from normal proteins, were finally detected together with tyrosine, and identified as responsible for 90% and 10%, respectively, of the total inhibitory activity as shown by experiments on LB and other unrelated tumors. The tumor inhibitory effects produced *in vitro* by m- and o-tyrosine were detectable even at low (micromolar) concentrations and those produced *in vivo*, were observed, not only on tumor implants but also on established metastases and growing vascular (s.c.) and avascular (ascitic) tumors, suggesting that they may have therapeutic potential based on a direct effect on tumor cells rather than an indirect effect on tumor vascularization.

The central paradox of CR, that is, the inhibition of secondary tumor implants together with the progressive growth of the primary tumor, has remained unsolved for more than a century. To account for this problem we showed that, as a primary tumor grows, relatively large amounts of most amino acids, including those that counteract the inhibitory effects of m- and o-tyrosine (phenylalanine, glutamic acid, aspartic acid, glutamine, and histidine), are accumulated in the tumor microenvironment while at distant sites, such as sites of putative secondary tumor implants, the content of amino acids is significantly lower. On this basis, a secondary tumor can be inhibited by circulating m- and o-tyrosine at the same time as the primary tumor is protected from their inhibitory effects by those counteracting amino acids and thus could continue to grow. Some years ago, on a theoretical ground, Prehn (33) anticipated this explanation which reconciles the 2 major nonimmunologic interpretations of CR that have been advanced in the past—the hypothesis of antiproliferative factors and the atrepsis theory. The intriguing observation that

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regeneration of normal tissues is usually not affected in tumor-bearing mice exhibiting the second peak of CR (13, 19), might also be explained by assuming that these regenerating tissues—but not secondary tumor implants—display a content of amino acids high enough to counteract the inhibitory effects produced by m- and o-tyrosine.

Up to date, m- and o-tyrosine have been studied, almost exclusively, as markers for oxidative damage associated with abnormal proteins detected in the blood of animals subjected to cardiac ischemia-reperfusion injury, mitochondria of exercised animals, atherosclerotic tissue of diabetic primates, aging lens of human beings, etc (34).

Most studies have assumed that m- and o-tyrosine are generated posttranslationally when L-phenylalanine present in proteins is exposed to hydroxyl radicals during oxidative damage. However, it has recently been suggested that oxidized amino acids, such as m-and o-tyrosine, might also be generated from free amino acids that could be subsequently incorporated into proteins during synthesis (34, 35). In fact, on the basis of our results showing that the serum antitumor activity attributed to m- and o-tyrosine was strongly inhibited by agents that reduce the number of MDSCs and the oxidative damage, and on the observation that, in tumor-bearing mice (including the LB tumor model used in this work) and in some cancer patients, MDSCs producing large amounts of ROS accumulate progressively in circulation (23, 36–38), we suggest that free m- and o-tyrosine present in serum from tumor-bearing mice would be produced, at least in part, when circulating molecules of phenylalanine are oxidized by hydroxyl radicals released by MDSC. The fact that anti-inflammatory agents also decreased that serum antitumor activity, is probably associated with their capacity to reduce the oxidative stress induced by inflammatory cytokines (39, 40) suggesting that oxidative stress could be critical to regulate the expression of CR.

Very few studies have previously reported antiproliferative effects mediated by m- and o-tyrosine. Gurer-Orhan and colleagues (34), while studying alternative mechanisms for oxidative stress and tissue injury during aging and disease, showed that free m-tyrosine and o-tyrosine were toxic to Chinese-hamster ovary cells when these cells were incubated *in vitro* with m- or o-tyrosine for 7 to 10 days. In the same way, Bertin and colleagues (35), while studying the development of more environmentally friendly weed management systems, showed that the unusual ability of many fine fescue grasses to outcompete or displace other neighboring plants was based on the phytotoxic properties of their root exudates and that more than 80% of the active fraction was m-tyrosine. Both authors hypothesized that one potential cytotoxicity mechanism could involve mischarging of tRNA and consequent misincorporation of these unnatural isomers of tyrosine into cellular proteins based on their structural similarities with phenylalanine or tyrosine. In turn, this misincorporation could cause structural disruption in proteins or could interfere with the functions of key enzymes such as DNA polymerase which might lead to errors in DNA replication and long-term consequences such as impaired cellular viability.

The mechanism of misincorporation into cellular proteins, claimed to be associated with long-lasting cytotoxicity effects on mammal and plant normal cells, could also be invoked to explain the short-lasting antiproliferative effects of m- and o-tyrosine on tumor cells described in this article. Although this alternative is possible, some of their antitumor effects might start before such misincorporation in proteins had a chance to occur. This is suggested by the rapid reversion of those effects, by the counteracting effects of amino acids (other than phenylalanine) that lack any obvious structural similarity with m- and o-tyrosine and in consequence with less possibilities to compete for the same tRNA, and by the very early inhibition of MAP/ERK signaling pathway which would drive tumor cells into a state of dormancy through a rapid decay of p-STAT3 and Ki-67 protein. Although a more profound understanding of the molecular mechanisms of tumor inhibition by m- and o-tyrosine is demanded, it is provoking, from an evolutionary point of view, that the same molecule, m-tyrosine, present in root exudates from many fescue grasses can inhibit the growth of competing neighboring plants and present in tumor-bearing mice can inhibit the growth of secondary tumor implants.

The phenomenon of CR has usually been neglected among researchers and clinicians probably because the idea that a primary tumor may exert inhibitory influences upon distant metastases meant that a tumor had to be considered an integrated, organ-like entity rather than a collection of independent atypical cells (15, 30, 33). In fact, hepatectomy stimulates mitosis in previously resting ectopic implants of hepatocytes in the same way that excision of a primary tumor induces mitosis in previously arrested secondary tumor implants (15, 41, 42). This and many other examples of CR-like phenomena associated with normal tissues and organs suggest that a tumor may mimic some aspects of organ homeostasis (30, 33, 43, 44). Along this new conceptual model of cancer, the discovery of m- and o-tyrosine as responsible for the most universal manifestation of CR, might contribute to unveil some of the control mechanisms of malignant and normal cell proliferation and also develop new and more harmless means to manage malignant diseases, especially by controlling the growth of metastases, before and after the removal of a primary tumor or after other surgical injuries or stressors that may promote the escape of metastases from dormancy (30, 45).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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