

# Involvement of T-Lymphocytes in the Stimulatory Effects of EMT<sub>6</sub> Tumors on Medullary Pluripotent Stem Cells of BALB/c Mice

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## ABSTRACT

Untreated EMT<sub>6</sub> tumors and bone marrow of tumor-bearing BALB/c mice secrete factors capable of stimulating quiescent medullary splenic colony-forming units into cycle. Since the tumor population is heterogeneous, it is of interest to determine the nature of the cells which are involved in this phenomenon. Experiments with conditioned media from tumors of BALB/c and athymic mice show that EMT<sub>6</sub> cells themselves may secrete a stimulating factor but that the presence of T-lymphocytes is necessary at some time in the development of the tumor. Likewise, the presence of the tumor is necessary for bone marrow to secrete these factors in our experimental model. This suggests reciprocal influences of the T-cells and the EMT<sub>6</sub> tumor cells in the phenomena that we describe.

## INTRODUCTION

Many studies have now demonstrated that nonhematological solid or ascites tumors determine hematological disorders in humans (17) and mice (3, 7, 12, 26). These disorders concern all the hematological compartments: peripheral blood counts are modified; unipotent granulocyte-macrophage stem cells are decreased in the marrow and increased in the spleen in Ehrlich ascites-bearing mice (19) and increased in the bone marrow of EMT<sub>6</sub> tumor-bearing mice (4); the pluripotent stem cell compartment (CFU-S)<sup>2</sup> is depleted in the bone marrow of mice with Ehrlich ascites carcinoma or NCTC tumor (5-7, 16, 22, 27) and increased in the blood and in the spleen from mice with Ehrlich ascites mammary carcinoma, transplanted fibrosarcoma, methylcholanthrene-induced carcinoma, and spontaneous mammary cancer (20, 21, 23, 25).

The kinetic status of CFU-S was studied by Croizat and Frindel (6) in NCTC tumor-bearing mice and by Lala (22) in Ehrlich ascites-bearing mice. In both cases, normally quiescent CFU-S were found to be in cycle during the development of the tumors.

In a previous paper (14), we have reported that diffusible factors are secreted by the EMT<sub>6</sub> tumors during the exponential growth period that are capable of triggering CFU-S into cycle. This factor(s) was not detectable when the tumor reached the plateau phase of growth. Bone marrow from the tumor-bearing mice was also found to secrete a stimulating factor(s) in parallel with those secreted by the tumor itself.

Stimulating factors have also been found in serum (24, 28), in the thymus and in the bone marrow (33) of leukemic animals, in tumor extracts (8-11, 23), and in ascitic fluid (16, 19).

The biochemical nature of the factor(s) will not be discussed in this paper.

The aim of this work is to determine the nature of the cells which secrete the stimulating factor(s) of CFU-S proliferation. It is well known that solid tumors are composed of a heterogeneous population containing macrophages, lymphocytes, etc. (2, 18, 30-32, 35, 36), and we wished to determine the eventual role of each one of the cell types concerned. Some experiments seem to exclude macrophages as being the secretory cells. The next candidates could be the lymphocytes or the tumor cells themselves. In order to verify the role of lymphocytes, we studied the behavior of EMT<sub>6</sub> tumors in nude mice which are athymic at birth and which are depleted of mature T-cells.

## MATERIALS AND METHODS

### Animals

A total of 1600 BALB/c OLA females or males (Bicester, England), 10 to 12 weeks old, were used for the CFU-S and bone marrow studies, and 180 BALB/c OLA females, 14 to 18 weeks old, plus 80 nude females or males, 10 to 13 weeks old (Swiss, IFFA-CREDO-France, or BALB OLA), were used for the tumor-bearing donors.

### Tumors

EMT<sub>6</sub> cells of the BALB/c-derived mammary carcinoma were passaged alternately *in vitro* and in solid form *in vivo*, as has been described previously (29). The solid tumors were obtained by a single s.c. injection of  $4 \times 10^5$  cells from the cell cultures.

The dimensions of the palpable tumors were measured, and the mice were killed when the tumor volume was 100 cu mm (7 days for the BALB/c mice and 6 days for the nude mice).

### In Vivo Experiments

The percentage of CFU-S in S phase of pooled bone marrow was determined for each group by the thymidine suicide technique of Becker and McCulloch (1).

Three mice (BALB/c or nude) were killed per experimental group. The cells were flushed from the femur and tibia of the donor mice using NCTC Medium 199. The cells were suspended in 1 ml of medium per leg and counted. Two  $\times 10^6$  cells were added into 2 vials. One contained 200  $\mu$ Ci of tritiated thymidine (specific activity, 25 Ci/mmol) in 2 ml of Medium 199; the second vial did not contain [<sup>3</sup>H]thymidine. After 20 min of incubation and dilution,  $5 \times 10^4$  bone marrow cells per mouse were injected i.v. into 8 recipient mice per vial. All recipients were BALB/c mice given, prior to the injection, a lethal dose of irradiation (7.40-Gray <sup>60</sup>Co source).

A linearity of the nude medullary CFU-S into the BALB/c recipient spleen was found, and  $5 \times 10^4$  cells were injected per mouse in every case. Sixteen recipient mice were injected per group. Mice were killed 9 days after the injection and the spleen colonies were counted.

### In Vivo-In Vitro Experiments

The secretion of soluble factors was assayed by 2 methods. **In vivo-In Vitro Method.** In this method, described by Frindel *et al.*

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<sup>2</sup> The abbreviation used is: CFU-S, splenic colony-forming units.  
Received October 22, 1981; accepted January 22, 1982.

(14, 15) previously, the system was composed of 2 cellular compartments separated by a Millipore filter (Millipore S. A., Malakoff, France). The filter permits the diffusion of molecules but isolates the 2 cell populations from direct contact.

Tissue fragments to be tested from nude or BALB/c mice were placed on the Millipore filter (Chart 1) with the bone marrow in the form of plugs (6/filter) and the tumor in the form of a slice (diameter, 8 mm).

The Millipore filter floated on the surface of tissue culture medium containing the responder cells. In all cases, these cells were harvested from bone marrow of normal BALB/c mice, and the CFU-S present in this responder population were quiescent at the time of incubation.

After 20 to 24 hr, filters and incubation medium were discarded, and the responder bone marrow cells were resuspended in order to assess the proliferation status of the CFU-S by the same suicide technique as for *in vivo* experiments. Eight × 10<sup>4</sup> responder cells were injected per BALB/c recipient mouse.

**Conditioned Medium.** This technique was used to test soluble factors from bone marrow cells and from cells of the peritoneal cavity of BALB/c mice during the exponential growth of the EMT<sub>6</sub> tumors. The bone marrow cells were obtained from tibias and femurs as in previous experiments. The cells of the peritoneal cavity were obtained by washing with physiological serum. After counting and dilution, 2 × 10<sup>5</sup> cells were seeded in Petri dishes; after 24 hr of incubation at 37°, the supernatant was harvested and filtered in order to eliminate the cells. The stimulating activity of the conditioned media was assayed by incubating bone marrow CFU-S of normal BALB/c for 24 hr. The proliferating status of CFU-S was assessed by the thymidine suicide technique.

The identical technique was used to test the eventual secretion of factors from the EMT<sub>6</sub> tumors growing on BALB/c or nude mice (BALB/c). The conditioned medium was harvested either from the primary cultures or after 7 weekly passages *in vitro*.

Conditioned media were harvested at 24 hr to 15 days of culture, filtered, and kept at -20° until use.

In some experiments, each individual clone was seeded in order to obtain a pure population of EMT<sub>6</sub> tumor cells. In this case, conditioned media were harvested after 5 days of culture.

The media were tested on normal BALB/c bone marrow *in vitro* containing 4 ml of conditioned medium, 1 ml of fresh Waymouth's medium (Eurobio, Paris, France), and 15% fetal calf serum (Sorga, Paris, France). The incubation at 37° lasted for 24 hr, and bone marrow CFU-S kinetics was assessed by the thymidine suicide technique of Becker *et al.*

**RESULTS**

**In Vivo Experiments**

Table 1 shows that CFU-S in BALB/c mice are quiescent (7.5% CFU-S in S phase). Seven days after injection of EMT<sub>6</sub> cells, when the tumor volume reached 100 cu mm, 43.2% CFU-S were in DNA synthesis. These results confirm our previous report (14).

In nude mice, CFU-S are quiescent (2.0% in S phase); 6 days after EMT<sub>6</sub> inoculation, when the tumor volume reached 100 cu mm, 5.7% CFU-S were in DNA synthesis.

**Soluble Factors**

**In Vivo-in Vitro Experiments.** Table 1 shows that the responder bone marrow CFU-S population is quiescent after 24 hr of incubation (10.2% in S phase). They remain quiescent when normal bone marrow plugs are floated on the Millipore filter (7.2% in S phase).

When tumor fragments from BALB/c mice were placed on the Millipore filter, the responder CFU-S were triggered into cycle (36.7% in S phase); when bone marrow fragments from

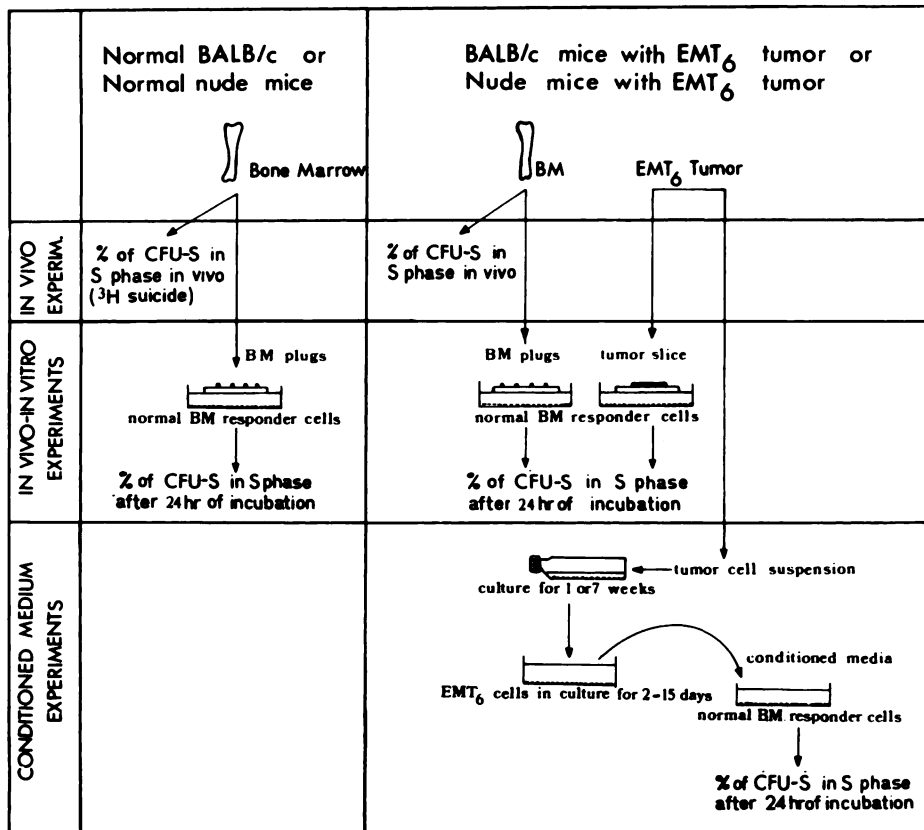


Chart 1. Experimental protocol. Three different procedures were used to test EMT<sub>6</sub> tumor influence on CFU-S kinetics.

Table 1  
Percentage of bone marrow CFU-S in DNA synthesis *in vivo* and in the responder population of the *in vivo-in vitro* experiments

	BALB/c mouse donors			Nude mouse donors			
	No. of experiments	% of CFU-S in S phase	$p^a$	No. of experiments and strain	% of CFU-S in S phase		$p$
					Individual experiment	Mean $\pm$ S.E.	
<i>In vivo</i> experiments							
Normal bone marrow	4	7.5 $\pm$ 4.5 <sup>b</sup>		3 (S) <sup>c</sup> (B) (B)	6 0 0	2.0 $\pm$ 2.0	NS
Bone marrow from 100-cu mm tumor-bearing mice	4	43.2 $\pm$ 10.1	<0.02	3 (S) (B) (B)	17 0 0		
<i>In vivo-in vitro</i> experiments							
Control (O/R cells)	4	10.2 $\pm$ 8.9					
NBM/R cells	4	7.2 $\pm$ 6.0	NS	3 (S)		7.7 $\pm$ 7.8	NS
EMT <sub>6</sub> BM/R cells	3	34.0 $\pm$ 9.1	<0.05	4 (S)		8.5 $\pm$ 6.4	NS
EMT <sub>6</sub> T/R cells	3	36.7 $\pm$ 6.2	<0.02	4 (S)		16.2 $\pm$ 2.0	NS

<sup>a</sup> t test.<sup>b</sup> Mean  $\pm$  S.E.<sup>c</sup> S, Swiss nude mice; B, BALB/c nude mice; NS, not significant; R cells, responder cells (always BALB/c mouse normal bone-marrow); NBM, normal bone marrow (BALB/c or nude mice); EMT<sub>6</sub> BM, bone marrow plugs from 100-cu mm EMT<sub>6</sub> tumor-bearing mice; EMT<sub>6</sub> T, slice of EMT<sub>6</sub> tumor (BALB/c or nude mice).

tumor-bearing mice were placed on the filter, 34.0% of the responder CFU-S were in cycle. This confirms our previous results.

However, when tumor fragments from nude mice or bone marrow from tumor-bearing nude mice were placed on the filter, the responder CFU-S cell population remained quiescent (16.2 and 8.5% of CFU-S in S phase, respectively).

**Conditioned Medium from Normal Tissue of Tumor-bearing Mice.** Table 2 shows that CFU-S enter the S phase after 24 hr of incubation with conditioned medium from bone marrow of EMT<sub>6</sub>-bearing BALB/c mice [21.7  $\pm$  1.9% (S.E.)]. After incubation of normal medullary cells with conditioned medium obtained from peritoneal cavity cells of tumor-bearing mice, the CFU-S remain quiescent (5.7  $\pm$  4.9%).

**Conditioned Medium from EMT<sub>6</sub> Cells.** Table 3 shows that, 24 hr after incubation with conditioned media from exponential growth phase of primary EMT<sub>6</sub> cultures and from long-term cultures of EMT<sub>6</sub> cells from BALB/c mice, CFU-S of the responder population are triggered into cell cycle (34.7 and 30.7% CFU-S in S phase, respectively). When conditioned medium is harvested from tumors in plateau phase, there is no stimulatory effect (2.3%).

When conditioned medium is harvested from tumors growing in nude mice (BALB/c), there is no stimulatory effect whatever experimental protocol is used (4.0 and 4.1%).

As can be seen in Table 4, 8 of 10 clones of the EMT<sub>6</sub> cells from BALB/c tumors have the capacity to secrete a factor stimulating CFU-S proliferation, whereas none of the clones of the EMT<sub>6</sub> cells from nude mice tumors secrete this factor.

## DISCUSSION

It has been shown by several authors (6, 20, 22, 34) that tumors stimulate CFU-S of the host marrow. We have previously reported that only during the exponential growth of EMT<sub>6</sub> tumors did the tumors and the bone marrow of tumor-bearing mice have this stimulatory effect. We have also reported that this effect is obtained via a diffusible humoral factor only when tumors were in the exponential rate of tumor growth (14).

Table 2  
Effect of conditioned media from medullary cells and cells of peritoneal cavity on responder CFU-S proliferation

Conditioned media	No. of experiments	% of CFU-S in S phase	$p^a$
Control culture media	4	3.0 $\pm$ 3 <sup>b</sup>	
Conditioned media of medullary cells	3	21.7 $\pm$ 1.9	<0.01
Conditioned media of peritoneal cavity cells	4	5.7 $\pm$ 4.9	NS <sup>c</sup>

<sup>a</sup> t test.<sup>b</sup> Mean  $\pm$  S.E.<sup>c</sup> NS, not significant.

The cells responsible for the secretion of these factors could be the tumor cells, macrophages, lymphocytes, or a cooperation between these different cell types, since these cells exist simultaneously in the tumors. In the bone marrow of tumor-bearing mice, macrophages and lymphocytes may also be involved.

**Macrophages.** Our results seem to suggest that macrophages are not directly involved in the secretion of factors stimulating CFU-S. (a) Macrophages exist in the nude mice, and yet neither the bone marrow nor the tumor of these mice secretes a factor capable of stimulating CFU-S into cycle. This cannot exclude the possibility that in nude mice macrophages are functionally different from those of BALB/c mice. (b) Steward and Beetham (35) have shown that the proportion of macrophages in the EMT<sub>6</sub> tumor increases with the size of the tumor. If the macrophage was responsible for the secretion of a stimulating factor, this secretion should increase with tumor growth. The contrary is observed. Moreover, preliminary results using a tumor<sup>3</sup> largely infiltrated with macrophages show no stimulatory effect.

We have shown that conditioned medium from peritoneal cavity cells of tumor-bearing BALB/c mice was incapable of stimulating CFU-S into DNA synthesis.

(c) Experiments with pure clones of EMT<sub>6</sub> tumors have shown

<sup>3</sup> Unpublished observations.

Table 3

Capacity of tumor cells to secrete CFU-S-stimulating factor after elimination of infiltrating tumor cells

EMT<sub>6</sub> cells are obtained from solid tumors grown in either BALB/c mice or BALB/c nude mice. Primary cultures: exponential phase, conditioned medium harvested 24 hr to 7 days after plating dispersed tumor cells; plateau phase, conditioned medium harvested 10 to 15 days after plating dispersed tumor cells. Long-term cultures (6 to 8 passages every 7 days): exponential phase, conditioned medium harvested 24 hr to 7 days after the last passage. Plateau phase, conditioned medium harvested 10 to 15 days after the last passage.

	No. of ex- periments	% of CFU-S in S phase	<i>p</i> <sup>a</sup>
Control	4	4.7 ± 2.8 <sup>b</sup>	
Primary EMT <sub>6</sub> tumor-conditioned media			
BALB/c mice			
Exponential-phase culture	3	34.7 ± 11.6	<0.02
Plateau-phase culture	1	0	
Nude mice			
Exponential-phase culture	3	4.0 ± 4.0	NS <sup>c</sup>
Plateau-phase culture	1	14.0	
Control	9	6.9 ± 2.5	
Long-term EMT <sub>6</sub> tumor-conditioned media			
BALB/c mice			
Exponential-phase culture	6	30.7 ± 4.4	<0.001
Plateau-phase culture	3	2.3 ± 2.4	NS
Nude mice			
Exponential phase culture	4	4.1 ± 2.4	NS
Plateau-phase culture	1	0	

<sup>a</sup> *t* test.

<sup>b</sup> Mean ± S.E.

<sup>c</sup> NS, not significant.

Table 4

Effect of "pure" EMT<sub>6</sub> cell culture on medullary CFU-S-proliferative activity

	% of CFU-S in S phase
Control culture media	16-0
EMT <sub>6</sub> cell-conditioned media from	
BALB/c tumor	
Clone B1	26
Clone B2	58
Clone B3	37
Clone B4	29
Clone B5	24
Clone B6	4
Clone B7	34
Clone B8	57
Clone B9	28
Clone B10	0
Nude mouse tumor	
Clone N1	0
Clone N2	9
Clone N3	0
Clone N4	0
Clone N5	0

that stimulation occurs and that macrophages are not directly involved in the phenomenon.

**Lymphocytes.** Since EMT<sub>6</sub> tumors are known to have lymphocyte infiltration and since lymphocytes are also present in the bone marrow which stimulate CFU-S responder cells, they should be the logical candidates for the secretory cell population. In order to verify this, we did parallel experiments on EMT<sub>6</sub> tumors from nude mice, known to be deficient in mature T-cells. Our results show that neither EMT<sub>6</sub> tumors nor bone marrow from tumor-bearing nude mice is capable of stimulating responder CFU-S.

These results suggest that T-lymphocytes play a role in the secretory process either by actually secreting the factors that stimulate CFU-S or by inducing tumor cells to secrete the factors. It is also possible that both of these phenomena co-exist.

In order to distinguish between these 2 possibilities, long-

term tissue cultures were set up. At least 7 passages were performed before the conditioned medium was tested, i.e., at a time when lymphocytes were no longer present in the culture (13).

Our results show that the stimulating activity of conditioned medium is retained after 7 passages when EMT<sub>6</sub> cells from BALB/c mice were originally harvested during exponential growth. This suggests that the "purified" population of tumor cells secretes the factors. However, in order to have unequivocal data concerning the "purity" of the cells, we cloned the EMT<sub>6</sub> tumors. Each clone was then individually seeded in separate dishes and conditioned medium was tested. Results show that 8 of 10 clones were capable of secreting the stimulating factors, thus suggesting that the majority of the cells retain the "memory" of a T-cell cooperation. On the contrary, when conditioned medium from clones of EMT<sub>6</sub> tumors growing on nude mice were tested, there was no stimulatory effect. In this case, the possibility of a suppressor in the nude mice could be evoked.

The results suggest that EMT<sub>6</sub> tumor cells themselves are capable of secreting stimulatory factors and that T-lymphocytes are involved. However, their presence is not necessary for the tumor cells to secrete the factor once the "message" has been delivered. The fact that bone marrow from tumor-bearing mice also secretes a stimulatory factor may mean that T-lymphocytes themselves may secrete factors in tumor-bearing mice and/or that some tumor cells infiltrate the bone marrow. The former suggestion may mean simply that the tumor acts as an antigen.

In conclusion, it seems that the tumor cell itself is capable of secreting a stimulatory factor(s) and that the T-cell is implicated in the phenomenon but that its presence at the time of secretion is not necessary. The next question is whether the tumor cell may be "educated" by some as yet unknown mechanism to secrete diffusible factor(s) capable of stimulating CFU-S into cycle.



Subsequent papers<sup>4</sup> will report experiments that seem to imply that this type of "education" is possible.

## ACKNOWLEDGMENTS

We greatly appreciate the excellent technical assistance of F. Le Pesteur.

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# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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*Cancer Res* 1982;42:1922-1926.

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