

Fast- and Slow-Growing Transplantable Tumors Derived from Spontaneous Mammary Tumors of the DBA/2 Ha-DD Mouse¹

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SUMMARY

Nineteen transplantable tumor lines were established from individual spontaneous mammary tumors of the DBA/2 Ha-DD mouse. Of these lines, 5 were classified as fast growing, 8 as medium growing, and 6 as slow growing, based on the time required for the tumors to reach a size of 10 mm in average diameter and on the average survival time of tumor-bearing syngeneic hosts. The relative differences in rate of growth among 5 of these lines remained stable during 11 to 19 transplant generations. In DBA/2J mice, a slow-growing and a fast-growing tumor line were cross-immunogenic. The differences in growth rate between these 2 tumor lines were not primarily related to differences in immunogenicity since they were not abolished in preirradiated hosts. The growth of cell populations from these 2 tumor lines in culture was comparable; however, cells from the fast-growing line had a plating efficiency about 4 times higher than those from the slow-growing line.

INTRODUCTION

Slow-growing SMT's⁴ in mice may provide model systems for an evaluation of antitumor agents with potential activity against relatively slow-growing solid tumors in man. Indeed, in analogy with the latter, mouse SMT's, either in the autochthonous host or implanted s.c. in syngeneic hosts, have been found to be relatively resistant to chemotherapy (5, 8, 12, 13, 18-20).

SMT's develop in 80% of retired female breeders of the DBA/2 Ha-DD mouse. Tumors of this type were among those used previously in this laboratory in the evaluation of new drugs prior to their clinical trial (14, 16). The variability in tumor growth rate and in response to drugs observed in these initial studies made it desirable to define more precisely some of the characteristics of such systems.

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⁴The abbreviations used are: SMT, spontaneous mammary tumors; TD₅₀, tumor cell number for 50% take; SMT-S, slow-growing SMT subline (Subline 6 in Table 1); SMT-F, fast-growing SMT subline (Subline 9 in Table 1); FCS, fetal calf serum; MTV, mammary tumor virus.

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Following the observation that cells from this type of SMT can grow as monolayers in culture (V. Ujhazy, M. Kitano, J. M. Ehrke, and E. Mihich, unpublished data), it became possible not only to define tumors according to their growth rates and immunogenicity in DBA/2 mice but also to study their behavior in culture. A model comparing the responses of the same tumor line to a drug in mice and in culture would provide useful information on the intrinsic sensitivity of target cells to the drug as compared to the selectivity of drug action in the animal, which is conditioned by additional biological and pharmacological phenomena.

In this study, several sublines of SMT were classified according to their growth rates, which were found to be retained upon repeated transplantation in syngeneic hosts. The definition of certain characteristics of these tumor sublines in mice and in culture is the subject of this report. Some of these data have been presented in a preliminary communication (7).

MATERIALS AND METHODS

Mice. Retired DBA/2 Ha-DD mouse breeders bearing SMT were obtained from the breeding colony of the Roswell Park Memorial Institute within 1 week after tumor detection. Female DBA/2 Ha-DD and DBA/2J mice, 6 to 10 weeks old, were also obtained from this colony; these mouse sublines are both *H-2^d* but differ by other histocompatibility characteristics (15).

Tumor Transplantation. SMT's were dissected aseptically and necrotic portions were removed. Tumor fragments measuring approximately 1 mm in diameter were transplanted s.c. in the axillary region by standard trocar techniques. In experiments designed to determine the TD₅₀, cell suspensions in Earl's solution were prepared by mechanical procedures without enzyme treatment, according to the method described by Vaage (22). Cell viability was determined by the trypan blue exclusion method and, in various preparations, ranged between 10 and 50% of the total number of cells. Determination of the inoculum size was in each case based on viable cells only.

Tumor Growth in Mice. Tumor growth was measured by standard caliper techniques; the 2 longest diameters perpendicular to each other were used to determine the average tumor diameter. Growth curves were based on mean values derived from the average diameter of 5 to 10 tumors in each group. For the determination of survival, mice bearing tumors were observed until death or for 70 days after tumor implantation.

TD₅₀. Serial dilutions of suspensions viable cells differing by 10-fold were inoculated s.c. into syngeneic mice. Usually the tumor cell inoculum varied from 10² to 10⁶ cells/mouse. The TD₅₀ was derived graphically from a plot relating the number of mice that developed tumors within 90 days from the day of tumor inoculation to the size of the inoculum.

Immunization of DBA/2J Mice. Cell suspensions from subline SMT-F (Subline 9 in Table 1) were prepared as described above, and 1 × 10⁶ viable cells were inoculated s.c. into the right axillary region of each mouse. The immunization with the subline SMT-S (Subline 6 in Table 1) was carried out by implanting tumor fragments s.c. into the right axillary region. Animals in which the tumor grew initially and was later rejected were considered "immunized" against SMT-F and SMT-S.

Tumor Cells in Tissue Culture. Eagle's minimum essential medium with Earl's balanced salt solution enriched 3 times in its content of amino acids and vitamins (3 times Eagle's) was used for the tissue culture studies. Tumor tissue free of obviously necrotic parts was minced with small scissors and was placed in a mixture of 1 part of 3 times Eagle's medium and 1 part of 0.25% trypsin solution (Grand Island Biological Co., Grand Island, N. Y.); trypsinization was carried out at 37° with gentle stirring for 60 min. The suspension was centrifuged at 150 × g and the cells were washed twice in 3 times Eagle's medium with 10% FCS. Cell viability in the final suspension was determined by the trypan blue exclusion method and was found to range between 50 and 70%.

Viable cells in predetermined numbers were inoculated into 35-mm Falcon 3001 plastic Petri dishes in a total volume of 2 ml/dish of 3 times Eagle's medium with 10% FCS and were incubated at 37° under 5% CO₂ in air. At different times after the beginning of the incubation, 3 Petri dishes in each experimental group were randomly selected for cell counts. After discarding the medium and washing once, the cells that remained attached to the bottom of the dishes were treated for 15 to 20 min with 0.25% trypsin solution containing 1% EDTA at 37°. These cells were then counted separately in each dish, and in each case more than 95% of the cells were found to be viable by the trypan blue exclusion method. The cells counted at 24 hr were considered as the cell number at 0 time. Thereafter, cell counting was performed every 24 hr.

Determination of Plating Efficiency. Viable tumor cells (10³) in suspensions prepared as described above were plated into 35-mm Petri dishes containing 2 ml of 3 times Eagle's medium with 10% FCS and incubated at 37° under 5% CO₂ in air. Four days later the dishes were washed carefully with 0.9% NaCl solution and the cells were fixed with absolute methanol and stained with Giemsa. Plaques of more than 8 cells were counted under the microscope independently by 2 investigators, and the average values were used since the individual measurements did not vary by more than 5%. The plating efficiency was calculated as the percentage of the average number of plaques in 3 or 4 dishes relative to the number of cells inoculated.

Histological Examination. Tumor tissue slides were prepared according to standard histological techniques and were stained with hematoxylin and eosin.

Total-Body Irradiation. Total-body irradiation was administered by a Model 250 General Electric Maxitron machine. The dose of 250 R was calculated in air and was given at 250 kVp and 30 ma. A filter of 0.5-mm copper and 1.0-mm aluminum was used. Mice were confined in polyethylene plastic containers with adequate air supply and were placed at a skin target distance of 30 cm.

RESULTS

Classification of Transplantable SMT. Primary SMT's from 19 DBA/2 Ha-DD mice, each bearing a single tumor, were transplanted into syngeneic female mice. All of these tumors could be established as transplantable lines, and their characteristics were investigated during the 1st 4 transplant generations according to the following parameters: the day when the tumor reached a size greater than 10 mm in average diameter, the mean survival time of host mice and TD₅₀. As shown in Table 1, 5 lines were classified as fast-growing tumors, 6 as slow-growing tumors, and the remaining 8 as medium-growing tumors. Significant differences were noted between the fast and slow lines in terms of time required for the tumor to reach a size greater than 10 mm in average diameter and of mean survival time of their host. There was no major difference among fast, medium, and slow lines in terms of TD₅₀. However, 2 of the fast lines (lines 9 and 15) required only 1 × 10³ and 5.6 × 10² cells,

Table 1
Growth characteristics of 19 lines of SMT in DBA/2 Ha-DD mice

Line of SMT	Tumor > 10 mm in av. diameter on Day ^a	Mean survival (days)	TD ₅₀ ^b	Classification
1	16	39 ± 1 ^c	4.7 ± 10 ⁴	F ^d
9	18	28 ± 11	1.0 × 10 ³	F
14	15	34 ± 8	1.0 × 10 ⁶	F
15	19	31 ± 6	5.6 × 10 ²	F
18	20	46 ± 10	2.4 × 10 ⁵	F
2	26	> 70	4.2 × 10 ³	M
3	28	> 70	4.2 × 10 ³	M
7	24	49 ± 10	4.1 × 10 ⁵	M
8	23	39 ± 15	6.8 × 10 ³	M
10	22	33 ± 6	4.2 × 10 ³	M
13	20	48 ± 6	NT	M
17	22	44 ± 4	3.7 × 10 ⁴	M
19	21	55 ± 13	2.4 × 10 ⁴	M
4	55	> 70	4.2 × 10 ⁴	S
5	31	53 ± 12	3.2 × 10 ⁴	S
6	36	57 ± 4	1.2 × 10 ⁵	S
11	38	70	3.2 × 10 ⁴	S
12	31	54 ± 17	3.5 × 10 ⁴	S
16	36	> 70	1.9 × 10 ⁴	S

^a Counted from the day of implantation, Day 0. This size was visualized on a growth curve that was based on average tumor diameters from 10 to 20 individual tumors within 2 to 4 successive transplant generations (5 tumors for each transplant generation). Transplantation s.c. was by standard trocar technique.

^b Average values based on 2 to 4 transplant generations as above.

^c Mean ± S.D. Counted from the day of implantation. The animals were kept under observations for 70 days. Average values based on 2 to 4 transplant generations as above.

^d F, fast growing; M, growing at a medium rate; S, slow growing; NT, not tested.

respectively, for 50% tumor take, in contrast with the 4×10^3 to 4×10^5 cells required by all the other lines. Line 4 had the slowest initial growth rate of all the lines examined.

Stability of the Growth Rate of SMT. Lines 9 and 15 (fast), lines 6 and 12 (slow), and Line 8 (medium) were kept for further comparison. The relative differences in the rates of growth of these tumors were retained in subsequent transplant generations. The growth curves of slow line 6 and of fast line 9 are shown in Chart 1. Although for both lines the growth rate was somewhat accelerated in subsequent transplant generations relative to that after the 1st to 3rd transplantations, the relative differences between these 2 lines were retained over a period of 11 to 19 transplant generations. In view of the consistency of behavior of the 5 lines studied, only lines 6 and 9 were selected for further investigation, and these are referred to as SMT-S and SMT-F, respectively.

Evidence for Cross-immunogenicity between SMT-F and SMT-S in Allogeneic Mice. After transplantation of tumor fragments by trocar, SMT-F grows progressively in both DBA/2 Ha-DD and DBA/2J mice, whereas SMT-S does not grow in DBA/2J mice, which differ from the DBA/2 Ha-DD mice by non-H-2 characteristics. SMT-F is also rejected in a certain proportion of DBA/2J mice, however, after implantation of 1×10^6 viable cells in suspension. In a preliminary experiment it was found that, of 28 DBA/2J mice given implants of SMT-F, 16 ultimately died bearing large tumors. In the remaining 12 mice, the tumor grew initially for 10 days, reaching a maximum size of 8 mm in average diameter, and regressed thereafter, with complete rejection occurring within 20 days. All the 20 mice implanted with SMT-S rejected the tumors completely within 20 days after the tumors had grown initially to reach a maximum size of 6 mm in average diameter on Day 10. Mice that rejected either tumor were resistant to reimplantation of the same tumor. In view of these results, cross-immunogenicity between the 2 tumor lines was studied in DBA/2J mice by evaluating the effects of immunization with SMT-F or SMT-S on the subsequent growth of each of these lines. As shown in Table 2, groups of immunized mice

were also given total-body irradiation prior to implantation of challenge tumor. Since SMT-S grows progressively in most DBA/2J mice only if these are immunosuppressed by total-body irradiation, it is possible to measure preexisting immunity only in such hosts, taking advantage of the fact that, in contrast to primary responses, established immunity is known to be relatively resistant to immunosuppression.

In irradiated mice that had been immunized with either SMT-F or SMT-S, SMT-S was rejected more rapidly than in nonimmunized, nonirradiated mice, and no differences could be noted between the 2 immunized groups. In nonimmunized, irradiated mice, this tumor grew progressively in most cases. After implantation of SMT-F, the mice died in about 32 days and had large tumors at the time of death (data not shown). In the irradiated mice, tumor-related death occurred within 20 days. In irradiated mice that had been immunized with either SMT-F or SMT-S, survival was prolonged and not all the mice died. As a whole, the results shown in Table 2, which were confirmed

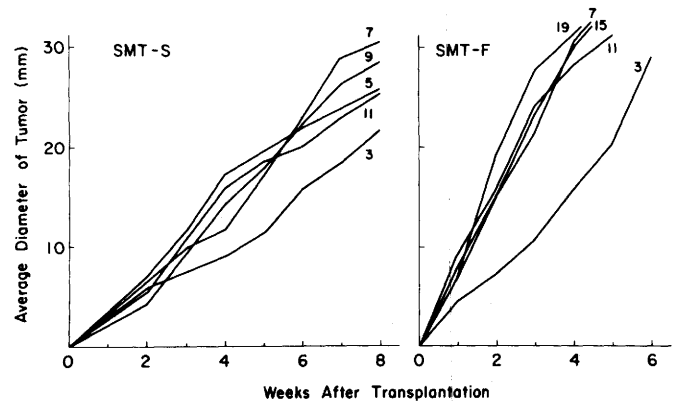


Chart 1. Growth curves of slow-growing Line 6 (SMT-S) and of fast-growing Line 9 (SMT-F) at different transplant generations in DBA/2 Ha-DD female mice (transplantation by standard trocar techniques). Each graph represents the average values from 5 mice. Numbers at the last weekly measurement indicate the successive transplant generation plotted and are counted from the 1st transplantation, which was transplant Generation 1.

Table 2
Effect of immunization on growth of SMT-F and SMT-S in DBA/2J mice

Tumor line used for immunization ^a	Tumor line used for challenge ^b	Irradiated mice ^c			Nonirradiated mice		
		Dead/total	% dead	Mean survival (days) ^d	Dead/total	% dead	Mean survival (days)
SMT-F	SMT-F	4/6	67	33 ± 13 ^e	NT ^f		
SMT-S		6/10	60	39 ± 13	0/5	0	
None		10/10	100	20 ± 8	25/30	83	32 ± 8
SMT-F	SMT-S	0/6	0		NT		
SMT-S		0/10	0		0/5	0	
None		8/10	80	59 ± 30	0/29	0	

^a Immunized mice are those that had rejected SMT-F or SMT-S (see text).

^b SMT-F and SMT-S were implanted s.c. in the left axillary region by standard trocar technique 21 days after immunization.

^c Total-body irradiation (250 R) was given the day prior to implantation of the challenge tumor.

^d Day following the day of challenge tumor implantation.

^e Mean ± S.D.

^f NT, not tested.

in another experiment not shown, indicate that SMT-S and SMT-F are cross-immunogenic in DBA/2J mice, at least within the limits of sensitivity of the transplantation system used.

Effect of Total-Body Irradiation on the Growth of SMT-F and SMT-S in Syngeneic Mice. The possibility that the differences in rate of growth between these 2 lines are due to major quantitative differences in immunogenicity was also examined. Viable cells (1×10^6) from either SMT-F or SMT-S were inoculated s.c. into syngeneic DBA/2 Ha-DD mice that had been given, or not given, 250 R total-body irradiation the day prior to tumor implantation. As shown in Chart 2, SMT-F grew in syngeneic mice at similar rates regardless of irradiation. In contrast, SMT-S grew somewhat more rapidly in the preirradiated mice, the differences in average diameter of tumors being statistically significant ($p < 0.05$) between Days 29 and 40. In comparing the slopes of the growth curves from Day 0 for SMT-F and from Day 18 for SMT-S, it appears that irradiation had no effect in the case of SMT-F (1.33 without X-ray and 1.26 with X-ray) but had an effect in the case of SMT-S (0.56 without X-ray and 0.72 with X-ray). In the case of SMT-S, the slopes were statistically different ($p < 0.001$). The differences in slopes between the tumor lines appear relatively minor, however, in comparison with the major differences in onset of measurable tumor growth that are due to the long latency period of SMT-S. In view of the major overall differences between the 2 lines that were seen also in preirradiated mice, it seems unlikely that the differences in biological behavior between SMT-S and SMT-F are due primarily to a greater immunogenicity of SMT-S cells.

Morphological Characteristics of SMT-F and SMT-S. Macroscopically, SMT-F is soft and fragile, with the center becoming readily necrotic during growth; in contrast,

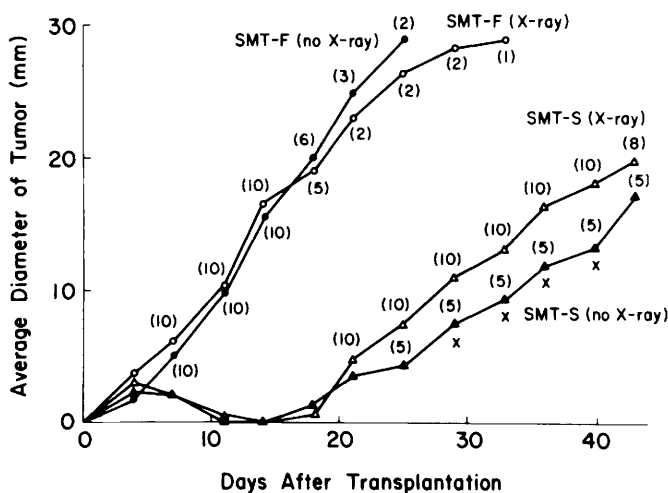


Chart 2. Effect of total body X-irradiation on the growth of SMT-S and SMT-F in syngeneic DBA/2Ha-DD female mice. Transplantation was by the s.c. inoculation of 10^6 viable tumor cells. Tumor cell suspensions were prepared by mechanical procedures without using trypsin (see "Materials and Methods" for details). Numbers in parentheses, number of mice surviving at each time point. The differences in average tumor size between SMT-S (X-ray) and SMT-S (no X-ray) curves were statistically significant ($p < 0.05$) between Days 29 and 40, and the corresponding time points are marked by x.

SMT-S is a hard tumor, and no necrotic portion is usually observed. No cystic formation was observed in either case. No metastasis was found with either tumor. The histological studies carried out at the 4th and 13th transplant generations for SMT-F and at the 2nd and 7th transplant generations for SMT-S did not reveal any qualitative difference between these tumors, both of which can be considered as type B solid adenocarcinoma according to the classification of Dunn (4). No infiltration of lymphocytes was noted in either tumor. An electron microscopic examination performed through the courtesy of Dr. U. Kim of the Roswell Park Memorial Institute showed that these tumors contain typical B-type virus particles, which are characteristic of MTV, as reported by Dalton (3).

Growth of SMT-F and SMT-S Cells in Culture. In an attempt to evaluate further the basis for the differences in growth observed in mice, the 2 lines of SMT were studied in tissue culture. The growth rates of SMT-F and SMT-S cells under these conditions are shown in Chart 3. The inoculum into each Petri dish was 5×10^5 cells for SMT-S and 2.5×10^5 cells for SMT-F; these numbers were based on the results of preliminary tests in which the appropriate inoculum size for each tumor in similar dishes had been determined. The dotted straight lines shown in Chart 3 are theoretical lines corresponding to a cell population doubling time of 48 hr. The growth curves of both SMT-S and SMT-F were not too dissimilar from this theoretical line, suggesting that the doubling time in culture for both of these cell lines is close to 48 hr. This similarity is at variance with the differences in rate of tumor growth observed in mice.

Plating Efficiency of SMT-F and SMT-S. The plating efficiency of these 2 lines is shown in Table 3. The mean plating efficiency of SMT-S cells was 4 times less than that of SMT-F cells. In each experiment, both cell lines were used in parallel so that the values of plating efficiency obtained could be compared directly. In each individual experiment, the plating efficiency of SMT-F cells was greater than that of SMT-S. A greater variation in plating efficiency was noted among experiments in Group A however, for which different batches of FCS were used, than among those in Group B, for which the same batch of FCS was used, suggesting that the plating efficiency was affected not only by the nature of the tumor cells tested but also by the conditions of the culture.

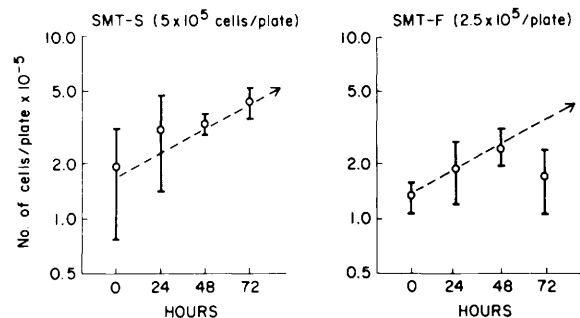


Chart 3. Growth in culture of cells from SMT-S and SMT-F. Points, mean cell counts derived from a total of 6 dishes in 2 experiments. Vertical bars, S.D. from the mean. - - -, theoretical curve based on an average cell number doubling time of 48 hr.

DISCUSSION

Of 19 transplantable lines derived from individual SMT's of the DBA/2 Ha-DD mouse, 5 were classified as fast-growing tumors, 8 as medium-growing tumors, and 6 as slow-growing tumors. The relative differences in rate of growth among 5 of these lines remained stable upon repeated transplantation in syngeneic mice. Two of these lines, a slow (SMT-S)- and a fast (SMT-F)-growing tumor, were studied further.

In transplantation experiments, SMT-F and SMT-S were found to be cross-immunogenic in allogeneic DBA/2J mice. The immunogenicity of these 2 tumor lines in this mouse is probably related to histocompatibility differences at loci other than *H-2* that occur between DBA/2 Ha-DD and DBA/2J mice but may be also due, at least in part, to MTV-related antigen (1, 2). Indeed, electron microscopic evidence for MTV had been obtained in the tumor studied; in addition, the DBA/2J mouse used has a relatively low incidence of SMT (0 to 50% of breeders) and thus could react to MTV antigen (6, 21). The possibility that these 2 lines of SMT also have cross-reacting, non-virus-related antigens cannot be excluded. These antigens have been detected by others in MTV-positive mice (10, 17, 23, 24) and in autochthonous hosts (25). Indeed, some immunity against SMT-S was apparent in the syngeneic DBA/2 Ha-DD mouse, as implied by the slight, but significant, differences in growth rates noted between nonirradiated and irradiated hosts.

SMT-S appeared to be somewhat more antigenic than did SMT-F in syngeneic mice, as indicated by the fact that SMT-S required more cells for TD_{50} than did SMT-F, and the growth of SMT-S, but not that of SMT-F, was accelerated in preirradiated hosts. Since major differences between SMT-F and SMT-S were also observed in preirradiated mice, however, they are probably not due primarily to a greater immunogenicity of SMT-S. In tissue culture studies, SMT-F and SMT-S cells grew at almost the same rate, with a doubling time of about 48 hr. Yet, when 1 to 5×10^6 of these cultured cells were implanted s.c. in syngeneic mice, their rates of growth still remained as different as the rates of growth of corresponding tumors that had been kept in mice continuously (unpublished data).

Nutritional factors may be a basis for the differences observed. SMT-F may require a smaller amount of an essential nutrient than does SMT-S, and this may become apparent in mice. Nevertheless, this possibly seems inconsistent with the reproducible finding that, at 72 hr, a large number of SMT-F cells dies in culture, in contrast with the lack of such finding for SMT-S cells (see Chart 3). Moreover, in the mouse, gross central tumor necrosis invariably occurs in SMT-F but not in SMT-S; this difference may be due to a relative inadequacy of nutrients supplied through vascularization (11) and would be consistent with a greater requirement for nutrients by SMT-F or with differences in vascularization between the 2 tumors.

The plating efficiency of SMT-F was found to be 4 times higher than that of SMT-S. This finding, and the similarities in growth rates in culture, would be consistent with the possibility that cells with greater "oncogenic potential" (9),

Table 3

Plating efficiency of SMT in primary culture

The inoculum was 1×10^8 cells/dish. A plaque was considered to be formed by a cluster of at least 8 cells. Plaques were counted 4 days after plating. The percentage of plaques values are based on the number of viable cells plated.

Group	% plaques	
	SMT-S	SMT-F
A ^a	0.66 ± 0.41 ^b (0.25-1.30) ^c	2.31 ± 1.65 (0.53-4.70)
B	0.65 ± 0.28 (0.40-0.87)	3.08 ± 1.44 (1.57-4.00)
A + B	0.65 ± 0.35 (0.25-1.30)	2.56 ± 1.4 (0.53-4.70)

^a A, 6 experiments carried out using different lots of FCS; B, 3 experiments carried out using the same lot of FCS.

^b Mean ± S.D.

^c Values in parentheses, minimum and maximum values obtained.

possibly present among SMT-F cell subpopulations, have the same doubling time as "similar" cells possibly present in SMT-S but that the frequency of cells with greater oncogenic potential is higher in SMT-F than in SMT-S, thus conditioning differences in overall tumor growth in mice. This hypothesis requires verification by cell kinetic studies *in vivo*.

In conclusion, the differences between the 2 lines of SMT established in DBA/2 Ha-DD mice are possibly due to the convergence of 2 phenomena, namely, a greater immunogenicity of cells from the slow line and a higher incidence of so-called "more oncogenic" cells in the fast line.

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