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Effect of Splenectomy on the Progression of Postoperative Pulmonary Metastases of the 3LL Tumor*

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Abstract—*Surgical excision of the local intrafootpad tumor of the 3LL lung carcinoma is followed by accelerated growth of its lung metastases. When, however, splenectomy was performed concomitantly with tumor excision, the acceleration of lung metastases was prevented. In cases where excision of the local tumor took place when it reached large sizes, concomitant splenectomy did not prevent the accelerated growth of the lung metastases. If, however, at these stages of tumor growth splenectomy was performed 3 days prior to the excision of the tumor, it did prevent the accelerated growth of metastases. Intrafootpad reinoculation of tumor cells following tumor excision and splenectomy caused further reduction in metastatic growth. The results suggest the existence of two possible distinct mechanisms which control metastatic growth: the local tumor might exert non-immunologically, an inhibitory effect on its lung metastases, and the spleen, possibly via suppressor lymphocytes, may suppress an immune effector activity against the tumor metastases, an activity which is manifested following splenectomy.*

INTRODUCTION

IN OUR previous studies we found that the surgical removal of the local 3LL Lewis lung carcinoma or B16 melanoma grown in syngeneic recipients led to an accelerated growth of lung metastases [1]. This accelerated growth of metastases in the tumor-excised mice was prevented if the tumor cells were reinoculated to the other footpad. Thus, the local tumor seems to exert a suppressive effect on its metastases. This effect seemed to have a non-immune component since it was manifested also in immune suppressed animals [2].

In these studies we observed that the local growth of the 3LL tumor was associated with a dramatic splenomegaly of the tumor-bearing mice. Spleen weights of such mice were higher by a factor of 3–4 compared to the spleens of normal mice. Such splenomegaly did not appear in tumor-excised mice.

Splenomegaly accompanying tumor growth has been reported by others [3–5]. It seemed to reflect both quantitative and qualitative shifts in the cellular composition of the lymphoid subpopulations similar to those occurring during antigenic stimulation [4–6]. The nature of the immune reactivities of the enlarged spleen in tumor-bearing animals is not clear. Thus, it was shown that such spleen cells, inoculated into syngeneic recipients together with tumor cells, can inhibit or enhance tumor growth depending on the stage of tumor development in the donor animals [7, 8]. Adult splenectomy was reported to cause inhibition of growth of a transplantable syngeneic tumor [9, 10] and a faster regression rate of allogeneic tumors [11]. The inhibition of tumor growth was generally attributed to the depletion of spleen cells which generate enhancing antibodies [12, 13], or to the depletion of suppressor cells [14].

Although a considerable amount of data has accumulated concerning the possible role of the spleen in controlling local tumor growth, almost no information concerning the possible involvement of the spleen in regulating metastasis spread is available. Such information might be of clinical relevance because of the abundance of elective splenectomy operations in cancer patients [15].

In this paper we report on studies on the

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Abbreviations used in this paper: [^{125}I]-UdR—^{125}iodo-deoxyuridine; FUdR—fluorodeoxyuridine; TBM—tumor-bearing mice; splx—splenectomy.

effect of splenectomy on the development of lung metastases in tumor-bearing and tumor-excised animals. We demonstrate that the accelerated metastatic growth observed following excision of the local tumor is prevented if splenectomy is performed simultaneously with tumor surgery. This suggests that two distinct contrasting mechanisms control metastatic growth: a non-immune suppression of metastasis exerted by the local tumor and immune enhancement of metastasis mediated by spleen suppressor cells, which may suppress anti-tumor effector lymphocytes.

MATERIALS AND METHODS

Animals

Inbred 6 to 8-week-old male and female C57BL/6 mice were supplied by the Animal Breeding Center of the Weizmann Institute.

Tumors

All experiments were done with the Lewis lung carcinoma (3LL). Single cell suspensions were prepared from solid tumors by treatment of minced tissue with a 0.3% trypsin solution (hog pancreas: Nutritional Biochemicals Corp., Cleveland, OH). After washings, the viability of the injected tumor cells, determined by dye exclusion, was 95–99%. In all experiments 10^5 3LL tumor cells were inoculated into the right hind footpad. Tumor size was measured by caliper and the volume calculated.

Splenectomy

Splenectomy was performed under nembutal anesthesia (60 mg veterinary nembutal per kg body weight). The solution injected contained 6 mg nembutal per 1 ml diluted in PBS. The spleen excision was made after heat ligation of the efferent and afferent blood vessels. Sham operations include all the above steps except spleen excision, and were done to all the control groups.

Tumor excision

Excisions were performed by amputating the tumor-bearing leg after ligation above the knee joint.

Metastasis development assay

Lung metastases were examined 10–13 days after tumor excision (as indicated). Because of the rapid and mostly confluent growth of lung metastases after tumor excision, it was impossible to count individual foci. To determine metastatic growth, we measured lung weights and the extent of ^{125}iodo-deoxyuridine ([^{125}I]UdR) incorporation into the replicating cells in the lungs. [^{125}I]UdR incorporation was performed according to Bonmassar *et al.* [16]. Twenty-four hr before the mice were killed, all animals (including intact controls) were injected with 25 μg/mouse of fluorodeoxyuridine (UFdR) intraperitoneally and 30 min later with 1 μCi/mouse [^{125}I]UdR (Radiochemical Center, Amersham, England; specific activity 5 Ci/mg). The degree of radioactivity incorporated to the lungs was measured using a Packard gamma spectrometer.

Statistics

The spread of metastases does not follow a normal distribution, probably because this process involves many independent biological factors. Although we used 8–13 animals per group, it happened that one or two mice gave extreme results.

If these were to be omitted, then the conventional *t*-test could be used. Yet, on the basis of statistical advice, we decided to include all the experimental results and employ a non-parametric significance test. We used the Kruskal–Wallis test and then determined the significance in a multiple comparisons test.

RESULTS

The first set of experiments was designed to investigate the effect of splenectomy on the development of metastases in tumor-bearing and tumor-excised mice. Both splenectomy and tumor excision were performed in animals bearing tumors of different sizes. Fourteen days following inoculation with 1×10^5 3LL tumor cells, mice were divided into three groups according to tumor size, as indicated in Table 1. Mice were either amputated or splenectomized, and one group underwent both tumor excision and splenectomy.

An acceleration of metastatic development was observed in the lungs of tumor-excised mice. When, however, amputation and splenectomy were performed simultaneously, the otherwise observed increase in metastatic growth following tumor excision was found only in the group of mice carrying large-size tumors (365–665 mm^3). The groups of mice possessing tumors of smaller size (108–256 mm^3) failed to show the otherwise observed increase in metastatic growth manifested following tumor excision.

We repeated this experiment using an additional method of determining the degree of metastatic growth, that of measuring the level of [^{125}I]UdR incorporation by replicating metastatic cells in the lungs (Table 1, Experiment 2). Tumor excision alone resulted in a

Table 1. Effect of simultaneous splenectomy and tumor excision on metastatic progression

No. of group	Treatment Splenectomy	Treatment Amputation	Experiment 1 108–256 mm³ tumors* Weight of lungs (mg)	Experiment 1 258–364 mm³ tumors* Weight of lungs (mg)	Experiment 1 365–665 mm³ tumors* Weight of lungs (mg)	Experiment 2 62–171 mm³ tumors* Weight of lungs (mg)	Experiment 2 62–171 mm³ tumors* CPM
1	–	–	250	261	280	236	3022
2	–	+	385†	405†	348‡	379†	14026†
3	+	–	262	300	265	234	3153
4	+	+	276	348	380	284	3544
	Intact mice					195	647

10^5 3LL tumor cells were inoculated into the hind right footpad of 6-week-old C57B1 females. When local tumors reached a certain size, animals were splenectomized, amputated, or underwent both treatments simultaneously. Ten animals were used in each tumor size groups. Experiment 1: weight of lungs with metastases was determined 10 days following tumor excision. Experiment 2: 10 days following tumor excision, mice were inoculated with 25 μg/mouse [^{125}I]UdR. Twenty-four hours later, the weight of lungs and level of radioactivity were determined.

*Size of amputated tumors.

†Significantly different from groups 1, 3 and 4 ($P < 0.05$).

‡Significantly different from groups 1 and 3 ($P < 0.05$).

marked increase in both the weight of lungs and level of [^{125}I]UdR uptake. When splenectomy was performed simultaneously with amputation, the increase in weight of lungs and [^{125}I]UdR uptake was completely abolished. In all experimental groups one finds a good correlation between the weight of lungs and [^{125}I]UdR uptake. The effect of splenectomy on subsequent progression of postoperative metastases as a function of the local tumor size is of particular interest regarding possible immune mechanisms controlled by the spleen. In order to test if the loss of the suppressive effect of splenectomy in animals bearing large tumors is due to changes in the tumor cell population or changes taking place in the spleen itself, the following experiments were performed: normal mice were inoculated into the hind footpad with 1×10^5 3LL tumor cells. Some of these animals were splenectomized when the tumor was 63–108 mm³ in volume and amputation was performed 3 days later, when the tumor had reached 365–515 mm³ in volume. Thirteen days later the mice were killed and their lungs were examined. Table 2 indicates that when splenectomy was performed 3 days prior to tumor excision, the acceleration of metastasis development was almost completely abolished, even though the tumors were permitted to grow to the size of 365–515 mm³ in volume. These results suggest that the failure to inhibit the progression of metastasis results from a functional failure in the spleen-derived effector cells rather than from changes in the resistance to immune injury of the tumor cells in large, developed metastatic foci.

In our previous work [1] we showed that one can abrogate the postsurgical acceleration of metastatic growth by reinoculation of live 3LL tumor cells into the tumor-excised animals. Therefore, the next set of experiments was designed to test whether the two phenomena,

Table 2. Splenectomy prior to excision of large tumor prevents accelerated progression of metastases

Treatment Splenectomy	Treatment Amputation	No. of mice per group	Weight of lungs (mg)
–	–	10	222
–	+	12	646*
+	–	9	256
+	+	13	406*

Splenectomy was performed when local tumors reached 63–108 mm³ in volume. Amputation was performed 3 days later, when tumors were 365–515 mm³ in volume. Lungs were examined 13 days following amputation.

*$P < 0.02$ in the comparison between the two groups marked with *.

Table 3. The effect of splenectomy and/or reinoculation of fresh tumor cells on the progression of lung metastases following excision of the primary tumor

No. of group	Treatment: Amputation	Splenectomy	Reinoculation	Mice per group: Experiment 1	Experiment 2	Volume of reinoculated tumor (mm^3): Experiment 1 mean ± S.E.	Experiment 2 mean ± S.E.	Weight of lungs (mg): Experiment 1	Experiment 2	[^{125}I]UdR uptake† CPM: Experiment 1	Experiment 2
1	−	−	−*	10	10	—	—	258	243	1995	1361
2	−	+	−	8	10	—	—	275	300	2661	2615
3	+	−	−	9	15	—	—	554‡	417‡	8842‡	4575‡
4	+	+	−	8	12	—	—	464§	343§	4686§	2443§
5	+	−	+	9	11	684 ± 92	579 ± 57	463§	303§	5351(4263)§	2560(1008)§
6	+	+	+	9	12	429 ± 59	256 ± 30	362‖	241‖	3058(1970)‖	1811(347)‖
7	Intact mice		+	12	10	1000 ± 119	1098 ± 135	189	182	1088	1464
8	Normal mice		−	—	7	—	—	—	191	—	499

Mice were injected with 10^5 3LL tumor cells into the right hind footpad. Amputation and splenectomy were performed simultaneously when tumor reached 63–108 mm^3 in volume. Some of the mice (as indicated in the table) were reinoculated with 3×10^6 3LL tumor cells into the other footpad. Eleven days later all mice were killed and their lungs assayed for the presence of metastases.

*These control animals underwent amputation of their right healthy leg.

†The numbers in parentheses are the calculated net numbers. They are obtained by deducting the number of CPM of the intact, reinoculated mice from the relevant CPM.

‡Significantly different from groups 1 and 2 ($P < 0.05$).

§Significantly different from group 3 ($P < 0.05$).

‖Significantly different from groups 4 and 5 ($P < 0.05$).

splenectomy and reinoculation, are related, i.e. if the reduction in metastatic growth following reinoculation of a second tumor is dependent on the presence of a functional spleen.

Mice were inoculated with 10^5 tumor cells into the right hind footpad. When tumors reached 63–168 mm^3 in volume, amputation, splenectomy and reinoculation of 3×10^6 tumor cells to the other footpad were performed in different combinations, as shown in Table 3. The results summarized in this table demonstrated again that amputation of the tumor-bearing leg caused a marked increase in metastatic growth and that this increase was abolished by either splenectomy or reinoculation of fresh tumor cells. When splenectomy and reinoculation of tumor cells were performed simultaneously, a clear additive inhibitory effect was observed, leading to a dramatic reduction in the weight of the lungs and [^{125}I]UdR uptake by the metastases in the lungs.

In the tumor-excised, reinoculated mice, some of the metastatic foci in the lungs have probably derived from the reinoculated tumor. In intact mice inoculated with the same number of tumor cells (3×10^6), a large tumor develops (1000–1098 mm^3) and the [^{125}I]UdR uptake is 1088–1464 cpm (vs 499 cpm in normal mice). If one calculates the 'net' level of radioactivity in the lungs of the tumor-excised, splenectomized and reinoculated mice, the inhibitory effect on the metastatic growth seems even more dramatic (Table 3). The growth of the local reinoculated tumor is also much slower in the splenectomized mice, and to a lesser extent in the non-splenectomized mice, when compared to the growth of the same number of tumor cells inoculated in intact mice. These results suggest that the inhibitory effects alleviated by excising the first local tumor and by splenectomy are due to two different mechanisms.

DISCUSSION

We have previously demonstrated that the surgical excision of the local 3LL tumor was followed by an accelerated growth of its lung metastasis. This accelerated metastatic growth was prevented if, following tumor excision, cells of the local tumor were reinoculated. It was, therefore, deduced that the local tumor exerts an inhibitory effect on its metastasis. Had this inhibition of metastatic growth been mediated just via the immune system, local tumors grown in immune suppressed animals should not exert an inhibitory effect on the growth of its lung metastasis. We demonstrated [2] that in immune suppressed animals, the growth of lung metastasis is significantly accelerated, thus indicating that host immune reactivity does control metastatic progression. Yet, surgical excision of the local tumor in such animals was followed by further acceleration of metastatic growth [2]. It thus appeared that the growth-suppressing effect which the local tumor exerts on its metastasis has a non-immune component. In the present study we demonstrated that splenectomy performed simultaneously with the excision of the local tumor prevented the otherwise accelerated growth of metastasis. It appears, therefore, that metastatic progression as a function of the local growth is controlled by two distinct mechanisms: a non-T cell, cell-mediated suppression of metastasis by the local tumor and a possible concomitant suppression of effector cytotoxic T cells by spleen suppressor cells. Splenectomy, in depleting the suppressor cell activity, may result in unmasking effector T cell activity which in turn inhibits metastatic growth. Splenectomy thus counteracts, by elevating the host's immune reactivity, the acceleration of metastatic growth otherwise observed after local tumor excision. Since the suppressing effect exerted by the local tumor may not be mediated by the immune system [17, 18], one would not expect it to depend on spleen cells. Indeed, we observed in the present study that following splenectomy and tumor excision, reinoculation of tumor cells resulted in further inhibition of metastatic growth. Sugarbaker suggested that the local tumor may secrete factors which inhibit non-immunologically metastatic growth [17]. Yet the actual existence of such factors remains to be demonstrated.

The involvement of the spleen in immune reactivity associated with tumor growth is well documented and tumor growth in experimental animals is usually accompanied by a marked splenomegaly with a concomitant shift in the spleens' cellular composition [3, 4]. Functional shifts were demonstrated in studies showing that spleen cells of 3LL tumor-bearing mice in early phases of tumor growth are capable of suppressing tumor growth when inoculated with tumor cells, but at later stages become strongly tumor enhancing, in all probability due to suppressor cells [7, 8].

Experiments where splenectomy was performed in hamsters prior to tumor transplantation (−7 days), with subsequent removal of the developed tumors at different sizes [14], led Gershon to suggest that the spleen appears to modulate the antitumor response by diminishing the production or capacity of

either killer cells or of suppressor cells at different stages of the development of a locally growing tumor. The results of the present study also suggest that the splenic lymphoid population is playing a role in controlling both the antitumor response and the subsequent progression of postoperative lung metastases. The nature of the splenic control was again dependent on the stage of tumor development: in early, but not late, stages of tumor growth, splenectomy resulted in strong inhibition of postoperative progression of lung metastases (Table 1). The failure of splenectomy to suppress the development of metastases following the excision of large tumors could be attributed to two distinct mechanisms: (a) at later stages of tumor growth, a large number of metastatic cells have progressed in the lungs, and at these stages they may no longer be susceptible to immunological controls exerted by the spleen; (b) as a result of changes in the composition of the spleen cell subpopulations usually observed in tumor-bearing animals, the spleen itself may lose its ability to generate appropriate immunocytes which can affect tumor growth.

Since our results (Table 2) show that splenectomy at early stages followed by tumor excision at later stages is effective in suppressing the progression of the postoperative metastases, the second mechanism is obviously favored. Such qualitative changes in the spleens' cell composition during tumor growth can explain why splenectomy affects the development of postoperative progression of metastases only in the first stages of the primary tumor growth: the spleen was shown to be the main source of both active suppressor T cells [19] and their precursors [20], while the precursors of killer and helper T cells, although abundant in the spleen, are not confined to this organ. Thus, if the spleen is removed in the first stages of tumor growth, the depletion of suppressor cells increases the anti-tumor effector response, while at later stages, when the suppressor cells have migrated out of the spleen, there is no protective effect of splenectomy. This is supported by the finding that in tumor-bearing mice one finds potentially active T killer cells, provided that their activity is unmasked by removing suppressor cells [8]. In this context it should be noted that if splenectomy is performed before tumor transplantation the local tumor appearance is inhibited [10, 12, 21]—suggesting again the depletion of suppressor cells or of cells that produce enhancing antibodies.

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