Immunotherapy of Cancer with Antibody

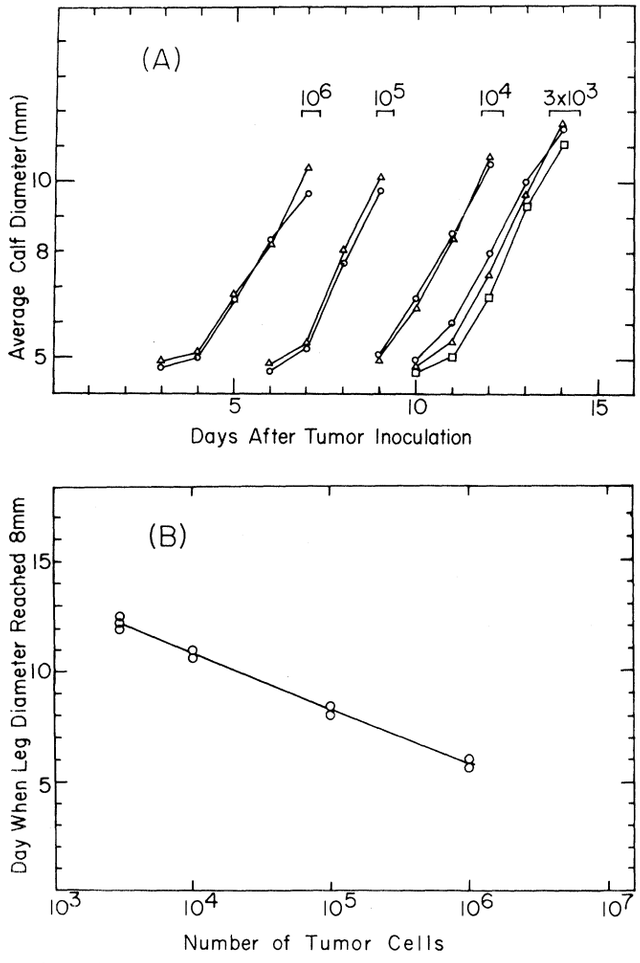
Abstract. *A long-term suppression of a transplanted solid tumor that has been growing in a syngeneic animal can be achieved by the administration of antibody against the tumor. The susceptibility of such growing tumor cells to antibody treatment is similar to that of a comparable number of freshly injected tumor cells.*

About 35 years ago, Gorer reported that antiserum directed against a tumor could suppress the growth of that tumor in an animal (*1*). Given this basic observation, which has been confirmed and studied by numerous investigators (*2, 3*), the specificity of antibody makes it an attractive agent for cancer therapy, provided that enough antibody can be raised, and that its administration is not accompanied by harmful side effects. In addition, the suppression by antibody should be permanent or long lasting and the treatment should be effective in suppressing a growing or established tumor.

In this study, we raised antibody by immunizing mice with an attenuated tissue culture line of syngeneic tumor cells (*4*). This antibody was used to cause a prolonged suppression of the growth of freshly inoculated tumor cells as well as a solid tumor that has resided in an animal for a period of time. The mechanism of this prolonged suppression did not operate through a transient cytostasis (*5*) or an increase in the cell division time.

C3H/HeN MTV$^-$ mice (abbreviated C3H) of either sex, aged 8 to 12 weeks, were used throughout the experiments. They were obtained from the National Cancer Institute through the courtesy of the Frederick Cancer Research Center, Frederick, Maryland. The 6C3HED lymphosarcoma, which originated in a C3H mouse, was obtained from the Jackson Laboratory, Bar Harbor, Maine, and was maintained in solid form by passage in C3H mice. To raise syngeneic antibody, C3H mice were immunized with a tissue culture line of the tumor (*4*).

To study the growth pattern of the tumor, mice were inoculated in the calf muscle with varying numbers of tumor cells. The growth of the tumor was followed by measuring the calf diameter with a vernier caliper (*3*). With fewer cells in the inoculum, it took longer for the tumors to become macroscopic (Fig. 1A). However, once the tumor became measurable, the slopes of growth curves were similar regardless of the number of tumor cells in the inoculum. The time required for leg diameters to reach 8 mm was plotted against the number of tumor cells in the inoculum (Fig. 1B); from this straight growth curve we calculated the in vivo doubling time of this tumor to be 18 hours. This type of linear relationship indicates that the generation time and the lag period (that is, the time elapsed between inoculation and the start of tumor



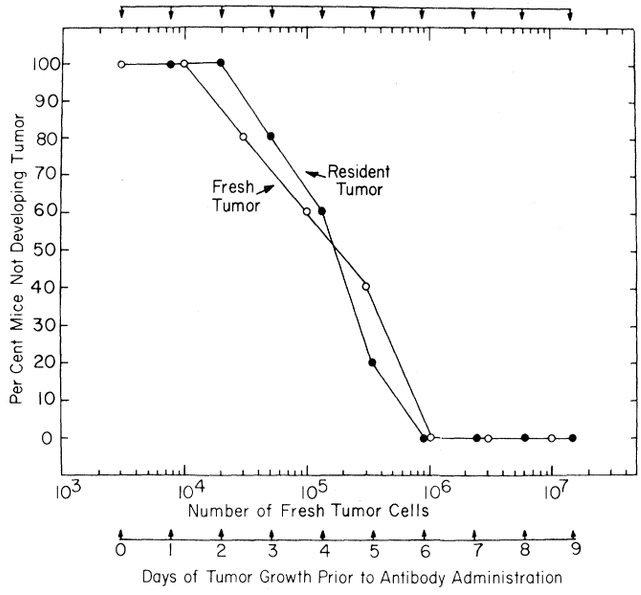


Fig. 1 (left). (A) The growth pattern of tumor cells in normal mice. The tumor cell suspension was made in RPMI 1640 tissue culture medium containing 0.1 percent normal mouse serum, and 10^6, 10^5, 10^4, and 3×10^3 tumor cells, as indicated, were injected into groups of five male mice. Duplicate groups were used for each inoculum except for triplicate groups of the 3×10^3 tumor cell inoculum. The increase in leg diameter was measured by a vernier caliper (*3*). Each point represents an average value from five mice. (B) The time (days) when the leg diameter reached 8 mm is plotted against the number of tumor cells in each inoculum on a semilogarithmic scale. It takes 2.5 days for the tumor cells to increase their number tenfold or 18 hours to double their number. The experiments were repeated three times with essentially the same results. Fig. 2 (right). Each of 45 male mice were inoculated with 3×10^3 tumor cells and tested for the susceptibility of growing tumor cells to a constant amount of antibody (0.5 ml of undiluted immune ascites fluid). Antibody was administered intraperitoneally on days 0, 1, 2, 3, 4, 5, 6, 7, 8, or 9. Animals not developing tumors during the 180-day period after tumor inoculation are shown on the ordinate. The lower abscissa shows the days after tumor inoculation and arrows indicate the predicted number of tumor cells, equivalent to the freshly injected tumor cells shown in the upper abscissa. The calculation was made on the basis of no lag period and a generation time of 18 hours. Also, on day 0, groups of five male mice were inoculated with 3×10^3, 10^4, 10^5, 10^6, or 10^7 tumor cells and tested immediately for the susceptibility to the same constant amount of antibody. (○) Freshly inoculated tumor cells; (●) previously inoculated growing or resident, tumor cells. Experiments like this were repeated two more times with essentially the same results.

cell division) of the tumor cells are constant, regardless of the number of tumor cells in the inoculum. Since the lag period for each inoculum would be the same, it was determined for a 10^6 tumor cell dose (*6*). Mice inoculated with 10^6 tumor cells each were given an intraperitoneal injection of tritiated thymidine 3 hours, 24 hours, or 5 days after tumor implantation. Autoradiographic preparations were made from histological sections of calf muscles containing tumor cells taken 3, 6, and 16 hours after labeling. The percentage of tumor cells labeled in a 3-hour-old tumor was 21, 41, and 63 percent at 3, 6, and 16 hours after labeling with tritiated thymidine for the times stated. Similarly, the percentages of labeling in 24-hour-old tumors and 5-day-old tumors were 23, 46, and 72; and 27, 38, and 68, respectively. The lag period for this tumor, therefore, was less than 3 hours.

Since tumor cells can begin to divide within less than 3 hours after injection into an animal and could double in number every 18 hours thereafter, we evaluated the antibody susceptibility of the tumor cells that had grown in mice for varying periods of time. Mice were inoculated with 3×10^3 tumor cells each, and the tumor cells were allowed to multiply. The suppressibility of the tumor was tested by administering a constant amount of antibody to each mouse at different times. All mice treated with antibody up to day 2 did not develop tumors (Fig. 2). In the group treated on day 5, only one mouse out of five did not develop a tumor. After day 5, all mice developed tumors. Our study shows that a growing, or resident tumor can be suppressed by antibody, but the effectiveness of antibody diminishes as the tumor grows. The number of tumor cells present in the mice at a given time can be predicted on the basis of an 18-hour generation time and a lag period shorter than 3 hours. Two reasons can be marshaled to explain why a tumor apparently becomes resistant to antibody treatment when residing in a host. The resistance can be explained solely on the basis of an increase in cell number; that is, more antibody is needed to suppress a larger number of tumor cells. Alternatively, some change in the properties or the histological arrangement of the tumor cells may occur with the passage of time. To clarify this problem, the susceptibility of varying numbers of freshly injected tumor cells was determined and compared to that of a growing tumor. All inoculations were made on the same day; the same pool of tumor cells was used both for the resident and the freshly injected cells. For the resident tumors, antibody was administered after tumor inoculation on the days given in Fig. 2. The susceptibility of resident tumors and a comparable number of freshly injected tumor cells is similar (Fig. 2). Therefore, the increase in the resistance can be explained simply on the basis of an increase in the tumor cell number.

Finally, we asked whether transient cytostasis plays an important role in the antibody-mediated suppression of tumor growth in vivo. In normal mice, a single viable tumor cell growing in the calf muscle should increase the leg diameter to 8 mm by 19 to 21 days (*7*). If a transient cytostasis or a lengthening of the generation time were to play an important role, one would expect that, in a group of mice treated with an antibody dose just enough to suppress tumor growth in some but not all mice, some mice would begin to develop tumors far beyond day 21. Of 72 mice belonging to this group, 37 developed tumor between day 12 and day 22 but no mice developed tumor beyond this. Those mice not developing tumor were under observation for 180 days from the beginning of the experiment without any sign of disease. Considering the short life span of mice and especially the short generation time of the tumor, antibody treatment can cause a prolonged tumor suppression.

Our studies show that tumor cells that have been growing in mice can be suppressed by antibody as efficiently as a comparable number of freshly injected tumor cells. Neither a transient cytostasis nor a lengthening of the generation time seems to play an important role in this suppression. Studies in vitro indicate that macrophages can cause cytostasis of sensitized tumor cells through a nonlytic and nonphagocytic mechanism (*8*). Our results suggest that such cytostasis, if also seen in vivo, must be relatively permanent or must be followed by killing of the target cells. This type of antibody-mediated tumor suppression can occur in thymectomized mice as effectively as in normal mice even though the thymectomized mice were deficient in their ability to reject an allograft and to produce antibody to sheep erythrocytes (*3*). It is possible that the immunotherapy of cancer with passive antibody may be accomplished without active immune responses on the part of the tumor-carrying host. In addition, since antibody may mediate tumor suppression in vivo through various effectors such as macrophages, lymphocytes, and platelets, a wide choice of treatment is available (*3*).

Two problems should be discussed concerning the use of antibody in a clinical situation. It has been repeatedly shown that passive antibody may interfere with the development or expression (or both) of active cellular and humoral immunity (*9*). Thus, the use of passive antibody, as with immunosuppressive cytotoxic drugs, if not completely successful, may worsen the host's immunity. This means that antibody should be used in such a way as to achieve complete suppression of the tumor. A second problem involves the number of tumor cells that can be suppressed by antibody. It has been observed that as the tumor cell number exceeds certain limits—for example, 10^6 tumor cells per mouse—a disproportionately larger amount of antibody is required to achieve tumor suppression because of the development of effector cell shortage (*10*). Until a way is found to overcome this effector shortage, it is not possible to ascertain how big a tumor load can potentially be suppressed by antibody. Recently, a factor (or factors) which is released by tumor cells and which can interfere with the normal function of macrophages has been described (*11*). Neutralization of such a factor may increase the efficacy of antibody in suppressing a large number of tumor cells. Meanwhile, the treatment with nonspecific cytotoxic agents such as drugs may be considered to reduce the number of tumor cells to a level treatable by antibody. The procedures and cytotoxic agents should be selected to promote the maximum effectiveness of antibody in an animal treated with such tumorcidal agents (*12*).

In our studies, we have used syngeneic antibody to minimize the possible complications caused by cross reaction with normal cells. As human tumor-specific antigens are characterized and purified (*13*), it may be possible to produce a sufficiently tumor-specific xenoantibody utilizing purified antigen for clinical trial. It is encouraging that only a few micrograms of antibody given systemically can cause prolonged if not permanent suppression of 10^5 pathogenic tumor cells in mice (*14*).

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preparation. When maintained in tissue culture, a line of cultured tumor cells became attenuated in the sense that, when such cells were injected into C3H mice, the tumor grew but later regressed in all animals. In contrast, uncultured parent tumor cells grew and killed all recipient mice. Mice rejecting the tissue culture line of tumor cells could now reject inoculated parent tumor cells. To raise syngeneic antibody of high titer, C3H mice were inoculated intraperitoneally at weekly intervals with 2×10^7 tumor cells of the tissue culture line in 50 percent saline–complete Freund's adjuvant emulsion. After 4 to 5 weeks, immune ascites developed, and the animals were tapped. After centrifugation to remove cells, the ascites fluids were heated at 56°C for 45 minutes and again centrifuged to remove debris. Tumor suppressive activity resided mainly in the γ1 class of antibody and was not absorbed by normal cells from spleen, thymus, or lymph node. Nonimmune ascites prepared from mice given only the complete Freund's adjuvant emulsion did not have any tumor suppressive activity. The term syngeneic was used only to denote that the tumor arose from a C3H mouse.

5. The term cytostasis was used to mean that cells do not divide but remain viable.
6. The lag period for the tumor cell was determined with a 10^6 cell inoculum; below this level, it is not possible to detect a significant number of tumor cells in histological sections. Twenty male mice were each inoculated with 10^6 tumor cells in the calf muscle. Groups of six mice were given an intraperitoneal injection of 0.25 ml of saline containing 25 μc of tritiated thymidine (20 c/mmole, New England Nuclear) 3 hours, 24 hours, or 5 days after tumor inoculation. Two mice in each group were killed at 3, 6, and 16 hours after the administration of tritiated thymidine. The calf muscles were fixed for 2 days in 10 percent formalin containing 1 percent calcium chloride. Tissue sections (4 μm thick) were processed for autoradiography as described in R. Baserga and D. Malamud [*Autoradiography: Techniques and Application* (Harper & Row, New York, 1969)]. Briefly, slides were dip-coated in Kodak NTB-2 emulsion. After a 20-day exposure they were developed in Kodak Dektol, fixed, and stained with hematoxylin and eosin. Cells with more than five grains over the nuclear area were scored as positive. Two hundred tumor cells were counted per mouse. Mice not given tritiated thymidine served as controls.
7. The time required for a single tumor cell to multiply and increase the leg diameter of a mouse to 8 mm can be determined by a limiting dilution method in which a tumor cell suspension containing a precisely known number of tumor cells is diluted to the point where only some mice receiving such diluted inoculums develop tumors. The tumor cell inoculums, containing an average of about five tumor cells, produced tumors in about 60 percent of recipients. In practice, however, it is not clear whether the inoculum contained the expected number of tumor cells since a loss of a few cells on the wall of a pipette, syringe, or needle would have seriously altered the actual number of tumor cells injected. Therefore, we took the longest time required for the tumor to grow at various limiting dilutions to be that required for a single cell. The longest time observed was 21 days. Another way to determine the value is to extrapolate a growth curve such as that shown in Fig. 1B to the one-cell level. An extrapolation of three such curves gave an average of 19.3 days.
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Endocrine-Dependent Rat Mammary Tumor Regression: Use of a Gonadotropin Releasing Hormone Analog

Abstract. *Long-term administration of [D-Leu^6, des-Gly-NH$_2$^{10}, Pro-ethylamide^9]-GnRH, an analog of gonadotropin releasing hormone, caused regression of neoplastic tissue in a rat bearing a spontaneous mammary adenocarcinoma and in rats in which tumors had been induced by treatment with dimethylbenzanthracene (DMBA). During two separate treatment periods with the analog, the tumor in the single animal regressed although it had previously grown spontaneously. After a third period of growth, ovariectomy also induced regression, suggesting endocrine dependency of the tumor. These observations were confirmed in the DMBA-induced tumor system, where tumor regression in the analog-treated rats was comparable to that observed in the ovariectomized rats, and in both cases the tumor regression was significant when compared to untreated controls.*

We have shown (*1*) that twice-daily injections of microgram quantities of the gonadotropin releasing hormone analog, [D-Leu^6, des-Gly-NH$_2$^{10}, Pro-ethylamide^9]-GnRH (A-43818) (analog **1**) (*2*, *3*), into female rats inhibits normal reproductive processes. When given to immature animals analog **1** postponed maturation of the reproductive organs, while in the mature animal analog **1** caused cessation of cycling and atrophy of the ovary and uterus. Since the primary anatomical findings in these animals were atrophy of the ovary and of the uterus, we speculated that the initial endocrinological effect of the analog is interference with the biosynthesis or the utilization of the ovarian steroid hormones. This view is in accord with the effects on reproductive function and upon reproductive tissues.

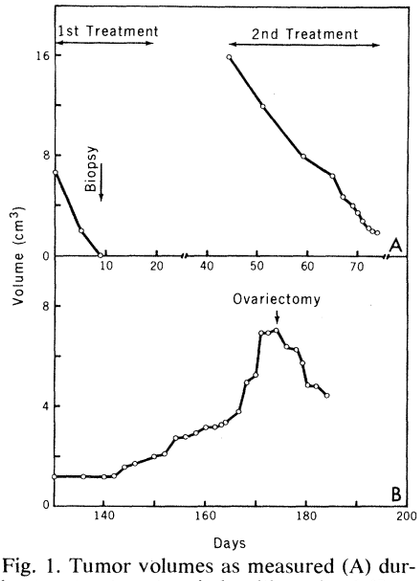

Fig. 1. Tumor volumes as measured (A) during two treatment periods with analog **1** (5 μg of analog **1** was injected subcutaneously twice a day), and (B) before and after ovariectomy.

We proposed that analog **1** might not only have important implications in reproduction control, but might also be applicable to the treatment of steroid-dependent neoplasms of the reproductive organs, especially the mammary gland. We tested this theory in a female rat bearing a large spontaneous tumor in the breast region, and in rats in which tumors had been induced by dimethylbenzanthracene (DMBA) (*4*).

The single animal bearing a spontaneous mammary tumor was given two periods of treatment with analog **1**. As shown in Fig. 1, the tumor volume was reduced in both treatment cycles. A biopsy specimen taken after the first treatment cycle indicated that the tumor was a mammary adenocarcinoma (*5*). Af-

Table 1. Comparison of DMBA-induced tumor responses in rats treated with analog **1**, ovariectomized rats, controls. Analog **1** [D-Leu^6, des-Gly-NH$_2$^{10}, Pro-ethylamide^9]-GnRH was administered subcutaneously (5 μg) twice a day. Ovariectomy was performed on the same day that the treatment with analog **1** was started. The ovariectomized and control animals received saline. The results are given as the numbers of animals showing tumor regression (R) or no regression (NR); R and NR were judged on the basis of the change in the total volume of the tumors on each animal. (*P*, probability; NS, not significant.)

Treatment	7 days			14 days			21 days			30 days		
	R	NR	*P**	R	NR	*P*	R	NR	*P*	R	NR	*P*
Control	0	6	NS	0	6	.001	0	6	.001	1	5	.01
Ovariectomized	2	4		6	0		6	0		6	0	
Control	0	6	.015	0	6	.002	0	6	.002	1	5	.01
Analog **1**	4	1		5	0		5	0		5	0	
Ovariectomized	2	4	NS	6	0	NS	6	0	NS	6	0	NS
Analog **1**	4	1		5	0		5	0		5	0	

*Calculated from Fisher's exact probability for 2 × 2 contingency tables (*8*).