ANTIBODY-MEDIATED SUPPRESSION OF GRAFTED LYMPHOMA

IV. Influence of Time of Tumor Residency In Vivo and Tumor Size Upon the Effectiveness of Suppression by Syngeneic Antibody*

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Antibody directed against a tumor was shown to cause the suppression of the growth of that tumor by Gorer (1). Many studies have been carried out to delineate the active classes of antibody and the effectors that participate in this reaction (2-12). In the series of experiments presented in this paper, we have quantitatively analyzed some of the factors which may influence the effectiveness of antibody in tumor suppression. The development of a macrophage shortage with increasing tumor size emerged as an important factor that can limit the effectiveness of the antibody.

Materials and Methods

Mice. Mice of the inbred strain C3H/HeN MTV- (C3H) of either sex, aged 8-12 wk were used throughout the experiments. They were obtained from the National Cancer Institute through the courtesy of the Frederick Cancer Research Center, Frederick, Md.

Tumor. The C3H lymphoma 6C3HED, obtained from the Jackson Laboratory, Bar Harbor, Maine, was maintained by inoculating 4 × 10^7 tumor cells into the calf muscle of syngeneic C3H mice. The term syngeneic is used only to indicate that the tumor originated in a C3H mouse. Tumor was harvested 12-14 days after inoculation and minced in RPMI 1640 tissue culture medium (Microbiological Associates, Bethesda, Md.) containing 0.1% C3H mouse serum (medium). The minced tumor was pressed through a stainless steel screen. The cells were washed three times in the same medium by centrifugation. After the last wash, the cells were suspended and 3 min was allowed for clumps to settle in the centrifuge tube. The top two-thirds of the supernate were collected and the cell count was adjusted to the desired numbers. All procedures were carried out under aseptic conditions with sterile instruments.

Preparation of Antibody. Antibody against the tumor was raised in syngeneic C3H mice according to the method of Pasternack et al. 2 Briefly, C3H mice were inoculated weekly with 2 × 10^7 tumor cells of a tissue culture line in 50% saline-complete Freund's adjuvant emulsion intraperitoneally. After 4-5 wk, immune ascites developed and the animals were tapped with a 16-

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1 Abbreviations used in this paper: C3H, C3H/HeN strain of mice; medium, RPMI 1640 tissue culture medium containing 0.1% C3H mouse serum.

2 Pasternack, G. R., R. J. Johnson, and H. S. Shin. Manuscript in preparation.
gauge needle. After centrifugation to remove cells, the ascites fluid was heated for 45 min at 56°C and again centrifuged at 40,000 g for 15 min to remove debris. The tumor suppressive activity resided mainly in the IgG1 class of antibody. To obtain nonimmune ascites, mice were injected with saline-complete Freund's adjuvant without tumor cells. It was heated and cleared in the same way as the immune ascites fluid.

**Experimental Tumor Inoculation.** In one calf muscle, 0.05 ml of a tumor cell suspension or a mixture of tumor and effector cells was injected. Tumor growth was followed by caliper measurement of the calf size in two diameters taken at right angles to each other. Values were recorded as the average of the sum of the two diameters for each measurement.

**Titration of Antibody.** All immune or nonimmune ascites fluids were administered to the mice intraperitoneally within 1 h of tumor inoculation unless otherwise mentioned. When suppressed by antibody, tumor growth was delayed. However, once tumor growth started after a delay, the slopes of the growth in individual mice were similar for control and antibody-suppressed groups. The delay of growth in individual mice was converted to percent suppression from the growth curve shown in Fig. 2. For example, if antibody delayed the tumor growth, i.e., time required for leg diameter to reach 8 mm, by 2.5 days, suppression was 90% (see the Suppression as a Function of Antibody Dose under Results). If tumor growth was suppressed over 22 days, the suppression was considered to be 100% since beyond this point none of the mice developed tumor for over 180 day observation period. In all experiments, groups of five mice, caged separately, were used as basic units. The percent suppression of the tumor in each of five mice was determined and the average and standard error were calculated. Often duplicate groups were used. In such cases either the average and standard error from 10 mice from duplicate groups or the average of the mean values from duplicate groups with a range of means were presented. Each time a titration was performed, a control experiment was done to obtain a growth curve for nonsuppressed tumor, like the one shown in Fig. 2.

**Antibody Activity.** For a standard titration, the amount of antibody required to suppress the growth of a $10^5$ tumor cell inoculum was measured. 1 suppressive U was defined as the amount of antibody that could suppress the growth of $10^5$ tumor cells by 50%. The titer of the same antibody measured on different days fluctuated due to unknown variables. Therefore, when required, the amount of antibody needed to suppress the growth of $10^5$ tumor cells by 50% was determined as a control for each experiment. Two lots of immune ascites fluid were used. The lots P-1 and P-2 each contained about 100 suppressive U of antibody per milliliter of ascites fluid.

**Effector Cells.** Lymphocytes were isolated from peritoneal exudates induced by injecting intraperitoneally 3 ml of 2% starch hydrolysate (Connaught Medical Laboratories, Toronto, Canada) dissolved in 0.15 M NaCl. Starch was dissolved by boiling the suspension for 5 min. 2 days after injection the mice were killed by asphyxiation with CO₂ and 5 ml of medium were injected intraperitoneally. The mice were shaken gently, the peritoneal fluid was aspirated, and its cells were washed once in the medium. Lymphocytes were purified on a glass bead column essentially as described by Shortman et al. (13). Such preparations contained more than 99% lymphocytes by morphology (5).

To obtain macrophages, mice were given 3 ml each of 2.95% thioglycolate (Difco Laboratories, Detroit, Mich.) intraperitoneally. 5 days after the injections, the mice received 500 R whole-body irradiation to reduce the number of lymphocytes in the peritoneal exudate. 30-40 h later the exudate was collected by washing the peritoneum with 5 ml medium. The recovered cells were washed twice in medium and adjusted to the desired concentrations. Greater than 95% of the cells were macrophages.

**Vasoactive Substances.** Bradykinin triacetate was purchased from ICN, Nutritional Biochemicals Corp., Cleveland, Ohio and serotonin from Sigma Chemical Co., St. Louis, Mo. An intradermal injection of 10 μg of bradykinin or 25 μg of serotonin, each in 0.05 ml of saline, caused edema with average diameters of 7.2 mm and 6.2 mm, respectively, when measured 20 min after the injection. Saline controls caused swelling with an average diameter of 5.3 mm.

**Autoradiography.** At various times after tumor inoculation, mice were given intraperitoneally 0.25 ml of saline containing 25 μCi of tritiated thymidine (20 Ci/mM, New England Nuclear, 3 Johnson, R. J., G. Pasternack, and H. S. Shin. Manuscript in preparation.
The mice were killed 3, 6, and 16 h after pulse labeling and the calf muscles were fixed for 2 days in a 10% formalin solution containing 1% calcium chloride. Tissue sections of 4 μm thickness were processed for autoradiography as described (14). 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. 200 tumor cells were counted per mouse.

Results

The Growth Rate of the Tumor Cells. Tumor cells ranging in numbers from $10^3$ to $10^6$ were inoculated into the calf muscles of mice and the increase in the leg diameters was followed. As shown in Fig. 1, the time period that the tumors remained macroscopically undetectable was inversely proportional to the size of the inoculum. Once the tumors became macroscopic, the slopes of the growth curves were the same for the different inocula. The plot of the time required for the leg diameter to reach 8 mm, the steepest portion of a growth curve, against the inoculum size on a semilogarithmic scale produced a straight line (Fig. 2). For each 10-fold difference in tumor cell number, a 2.5-day difference in reaching the 8-mm leg diameter was seen. The generation time of 18 h was calculated from the formula $G = \frac{0.3t}{(\log_{10} B - \log_{10} b)}$ where $G$ represents generation time, $B$ the total cell number at the end of a given period of time, $t$, and $b$ the initial cell number.

The Lag Period for a Freshly Inoculated Tumor. When freshly inoculated into an animal, the tumor cells may stay dormant for a period of time before they begin to divide. The straight line shown in Fig. 2 implies that the dormant or lag period and the generation time stay constant regardless of the inoculum size. The lag period was determined for a $10^6$ tumor cell inoculum. Mice inoculated with $10^6$ tumor cells were given tritiated thymidine at various times after tumor implantation. As shown in Fig. 3, the tumor cells began to incorporate the label as early as 3 h after tumor implantation and the rate of the label incorporation was similar among tumors which resided in the animals for different periods of time. Therefore, the lag period was less than 3 h. A direct confirmation that an inoculum containing fewer cell numbers also had a lag period of less than 3 h, as predicted by the growth curve, could not be carried out since when fewer cells were inoculated, significant numbers of tumor cells could not be detected in histological sections.

Tumor Suppression as a Function of Antibody Dose. The suppression of $10^5$ tumor cells by different doses of intraperitoneally administered antibody was measured. In normal mice, $10^5$ tumor cells caused an increase in the leg diameter to 8 mm by eight days. When antibody was administered, tumor growth was delayed but the slopes of tumor growth in individual mice were the same once growth started in a group suppressed by antibody. The delay in growth was converted to percent tumor suppression. For example, if the growth was delayed by 18 h, from the growth curve shown in Fig. 2, one could conclude that tumor was suppressed from $10^5$ to an equivalent of $5 \times 10^4$; this then was 50% suppression. As shown in Fig. 4, the dose response curve seemed to be concave to the abscissa. Higher doses of antibody caused more suppression. Nonimmune ascites at comparable amounts to immune ascites did not have any suppressive activity.
Fig. 1. Growth pattern of the tumor cells in normal C3H mice. Each point represents an average value from a group of five mice. Experiment 1, •--•, experiment 2, △--△, experiment 3, □--□, and experiment 4, ○--○, were performed on different days.

Fig. 2. Semi-logarithmic plot of the day the mean leg diameters reached 8 mm against the number of tumor cells in each inoculum. Data from Fig. 1. Symbols are the same as in Fig. 1.

Susceptibility of Tumor Cells that have Resided in an Animal as Compared to Freshly Injected Tumor Cells. It is possible that tumor cells that have been growing for a period of time in vivo are different from freshly injected tumor cells for various reasons. For example, fresh injection causes tissue damage and inflammation. In addition, freshly injected tumor cells are dispersed as single cells, but dividing tumor cells may form cell-nests which are not readily accessible to antibody and effectors. In the experiment presented in Fig. 5, 3 × 10³ tumor cells were inoculated and allowed to grow. On day 0, 1, 2, 3, 4, 5, 6, 7, 8, or 9, 0.5 ml of immune ascites containing about 50 suppressive U of antibody were administered to test the susceptibility of the growing tumor. Again the delay in tumor growth was converted into percent suppression. As shown in Fig. 5, the percentage of tumor cells suppressed by antibody decreased as antibody was
Mice inoculated with $10^6$ tumor cells were pulsed with 25 $\mu$Ci of tritiated thymidine given intraperitoneally 3 h, 24 h, or 5 days after tumor implantation. Two mice from each group were killed at 3, 6, or 16 h after administration of the label. The calf muscles containing tumor cells were processed for autoradiography. Each point represents the mean value from two mice. The arrows indicate the time of tritiated thymidine injection.

Percent tumor suppression as a function of antibody dose. Each point represents mean value from 10 mice in two duplicate groups. The bars indicate the standard errors of the means.

given later. The cause for the diminished suppression might lie in some of the possibilities mentioned. Alternatively, the diminished suppression could be explained entirely on the basis of an increase in tumor cell number with passage of time, i.e., more antibody is needed to suppress a larger number of tumor cells. On the basis of no lag period and an 18 h generation time, the number of tumor cells which must be present at any given time in an animal initially inoculated with $3 \times 10^3$ cells can be predicted. The predicted numbers are indicated by arrows in the lower abscissa. The suppression of freshly injected tumor cells, ranging in numbers from $3 \times 10^3$ to $10^7$, was compared to that of growing tumor. As also shown in Fig. 5, the susceptibilities are virtually the same. The experi-
ment was repeated twice with similar results. Therefore, in the case of this particular tumor, and within the range of tumor cell numbers and antibody doses tested, there is no reason to believe that a growing or established tumor is different from a freshly injected tumor in its susceptibility to antibody-mediated suppression on a per cell basis.

Tumor Size as a Factor Influencing the Effectiveness of Antibody in Tumor Suppression. The amount of antibody needed to suppress 50% of the tumor cells in inocula containing $10^5$ or $10^6$ cells was compared. For the experiments, the immune ascites was administered 2 h before tumor inoculation to allow the antibody to equilibrate in the mice. One suppressive U of antibody, by definition, was needed to suppress 50% of $10^5$ tumor cells. If the amount of antibody needed to suppress 50% of the tumor cells is directly proportional to the number of tumor cells, in the same experiment one would expect the dose of antibody required to suppress 50% of $10^6$ cells to be 10 suppressive U. In other words, the ratio of the number of tumor cells in an inoculum to the amount of antibody needed for 50% suppression would be constant regardless of the number of tumor cells present in the inoculum. Instead, as shown in Fig. 6, 30 suppressive U of antibody were needed to achieve 50% suppression of $10^6$ cells. When tumor cells premixed with macrophages were tested, however, about 10 suppressive U were needed. With the same method of titration as shown in Fig. 6, in a series of experiments we evaluated the effectiveness of antibody in suppressing $10^4$, $3 \times 10^4$, $10^5$, or $10^6$ tumor cells with or without premixed macrophages, lymphocytes, bradykinin, or serotonin. The results are shown in Fig. 7 in which effectiveness was expressed by the effectiveness index, defined as (number of tumor cells in
Fig. 6. The suppression of $10^8$ tumor cells with or without exogenous macrophages. No added exogenous macrophages, $\bullet - \bullet$, exogenous macrophages to tumor cell ratio of 1:1, $\Delta - \Delta$, and 1:3, $\blacktriangle - \blacktriangle$. Each point represents the mean value of 10 mice. The bars indicate the standard errors of the means.

Fig. 7. Effectiveness of antibody in suppressing varying numbers of tumor cells without exogenous vasoactive substances or effector cells (C), with 10 µg of bradykinin (B), with 25 µg of serotonin (S), with exogenous macrophages equal in number to the tumor cells (M) and with exogenous lymphocytes twice the number of tumor cells (L). Duplicate experiments were carried out on different days and the average values are given. The bars indicate the range of two values. The ordinate shows tumor cell number as well as the antibody effectiveness index defined as $(\text{tumor cell number in the inoculum})/(\text{dose of antibody needed for 50% suppression in suppressive units} \times 10^5)$. * No range of values is shown since the effectiveness of suppressing $10^5$ tumor cells is always $1 \times 10^{-5}$ by definition.

inoculum)/(dose of antibody needed for 50% suppression in suppressive units $\times 10^5$). Since 1 suppressive U was defined as the amount of antibody needed to suppress 50% of a $10^8$ tumor cell inoculum, the suppressive index for $10^8$ tumor cell inoculum is always 1. The titer of antibody measured on different days fluctuated. For this reason, the amount of antibody needed to cause 50% suppression of a $10^8$ tumor cell inoculum was determined for each experiment as a control. Then the amounts of antibody required to suppress 50% of various other tumor cell preparations could be compared to this control value. The effectiveness indices were approximately 1 for $10^4$ and $3 \times 10^4$ tumor cells but dropped to 0.3 for $10^6$ tumor cells. The effectiveness index was restored to about 1
when 10^6 tumor cells premixed with macrophages were tested. Bradykinin and serotonin did not affect the effectiveness of antibody in suppressing 10^5 or 10^6 tumor cells. Lymphocytes, unlike macrophages, did not improve the effectiveness in the suppression of 10^6 tumor cells. Exogenous macrophages added to 10^6 tumor cells diminished the effectiveness index to the level of 0.7. The results indicate that at the 10^5 tumor cell level, the suppression by antibody takes place in an optimal manner in the sense that added macrophages or vasoactive substances do not improve the effectiveness of antibody. If anything, added macrophages decreased the effectiveness. However, at the 10^6 tumor cell level effectiveness diminishes due to the development of a macrophage shortage; a shortage of lymphocytes was not detected. Injection of agents that increase vascular permeability did not restore the effectiveness. It is important to point out that the growth rates of tumor cells premixed with macrophages, lymphocytes, bradykinin, or serotonin were the same as the growth rates of a comparable number of tumor cells alone indicating that under the experimental conditions mentioned these effector cells and vasoactive substances do not have suppressive activity without antibody.

Discussion

In our study we evaluated some of the factors which might potentially influence the effectiveness of antibody in mediating tumor suppression. The effect of the time of tumor residency in vivo was examined first for the following reasons. Under the usual experimental conditions, the tumor cells were harvested from donors, dispersed into single cell suspensions, counted and injected into recipients. Injection causes trauma. When tested for their susceptibility to antibody, such cells existing as single cells in an inflamed area may respond differently than a more “natural” tumor that has grown in an animal for a period of time without a significant traumatic inflammatory reaction and has formed nests of cells from the initially isolated single cells. Experimental results indicate that whatever difference there may be between growing tumor cells and a comparable number of freshly injected tumor cells, that difference per se did not alter the effectiveness of antibody in causing tumor suppression within the range of the tumor cell numbers and antibody doses tested. On the other hand, when the tumor size became large, the effectiveness of antibody diminished due to the development of a macrophage shortage. Substances that increase vascular permeability or exogenous lymphocytes did not correct this diminished antibody effectiveness, indicating that diffusion of antibody or a shortage of lymphocytes were not problems. The ineffectiveness of lymphocytes might be due to two reasons. Either a lymphocyte shortage truly was not a problem, or alternatively, the immune ascites used in this experiment did not contain the appropriate class of antibody to cooperate with lymphocytes. Johnson and Shin have shown that mouse IgG1 caused tumor suppression in cooperation with macrophages but not with lymphocytes (9). Preliminary studies indicate that the tumor suppressive activity of the immune ascites resides mainly in the IgG1 class of antibody making the latter possibility a likely one. Classes of antibody that can suppress tumor in cooperation with lymphocytes are needed to determine whether a shortage of lymphocytes may also develop at high tumor cell numbers. In any case, one of the major obstacles in suppressing a large number
of tumor cells is the development of a macrophage shortage. It is not known whether this problem is due to a factor(s) from the tumor that interferes with macrophage functions (15–17). The loss of effectiveness of antibody in tumor suppression due to the development of an effector shortage suggests that, depending on the situation, the suppressive activity or enhancing activity of a class of antibody may prevail. Thus, the IgG1, IgG2, and IgM classes of antibody were shown to have both suppressive and enhancing activities (8, 9, 18, 19). At low tumor cell numbers, the suppressive activity of such antibody will be sufficient to cause tumor suppression even after the expression and (or) development of the host’s active immunity has been blocked. Therefore, enhancing activity of antibody may be masked. However, at large cell numbers, due to the development of effector shortage, suppressive activity of antibody will sharply diminish. Under such conditions only the enhancing or blocking activity will manifest itself.

At the $10^5$ tumor cell level, suppression takes place in an optimal manner in the sense that added effector cells or the substances that increase vascular permeability did not make the suppression any more effective. In addition, below the $10^5$ tumor cell level, the amount of antibody needed to achieve the same fraction of tumor suppression is directly proportional to the number of tumor cells in the inoculum, unlike many tumoricidal agents which operate in a "first-order fashion" and therefore require a constant dose to eliminate a constant fraction, regardless of tumor cell number (20). Furthermore, it takes only a few micrograms of antibody to cause a prolonged, if not permanent, suppression of $10^5$ tumor cells. Suppression can occur even in the absence of a host’s active immune responses (5). These attributes of antibody-mediated suppression make immunotherapy of a small number of tumor cells by passive antibody an attractive possibility.

Summary

In the suppression of the growth of a mouse lymphoma 6C3HED by antibody, the effectiveness of antibody in suppressing growing or established tumor cells and comparable number of freshly injected tumor cells is quantitatively similar. The effectiveness of antibody diminishes markedly when the number of tumor cells per mouse reaches the level of $10^6$ due to the development of a macrophage shortage. At the $10^5$ tumor cell level, antibody-mediated suppression takes place in an optimal manner and between $10^5$ and $10^4$ tumor cell numbers, the amount of antibody required to suppress 50% of the tumor cells is directly proportional to the number of tumor cells suppressed.

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