Blood Level of B and CD4+ Lymphocytes Measured before Induction of an Experimental Tumor in Rats Predicts Tumor Progression and Survival¹

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Abstract
After an initial series of experiments indicated that early responses of B lymphocytes were important in controlling tumor metastases in two rat models of cancer (N. Quan et al., Cancer Res., 59: 1080–1089, 1999), the present study assessed whether differences in the number of B lymphocytes that are normally present in different individual rats before any tumor development could predict tumor growth, metastases, and length of survival when tumor challenge subsequently occurred. Repeated baseline measurements of several circulating lymphocyte subtypes (i.e., natural killer, B, CD4+, CD8+ lymphocytes) were made in individual inbred WAG rats before any introduction of tumor cells, and stable baselines for these subtypes were found. Animals were then injected with 2 × 10⁶ CC531 tumor cells (a syngeneic tumor) into the leg, and the size of the resulting primary tumor measured. Primary tumors were surgically removed 6–7 weeks after tumor-cell injection, and animals then followed until death from metastases. In two experiments, the size of the primary tumor as well as the length of time that animals survived correlated with the pretumor percentage of certain lymphocyte subtypes in peripheral blood before tumor-cell injection. Baseline percentage of B lymphocytes was significantly negatively correlated with the size of the primary tumor and was positively correlated with the duration of survival. Baseline percentage of CD4+ lymphocytes showed the opposite relationship, being positively correlated with tumor size and negatively correlated with survival time, although these correlations were lower than those for B lymphocytes. Percent B lymphocytes in circulation also declined during tumor development. In summary, a high percentage of endogenous peripheral blood B lymphocytes predicted growth of smaller primary tumors and longer survival after experimental tumor induction in a rat model, further suggesting that B lymphocytes are involved in protection against development of certain tumors.

Introduction
Although development of various experimental tumors is apparently controlled by NK⁴ cells [e.g., the studies of Gorelik et al. (¹) and Barlozzari et al. (²)], we recently found evidence pointing to the direct involvement of B lymphocytes in the control of some of these tumors (³). In this paper, we reported that the most marked change in a lymphocyte subtype that occurred in different compartments after i.v. injection of tumor cells that metastasize to lung was a rapid accumulation of B lymphocytes in the lung. Two further manipulations then suggested that B lymphocytes were involved in the control of tumor development. First, immunoneutralization of B lymphocytes by injection of a high dose of antibody to rat B lymphocytes increased development of lung tumors after i.v. tumor-cell administration. Second, inoculation of the Fischer 344 rats with MADB106 tumor given into the leg, which makes these rats resistant to development of lung tumors when MADB106 is subsequently given i.v., resulted in an increased level of B lymphocytes in the lung of these “lung-tumor resistant” animals.

Experimental studies from other laboratories have also implicated B lymphocytes in the control of tumor growth and metastasis. Yuhas (⁴) found that infusion of splenic B lymphocytes into BALB/c mice reversed radiation-induced enhancement of alveolar cell carcinoma metastases. Guo et al. (⁵) reported that injection of cells produced by the fusion of activated B lymphocytes with hepatocellular carcinoma cells protected against subsequent tumor challenge and had antitumor activity against established tumors in Wistar rats. Also, effects of antitumor agents in nude versus SCID mice point to the importance of B lymphocytes in tumor surveillance. Because SCID mice lack both B and T lymphocytes, whereas nude mice only lack T lymphocytes, differential responses of these strains have been used to assess the contribution of B lymphocytes in tumor-related phenomena. Quin and Blankenstein (⁶) found that lymphotoxin produced by transfected J588L cells led to tumor rejection in syngeneic BALB/c mice, partial rejection in nude mice, and lack of rejection in SCID mice. The differential antitumor effect of lymphotoxin in nude and SCID mice sug-

¹ The abbreviation used is: NK, natural killer.
suggests the involvement of B lymphocytes in antitumor action of lymphotoxin. Similarly, Reisfeld et al. (7) reported that an antibody-lymphotoxin fusion protein inhibited metastases in athymic nude mice but not in SCID mice.

The experiments described in this paper attempted to extend our earlier findings. Whereas we previously used immunoneutralization to manipulate B lymphocytes (3), the present study examined whether naturally occurring individual differences in B lymphocytes might be related to tumor development and survival. In these studies, inbred WAG rats were injected with syngeneic CC531 tumor cells into the ventral aspect of the hind leg so they would develop a primary tumor at this site. Before tumor cells were injected, blood samples were taken from each rat, and the percentage of various lymphocyte subtypes (NK, B, CD4+, and CD8+) were measured so that a pretumor circulating level of these lymphocyte subtypes was determined for each animal. After tumor cells were injected, the size of the primary tumor was measured at various intervals for 6–7 weeks, at which time the primary tumor was removed and the length of time that animals survived was measured. The intent of the study was to determine if naturally occurring differences in the percentage of any blood lymphocyte subtype present before tumor cells were injected would prove to be related to (i.e., correlated with) the development of the primary tumor (i.e., size of tumor), development of metastases, and duration of survival.

Materials and Methods

Animals. Female inbred WAG (Harlan/CPB, Zeist, the Netherlands) rats >8 months of age were used. Animals were housed four to a cage in microisolator cages under positive-pressure ventilation, with cages maintained on closed-shelf, laminar-flow racks. Thus, rats in any given cage were not in contact with pathogens, odors, or noises of other animals in the colony. The animal colony room was maintained at 22°C ± 1°C on a 12/12 light/dark cycle with food and water available ad libitum. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee.

Blood Sampling Procedure. Samples of peripheral blood were collected in heparinized Natelson tubes by nipping the end of the subjects’ tail with a sterile scalpel and gentle milking of the tail. Blood samples were collected beginning ~3.0 h after lights-on in the colony. For sampling, a cage containing four animals was removed from the rack, and each animal was taken individually into an adjoining room where the blood sample was taken. After taking of the blood sample, each animal from a cage was placed into a holding cage in the adjoining room until all four animals of the cage had been sampled, at which time the four animals were returned to their home cage and the cage was replaced into the rack. Completing blood sampling for a cage required 8–10 min. Blood was drawn from the animals of a cage in the same order on each occasion when sampling was done; subsequent analysis determined that the percent of lymphocyte subtypes found in blood was unrelated to the order of blood sampling of the animals in a cage. Completing blood sampling from all animals in a study required ~1 h.

Percent Lymphocyte Determination. Two samples of 200 μl each were taken for florescent labeling of lymphocytes and the quantification of lymphocyte subtypes [NK, B (CD45RA), CD4+, CD8+] on a flow cytometer using standard techniques. Details of the procedure used were given previously in Quan et al. (3). Briefly, each sample was incubated for 45 min at 4°C after the addition of 50 ml of 1:200 dilution of monoclonal antibodies to FITC-conjugated antiCD4 (OX-38) and R-phycocerythrin-conjugated antiCD8 (OX-8) to one sample and R-phycocerythrin-conjugated antiCD45RA (OX-33) and 1:300 dilution of FITC-conjugated anti-NK (NK 3.2.3) to the other sample. (Dilutions used were determined by serial dilution to give the best staining of the above lymphocyte surface markers.) Samples were analyzed on a fluorescence-activated cell sorting CALIBER (Becton Dickinson) cytometer. Positive staining was gated by subtracting autofluorescence from unstained control samples.

Tumor Cell Culture. The syngeneic CC531 cell line, derived from a 1.2-dimethylhydrazine-induced adenocarcinoma of the colon of a WAG rat (8), was used (generously supplied by Dr. Peter Kuppen [Leiden, the Netherlands]). Cell lines were maintained in 5% CO2 at 37°C in monolayer cultures in complete media (RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum, 0.01 mg/ml gentamicin, 2 mM l-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Cultures were harvested with 0.25% EDTA in HBSS, washed, and suspended in Dulbecco’s PBS [8 g/liter NaCl, 1.15 g/liter NaHPO4, 0.15 g/liter NaCl, 0.45 g/liter NaHCO3 (PBS)] before injecting into animals. Cell cultures used had undergone <10 passages.

Induction of Tumor, Measurement of Primary Tumor Size, and Assessment of Tumor Metastases. Rats were lightly anesthetized with halothane, and 2 × 106 tumor cells in 1 ml of PBS were injected s.c. on the ventral surface into the upper thigh region of the left leg. The size of the primary tumor that developed at this site was determined by lightly anesthetizing the animal, palpating the tumor to determine its extent, and then measuring the tumor with microcalipers. The diameter was calculated by averaging the length and width measurements, which gave an accurate representation of the tumor size as evidenced by tumor diameter being correlated with tumor weight in grams in experiment 2. For removal of the primary tumor, animals were anesthetized with halothane, an incision was made in the leg skin to expose the s.c. tumor, and the tumor was dissectioned out. The wound was then closed with surgical staples, and following a brief recovery period, the animal was returned to its home cage. Animals remained in the home cage until death appeared imminent (indicated by weight loss, decrease in grooming and mobility, and difficulty breathing) at which time the animals were sacrificed by opening of the chest cavity under halothane anesthesia. At this time, animals were necropsied for presence of metastases throughout the body. Lung metastasis was quantified using the method described in Wexler (9). For this procedure, after opening of the chest cavity, the lungs were infused with 15% India ink through the trachea, which causes the lung surface to stain black except for tumors that resist dye and thus are white. The number of metastasis on the lung surface was subsequently counted.

Statistical Analysis. Correlations reported are Pearson Product Moment correlations. Comparisons between groups were done by t tests.

Procedure of Experiment 1. Four blood samples (with a 2-week interval between each sample) were taken before tumor-cell injection and analyzed for composition of lymphocyte subtypes. Sixteen animals were used. After collection of the fourth sample from all animals, each animal was anesthetized as described, and tumor cells were injected. On days 21 and 47 after tumor-cell injection, a blood sample was taken after which the size of the primary (leg) tumor was measured. On day 49, the primary tumor was surgically removed. Thereafter, animals remained undisturbed in the home cage until death appeared imminent, at which time the animal was removed and sacrific-
phocyte subtypes (NK, B, CD4, CD8) were taken (indicated by sample number) with a 2-week interval between each sample. The table shows the correlations between the percent of different lymphocyte subtypes (NK, B, CD4+, CD8+) in the different blood samples. Not statistically significant. All of the other correlations shown in this table are statistically significant (at least P < 0.05).

### Procedure of Experiment 2
The procedure of this experiment was the same as in experiment 1 except that the primary tumor was removed earlier than in the previous experiment (day 41 after tumor-cell injection) to reduce the possibility of any mortality arising directly from the primary tumor and thus increase longevity in the experiment. Sixteen animals were used. In addition, three other animals were treated exactly as the 16 animals described above, including surgery on day 41, but these animals were not injected with vehicle containing no tumor cells. These animals given no tumor cells were included to determine whether changes in blood lymphocyte subtypes that occurred after tumor-cell injection were attributable to the presence of tumor or occurred simply because of repeated sampling or other manipulations. As in experiment 1, animals remained undisturbed in their home cage after tumor-removal surgery until death appeared imminent, at which time the animal was sacrificed, and a necropsy was conducted.

### Results

#### Baseline Measurement of Lymphocyte Subsets
Table 1 shows, for each of the lymphocyte subtypes that was measured, the correlation between each of the four blood samples taken before tumor cells were injected. In both experiments, all of these correlation coefficients were statistically significant (P < 0.05) except one. That these correlation coefficients were significant indicates that the composition of blood lymphocyte subtypes found in individual animals was similar from one sampling to another, and thus individual animals show statistically significant stability with respect to these subtypes of circulating lymphocytes. For each lymphocyte subtype, the average of the four measures made before tumor-cell injection was computed for each animal, and this constituted each animal’s “baseline” value for that lymphocyte subtype.

#### Growth of Primary Tumor
On the day that the fourth blood sample was taken, tumor cells were injected into the leg, after which, as expected, a primary tumor subsequently developed at the site of the injection. In experiment 1, the average tumor size on day 21 after injection was 20.2 (± 1.6) mm in diameter, and this progressed to 34.4 (± 1.7) mm by day 47. The validity of the measurement technique used to measure the size of the primary tumor in vivo was confirmed when the tumor was removed on day 49, weighed, and the weight of the excised tumor correlated with the measurements of size. The average tumor weight on day 49 was 16.6 (± 2.0) g, and, more important, the weight of the individual tumors correlated r = +.93 with measurement of diameter made on day 47. In experiment 2, smaller primary tumors developed than were seen in experiment 1. On day 21 after injection, average tumor size was 13.4 (± 1.5) mm, and this progressed to 20.5 (± 4.6) mm on day 41. The weight of the tumor when it was removed on day 41 again correlated highly (r = +.90) with the size (diameter) measured in vivo on that day. It can be noted that in experiment 2, considerable variability of tumor size was seen on day 41 because in this study, some tumors actually became smaller.

### Table 1  Correlations between percent of different lymphocyte subtypes (NK, B, CD4+, CD8+) measured in blood samples taken at 2-week intervals

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
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<tr>
<td>NK Cells</td>
<td></td>
<td>.79</td>
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<tr>
<td>B lymphocytes</td>
<td></td>
<td>.74</td>
</tr>
<tr>
<td>CD4+ lymphocytes</td>
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<td>.69</td>
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<tr>
<td>CD8+ lymphocytes</td>
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<td>.66</td>
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Tail blood was sampled from 16 rats in each of two experiments; four samples were taken (indicated by sample number) with a 2-week interval between each sample. Table 2 shows the correlations between percent of different lymphocyte subtypes (NK, B, CD4+, CD8+) in the different blood samples.

### Table 2  Correlations between blood lymphocyte subtype and primary tumor size

<table>
<thead>
<tr>
<th>Correlation between</th>
<th>B</th>
<th>NK</th>
<th>CD4+</th>
<th>CD8+</th>
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<tbody>
<tr>
<td>% of subtype at baseline</td>
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<td></td>
<td></td>
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<tr>
<td>and tumor size on day 21&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Experiment 1</td>
<td>−.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.25</td>
<td>+.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.36</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>−.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.13</td>
<td>+.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.31</td>
</tr>
<tr>
<td>% of subtype at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>and tumor size on day 47 (experiment 1)</td>
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<tr>
<td>Experiment 1</td>
<td>−.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.22</td>
<td>+.36</td>
<td>−.25</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>−.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.12</td>
<td>+.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.42</td>
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<tr>
<td>% of subtype at baseline</td>
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<tr>
<td>and tumor weight on day 49 (experiment 1)</td>
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<tr>
<td>Experiment 1</td>
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<td>−.43&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>−.17</td>
<td>+.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.47&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>% of subtype on day 21</td>
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<td>and tumor size on day 21</td>
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<tr>
<td>Experiment 1</td>
<td>−.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>−.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.02</td>
<td>+.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.30</td>
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<tr>
<td>% of subtype on day 47 (experiment 1)</td>
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<td>or 41 (experiment 2)</td>
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<tr>
<td>Experiment 1</td>
<td>−.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−.46&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>−.11</td>
<td>+.10</td>
<td>−.16</td>
</tr>
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</table>

<sup>a</sup>Tail blood was sampled from 16 rats in each of the two experiments; four samples were taken at 2-week intervals before tumor cells were injected, and a sample was also taken when the size of the primary tumor was measured at 3 weeks after tumor-cell injection (day 21) and at 67 weeks after tumor-cell injection (day 41 or 47/49). For each of the lymphocyte subtypes measured (NK, B, CD4+, CD8+), the correlation between the size of each animal’s primary tumor (or the weight of tumor on the day it was surgically removed) and the percentage of that lymphocyte subtype found in its blood before the injection of any tumor cells, i.e., at baseline (average of the four samples), or in the blood on the day when the size of the primary tumor was measured.

<sup>b</sup>Statistically significant (at least P < 0.05).
than they had been on day 21—whereas in experiment 1 every animal showed a larger primary tumor on the second measurement than the first, six animals in experiment 2 showed a decrease in primary tumor size on the second measurement. Of these, two animals in experiment 2 apparently had resolved the primary completely by the second measurement; their primary tumor, which had been of measurable mass on day 21, was no longer evident on day 41 either by palpation or on inspection of the region when it was opened surgically for tumor removal.

**Relationship of Lymphocyte Subtypes in Blood before Injection of Tumor Cells to Size of Primary Tumor.** The top six rows in Table 2 show correlations between the baseline value of each lymphocyte subtype (i.e., the average value computed from the four blood samples taken before injection of tumor cells) and the size of the primary tumor measured in each animal in vivo on day 21 and on day 47 (experiment 1) or 41 (experiment 2) after tumor-cell injection, as well as the weight of this tumor when it was removed. In both experiments 1 and 2, a significant relationship was seen between the baseline value for B lymphocytes and the size of the tumor on day 21 after tumor-cell injection; these correlations were the largest observed in the study. These relationships were negative, indicating that a high percentage of B lymphocytes at baseline was associated with small tumor size. The baseline value of B lymphocytes was also significantly correlated with tumor size on days 47 or 41 and weight of tumor when it was removed, although these correlations were not as large as those with tumor size on day 21. The other lymphocyte subtype whose baseline value was related to tumor size was CD4+ lymphocytes. The percent of these lymphocytes measured before tumor-cell injection was positively related to tumor size, meaning that a high percentage of CD4+ lymphocytes was associated with large tumor size. Interestingly, the baseline value for NK cells showed no relationship to subsequent tumor size despite the fact that growth of the CC531 tumor has been found to be under NK surveillance. Baseline values of CD8+ cells also were not significantly related to tumor size (except in one instance).

Percentages of lymphocyte subtypes in circulation on the days that tumor size was measured were also correlated with the size of the tumor measured on those days, and these correlations are shown in the lower four rows of Table 2. On days when tumor was measured, the percent of B and CD4+ lymphocytes in circulation on those days had a significant relationship to tumor size, whereas NK and CD8+ lymphocytes also showed this relationship but only in the first experiment.

**Relationship of Lymphocyte Subtypes in Blood before Injection of Tumor Cells to Duration of Survival.** Table 3 shows the frequency and location of metastases found at the necropsy of the animals in experiments 1 and 2. All animals that were necropsied, which was done when an animal approached death, had extensive metastases in lung, often >1000 colonies in the lung, and this was the apparent cause of death in all cases. Metastases were also found in lymph nodes, colon, and thymus. Four animals in experiment 2 lived beyond the 6-month cutoff for the study and consequently metastases were not assessed in these animals.

An important focus of the study was to determine whether baseline measures of lymphocyte subtype (i.e., pretumor measures) might be related to the length of time that animals survived when they were subsequently injected with tumor cells. To assess this relationship in experiment 1, the animals were divided into groups representing the extremes of survival. In experiment 1, no animal survived beyond 116 days after tumor cells were injected. Six rats survived the longest, all of these living >90 days after tumor-cell injection; these were designated as “long survivors.” Five rats in this study formed a group that survived for the least duration, all of these living for <65 days after tumor-cell injection; these were designated as “short survivors.” Fig. 1 shows average baseline lymphocyte values of the long and short survivors. Whereas no difference for any subtype reached statistical significance, the baseline value of B lymphocytes showed the largest difference between long and short survivors, with long survivors tending to have higher baseline B lymphocytes than did short survivors. This difference approached, but did not reach, statistical significance.

As pointed out in the procedure, in experiment 2 the primary tumor was removed earlier than in experiment 1 (i.e., 41 days after tumor cells were injected versus 49 days) to attempt to increase differences in longevity between individual animals and thus permit better evaluation of any relationship between lymphocyte baseline values and duration of survival. Baseline values for two lymphocyte subtypes, B and CD4+, were found to be related to survival duration in experiment 2. Fig. 2 shows the relationship between the baseline value for B lymphocytes and survival duration for each animal in the study, and Fig. 3 shows the same relationship for CD4+ lymphocytes. As Fig. 2 shows, high baseline values of B lymphocytes were associated with long survival; in fact, those animals with the highest pretumor levels of B lymphocytes were still alive 180 days (6 months) after tumor-cell injection. The correlation of baseline B-lymphocyte value and number of days of survival shown in Fig. 1 was \( r = +.63 \) (\( P < .05 \)). Survival duration was also related to baseline CD4+ lymphocytes, which is shown in Fig. 3. The baseline value of CD4+ lymphocytes was negatively related to survival—animals with low CD4+ lymphocytes before tumor-cell injection lived longer than animals with high CD4+ lymphocytes. The correlation of baseline CD4+ lymphocyte values and number of days of survival was \( r = -.58 \) (\( P < .05 \)). Not significantly related to survival were baseline values of NK cells (\( r = -.04 \)) or CD8+ cells (\( r = +.30 \)).

**Changes in Circulating Lymphocyte Subtypes during Tumor Development.** Because blood samples were taken and lymphocyte subtypes were measured on the days that tumor...
size was determined, changes in circulating lymphocyte subtypes occurring in the course of tumor development also could be assessed. Fig. 4 shows the percentage of lymphocyte subtypes on the days that tumor size was measured in both experiments 1 and 2; baseline values are also shown to permit evaluation of change as tumor developed. Considering that changes in blood lymphocyte subtypes might vary depending on rate of tumor growth and duration of survival, these data are presented for long and short survivors—for experiment 1, these groups are defined as described in the previous section, whereas for experiment 2, six animals survived >100 days (long survivors) and the remaining 10 animals survived no more than 80 days (short survivors; see Fig. 2).

In experiment 1, similar changes occurred in both long and short survivors, although some changes were more pronounced in the short survivors than in the long survivors. The percentage of B and CD8+ lymphocytes markedly decreased as tumors developed. NK cells were also decreased in tumor-bearing animals relative to baseline, but the decrease was not progressive as the percentage of these lymphocytes increased somewhat on day 47. CD4+ lymphocytes, in contrast to the other lymphocyte subtypes, showed a marked increase with tumor progression. In experiment 2, a small number of additional animals that were not injected with tumor (n = 3) were included in the study to assess the possibility that changes seen in lymphocyte percentage might be simply attributable to repeated sampling or assay variability. Changes in circulating lymphocytes during tumor development were considerably less pronounced in experiment 2 than in experiment 1. The only change seen in tumor-bearing animals in experiment 2 that was (a) similar to what was seen in experiment 1, and (b) also did not occur in “no tumor” animals and thus could be attributed to progression of tumor was a decline in circulating B lymphocytes—a highly significant fall from baseline occurred in circulating B-lymphocyte percent in short survivors, with much less decline occurring in long survivors (whose average primary tumor size actually decreased on the second measurement relative to the first; see lowest panel of Fig. 4).

Discussion
The results described in this paper showed that the percentage of certain lymphocyte subtypes measured in blood before the induction of an experimental tumor predicted the growth (size) of the primary tumor as well as the development of metastases and duration that animals survived after the tumor-cell injection. The present study was stimulated by our earlier results, which suggested that B lymphocytes exert an inhibitory influence over tumor development in certain animal models (3).

Consistent with these earlier findings, differences between individual rats in the percentage of endogenous circulating B lymphocytes showed a significant negative relationship to the size of a CC531 primary tumor that WAG rats developed when tumor cells were injected as well as to the time of death from metastases. Thus, animals with the highest percentages of circulating B lymphocytes before tumor-cell injection developed the smallest primary tumors and lived longest after tumor cells had been injected. Even across the two experiments that were conducted, results consistent with this relationship appeared—the animals in experiment 1 had significantly lower baseline levels of circulating B lymphocytes than did the animals in experiment 2, and the animals in experiment 1 developed larger primary tumors than did the animals in experiment 2 and also died earlier. The results described in this paper contribute further evidence suggesting that B lymphocytes affect certain experimental tumors by showing the importance of endogenous B lymphocytes, thereby adding to previously reported findings that had shown this phenomenon by using experimental manipulation of B lymphocytes (i.e., immunoneutralization) to affect tumor growth.

It can be noted that the relationship between B lymphocytes and tumor development reported in this paper may not be unique to the WAG rat-CC531 tumor model. In a previous study, we injected Fischer 344 rats s.c. with MADB106 tumor cells to produce a primary tumor of this type (3). In that study, one of the animals completely resolved its s.c. MADB106 tumor. When B lymphocytes in lung were assessed (only lung was measured in this study), this animal was found to have by far the highest percentage of B lymphocytes of any animal in the study.

The mechanism through which B lymphocytes influence tumor development, however, is not yet evident from these studies. Of considerable importance, the earlier study (3) showed that immunoneutralization of B lymphocytes had to occur in very close time proximity to the introduction of tumor cells to augment tumor development. Delaying administration of the neutralizing antibody to B lymphocytes by even 1 or 2 h after the tumor cells were injected resulted in significant and time-related decreases in the ability of immunoneutralization to increase tumor development, with a delay of 24 h resulting in no effect of the immunoneutralization. This finding indicates that antitumor effects of B lymphocytes depend on events that occur very soon after tumor cells are present at the site of tumor progression. In that any production of antibodies to the tumor by B lymphocytes would occur at moderately long time intervals after the introduction of tumor antigen (i.e., days), the observed time course of the effects of immunoneutralization
indicates that antitumor actions of B lymphocytes are mediated, at least in part, by functions of B lymphocytes other than production of antitumor antibodies. Various other functions of B lymphocytes can be suggested to underlie their antitumor activity. First, B lymphocytes might enhance cytotoxicity of NK cells, either by increasing production of IFN-γ by NK cells (10) or by themselves releasing interleukin 12, which markedly potentiates NK cell cytotoxicity (11). Second, antitumor activity of B lymphocytes may derive from their functioning as antigen-presenting cells, in that antigen presentation is important in tumor killing (12, 13). B lymphocytes have been found to bind, internalize, and present antigens (reviewed by Pernis and Weber (14)).

CD4+ lymphocytes also were related to tumor growth, but this lymphocyte subtype showed the opposite relationship from that shown by B lymphocytes, i.e., baseline CD4+ lymphocyte percentage was positively related to primary tumor size and negatively related to length of survival. Thus, animals with high percentages of CD4+ lymphocytes before tumor-cell injection developed the largest primary tumors and died soonest after being injected with the tumor. An obvious question is whether the reciprocal relationship between B and CD4+ lymphocytes is an artifact of the measure used, which was the percentage of total lymphocytes. It can be asked whether a high percentage of CD4+ lymphocytes might appear to be related to tumor growth simply because this measure would necessarily rise in any instance where the percentage of B lymphocytes is reduced, or vice versa. However, analysis of the data shows that this is not the case, but that both B and CD4+ lymphocytes make a separate significant contribution to predicting tumor growth and mortality. For example, in experiment 1, baseline percent B lymphocytes correlated −.63 with tumor size of day 21, and baseline percent CD4+ lymphocytes correlated +.51. Calculation of a multiple correlation using the explanatory value of both B and CD4+ baselines yielded a multiple r of .88, which meant that using both B and CD4+ baselines explained 77% of the variance in tumor size on day 21, whereas the B lymphocyte baseline alone explained 40% of the variance. Thus, adding baseline percent of CD4+ cells increased the amount of variance that was explained by the largest of the individual correlations (i.e., that of baseline percent B lymphocyte) by 37%, which is a highly statistically significant addition (P < .01).
CD4+ lymphocytes each made a statistically significant independent contribution to predicting tumor size shows that the two measures do not owe their ability to predict tumor size to their being correlated with one another.

It was also observed that the percentage of B lymphocytes in circulation declined as the experimental tumor grew. A fall in circulating NK and CD8+ lymphocytes was seen in experiment 1, but only the fall in B lymphocytes appeared consistently in both experiments. A possible explanation for why this occurs is that B lymphocytes move out of circulation as part of the host response to control tumor development and that B lymphocytes then accumulate in tumor sites and/or lymph nodes during the growth process because these lymphocytes are directly involved with tumor surveillance at the site of tumor cells. This possibility would be consistent with our previous findings (3) reporting accumulation of B lymphocytes in lung when tumors that metastasize in lung were introduced. It also would explain why animals having a high percentage of circulating B lymphocytes develop smaller tumors, reduced metastases, and survive longer, in that such animals would have large numbers of circulating B lymphocytes available for combating tumor development.

The results described here relate to previous clinical findings regarding B lymphocytes in cancer development. In the 1970s, studies examined lymphocyte number and subtype composition in peripheral blood of cancer patients, and several of these studies reported that a reduced level of B lymphocytes in the peripheral blood of cancer patients was prognostic of poor outcome [reviewed in Lee (15)]. Similar findings were subsequently reported by Domagala et al. (16) and Wood and Neff (17), the latter investigators arguing that the decrease in B lymphocytes could not be accounted for by monocytosis in the cancer patients. Recently, Kay et al. (18) reported that patients with multiple myeloma showed low circulating levels of CD19+ B lymphocytes in clinical stage III and that high circulating levels of CD19+ B lymphocytes were associated with longer event-free survival and clinical response. Similarly, Rawstron et al. (19) found a suppression of CD19+ B lymphocytes in presentation or relapse stages of multiple myeloma. The finding reported in this paper that circulating B lymphocytes declined in the WAG rat during tumor development would appear consistent with the findings reported in these papers. However, it should be noted that not all investigations have observed a decrease in circulating B lymphocytes during cancer development because Stein et al. (20) reported elevated levels of circulating CD5+ lymphocytes in cancer patients with
a variety of tumors. On the other hand, this last finding might not be contradictory but instead indicate that different subpopulations of B lymphocytes are affected differently during cancer development. It should be noted that these clinical findings were obtained in patients who already had cancer, whereas the present study is prospective in design and demonstrates for the

Fig. 4. Percent of lymphocyte subtypes (B, NK, CD4+, CD8+) in blood at baseline (i.e., average of four blood samples taken at 2-week intervals before tumor-cell injection) and on days 21 and 47 (experiment 1) or 41 (experiment 2) after tumor-cell injection in short and long survivors in both experiments 1 and 2. See “Results” for the definition of short and long survivors. Experiment 2 also included rats measured at each time point but not injected with any tumor. Also shown (bottom) is the size of the primary tumor. Means and SEs are presented. * differs significantly (at least P < .05) from baseline (by dependent t test comparing each animal’s level with its own baseline value).
first time that lymphocyte subtypes in peripheral blood before cancer development are predictive of survival after tumor challenge. Further research is needed in animal models to define the nature of the role that the B lymphocyte plays in the progression of some tumors and in human populations to determine if monitoring of lymphocyte subtypes is clinically relevant for predicting tumor development and metastases.

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Blood Level of B and CD4+ Lymphocytes Measured before Induction of an Experimental Tumor in Rats Predicts Tumor Progression and Survival

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