

# Evidence for Involvement of B Lymphocytes in the Surveillance of Lung Metastasis in the Rat<sup>1</sup>

Ning Quan, Zhao-bin Zhang, Melissa K. Demetrikopoulos, Richard P. Kitson, William H. Chambers, Ronald H. Goldfarb, and Jay M. Weiss<sup>2</sup>

Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, Georgia 30322 [Z.-b. Z., M. K. D., J. M. W.]; Department of Pathology, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213 [W. H. C.]; Department of Molecular Biology and Immunology, University of North Texas Health Science Center at Fort Worth, Fort Worth, Texas 76107 [R. P. K., R. H. G.]; and National Institute of Mental Health, Section on Functional Neuroanatomy, Bethesda, Maryland 20892 [N. Q.]

## ABSTRACT

These studies examined the composition of lymphocytes within the lung after the introduction of tumor cells that metastasize to the lung in rats. *i.v.* delivery of MADB106 tumor cells into syngeneic Fischer 344 rats caused dose- and time-dependent development of lung tumors, with surface metastases evident 7 days after injection and markedly increased 11 days after injection. The total number of lymphocytes recovered from the lung was increased 11 days after injection but not 7 days after injection. When lymphocytes from the lung, spleen, and blood were subjected to fluorescence-activated cell sorting analysis, the most conspicuous change was an increase in the percentage of CD45RA+ cells (*i.e.*, B lymphocytes in the rat) in the lung, with no changes seen in the percentage of natural killer (NKR-P1+), CD4+, or CD8+ cells in the lung. Analysis of the time course showed that B lymphocytes increased in the lung soon after *i.v.* tumor injection, with an initial peak seen 6 h after injection. Rapid influx of B lymphocytes into lung after *i.v.* tumor cell injection was also observed in another syngeneic tumor model, *i.e.*, after injection of CC531 cells into WAG rats. To determine whether the influx of B lymphocytes into the lung might participate in tumor surveillance, a high dose of antibody (100  $\mu$ g) to rat B lymphocytes was given to immunoneutralize these cells; this produced an increase in lung tumors in both models. Finally, Fischer 344 rats were given a *s.c.* injection of MADB106 tumor cells that made them resistant to lung tumors when given a later *i.v.* injection of these tumor cells. These animals were found to have an elevated level of B lymphocytes residing in the lung associated with the resistance to lung tumor. These findings suggest that early responses of B lymphocytes are important in protection against tumor development in two rat models of cancer.

## INTRODUCTION

MADB106 tumor, a chemically induced mammary adenocarcinoma of Fischer 344 rats, has been used in several studies to investigate the effects of NK<sup>3</sup> cells on tumor metastasis in a syngeneic model. MADB106 cells primarily metastasize to lung after *i.v.* administration, and the development of this tumor is sensitive to NK cells. Barlozzari *et al.* (1) showed that reducing NK activity by giving an antiserum to NK cell surface marker asialo GM<sub>1</sub> markedly decreased lung clearance of radiolabeled MADB106 cells and increased surface lung metastases in rats given injections of the tumor cells. These effects were prevented by adoptive transfer of purified NK cells, but not T cells, before tumor injection. To further confirm that the effects were due to NK cells, the investigators showed that alveolar macrophages were asialo GM<sub>1</sub>-negative and that

their tumoricidal activity against MADB106 tumor was not affected by the antiserum treatment. The sensitivity of MADB106 to NK cells has since been confirmed in similar studies using mAb 3.2.3 to eliminate NK cells (2–4).

The original purpose of the study described here was to measure the number of NK cells in lung after Fischer 344 rats received injections of MADB106 tumor cells to determine whether the number of NK cells would be increased in lung after tumor cell injection, the expectation being that NK cells would migrate to lung in the process of tumor defense. The initial studies assessed lymphocyte subsets at different times after *i.v.* tumor cell injection, examining lymphocytes recovered from lung, spleen, and blood. Surprisingly, the most marked change observed was a significant increase in the number of B lymphocytes appearing in lung, with no apparent change occurring in the NK cell number in lung. Changes in lung lymphocytes were then examined in another tumor model—the injection of CC531 tumor cells into WAG rats—to determine whether similar changes would be seen here as well, and an increase in B lymphocytes in lung was also observed in this tumor model. To test the possibility that B lymphocyte function might be involved in antitumor activity, animals were treated with an anti-B lymphocyte antibody, and the effect on the development of lung metastases was assessed in both models. Giving an antibody to B lymphocytes in conjunction with *i.v.* injection of tumor cells increased lung metastases markedly in both tumor models. The final studies reported here examined lung lymphocytes in Fischer 344 rats that were made resistant to the development of lung metastases after *i.v.* injection of MADB106 tumor cells by being given an earlier *s.c.* injection of MADB106 tumor cells. In these “resistant” animals, the percentage of B lymphocytes in lung was found to be markedly elevated before they received the *i.v.* injection of tumor cells. On the basis of the findings of these studies, we conclude that B lymphocytes seem to participate in the surveillance of lung tumor metastases in the rat models examined.

## MATERIALS AND METHODS

**Animals.** Male inbred Fischer 344 (Charles River) and WAG (Harlan/CPB, Zeist, the Netherlands) rats weighing 200–300 g were used in this study. Animals were housed three to a cage in enclosed microisolator cages under positive-pressure ventilation with the cages maintained on closed-shelf, laminar-flow racks. Thus, rats in any given cage were not in contact with any pathogens, odors, or noises of other animals in the colony. The temperature of the animal room was maintained at  $22 \pm 1^\circ\text{C}$ ; light and darkness alternated, with the light on from 7:00 a.m. to 7:00 p.m. The animals were given sterilized food and water *ad libitum*.

**Tumor Cell Culture.** MADB106 is a selected variant cell line obtained from pulmonary metastasis of a mammary adenocarcinoma (MADB100) that was chemically (9,10-dimethyl-1,2-benzanthracene[DMBA]) induced in the Fischer 344 rat (1). The cell line used here was checked for mycoplasma contamination and found negative using a mycoplasma detection kit (Boehringer Mannheim, Indianapolis, Ind.). When the initial cells used produced a low number of lung metastases after *i.v.* injection, the cell line was rederived by passage through animals pretreated with cyclophosphamide to ablate NK

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<sup>2</sup> To whom requests for reprints should be addressed: Jay M. Weiss, Emory West Campus, 1256 Briarcliff Road NE, Atlanta, GA 30306. Phone: (404) 894-5948; Fax: (404) 894-5901; E-mail: jweiss01@emory.edu.

<sup>3</sup> The abbreviations used are: NK, natural killer; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody; FITC, Fluorescein Isothiocyanate; PE, R-Phycocerythrin.

activity; tumors were removed from lungs and cultured, and the resulting cell line produced increased and relatively consistent numbers of lung metastases. CC531 is a cell line derived from a 1,2 dimethylhydrazine (DMH)-induced adenocarcinoma of the colon of the WAG rat (5); the cell line used was obtained from Dr. Peter Kuppen (University of Leiden, Leiden, The Netherlands). Cell lines were maintained in 5% CO<sub>2</sub> 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 acid, and 1 mM sodium pyruvate. To control for possible mutation of the cell lines, only the cultures that had undergone less than 10 passages (from the original batch that we obtained) were used in the experiments. Cultures were harvested from the flask with 0.25% EDTA in HBSS, washed, and suspended in PBS before being injected into animals.

**Induction and Assessment of Lung Tumor Metastases.** Rats were lightly anesthetized with halothane, and tumor cells were injected into the lateral tail vein in a volume of 1.0 ml of PBS. At various time intervals after injection, animals were killed by the opening of the chest cavity under halothane anesthesia. To quantify metastases, the method described by Wexler (6) was used. Lungs were removed and infused via the trachea with a distilled water solution containing 15% India ink. Lung tissue was then placed in a dedying solution consisting of (by volume) 70% ETOH, 10% formaldehyde, 5% glacial acetic acid, and 15% distilled water. After 48 h in the dedying solution, normal lung surface retains black stain, whereas the tumor (which resists the dye) appears as white nodules on the surface of the lung. This method has been shown to be equally sensitive to the use of radiolabeled tumor cells for detecting lung metastasis of MADB106 (1). The number of metastases on the surface of the lung was quantified by the following procedure: as stated above, counting of metastases was done on lung removed from the dedying solution after 48 hours. For the counting procedure, lungs were coded so that the person doing the counting was unaware of the condition represented by the tissue being quantified. For the lung tissue from any given animal, three surface areas, each 1.0 cm<sup>2</sup> in size, were demarcated, and the number of metastases in each of these areas were counted; the average of the three areas was computed and represented the number of metastases (per cm<sup>2</sup>) for that subject. The three areas to be counted were selected by the individual doing the counting on the basis of their appearing representative of the entire lung; in some instances the number of metastases on the entire lung surface was also counted in addition, and the resultant count was found to correspond closely with the estimate obtained by sampling three regions.

**Lymphocytes Collection from Lung, Blood, and Spleen.** Lymphocytes were collected by a modification of the method of Thivierge and Rolapleszcynski (7). Briefly, after animals were anesthetized with halothane, they were perfused with an artificial interstitial fluid buffer (0.12 M NaCl, 0.047 M K Cl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.025 M NaHCO<sub>3</sub>, 2.4 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.2 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 10 mM glucose) supplemented with 1.5% BSA via the insertion of an input cannula into the left ventricle of the heart. Heparin (10 units/ml) was added to prevent blood coagulation. Blood was collected from an output cannula inserted into the right atrium. The lungs and spleens were then dissected out. The lungs were further perfused with a similar buffer containing 0.6 mg/ml protease (Sigma; Type XXIV:bacterial) and 1 mM EDTA to break down connective tissue in order to facilitate release of lymphocytes. Lungs were then incubated for 10 min in protease, after which the tissue was cut into small pieces in the artificial interstitial fluid supplemented with DNAase (80 mg/ml). Spleen was also cut into small pieces in RPMI culture. The cells of the lung and spleen were then dispersed and filtered through a mesh nylon cloth of 210 · mm pore size. WBCs were collected after blood samples were centrifuged at 300 × g for 30 min and then suspended in RPMI. After cell suspensions of blood, lung, and spleen in RPMI, were washed twice, they were gently layered over a gradient of Ficoll-Hypaque and centrifuged at 500 × g for 30 min. The lymphocytes thus separated on the gradient were used for FACS analysis of lymphocyte subsets.

**FACS Analysis.** Lymphocytes that were recovered in each compartment were counted on a hemacytometer and resuspended in PBS containing 10% goat serum at a concentration of 5 × 10<sup>6</sup> cells/ml. Two samples of 100 μl each were then taken for double labeling of lymphocytes, which was done by incubating each sample for 45 min at 4°C after the addition of 50 μl of 1:200 dilution of FITC-conjugated mAbs to CD4+ cells (OX38; Pharmingen) and PE-conjugated anti-CD45RA+ (OX33; Pharmingen) to one sample, and 50 μl of 1:300 dilution of anti-NKR-P1-FITC (3.2.3; Ref. 8) and anti-CD8+PE

(OX8; Pharmingen) to the other sample. The dilutions used were determined by initially carrying out serial dilutions to ascertain the best staining of lymphocytes for these surface markers. Each sample was then washed twice with 3 ml of 10% goat serum in PBS, resuspended in 200 μl of this solution, and immediately analyzed on a FACSscan (Becton-Dickinson) using the consort program. Positive staining was gated by subtracting autofluorescence from unstained control samples.

Concerning the antibodies selected for labeling, OX33 has been found to bind only to a large cell surface glycoprotein, CD45RA+, which, in the rat, has been found only on purified B lymphocytes (9). To further confirm that this mAb was specific for rat B lymphocytes, blood and splenic lymphocytes from both Fischer 344 and WAG rats were double-labeled with OX33 and either anti-NKR-P1 (3.2.3) or anti-CD8+ (OX8); this was done in addition to double-labeling with OX33 and anti-CD4+ that is normally carried out in the FACS analysis (see preceding description). Also, additional lymphocytes were double-labeled with OX33 and an anti-IgM antibody. Essentially no cells (*i.e.*, less than 0.5% in all cases) were double-labeled by OX33 and either the anti-NKR-P1 or anti-CD8+ antibody, as is also the case for double-labeling with OX33 and anti-CD4+. In contrast, more than 90% of lymphocytes that were labeled with OX33 were found to be labeled with anti-IgM.

**Statistical Analysis.** Differences between control and experimental conditions were assessed by one-way ANOVA (in cases in which more than two groups were included in the experiment) or by *t*-tests (for comparison of a single experimental condition with the control condition). When an ANOVA was conducted, a comparison of each individual experimental condition with the control condition was then conducted using Dunnett's test when a significant overall main effect attributable to experimental condition was found. Statistical significance reported in the text and figures are based on these analyses.

## RESULTS

**Characterization of Lung Tumor Metastasis after i.v. Injection of MADB106 Tumor Cells.** One ml of PBS vehicle containing MADB106 at either 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> cells/ml was injected into the tail vein of different groups of Fischer 344 rats. Seven days after the injection, these animals were killed to measure tumor metastases in the lung. Fig. 1 (*left*) shows that the injection of MADB106 tumor cells caused dose- and time-dependent development of lung tumor metastases. When killed on day 7 after injection, 10<sup>4</sup> tumor cells did not induce any visible lung surface metastasis, whereas 10<sup>5</sup> and 10<sup>6</sup> tumor cells induced a mean (±SE) of 2.8 ± 1.1, and 11.8 ± 2.4 metastases/cm<sup>2</sup>, respectively. In animals injected with 10<sup>6</sup> tumor cells and killed on day 11 after the injection, the number of metastases resulting from 10<sup>6</sup> tumor cells had increased 6-fold (to 67.6 ± 3.8 metastases/cm<sup>2</sup>) from the number seen on day 7.

**Number of Lymphocytes Recovered from Lung, Spleen, and Blood after Tumor Cell Injection.** On day 7 and 11 after the animals were injected with MADB106 tumor cells, lymphocytes were harvested from lung, spleen, and blood, and subjected to FACS analysis to determine lymphocyte subtypes present in these compartments. The number of lymphocytes recovered from spleen and blood on day 7 or 11 after the injection was not affected by the injection of any dose of tumor cells. The mean (and SE) number of lymphocytes recovered from vehicle-injected animals was 57.6 ± 5.2 (× 10<sup>6</sup>) from spleen and 10.2 ± 1.2 (× 10<sup>6</sup>) from blood (10 ml). The number of lymphocytes recovered from lung are shown in Fig. 1 (*right*). Approximately 2.0 × 10<sup>6</sup> lymphocytes were obtained from the lungs of vehicle-injected control animals. On day 7 after tumor cells were injected, the number of lymphocytes recovered from the lungs was not significantly affected by any dose of tumor cells. On day 11, however, the number of lymphocytes recovered from the lungs was significantly increased in animals that had been injected with 10<sup>5</sup> and 10<sup>6</sup> tumor cells.

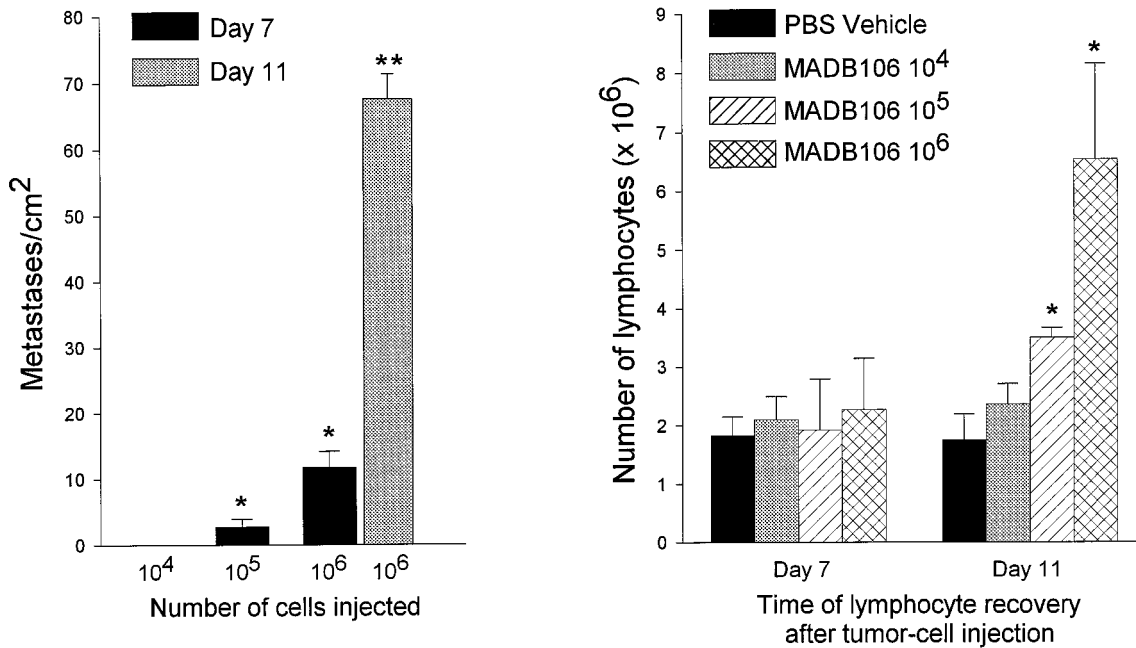


Fig. 1. *Left*, the mean number of lung tumor metastases counted on day 7 and day 11 after Fischer 344 rats received an injection into the lateral tail vein (i.v.) with various doses of MADB106 tumor cells. *Bar*, SE; \*, significantly different (at least  $P < 0.05$ ) from the number of metastases counted after injection of  $10^4$  tumor cells. *Right*, the mean number of lymphocytes recovered from lung on day 7 and on day 11 after rats received injections with various doses of MADB106 tumor cells. \*, significantly different (at least  $P < 0.05$ ) from saline-injected animals (PBS Vehicle). *Bar*, SE.  $n = 4$  for all groups shown.

**Subsets of Lymphocytes in Lung at 7 and 11 Days after Tumor Injection.** Fig. 2 shows the results of FACS analysis, indicating the percentage of lymphocyte subsets found in lung, blood, and spleen 7 days (*left*) and 11 days (*right*) after tumor cell injection. Blood and splenic lymphocytes showed no evidence of changes in the percentage of NK, CD4+, CD8+, and B lymphocytes in these compartments. After tumor cell injection in lung, however, a change in the percentage of B lymphocytes was seen. In vehicle-injected control animals, the B lymphocyte percentage in the lungs was very low, usually less than 1% of lung lymphocytes ( $0.6 \pm 0.3\%$  for all control animals at both time points). As is evident in Fig. 2, B lymphocyte percentage after tumor cell injection, although never becoming a large percentage of lymphocytes in the lungs, was elevated appreciably and significantly 7 and 11 days after injection.

**Subsets of Lymphocytes in Lung at 2, 6, and 18 h after Tumor Injection.** To study the onset of the B-lymphocyte increase in the lungs, lymphocyte subsets were analyzed at 2, 6, and 18 h after the i.v. injection of  $10^6$  tumor cells. The total number of lymphocytes recovered from the lungs at 2, 6, and 18 h after the tumor injection was, respectively,  $1.9 \pm 0.6$ ,  $2.0 \pm 0.5$ , and  $2.5 \pm 0.6 \times 10^6$  cells per lung, which did not differ from the number of lymphocytes found in control animals killed in conjunction with the tumor-injected animals of this study (*i.e.*,  $2.1 \pm 0.4 \times 10^6$ ); also, no differences in the total number of lymphocytes obtained from the blood or spleen were noted at any of these time points.

The percentages of lymphocyte subsets found in lung, blood, and spleen are shown in Fig. 3. As was seen on days 7 and 11 after tumor cell injection, analysis of blood and splenic lymphocytes also recovered at time intervals more proximal to the tumor cell injection showed percentages of NK, CD4+, CD8+, and B lymphocytes to be unaltered by tumor cell injection. However, changes were again seen in the composition of lung lymphocytes. As shown in Fig. 3, the B lymphocyte percentage was significantly elevated 6 h after tumor cell injection. These findings indicate that the accumulation of B lymphocytes in lung begins quite early after tumor cells are injected (*i.e.*,

some elevation of B lymphocytes in lung was present 2 h after injection). These results also suggest that the early influx of B lymphocytes may be declining by 18 h postinjection. In addition, a small but statistically significant increase in the percentage of CD4+ cells in lung was seen 6 h after injection, as was a small decline in CD8+ cells 2 h postinjection.

A summary of the time course of changes in the percentage of B lymphocytes seen in lung after i.v. injection of MADB106 tumor cells is shown in Fig. 4. In addition to the various time points described previously, Fig. 4 also shows the percentage of B lymphocytes found in lung 2 days after tumor cell injection, a time point that was also measured.

**Effects not due to Contaminants or Lung Irritation.** To determine whether the B lymphocyte influx into lung described above may have occurred because of some contaminant in the tumor-cell culture media, 1.0 ml of PBS containing  $10^6$  MADB106 cells was lysed by ultrasound sonication [by Kontes sonicator set at 40% of full scale (25 watts) power, 20 kHz frequency; exposure of cells three times for 30 s each, with 20 s between exposures] and was injected into the tail vein; when this was done, the percentage of B lymphocytes in lung that were seen 6 h after injection (*i.e.*,  $0.2 \pm 0.1\%$ ;  $n = 3$ ) did not differ from control levels, thereby indicating that a contaminant in the media was not responsible for the effect. Second, to determine whether the increase in B lymphocytes into lung after tumor cell injection might have depended on the use of inhaled anesthetic (*i.e.*, halothane) that irritated lung tissue,  $10^6$  MADB106 tumor cells were injected into nembuthal-anesthetized animals. Six h after injection, the percentage of B lymphocytes in lung was similar to that observed earlier (*i.e.*,  $8.5 \pm 1.4\%$ ;  $n = 3$ ), thus indicating that halothane anesthesia was not a critical factor.

**Subsets of Lymphocytes in WAG Rats Injected with CC531 Tumor Cells.** To determine whether the changes seen in lung that are described above also would characterize another tumor model, CC531 tumor cells were injected into syngeneic WAG rats. CC531, like MADB106, produces lung metastases when injected i.v. (5, 10).

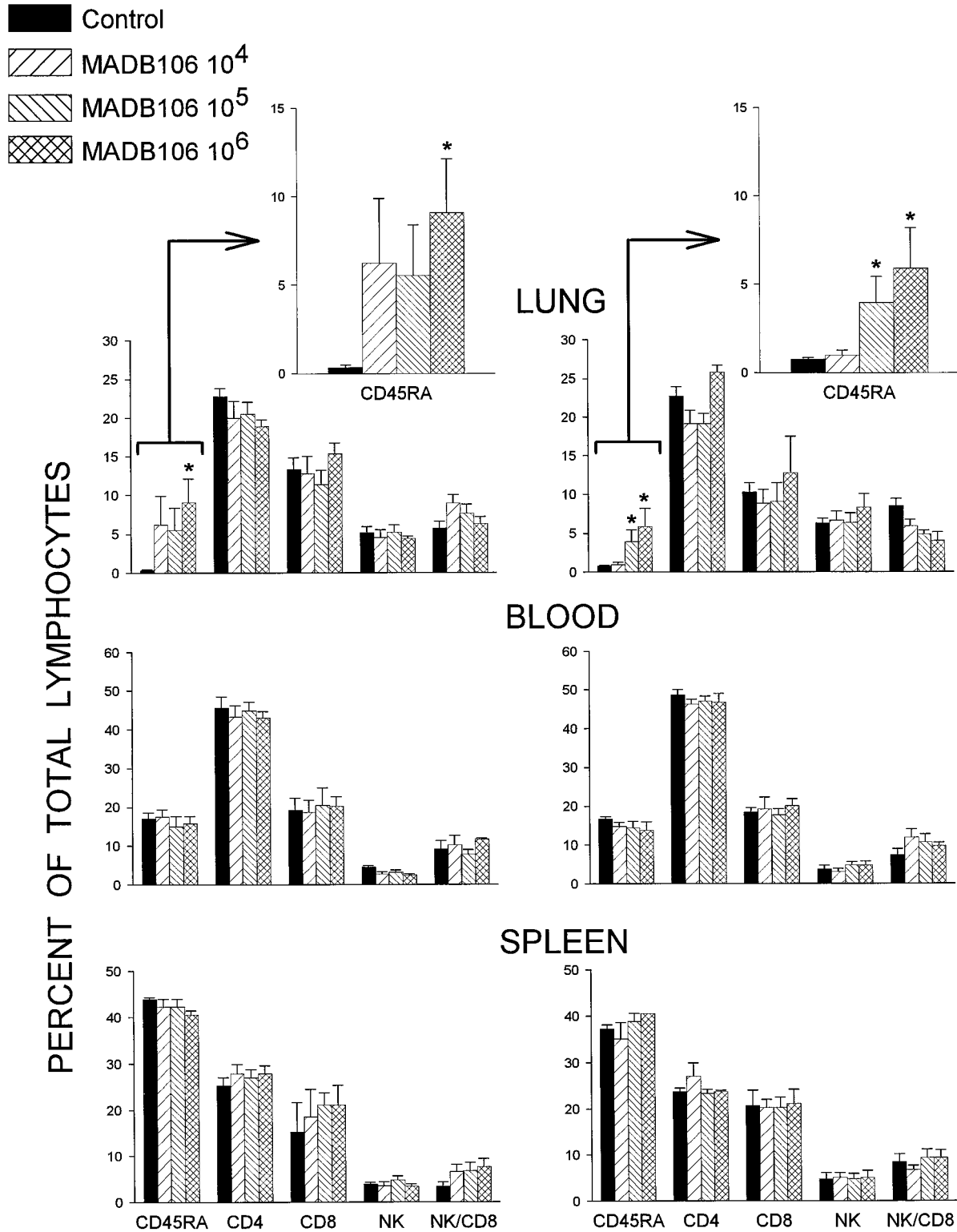


Fig. 2. The percentage of lymphocyte subsets found in lung, blood, and spleen on day 7 (left) and day 11 (right) after Fischer 344 rats were given an injection i.v. with PBS vehicle (Control) or various doses of MADB106 tumor cells. Inserts (arrow) magnify results for CD45RA+ cells in lung shown below the bracket. Means and SEs (bars) are presented.  $n = 4$  in each group. \*, significantly different (at least  $P < 0.05$ ) from Control. This figure shows that, for the various lymphocyte subtypes measured in lung, spleen, and blood, only CD45RA+ cells (B lymphocytes) in the lung were observed to change significantly after i.v. injection with MADB106 tumor cells.

Findings from the analysis of lymphocytes recovered 6 h after tail-vein injection of  $10^6$  CC531 tumor cells are shown in Fig. 5. After the injection of CC531 tumor cells, a statistically significant increase in the percentage of B lymphocytes was also seen in the lungs of WAG rats. Whereas no B lymphocytes could be detected in the lungs of

control WAG rats, the B lymphocyte percentage was  $2.3 \pm 0.2\%$  6 h after tumor cell injection. The small magnitude of this increase may be due to the fact that this particular strain of rat has a very low percentage of B lymphocytes in various compartments under normal conditions (i.e., blood B lymphocytes were  $2.2 \pm 0.4\%$  as compared

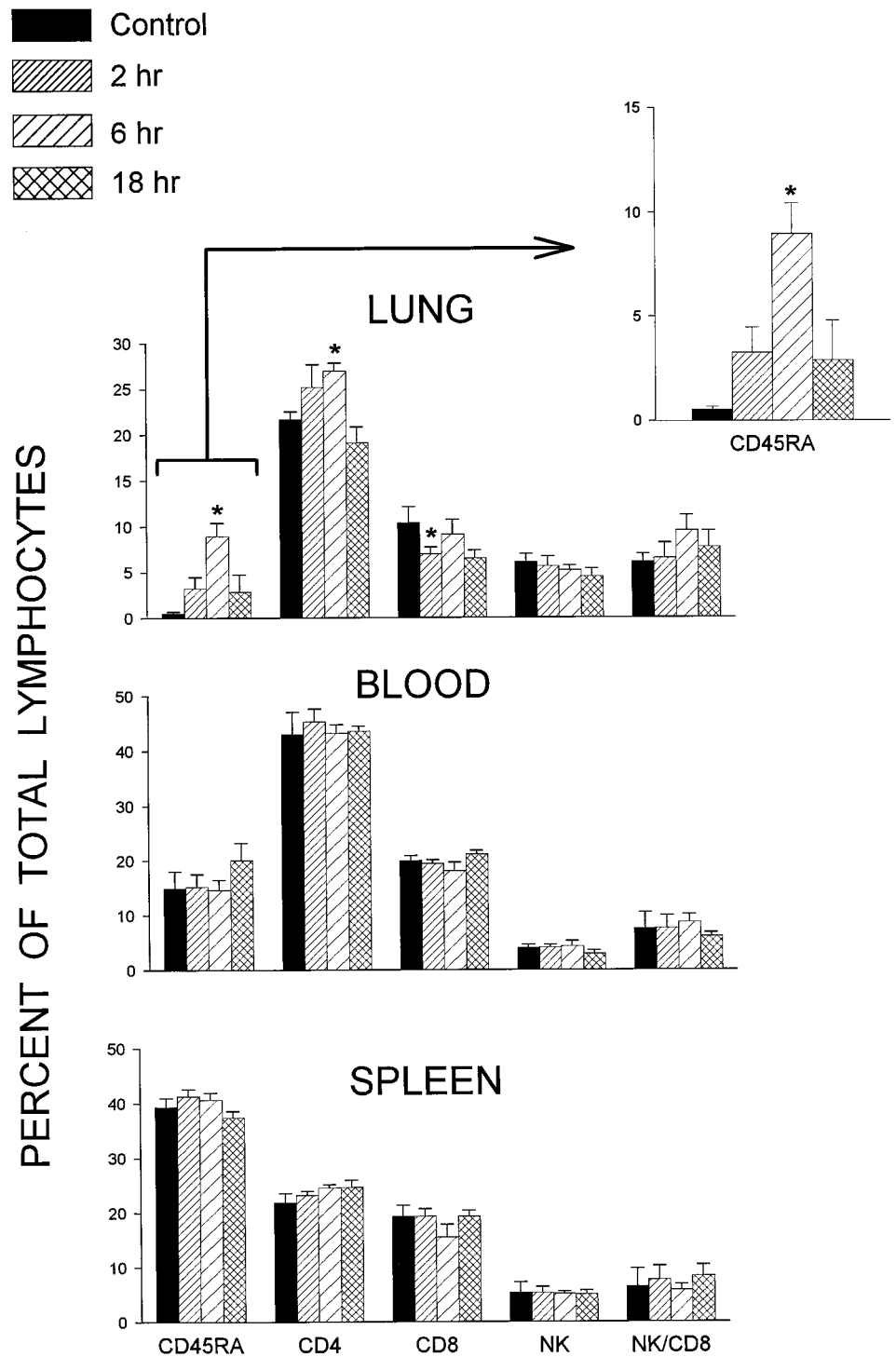


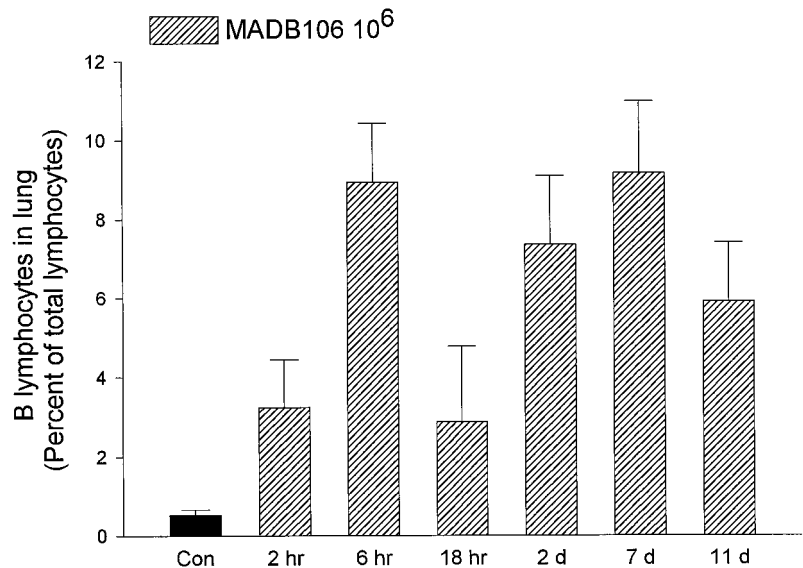
Fig. 3. The percentage of lymphocyte subsets found in lung, blood, and spleen at 2, 6, and 18 h after Fischer 344 rats received an injection of  $10^6$  MADB106 tumor cells. Insert (arrow) magnifies results for CD45RA+ cells in lung shown below the bracket. Means and SEs (bars) are presented. \*, significantly different (at least  $P < 0.05$ ) from Control (noninjected animals).  $n = 4$  in each group. This figure shows that the percentage of CD45RA+ cells (B lymphocytes) was significantly increased in lung 6 h after the i.v. injection with MADB106 tumor cells.

to the  $17.1 \pm 1.5\%$  in Fischer 344 rats and  $21.6 \pm 1.8\%$  in Sprague Dawley rats). Interestingly, the percentage of NK cells in the lungs of WAG rats also was found to be increased significantly (from  $7.3 \pm 1.1\%$  to  $17.4 \pm 1.9\%$ ) 6 h after tumor injection. The total number of lymphocytes recovered from the lungs of control rats ( $3.5 \pm 1.7 \times 10^6$  cells) and of tumor-injected ( $4.1 \pm 1.8 \times 10^6$ ) WAG rats was not different.

**Effect of High-Dose i.v. Injection of OX33 Injection on Lung Tumor Metastasis.** To determine whether B lymphocytes might participate in antitumor activity directed against the experimental tumors studied here, antibody to B lymphocytes (OX33) was injected

i.v. in a high dose ( $100 \mu\text{g}$ ) to attempt to immunoneutralize B lymphocytes and to ascertain whether this would affect development of lung metastases resulting from the i.v. injection of tumor cells. Fischer 344 rats received an i.v. injection with either (a) anti-B lymphocyte antibody (OX33,  $100 \mu\text{g}$  IgG) + MADB106 ( $10^6$  cells) or (b) an isotype control antibody for OX33 (mouse antirat IgG1;  $100 \mu\text{g}$ ) + MADB106. In addition, another group received an injection with MADB106 alone and then were given the OX33 ( $100 \mu\text{g}$ ) i.v. 24 h later, this was done to assess whether effects might be sensitive to the time of antibody administration. Lung tumor metastases were then counted 7 days after tumor injection. Fig. 6 (left) shows that

Fig. 4. Summary of the time course of the appearance of CD45RA+ cells (B lymphocytes) in lung after Fischer 344 rats received an injection of  $10^6$  MADB106 tumor cells. Means and SEs (bars) are shown. This figure shows that the percentage of B lymphocytes in lung was somewhat increased 2 h after the tumor injection, peaked at 6 h postinjection, and then decreased at 18 h postinjection. A second elevation of B lymphocytes was seen at day 2 postinjection, and this elevation remained on day 7 and 11.



animals that received tumor cells without being given the antibody to B lymphocytes (*i.e.*, tumor cells + isotype) developed  $2.1 \pm 0.3$  metastases/cm<sup>2</sup>, whereas animals that received B lymphocyte antibody together with tumor cells developed significantly more metastases ( $19.9 \pm 3.0$ /cm<sup>2</sup>). The ability of B lymphocyte antibody administration to augment lung metastases was short-lived; in those animals given tumor cells followed 24 h later by B lymphocyte antibody, no increase in metastases was seen (*i.e.*,  $2.0 \pm 0.5$  metastases/cm<sup>2</sup>).

The effect of treatment with high-dose antibody to B lymphocytes was also assessed in WAG rats given CC531 tumor cells. Thus, WAG rats also received either B lymphocyte antibody (100  $\mu$ g OX33) + CC531 tumor cells ( $1.5 \times 10^6$ ) or isotype (100  $\mu$ g IgG1) + CC531 tumor cells. The results are shown in Fig. 6 (*right*). As was found for Fischer rats that received MADB106 tumor cells, treatment with OX33 antibody to immunoneutralize B lymphocytes resulted in a marked increase in the development of lung tumors in the WAG rat model as well.

Further examination of the time course of metastases-promoting effects of injecting antibody to B lymphocytes was also carried out. In the *left panel* of Fig. 7 are shown the number of lung metastases that developed after i.v. injection of  $10^6$  MADB106 tumor cells either alone or in conjunction with 100  $\mu$ g OX33 at different times after tumor cells were injected; OX33 was given simultaneously (*i.e.*, "0" h) or 1 or 2 h after i.v. injection of tumor cells. Animals were then killed 9 days later. When antibody to B lymphocytes was given simultaneously with MADB106 tumor cells, lung metastases were significantly increased. When antibody was injected either 1 or 2 h after i.v. injection of tumor cells, the ability of anti-B-lymphocyte antibody to increase lung metastases decreased progressively as a function of time after tumor cells had been injected, which indicated that the tumor-enhancing effects of disrupting B lymphocyte function with antibody decline rapidly after MADB106 cells have been introduced into the host. For comparison, a similar experiment was conducted using antibody to NK cells (3.2.3 antibody). As pointed out in the "Introduction," MADB106 metastases are highly sensitive to the action of NK cells and are increased by the *in vivo* presence of anti-NK antibodies (1, 3). A similar effect as was observed for anti-B-lymphocyte antibody was found with the use of antibody to NK; *i.e.*, injection of the antibody together with tumor cells resulted in an increased development of lung metastases, and the ability of antibody to produce this effect diminished as the time of antibody

injection was delayed after the tumor cells were given. These results are shown at the *right side* of Fig. 7. Although the delays between i.v. injection of MADB106 tumor cells and of 3.2.3 antibody were longer than the delays between tumor cell and OX33 injections, these data indicate that the effectiveness of anti-NK antibody also requires the introduction of antibody in close temporal proximity with the introduction of tumor cells to affect the development of lung metastases after i.v. injection of tumor cells.

**Tumor Metastases and Lymphocyte Subsets in Fischer 344 Rats Bearing Primary Subcutaneous Tumor.** Lymphocyte subsets associated with resistance to the development of lung tumor metastases were also studied. Fischer 344 animals that have primary tumors caused by s.c. injection of MADB106 tumor cells are resistant to the development of lung metastases when MADB106 tumor cells are subsequently given i.v. For this study, 20 Fischer 344 rats received an injection of  $10^6$  MADB106 cells s.c. (under the thigh of the rear left leg), as a result of which 19 rats developed s.c. tumors within 10 days, and 1 animal never developed visible or palpable subcutaneous tumor before being killed. One month after the animals had received the s.c. injection of tumor cells, eight tumor-bearing animals were given an i.v. injection of  $10^6$  MADB106 cells as were eight normal control Fischer 344 animals. One-half of the animals in each group were then killed 7 days after the i.v. injection, and the remaining one-half were killed 14 days after the i.v. injection. Whereas i.v. tumor cell injection normally produced lung metastases in the control animals (mean number of lung metastases/cm<sup>2</sup> was  $4.4 \pm 0.4$  at 7 days and  $>50$  at 14 days after i.v. injection of tumor cells), animals with previously developed s.c. tumors showed no visible lung metastases at death. Also, four additional tumor-bearing animals did not receive any further experimental treatment after injection of s.c. tumor, and each died with a large primary s.c. tumor approximately 3 months later; these animals were used for the determination of tumor metastases when the disease was also allowed to progress until death. No lung metastases were found in these animals either. Thus, animals bearing s.c. MADB106 tumors showed resistance to normal development of lung tumors when they subsequently received an i.v. injection of MADB106 tumor cells; in addition, animals with s.c. tumor did not develop lung metastases before death if untreated.

To determine what changes in lymphocyte subsets might accompany the resistance to lung metastases seen in s.c. tumor-bearing animals, lymphocyte subsets were analyzed in lung, blood, and

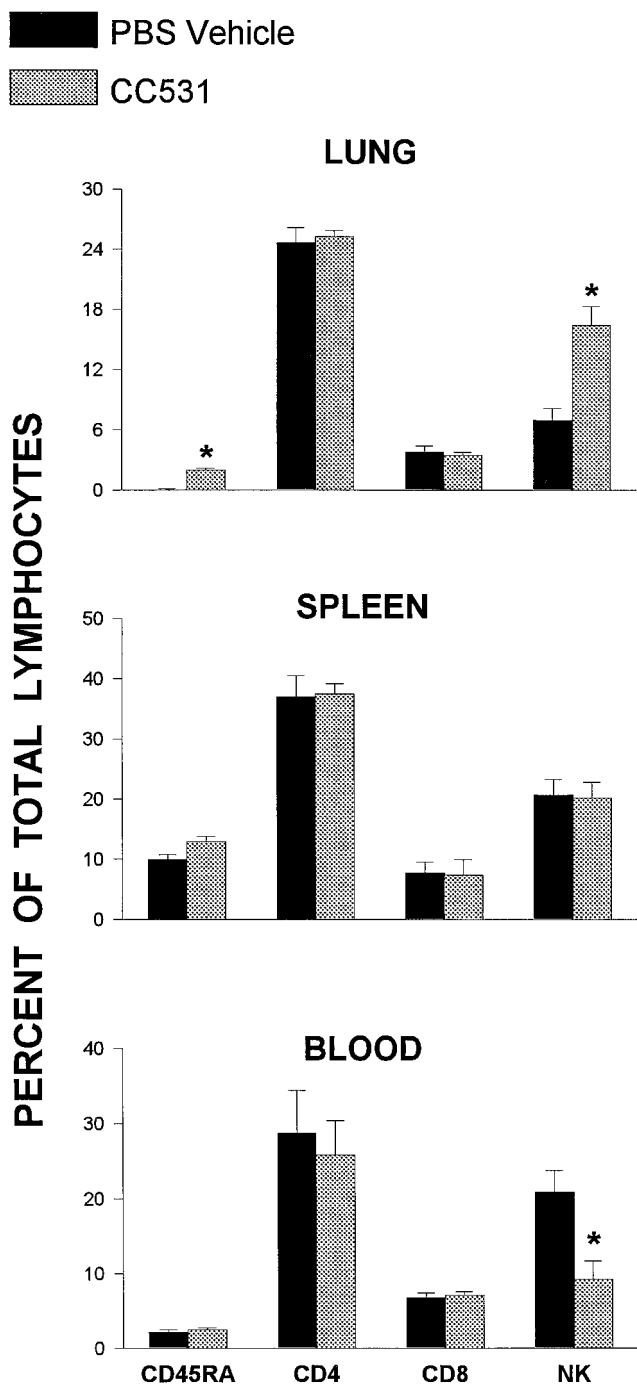


Fig. 5. The percentage of lymphocyte subsets found in lung, spleen, and blood at 6 h after WAG rats were given an i.v. injection with  $10^6$  CC531 tumor cells. Means and SEs (bars) are presented. \*, significantly different (at least  $P < 0.05$ ) from saline-injected controls.  $n = 4$  in each group. This figure shows that the percentage of CD45RA+ (B lymphocytes) and NK cells increased in lung 6 h after i.v. injection of CC531 tumor cells into WAG rats, and NK cells also were decreased in blood at this time.

spleen. One month after s.c. injection of tumor, lymphocytes were harvested from a group of tumor-bearing animals ( $n = 4$ ), and a group of normal control Fischer 344 rats ( $n = 4$ ). Lymphocytes were also harvested from the remaining s.c. tumor-bearing animals 6 h after they were injected i.v. with  $10^6$  MADB106 tumor cells ( $n = 4$ , including the subject that did not develop visible or palpable s.c. tumor) as well as from normal control animals after they received a similar i.v. injection of tumor cells ( $n = 4$ ). Fig. 8 shows the results of the FACS analysis of lymphocyte subsets recovered from the lung of animals

bearing s.c. tumor contrasted with animals that did not have s.c. tumor. As was observed previously, normal animals (*i.e.*, those without s.c. tumor) had a very low percentage of B lymphocytes in the lung ( $0.5 \pm 0.1\%$ ), which was increased in normal animals to  $8.9 \pm 1.5\%$  6 h after the i.v. tumor injection. By contrast, animals bearing s.c. tumor were found to have, without any introduction of tumor cells by i.v. injection,  $10.8 \pm 1.0\%$  B lymphocytes in the lung. The percentage of B lymphocytes in the lung of s.c. tumor-bearing animals that were injected i.v. with tumor cells ( $n = 3$ , which excludes the one animal that did not develop an s.c. tumor after being injected s.c. with tumor cells) was  $5.6 \pm 1.9\%$  6 h after i.v. tumor injection, a value somewhat lower (but not significantly so) than the  $10.8 \pm 1.0\%$  found in the s.c. tumor-bearing animals that received no i.v. injection of tumor cells but still a value significantly higher than the typically low value ( $0.5 \pm 0.1\%$ ) that was found in normal uninjected animals. It should be noted that the one animal that did not develop visible or palpable s.c. tumor and that was also given an i.v. injection of tumor cells was found to have 26.0% B lymphocytes in its lung 6 h after the i.v. injection of MADB106; this was the highest percentage of B lymphocytes that we have ever observed in the lung of any Fischer 344 rat. In addition to these differences in lung B lymphocytes, animals with s.c. tumor had a higher percentage of CD4+ cells in lung than did animals without s.c. tumor; this percentage was further increased 6 h after the i.v. tumor injection. Finally, the total number of lymphocytes recovered from the lungs of animals bearing s.c. tumor was  $2.3 \pm 0.52 \times 10^6$ . This number did not change significantly 6 h after the i.v. tumor injection ( $2.1 \pm 0.5 \times 10^6$ ) and was not different from animals without s.c. tumor ( $2.1 \pm 0.4 \times 10^6$  and  $2.0 \pm 0.5 \times 10^6$ , respectively).

## DISCUSSION

The experiments described above were initiated to determine the lymphocyte subtypes that would accumulate in lung after the introduction into the rat of tumor cells that metastasize to lung. In that the initial studies used tumor cells that generate NK-sensitive tumors, it was anticipated that significant changes, and possibly the major changes, would occur in a number of NK cells accumulating in lung. Instead, the most consistent changes seen in lung occurred with respect to B lymphocytes. Whereas B lymphocytes were found to be normally very low in the animals used in the models studied here, the number of such cells accumulating in lung increased approximately 10-fold by 6 h after i.v. tumor injection. This was seen in two tumor models, with: (a) MADB106 tumor cells that are syngeneic for Fischer 344 rats; and (b) CC531 cells that are syngeneic for WAG rats. Furthermore, it was found that in Fischer 344 rats that have been made resistant to the development of MADB106 lung metastases when tumor cells are injected i.v. because they have been previously injected s.c. with MADB106 tumor cells and consequently bear s.c. MADB106 tumors, an elevation in B lymphocytes was found residing in the lungs of such animals without i.v. introduction of tumor cells.

The findings described above indicate that B lymphocytes could be involved in host defense against metastases. As a first attempt to test this, antibody to B lymphocytes was injected to attempt to neutralize B lymphocytes and to ascertain whether this would affect the development of lung tumor metastases. When antibody was injected simultaneously with tumor cells, the number of lung metastases observed at both 7 and 9 days postinjection was markedly increased, which suggests that the disturbance of B lymphocyte activity at the time that tumor cells are injected increases the vulnerability of the host to MADB106 metastases to lung. The ability of B lymphocyte antibody to increase the lung metastases

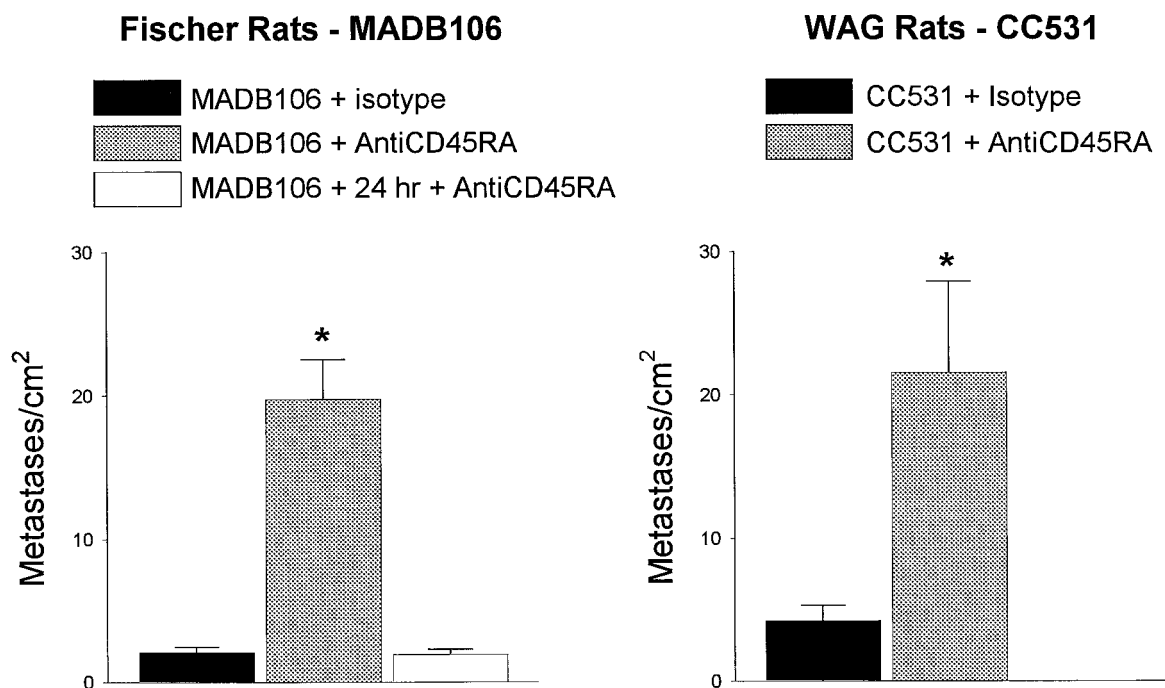


Fig. 6. The effect of giving a high dose of anti-CD45RA+ mAb on the development of lung metastases produced by i.v. MADB106 tumor cells in Fischer 344 rats and by CC531 tumor cells in WAG rats. *Left*, number of tumor metastases found in lung 7 days after Fischer 344 rats received i.v. injection of  $10^6$  MADB106 tumor cells together with either: (a) 100  $\mu$ g of isotype control antibody (*MADB106 + isotype*;  $n = 6$ ); (b) 100  $\mu$ g of anti-CD45RA+ antibody (*MADB106 + AntiCD45RA+*;  $n = 8$ ); or (c) 100  $\mu$ g of anti-CD45RA+ antibody given 24 h after tumor cell injection (*MADB106 + 24 hr + AntiCD45RA+*;  $n = 4$ ). *Right*, the number of tumor metastases found in lung 45 days after WAG rats received i.v. injection of  $1.5 \times 10^6$  CC531 tumor cells together with: (a) 100  $\mu$ g of isotype control antibody (*CC531 + isotype*;  $n = 8$ ); or (b) 100  $\mu$ g of anti-CD45RA+ antibody (*CC531 + AntiCD45RA+*;  $n = 9$ ). (In this study, the anti-CD45RA+ antibody given to four of the animals was treated to remove sodium azide; this produced no difference from the results seen in the five animals given anti-CD45RA+ antibody that was not treated in this manner.) Means and SEs (bars) are presented. \*, significantly different (at least  $P < 0.05$ ) from the number of metastases seen in the group receiving isotype control antibody.

declined rapidly when the anti-B-lymphocyte antibody injection was given at different times after tumor cells had been injected i.v.; this indicates that the antitumor influence of B lymphocytes that the antibody disrupts occurs very soon after the tumor cells reach the lung, and that a delay of even a few hours is sufficient to remove this influence from the tumor surveillance schema. However, this time course is apparently not unique to the B-lymphocyte influence on metastases development inasmuch as a similar time course was found with respect to NK cells. MADB106 is known to

be a NK-sensitive tumor (1, 11), and when the antibody to NK cells was injected simultaneously with MADB106 tumor cells, lung metastases were subsequently found to be greatly increased. However, when the injection of anti-NK antibody was made 6 h after i.v. tumor cell injection, the antibody failed to augment development of lung metastases. Thus, a similar time course indicating that critical events regarding tumor surveillance occur within a short time after tumor cells are injected i.v. can be surmised from the findings with respect to both B and NK lymphocytes.

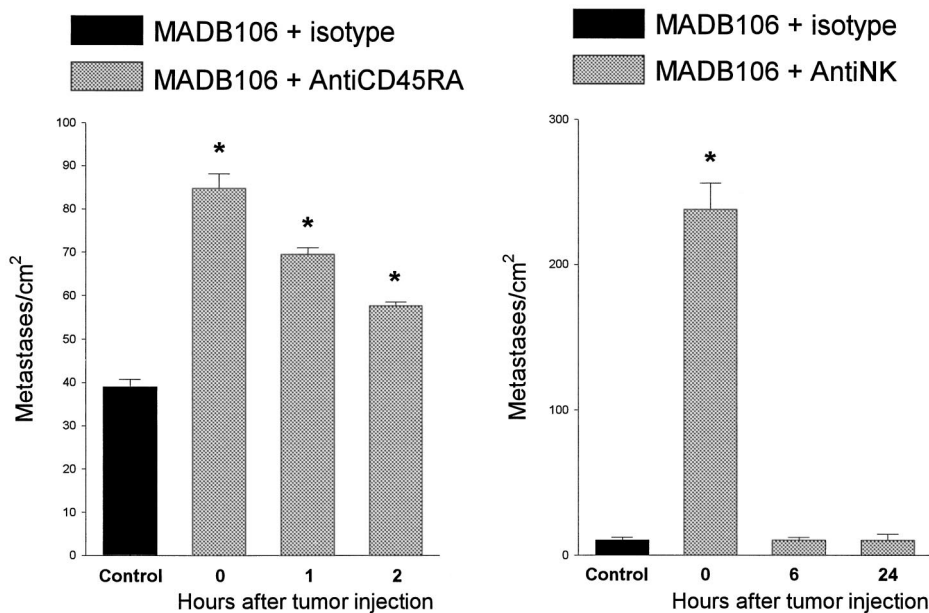
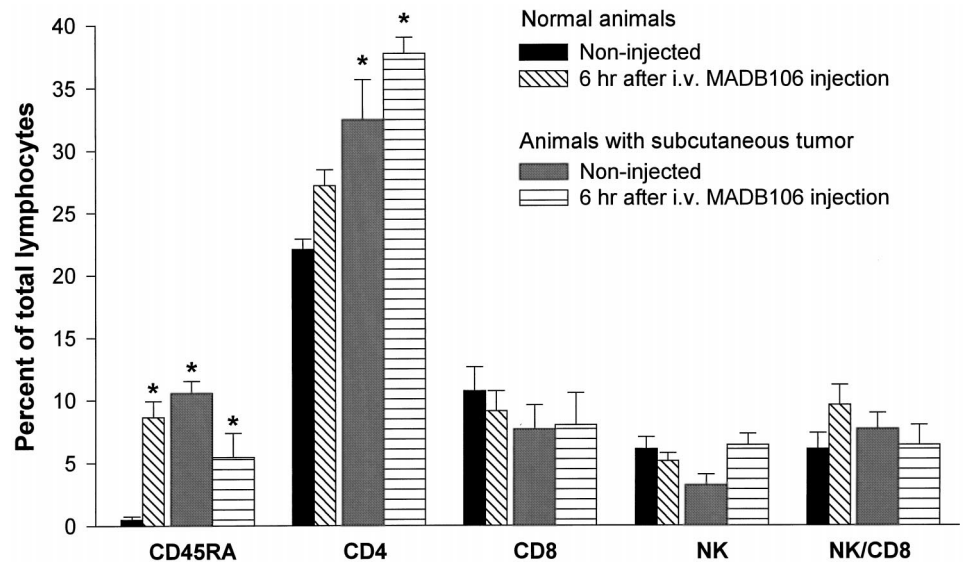


Fig. 7. The number of tumor metastases in the lung 9 days after Fischer 344 rats received an i.v. injection of  $10^6$  MADB106 tumor cells and a second injection of 100  $\mu$ g of anti-CD45RA+ antibody (*left*) or anti-NK antibody (*right*) at various time points after the tumor injection. 0, simultaneous injection of antibody and tumor cells; other numbers (e.g., 1, 2, 6, and 24) indicate delay (in hours) in antibody injection after tumor cells had been given. In isotype control condition (*MADB106 + isotype*), one animal received 100  $\mu$ g of isotype antibody at each time point shown. Means and SEs (bars) are presented.  $n = 3$  in each group. \*, significantly different (at least  $P < 0.05$ ) from animals receiving isotype control antibody.



Fig. 8. The percentage of lymphocyte subsets in lung of Fischer 344 rats bearing s.c. MADB106 tumor (*Animals with subcutaneous tumor*) or having no s.c. tumor (*Normal animals*). Lung lymphocyte subsets of these animals were assessed 6 h after they had received an i.v. injection of  $10^6$  MADB106 tumor cells (*6 h after i.v. MADB106 injection*) or without an i.v. injection having been given (*Non-injected*). Means and SEs (bars) are presented. \*, significantly different (at least  $P < 0.05$ ) from normal animals (*i.e.*, *Normal animals, Non-injected*).  $n = 4$  in each group. This figure shows that animals bearing s.c. MADB106 tumors, which are resistant to the development of lung metastases when MADB106 tumor cells are given i.v., have a markedly elevated percentage of CD45RA<sup>+</sup> cells (B lymphocytes) in the lung when tumor cells are injected i.v.



It is not yet known whether injecting antibody to B lymphocytes blocks the ability of B lymphocytes to accumulate in the lung within the first few hours after tumor cell injection. To address this question, it will be necessary to label B lymphocytes for FACS analysis with antibody other than OX33, because OX33 will be given simultaneously with the tumor cell injection to assess whether this treatment would block B lymphocyte influx into the lung, and hence the CD45RA<sup>+</sup> antigen would already be occupied before labeling. To label rat B lymphocytes at another antigenic site, we used a polyclonal anti-IgM (Pharmingen). Preliminary findings using this label indicated that treating Fischer 344 rats with OX33 at the time of tumor cell injection did not prevent B lymphocytes from accumulating in the lung in the first few hours after i.v. injection of MADB106 tumor cells. But because the anti-IgM polyclonal antibody label may not be specific for B lymphocytes (*e.g.*, it may bind to cells that have secreted IgM attached to them), this observation is not conclusive. Of course, it will be recognized that OX33 could block the antitumor activity of B lymphocytes by blocking some function of B lymphocytes without affecting their accumulation in the lung after i.v. tumor cell injection; this is what the preliminary data (described here) suggest occurs. Finally, it can be noted that an additional experiment was conducted in which we injected tumor cells rendered inactive by mitomycin treatment (incubation of  $10^6$  cells with  $50 \mu\text{g/ml}$  mitomycin at  $37^\circ\text{C}$  for 1 h). This resulted in a similar influx of B lymphocytes into the lung as seen with the injection of normal tumor cells (*i.e.*,  $8.7 \pm 0.6\%$ ;  $n = 3$ ), which suggests that surface membrane characteristics and not the functional activity of the tumor cells is sufficient to stimulate the influx of B lymphocytes into the lung that is reported here.

An important concern regarding the finding that the percentage of B lymphocytes rose markedly in the lung after i.v. injection of MADB106 and CC531 tumor cells was whether B lymphocytes accumulated in the lung simply because of mechanical phenomena. It can be envisioned that tumor cells injected into the tail vein would become lodged in small blood vessels in the lung, causing blockage in these vessels that, in turn, would block B lymphocytes from flowing through the lung, thereby increasing B lymphocytes in the lung. This possibility, however, is not consistent with the observed results. In the Fischer 344 rats, no lymphocyte subtype other than B lymphocytes were increased in the lung after i.v. tumor injection; mechanical blockade would predict increases in other lymphocyte subtypes of the size as B lymphocytes, *e.g.*, NK cells. Furthermore, the finding that B

lymphocytes in the lung were elevated in Fischer 344 rats bearing s.c. tumor without any i.v. tumor having been given also indicates that the influx of B lymphocytes to the lung is not a mechanical event caused by an i.v. injection of tumor cells.

How the accumulation of B lymphocytes in the lung may contribute to controlling tumor metastasis can only be suggested at this time. Various studies (1–4) using the Fischer 344-MADB106 model, including the present one, have shown that lung tumor metastases in this model are regulated by NK cells *in vivo*. It has been reported that B lymphocytes can directly interact with NK cells, inducing NK cells to increase production of IFN- $\gamma$  (12). Because IFN- $\gamma$  can increase NK activity (13), one possibility is that B lymphocytes may augment NK activity via the release of this cytokine. Also, activated B lymphocytes were found to be more potent in promoting IFN- $\gamma$  production by NK cells than were B lymphocytes at rest (12). Thus, the prevention of lung metastasis, particularly in animals bearing s.c. tumor, could be due to the presence of tumor antigen-activated B lymphocytes.

Other possibilities by which B lymphocytes may contribute to host protection can be suggested as well. B lymphocytes may release interleukin 12 at the site of tumor. Activated B lymphocytes have been found to release this cytokine (14), and interleukin 12 has been reported to potentially enhance the activity of antitumor effector cells (15). Also, antitumor action of B lymphocytes may derive from their function as antigen-presenting cells. Tumor antigen presentation recently has been found to be significant in tumor killing (16, 17). Undergoing clonal expansion in response to antigen, B lymphocytes have been shown to bind, internalize, and present antigens in the context of Class II MHC (18). When Guo *et al.* (19) reported that a tumor vaccine could be generated by fusion of certain hepatoma cells with activated B lymphocytes, these authors speculated that the antigen presentation machinery of B lymphocytes may be key to the induction of tumor-cell killing.

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## Evidence for Involvement of B Lymphocytes in the Surveillance of Lung Metastasis in the Rat

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