EFFECT OF BLOOD TRANSFUSIONS ON IMMUNE FUNCTION. PART VI. EFFECT ON IMMUNOLOGIC RESPONSE TO TUMOR

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Effect of blood transfusions on immune function. Part VI. Effect on immunologic response to tumor

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Transfusions are reported to increase the incidence of tumor metastasis in clinical studies and primary tumor growth in animal studies. We evaluated the effect of transfusions on immunologic response to primary and metastatic tumors in multiple rat models. One half of the animals were administered lactated Ringer's solution and one half ACI rat blood at the time of tumor challenge. In 80 rats a slow-growing colon tumor was implanted subcutaneously. At 4 months there were no significant differences in tumor size or leukocyte infiltration of the tumor. Similar results were obtained with a rapidly growing colon cancer. Analysis of T-lymphocyte subpopulations in both groups showed no differences. Rats transfused at the time of intravenous challenge with a suspension of 1×10^6 tumor cells had a mean survival time of 38.3 ± 0.8 days and the control group had a mean survival time of 41.1 ± 0.8 days ($p = 0.016$). One week after transfusion, natural killer cell lysis of tumor cells at a 100:1 effector/target cell ratio wa 18.0% ± 1.8% in the transfusion group and 23.0% ± 1.3% in the control group ($p = 0.034$). In conclusion, transfusions in multiple rat cancer models did not affect primary tumor growth or the host's immunologic response to it but did significantly impair natural killer cell function and survival with tumor metastases. (SURGERY 1990;108:172-8.)

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BEFORE THE 1970s it was generally believed that administration of blood products to patients would result in immunostimulation in the recipient.[1] This belief was based on the demonstration by Medawar[2, 3] that the administration of blood products resulted in a specific donor immunization in a rabbit skin allograft model. This led transplant surgeons to avoid administering blood transfusions to patients awaiting solid organ transplantation in the belief that such transfusions would increase the immune responsiveness of the recipient and lead to an eventual rejection of the allograft.[4] This policy was reversed in the early 1970s when a number of publications reported that transfusions administered before transplantation decreased the rate of allograft rejection.[5-8]

With the acceptance of the concept that blood transfusions cause immunosuppression in transplant recipients, surgeons in the 1980s have addressed the question of whether this transfusion-induced immunosuppression is also present in patients not undergoing transplantation. There has been a large number of reports that indicate that perioperative blood transfusions increase the rate of tumor growth and metastasis in patients undergoing oncologic surgery.[9] These reports involved retrospective patient analyses. Therefore the possibility exists that the patients with more advanced

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tumors received a greater proportion of the blood transfusions. If true, this would indicate that the detrimental effect observed with blood transfusion was not the result of the transfusion itself but rather the more advanced nature of the patient's tumor.

To evaluate this possibility in a controlled prospective study, several investigators have investigated the effect of blood transfusions on primary tumor growth in animal models. The majority of these studies have demonstrated an enhanced rate of primary tumor growth and a diminished long-term survival in animals receiving allogeneic transfusions.[9] In clinical practice, control of the primary tumor can usually be achieved through surgical intervention. It is rather the metastasis of the tumor that results in the eventual death of the patient. We have evaluated the effect of blood transfusion on host response to both primary tumor growth and metastic tumor growth.

MATERIAL AND METHODS

Animals. Two hundred fifty adult male Wistar-Furth rats weighing ±250 gm were used as tumor recipients. Twenty adult male ACI rats were used as blood donors. All animals were housed in individual cages and were allowed food and water ad libitum throughout the study. Animals were observed for 1 week before entry into the study to exclude the possibility of any preexisting diseases.

The care of all animals was in accordance with the guidelines set forth by the Animal Welfare Act and other federal statutes and regulations relating to animals and studies involving animals, and with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication 86-23).

Transfusion protocol. Blood was obtained from the donor animals by vena cava puncture and mixed at 4:1 volume ratio with citrate-phosphate-dextrose-adenine 1 anticoagulant. Animals in the control group were given 3 ml of lactated Ringer's solution and those in the transfusion group were given 1 ml of ACI blood intravenously at the time of primary tumor implantation. The increased volume of lactated Ringer's solution was chosen because it was believed that it approximated the intravascular volume changes achieved in the animals receiving 1 ml of whole blood.

Tumor protocol. Three tumor models were used to evaluate the transfusion effect. In the first model, 80 rats (saline, 40 rats; transfusion, 40 rats) were anesthetized with sodium pentobarbital (35 mg/kg intraperitoneally) and a 1 cm incision was made in the thigh of the left lower extremity. A 2 mm^3 aliquot of a 1,2-dimethylhydrazine-induced, poorly differentiated syngeneic colon adenocarcinoma was implanted into the thigh. This tumor was supplied by Drs. Ravikumar and Steele of the New England Deaconess Hospital, Boston. The incision was closed with surgical clips and the tumor was allowed to grow for a period of 4 months. The animals were then sacrificed with a lethal dose of sodium pentobarbital (600 mg/kg intraperitoneally) and the tumor was excised and weighed. A biopsy specimen of the tumor obtained at its periphery was fixed in formalin and stained with a hematoxylin and eosin stain for later assay of leukocyte infiltration.

The second tumor model had the same size aliquot of a more rapidly growing 1,2-dimethylhydrazine-induced, poorly differentiated syngeneic colon carcinoma implanted in the subcutaneous tissue overlying the thoracic vertebrae. This tumor was also supplied by Drs. Ravikumar and Steele.

In 20 of these animals (saline, 10 rats; transfusion, 10 rats) the abdomens were painted with 1 ml of a 0.5% dinitrofluorobenzene solution in a 4:1 volume ratio of acetone/olive oil. The tumors were allowed to grow for 1 week. On the day before sacrifice, the ear thickness of each rat was determined with an engineer's caliper. The ear was then painted with 0.1 ml of the same 0.5% dinitrofluorobenzene solution. Twenty-four hours later, the animals were anesthetized with sodium pentobarbital (35 mg/kg intraperitoneally). The tumors were excised and weighed, and peripheral biopsy specimens of the tumors were fixed in formalin and stained with hematoxylin and eosin for later analysis of leukocyte infiltration. Ear thickness was again measured and the percent ear swelling in comparison with the measurements on the preceding day was determined. Ear swelling measured by this technique has previously been shown to correlate with cell-mediated immunity.[10] The ears were amputated, fixed in formalin, stained with hematoxylin and eosin, and histologically assayed for leukocyte infiltration in response to the dinitrofluorobenzene. Finally, the spleens were removed and lymphocytes were harvested by Ficoll-Hypaque centrifugation. The lymphocyte preparations so obtained were stained with antilymphocyte monoclonal antibody preparations, washed, and reacted with affinity-purified fluorescent-labeled, goat antimouse immunoglobulin G as a second-step reagent. Fluorescent-labeled cells were analyzed by flow cytometry. For each sample, 5000 cells were assayed and the numbers of cells labeled by the monoclonal antibodies specific for pan-T lymphocytes (OX-19), helper/inducer lymphocytes (W3/25), and suppressor/cytotoxic lymphocytes (OX-8) cell surface markers were determined. For each sample a negative control with a monoclonal antibody of the same isotype (immunoglobulin G_1) to human T cell (anti-Leu-2) was run to determine the cutoffs. The posi-

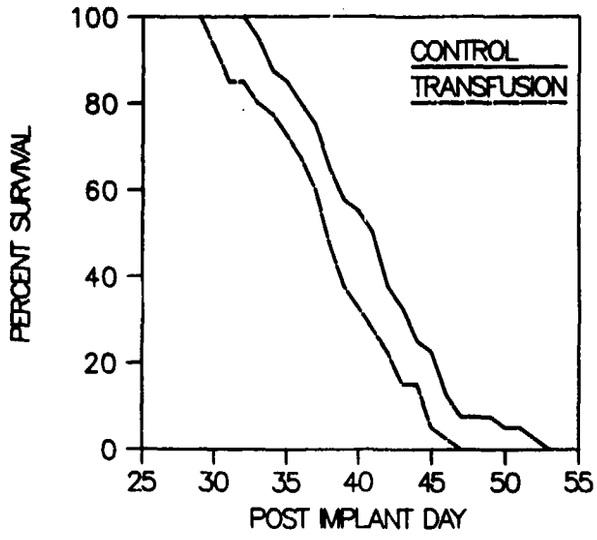

Fig. 1. Survival curves for rats administered blood transfusions or lactated Ringer's solution at the time of challenge with 1×10^6 tumor cells administered intravenously.

tive cutoff was set at a point determining the upper 2% or less of background control and the number of background control cells was subtracted from each count. Nonlymphoid cell contamination was assayed by analysis of forward and 90-degree light scatter. Cells with light-scattering intensities outside limits established for normal lymphocytes were removed from analysis. The percentage of lymphocytes bearing interleukin-2 and transferrin receptors was also determined.

An additional 40 Wistar-Furth rats (saline, 20 rats; transfusion, 20 rats) with the same tumor were followed until death to determine mean survival times. Weekly tumor size determinations were made in these animals according to the technique of Chance et al.[11]

For the final tumor model, a tumor-cell suspension was prepared from the rapidly growing 1,2-dimethylhydrazine–induced solid tumor. Briefly, viable tumor was obtained by excision of the rapidly growing tumor from a Wistar-Furth rat. The tumor was mechanically disaggregated by first slicing the tumor into approximately 1 mm^3 pieces and then vigorously shaking the suspension in complete RPMI-1640 medium with penicillin, streptomycin, and 10% fetal calf serum. The cells were washed three times in the same medium. A sample of this suspension was stained with trypan blue and the number of viable tumor cells was determined. The cells were then resuspended in sufficient RPMI medium to achieve a final concentration of 1×10^6 cells/ml. A 1 ml aliquot of this suspension was injected intravenously into 80 Wistar-Furth rats immediately after the

Table I. Tumor weights 8, 15, and 22 days after tumor implantation for control and transfusion groups

Post-tumor implantation day	*Tumor weight (gm)*		
	Control group	*Transfusion group*	*p Value*
8	18.1 ± 0.9	21.2 ± 2.1	0.130
15	65.1 ± 1.9	64.4 ± 3.5	0.865
22	73.4 ± 2.1	73.2 ± 2.0	0.946

administration of either lactated Ringer's solution ($n = 40$) or blood ($n = 40$). The animals were followed until death and mean survival times were determined. Necropsy was performed in all animals and biopsy specimens of the pulmonary metastases were fixed in formalin and stained with hematoxylin and eosin for later analysis of leukocyte infiltration.

Leukocyte infiltration. Leukocyte infiltrates were quantified by enumeration of white blood cells in the tumor periphery or the dermis of the DNFB-treated ear. Fifteen high-power fields (hpf) were counted in each specimen with an image-analysis system (Optomax 40-10, Optomax, Inc., Hollis, N.H.) as described previously.[12] An average was obtained for these 15 hpf.

Natural killer cell function. The effect of blood transfusions on natural killer cell function was assayed at two points in relation to the time of blood administration. Forty animals (control, 20 rats; transfusion, 20 rats) were sacrificed by decapitation 1 week after the administration of blood or lactated Ringer's solution, and 20 animals (control, 10 rats; transfusion, 10 rats) were sacrificed 2 weeks after the administration of blood or lactated Ringer's solution. After decapitation, the spleens were rapidly removed and homogenized in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The number of viable nucleated cells in each homogenate was determined in dilutions appropriate to the desired concentration of mononuclear cells. Dilutions were done to achieve effector/target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Then 1×10^4 YAC target cells that had previously been labeled with sodium (chromium 51) chromate were added to each well, with a final volume of 0.2 ml/well in V-bottom microfilter plates. After centrifugation at 40 *g* for 2 minutes, the plates were incubated at 37° C in 5% CO_2 for 4 hours. The plates were then centrifuged at 500 *g* for 5 minutes and a 100 μl aliquot of the supernatant was collected and assayed for ^{51}Cr on a γ-counter. The percentage of cell lysis was calculated as the mean counts per minute released in the presence of effector cells minus the mean counts per minute

spontaneously released by target cells incubated with medium alone divided by the counts per minute released after treating target cells with Triton X-100 (1:100 dilution) minus the counts per minute spontaneously released with medium alone; the quotient was multiplied by 100.

All data are expressed as mean ± SE. Comparisons among groups were made with the Savage (Mantel-Cox) test for survival time and analysis of variance for all other data. Significance was assumed at $p < 0.05$.

RESULTS

Blood transfusion was found to have no effect on the size of the primary tumor in the slow-growing colon carcinoma model. The mean tumor weight in the saline-treated animals was 26.0 ± 1.1 gm and in the transfused animals 28.1 ± 1.4 gm ($p = 0.098$). Assays of leukocyte infiltration into the tumors failed to demonstrate any effect of blood transfusion. With the slow-growing tumor, there were 171.0 ± 12.6 cells/hpf in the control group and 181.6 ± 16.6 cells/hpf in the animals that had undergone transfusion ($p = 0.6$).

The tumor weights over time in the rapidly growing tumor model are shown in Table I. None of these differences were statistically significant. The average tumor weights at the time of death for the two groups were 69 4 ± 4.1 gm in the control group and 62.2 ± 2.1 gm in the transfusion group ($p = 0.135$). The lower tumor weight recorded at the time of death compared with that measured 22 days after tumor implantation is the result of two factors. First, a significant percentage of the animals had died of the tumor before this time, and thus there was a difference in the populations. Second, the tumor weight 8, 15, and 22 days after transplantation was a calculated value, whereas the value at the time of death was a direct measurement. Blood transfusions also did not affect ear swelling in this model. The control group had a mean of 33.6% ± 5.8% ear swelling and the animals that had undergone transfusion had a mean of 42.2% ± 7.1% ear swelling ($p = 0.358$). There were 98.3 ± 6.3 cells/hpf in the rapidly growing tumor from the control group and 87.1 ± 6.1 cells/hpf in the tumor from animals that had undergone transfusion ($p = 0.21$). Analysis of leukocyte infiltration in the dinitrofluorobenzene-treated ears of the animals revealed 97.5 ± 6.9 cells/hpf in the control group and 113.3 ± 5.6 cells/hpf in the animals that had undergone transfusion ($p = 0.087$). The mean survival times in the rapidly growing, subcutaneously implanted tumor model were 23.6 ± 0.5 days for the control group and 22.0 ± 0.7 days for the group that had undergone transfusion ($p = 0.127$).

Survival curves of the tumor metastasis model are shown in Fig. 1. The mean survival time for the control group was 41.1 ± 0.8 days and for the transfusion group 38.3 ± 0.8 days. This difference was statistically significant ($p = 0.016$). Two animals in the transfusion group were noted to have tumor metastases to supraclavicular lymph nodes at the time of death. In the control group, tumor metastases were confined to the lungs. Analysis of the leukocyte infiltration of the metastatic pulmonary tumor nodules revealed an average of 281.2 ± 13.0 cells/hpf for the control group. The transfusion group was found to have an average of 269.0 ± 8.2 cells/hpf ($p = 0.42$ vs control group).

Table II. Natural killer cell cytotoxicity as measured by percent YAC cell lysis 1 and 2 weeks after transfusion

Effector/target ratio	*Control group*	*Transfusion group*	*p Value*
1 wk after transfusion			
100:1	23.0 ± 1.3	18.0 ± 1.8	0.034
50:1	15.2 ± 0.9	12.4 ± 1.8	0.176
25:1	10.1 ± 1.3	8.8 ± 1.6	0.552
12.5:1	5.5 ± 0.8	4.6 ± 1.0	0.487
2 wk after transfustion			
100:1	25.9 ± 1.7	17.9 ± 2.7	0.025
50:1	20.9 ± 2.3	15.1 ± 3.4	0.175
25:1	11.9 ± 2.5	6.6 ± 3.0	0.184
12.5:1	4.1 ± 1.2	2.1 ± 1.2	0.255

Blood transfusions depressed natural killer cell function as measured at the 100 effector cell/tumor cell ratio both 1 and 2 weeks after transfusion (Table II). One week after transfusion, the percent cell lysis at a 100:1 ratio with cells obtained from the control group was 23.0% ± 1.3% and in the group that underwent transfusion 18.0% ± 1.8% ($p = 0.034$). Two weeks after transfusion, the cell lysis in the control group was 25.9% ± 1.7% and in the transfused group 17.9% ± 2.7% ($p = 0.025$).

The percentages of pan-T lymphocytes (OX-19), helper/inducer T lymphocytes (W3/25), and suppressor/cytotoxic T lymphocytes (OX-8) among splenocytes obtained from control and transfused animals are shown in Table III. The percentage of lymphocytes bearing interleukin-2 and transferrin receptors are also shown in Table III. Transfusions did not significantly alter these percentages.

DISCUSSION

Surgical intervention is often able to control the primary site of common solid malignant neoplasms. It is commonly the distant metastasis that eventually lead to

Table III. T-lymphocyte subset populations plus lymphocytes bearing interleukin-2 and transferrin receptors in control and transfused rat splenocytes

	Control Group (%)	*Transfusion group (%)*	*p Value*
Pan-T cells	74.5 ± 3.0	80.2 ± 2.6	0.1634
Helper/inducer T cells	51.8 ± 1.6	52.1 ± 1.0	0.8703
Suppressor/cytotoxic T cells	22.0 ± 1.0	25.0 ± 1.3	0.0807
Interleukin-2 receptors	2.01 ± 0.34	2.72 ± 0.32	0.1400
Transferrin receptors	0.94 ± 0.12	1.09 ± 0.15	0.4599

a fatal outcome. Such metastases can take place by three primary methods: migration through coelomic cavities, spread through lympathic vessels, and spread through blood vessels. Tumor spread by these methods does not always result in metastasis. For such tumor spread to eventually become a successful metastasis, the tumor cell, or group of tumor cells, must implant in a distant site and then escape control by the body's immune system.

The tumor host generates a complex immunologic response to both the primary and metastatic tumor cells. Among the more important components of this response are cytotoxic macrophages, helper/inducer T lymphocytes, cytotoxic T lymphocytes, and natural killer cells. The natural killer cell appears to be particularly important in controlling tumor cells that are disseminated from the primary tumor source.[13, 14] The natural killer cell is able to attack tumor cells without any major histocompatibility complex restrictions and is also able to initiate tumor lysis on initial exposure to the tumor cells. In contrast, cytotoxic T lymphocytes require a period of 7 to 10 days after exposure to tumor cells before the onset of tumor cell lysis.

This study attempts to delineate the effect of blood transfusions on host response to tumor, with specific emphasis on the immunologic response to metastases. We were unable to demonstrate any effect of blood transfusions on primary tumor growth with two different colon cancer models. This finding differs from our earlier report.[15] However, there have been other reports of studies demonstrating no effect of blood transfusions on primary tumor growth.[9] These contradictory findings indicate the complex nature of the host response to blood transfusions, the host response to tumor, and the variety of animal tumors with regard to antigenicity and growth characteristics.

Our study failed to demonstrate any effect of blood transfusions on T-lymphocyte- and macrophage-mediated immunologic parameters including tumor infiltration by white blood cells. Such white cells are usually predominantly T lymphocytes.[16] Because immunologic control of primary tumor growth is principally dependent on macrophage and T-lymphocyte activity, our failure to demonstrate any effect of transfusion on primary tumor growth and T-lymphocyte or macrophage functions is entirely consistent.

Our study did demonstrate a significant effect of blood transfusions on natural killer cell function, both 1 and 2 weeks after transfusion. It has previously been reported that blood transfusions impair natural killer cell function in humans,[17] but this study was flawed in that the transfusions were administered to patients with blood dyscrasias to correct anemia. The possibility therefore exists that the sicker patients received a greater proportion of the transfusions and the diminished natural killer cell function observed in the patients that had undergone transfusion was caused by more severe preexisting disease. This study indicates that this was probably not the sole reason for the diminished natural killer cell function in the patients that had undergone transfusion.

The finding of diminished survival time with tumor metastases in transfused rats is consistent with the natural killer cell findings. Because natural killer cells are of primary importance in controlling metastases, impairment of natural killer cell function should enhance tumor metastasis and growth. These findings thus support the retrospective clinical studies in patients with tumors that report increased rates of tumor metastases in patients receiving perioperative blood transfusions.

The mechanism by which blood transfusion impairs natural killer cell function has not yet been determined. One possibility is the previous demonstration that blood transfusions decrease interleukin-2 production.[18] Interleukin-2 has been shown to be necessary for optimal natural killer cell activity.[19] If this is found to be the case, concomitant administration of interleukin-2 with blood transfusions might block or correct the immunosuppressive transfusion effect. Other immunologic interventions that might be used at the time of transfusion to prevent posttransfusion immunosuppression include the use of the H_2 blocker, ranitidine,[20] or cyclooxgenase inhibitors.[21]

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DISCUSSION

Dr. Mark A. Malangoni (Louisville, Ky.). You have shown decreased survival in the animal model at less than 10% blood volume transfusion, and you support your conclusions with a single in vitro observation. Do you have any evidence that you actually had decreased numbers of pulmonary metastases? Did you count these?

You used whole blood. Very often when we give transfusions, we do not give whole blood, we give packed cells. What incompatibilities may be present in the whole blood that may be absent when you give packed-cell transfusions? Is this incompatibility specific to the Wistar and ACI rats, or can it occur in other situations?

Most patients who die of colon cancer die of hepatic metastases rather than pulmonary metastases. Is the effect that you have seen specific for pulmonary metastasis development, or can you demonstrate that the effect also occurs in a model of hepatic metastases or peritoneal metastases, both of which would be more common than pulmonary metastases in the clinical setting?

Dr. Thomas C. Moore (Torrance, Calif.). Did you use anesthesia or heavy sedation in either drawing or administering the blood. This is of importance because of the multiple potent immunosuppressive effects of anesthesia, including depression of peripheral lymph node lymphocyte traffic and elevation in nodal efferent lymph prostaglandinE_2 levels in the sheep.

Dr. Donald E. Fry (Albuquerque, N.M.). Several issues in the manuscript are debatable. What is a slow-growing tumor? If it grows in 4 months from nothing to 12% of the carcass weight of the animal, then I hope my slow-growing cancer does not work like that. Some very important questions need to be raised including even the validity of the tumor metastasis model, since the data indicate that the difference in survival is 41 days versus 38 days. That may be statistically significant, but it is not very exciting.

Why a 1 ml transfusion? One milliliter represents one-twentieth of the blood volume of a rat that weighs 250 gm. One milliliter of blood would be the equivalent of one half of a unit of blood if we wanted to extrapolate it to a human being. Is there an oversight here in the interpretation of these data by virtue of not giving a larger transfusion or not looking for some kind of a dose-effect response?

Similarly, from a methodologic point of view relative to the 100:1 effector/target cell ratio, where did the ratio come from? Does there need to be more of an effector/target cell variability in examining things to identify what these relationships are?

Dr. Donald L. Morton (Los Angeles, Calif.). What about the immunogenicity of the tumor model? If the model you are studying does not have tumor-specific transplantation antigens, one might not expect anything except a natural killer cell effect.

Dr. Waymack (closing). We were unable to quantitate the

number of pulmonary metastases. The cause of death in the animals was primarily hemorrhage into the lung from the tumors and, as such, it was impossible to either accurately weigh or count the nodules in the lung.

Whole blood was chosen simply because it was easier to prepare in our laboratory. The components in blood, which based on transplant literature are believed to be immunosuppressed, are primarily the white blood cells, and with this model we were giving the white blood cells.

As to the question on hepatic meatastases, we are unable to perform that study with this tumor cell line, which is extremely sensitive to macrophage cytotoxicity. This tumor cell has difficulty binding to hepatic endothelial cells. The injection of even 1×10^7 tumor cells into the portal vein does not result in successful hepatic metastases.

The question on the peritoneal metastases is an interesting one. We have performed this study. When 1×10^6 tumor cells were injected intraperitoneally, there was no effect of the transfusion on long-term survival. This was somewhat surprising, so we assayed macrophages from groups that had been transfused and groups that had not been transfused for their cytotoxicity capacities against tumors cells, and we found no effect of the transfusions on macrophage cytotoxic capabilities, which may explain the lack of an effect in the in vivo peritoneal model.

The question about anesthesia is important. We were aware of these findings; when we administered the blood to the animals, they were merely restrained and the blood was injected through the dorsal penile vein. This is a very efficient, rapid model. The same was true for our natural killer cell assay protocol since it has been shown that anesthesia impairs natural killer cell function.

Dr. Fry, survival decreasing from 41 to 38 days has clinical significance. If one were to extrapolate the 3-day difference to human studies that are based on theoretic life expectancy from the time of initial tumor adherence to pulmonary endothelial cells, this would probably translate into something in the order of 3 to 6 months. Such a time period has some degree of clinical importance. As to the question about the volume of blood administered, we chose 1 ml merely to try and avoid any possible hemodynamic changes in the rat.

The 100:1 ratio for effector/target cells was not the only ratio tested. The manuscript describes ratios ranging from 12.5:1 to 100:1. The trend was the same in all of the ratios. The 100:1 ratio was used in the presentation today simply because it demonstrated the most significance. Finally, the tumor is only weakly immunologic.