Specificity of *In Vivo* Tumor Rejection Assessed by Mixing Immune Spleen Cells with Target and Unrelated Tumor Cells[1,2] (37688)

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Immune rejection of tumors *in vivo* is a complex process that may depend on the balance of various types of immune responses. It is now quite evident that there are multiple mechanisms of immune responses that may be arbitrarily divided into antibody-dependent and cell-mediated. This paper deals only with the second aspect and considers the specificity of the reaction. Recent experiments have suggested that a two-step mechanism may be involved: firstly an immunologically specific reaction and then a second nonspecific tumor-cell killing (1, 2). It was proposed for the first step that lymphocytes recognize antigenic determinants on the membrane of target cells through specific receptor interaction, whereas in the second step, the target cells are then destroyed in a nonspecific process. Immune reactions to other antigens such as BCG, when present in the local tumor site, may also lead to a nonspecific killing of the tumor cells (3). This concept is further supported by experiments in which the growth of a hepatoma was suppressed at the site of delayed hypersensitivity skin reactions to another antigenically different hepatoma (1). In other studies (4), some nonspecific killing was observed when "specific" and "bystander" fibroblast cells were used as target cells for assaying the cytotoxic capacity of lymphocytes immunized against alloantigens, although the specific killing was found to be always considerably more pronounced. Phytohemagglutinin-transformed lymphoblasts have been shown to damage target cells nonspecifically (5) and to liberate lymphotoxins, which in other studies (6) have been demonstrated to destroy target cells in a nonspecific manner.

On the other hand, there is clear evidence in the literature that cytotoxic lymphocytes, immunized *in vivo* or *in vitro* across an H-2 barrier, will kill ^{51}Cr-labeled target cells in a very specific way (7, 8), and that *in vivo* homograft rejection, tested by injection of *in vitro* mixtures of H-2 compatible and H-2 incompatible tumor cells in recipient mice, is a highly immunologically specific process (9, 10). Our own experiments concerning the rejection of plasma cell tumors in mice have similarly suggested a specific tumor rejection mechanism (11).

In the present studies, the question has been further investigated by using a syngeneic transfer system consisting of mouse fibrosarcoma cells, spleen cells from mice immune to this tumor, unrelated myelomonocytic leukemia cells, and sublethally irradiated recipient mice. Evidence will be presented macroscopically and microscopically that the tumor rejection is a very specific process, in that in transfer experiments the unrelated leukemic tumor cells always grew when admixed with immune spleen cells and target fibrosarcoma cells, whereas the latter did not. Furthermore, the use of AKR anti-θ serum enabled us to show that T lymphocytes are responsible for the specific tumor immunity, extending previous observations on the immune response to plasma cell tumors (11).

Materials and Methods. Animals. BALB/c/

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and Bradley Wehi mice, aged 60–140 days, were used throughout.

Tumors. The tumors designated WEHI 164 and WEHI 167 are fibrosarcomas, induced by injection of 200 μg 3-methylcholanthrene (MC) in 50 μl trioctanion in the subcutaneous tissues of BALB/c mice. Tumor WEHI 3 is a mineral oil-induced myelomonocytic leukemia of BALB/c origin (12). The tumors were maintained *in vivo* by serial subcutaneous transplantation or stored in liquid nitrogen and thawed when required. During these experiments, tumor WEHI 164 was in its 3rd–7th transplant generation, tumor WEHI 167 in its 4th–8th transplant generation, and tumor WEHI 3 had been transplanted between 20–40 times previously.

Tissue culture lines. Tumors WEHI 164 and WEHI 167 were established in tissue culture using single-cell suspensions derived from 0.25% trypsin digests of minced tumor. The cultures were designated WEHI 164 TC and WEHI 167 TC. Both tumor lines were in their 3rd–10th passage and were grown in 100 × 20 mm tissue culture dishes (Nr 3003, Falcon Plastics, Oxhard, CA) in Dulbecco's modified Eagle's minimal essential medium with penicillin (100 U/ml) and streptomycin (100 μg/ml) supplemented with 10% fetal calf serum (FCS). In order to obtain consistent tumor growth within 10 days of inoculation into sublethally irradiated BALB/c mice, the following tumor doses were found to be required: 5×10^4 WEHI 3 cells, 5×10^4 WEHI 164 TC cells, and 1×10^5 WEHI 167 TC cells.

Immunization. BALB/c mice were immunized to tumor WEHI 164 or WEHI 167 using a technique described earlier (13). In brief, 10^5 viable tumor cells WEHI 164 TC and WEHI 167 TC were injected intramuscularly into the gastrocnemius muscle of the right limb. Approximately 10 days later, the tumor-bearing limb was removed under ether anesthesia.

Cell suspensions. Tumor-bearing mice were killed by cervical dislocation, tumors removed aseptically, cut into small pieces, and suspended in Eisen's balanced salt solution (EBSS) containing 10% FCS. Following gentle teasing through a stainless steel sieve, tumor cells were washed twice and suspended in EBSS containing 10% FCS. Cell clumps were removed by layering the cell suspension over 2 ml of FCS for 5 min, after which the number of viable cells was determined by eosin dye exclusion. Spleen cells were prepared by the same procedure.

Cellular transfer of tumor immunity. Mixtures of single-cell suspensions of spleen cells from immunized mice and tumor cells were prepared *in vitro* with different ratios of the cell types, then the mixtures were injected subcutaneously in sublethally irradiated (400 R) BALB/c mice. The mean diameter of the tumor was measured at 3-day intervals, and tumor growth was recorded. The results presented record the incidence of detectable tumors at the time of peak tumor growth in the control mice.

Treatment of spleen cells with AKR anti-θ serum. AKR anti-θ serum was prepared according to the method of Reif and Allen (14). The cytotoxic titer against thymocytes of BALB/c origin was 1:90. In order to lyse the θ-positive cells in a given spleen cell population, 50×10^6 viable cells were suspended in 1-ml heat-inactivated AKR anti-θ serum or serum dilution, incubated for 30 min at 4°, washed twice, and resuspended in 1.5 ml of agarose-absorbed guinea pig complement. After incubation for an additional 30 min at 37°, the cells were washed twice, and then used for further experimental procedures.

Irradiation. Mice were exposed to total body irradiation using a Philips (RT 250) X-ray machine operating under conditions of 250 kV, 15 mA, and an HVL (half-value layer) of 0.8 mm Cu. The sublethal dose used for BALB/c mice was 400 rads to midpoint with maximum back scatter.

Results. Cellular transfer of immunity to a methylcholanthrene-induced fibrosarcoma. Preliminary observations during routine tumor passages had indicated that tumor WEHI 164 had a tendency to spontaneously regress, suggesting it carried strong tumor-associated transplantation antigens (TATA). Consequently, this tumor and fibrosarcoma WEHI 167 were selected and studied for immunogenicity. Syngeneic BALB/c mice were immunized with either 10^5 viable WEHI 164 TC cells or WEHI 167 TC cells as described

TABLE I. Adoptive Transfer of Immunity to Fibrosarcoma WEHI 164 and WEHI 167.

Spleen cells	Ratio of spleen cells to tumor cells	WEHI 164 tumor growth[a]	WEHI 167 tumor growth[a]
Nonimmune	100:1	6/6	6/6
Immunized to WEHI 164	100:1	0/6	6/6
	10:1	5/6	—
	1:1	6/6	—
Immunized to WEHI 167	100:1	6/6	6/6
	10:1	—	6/6
	1:1	—	6/6

[a] Incidence of detectable tumor growth 14 days later.

in *Methods*; 10 days after tumor removal, spleen cell suspensions from immunized and control mice were prepared. Graded numbers of spleen cells were mixed with a constant number of tumor cells—5×10^4 WEHI 164 TC cells and 10^5 WEHI 167 TC cells. The mixtures were injected subcutaneously into sublethally (400 R) irradiated BALB/c recipient mice. Control groups received tumor cells mixed with spleen cells from nonimmune sham-operated animals. The results (Table I) show that only WEHI 164 was immunogenic under the conditions tested, since spleen cells from animals immunized to WEHI 164 completely prevented WEHI 164 tumor growth when mixed at a ratio of 100 immune spleen cells to 1 tumor cell. The adoptive immunity was immunologically specific in that WEHI 164 immune spleen did not inhibit WEHI 167 tumor growth. Spleen cells from BALB/c mice immunized with WEHI 167 did not prevent WEHI 167 tumor growth in the adoptive transfer experiment, indicating lack of detectable TATA on this tumor. These experiments were successfully repeated several times, using *in vitro*- or *in vivo*-grown fibrosarcoma cells.

Specificity of tumor rejection. To further evaluate the specificity of *in vivo* tumor rejection, the following experiment was performed. Two tumors of BALB/c origin were selected which could be easily distinguished histologically: WEHI 3 is a myelomonocytic leukemia characterized by a mixed picture of early monocytic and myelocytic cells (12) and WEHI 164 a fibrosarcoma with typical spindle-shaped cells. If WEHI 164 tumor rejection is a specific immune process, then it would be expected that lymphoid cells immunized against WEHI 164 and mixed with both tumors WEHI 3 and WEHI 164 would only prevent WEHI 164, but not WEHI 3, tumor growth. The result could be verified histologically.

Spleen cells from either mice immunized against WEHI 164 or nonimmunized control mice were mixed with either 5×10^4 WEHI 164 cells, 5×10^4 WEHI 3 cells, or with both 5×10^4 WEHI 164 and 5×10^4 WEHI 3 cells; cell mixtures were then injected subcutaneously into sublethally irradiated BALB/c animals. The ratio of spleen cells to tumor cells was 100:1. The incidence of macroscopically detectable tumor growth was recorded 14 days later, and the area of tumor cell injection was taken for histological investigation. The macroscopic results are depicted in Table II and clearly show: (i)

TABLE II. Specificity of Rejection of WEHI 164 Tumor, Shown by Mixing WEHI 3 Tumor Cells and WEHI 164 Cells with WEHI 164 Immune Spleen Cells.

Group no.	Spleen cells[a]	Tumor I	Tumor II	Tumor growth[b]
1	nonimmune	WEHI 3	—	6/6
2		—	WEHI 164	6/6
3		WEHI 3	WEHI 164	6/6
4	immunized to WEHI 164	WEHI 3	—	6/6
5		—	WEHI 164	0/6
6		WEHI 3	WEHI 164	6/6

[a] WEHI 164 immune spleen cells of BALB/c mice were mixed with the indicated tumor cells *in vitro* at a ratio of 100 spleen cells to 1 tumor cell. The dose of WEHI 3 and WEHI 164 was both 5×10^4 cells/inoculum. The mixture was injected subcutaneously into sublethally irradiated (400 R) BALB/c mice.

[b] Incidence of detectable tumor growth 14 days later.

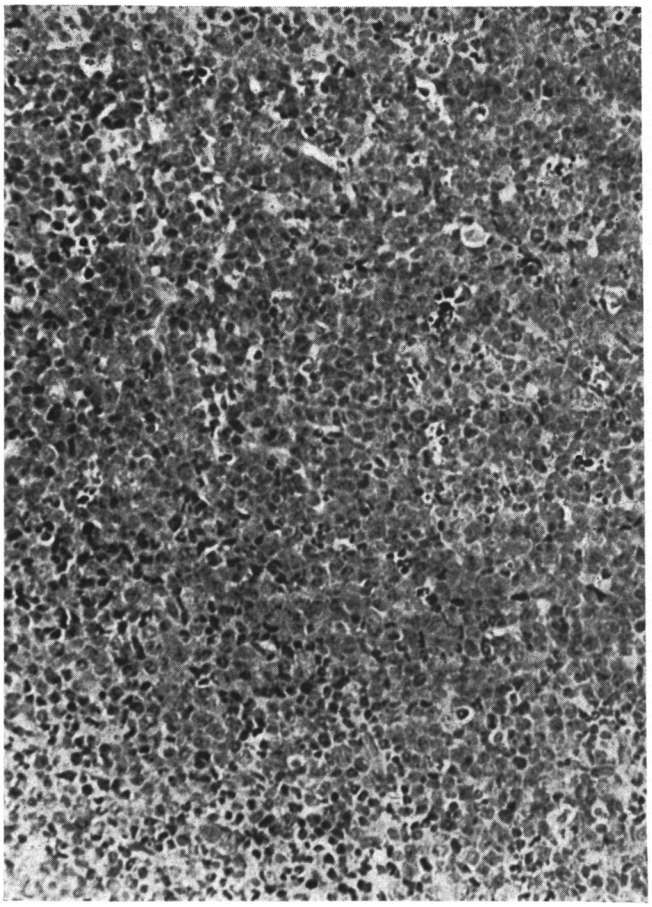

FIG. 1. Monomyelocytic leukemia WEHI 3 (× 100).

WEHI 3 and WEHI 164, together or individually, mixed with nonimmune syngeneic BALB/c cells always produced tumors; (ii) WEHI 164 immune spleen cells prevented only WEHI 164 (group 5), but not WEHI 3, tumor growth (group 4); and (iii) when WEHI 164 immune spleen cells are mixed with WEHI 3 and WEHI 164 cells, a tumor grew in all recipients and mice (group 6). The histological sections of group 1 showed the typical picture of the WEHI 3 leukemic (Fig. 1) and those of group 2 all the characteristics of the fibrosarcoma WEHI 164 (Fig. 2). The serial sections of group 3 revealed unequivocally that the tumors were all mixtures of mononucleated leukemic cells and fibrosarcoma cells (Fig. 3). The sections of the tumors of group 4 were almost indistinguishable from those of group 1. In the sections of group 5, no tumor cells were detected. The serial sections of the tumors of group 6 showed only masses of mononucleated tumor cells almost identical to Fig. 1, and no fibrosarcoma cells at all, indicating that the immune lymphoid cells selectively blocked the growth of WEHI 164 fibrosarcoma cells but not the WEHI 3 monomyelocytic leukemic cells.

Effect of AKR anti-θ serum on WEHI 164 immune spleen cells. The role of T cells in the adoptive transfer of immunity to WEHI 164 was tested using anti-θ serum treatment. Pretreatment of WEHI 164 immune spleen cells with AKR anti-θ serum at dilutions of 1:1 and 1:4 prevented the transfer of immunity to WEHI 164, indicating an essential role of T cells in this immune response.

Discussion. The results presented in this paper demonstrate that *in vivo* tumor rejec-

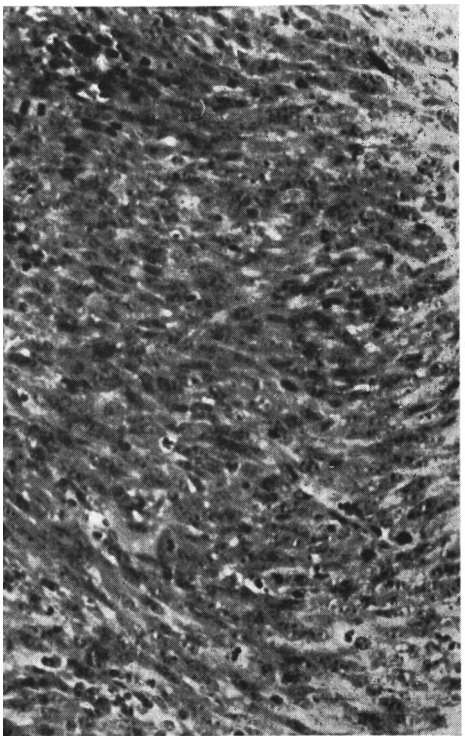

FIG. 2. Fibrosarcoma WEHI 164 (× 100).

tion by immune lymphoid cells can be a very specific response.

It was first established that the fibrosarcoma line used was antigenic and that immunity to this tumor could be transferred to nonimmune recipients by spleen cells from immunized syngeneic animals (Table I). The capacity of these *in vivo* immunized spleen cells to transfer tumor immunity was shown to be dependent on θ antigen bearing T lymphocytes (Table III). This result lends further support to the concept that at least one form of tumor immunity is very dependent on T lymphocytes. This has been discussed previously in the context of immune responses to murine plasma cell tumors (11).

The main aspect of this report concerns whether tumor rejection is a specific immune process, or whether nonspecific components are always present. It was found that in this system tumor rejection is mediated by *specific* immune lymphoid cells. Myelomonocytic leukemia cells were added to mixtures of *in vivo* immunized spleen cells and their target fibrosarcoma cells, and this mixture was subcutaneously injected into sublethally

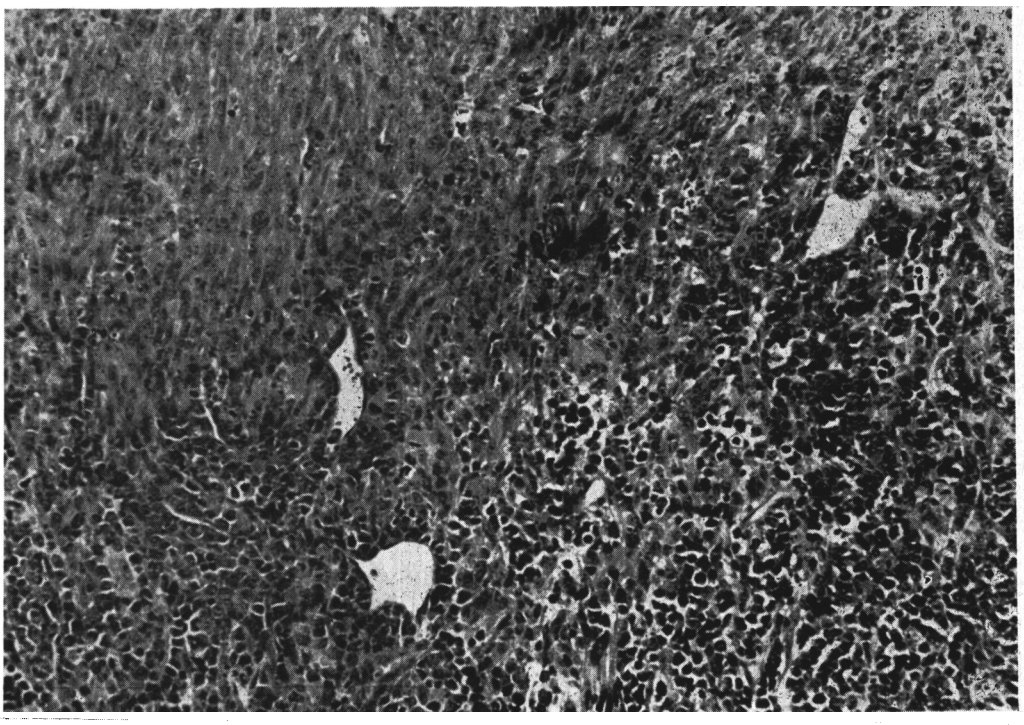

FIG. 3. Mixture of monomyelocytic leukemia WEHI 3 and fibrosarcoma WEHI 164 (× 100).

TABLE III. Effect of AKR anti θ Serum on WEHI 164 Immune Spleen Cells.

Spleen cells	Serum pretreatment and dilution[a]	WEHI 164 tumor growth[b]
Nonimmune	—	6/6
WEHI 164 immune	—	0/6
	normal AKR	0/6
	AKR anti θ 1:1	6/6
	AKR anti θ 1:4	5/6
	AKR anti θ 1:16	0/6

[a] Equal aliquots of WEHI 164 immune spleen cells were treated with AKR anti θ serum on normal AKR serum plus C as described in *Methods*, diluted, and mixed *in vitro* with WEHI 164 cells which resulted for the mixture of spleen cells pretreated with normal AKR serum in a ratio of lymphoid cells to tumor cells of 100:1. The mixture was injected subcutaneously into sublethally irradiated (400 R) BALB/c recipients.

[b] Incidence of detectable tumor growth 14–21 days later.

irradiated syngeneic mice. It was consistently found (group 6) (Table II) that a tumor grew within 10–14 days later, and consisted of masses of leukemic cells without any detectable fibrosarcoma cells, indicating that the spleen cells immune to the fibrosarcoma had mediated complete fibrosarcoma rejection, but that this response had not interfered with the growth of the leukemic cells. This finding suggested that fibrosarcoma inhibition was due to the direct specific effect of immune lymphoid spleen cells, and that nonspecific effector products were not involved.

Our results do not support the concept that a two-step process (1, 2) involving an immunologically specific recognition of antigen followed by release of nonspecific effector substances, is the exclusive mechanism of cell-mediated immunity to tumors. Our results do not deny the possible existence of such a mechanism. However, they emphasize that in an *in vivo* model of the development of immunity to a growing tumor, in the absence of any deliberate boosting of immunity, the natural immune response may solely involve a purely *specific* lymphoid cell process. The present results extend our previous observations with immunity to plasma cell tumors (11) and other *in vivo* observations (9, 10).

The nonspecific growth inhibition of tumor cells induced by bacterial adjuvant inoculation (3) and specific tumor immune rejections are very likely to involve different mechanisms, and for each given tumor-immunity situation, the possible role of either mechanism must be evaluated.

Summary. When myelomonocytic leukemia cells were admixed to spleen cells immune to a fibrosarcoma and the fibrosarcoma target cells, the growth of the leukemic cells was not inhibited. This was demonstrated in *in vivo* adoptive transfer experiments involving syngeneic animals. It was also shown that T cells are essential for the transfer of immunity to the fibrosarcoma. The results demonstrate that tumor cell rejection was a completely immunologically specific process.

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1. Zbar, B., Wepsic, H. T., Borsos, T., and Rapp, H. J., J. Nat. Cancer Inst. **44**, 473 (1970).

2. Evans, R., and Alexander, P., Nature (London) **236**, 168 (1972).

3. Zbar, B., Nat. Cancer Inst. Monogr. **35**, 341 (1972).

4. Cohen, I. R., and Feldmann, M., Cell. Immunol. **1**, 521 (1971).

5. Holm, G., Perlmann, P., and Werner, B., Nature (London) **203**, 841 (1964).

6. Granger, G. A., and Kolb, W. P., J. Immunol. **101**, 111 (1968).

7. Brunner, K. T., Mauel, J., Rudolf, H., and Chapius, B., Immunology **18**, 501 (1970).

8. Wagner, H., and Feldmann, M., Cell. Immunol. **3**, 405 (1972).

9. Klein, G., and Klein, E., Nature (London) **178**, 1389 (1956).

10. Klein, E., and Klein, G., Cell. Immunol. **5**, 201 (1972).

11. Rouse, B. T., Röllinghoff, M., and Warner, N. L., Eur. J. Immunol., in press (1973).

12. Warner, N. L., Moore, M. A. S., and Metcalf, D., J. Nat. Cancer Inst. **43**, 963 (1969).

13. Röllinghoff, M., Rouse, B. T., and Warner, N. L., J. Nat. Cancer Inst. **50**, 159 (1973).

14. Reif, A. G., and Allen, J. M. V., J. Exp. Med. **120**, 413 (1964).

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