LEUKOCYTE SUBPOPULATIONS ELICITED BY A NONTUMORIGENIC VARIANT OF B16 MELANOMA: THEIR ROLE IN DIRECT REJECTION OF THE MELANOMA AND IN PREVENTION OF TUMORIGENESIS IN WINN ASSAYS

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A variant of the B16 melanoma, clone C_3471, which is nontumorigenic in immunocompetent mice, is capable of immunizing such mice against the parental, tumorigenic clone B_559 (1, 2). Clone B_559 is not immunogenic and does not induce concomitant tumor immunity (3). The immunogenic C_3471 variant was derived from B_559 cells by continuous growth in the thymidine analog, 5-bromodeoxyuridine (BrdUrd).[1] C_3471 cells have undergone numerous phenotypic changes, including loss of tumorigenicity, pigmentation and plasminogen activator activity, as well as induction of endogenous retrovirus (1, 4–6). Other investigators have also produced immunogenic alterations of tumor cells by xenogenization with viruses (7–9), haptens (10, 11), mutagens (12–15), and with antineoplastic drugs (16–19).

The inability of C_3471 cells to form tumors reflects alteration(s) in interactions of these cells with the host's immune system, rather than an intrinsic loss of tumorigenic potential of C_3471 cells (20). Immunocompetent mice mount an inflammatory response to C_3471 cells within 24 h after inoculation, and inhibit tumor formation by these cells (2, 21). Mice immunologically compromised by treatment with anti-mouse thymocyte serum (ATS) or X irradiation support tumor formation by C_3471 cells.

In the experiments reported here, we have mixed B_559 cells with peritoneal cells from C_3471 cell-immunized mice and inoculated the mixtures into normal and immunosuppressed mice (Winn-type assay) to analyze leukocyte populations responsible for immunity to B_559 melanomas. Our results show that normal mice do not develop tumors when inoculated with a mixture of B_559 cells and peritoneal cells

* Part of this work was carried out during the tenure of postdoctoral fellowship 5F 32 CA 06201 from the National Institutes of Health.

‡ Recipient of a Special Fellowship of the Leukemia Society of America. Part of this work was carried out during the tenure of a postdoctoral fellowship from the Helen Hay Whitney Foundation, New York.

§ Recipient of grants AI 08697 and CA 30198 from the National Institutes of Health.

‖ Recipient of grant CA 10095 from the National Institutes of Health and grants from the Hahnloser-Bak Fund and the Esther and Morris Grossman Foundation.

[1] *Abbreviations used in this paper:* ATS, rabbit anti-mouse thymocyte serum; BCG, bacillus Calmette-Guerin; BrdUrd, 5-bromodeoxyuridine; FBS, fetal bovine serum; IgG-GRBC, IgG-coated, glutaraldehyde-fixed sheep erythrocytes; MLP, mean latent period; NRS, normal rabbit serum; PBS, phosphate-buffered saline without Ca^{++} or Mg^{++}; PEC, peritoneal exudate cells (leukocytes).

from C_3471 cell-immunized mice, and that a high percentage of these mice retain long-lasting immunity to $B_5$59 cells. Thy-1.2$^+$ peritoneal lymphocytes, or peritoneal macrophages from C_3471 cell-immunized mice each protect normal mice against melanoma formation when coinoculated with $B_5$59 cells. Thy-1.2$^+$ lymphocytes are required for both the generation and maintenance of tumoricidal macrophages in this system. Significantly, tumoricidal macrophages from C_3471-immune mice do not protect antithymocyte serum treated recipient mice against $B_5$59 melanomas, indicating that recipient thymus-dependent leukocytes are required to effect tumor eradication.

Materials and Methods

Cells. The derivation and maintenance of the melanotic tumorigenic $B_5$59 clone and the amelanotic, nontumorigenic C_3471 subclone of the B16 melanoma have been described previously (6, 22). Briefly, cells were maintained in monolayer culture in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, MD), 50 μg/ml gentamycin (Schering Corp., Kenilworth, NJ), 50 μg/ml aureomycin (Lederle Laboratories, Wayne, NJ), and 2.5 μg/ml fungizone (E. R. Squibb & Sons, Inc., Princeton, NJ). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells of the C_3471 subclone were grown continuously in the presence of 1 μg/ml BrdUrd added to the maintenance medium. Semiconfluent monolayers were dispersed using 0.0125% trypsin (Worthington Biochemical Corp., Freehold, NJ) with 2.6 mM EDTA. Total cell counts were determined with a Coulter counter and cell viability measured by trypan blue dye exclusion using a hemocytometer. Cells were checked periodically for mycoplasma using the Hoechst stain method (23); no mycoplasma were detected.

Mice. Female C57BL/6 mice, 5–7 wk of age, were obtained from the Mammalian Genetics Animal Production Section, Division of Cancer Treatment, National Cancer Institute at the Charles River Breeding Laboratories, Inc., Wilmington, MA.

Immunization. C_3471 cells used for immunizing mice were dispersed with trypsin, washed with serum-free MEM, and resuspended at 5×10^6 viable cells/ml MEM for inoculation. All mice used as donors of immune leukocytes for Winn assays were immunized by three intraperitoneal inoculations of 10^6 viable cells at weekly intervals.

To determine the effect of immunization route on the capacity of the animal to reject a tumor challenge, a similar immunization schedule was followed using subcutaneous injection of C_3471 cells into the flank, followed by inoculation of 2×10^5 viable $B_5$59 cells (the minimal number required to yield tumors in 100% of mice injected) 7 d after the last immunization, either intraperitoneally or subcutaneously, into the opposite flank. Mice receiving subcutaneous challenge with $B_5$59 cells were monitored for tumor formation by examination of the inoculation site for palpable melanoma nodules. Tumor development in the peritoneal cavity was monitored initially by swelling of the abdomen, and confirmed by dissection 40 d after challenge. Intraperitoneal $B_5$59 tumors are easily visualized macroscopically as pigmented masses or extensive "peppering" of the mesentery.

Irradiation. Mice were exposed to 650 rad from a 2,400 Ci caesium source gamma cell (located at Memorial Sloan-Kettering Cancer Center, New York), and were maintained in sterile bedding and provided with sterile drinking water acidified to pH 2.0 with 1 N HCl.

In Vivo Depletion of T Lymphocytes. Naïve or C_3471 cell-immunized mice were inoculated intraperitoneally with 0.2 ml rabbit ATS (M. A. Bioproducts) on days 6, 5, 3, 2, and 1 before use in an experiment. On day 0, mice were either inoculated with 2×10^5 $B_5$59 tumor cells, inoculated with mixtures of peritoneal cells and $B_5$59 cells for Winn assays, or killed for harvest of peritoneal exudate cells (PEC). Wright-Giemsa staining of cytocentrifuged PEC from ATS-treated C_3471-immunized mice revealed 65% macrophages, 2% lymphocytes, 16% neutrophils, 17% eosinophils, and 0.5% mast cells.

PEC. PEC were harvested by lavage of the peritoneal cavities of naïve or C_3471 cell-immunized mice with balanced salt solution containing 1 mM EDTA and 1 mM Hepes

according to a modification of the procedures of Cohn and Benson (24) as described by Michl et al. (25). Immune PEC were collected 7 d after the last inoculation of $C_3$471 cells. Lysis of contaminating erythrocytes was done routinely using hypotonic salt solutions. The leukocytes were then centrifuged (600 *g*/12 min/4°C) and resuspended in MEM without serum. Cells were enumerated using a hemocytometer and viability determined by trypan blue dye exclusion. Wright-Giemsa staining of cytocentrifuged PEC from $C_3$471 cell-immunized mice revealed 82% mononuclear leukocytes (macrophages and lymphocytes), 6% eosinophils, 3% neutrophils, and 3% mast cells.

Fractionation of PEC Subpopulations

GLASS ADHERENT AND NONADHERENT. Subpopulations of $C_3$471-immune PEC were separated on the basis of adherence to glass. Glass-adherent leukocytes were separated from nonadherent cells by incubation of 3–4 × 10^7 PEC in 100-mm sterile glass petri dishes with MEM supplemented with 15–20% FBS for 2–2.5 h at 37°C in a humidified atmosphere containing 5% CO_2. Approximately 95% of PEC that adhered to glass in a 6-h incubation adhered in the first 2.5 h after plating. An average of 47% (± 5%) $C_3$471-immune PEC are glass adherent within 2.5 h. Nonadherent PEC were transferred to tubes on ice. The adherent PEC monolayers were washed three times each with 5 ml of MEM at 25°C. Adherent PEC were washed twice more with ice-cold PBS containing 1 mM EDTA and 1 mM Hepes (Sigma Chemical Co., St. Louis, MO). After discarding these fluids, adherent cell monolayers were incubated with cold PBS containing EDTA-Hepes for 5–10 min at 4°C. Adherent PEC were then gently scraped using a sterile rubber policeman and transferred to tubes on ice. Both adherent and nonadherent PEC were pelleted (600 *g*/12 min/4°C) and resuspended in MEM without serum for counting and viability determinations as above. Wright-Giemsa staining of cytocentrifuged preparations of glass-adherent immune PEC revealed 97% mononuclear leukocytes, 1% mast cells, and 1% neutrophils. Over 95% of these mononuclear leukocytes were macrophages as measured by their capacity to bind and/or ingest glutaraldehyde fixed erythrocytes coated with IgG (Table I). Similar staining of leukocytes in the fraction not adherent to glass revealed 90% mononuclear leukocytes, 3% mast cells, and 5% eosinophils. Less than 7% of these leukocytes ingested any IgG-coated erythrocytes.

FRACTIONATION ON NYLON WOOL. Nonadherent PEC were further purified for enrichment of T lymphocytes by fractionation on nylon wool columns based on the method of Julius et al. (26). Nylon wool from Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Deerfield, IL) was soaked in 0.2 N HCl, boiled in distilled water, and autoclaved. Before addition of leukocytes, the columns were washed and preincubated with PBS containing 5% heat-inactivated FBS prewarmed at 37°C. After addition of 0.5–1.5 × 10^8 viable glass-nonadherent PEC suspended in prewarmed PBS + 5% FBS, columns were incubated sealed for 1 h at 37°C. PEC not adhering to the nylon wool were eluted dropwise with at least two column volumes of warm PBS + 5% FBS into centrifuge tubes, and cell number and viabilities were determined. Wright-Giemsa staining of cytocentrifuged nylon wool eluate cells revealed a virtually pure (98%) population of lymphocytes.

Activation of Adherent PEC In Vitro. PEC harvested from naïve C57BL/6 mice or from ATS-treated $C_3$471-immunized mice were plated on glass petri dishes to separate glass adherent and nonadherent fractions as above. Adherent cell monolayers were washed to remove nonadherent leukocytes, and PEC in the glass-nonadherent or nylon wool-purified fractions from either unimmunized normal C57BL/6 mice or from $C_3$471-immunized C57BL/6 mice were incubated

TABLE I

Binding and Phagocytosis of IgG-Coated Erythrocytes by Glass-Adherent PEC

	Ingested or bound particle	Phagocytic index*	Percent control	Binding index*	Percent control
Resident	IgG-GRBC	442.5 (92)‡	100	937.2 (96)‡	100
$C_3$471-immune	IgG-GRBC	738.7 (95)	165	1324.8 (96)	141

* Phagocytic and binding indices were determined as described previously (3).

‡ Percent macrophages binding or ingesting IgG-GRBC.

with the glass-adherent cells for ~18 h in MEM containing 10% FBS. At the end of incubation, nonadherent PEC were removed and adherent cells were washed with MEM, PBS, and then PBS containing 1 mM EDTA and 1 mM Hepes, to minimize contamination with nonadherent leukocytes. PEC still adherent to the glass petri dishes were then harvested by scraping, as above, counted and coinjected with $B_5$59 cells in Winn assays.

Antisera. Immune lysis of T lymphocytes from unfractionated and fractionated PEC was carried out using either rabbit ATS having a cytotoxic titer of 1:3,200, or monoclonal mouse anti-Thy-1.2 (gift of Dr. U. Hämmerling, Memorial Sloan-Kettering Cancer Center) having a titer of 1:10,000. 4–6-wk-old rabbit complement obtained from Pel-Freez Biological, Inc. (Rogers, AZ) was used at a 1:4 dilution to effect lysis. PEC pellets containing 0.25–2.5 × 10^7 viable cells were resuspended in a total volume of no more than 200 μl of either rabbit ATS or anti-Thy-1.2 in PBS (with Ca^{++} and Mg^{++}), incubated on ice for 30–40 min, washed in PBS, resuspended in 200 μl PBS containing rabbit complement at 1:4 dilution, and incubated for 20 min at 37°C in a CO_2 incubator. Cell viability was estimated by trypan blue dye exclusion.

Tumor Neutralization Assay. A modification of the adoptive tumor neutralization assay described by Winn (27) was used to evaluate the effectiveness of immune peritoneal cell populations in preventing tumor formation by $B_5$59 melanoma cells. Unfractionated PEC, harvested from naïve or immune mice as described above, were washed in PBS and resuspended in MEM without serum. Subpopulations of immune PEC were similarly prepared after fractionation. PEC were mixed with freshly harvested $B_5$59 cells to give the appropriate PC/$B_5$59 cell ratio in a final volume of 0.2 ml. This cell mixture was inoculated subcutaneously into the flank of C57BL/6 mice and animals were checked two to three times weekly for appearance of palpable tumor nodules. Tumor formation is reported for all animals used in each experiment.

In Vitro Cytotoxicity Assay. A colony assay (V. H. Freedman, G. Kaplan, C. S. Copeland, and S. C. Silverstein, manuscript in preparation) was used to measure cytotoxicity. Briefly, tumor cells were plated in 16-mm Costar wells (Costar, Data Packaging, Cambridge, MA), and overlaid with PEC from immunized mice. After 24 h incubation, the tumor cells were trypsinized, diluted, and plated in 60-mm petri dishes. Colonies were counted after 7 d. The percent $B_5$59 cells killed was calculated as number of colonies in experimental sample divided by the number of colonies in control sample times 100.

Results

Effect of Immunization Route on Rejection of $B_5$59 Melanoma. We compared the efficacy of subcutaneous vs. intraperitoneal immunization with $C_3$471 cells in protecting C57BL/6 mice against challenge with various numbers of malignant $B_5$59 melanoma cells. Fig. 1 shows that at all tumor cell doses tested, mice immunized intraperitoneally were more resistant to intraperitoneal challenge with $B_5$59 cells than were mice immunized subcutaneously. Subcutaneous immunization provided good protection against subcutaneous or intraperitoneal challenge only at the lowest tumorigenic dose (2 × 10^5 $B_5$59 cells). $B_5$59 tumors developed in 100% of unimmunized animals. As will become evident, the peritoneal cavities of mice immunized with $C_3$471 cells contain tumoricidal leukocytes. Fig. 1 shows that repeated intraperitoneal immunization with $C_3$471 cells was the most efficient method of eliciting these leukocytes. Therefore, all subsequent studies were performed using peritoneal leukocytes elicited by intraperitoneal immunization.

Effects of Immunosuppression on Rejection of $B_5$59 Melanoma by $C_3$471 Cell-immunized Mice. C57BL/6 mice treated with ATS before inoculation with $C_3$471 cells develop progressive amelanotic $C_3$471 tumors (20, 21, 28). To determine whether ATS alters established immunity to the malignant parental melanoma (clone $B_5$59), mice immunized with $C_3$471 cells were treated with ATS before subcutaneous challenge with $B_5$59 cells. All of these mice developed melanotic tumors. In contrast, all of the immunized control mice that received normal rabbit serum (NRS) were protected

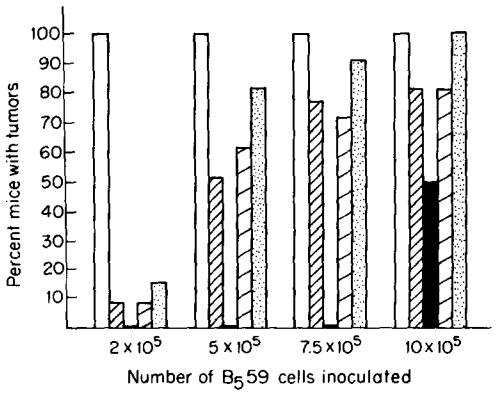

FIG. 1. Effect of immunization route on rejection of subcutaneous vs. intraperitoneal challenge with $B_5$59 melanoma cells. C57BL/6 mice were immunized by either three subcutaneous (s.c.) or intraperitoneal (i.p.) inoculations of 10^6 $C_3$471 cells, each 7 d apart. Immunized mice were challenged by either subcutaneous or intraperitoneal injection of the indicated numbers of $B_5$59 melanoma cells, 7 d after the last $C_3$471 cell inoculation. □, Unimmunized C57BL/6 control mice challenged either i.p. or s.c.; ▨, s.c. immunized mice challenge i.p.; ■, i.p. immunized mice challenged i.p.; ▨, s.c. immunized mice challenged s.c.; ▨, i.p. immunized mice challenge s.c. Mice were monitored for tumor formation as described in Materials and Methods. Data represent averages from four experiments.

TABLE II

Lack of Protection Against $B_5$59 Melanoma in ATS-treated $C_3$471-immunized Mice*

Mice injected subcutaneously with 2×10^5 $B_5$59 cells	Mice with tumors/mice inoculated	MLP ± SD
$C_3$471-immunized	0/8	—
ATS-treated, $C_3$471-immunized	12/12	9.9 ± 2.1
NRS-treated, $C_3$471-immunized	0/4	—
Unimmunized controls	12/12	12.9 ± 7.6

* Rabbit ATS or normal rabbit serum injected intraperitoneally into C57BL/6 mice immunized with $C_3$471 cells (Materials and Methods).

against tumor formation (Table II). These results indicate that established immunity can be abrogated by ATS treatment; they suggest that ATS-sensitive leukocytes are involved in both the afferent and efferent pathways of $C_3$471 cell-elicited host defense.

To evaluate the radiosensitivity of the cells that mediate $B_5$59 tumor cell rejection, C57BL/6 mice were immunized with $C_3$471 cells and exposed to 650 rad of X irradiation 24 h before inoculation with $B_5$59 cells. These mice were unable to reject the $B_5$59 cells (data not shown), indicating that radiosensitive effector cell(s) are required for destruction of $B_5$59 cells in $C_3$471 cell-immune mice.

Characterization of the Leukocytes Elicited by $C_3$471 Cells. To confirm that leukocytes from $C_3$471 cell-immunized mice are responsible for the rejection of $B_5$59 cells, we mixed varying numbers of $C_3$471-immune peritoneal leukocytes with 2×10^5 $B_5$59 cells and inoculated them subcutaneously into naïve C57BL/6 mice. $C_3$471 cell-immune PEC markedly inhibited tumorigenesis by $B_5$59 cells, completely abrogating tumor formation at PEC/melanoma cell ratios of 30:1 or greater, and inhibiting tumor formation in 90% of mice inoculated with a 25:1 ratio. The latter is therefore the ratio of PEC/melanoma cells used for all subsequent Winn assays. In those few

animals that did develop tumors, the mean latent period (MLP) for development of palpable subcutaneous nodules increased progressively as the ratio of PEC/melanoma cells decreased (Fig. 2). As expected, at all ratios tested (10:1 to 40:1), PEC from unimmunized C57BL/6 mice had no inhibitory effect on tumor formation by $B_5$59 cells (data not shown).

To identify the kinds of leukocytes responsible for the tumoricidal effects of $C_3$471-immune PEC, we carried out tumor neutralization assays using glass-adherent (macrophage-enriched) and glass-nonadherent (lymphocyte-enriched) subpopulations. 5×10^6 leukocytes from each subpopulation were as effective in protecting mice against $B_5$59 melanoma as unfractionated immune PEC (Table III). For those few mice that developed $B_5$59 tumors, the MLP was nearly doubled. Similar protection was obtained when mice were inoculated with a mixture of 2.5×10^6 each of glass-

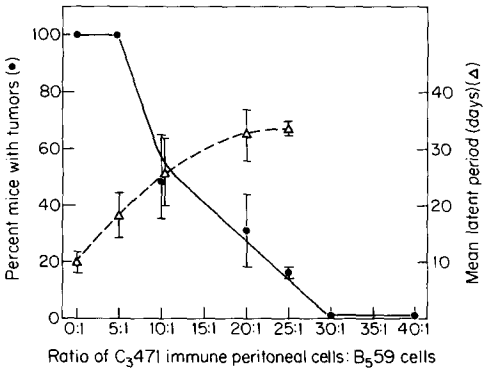

FIG. 2. Inhibition of $B_5$59 tumor formation by varying numbers of $C_3$471-immune PEC. Peritoneal leukocytes, from mice immunized by three successive weekly intraperitoneal inoculations of 10^6 viable $C_3$471 cells, were co-injected subcutaneously, in varying ratios with 2×10^5 viable $B_5$59 melanoma cells into immunocompetent naïve C57BL/6 mice. Mice were monitored for $B_5$59 tumor formation by palpation of injection sites two to three times weekly. Data represent averages from seven experiments.

TABLE III

PEC from $C_3$471 Cell-immunized Mice Inhibit Tumor Formation by $B_5$59 Cells

$C_3$471-immune PEC*	Mice with tumors/ mice inoculated	MLP ± SD
Unfractionated	5/26	20.6 ± 15.5
Glass adherent	4/30	21.0 ± 9.9
Glass nonadherent	6/33	21.3 ± 6.1
Glass adherent + glass nonadherent‡	5/18	18.0 ± 9.8
Controls§		
Unfractionated normal C57BL/6 PEC + $B_5$59 cells	29/32 11/12	13.7 ± 8.4 11.6 ± 4.2

* PEC from $C_3$471-immunized mice inoculated in a mixture with $B_5$59 melanoma cells at a 25:1 ratio using 2×10^5 viable melanoma cells.

‡ Glass adherent and nonadherent immune PEC mixed together at a 1:1 ratio after separation on glass as described in Materials and Methods.

§ 2×10^5 $B_5$59 cells inoculated either alone or together with 5×10^6 PEC from naïve mice.

adherent and nonadherent fractions together with $B_5$59 cells (Table III). These findings differ from those we reported previously using glass-adherent and nonadherent fractions of PEC elicited by immunization with heat-killed bacillus Calmette-Guerin (BCG) (3) In those experiments, only the glass-adherent leukocytes were able to confer protection against $B_5$59 tumor formation.

To characterize the cells in the nonadherent PEC population that were responsible for rejection of $B_5$59 melanomas, nonadherent PEC from $C_3$471 immune mice were treated with anti-thymocyte serum or anti-Thy-1.2 and complement and then coinjected with $B_5$59 cells into naïve mice. This treatment completely abrogated the capacity of immune nonadherent PEC to protect mice against $B_5$59 melanoma (Table IV). To confirm that the T cells alone were capable of inhibiting melanoma formation, immune T cells were purified by passage of $C_3$471-immune nonadherent PEC over a nylon wool column. Mice inoculated with a mixture of these T cells and $B_5$59 melanoma cells were protected against $B_5$59 melanomas; this protection was abolished by treatment of the immune T cells with anti-thymocyte serum or anti-Thy-1.2 and complement (Table IV). In contrast, treatment of $C_3$471 immune glass-adherent PEC with ATS or anti-Thy-1.2 and complement had no effect on the capacity of these leukocytes to inhibit $B_5$59 tumorigenesis (Table IV). These results indicate macrophages and thymus-derived lymphocytes are responsible for the in vivo tumor inhibitory effects of the corresponding glass-adherent and nonadherent $C_3$471 cell-immune PEC fractions; and that macrophages from $C_3$471 immunized mice retain their tumoricidal activity in the absence of immune donor T cells.

Participation of Recipient ATS-sensitive Leukocytes in Rejection of $B_5$59 Melanoma Cells. ATS-sensitive lymphocytes can be removed from either unfractionated or glass-adherent immune PEC without impairing their capacity to cause rejection of $B_5$59 cells in Winn assays in normal mice (Table IV). Therefore, both unseparated and adherent PEC should yield a high degree of protection if recipient T cells do not

TABLE IV

Effects of In Vitro Treatment of $C_3$471-immune PEC With Anti-Thy-1.2 or Rabbit ATS on Their Tumor Inhibitory Properties

$C_3$471-immune PEC subpopulation*	Treatment					
	C′ Alone‡		Anti-Thy-1.2§ + C′		ATS‖ + C′	
	Percent lysis	Mice with tumors/ mice inoculated¶	Percent lysis	Mice with tumors/ mice inoculated¶	Percent lysis	Mice with tumors/ mice inoculated¶
Unfractionated	1.6	0/6	18.8	1/6	20.7	0/8
Glass adherent	0.9	0/4	3.5	0/5	4.2	1/5
Glass nonadherent	1.1	0/4	47.1	6/6	49.4	4/4
Nylon wool nonadherent	0.9	1/21	94.5	8/8	95.3	4/4

* PEC were obtained and fractionated as described in Materials and Methods. The nylon wool-nonadherent cells were obtained by passage of glass-nonadherent cells through nylon wool columns as described in Materials and Methods.

‡ Rabbit complement diluted 1:4.

§ Monoclonal mouse anti-Thy-1.2 diluted 1:200.

‖ Rabbit anti-mouse thymocyte serum diluted 1:100.

¶ PEC inoculated with $B_5$59 melanoma cells at 25:1 ratio using 2×10^5 viable melanoma cells and 5×10^6 PEC.

participate in the rejection of $B_5$59 cells in Winn assays. To examine this question, naïve mice were treated with ATS before inoculation with $C_3$471 cell-immune PEC and $B_5$59 cells. ATS treatment was suspended 24 h before leukocyte transfer to minimize effects of circulating anti-T cell antibody on immune donor PEC. As expected, the glass-nonadherent fraction was unable to protect against tumor formation in the ATS-treated recipients. Surprisingly, however, neither unfractionated $C_3$471-immune PEC (containing macrophages and T cells), nor the glass-adherent subfraction (comprised primarily of macrophages), was able to convey protection against $B_5$59 melanoma upon adoptive transfer into ATS-treated syngeneic mice (Table V).

The inability of the macrophage-enriched fraction to protect ATS-treated recipients against $B_5$59 melanoma suggested that recipient T cells participate in tumor neutralization by $C_3$471-immune macrophages. This hypothesis is tenable only if the ATS used in these experiments did not inhibit macrophage recruitment, viability, or tumoricidal effector functions. Two lines of evidence show that it did not. First, mice immunized intraperitoneally with $C_3$471 cells for 3 successive wk were treated with ATS as described in Materials and Methods. Recovery of total PEC from these mice was invariably nearly twice that from untreated immune mice (7.6×10^6 vs. 4.5×10^6), and Wright-Giemsa-stained cytocentrifuge preparations of these PEC contained 65% macrophages, 2% lymphocytes, 16% neutrophils, and 17% eosinophils. PEC from ATS-treated immune mice contained more macrophages than PEC from immune mice (4.9×10^6 vs. 2.25×10^6). Second, ATS in the presence (Table IV) or absence of complement (Tables VI and VII) did not inhibit the tumoricidal activity of immune macrophages in vitro (Table VI) or in vivo (Tables IV and VII). That the concentration of ATS used exceeded that in the serum of ATS pretreated mice at the time of Winn assay was determined as follows: ATS at a dilution of 1:100 lysed 95% of $C_3$471-immune nylon wool-purified T cells (Table IV), whereas undiluted serum from mice treated with ATS (as in Table V) plus complement lysed only 30–37% of these T cells (data not shown). No detectable lysis of $C_3$471-immune macrophages was observed when these cells were incubated with undiluted serum from ATS treated

TABLE V

Treatment of Recipient Mice with ATS Blocks the Capacity of $C_3$471-Immune PEC to Inhibit $B_5$59 Melanoma Formation in Winn Assays

$C_3$471-immune subpopulation*	Untreated recipients‡		ATS-treated recipients§	
	Mice with tumors/mice inoculated	MLP ± SD	Mice with tumors/mice inoculated	MLP ± SD
Unfractionated	0/11	—	14/14	10.4 ± 2.0
Glass adherent	1/6	19.0 ± 0	7/7	14.5 ± 1.7
Glass nonadherent	1/7	26.0 ± 0	7/7	15.7 ± 3.8
$B_5$59 Controls	11/11	9.4 ± 2.4	12/12	8.3 ± 3.1

* As in Table IV.

‡ Normal C57BL/6 mice.

§ Normal C57BL/6 mice pretreated with rabbit ATS. These mice received five 0.2-cc inoculations of ATS intraperitoneally on days −6, −5, −4, −2, and −1 before subcutaneous inoculation of immune PEC with melanoma cells for Winn assay.

TABLE VI

ATS Does Not Inhibit Killing of $B_5$59 Melanoma Cells by $C_3$471-immune Adherent PEC In Vitro

Number of adherent PEC*/well	ATS‡	Percent $B_5$59 cells viable§
5×10^6	−	0.01%
5×10^6	+	0.01%
2×10^6	−	0.5%
2×10^6	+	0.5%
—	−	100%
—	+	100%

* Glass-adherent PEC from $C_3$471-immunized mice harvested as described in Materials and Methods.

‡ ATS at a 1:100 concentration in MEM with 10% FBS.

§ Percentage of $B_5$59 melanoma cells killed is based upon the number of colony-forming units of $B_5$59 cells remaining after 24 h incubation at 37° of a mixture of 1×10^5 $B_5$59 cells and the indicated number of $C_3$471-immune PEC. $B_5$59 cells were harvested and plated; colonies were enumerated as described in Materials and Methods. 100% represents the average number of $B_5$59 colonies recovered from control wells.

TABLE VII

Co-Injection of ATS With $C_3$471-immune Macrophages Does Not Inhibit Their Tumoricidal Activity

	Mice with tumors/ mice inoculated	MLP ± SD
$C_3$471-immune adherent PEC*	0/4	—
$C_3$471-immune adherent PEC + ATS‡	0/6	—
$B_5$59 controls	5/6	16.4 ± 3.5

* Each mouse was inoculated subcutaneously with 0.5 ml MEM containing 5×10^6 viable glass-adherent PEC from $C_3$471-immunized mice and 2×10^5 $B_5$59 melanoma cells.

‡ Each mouse was inoculated subcutaneously with 0.2 ml MEM containing 5×10^6 viable glass-adherent PEC from $C_3$471-immunized mice, 2×10^5 $B_5$59 melanoma cells, and 2 μl ATS. The final ATS concentration was 1:100.

TABLE VIII

*Effect of ATS Treatment In Vivo on Tumoricidal Activity of PEC of $C_3$471-Immunized Mice**

$C_3$471-immune subpopulation	Mice with tumors/mice inoculated		
	From untreated immune donors‡	From ATS-treated immune donors§	From NRS-treated immune donors‖
Unfractionated	2/29	29/30	0/5
Glass adherent	0/10	14/16	0/8
Glass nonadherent	0/12	11/12	0/8
$B_5$59 Controls¶	35/36		

* 5×10^6 PEC harvested from $C_3$471-immune mice, prepared as described in Materials and Methods, were inoculated together with 2×10^5 $B_5$59 cells into naïve C57BL/6 mice.

‡ PEC from untreated $C_3$471-immunized mice as in Table IV.

§ PEC from $C_3$471-immunized mice as above harvested 24 h after the last inoculation of rabbit ATS. Mice were treated with ATS for 3 d (days −6, −5, and −4) after their last immunization with $C_3$471 cells, and again on the 2 d (days −2 and −1) before harvest of their PEC as described in Materials and Methods.

‖ Mice were treated with NRS following the schedule described above (§).

¶ 2×10^5 $B_5$59 cells inoculated subcutaneously into naïve C57BL/6 mice.

TABLE IX

Activation of Macrophage Tumoricidal Activity by C_3471-Immune Lymphocytes

Source of glass-adherent PEC used in Winn Assays*	Cells incubated with glass adherent PEC	Mice with tumors/mice inoculated	MLP ± SD
ATS-treated, C_3471-immunized	Glass nonadherent from normal C57BL/6 mice	7/7	11.4 ± 3.5
	Nylon wool eluate from normal C57BL/6 mice	5/5	18.3 ± 7.9
	Glass nonadherent from C_3471-immunized mice	2/21	17.0 ± 1.4
	Nylon wool eluate from C_3471-immunized mice	1/5	26.0
Normal C57BL/6 mice	Glass nonadherent from normal C57BL/6 mice	3/3	22.3 ± 9.8
	Nylon wool eluate from normal C57BL/6 mice	4/4	15.8 ± 5.3
	Glass nonadherent from C_3471-immunized mice	0/5	—
	Nylon wool eluate from C_3471-immunized mice	1/4	18.0
B_559 cells	None	13/14	12.6 ± 4.1

* Glass-adherent PEC were harvested from either naïve, untreated C57BL/6 mice or from C_3471-immunized mice injected intraperitoneally with rabbit ATS after the last inoculation of C_3471 cells and before harvest of PEC as in Table VIII and as described in Materials and Methods. The nonadherent cells were incubated with the adherent PEC for 24 h and then removed by washing. The adherent cells were removed from the monolayer, mixed with B_559 cells, and inoculated into naïve mice at a ratio of 5×10^6 viable adherent cells: 2×10^5 B_559 cells as described in Materials and Methods.

mice in the presence of complement for 1–3 h at 37°C (data not shown).

ATS-sensitive T Lymphocytes are Required for the Maintenance of Tumoricidal Macrophages in C_3471 Cell-immune Mice. To determine whether T cell functions are required for the maintenance of macrophage cytotoxic effector functions in vivo C_3471 cell-immunized mice were treated with ATS after their last immunization with C_3471 cells. Their PEC were then harvested and co-inoculated together with B_559 cells into naïve C57BL/6 mice. ATS treatment of the immunized donor mice resulted in abrogation of the tumor protective capacity of their PEC; both adherent and nonadherent PEC were inactive. Normal rabbit serum had no effect (Table VIII). These results suggested that the inhibitory action of ATS on the maintenance of macrophage tumoricidal activity was an indirect one, a consequence of ATS-mediated destruction of T lymphocytes. If so, then addition of C_3471-immune lymphocytes to these macrophages should restore their tumoricidal activity. To test this hypothesis, glass-adherent PEC harvested from ATS treated immune mice, were washed to remove nonadherent leukocytes, and incubated for 18 h with nonadherent or nylon wool-purified leukocytes from C_3471-immunized donors or from nonimmunized C57BL/6 mice. Nonadherent PEC were removed by extensive washing. 92% of the glass-adherent cells ingested IgG-coated glutaraldehyde-fixed sheep erythrocytes, showing that the washing procedure was sufficient to remove virtually all added immune lymphocytes. The macrophages were harvested and injected together with

$B_5$59 melanoma cells into naïve C57BL/6 mice. Macrophages incubated with either glass nonadherent or nylon wool-purified leukocytes from immunocompetent $C_3$471-immune mice, conferred a high degree of protection against $B_5$59 tumor formation to naïve Winn assay recipients (Table IX). Macrophages incubated with either nonadherent or nylon wool-purified cells from unimmunized C57BL/6 mice provided no protection against tumor formation (Table IX). These results confirm that T lymphocytes from $C_3$471-immune mice are essential for the generation and maintenance of the tumor inhibitory properties of macrophages.

To determine whether lymphocytes from $C_3$471 cell-immune animals could stimulate macrophage tumoricidal activity in the absence of tumor antigens in the adherent cell population, macrophages from normal, nonimmunized C57BL/6 mice were incubated with glass nonadherent or nylon wool eluate PEC from $C_3$471-immunized mice. At the end of the incubation, the nonadherent cells were removed by extensive washing. (Over 90% of the remaining adherent leukocytes were macrophages as measured by their ingestion of IgG-coated glutaraldehyde-fixed erythrocytes [IgG-GRBC].) The adherent leukocytes were detached from the plates and coinjected with $B_5$59 melanoma cells into naïve mice. These adherent cells prevented tumor formation in eight of nine recipients (Table IX). All animals receiving macrophages that had been incubated with glass nonadherent or nylon wool eluate cells from unimmunized mice developed lethal melanomas (Table IX).

Acquisition of Tumor Immunity by Winn Assay Recipients. $B_5$59 melanoma cells are not immunogenic. $B_5$59 tumor formation is not prevented by inoculation of mitomycin-treated or radiation-killed $B_5$59 cells (1, 29), or by repeated inoculation of crude membranes prepared from cultured $B_5$59 cells (29) into syngeneic mice. Also, animals bearing $B_5$59 tumors do not exhibit concomitant tumor immunity (3). Efforts to transfer immunity against $B_5$59 melanoma to naïve syngeneic mice with $C_3$471-immune leukocytes have been unsuccessful. Neither 10^7 peritoneal nor 10^7 spleen cells from $C_3$471-immunized mice conferred any protection against $B_5$59 tumor formation even when administered on multiple occasions, subcutaneously, or intravenously to normal or sublethally irradiated naïve C57BL/6 mice. In these experiments 27 of 28 normal mice and 22 of 23 irradiated mice receiving $C_3$471-immune leukocytes developed lethal melanomas when challenged with 2×10^5 $B_5$59 cells. However, when animals that had received 5×10^6 unfractionated $C_3$471-immune PEC together with the melanoma cells in a Winn assay were tested by a second injection of $B_5$59 cells 40–60 d later, 12 of 21 animals remained free of tumors 40 d after this second challenge. Thus, a significant degree of systemic immunity to $B_5$59 melanoma can be adoptively transferred by immune peritoneal leukocytes in the presence of tumor cells.

Discussion

We have examined the mechanism of rejection of clone $B_5$59 of B16 melanoma by syngeneic C57BL/6 mice immunized with an immunogenic variant (clone $C_3$471) of the parental melanoma. Immunization with $C_3$471 cells results in systemic immunity to $B_5$59 melanoma (1, 2). The protective effect of immunization is most pronounced when immunization and subsequent challenge are within the same tissue compartment (the peritoneal cavity); nonetheless, significant systemic resistance to tumor challenge is observed when the sites of immunization and/or tumor challenge lie outside the peritoneum (Fig. 1). Treatment of naïve mice with ATS before $C_3$471

inoculation permits the development of C_3471 tumors (20), and established immunity to B_559 cells can be abrogated by ATS treatment (Tables II and V) or X irradiation of the immune mice. These findings indicate that intact T cell function is required for the establishment and maintenance of immunity to this melanoma. This conclusion was substantiated when leukocyte subpopulations from immune mice were coinjected with B_559 cells into naïve or ATS-treated recipients. Macrophage or T cell enriched populations prevented tumor growth in naive mice but not in ATS-treated mice (Table V).

There are two distinct pathways via which immune T cells might act in this system. T cells might function as cytotoxic effector cells or as amplifier and helper cells. We do not know whether C_3471-immune T cells are themselves cytotoxic. Further work is needed to resolve this issue. However, our data do indicate that these T cells act as amplifiers of the immune reaction, promoting the activation of mononuclear phagocytes that are tumoricidal in vivo (Table IX). A requirement for cooperation between mononuclear phagocytes and immune T cells is further emphasized by the inability of macrophages from C_3471-immunized mice to prevent tumors in ATS-treated Winn assay recipients (Table V).

Previous work with macrophages elicited by BCG immunization showed that co-injection of BCG-activated macrophages together with B_559 cells inhibited melanoma formation in normal, X-irradiated and nude mice (3). Thus it was surprising to find that demonstrably tumoricidal macrophages from C_3471-immune mice did not inhibit B_559 melanoma formation in mice treated with ATS (Table V). Careful examination of the effects of ATS showed that this serum did not inhibit macrophage recruitment in vivo, or macrophage viability or tumoricidal activity in vitro (Tables IV, VI, and VII). These findings indicate that the inhibitory effect of ATS on macrophage tumoricidal activity in vivo is not due to a direct action of this serum on the macrophages. We conclude that T cell functions are required for the full expression of macrophage tumoricidal activity in vivo.

In addition to a requirement for T cells and macrophages, other cell types may be involved in the rejection of B_559 cells by C_3471 cell-immunized mice. Beige mice immunized with C_3471 cells fail to reject B_559 melanoma (38), suggesting that natural killer and/or other leukocyte subpopulations that are defective in beige mice (39–41) may be required.

Approximately half of the animals that reject B_559 cells after co-injection of these melanoma cells with immune PEC developed long lasting immunity to B_559 cells as measured by their ability to reject a challenge with the tumorigenic melanoma cells. In contrast, as we reported previously (3), mice that did not develop tumors after co-injection of BCG-immune PEC with B_559 cells remained completely susceptible to subsequent challenge with B_559 cells. Thus, the ability of the mice that received C_3471-immune peritoneal leukocytes and B_559 melanoma cells to resist a second challenge 40–60 d later appears to be due to stimulatory effects on the host immune system of adoptively transferred immune leucocytes in the presence of antigen (B_559 cells).

The BrdUrd-induced changes responsible for the immunizing properties of C_3471 cells have not been defined. However, virus production by C_3471 cells seems to play a key role. C57BL/6 mice immunized with nontumorigenic feral mouse cells chronically infected with C_3471 cell-derived retrovirus are protected against challenge with

$B_5$59 melanoma cells (38).

In summary, three novel findings emerge from this work. First, that an intact T lymphocyte system is needed to elicit tumoricidal macrophages by immunization with the nontumorigenic BrdUrd-grown melanoma variant. Second, that the tumoricidal macrophages elicited are unable to mediate rejection of $B_5$59 melanoma cells in a Winn assay in the absence of intact recipient T lymphocyte function, further emphasizing a requirement for cooperation of T cells with other leukocyte populations. Third, that a significant proportion of naïve C57BL/6 mice can be immunized against $B_5$59 melanoma when these tumor cells are co-injected with peritoneal cells from $C_3$471 cell-immune mice. This last finding suggests one approach to immunization against nonimmunogenic syngeneic tumors.

Summary

The mechanisms by which various leukocyte subpopulations elicited by an immunogenic, nontumorigenic subclone ($C_3$471) of B16 melanoma caused rejection of the tumorigenic parental melanoma ($B_5$59), were investigated. Leukocytes from $C_3$471-immune mice were co-injected with $B_5$59 tumor cells in Winn assays into normal syngeneic recipients. Tumor formation by $B_5$59 cells was prevented when $C_3$471-immune (*a*) unfractionated peritoneal leukocytes, or (*b*) glass-adherent peritoneal cells (90% macrophages), or (*c*) nylon wool purified nonadherent cells (95% Thy-1.2$^+$) were used in the Winn assays. If the $C_3$471-immunized mice were treated with antithymocyte serum before harvest of their peritoneal cells, none of these leukocyte populations were effective in the Winn assay. However, macrophages from these immunologically compromised donors regained their tumoricidal activity after incubation in vitro with T lymphocytes from untreated $C_3$471-immune donors; similarly, $C_3$471-immune lymphocytes rendered normal resident peritoneal macrophages tumoricidal in Winn assays. When $C_3$471-immunized mice were irradiated or treated with antithymocyte serum before direct challenge with $B_5$59 cells, melanomas developed, thus providing additional evidence for the need for intanct T cell function to establish immunity against the melanoma. Furthermore, when Winn assay recipients were treated with antithymocyte serum, neither $C_3$471-immune macrophages nor T cells were able to prevent tumor formation. These findings indicate that antithymocyte serum-sensitive (Thy-1.2$^+$) lymphocytes are necessary both for the generation of tumoricidal leukocytes in $C_3$471-immunized mice, and for the rejection of $B_5$59 melanoma by demonstrably tumoricidal macrophages in Winn assay recipients. In addition, long-lasting immunity developed in 50% of the normal mice that had received both $C_3$471-immune peritoneal cells and $B_5$59 tumor cells, as manifested by their capacity to reject a second challenge with $B_5$59 cells 40–60 d later.

The authors gratefully acknowledge the skillful technical assistance of Nance Hamel and Nenita G. Cortez. The authors thank Dr. Ulrich Hämmerling for his gift of monoclonal anti-Thy-1.2 antibody and Dr. Ralph Steinman for a critical reading of the manuscript.

Received for publication 29 July 1982 and in revised form 3 September 1982.

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