A Model for Immunity to Melanomas in Mice[1,2] (37605)

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Tumors express specific antigens which stimulate both humoral and cellular immunity in the host (1–3). Tumor regression or protection from tumor cell challenge *in vivo* has been attributed to the cellular arm of the stimulated immunity (4–6). Moreover, lymphocytes from tumor-bearing animals and human patients have a specific cytotoxic effect on cultivated tumor cells from the same individual (7, 8). Progressive tumor growth in individuals with strong cellular immunity to their tumors has been attributed to specific inhibitory factors in their serum. These specific inhibitory factors may include enhancing antibodies (9), immune complexes (10), or soluble tumor antigens (11). Depending upon the relative importance of the specific blocking factors, tumor immunity could be blocked at the tumor cell level (antibodies or immune complexes) or at the immune lymphocyte level (immune complexes or soluble tumor antigens).

The purpose of this study was (1) to develop a model in the mouse whereby tumor immunity to melanomas can be studied and (2) to investigate the relationship between humoral and cellular immunity to protection from development of the melanoma tumor.

Materials and Methods. Melanoma cells. A melanoma cell line, designated P51, was derived in 1968 from a transplantable B16 mouse melanoma (12) and has been maintained by serial subculture in our laboratory since then (13, 14). Cultures were grown at 37° in medium 199 supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.).

Immunity to P51 cells. Groups of C57BL/6 mice were immunized subcutaneously in each footpad and the nape of the neck with 2×10^7 killed (frozen and thawed in PBS) P51 cells in 0.5 ml complete Freund's adjuvant. Control mice were immunized with 0.5 ml complete Freund's adjuvant alone. Antimelonoma antibody was detected by chromium release from labeled P51 cells as previously described by others (15, 16). Briefly, 250 μCi ^{51}Cr, specific activity, 0.2–0.3 mCi/mmole (Squibb, Detroit, Michigan) were added to approximately 5×10^6 P51 cells (log phase) in 10 ml medium 199 with 20% heated (56° for 30 min) fetal calf serum. After 30 min at 37°, the medium was replaced with 2 ml 0.25% trypsin–Eagle's solution (17). After 5–10 min at 37°, the labeled cells were harvested, washed 3 times, incubated at 4° for 30 min, washed again, and resuspended in medium 199. To 10^5 cells in 0.1 ml medium 199 were added 0.1 ml test serum and 0.1 ml rabbit complement. The reaction tubes were then incubated 60 min at 37° with agitation and centrifuged at 500*g*, and the supernatant fluid was collected and counted in a 1 MEV window (Packard Auto Gamma Counter). The amount of ^{51}Cr released from 5 freeze-thaw cycles of labeled cells in distilled water was regarded as maximum; background release was the ^{51}Cr released from cells incubated with normal serum. When ^{51}Cr release exceeded 20% of background release, the reactions were positive; when less than 10%, the reactions were negative.

Cell-mediated immunity in mice was

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evaluated by indirect migration inhibition. For this assay 10^6 P51 cells or mouse L cells were incubated with 3×10^6 mouse lymph node cells from untreated or immunized animals for 48 hr at 37° in medium 199. The culture supernatants were tested for inhibitory action on guinea pig peritoneal exudative cells (18). Less than 20% macrophage migration inhibition was considered negative, more than 40% positive.

Passive transfer. Peritoneal exudative cells were collected in three 2-ml washes of the abdominal cavity with Hanks' balanced salt solution, and pooled, and centrifuged at 150*g* for 15 min. Suprascapular, axillary, tracheal, inguinal, and mesenteric lymph nodes as well as spleens were dissected from the animals, trimmed and minced, and the cells expressed through a stainless steel screen. Cells from each cell source were pooled separately, and the total cells of a particular cell source from 10 donor mice were injected intraperitoneally into 3 recipient animals. At either 48 hr or 16 days after cell transfer, recipient mice were challenged with various doses of viable P51 tumor cells and carefully observed for 20 days; observations during this period were recorded. The animals were then sacrificed and each tumor was dissected and its weight recorded.

Results. Tumor growth. To determine the dose of P51 cells for subsequent testing, melanomas were produced by the subcutaneous injection of 10^5, 5×10^5, 10^6, or 5×10^6 live cells suspended in 0.1 ml phosphate buffered saline into five 6-week-old C57BL/6 male mice. When 10^6 cells were used, all tumors were palpable from the 10th to the 12th day and visible by the 14th to 15th day after injection. On the 20th day, the tumors were about 2 cm in diameter and weighed 2.5 ± 0.37 g. When the number of P51 cells was less than 10^6 inconsistent results were obtained. Therefore, 10^6 viable cells were used in most subsequent inoculations.

Immunity to P51 cells. The effect of a single immunization on tumor growth and weight at 20 days was compared in untreated, adjuvant-treated, and immunized mice (10 animals/group). Fourteen days after being inoculated with 10^6 viable P51 cells, 10 of the untreated animals, 9 of the 10 adjuvant-treated animals, but no immunized animals had visible tumors (Table I). By 20 days, visible tumors were observed in 9 of the 10 immunized animals. Despite the delayed tumor growth in immunized mice, mean tumor weights at 20 days did not show a significant difference between the control and immunized groups. Although all adjuvant-treated animals developed visible tumors at about the same time as the untreated group, their tumor weight at 20 days was significantly less ($p < 0.05$, Table I) than that of the other groups. Humoral and cellular immunity was also measured in these mice. No matter what their treatment schedules, all animals with tumors weighing more than 3 g developed detectable tumor specific antibody. Furthermore, animals in the immunized group with tumors less than 3 g also developed detectable levels of antibody. When tumor weight was less than 1 g, however, antibody was not found (Table II).

Cellular immunity in these groups was

TABLE I. Tumor Growth in Untreated Mice and Mice Immunized with Melanoma Cells.

Group[a]	Fraction of mice with visible tumors on day: 10	14	17	20	Tumor weight (g) at day 20
Untreated	0/10	10/10	10/10	10/10	2.7 ± 0.73[b]
Adjuvant alone	0/10	9/10	10/10	10/10	1.9 ± 0.78
Immunized	0/10	0/10	5/10	9/10	2.3 ± 1.3

[a] Untreated = mice held for 30 days before inoculation with melanoma cells; Adjuvant alone = mice immunized with 0.2 ml complete Freund's adjuvant and inoculated 30 days later with 10^6 viable melanoma cells; Immunized = mice immunized with 2×10^7 killed melanoma cells in 0.2 ml complete Freund's adjuvant and inoculated 30 days later with 10^6 viable melanoma cells.

[b] Mean ± SD.

TABLE II. Humoral and Cellular Immunity in Untreated and Immunized Mice After Melanoma Cell Challenge.

Group[a]	Tumor weight (g) at day 20	Fraction of mice with detectable antibody	Migration inhibition[b]	Antibody titer[c]
Untreated	1–2	0/2	<20	—
	2–3	0/5	<20	—
	>3	3/3	<20	4,8,8
Adjuvant alone	1–2	0/7	<20	—
	2–3	0/2	<20	—
	>3	1/1	<20	4
Actively immunized	<1	0/1	>50	—
	1–2	4/4	>50	8,16,16,32
	2–3	3/3	>50	16,16,32
	>3	2/2	>50	16,64
Passive transfer recipients	<2	0/15	ND	—
	2–3	0/1	ND	—
	>3	1/1	ND	8

[a] Untreated = mice held for 30 days before inoculation with melanoma cells; Adjuvant alone = mice immunized with 0.2 ml complete Freund's adjuvant and inoculated 30 days later with 10^6 viable melanoma cells; Immunized = mice immunized with 2×10^7 killed melanoma cells in 0.2 ml complete Freund's adjuvant and inoculated 30 days later with 10^6 viable melanoma cells.

[b] 20% inhibition was not significant; 50% was significant; ND = not done.

[c] Reciprocal of highest serum dilution that gave significant chromium release in microcytotoxicity test.

assayed by indirect migration inhibition. Migration inhibition was always less than 20% in untreated or adjuvant-treated animals but greater than 50% in immunized animals (Table II). Culture supernatant fluids from animals immune to P51 cells and tested with mouse L cells also exhibited less than 20% migration inhibition.

Passive transfer of tumor immunity. The lack of significant tumor protection in immunized animals may result from competition between humoral and cellular immunity. To test this hypothesis, we carried out passive transfer experiments. Peritoneal exudative cells, lymph node cells, and spleen cells were harvested from immunized donors, washed, and transferred to previously untreated mice. Recipient animals were challenged (1) with 10^6 viable P51 cells 2 days after cell transfer, (2) with 10^6 viable cells 16 days after cell transfer, and (3) with 5×10^6–10^7 viable P51 cells 2 days after cell transfer. Progressive tumor growth and tumor weight at 20 days after injection with the P51 cells were recorded. The mean tumor weight in nonimmune control animals was 2.7 ± 0.73 g; in all animals receiving peritoneal exudative, lymph node, and spleen cells from immunized mice and challenged 2 days later with 10^6 P51 cells, it was 0.4 ± 0.26 g (Table III). Recipient animals receiving cell transfers from the above groups of immunized mice but challenged with 10^6 P51 cells 16 days after cell transfer showed some protection, but the results were less consistent. In this group the recipients of peritoneal exudative cells demonstrated the best protective immunity (mean tumor weight 0.5 ± 0.57 g). When cell-recipient animals were challenged 2 days after transfer with 5×10^6–10^7 P51 cells, protection was most notable at 14 days after challenge: Only one of 18 animals compared with 10/10 controls had a visible tumor. However, at day 20 all animals had visible tumors and only the animals that had received peritoneal exudative cells showed a statistically significant difference in mean tumor weight from that of control animals ($p < 0.01$) (Table III).

Discussion. Although immunization of mice

TABLE III. Tumor Growth in Mice Receiving Cell Transfers.

Group[a]	Cell source[b]	Fraction of mice with visible tumors on day: 10	14	17	20	Tumor weight (g) at day 20
Early challenge	None	0/10	10/10	10/10	10/10	2.7 ± 0.73[c]
	PE	0/3	0/3	0/3	0/3	0.33 ± 0.15
	LN	0/3	0/3	0/3	2/3	0.50 ± 0.46
	SP	0/3	0/3	0/3	0/3	0.35 ± 0.15
Late challenge	PE	0/2	1/2	1/2	2/2	0.50 ± 0.57
	LN	0/3	0/3	1/3	1/3	2.1 ± 2.1
	SP	0/3	0/3	1/3	2/3	1.8 ± 0.95
Early challenge (large dose)	None	0/10	10/10	10/10	10/10	4.6 ± 0.54
	PE	0/6	0/6	4/6	6/6	2.4 ± 0.72
	LN	0/6	0/6	6/6	6/6	5.0 ± 0.73
	SP	0/6	1/6	6/6	6/6	4.0 ± 0.57

[a] Early challenge = Mice inoculated with 10^6 viable melanoma cells 2 days after receiving immune cells from donors. Late challenge = Mice inoculated with 10^6 viable melanoma cells 16 days after receiving immune cells from donors. Early challenge (large dose) = Mice inoculated with 5×10^6–10^7 viable melanoma cells 2 days after receiving immune cells from donors.

[b] PE = peritoneal exudate cells; LN = lymph node cells; SP = spleen cells.

[c] Mean ± SD.

with P51 cells stimulated both humoral and cellular immunity (Table II), it did not protect these animals from tumor cell challenge 30 days later. The mean tumor weights of untreated and immunized mice 20 days after melanoma challenge were not statistically different (Table I). However, there seemed to be a direct relationship between tumor size and anti-P51 antibody. Animals with tumors greater than 3 g always developed detectable anti-P51 antibody. Thus, antitumor antibody may have a tumor-enhancing function; such enhancement has been described by others *in vitro* (9) and *in vivo* (19). These observations suggest that humoral antibody may interfere with cell-mediated immunity directed against the P51 melanoma. Competition between humoral and cellular immunity for tumor cell determinants has been suggested as a mechanism that favors tumor survival (20). To test this hypothesis in this animal model, we carried out passive transfer experiments. Animals receiving cell transfers (peritoneal exudate, lymph node, and spleen) from immunized mice and challenged with P51 tumor cells 2 days after transfer showed not only a retarded rate of tumor development but also significantly reduced tumor weights than controls (Table III). On the other hand, when recipient animals were challenged with P51 cells 16 days after cell transfer, only the recipients of immune peritoneal exudate cells showed a significant reduction in tumor development. In addition, the peritoneal cells were more effective than lymph node or spleen cells when larger doses of P51 cells were used to challenge recipient mice (Table III). This may reflect the comparative efficiency of our harvesting procedures, the timing of the cell collection from immunized donors, or the diversity of the peritoneal cell population itself.

In all experiments, mice were observed up to 20 days after tumor cell challenge. Protection from tumor cell challenge was measured by tumor appearance and tumor weight at 20 days. Although some animals were completely protected from detectable tumor growth at 14 days, by 20 days all animals had some tumor development which suggests that long observation periods are vital in experiments on tumor immunity. Although complete tumor protection can be observed in short periods, the differences between test and control groups at later time periods may

not be so evident.

In this model, tumor survival or control utimately depends on a balance between humoral and cellular immunity. The passive transfer experiments favor cellular immunity and result in a significant degree of tumor protection in the recipient animals. However, the incomplete protection may reflect the transfer of immunoglobulin-producing cells or antibody bound to the transferred cells. To avoid the transfer of antibody or immune globulin-producing cells, passive transfer experiments with tumor-specific transfer factor isolated from immune cells are in progress. Since tumor-specific transfer factor should confer only a cellular immunity (21), complete tumor protection may be possible.

Summary. C57BL mice immunized with cultured B16 melanoma cells developed both humoral and cellular immunity to melanoma antigens but were not resistant to melanoma cell challenge. Spleen, lymph node, and peritoneal exudative cells from immunized mice, however, passively transferred melanoma immunity to normal mice. Recipient mice were melanoma resistant if challenged with tumor cells 2 days, rather than 16 days, after adoptive transfer. Peritoneal exudative cells were more effective in the cell transfer experiments than the spleen or lymph node cells. Antimelanoma antibody was detected in unimmunized mice with large tumors and all immunized mice. In unimmunized mice antimelanoma antibody appeared to be associated with progressive tumor growth.

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