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# Tumor Specific Immunity to Mouse Melanoma *In Vitro*[1] (40399)

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In the past, most *in vitro* methods used in the study of tumor immunity have measured direct tumor cell killing by immune cells of the tumor host (1, 2). However, some assays for tumor immunity have been developed that measure the ability of immune cells to inhibit tumor cell growth *in vitro*, as measured by decreased [$^3$H]thymidine uptake of tumor cells (3–7); this latter type of assay, which can be used to measure both cell killing and stasis, is referred to as an IDS (inhibition of tumor cell DNA synthesis) assay and has often been employed in the measurement of immunity of immunized animals to allogeneic tumor cells (3, 6). We have utilized the IDS assay to study reactivity of C57B1/6 mice to a transplantable syngeneic melanoma cell line. Changes in nonspecific lymphocyte reactivity in the course of tumor growth were monitored by tests of mitogen transformation of spleen cells from tumor-bearing animals.

*Materials and methods. Animals.* Strain C57B1/6 male mice, 4–6 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Maine.

*Tumor lines.* The P-51 cell line was derived from a spontaneous B-16 melanoma in a C57B1/6 mouse and has been maintained in continuous culture (8). The P-51 line is highly pigmented and more tumorigenic than the standard B-16 melanoma line. Cultures were free of mycoplasma and viruses. P-51 cells for tumor transfer and IDS assay were grown until confluent, trypsinized, and washed three times in RPMI-1640. Since cultured cells were used both for the induction of tumors and for the IDS assay, the problem of genetic drift of cultured melanoma cells was eliminated. Mice injected subcutaneously with $10^6$ cultured B-16 (P-51) cells develop palpable tumors within 2–3 weeks and start to die after 5 weeks. EL4 leukemia cells, which are syngeneic to C57B1/6 mice, were obtained from Dr. E. Benjamini (University of California, Davis) and were grown intraperitoneally (ascites) in mice.

*Spleen cells.* Cells were washed three times in RPMI-1640 before use in assays. The method of Julius *et al.* (9) was used in the filtering of spleen cells through nylon wool. This procedure was shown to remove B cell activity, as measured by mitogenic response, to bacterial lipopolysaccharide (LPS). For irradiation experiments spleen cell suspensions in RPMI-1640 were irradiated with 3000 R.

*Cultures of spleen cells with mitogens.* Spleen cells were cultured with a wide range of different concentrations of the mitogens concanavalin A (Con A, Miles Laboratories, Kankakee, IL), phytohemagglutinin (PHA-P, Difco, Detroit, Michigan), *Salmonella typhimurium* LPS (LPS No. 594136, Difco), and pokeweed mitogen (PWM, Gibco, Grand Island, NY). Cultures were carried out by the method of Fairchild *et al.* (10) using $6 \times 10^5$ cells per culture.

*IDS Assays.* Assays were done as previously described by Wilson *et al.* (7) in Falcon Microtest II plates. Briefly, $5 \times 10^5$ normal or immune (tumor bearers') spleen cells and $2.5 \times 10^4$ washed cultured B-16 tumor cells in 0.2 ml of medium containing 10% fetal calf serum and antibiotics were dispensed into flat-bottomed microculture wells (Falcon Plastics, Cockeysville, MD). These cell concentrations and ratios had been shown to give maximum IDS that was demonstrable only with B-16 tumor cells and virtually no IDS with syngeneic EL4 tumor cells (when using B-16 immune spleen cells). Control cultures contained only tumor cells or spleen cells. After 24 hr of incubation at 37° in 95% air and 5% $CO_2$, 1 $\mu$Ci of [$^3$H]thymidine was added to

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each culture well. After 12 additional hr of incubation, cultures were harvested. The IDS and specific IDS were calculated as follows:

$$\text{IDS} = 1 - \left[\frac{^{\text{cpm}}\text{tumor cells + spleen cells}}{^{\text{cpm}}\text{tumor cells}}\right] \times 100$$

$$\text{Specific IDS} = (^{\text{IDS}}\text{immune spleen cells}) - (^{\text{IDS}}\text{normal spleen cells})$$

The amounts of isotope incorporated by either normal or immune spleen cells alone were negligible with respect to calculation of IDS.

*Fetal calf experiment.* To determine whether injection of fetal calf sera alone affected the inhibitory activity of normal spleen cells, 50 animals were injected with 0.1 ml RPMI containing 10% fetal calf sera, the maximum amount that was administered to any of the test mice with the B-16 tumor. At weekly intervals 10 mice were sacrificed and spleen cells removed and pooled as described above, and assayed for IDS activity.

*Results.* The IDS assay and mitogen response studies were used in the evaluation of the reactivity of normal and tumor-bearing mice at varying stages of tumor growth (7–35 days). Spleen cells from groups of 3–6 mice were used in each assay. The results of specific IDS assays are shown in Fig. 1. Specific IDS was first apparent 2 weeks after the B-16 tumors had begun to grow in the hosts; it reached a maximum during the 3rd week and remained at this level for an additional 2 weeks. Spleen cells from animals with 21–35 days of tumor growth could inhibit tumor cell DNA synthesis to a significantly greater extent ($P < .001$) than those cells from normal mice or animals with 1–2 weeks of tumor growth.

Nonspecific reactivity of spleen cells was measured by mitogenic responses in culture. The mitogens Con A and PHA were used in the evaluation of T lymphocyte function; LPS was used in the study of B lymphocyte function; and PWM was used in the measurement of reactivity of both B and T cells. The results of a typical experiment are shown in Fig. 2. Unstimulated control cultures usually showed an uptake of less than 5000 cpm. The

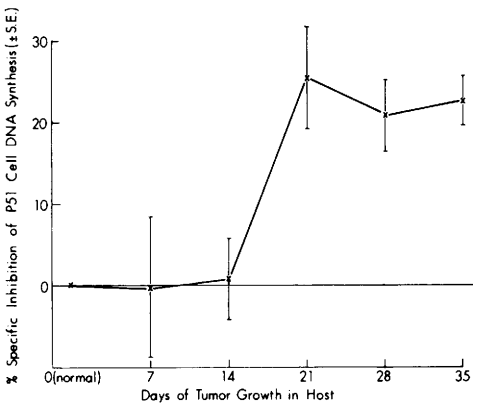


FIG. 1. *In vitro* inhibition of B-16 melanoma cell DNA synthesis by spleen cells from tumor-bearing C57Bl/6 mice. Results are averages of 19 experiments and are expressed as specific IDS (Materials and Methods Section). Spleen cells were taken from both normal mice and mice that had been injected with $10^6$ B-16 cells 7–35 days earlier. Specific IDS was significant ($P < .02$) at 14 and 21 days and highly significant ($P < .001$) at 35 days.

Con A response decreased gradually with tumor growth and was significantly lower ($P < .001$) than normal by day 35 of tumor growth. In contrast, the PHA response increased significantly ($P < .001$) from days 7 to 21 of tumor growth but decreased to normal by the 35th day. Responses to both LPS and PWM increased in the early stages and remained slightly elevated even at 35 days. Thus, all responses except that of Con A were normal or elevated after tumor growth became established. Experiments performed with other concentrations of mitogens gave qualitatively similar results.

A previous study utilizing an allogeneic system reported that IDS is mediated by immune T cells (6). In a separate set of experiments, an attempt was made to remove nonspecific IDS exhibited by spleen cell populations, by filtering effector cells through nylon wool columns. In comparison with untreated spleen cells, nylon wool filtered cells showed a lower degree of nonspecific IDS (about 16–17% less), but specific IDS was identical when either type of effector cells preparation was used (Table I).

To determine if spleen cell proliferation is necessary in the IDS assay, we irradiated these cells prior to mixing them with tumor cells. Results suggested that irradiated im-

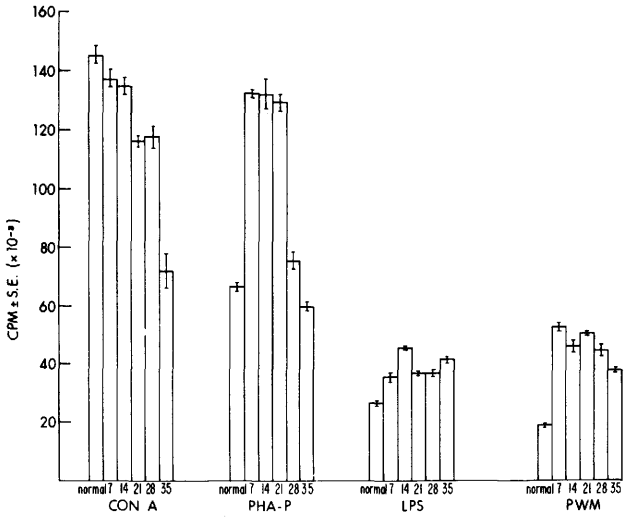


FIG. 2. Mitogenic responses of spleen cells from normal and tumor-bearing mice. Spleen cells from normal B57Bl/6 mice and mice that were injected with $10^6$ B-16 tumor cells 7, 14, 21, 28, and 35 days earlier were cultured with optimal levels of mitogens. Cell backgrounds have been subtracted from the values shown.

TABLE I. EFFECT OF DEPLETING B CELLS FROM SPLEEN CELL POPULATIONS USED IN THE IDS ASSAY.

| Cells | cpm ± SE | IDS (%) | Specific IDS (%) |
|---|---|---|---|
| Controls | | | |
| B-16 cells | 155,559 ±6,144 | — | — |
| Spleen cells—normal | 2,475 ±70 | — | — |
| Spleen cells—normal, B cell depleted[a] | 458 ±51 | — | — |
| Spleen cells—24-day[b] | 3,123 ±254 | — | — |
| Spleen cells—24-day,[b] B cell depleted[a] | 1,307 ±235 | — | — |
| Tests: | | | |
| Spleen cells—normal + B-16 cells | 82,126 ±2,166 | 47.2 | — |
| Spleen cells—24-day + B-16 cells | 58,810 ±461 | 62.2 | 15.0[c] |
| Spleen cells—normal, B cell depleted + B-16 cells | 106,743 ±4,479 | 31.3 | — |
| Spleen cells—24-day, B cell depleted + B-16 cells | 83,894 ±6,468 | 46.1 | 14.8[c] |

[a] B cells were depleted by passage of spleen cells through nylon wool columns.

[b] Spleen cells from C57Bl/6 mice injected with $10^6$ B-16 melanoma cells subcutaneously 24 days earlier.

[c] With both populations of 24-day spleen cells there was a significant level ($P < .05$) of specific IDS.

TABLE II. EFFECTS OF IRRADIATION OF SPLEEN CELLS ON THEIR ABILITY TO INHIBIT B-16 MELANOMA CELLS *in Vitro*.

| Cells | cpm ± SE | IDS (%) | Specific IDS (%) |
|---|---|---|---|
| Controls: | | | |
| B-16 cells | 118,541 ±2,975 | — | — |
| Spleen cells—normal | 6,201 ±449 | — | — |
| Spleen cells—normal, irradiated (3000 R) | 712 ±95 | — | — |
| Spleen cells—35-day[a] | 6,471 ±443 | — | — |
| Spleen cells—35-day, irradiated (3000 R) | 699 ±52 | — | — |
| Tests: | | | |
| Spleen cells—normal + B-16 cells | 67,072 ±1,489 | 43.4 | — |
| Spleen cells—35-day + B-16 cells | 52,981 ±2,593 | 55.3 | 11.9[b] |
| Spleen cells—normal, irradiated + B-16 cells | 67,530 ±2,488 | 43.0 | — |
| Spleen cells—35-day, irradiated + B-16 cells | 45,834 ±2,576 | 61.3 | 18.3[b] |

[a] Spleen cells from C57Bl/6 mice injected with $10^6$ B-16 melanoma cells subcutaneously 35 days earlier.

[b] With both populations of 35-day spleen cells there was a significant level ($P < .02$) of specific IDS.

mune spleen cells are still capable of specifically inhibiting B-16 cells' DNA synthesis in the assay (Table II). Untreated immune spleen cells and irradiated cells gave 11.9% and 18.3% specific IDS, respectively.

A comparison of IDS activity between spleen cells of normal mice and those injected

with 0.1 ml of 10% fetal calf sera in RPMI media show that there was no statistical difference ($t$ test at .05 level) between these groups (Table III).

*Discussion.* The experiments reported here have demonstrated that spleen cells from 2- to 5-week-old B-16 tumor-bearing mice are capable of inhibiting DNA synthesis of syngeneic B-16 tumor cells *in vitro*. When compared to normal spleen cells, these spleen cells from tumor-bearing mice showed normal or enhanced reactivity to PHA, LPS, and PWM mitogens and an impaired reactivity to Con A *in vitro*. Konda *et al.* (11) have also reported finding enhanced spleen cell mitogen reactivity with PHA and LPS in C57B1/6 tumor-bearing mice. The discrepancy we observed between Con A and PHA responses of tumor bearers' spleen cells could be due to the fact that each of these mitogens reacts with different mouse T cell subpopulations (12). Since Con A reactivity is depressed, it would appear that fully intact T cell immunocompetence is not required by the effector cell population in the IDS assay. Other investigators studying immunocompetence in either human or animal tumor hosts have reported finding impaired mitogen responses during advanced stages of tumor growth; they attribute these impaired responses to serum blocking factors (when cells were cultured with autologous serum) or suppressor cells (13, 14). Our observation that the spleen cells of these B-16 tumor-bearing mice (in medium supplemented with fetal calf serum) showed normal or augmented response to most mitogens and were capable of inhibiting tumor cell growth, even at late stages of the disease, suggests that blocking factors may be responsible for continued tumor growth in vivo. Wilson *et al.* (7) have shown that serum blocking factors that inhibit IDS activity are in fact present in mice with B-16 melanoma tumors. Blocking by serum of tumor-bearing animals was not demonstrable in the cell concentration range at which there are significant differences in IDS of normal and tumor-bearing spleen cells. We suspect that use of washed cells cultured in fetal calf serum instead of in autologous serum reduced or eliminated the influence of blocking factors in the mitogen and IDS assays.

The IDS assay appears to measure both specific reactivity and nonspecific inhibition of DNA synthesis; therefore, the terms "IDS" and "specific IDS" have been used. A considerable amount of nonspecific IDS of B-16 cells was effected by normal spleen cells. A portion of this nonspecific activity could be removed by nylon wool filtration of spleen cells, a procedure that depletes B cell activity as measured by LPS response. Nonspecific IDS may represent natural immunity directed against tumor antigens or normal tissue antigens mediated by B cells not removed by nylon wool, T cells, macrophages, or other cells.

DNA synthesis and cell division by the spleen cells that mediate IDS are not required. Apparently the effector cell in our assay is somewhat different from that in the IDS assay described by Oppenheim *et al.* since their effector cell was radiosensitive (4). Their assay required 44–92 hr and appeared to be dependent upon effector cell DNA synthesis and cell division. Our short assay time (36 hours) makes cell division unnecessary.

We have yet to determine the actual nature of the specific IDS effector cell in this syngeneic system, but our B cell depletion exper-

TABLE III. INJECTION OF MICE WITH FETAL CALF SERA ALONE DOES NOT AFFECT IDS.[a]

| | B-16 tumor cells | B-16 tumor cells + normal spleen cells | | B-16 tumor cells + spleen cells FCS treated mice | | |
|---|---|---|---|---|---|---|
| Day | Average (cpm) | Average (cpm) | (% IDS) | Average (cpm) | (%IDS) | Specific (IDS) |
| 7 | 73,328 | 50,300 | 32 | 49,172 | 33 | 1 |
| 21 | 108,746 | 97,559 | 11 | 98,679 | 10 | −1 |
| 28 | 153,576 | 158,518 | −3 | 144,336 | 7 | 10 |
| 35 | 47,499 | 50,882 | −7 | 49,369 | −3 | 4 |

[a] Spleen cells were collected from normal C57Bl/6 mice or mice injected with 0.10 ml of a 10% fetal calf sera suspension at weekly intervals. $5 \times 10^5$ spleen cells were cocultured with $2.5 \times 10^4$ B-16 melanoma cells for 48 hr. Twelve hours prior to termination of culture, 1 $\mu$Ci/well of [$^3$H]thymidine was added. Controls included tumor cells alone and spleen cells alone.

iments suggest it is not a B cell. Williams *et al.* (6) have shown, with a similar type of assay in an allogeneic system, that the primary effector cells are T lymphocytes.

As illustrated by our experiments, the advantages of the IDS assay are its ability to measure both specific tumor immunity and nonspecific inhibition of tumor cell DNA synthesis in a syngeneic system, the rapidity of the test, and the small numbers of cells required. In the future, it may be possible to develop an assay for tumor specific antigens based on the ability of soluble antigens to block IDS by immune cells.

*Summary.* An *in vitro* assay that measures the ability of mouse spleen cells to inhibit DNA synthesis (IDS) of syngeneic melanoma tumor cells was used in the evaluation of tumor immunity. The IDS assay was used in the measurement of both specific immunity mediated by spleen cells from tumor-bearing mice and nonspecific tumor cell growth inhibition mediated by normal spleen cells. Specific IDS was first demonstrable 2 weeks after injection of $10^6$ B-16 tumor cells subcutaneously and persisted during advanced stages of disease. Changes in spleen cell IDS activity were correlated with changing mitogen reactivity during the course of tumor growth. A portion of the spleen cells mediating nonspecific IDS was removed by nylon wool filtration, but the effector cells exhibiting specific immunity were not removed by this procedure. Immune spleen cells did not need to synthesize DNA or divide to inhibit tumor cell DNA synthesis.

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