Harding–Passey Melanoma in the BALB/C Mouse as a Model for Studying the Interactions Between Host Macrophages and Tumor Cells

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The murine Harding–Passey melanoma (HPM) has been transplanted for 47 yr in allogeneic mice. It was found to be a very stable system for both melanin production and cell populations. The intense melanin production was shown to be typical by electron microscopy (1), and was of the "brown" type corresponding to the original brown mouse in which it arose (2). In their first paper, Harding and Passey (2) described two cell types. They suggested that one was "phagocytic in character." Previous work (1) showed that transplants and primary cultures consisted of two cell types: the melanocyte and the phagocyte. It appeared that tumor phagocytes and peritoneal macrophages fed with melanin were identical (3).

Many authors found that macrophages are the effectors of tumor rejection in vivo and in vitro in allogeneic systems (4–7) and in vitro in a syngeneic system (8). In this work we report several experiments on the host–melanoma interaction with a special reference to macrophage–HPM cell interaction.

MATERIALS AND METHODS

Mice. Inbred Balb/C females from the Chester Beatty Research Institute stock, 80–120 days old were used in all the experiments.

Melanoma transplants. HPM was implanted intraperitoneally (ip) as it produced clean small tumors, covered with the visceral peritoneum. Such tumors were very suitable for culturing and offered an elegant double compartment system with the peritoneal cavity (3).

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Melanoma cultures. Primary cultures were prepared from peritoneal nodules chopped and treated with trypsin and EDTA. The resulting cell suspension was cultured as a monolayer in Eagle's Dulbecco medium or 199 medium supplemented with 15% heat inactivated Fetal Bovine Serum, with penicillin and streptomycin, in Falcon plastic Petri dishes (3.5 cm diam) or flasks (25 cm²). Details will be published elsewhere (3).

Macrophage cultures. Mouse peritoneal macrophages (PM) were obtained by injecting, along the midline of the abdomen (the skin was stripped off) 2 ml of 199 medium ip and withdrawing the fluid from both flanks with a syringe. After seeding 1 × 10⁶ to 2 × 10⁶ PM for 2 hr, the unattached cells, mostly lymphocytes, were removed by six intense washings, according to Evans (9). Then the monolayer was cultured in complete medium.

Electron microscopy of cultures. The cultured cells were fixed, embedded, and cut in situ. At a suitable time, the culture medium was aspirated and the cultures washed with 199 devoid of serum. Then they were immersed for 30 min at 0°C in glutaraldehyde–osmium tetroxide mixture according to the method of Hirsch and Fedorko (10) and washed three to four times in cold 0.1 M cacodylate buffer. The cells were then dehydrated in cold methyl alcohol, embedded with two changes of Epon and incubated at 60°C for 48 hr. Randomly selected areas were cut out in small blocks with a saw, the plastic cap overlying the monolayer was pulled off, and the monolayer cut parallel to the surface using a Diamond knife. On some occasions cell suspensions were processed in the same way but embedded in capsules. The sections were contrasted with uranyl acetate and lead citrate or with lead monoxide.

Cytochemistry of cultures. Cultures were fixed in 1% formaldehyde in cacodylate buffer 0.1 M pH 7.4 for 4 hr at 4°C. The acid phosphatase reaction was performed according to Barka and Anderson (11) using Naphthol AS-BI phosphate and hexazonium–pararosanilin as substrate. The tyrosinase reaction was performed by incubating the fixed cells in 0.1% DL-3,4 dihydroxyphenylalanine (dopa) in cacodylate buffer 0.1 M pH 7.4 at 37°C for 4 hr. The combined acid phosphatase-dopa reaction was performed according to the method of Mishima (12).

Tritiated thymidine uptake in cultures. Two hours before the time, duplicate cultures were incubated with complete medium containing 2 μCi[³H] TdR (sp act 5 Ci/mM Amersham) at 37°C. Then the process was performed according to Temin (13).

Spleen nucleated cell isolation. The spleens were minced in 199 and the cell suspension layered onto a Ficoll–Trisosil mixture and centrifuged (14).

Immunization schedule. Tumor cells were irradiated with 10,000 rads using a ⁶⁰Co source. The tumor cells consisted either of HPM for specific immunization or L-cells or mammary carcinoma cells (MTV-syngeneic) for nonspecific immunization; 10⁶ irradiated cells were suspended in 1 ml 199 without serum and injected ip three times at 10-day intervals. Controls received 1 ml of 199 alone. For xenogeneic immunization 10⁶ live cells from the MDCK continuous line were injected.

RESULTS

Primary cultures of HPM peritoneal transplants. Phase contrast microscopy revealed two cell types which are illustrated in Fig. 1. The first type was more numer-
ous and exhibited long, slim processes. In contrast, the second type consisted of cells of a more regular shape, containing large brown vacuoles. Electron microscopy showed that the first contained single melanosomes (Fig. 2) and the second, melanosomes enclosed in phagolysosomes only (Fig. 3). The former cell was considered to be a malignant melanocyte and the latter a phagocyte. The major difference between these two cell types resulting from six different methods are summarized in Table 1.

**Subcultures of the HPM.** After three in vitro passages the resulting trypsinized population was found to consist only of the melanocyte type as judged from the above criteria (Table 1). Cells left in the primary culture after trypsinization were recognized as macrophages according to the same criteria.

**Tumor growth after injecting subcultured HPM ip.** The purified continuous line of HPM was injected ip into five batches of ten mice each at the doses of, respectively $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ HPM cells. On day 30, the mice were killed, the presence of growth checked, and the tumor weight assessed by addition of the

| Method                        | Harding-Passey melanoma | Melanocyte           | Phagocyte       |
|-------------------------------|-------------------------|----------------------|-----------------|
| 1. Electron microscopy        | Single melanosomes      | Melanin in lysosomes |
| 2. Acid phosphatase           | Weak, diffuse           | Strong, granular     |
| 3. Tyrosinase                 | Strongly positive       | Background           |
| 4. Morphology in culture      | Long processes          | Short processes      |
| 5. Trypsin removal in culture | No resistance           | Resistance           |
| 6. Phagocytosis               | Absent                  | Present              |
| 7. Thymidine uptake           | Present                 | Absent               |

**Fig. 1.** Primary culture of HPM. Phase contrast microscopy after combined acid phosphatase and tyrosinase reactions. One round phagocyte (P: in the center, strongly positive for acid phosphatase) is surrounded by melanocytes (strongly positive for tyrosinase) exhibiting long thin processes. $\times 130$
Fig. 2. HPM melanocyte. Electron micrograph showing many typical single melanosomes containing osmiophilic granular material of a variable density, enclosed by a single membrane. Mit: mitochondria. $\times 50,000$
Fig. 3. HPM phagocyte. Electron micrograph showing that all melanosomes are accumulated in phagolysosomes (PL). There is a prominent golgi (G) and several lysosomes (L). Ip = liposomes. N = nucleus. ×14,000
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Fig. 4. Percentage of take of tumor graft plotted against the number of cultured HPM melanocytes injected intraperitoneally (ip) 30 days before.

Fig. 5. Tumor weight (in grams) plotted against the number of cultured HPM melanocytes injected ip 30 days before.

many peritoneal nodules easily removed from the mesentery. Figure 4 shows that there was a threshold of 100% take at $10^5$ and that fewer than $10^3$ HPM failed to grow. Figure 5 shows the correlation between tumor cell dose and resulting growth.

Study of PM infiltration of HPM nodules and PM population. The degree of macrophage infiltration in tumors from individual mice was assessed semiquantitatively and the number of acid phosphatase positive peritoneal cells (PM) counted in a hemocytometer. They were recorded according to the time of growth of $10^5$ ip inoculated HPM subcultured cells. Figure 6 shows that after 4–5 wk the small tumor growth was accompanied by an increased PM population in both tumors and peritoneal cavity (7). Large tumor growth had intense macrophage infiltration but the peritoneal population was decreased. Lymphocytes were only occasionally
Fig. 6. Peritoneal macrophages (PM) and tumor macrophages (HPM phagocytes). Number of PM (acid phosphatase positive cells) in the peritoneal cavity of individual mice, plotted against the length of the HPM growth and the tumor weight. Semiquantitative assessment of PM infiltration in tumors (Δ = large infiltration) and lymphocyte rim around tumor (● = presence). The mice (except “no tumor”) were injected ip with 10⁶ cultured HPM cells.

found around the tumors and never inside. There was no correlation with macrophage infiltration.

**Immunization of mice against HPM.** As shown in Fig. 8, a consistent degree of protection against tumor graft was achieved by repeated inoculations of irradiated HPM cells; 28 days after the last shot all mice injected with 10⁶ living HPM grew tumors, but survival on day 26 of immunized mice was much better. In the case of mice injected 33 days before with 10⁵ living HPM (which is a threshold dose, Fig. 4) only one in five mice had a small tumor.

**Effect of Immunocompetent Cells on the Tritiated Thymidine Uptake by HPM in Mixed Cultures**

a. **Effect of spleen cells.** 8 × 10⁶ spleen cells (SC) from immune and control animals were seeded onto 4 × 10⁴ HPM cells (ratio 200:1) and cultured for 72 hr. Then the SC were washed off and a 2-hr tritiated thymidine incubation was performed with HPM cells. Seven days after immunization, there was a slight but specific inhibition of thymidine uptake (Fig. 9 left). However, 31 days after immunization, both SC, from mice immunized against HPM and MT, produced a strong inhibition. In addition the specific SC gave a slightly stronger inhibition than the nonspecific SC (Fig. 9 right).

b. **Effect of peritoneal macrophages.** As shown in Fig. 10, normal unstimulated PM had a marked inhibitory effect on thymidine uptake. In contrast specifically immunized PM were less inhibitory than control PM 7 days after immunization and just as inhibiting 31 days after immunization.

**Electron microscopy of PM after irradiated tumor cell injections.** The repeated ip injection of irradiated HPM cells was found to produce an enlargement of PM
Fig. 7a. Light micrograph of tumor of less than 0.5 g 2-3 wk after $10^6$ HPM cells. Same experiment as in Fig. 6. b. Tumor of less than 0.5 g 4-5 wk after $10^6$. 
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Fig. 8. Protection of mice after immunization. Left—number of control and immunized mice surviving 26 days after ip injection of $10^6$ HPM. Middle—tumor weight of individual mice 33 days after $10^6$ HPM ip. Right—number of tumor graft take 33 days after $10^6$ HPM ip.

Fig. 9. Tritiated thymidine uptake by cultured HPM exposed to spleen cells (SC). Left—SC from control mice (C) and from mice immunized 7 days before against HPM (a HPM) and against MDCK cells (a MDCK). Right—SC from control and immunized mice 31 days before against HPM and against mammary tumour (a MT). Mean count per minute between two determinations.

(Fig. 13) as compared with the control (Figs. 11, 12). This was due to the phagocytosis of melanin; 7 days after the last shot the melanin-loaded phagolysosomes filled the cytoplasm completely (Fig. 14); 31 days after, only several phagolysosomes with less melanin were found.

DISCUSSION

The present data indicate that the phagocytes which invade the Harding–Passey melanoma are host macrophages identical to those of the peritoneum macrophages. The longer the tumor growth, the more macrophages were found in the tumors.
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However, when the tumor demand was intense following tumor growth, the number of PM in the peritoneal cavity was decreased. This suggests the existence of a balance between two populations of peritoneal macrophages (PM): tumoral and peritoneal. The possibility of an immunological role of the PM in our system was investigated by using PM collected from animals rendered immune. This assumption was proved by in vivo assays showing protection against HPM grafts and by in vitro assays showing the inhibitory effect of spleen lymphocytes. In contrast the in vitro experiments with PM showed an unexplained inhibitory effect of normal, unstimulated PM. Moreover, PM from specifically immunized animals exhibited a decrease in this inherent inhibitory effect when taken 7 days after the last shot. A return to the normal response was observed 31 days afterward. In addition, PM from nonspecific immunization (aMT) produced more inhibition than specific PM on day 31. Therefore our results showed a great deal of nonspecificity in immune conditions and a possible inherent inhibitory capacity of normal macrophages. That macrophages can exert specific inhibition of lymphoma cells was shown in syngeneic (9) and in allogeneic (6) systems. In contrast, using solid tumor systems, others (15, 16) found nonspecific macrophage cytotoxicity. Moreover, a paper presented at this conference dealt with an in vivo protection against HPM by nonspecific immunization against histocompatibility antigens (17). In a previous work we found an increase of lysosomes in macrophages from animals immunized against a syngeneic lymphoma (18). No direct involvement of lysosomes with specific lymphoma inhibition could be found. In the present system, which behaves like a syngeneic system, the macrophages from immune animals exhibited lysosomes filled with melanin. The quantity of such phagolysosomes was much higher in macrophages obtained 7 days after the last shot as compared with 31 days after, presumably because of melanin degradation (19). It was expected
FIG. 11. Electron micrograph at low magnification of peritoneal exudate from control mouse showing peritoneal macrophages (PM), peritoneal lymphocytes (PL) mast cell (MC). ×4000
that melanin would increase the shape and number of lysosomes in macrophages. Indeed, Cohn and co-workers (20) have established that endocytosis is the main lysosome inducer in macrophages. Therefore the overloading of macrophage lyso-

Fig. 12. Electron micrograph of control PM with kidney-shaped nucleus (sometimes lobulated), typical dendritic processes (DP), and lysosomes (L). Peritoneal lymphocytes (PL) are smaller with coarse chromatin and smooth membrane. ×5300
Fig. 13. Electron micrograph of PM 7 days after last immunization with irradiated HPM cells. As compared with Fig. 12, the cells are larger and have a cytoplasm filled with melanosomes enclosed in phagolysosomes (PL). ×5300

Somomes with HPM melanin might be interpreted as a harmful effect on macrophages of both immunization procedure and HPM growth. Such overloading could also be produced by an intense engulfment of xenogeneic cell debris (MDCK) reported
Fig. 14a. High magnification of control PM with typical dendritic processes (DP), pinocytic vesicles (PV), small dark lysosomes (L), and golgi vesicles and saccules (G) x13,000. 

b. Same magnification as in Fig. 14a. PM fed with melanosomes 7 days after last immunization. The cytoplasm is filled with many phagolysosomes (PL) containing aggregates of melanosomes. There are still several small lysosomes (L).
in this work. Our results strongly suggest that these phenomena are to be related to the general concept of “Reticuloendothelial Blockade” (21).

The finding that normal macrophages had an inherent thymidine uptake inhibitory effect remains to be carefully explored, because of the existence of bacterial and toxin arming phenomenon (22) and antibody-dependent cell-mediated cytotoxicity (23).

SUMMARY

Transplants and primary cultures of the Harding–Passey Melanoma (HPM) were found to consist of melanocytes and host macrophages. A pure population of melanocytes (obtained by subculturing) produced tumors when injected intraperitoneally (ip) into Balb/c mice. There was a progressive infiltration of the tumors by peritoneal macrophages (PM). Repeated inoculations of irradiated HPM cells ip induced in vivo a protection against HPM graft. In vitro spleen cells produced a nonspecific inhibition of thymidine uptake by HPM. Normal PM were found to exhibit a spontaneous nonspecific inhibition. This capacity was impaired in PM taken from immune animals 7 days after immunization and recovered 31 days after. The relevance of such findings to cell-mediated immunity and lysosome function are discussed.

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