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Tumor Facilitation in a Syngeneic Host–Tumor System (38430)

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Mice challenged with sufficient numbers of syngeneic neoplastic cells develop progressively growing tumors. For this reason, detection of tumor enhancement in syngeneic systems has been difficult. Parameters of syngeneic tumor enhancement have been limited to comparisons between groups of animals, all of which have progressively growing tumors (1–3). It has therefore been necessary to measure syngeneic enhancement in terms of differences in tumor volume (1) and differences in tumor growth patterns (2, 3). In contradistinction, enhancement across histocompatability barriers has been much more readily demonstrable because enhanced tumors usually grow progressively whereas controls usually regress.

A polyoma virus-induced fibrosarcoma cell line (Py-8914b) which lost its oncogenic potential after 110 passages *in vitro* while retaining detectable polyoma antigens as well as appropriate H-2 antigens was developed in this laboratory. This report describes active tumor facilitation in a syngeneic system using this variant subline Py-8914b as the immunogen and the oncogenic parental line Py-89 (9) as the target cell. In addition, the kinetics of active tumor facilitation are described and correlated with the development of cell-mediated immunity (CMI) as measured by the microcytotoxicity assay (MCA) and the ^{51}Cr release, cell-mediated cytotoxicity assay (CRA).

Materials and Methods. Polyoma cell lines. Py-89 is a polyoma virus-induced fibrosarcoma derived from C57Bl/Ka mice and adapted to grow *in vitro* by Ting (5). Py-89 began to lose its ability to cause tumors in syngeneic recipients after 80 passages *in vitro*, and after 110 passages Py-89 had lost all of its oncogenic potential. The nononcogenic Py-89 cell line was designated Py-8914b to distinguish it from the parental oncogenic Py-89 cell line (5). Py-8914b cells were capable of inducing strong, immunologically specific, transplantation immunity to Py-89 cells in syngeneic recipients under certain conditions (4). Py-8914b cells also served as an excellent target cell in the MCA. In addition, Py-8914b cells were also found to be usable although not ideal in the CRA.

Immunizations. Ten-week-old female C57Bl/KaLw mice were divided into six groups: One group received no immunizations (NI); four groups received one intraperitoneal (ip) injection of 2×10^7 Py-8914b cells at 1 (I), 2 (II), 3 (III), and 4 (IV) weeks prior to challenge *in vivo* and use in *in vitro* testing; one additional group received 6 weekly ip injections of 2×10^6 Py-8914b cells (MI) with the last immunization occurring 1 week prior to challenge and *in vitro* testing.

Tumor challenge. Mice from each of the above groups were divided into three subgroups and challenged subcutaneously with 10^5, 10^6, or 10^7 Py-89 cells which had become only partially oncogenic following *in vitro* passage (passages 90–95). Mice were followed for 20 weeks, and tumors greater than 1 mm in diameter were scored as positive only if they progressed to cause death of the animal.

Microcytotoxicity assay. This method was adapted from that of the Hellströms (6). Sixty Py-8914b cells were seeded into each well of a Falcon Microtest-II plate (Falcon Plastics) in Ham's F-12 media supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). The Py-8914b target cells (TC) were allowed to attach to the bottom of the well and then 10^5 lymph node attacking cells (AC) in replicates of eight were added in 0.2 ml of Ham's F-12 medium supplemented with penicillin (10 μm/ml) and streptomycin (10 μg/ml). Each plate was incu-

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bated for 45 min at 37° in CO_2, after which 0.05 ml of a 50% mixture of fetal calf serum and F-12 was added to each well followed by a 40-hr incubation at 37° in CO_2. The assay was terminated by washing the wells with phosphate buffered saline plus 10% fetal calf serum and then fixing the remaining TC with methanol and staining these cells with Giemsa and crystal violet. The remaining TC were counted by an observer who had no knowledge of the experimental protocol. The number of remaining TC in the treated groups was compared to those in nontreated groups by means of the Student's t test. The percent reduction was determined by subtracting the remaining TC in the treatment groups from those in the nontreatment and dividing the result by the TC remaining in the nontreatment group. This figure was then multiplied by 100.

^{51}Cr release cytotoxicity assay (cell-mediated). This assay was adapted from that of Berke *et al.* (7) and is more completely described by Cohen *et al.* (8). Py-8914b cells were labeled with ^{51}Cr while growing *in vitro*. The night preceding the assay, a flask of labeled cells (approximately 10^6) was trypsinized and placed in spinner culture with some additional ^{51}Cr and the cells were incubated overnight. The TC were removed from spinner culture, washed, and diluted to $2.5 \times 10^4/0.05$ ml. AC were peritoneal exudate cells (PEC) which were stimulated by injecting 3 ml of 12% autoclaved sodium caseinate in phosphate buffered saline (Difco) 15 hr prior to harvest. PEC uniformly consisted of 50% lymphocytes, 48% macrophages, and 2% neutrophils. The PEC were washed and diluted to $10^7/0.5$ ml in RPMI-1640 media, 20% fetal calf serum (immunoprecititin free, Gibco), penicillin (10 μg/ml), and streptomycin (10 μg/ml). Glass tubes (10×75 mm) received 0.5 ml of AC and 0.05 ml of TC (200AC:1TC). The tubes (quadruplicates) were sealed and rocked at 6 rocks/min in a horizontal position for 4.5 hr. The assay was ended by adding 2 ml of 4° media to each tube. Cells were centrifuged and the radioactivity in an aliquot of supernatant was determined in a gamma counter (Nuclear Chicago). Cytotoxic percentage was determined by subtracting the counts per minute released by the untreated PEC from the counts per minute released by PEC from the treated groups. This difference was divided by the maximum possible release as determined by freeze thawing four times. This figure was then multiplied by 100.

Results. Tumor facilitation. 24% of the NI mice developed progressively growing tumors resulting in death of the animal (Fig. 1). Mice challenged with the partially oncogenic Py-89 cells at 2 (II) and 3 (III) weeks after immunization developed progressively growing tumors at a frequency of 62 and 78%, respectively, both of which were significantly different from the frequency of "takes" of the NI mice ($P < 0.01$). Mice challenged at 1 week (I) after immunization developed progressively growing tumors at a rate of 46% ($0.05 < P < 0.10$). Mice immunized with multiple small doses of Py-8914b cells (MI) exhibited progressively growing tumors at a rate of 48% ($0.05 < P < 0.10$). Mice challenged 4 weeks (IV) after immunization behaved essentially the same as the NI group. Tumor facilitation of Py-89 was readily demonstrable therefore at 2 and 3 weeks after immunization with the variant Py-8914b line; facilitation was not as clearcut at 1 week after immunization and following multiple small immunizations. Neither facilitation nor protection could be demonstrated at 4 weeks after immunization.

Cell-mediated immunity. CMI as measured by the CRA was first noted 1 week after immunization (Fig. 2). CMI became undectable by 14 days postimmunization, and no CMI could be detected using the CRA in groups II, III, IV, and MI. In the MCA assay, CMI did not become detectable until 12 days postimmunization, but once detectable, it remained so for at least 35 days, after which it was not further tested. Groups II, III, IV, and MI therefore displayed CMI using the MCA.

Correlations. There appeared to be an inverse relationship between the two assays for CMI. As CMI became detectable in the MCA, it waned and disappeared in the CRA. One possible reason for this discrepancy may be the difference in attacking cell populations (lymph node cells in the MCA and PEC in the CRA). To resolve this difference, one group of mice donated AC for both assays 12 days after a single immunization so that both assays would show activity. Lymph node cells were used as AC in both assays while PEC could only be used in the CRA because the macrophages present in PEC adhered to the glass in the MCA and were indistinguishable from adherent tumor cells during the scoring phase. Lymph node cells were positive in both assays

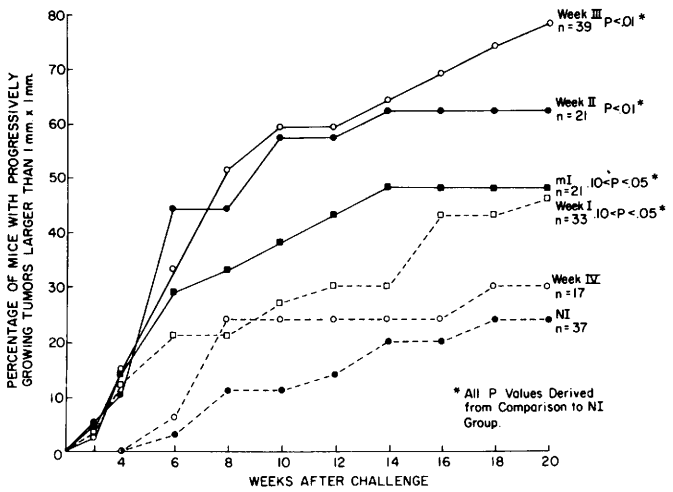

FIG. 1. Tumor facilitation. Percentages of mice with progressively growing tumors resulting in death of the animal. Groups I–IV refer to the time of challenge in weeks after last immunization. MI refers to multiple small immunizations prior to challenge and NI refers to mice receiving a challenge without prior immunization. All P values were derived from standard χ^2 determinations (one tail). Mice receiving 10^5–10^7 Py-89 cells did not differ in frequency and are therefore combined.

(Table I), yielding a low but significant cytotoxic percentage in the CRA and 33% suppression in the MCA. PEC also produced a low but significant cytotoxic percentage which was comparable to that produced by the lymph node cells. Therefore, it appeared that the kinetic differences between the two assays was not a result of differences in attacking cell populations.

Tumor facilitation was weakly detectable at 1 week postimmunization, peaked at 2 and 3 weeks postimmunization, and was no longer detectable at 4 weeks postimmunization. Thus, tumor facilitation *in vivo* appeared to have no apparent relationship to the *in vitro* development of CMI.

Discussion. Syngeneic tumor enhancement has been described using growth pattern discrepancies (2, 3) and differences in actual tumor dimensions (1). These tedious parameters are necessary in a syngeneic system in which tumors grow readily. By challenging mice with Py-89 cells that were of low oncogenic potential, facili-

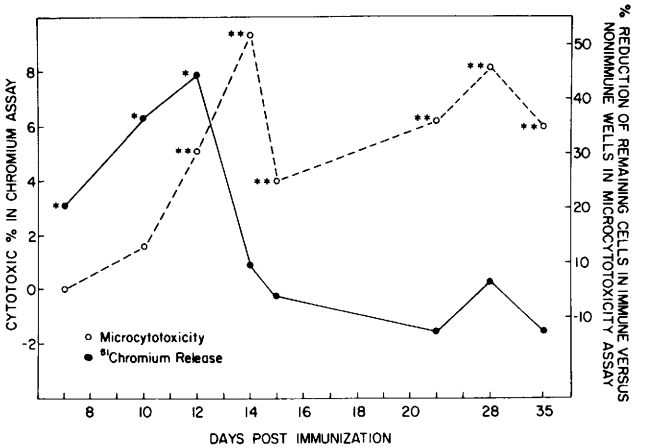

FIG. 2. Comparison of the developmental kinetics of cell-mediated immunity using both the ^{51}Cr release cell-mediated cytotoxicity assay and the microcytotoxicity assay. All P values were derived from Student's t test. * = $P < 0.05$; ** = $P < 0.001$.

TABLE I. Results of the Microcytotoxicity Assay and the ^{51}Cr Release Cell-Mediated Cytotoxicity Assay. Aliquots of the Same Pools of Attacking Cells Used.

Microcytotoxicity assay		^{51}Cr release assay	
Source of attacker cell pools	Number of adherent cells remaining in wells ± SEM	Source of attacker cell pools	Cytotoxic % ± SEM
Lymph node cells			
nonimmune	79 ± 4.8		
immune	53 ± 3.6[a]	Lymph node cells	2.6 ± 0.7[b]
Peritoneal exudate cells	not tested[c]	Peritoneal Exudate cells	3.5 ± 0.6[b]

[a] $P < 0.001$ (percentage reduction was 32.9%).

[b] $P < 0.05$.

[c] Peritoneal exudate cells contain numerous macrophages which adhere to the wells in the microcytotoxicity assay and make the scoring in that assay impossible.

tation was demonstrated rather easily by noting the differences in incidence of progressively growing tumors between the NI mice and those immunized in various ways. Thus previous difficulties with accurately measuring tumor volumes and charting growth curves were obviated in this study.

C57 mice receiving five small immunizations with Py-8914b demonstrated some tumor facilitation when challenged 1 week after their last immunization. When 5 weeks elapsed between the last immunization and the challenge, impressive protection from tumor growth was demonstrated as shown previously (4). When 4 weeks elapsed between immunization and challenge, neither facilitation nor protection was noted. It appeared, therefore, that the proximity of challenge to the time of the last immunization was one of the factors which determined whether tumor facilitation or rejection occurred. If facilitation of Py-89 represents specific immunologic enhancement, the kinetics of the response parallels the kinetics of antibody induction in the classic Sarcoma I System. Kaliss observed peak production of enhancing antiserum 2–3 weeks after immunization (11). On the other hand facilitation waned after this time in our syngeneic system whereas Kaliss found persistence of high titers of antiserum for as long as 8 mo in an allogeneic system.

The development of CMI as measured by MCA and CRA appeared to follow separate patterns. These assays have been compared by Wright *et al.* (9) and De Landazuri and Herberman (10) in other host–tumor systems. These authors reported that activity in the CRA which in their hands disappeared in 14–20 days could be detected, after having apparently disappeared, by incubating the AC overnight *in vitro*. Wright *et al.* (9) felt that both assays reflected the same basic immune process realizing that truly rigorous comparison was not possible due to technical differences in the assays. The data for both assays in this report paralleled those of Wright *et al.* (9) although overnight *in vitro* activation of AC was not attempted in this study. One advantage of using Py-8914b cells was their suitability as target cells in both assays thereby making inter-assay comparisons somewhat more rigorous.

Tumor facilitation appears to be a nonstatic phenomenon depending largely on when mice are challenged in relation to their last immunization. CMI develops independently of tumor facilitation. Whether or not CMI can be effective in rejecting tumors may be related to whether or not neoplastic cells arise at a time when an atmosphere of facilitation is prevalent.

We do not distinguish in this study facilitation from true immunologic enhancement. Specificity controls were not used nor did we attempt passive transfer with serum. There is observed a striking correlation between the titer of cytotoxic antibody as detected in a ^{51}Cr release assay and immunologic enhancement of tumor in allogeneic systems, differing at H-2 alloantigens (12); unfortunately we were unable to detect cytotoxic antibody against polyma antigen in our syngeneic system.

Summary. Active tumor facilitation in a syngeneic system is described using a polyoma-induced immunogenic cell line to facilitate growth of the oncogenic cell line. Criteria for facilitation are those usually reserved only for

allogeneic tumor enhancement. The kinetics of tumor facilitation are described and correlated with the development of cell-mediated immunity as measured by two separate *in vitro* assay systems. The results and their implications are discussed.

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