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J Immunol (1980) 124 (4): 1642–1647.
https://doi.org/10.4049/jimmunol.124.4.1642

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THE JOURNAL OF IMMUNOLOGY
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Vol. 124, No. 4, April 1980
Printed in U.S.A.

IN VIVO LYSIS OF L5178Y CELLS IN THE ESTABLISHMENT OF THE TUMOR-DORMANT STATE IN DBA/2 MICE[1]

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In vivo **lysis of L5178Y cells was studied at an early point in the establishment of the tumor-dormant state in DBA/2 mice. We evaluated** ***in vivo*** **lysis 4 days after L5178Y cell challenge of immunized mice (immune mice), a time when** ***in vitro*** **anti-tumor cytolytic activity of harvested peritoneal cells is maximal. L5178Y cells were labeled with ^{125}IUDR** ***in vitro*****, inoculated into the peritoneal cavity of normal and immune mice, and the kinetics of lysis was determined by measuring the amount of radioactivity persisting in these mice at various intervals thereafter. We found that L5178Y cells were rapidly lysed in immune mice compared with normal mice. Excretion of ^{125}I from immune mice inoculated with intact labeled L5178Y cells was as rapid as from immune mice inoculated with lysed labeled L5178Y cells, indicating that lysis of L5178Y cells in the peritoneal cavity of experimental mice was an extremely rapid event. This lysis was shown to be specific in that another DBA/2 lymphoma, the L1210 cell line, was not lysed when inoculated into L5178Y cell immune mice. The** ***in vivo*** **lysis of L5178Y cells was shown to require host-tumor cell contact since labeled L5178Y cells, contained in diffusion chambers implanted in the peritoneal cavity of immune mice, were not lysed during a 5-hr incubation period. It was also found that L5178Y cells enclosed in diffusion chambers and implanted in the peritoneal cavity for 6 days grew as rapidly in immune mice as in normal mice, indicating that soluble anti-tumor factors do not inhibit tumor cell growth in the absence of host-tumor cell contact.**

The fate of tumor cells inoculated into an animal host has, in general, been evaluated with qualitative experimental endpoints, i.e., the tumor graft either grows progressively or, if not, considered to be rejected. However, such endpoints are inadequate in that an increasing number of reports now indicate that tumor cells can remain in a dormant state in a clinically normal host for prolonged periods of time and eventually grow out rapidly and kill the host (1-7). In the tumor-dormant state, tumor cells persist for prolonged periods of time in a clinically normal host with changes in the size of the tumor cell populations occurring slowly. In patients, tumor-dormant states are manifested by the recurrence of tumor years after a long clinical remission following treatment of the primary tumor (8-10).

We have previously reported that tumor dormancy is routinely established in DBA/2 mice after subcutaneous implantation of L5178Y cells, subsequent tumor nodule excision, and i.p. L5178Y cell challenge (11, 12). The L5178Y cells contained in the challenge inoculum grow at a logarithmic rate for 4 days and then undergo a rapid decline in tumor cell numbers. The period of decline in tumor cell numbers coincides temporally with the appearance in the peritoneum of T cells, which, when assayed *in vitro*, cause release of radiolabel from ^{51}Cr-labeled L5178Y target cells (11). However, the relevance of such *in vitro* cytolytic assays is open to question since they represent interactions that take place in an environment that is unavoidably quite different from that existing *in vivo*. To understand better the immune mechanisms involved in the establishment of the tumor-dormant state in the intact host, we carried out and report here a series of *in vivo* studies in which we evaluated the ability of L5178Y-immunized and challenged mice to destroy tumor cells.

MATERIALS AND METHODS

Animals. Female DBA/2 mice (8 weeks of age) were obtained from the Jackson Laboratories, Bar Harbor, Maine.

Tumor cells. L5178Y and L1210, both DBA/2-derived methylcholanthrene-induced lymphoma lines (13, 14), were used in these experiments. Both cell lines were maintained as suspension cultures in medium consisting of RPMI 1640 (Grand Island Biologicals, Grand Island, N. Y.) supplemented with 15% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM glutamine, and 15 mM HEPES[2] buffer. This medium will be subsequently referred to as "culture medium." Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere.

Tumor cell immunization and challenge. DBA/2 mice were immunized against L5178Y as previously described (11). Briefly, 10^6 L5178Y cells were implanted subcutaneously (s.c.) on the mid-ventral surface of DBA/2 mice. Ten days later the resultant 1-cm tumor nodules were surgically excised. Mice were then challenged 7 days later with an i.p. inoculation of 5×10^4 L5178Y cells.

Tumor cell labeling. Tumor cells used for *in vivo* cytolysis experiments were labeled with ^{125}IUDR according to the following procedure. One hundred microcuries of ^{125}IUDR (5 Ci/mg, Amersham Corporation, Chicago, Ill.) was added to log phase cultures containing 3 to 4×10^7 tumor cells in 200 ml of culture medium. Cultures were then incubated for 26 to 30 hr at 37°C

Received for publication September 24, 1979.

Accepted for publication December 19, 1979.

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[1] This work was supported by Grant 1899S, awarded by the National Cancer Institute, Department of Health, Education, and Welfare and by Biomedical Research Support Grant 1 507 RR-05414.

[2] Abbreviations used in this paper: HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid: s.c., subcutaneous.

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in a humidified 5% CO_2 atmosphere. The cell suspensions were then washed three times in culture medium, and cell viability and isotope incorporation were determined. Only those labeled cell cultures containing 80% viable cells were used in experiments. The above labeling conditions routinely resulted in incorporation of 0.5 cpm ^{125}IUDR/cell. Autoradiographic analysis of these suspensions revealed uniform labeling of all cells within the culture (data not shown). We used ^{51}Cr rather than ^{125}IUDR as a cell label for diffusion chamber experiments since we had previously determined that ^{51}Cr released from lysed cells diffused out of the chambers much more rapidly than did ^{125}I (unpublished results). Chromium labeling of tumor cells was performed as previously described (11).

In vivo cytolysis assays. All mice received drinking water containing 0.1% NaI for 3 days before their use in an assay. Normal and immune mice were inoculated i.p. with 2×10^6 ^{125}IUDR-labeled tumor cells in a 0.2 ml volume. In experiments in which disrupted cells were used, mice were inoculated i.p. with 0.2 ml of a cell lysate of 2×10^6 cells obtained by four freeze-thaw cycles in liquid nitrogen. Immediately after injection of either labeled tumor cells or lysates, mice were placed in plastic specimen containers (Pharmseal, Glendale, Calif.) ventral side down, and whole body radioactivity was determined by using a Baird Scintillation Detector equipped with a 1¾ in. crystal (Baird Atomic, Cambridge, Mass.). This served as the zero timepoint (i.e., 100% retention of label) in all experiments. The same mice were then counted at various intervals thereafter. Subsequent determinations of whole body radiation were expressed as % radioactivity retained according to the formula:

$$\% \text{ Retention} = \frac{\text{cpm at x hr}}{\text{cpm at 0 hr}} \times 100$$

Diffusion chambers. Gelman 13-μ 0.20-μ filters, a pore size small enough to exclude entry of host cells, were glued onto diffusion chambers (Millipore Corp., Bedford, Mass.) with Millipore MF Cement No. 1 and the assembled chambers were sterilized in ethylene oxide. Before use, the chambers were soaked overnight in culture medium at 37°C. The chambers were loaded with 1×10^5 ^{51}Cr-labeled L5178Y cells in a volume of 0.1 ml and sealed with a nylon plug and cement. In certain experiments, ^{51}Cr-labeled L5178Y cells were sensitized with alloantiserum before being loaded into the chambers. The initial radioactivity of each chamber was determined and recorded. Chambers were then implanted into the peritoneal cavity of recipient animals. At the end of the incubation period, chambers were recovered and retained radiolabel was measured.

In experiments in which growth of L5178Y cells in diffusion chambers was investigated, 5×10^4 unlabeled L5178Y cells were loaded into chambers that were subsequently implanted i.p. into normal and immune mice. Chambers were removed at daily intervals up to 4 days and tumor cells were recovered by incubating the chambers at 37°C in serum-free medium containing 2 mg/ml protease (Sigma Chemicals Inc., St. Louis, Mo.) for 3 hr. This procedure completely digests the fibrous clot that invariably forms within chambers implanted in mice for greater than 5 hr (unpublished observation). Cells were then washed out of the chambers and counted in a hemacytometer. The number of cells recovered from these chambers capable of outgrowth was determined as follows: Single-cell suspensions were prepared in 0.4 ml of culture medium and one-half of each suspension was added into the first well of each of two rows of flat-bottomed microtiter plates. A series of 2-fold dilutions of each suspension was made through a total of 24 wells in each plate. The plates were incubated for 14 days at 37°C in a 5% CO_2 humidified atmosphere. Cultures were refed with fresh culture medium twice throughout this period. After the 14 days incubation, the highest dilution of each cell suspension that yielded positive tumor outgrowth was identified, and the number of tumor cells in the original suspension removed from the diffusion chambers capable of outgrowth was determined by endpoint dilution analysis. Culture wells were scored positive for tumor outgrowth when microscopic observation showed increasing numbers of large lymphoblastoid cells that continued to proliferate after transfer to new culture wells. Cells from such positive wells were invariably tumorigenic when inoculated i.p. into normal DBA/2 mice. The sensitivity of this enumeration procedure is based upon observations in our laboratory that as few as three L5178Y cells are capable of outgrowth under these culture conditions.

RESULTS

Resistance of L5178Y cell immunized and challenged mice to a secondary challenge with L5178Y cells. To evaluate *in vivo* lysis of L5178Y cells within the peritoneal cavity of DBA/2 mice, we utilized a technique originally described by Hofer *et al.* (15). Tumor cells were labeled *in vitro* with ^{125}IUDR, inoculated i.p. and *in vivo* cytolysis was determined by measuring residual radioactivity in these mice at selected times after cell inoculations. Since the highest level of *in vitro* cytolytic activity is found in peritoneal cell populations removed from mice 4 days after L5178Y cell challenge (11), and since this is also the time at which there is onset of a rapid decline in the number of tumor cells in the peritoneal cavity, we chose this timepoint for the assessment of *in vivo* cytolysis in our experiments.

The *in vivo* cytolysis experiments to be presented below required an inoculation of 2×10^6 ^{125}IUDR-labeled L5178Y cells on the 4th day after L5178Y cell challenge. To be certain that this secondary challenge did not overwhelm the anti-tumor effector systems that suppressed L5178Y cells to a dormant state, we evaluated the resistance of immune (4-day post L5178Y cell challenge) mice to an inoculation of 2×10^6 unlabeled L5178Y cells. We found that all normal mice inoculated with 2×10^6 L5178Y cells died within 17 to 20 days, whereas the range of tumor-associated deaths in immune mice was 66 to 162 days post inoculation.

Comparison of lysis of L5178Y cells in normal and immune mice. We next inoculated 2×10^6 intact ^{125}IUDR-labeled L5178Y cells into normal and immune mice and measured retained ^{125}I at selected times after inoculation. Figure 1 reveals that 97% of ^{125}I had been excreted from immune mice by 11 hr after inoculation, indicating that lysis of the great majority of the tumor cells had occurred. In contrast, only 20% of ^{125}I had been excreted by normal mice by 11 hr and 40% of label was still retained by 48 hr.

To be certain that normal and immune mice had equivalent rates of excretion of ^{125}I released from lysed ^{125}IUDR-labeled tumor cells, we inoculated lysed L5178Y, previously labeled with ^{125}IUDR, into normal and immune mice. Figure 2 shows that there is no difference in the rates of excretion, as expressed by % retention, of ^{125}I from these two groups of mice. The immunization procedure, therefore, had no effect on the ability of mice to excrete ^{125}I released from lysed cells.

Rate of excretion of ^{125}I from immune mice inoculated with intact or disrupted ^{125}IUDR-labeled L5178Y cells. Since L5178Y cells were rapidly lysed in immune mice we compared the rates of excretion, as expressed by % retention, of ^{125}I from mice inoculated with L5178Y cells that were either intact or

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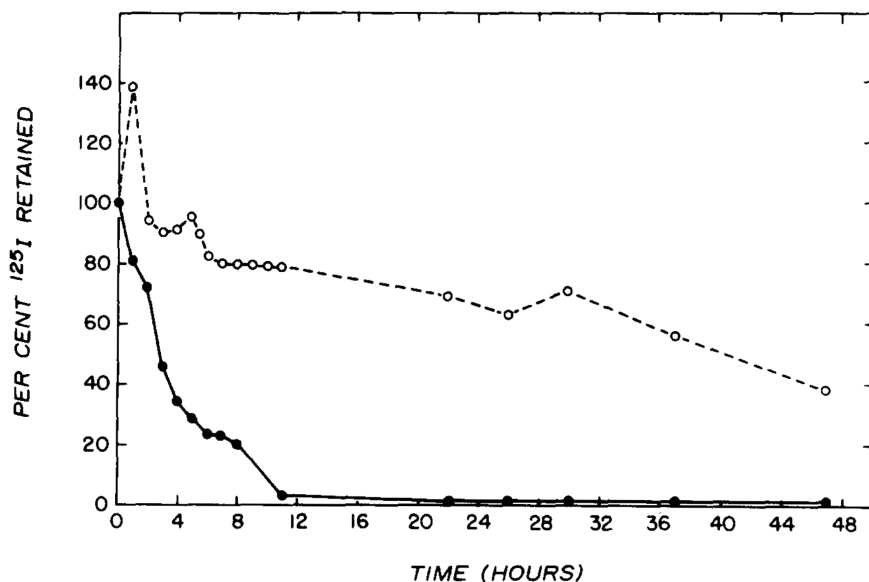

Figure 1. Lysis of ^{125}IUDR-labeled L5178Y cells in normal (O-----O) and immune (●——●) mice (expressed as % ^{125}I retention).

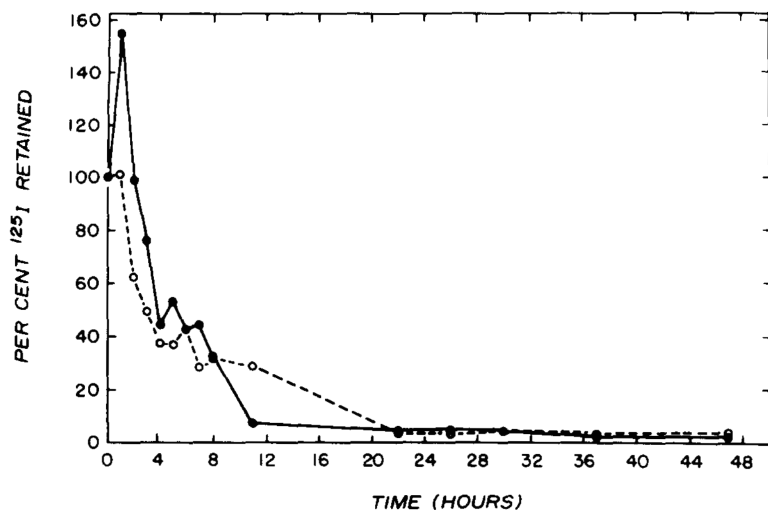

Figure 2. Excretion of ^{125}I from normal (O-----O) and immune (●——●) mice inoculated with disrupted ^{125}IUDR-labeled L5178Y cells (expressed as % ^{125}I retention).

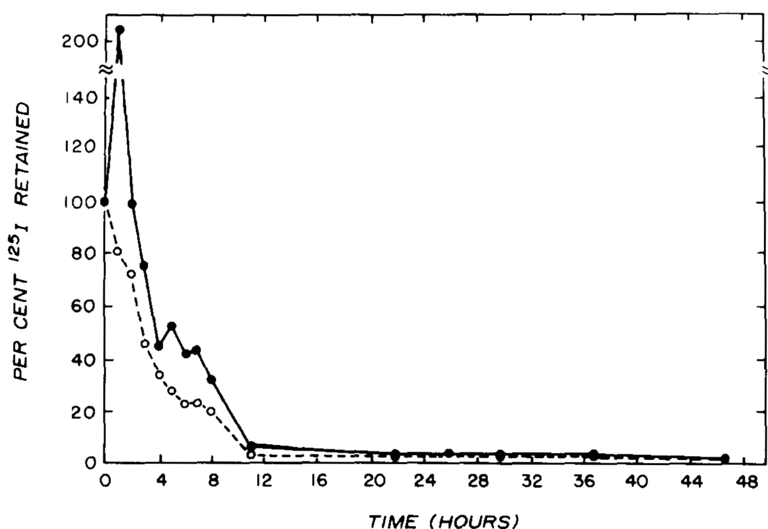

Figure 3. Excretion of ^{125}I from immune mice inoculated with intact (O-----O) or disrupted (●——●) ^{125}IUDR-labeled L5178Y cells (expressed as % ^{125}I retention).

previously lysed *in vitro*. As seen in Figure 3, there was little difference in ^{125}I excretion from immune mice inoculated with these two cell preparations, indicating that *in vivo* lysis of i.p. inoculated tumor cells is a very rapid event.

Specificity of cytolytic activity in immune mice. In order to ascertain the specificity of the lytic activity for L5178Y cells in immune mice, we labeled L1210 cells with ^{125}IUDR, and evaluated their lysis *in vivo*. As seen in Table I, little if any lysis of L1210 cells occurred in L5178Y immune mice.

Susceptibility of L5178Y cells in diffusion chambers to lysis by soluble factors outside the chambers. To determine whether the rapid *in vivo* lysis of i.p. inoculated L5178Y cells observed

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TABLE I

Specificity of L5178Y cell lysis in L5178Y immune mice

Hours after Injection of Cells	% ^{125}I Retained in Mice[a]			
	Expt. 1: L5178Y cells		Expt. 2: L1210 cells	
	Normal mice	Immune mice	Normal mice	Immune mice
	%		%	
5	98	34[b]	98	100
10	85	27[b]	92	100
15	78	20[b]	92	85
24	58	8[b]	76	71

[a] 2×10^6 ^{125}IUDR-labeled L5178Y or L1210 cells were inoculated i.p. into normal and immune and the percentage of ^{125}I retained in the mice measured at the indicated times.

[b] Significantly different from L1210 lysis in immune mice.

TABLE II

Susceptibility of L5178Y cells in diffusion chambers in vitro and in vivo to lysis by soluble factors outside the chambers

Experiment	% ^{51}Cr ± S.E.M. Remaining in Chambers after a 5-hr Incubation		
	Target cells in chambers	Experimental conditions outside chambers	
		Medium control	Ab + C
In vitro[a]	^{51}Cr-labeled L5178Y	86.3 ± 5.6	35.6 ± 5.5
In vivo[b]	Antibody-sensitized ^{51}Cr-labeled L5178Y	63.5 ± 4.1	36.7 ± 0.8

[a] Four diffusion chambers, each containing 1×10^5 ^{51}Cr-labeled L5178Y cells were individually incubated at 37°C in culture medium alone and four additional chambers were similarly incubated in culture medium containing C57BL/6 anti-DBA/2 serum (1:50 final) and guinea pig C (1:10 final). Retained radiolabel was measured for each chamber at the end of a 5-hr incubation period and calculated as a percentage of the original label present at time zero.

[b] Eight diffusion chambers, each containing 1×10^5 ^{51}Cr-labeled L5178Y cells previously sensitized by a 45-min incubation in a 1:100 dilution of C57 anti-DBA/2 serum, were implanted into eight immune mice. Four of the mice were immediately inoculated i.p. with 1 ml guinea pig C per mouse and the remaining four mice were inoculated with 1 ml of a medium (PBS) control.

in the immune mice was mediated by cellular or soluble effector systems, we labeled L5178Y cells with ^{51}Cr, placed them within diffusion chambers, and implanted the chambers into the peritoneal cavity of normal and immune mice.

Preliminary experiments revealed that a clot, which would inhibit diffusion of soluble factors, formed within the chambers between the 5th and 18th hr of incubation in the peritoneal cavity. Given this limitation in the use of the chambers, we evaluated the ability of soluble factors to enter the chamber and lyse labeled L5178Y cells within a 5-hr period, first under *in vitro* conditions and then *in vivo*.

One-hundred thousand ^{51}Cr-labeled L5178Y cells were enclosed in a number of chambers, half of which were then placed in culture vessels containing growth medium, and the other half placed in vessels containing medium with C57BL/6 anti-DBA/2 serum and guinea pig complement (C). The chambers were then incubated *in vitro* for 5 hr at 37°C and the radioactivity retained in the chambers was measured. As seen in Table II, significant lysis of L5178Y cells by antibody and C *in vitro* occurred within 5 hr. Thus, humoral factors could enter the chambers, lyse the cells, and the released label could exit within a 5-hr period. In the *in vivo* experiments, diffusion chambers were filled with labeled L5178Y cells that had been sensitized *in vitro* by incubation with C57 anti-DBA/2 serum, and implanted into immune mice. These mice were then inoculated i.p. with either 1 ml of guinea pig serum or of PBS. The remaining radioactivity in each chamber was counted after 5 hr. As seen in Table II (*in vivo* experiment), there was a marked lysis of sensitized labeled cells in chambers exposed to C *in vivo* compared with chambers not exposed to C, indicating that a soluble factor such as C outside the chambers could diffuse into the chamber in the peritoneal cavity and lyse the cells within 5 hr.

Lysis of L5178Y cells within diffusion chambers implanted in immune mice. ^{51}Cr-labeled L5178Y cells were enclosed in diffusion chambers and implanted into four normal and four immune mice. After 5 hr the chambers were removed and the remaining radioactivity in each chamber was determined. Table III shows that there was no significant lysis of L5178Y cells in diffusion chambers implanted into immune mice compared with normal mice. The absence of lysis indicates that lysis is not accomplished by soluble factors alone but requires tumor-host cell contact. Such experiments, however, do not exclude the participation of a humoral component in the *in vivo* cell-mediated lysis.

Cellular basis of resistance to L5178Y cell outgrowth. The

TABLE III

Susceptibility to lysis of L5178Y cells in diffusion chambers in the peritoneal cavity of immune mice

Time	^{51}Cr ± S.E.M. remaining in Chambers after a 5-hr Incubation[a]			
	Normal mice		Immune mice	
	Mean counts	% Input	Mean counts	% Input
hr				
0	5391 ± 491	70.4 ± 3.9	4998 ± 265	72.7 ± 3.7[b]
5	3738 ± 186		3631 ± 255	

[a] 1×10^5 ^{51}Cr-labeled L5178Y cells were enclosed in each diffusion chamber and radioactivity of each chamber counted at onset of experiment and 5 hr thereafter.

[b] Not statistically different from normal mice controls.

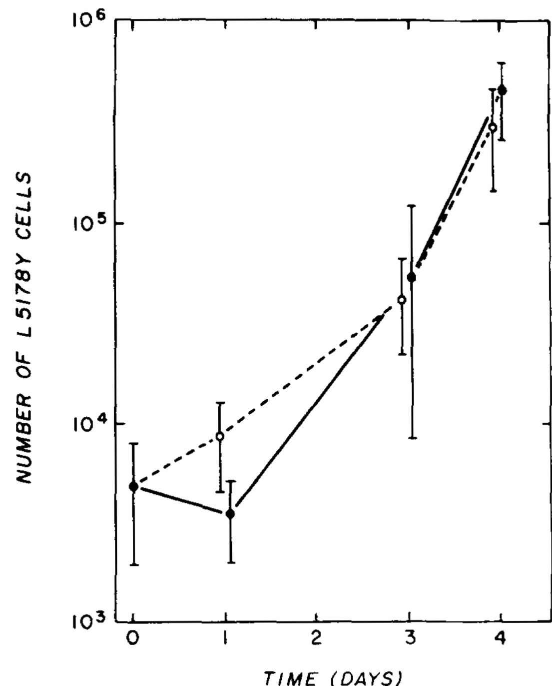

Figure 4. Growth of L5178Y cells in diffusion chambers implanted in normal (O-----O) or immune (●——●) mice.

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mechanism by which immune mice suppress tumor cell outgrowth was studied by comparing L5178Y cell growth in diffusion chambers implanted into normal and immune mice. The chambers were filled with 20,000 unlabeled L5178Y cells, implanted into the peritoneal cavity of the two groups of mice, and on days thereafter, four chambers from each group were removed. The intact chamber contents were digested with pronase (to remove the clot) and the number of L5178Y cells capable of outgrowth in each chamber was determined by titration in microtiter plate wells. As seen in Figure 4, there was no difference in cell growth between chambers implanted in the normal and immune groups of mice, indicating that suppression of tumor cell outgrowth also requires tumor-host cell contact.

DISCUSSION

Reduction of the tumor burden is an essential feature in the establishment of the L5178Y cell tumor-dormant state in DBA/2 mice. Elimination of all tumor cells does not occur, however, and a small number of residual cells persist under growth restraint throughout a prolonged clinically normal period (11). Previous analysis of this tumor-dormant model revealed that an i.p. L5178Y tumor cell challenge, given 7 days after surgical excision of the primary subcutaneous L5178Y tumor nodule stimulates a secondary cell-mediated immune response as evidenced by accumulation of T cells in the peritoneal cavity, which, when tested *in vitro*, are capable of lysing L5178Y cell targets (11). This cell-mediated immune response reaches maximum levels on the 4th day post L5178Y cell challenge (11). No L5178Y cell lytic antibody was detected during the establishment of the L5178Y dormant state (11). Since *in vitro* cytolytic assays used to measure anti-tumor responses are performed under conditions that are unavoidably quite different from the *in vivo* environment with regard to density of interacting cells, the ratio of effector to target cells, the possible presence of humoral blocking or enhancing factors *in vivo*, incubation conditions, etc., we sought to carry out an *in vivo* analysis of the host-tumor interactions occurring during the period when the tumor-dormant state is being established.

To accomplish such an *in vivo* analysis, we utilized a cytolytic assay, first described by Hofer *et al.* in 1969 (15). ^{125}IUDR is incorporated into the DNA of cells as an analog of thymidine and the isotope is contained within cells until their death (15–17). Lysis of the cells results in ^{125}I being split off from the nucleotide in a nonreutilizable form, which is rapidly transported to the kidney and excreted (15). In this assay the loss of ^{125}I from an animal inoculated with ^{125}IUDR-labeled L5178Y cells can be detected within a few hours after lysis of the labeled cells. We inoculated ^{125}IUDR-labeled L5178Y cells into the peritoneal cavity of mice 4 days post i.p. L5178Y cell challenge, a time at which the number of L5178Y cells begins to rapidly decline (11). Thus, lysis of the labeled L5178Y cells would occur under conditions identical to those that result in the destruction of the majority of cells used in the standard L5178Y cell challenge. A preliminary experiment demonstrating that normal and immune mice excrete label at comparable rates when inoculated i.p. with a cell lysate prepared from ^{125}IUDR-labeled L5178Y cells showed that the *in vivo* ^{125}I cytolytic assay could be used in our experimental system.

Comparison of lysis in normal and immune mice inoculated with intact labeled L5178Y cells revealed a greater lysis of L5178Y cells in the immune mice. In addition, a subsequent experiment showed that the extensive lysis of L5178Y cells in the immune group must occur very shortly after their inoculation, since the rate of loss of label from immune mice inoculated with intact cells was indistinguishable from the rate of loss after inoculation with *in vitro* disrupted cells. It should be noted that on occasion after inoculation of either intact or disrupted ^{125}IUDR-labeled cells, the whole body radioactivity determined at 1 hr greatly exceeded the radioactivity measured immediately after injection. This occurred randomly and, to our knowledge, has not been reported previously, possibly because few studies measured retention of label before 12 hr. We have not thoroughly investigated this phenomenon, and, at present, speculate that it may represent decreased shielding of radioactivity by organs within the animal as the released label enters the circulation and is concentrated in the kidney.

The cell-mediated cytolytic response generated during establishment of the L5178Y cell-dormant state was previously demonstrated by *in vitro* assays to be T cell mediated (11). Although we have not in the present communication identified the host cell responsible for *in vivo* cytolysis, we have found that the activity is immune specific since L5178Y cells, but not L1210 cells, are lysed. These findings correlate well with the *in vitro* specificity of tumor cell killing by peritoneal T cells taken from immune mice 4 days after L5178Y cell challenge (11).

We next demonstrated that direct cell-cell contact was required for lysis of L5178Y cells during the establishment of the dormant state. This conclusion was based on the observation that L5178Y cells enclosed in diffusion chambers, which prevented influx of host cells, were not lysed when such chambers were implanted into immune mice. Assurance that cytolytic soluble factors, if present outside the chamber, could lyse cells within chambers was provided by the demonstration *in vitro* that antibody and C could enter diffusion chambers and lyse L5178Y cells, and *in vivo* that C could enter diffusion chambers and lyse antibody-sensitized L5178Y cells. The use of exogenous guinea pig C was necessary in the *in vivo* experiments since the DBA/2 mouse is deficient in C5 (18, 19).

The 6-day L5178Y growth experiments were performed in diffusion chambers in the presence of a clot that routinely forms in all chambers between the 5 and 18 hr of incubation in the peritoneal cavity. However, soluble factors such as antibody and C can still diffuse into the diffusion chambers containing such clots, albeit at a slower rate, and lyse L5178Y cells with resultant release of radiolabel (unpublished results). Also, antibody can diffuse out of diffusion chambers containing antibody-secreting hybridoma cells for at least 10 days after their implantation into mice (T. Wiktor & L. Goldstein, unpublished observation). We can also conclude, therefore, that no soluble factors capable, by themselves, of inhibiting L5178Y cell growth are present in the peritoneal cavity during the establishment of the dormant state.

During establishment of the L5178Y tumor-dormant state, over 99% of tumor cells are lysed, and the surviving cells are restrained from rapid outgrowth, persisting in a dormant state for many weeks before growing out to form an ascitic tumor. The ability of a small percentage of cells of a primary tumor to escape the initial treatment of the tumor and persist in a dormant state for prolonged periods of time thus represents a major impediment to the cure of cancer. Development of ways to eradicate such tumor cells in the DBA/2-L5178Y tumor-dormant model can serve as a basis for development of an effective cure of tumor-dormant states in man.

Acknowledgments. The excellent technical assistance of Janis Lariviere and secretarial assistance of Dottie Schmidt is gratefully acknowledged.

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