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In Vivo Function of Immune Murine Peritoneal Exudate Cells after Freezing and Thawing[1] (40723)

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The preservation of cells by freezing is a common procedure in laboratories of varying research interests. Recent emphasis has been directed toward using freezing procedures which preserve the functional capacity of immunocompetent cells (1). Additional reports have described *in vitro* effects of freezing and thawing on lymphocytes, macrophages, and spleen cells (1–7). Hem (4, 5) showed that lymphocytes can produce antibody against SRBC post-thawing as well as give a good response to mitogens. In another communication (7), macrophages were shown to exhibit a general loss in the number of some receptor sites though phagocytosis by these cells appeared unaffected. Each of these studies, however, represents *in vitro* analysis of functional changes occurring post-thawing. None of these reports gave evidence that effector cells sensitized to tumor-specific antigens were capable of effecting transplantation resistance.

Results in our laboratory demonstrated that lymph node cells (LNC) and peritoneal exudate cells (PEC) were capable of passively conferring tumor resistance when freshly harvested from tumor resistant hamsters (8) or mice (9) injected with homologous tumor cells. Lymphocytes from a thioglycolate stimulated peritoneal exudate have been shown to be highly reactive against antigen (10), tumors (11), and allogeneic cells (12). In guinea pigs, these cytotoxic lymphocytes from the peritoneal cavity have been shown to be more reactive than lymph node cells (10). We have shown the same working with hamsters. The reason for this is not understood. However, several investigators suggest that the peritoneal exudate consists of an enriched subset of stimulated T lymphocytes which migrate into the irritated peritoneal cavity (13–15). It can also be shown that such thioglycolate stimulated peritoneal exudates consist of a large proportion of macrophages. Therefore, the use of peritoneal exudate cells from animals previously immunized against a particular antigen is more advantageous than lymph node cells.

There are many occasions when it is desirable to compare the protective potential of effector cells harvested at different times during the growth of a tumor to confer tumor resistance in a subsequent parallel challenge experiment. We conducted studies to determine the functional ability of peritoneal exudate cells from mice previously immunized with irradiated tumor cells following freezing and thawing against live tumor cells. This Winn-type bioassay of tumor mediated tumor immune reactivity *in vivo* to detect adoptively transferred anti-tumor reactivity is one of the most stringent bioassays in tumor immunology since one unkilled tumor cell can be potentially oncogenic.

Materials and Methods. Animals. Male Balb/c mice, 5 to 7 weeks old, were obtained from the Jackson Laboratory, Bar Harbor, Maine, and from Leo Goodwin Institute, Ft. Lauderdale, Florida.

Tumor cells. A methylcholanthrene-induced tumor cell line, 1315, derived in Balb/c mice was provided to us by Dr. I. Hellstrom, University of Washington Medical School, Seattle, Washington. Cells were

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harvested from *in vitro* cultivation in Eagle's essential minimum medium (EMEM, Flow Laboratories, Rockville, Md.)[2] plus 10% fetal calf serum (Flow Laboratories) and washed twice in Hank's balanced salt solution (HBSS, Flow Laboratories) to remove serum components.

Immunization. Tumor cells suspended in HBSS were X-irradiated with 5000 R (A.E.C.L. Telethon Jr., Model 80, 7000 Ci source of Co-60). Fifty mice were immunized weekly for 4 weeks, ip, with $3-5 \times 10^6$ irradiated tumor cells.

Peritoneal exudate cells. PEC were harvested from immunized mice as previously described by Leffell and Coggin (9). Briefly, 2 days following the last immunization, the mice received 1.0 ml of 3% thioglycolate, ip, to stimulate the influx of PEC into the abdominal cavity. Five days later, the mice were killed by cervical dislocation. PEC were collected by washing the peritoneal cavity two or three times with 8–10 ml HBSS injected from a syringe with an 18-gauge needle. The cells were centrifuged (425g in an IEC rotor No. 269 for 5 min at 4°C) and washed twice in fresh HBSS.

Freezing and Thawing. Following the final centrifugation, the cell pellet was resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 20% fetal calf serum (Flow Laboratories), 1% nonessential amino acids (Flow Laboratories), 2 m*M* glutamine (Flow Laboratories), 1 m*M* sodium pyruvate, penicillin (50 μg/ml), streptomycin (50 μg/ml), and fungizone (2.5 μg/ml) at a final concentration of $3.5-4.0 \times 10^7$ cells/ml. One-milliliter aliquots of the suspension were placed into separate sterile vials (Wheaton Scientific Co., Millville, N.J.) chilled in an ice bath to 4°C. Dimethylsulfoxide (DMSO, Mallinckrodt, Inc., St. Louis, Mo.) was then added to give a final concentration of 10% (v/v). The vials were placed at 4°C for 25 min, at −20°C for 20 min, and then stored at −80°C. PEC were thawed 1, 4, 7, 10, or 30 days after freezing by rapid agitation in a 37°C water bath. Cells were centrifuged gently (175*g* for 3–5 min at 37°C) to form a pellet and washed twice in HBSS by centrifugation. Viability was determined by trypan blue dye exclusion using a hemocytometer.

In vivo test. Fresh or frozen–thawed PEC were admixed with freshly harvested 1315 tumor cells at a ratio of 100:1 or 1000:1 and incubated 30 min at 37°C. An injection of $3-5 \times 10^6$ PEC–tumor cell mixture was administered, sc, to 20 mice per group. Mice were then palpated, weekly, for tumor appearance for 48 days. Control groups received 5×10^4 1315 tumor cells/ml, an admixture of freshly collected PEC and tumor cells in a ratio of 100:1, or an admixture of freshly collected PEC and tumor cells in a ratio of 1000:1.

Results. Peritoneal exudate cells were scored for viability using the trypan blue exclusion method before and after freezing in order to determine cell death attributed to the actual freezing process. Viability and the total number of cells recovered, as seen in Table I, decreased with increasing time of storage. The mean recovery of cells thawed on different days ranged from 60 to 80%. These results are in line with results obtained using a variety of tumor and normal cells in our lab.

All mice injected with only tumor cells (5×10^4) developed 100% tumors in 13–16 days. When the same number of tumor cells was admixed with frozen and thawed PEC harvested from mice receiving immunization against MCA-1315 and injected into

TABLE I. EFFECTS OF FREEZING ON VIABILITY AND RECOVERY OF PEC

Days frozen	Recovery[a] (%)	Viability[a] (%)
0 (Control)	—	100
1	80.1	96
4	78.0	90
7	75.6	89
10	71.3	84
30	59.7	80

[a] Data represents mean of triplicate studies.

[2] Abbreviations used: MCA, methylcholanthrene; PEC, peritoneal exudate cell; EMEM, Eagle's essential minimum medium; HBSS, Hank's balanced salt solution.

mice, tumorigenesis was delayed and tumor incidence was decreased when the ratio of PEC to tumor cells was 100:1. The first tumors did not appear until Day 19 and at the end of 48 days less than 10% of the experimental mice developed tumors. More significantly when the frozen PEC to tumor cell ratio was increased to 1000:1, only three tumors, less than 3% of the total number of mice injected, appeared in 48 days among all experimental groups (Table II). Mice receiving freshly harvested unfrozen PEC admixed with tumor cells developed no tumors even at the lower ratio in concurrence with results of Leffell and Coggin (9).

Discussion. Investigators (1–6) have reported that subpopulations of lymphocytes may be differentially damaged by freezing and thawing. Hem and Munthe-Kaas (7) suggested that freezing caused a generalized loss of surface receptors on macrophages thus affecting *in vitro* function. Jennings *et al.* (1) in turn suggested that any loss of function may simply be due to a decreased number of cells recovered. Our results show that PEC from mice immunized against a chemically induced tumor can be frozen, subsequently thawed, and used in an adoptive transfer assay at a 1000:1 ratio produce comparable results, once corrected for yield, to assays using freshly harvested PEC. Immune, frozen–thawed PEC did interrupt tumorigenesis and tumorigenesis was greatly decreased from control groups when the ratio of PEC to tumor cells was 100:1. This protection is less than reported by Leffell and Coggin (9) who showed that fresh PECs sensitized to MCA-1315 tumor cells conferred 100% protection in an adoptive transfer assay. Increasing the number of PEC 10-fold virtually inhibited the formation of tumors (3 in 110 mice) thus indicating that the trauma of freezing, resulting in reduced viability does result in a quantitative decrease in function. The exact nature of the presumed cell damage which affects the functional capacity of the PEC, seen more at the 100:1 ratio than the 1000:1 ratio, is not examined here. In addition, which cell population affected is also not determined. What is shown is that after freezing there are enough specifically sensitized cells unaffected by the freezing and thawing to be cytotoxic to tumor cells *in vivo*. Since the length of time frozen had no appreciable effect on further decreasing the effectiveness of PEC in the adoptive transfer assay, it can be concluded that these cells can be frozen for future use in *in vivo* immunological studies provided the investigator compensates for some generalized cell damage by increasing the number of cells used. The procedure seems especially amenable to time course studies of tumor development where the assay of effector lymphocytes and macrophages need to be assessed over a long time span using multiple collections of PECs on a single trial in an adoptive transfer assay.

Summary. Peritoneal exudate cells (PEC) were collected from Balb/c mice immunized against a 3-methylcholanthrene-induced (3-MCA) tumor and known to be capable of conferring tumor transplantation resistance *in vivo* in syngeneic recipients. These PEC were frozen in RPMI 1640 supplemented with 20% fetal calf serum, 1% nonessential amino acids, 2 m*M* glutamine, and 1 m*M*

TABLE II. EFFECT OF PEC TO TUMOR CELL RATIO ON TUMOR DEVELOPMENT

	PEC: Tumor cell (100:1)		PEC:Tumor cell (1000:1)	
Days frozen	No. of tumors	Percentage protection	No. of tumors	Percentage protection
0 (Control)	0/10	100	0/10	100
1	6/20	70	2/20	90
4	2/20	90	0/20	100
7	2/20	90	0/20	100
10	2/20	90	1/20	95
30	1/20	95	0/20	100

sodium pyruvate using dimethylsulfoxide as the cryopreservative agent. Adoptive transfer of tumor resistance in syngeneic recipients challenged with homologous 3-MCA sarcoma cells was attempted using these frozen exudate cells. Cells were thawed 1, 4, 7, 10, or 30 days after freezing and admixed with tumor cells in ratios of 100:1 or 1000:1 before injecting into mice. Tumorigenesis was decreased and delayed in groups receiving the 100:1 ratio. Less than 3% of the mice developed tumors in groups receiving the 1000:1 ratio. The number of cells recovered post-thawing ranged from 60 to 80%; viability of post-thawed cells ranged from 80 to 96%.

1. Jennings, J. J., Yanari, S. S., and Reid, R., Cryobiology **15**, 272 (1978).
2. Goodman, J. W., Blood **21**, 777 (1963).
3. Ashwood-Smith, M. J., Blood **23**, 494 (1964).
4. Hem, E., Cryobiology **13**, 134 (1976).
5. Hem, E., Pathol. Microbiol. Scand. C **84**, 489 (1976).
6. Hem, E., and Sokolowski, J., Cryobiology **13**, 537 (1976).
7. Hem, E., and Munthe-Kass, A. C., Cryobiology **14**, 649 (1977).
8. Coggin, J. H., Larson, V. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **124**, 1295 (1967).
9. Leffell, M. S., and Coggin, J. H., Cancer Res. **37**, 4112 (1977).
10. Rosenstreich, D. L., Blake, J. T., and Rosenthal, A. S., J. Exp. Med. **134**, 1170 (1971).
11. Berke, G., Sullivan, K. A., and Amos, B., J. Exp. Med. **135**, 1334 (1972).
12. Wernet, D., and Lilly, F., J. Exp. Med. **141**, 573 (1975).
13. Koster, F. T., McGregor, D. D., and Mackaness, G. B., J. Exp. Med. **133**, 400 (1971).
14. Koster, F. T., and McGregor, D. D., J. Exp. Med. **133**, 864 (1971).
15. Schwartz, R. H., Jackson, L., and Pavl, W. E., J. Immunol. **115**, 1330 (1975).

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