# Preservation of Antibody-Producing Cells at Low Temperatures: A Method of Storage that Allows Complete Recovery of Activity. (28711)

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Progress in extending our knowledge of mechanisms of antibody synthesis and other immune processes depends, in part, upon a system for rapid and reproducible assessment of antibody production. For the past several years we have been using an *in vivo* system of culturing antibody-producing cells wherein functional cells are transferred to isologous, irradiated recipients(1). A variety of factors which affect elaboration of antibody has been quantitatively evaluated: *e.g.,* number of cells placed in culture(2), degree of antigen priming(3), dosage of stimulating antigen(4), age of the animal donating cells(5), and inactivation by X-irradiation(6). It has, therefore, become important to have a method for collecting and storing, with little or no loss in functional capacity, cells from donors which have been subjected to various treatments. A method is reported here which accomplishes this purpose and which utilizes techniques and information already well known to students of cryobiology.

Previous studies on low-temperature preservation of mammalian cells capable of differentiation and sustained proliferation(7-14) have not included rigorous estimates of retention of *functional* capacity. Unresolved questions remain concerning the most suitable temperatures for long-term storage of cells and types and optimum concentrations of protective substances which should be used. While these conditions may be expected to vary with various types of cells, our results answer some of these questions with respect to cells engaged in antibody response.

*Methods and materials. Scheme of experiments.* There is a direct relationship between amount of antibody produced and number of active cells placed in the *in vivo* culture(2). A plot of $\log_2$ titer ($\log_2$ the reciprocal of the greatest dilution which still provides visible hemagglutination) as a function of cell number results in a straight line with a slope of 1, provided that serum antibody titer is measured during the late log or early stationary phase of the immune response. We have recently shown that active antibody-producing cells are derived through cellular division and maturation from relatively undifferentiated progenitors and that there is a direct relationship between antibody titer and number of antibody-containing cells(4,15,16). It follows, therefore, that when the amount of antibody produced by a given number of cultured cells is less than that in positive controls, the depressed response may be attributed to loss of active cells. The extent of antibody response in test cultures may be estimated from the following expression:

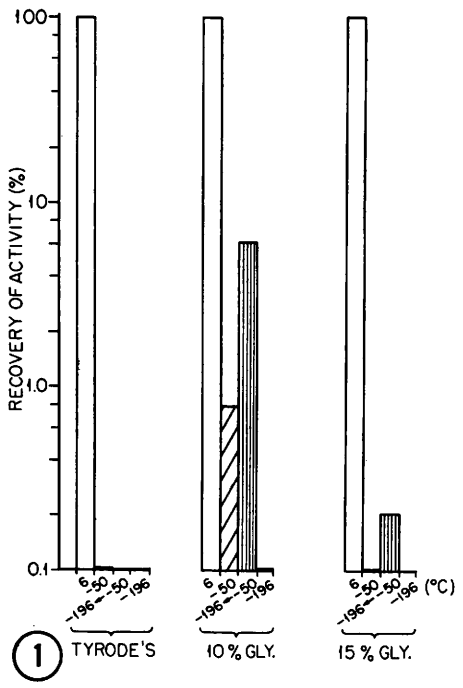
$$\text{Relative antibody } (\%) = \frac{1}{2^{s(T_c - T_{exp})}} \times 100$$

where: $s$ = slope of line relating to titer to cell number (1.0)
$T_c$ = $\log_2$ titer of positive control culture
$T_{exp}$ = $\log_2$ titer of experimental culture

In this manner reduction in antibody produced by a fixed number of cells resulting from the freezing and storage may be equated to damage and destruction of antibody-producing cells (Fig. 1 and 2).

Spleens were removed from mice preimmunized with sheep erythrocytes. The spleens were teased in Tyrode's solution (to which was added mouse serum in 1% concentration). The resulting cell suspension was counted in a hemacytometer, and the volume was adjusted to give a suspension containing $2 \times 10^8$ nucleated cells per ml. One aliquot of the cell suspension was immediately further diluted with Tyrode's so as to contain $2 \times 10^7$ cells per ml, and 1-ml portions were injected into irradiated culture mice (i.v.).

* Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.



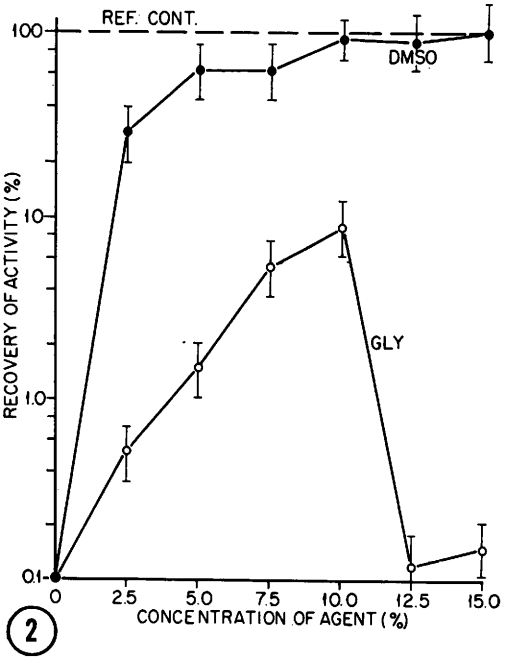




FIG. 1. Antibody-producing capacity of spleen cells suspended in 10% or 15% GLY and frozen by decreasing temperature at various rates. ▢ = Positive control, cells stored unfrozen at 6°C; ▨ (diagonal lined) = temperature decreased 1°C/min. to −50°C, stored at −50°C; ▥ (vertical lined) = temperature decreased 1°C/min. to −50°C, then to −196°C by immersion in liquid nitrogen, stored at −196°C; ■ = temperature rapidly decreased to −196°C by immersion in liquid nitrogen, stored at −196°C. Results based on 2 experiments utilizing 10 to 20 recipient mice for each condition.

FIG. 2. Antibody production by spleen cells frozen by decreasing temperature by 1°C/min. to −50°C followed by storage for 1 hr at −196°C. Cells suspended in varying concentrations of either GLY or DMSO. Results based on 3 experiments utilizing 10 or 20 recipient mice per point. 95% confidence limits shown.

A second aliquot was diluted with Tyrode's so as to contain $10^8$ cells per ml, and was set aside in the refrigerator at ~6°C until the end of the experiment. It was used as a control to determine whether there was loss in antibody-producing capacity with time of storage. Aliquots of a third group, containing $2 \times 10^8$ cells per ml, were diluted with equal volumes of the solutions (containing twice the desired final concentrations) which were expected to allay freezing damage to the cells. They were held at ~6°C until the close of the experiments when they, along with aliquots of the second group, were diluted with an additional volume of Tyrode's to provide a suspension of cells containing $2 \times 10^7$ cells per ml. Irradiated culture mice received i.v. injections of 1 ml each of the various suspensions. Finally, aliquots of a fourth group, containing $2 \times 10^8$ cells per ml were mixed with an equal volume of the solutions intended to prevent freezing damage such that the final suspension contained $10^8$ cells per ml in the proper desired concentration of freezing preservative. These suspensions were frozen and stored for a period of 1 hour. They were then thawed and further diluted with Tyrode's to a final concentration of $2 \times 10^7$ cells per ml (with respect to the initial, prefreezing cell concentration). One-ml portions were injected i.v. into irradiated culture mice.

All mice in which the various preparations of cells were cultured received i.p. injections of 1 ml of $2 \times 10^8$ sheep erythrocytes which, from the standpoint of the cultured cells, constituted a secondary antigen stimulus. After 6 days of culture of treated or untreated donor cells, *i.e.*, during the stationary phase of the antibody response, culture mice were bled, sera were collected and stored at

—20°C for later titration for hemagglutinating antibodies by our routine method(17).

Controls consisted of groups of irradiated mice treated in one of 3 ways: (a) one group received fresh, unfrozen spleen cells from preimmunized donors but no antigen stimulation, (b) a second group received antigen only, (c) a third group received neither spleen cells nor antigen and served as irradiation controls.

*Animals.* All mice employed were hybrid stock (C3H/Anf Cum ♀ × 101/Cum ♂ $F_1$ obtained from Cumberland View Farms, Clinton, Tenn. Males and females were used indiscriminately. They were housed in plastic cages and allowed free access to food and water.

*Irradiation conditions.* A General Electric X-ray machine was used. Recipient, culture mice received an 800-r exposure under the following operating conditions: 300 kvp at 20 ma; 180 r/min at 70 cm; inherent filtration, 4.75 mm of Be; added filtration 2 mm of Al; hvl, 0.47 mm of Cu.

*Freezing and thawing procedures.* All preparations which were frozen contained $10^8$ nucleated cells per ml suspended in the desired medium. One-ml aliquots were placed in glass ampules (1.2 ml volume) which were carefully sealed with a flame. Three conditions of freezing and thawing were tried. As will be shown, best results were achieved when the temperature of cell suspensions was decreased at the rate of 1°C/min from 4°C to —50°C (Slow-Freeze Unit, Canal Industrial Corp., Bethesda, Md.) followed by transfer to liquid nitrogen and storage at —196°C. Other conditions which were tried were: (a) decreasing the suspension temperature at the rate of 1°C/min to —50°C followed by storage at —50°C and (b) immediately immersing the suspensions in liquid nitrogen at —196°C followed by storage at this temperature. At the end of 1 hour of storage at the desired temperature, suspensions were always thawed quickly by placing them in a water bath at 37°C.

*Additives used to allay freezing damage.* Analytical grade glycerol (GLY) and dimethyl sulfoxide (DMSO, Matheson, Coleman & Bell, Norwood, Ohio) were employed as protective agents. Solutions, varying in concentration from 5.0% (v/v) to 30% were prepared in Tyrode's (containing 1% mouse serum). These solutions were then slowly added to equal volumes of mouse spleen cell suspensions containing $2 \times 10^8$ cells/ml so that the final preparations contained $10^8$ cells/ml and concentrations of additives ranging from 2.5% to 15%. Following the freezing and thawing cycle, the suspensions were diluted with 4 volumes of Tyrode's to provide preparations containing $2 \times 10^7$ cells/ml and concentrations of the additives low enough to be nonlethal for the mice into which they were injected. The dilutions with Tyrode's was carried out by slowly adding 0.5-ml portions to the cell suspensions at intervals of 2 minutes with frequent agitation because preliminary experiments showed that rapid dilution was detrimental to the cells owing possibly to osmotic shock.

*Results. Studies on toxicity of media for cells stored in an unfrozen state.* Before initiating experiments concerned with survival of cells maintained in frozen condition, it was necessary to determine the extent to which the additives, GLY and DMSO at various concentrations, might be toxic for antibody-producing cells. We first established that antibody-synthesizing cells could be maintained for up to 6 hours without loss of their activity when suspended in Tyrode's solution and stored in the refrigerator at ~6°C. It was then found that under the same conditions cells could be stored equally well when suspended in any of several concentrations of either GLY or DMSO (Table I). Thus, it

TABLE I. Antibody Production by Spleen Cells Stored at 6°C for up to 6 Hr in Varying Concentrations of GLY and DMSO.

| | GLY | | DMSO | |
|---|---|---|---|---|
| Medium conc (%) | Mean titers ($log_2$) | No. of samples | Mean titers ($log_2$) | No. of samples |
| -(Tyrode's) | 10.2 | 19 | 11.8 | 10 |
| 1.2 | 10.9 | 10 | 11.7 | 10 |
| 2.5 | 10.8 | 5 | 12.0 | 10 |
| 5.0 | 10.7 | 10 | 11.1 | 10 |
| 10.0 | 11.0 | 25 | 12.1 | 14 |
| 15.0 | 10.1 | 17 | 11.4 | 10 |

was possible to proceed directly to studies on the effects of freezing without concern as to possible complications resulting from either toxic effects of preservative media or decay of activity with time of *in vitro* storage.

*Evaluation of different rates of freezing and different storage temperatures.* It is known that most cells are more apt to survive freezing if they are cooled at a gradual rate of temperature decline and, after freezing, thawed rapidly(9,18,19). This also appears to be true of antibody-cell survival as shown in Fig. 1. In these initial experiments cells were suspended in each of 3 media: Tyrode's, 10% GLY, and 15% GLY. Portions of the various suspensions were either stored unfrozen at 6°C or frozen and stored under one of the conditions indicated in Fig. 1. The most satisfactory procedure was to decrease the temperature of the cell suspension at the rate of 1°C/min to —50°C. Recovery of activity was considerably greater when the cells were then stored for 1 hour at —196°C than at the higher temperature of —50°C.

*Comparison of GLY and DMSO as agents for allaying cell damage by freezing.* Of a number of substances which have been tested by various investigators for effectiveness in alleviating damage to mammalian cells by freezing, GLY and DMSO generally protect the best. Our results (Fig. 2) reveal a striking superiority of DMSO over GLY for protection of antibody-producing cells. In fact, as is shown in Fig. 2, concentrations of DMSO between 10% and 15% completely prevent loss of antibody-producing capacity of cells stored at —196°C for 1 hour. These preparations were, of course, frozen slowly (1°C/min to —50°C) and thawed rapidly. GLY was most effective at a concentration of 10% but even then less than 10% of antibody-forming potential was recovered. Either above or below (particularly above) 10% concentration GLY effectiveness sharply declined.

*Prolonged storage of cells in DMSO.* We have found that cells can be stored in 10% DMSO at —196°C for 135 days with no loss in their capacity to produce antibodies. The data are summarized in Table II.

TABLE II. Antibody Production by Spleen Cells Stored for Prolonged Periods in 10% DMSO at -196°C.

| Time of storage | Mean titer ($log_2$) | No. of samples |
|---|---|---|
| 0 | 11.3 | 15 |
| 1 hr | 11.2 | 10 |
| 1 day | 11.2 | 10 |
| 4 " | 10.9 | 11 |
| 8 " | 11.4 | 11 |
| 135 " | 11.6 | 15 |

*Discussion.* We have recently estimated that in the spleen of the optimally preimmunized mouse there is one potentially competent, anti-producing (PC) cell per test antigen in $10^5$ total spleen cells(4). Equating loss in antibody-forming capacity to cell destruction, it can be seen that, of the 200 PC cells ($2 \times 10^7$ spleen cells) injected into recipient mice, only about 18 (8.8%) survived freezing and thawing at the optimum GLY concentration of 10%. At GLY concentrations above 10% and below 5% less than 2 of the PC cells survived freezing. In contrast, DMSO concentrations of 10% to 15% permitted virtually all of the PC cells to survive. That there was no decline in recoverable activity of PC cells during prolonged storage in 10% DMSO at —196°C indicates that antibody-producing cells may be stored under these conditions indefinitely.

Our results do not entirely agree with those of other investigators who have studied methods for preservation of bone marrow cells. Thus Ferrebee *et al*(7) and Tran and Bender (9) reported that 15% concentration of GLY was most suitable for preservation of cells capable of alleviating irradiation mortality. Persidsky and Richards(12) reported marked toxicity of DMSO for bone marrow cells as judged by phase microscopic examinations and cell culture. One possibility is that cells of the antibody-producing series react differently to freezing and toxic factors than do bone marrow cells. Another possible cause of the discrepancies lies in the high variability inherent in methods employed by other investigators for assessment of functional survival (*e.g.*, protection from irradiation death, vital dye uptake) in comparison to the culturing and antibody-titration procedures.

Our data are not sufficient to draw definitive conclusions concerning the mechanisms by which spleen cells are injured during freezing and thawing or by which DMSO exerts its striking protective effect. Lovelock and Bishop(20) who originated the use of DMSO showed that it rapidly penetrates erythrocytes, a fact consistent with Lovelock's theory(21) that additives such as DMSO and GLY protect by virtue of their colligative properties, *i.e.*, that they protect by reducing the concentration of intra- and extracellular electrolytes at a given temperature. It has been pointed out elsewhere(22) that this hypothesis leads to the conclusion that rapid cooling should be less or no more harmful than slow cooling since rapid cooling would minimize the length of exposure to concentrated electrolytes. This was not true of spleen cells in 10% GLY; rapid cooling resulted in no functional survival whereas slow cooling did. This result is consistent with the view that slow cooling protects by minimizing the likelihood of intracellular ice formation(18,23). Nash(24) has suggested still another mechanism of protection, namely that it is related to the capacity of the additive to form hydrogen bonds. Our data provide no information on this possibility.

*Summary.* Methods for prolonged storage at low temperatures and recovery of antibody-producing cells from spleens of preimmunized mice were investigated. A method was devised which permits the cells to be stored with no decline in their capacity to synthesize antibodies upon subsequent *in vivo* culture. The method consists of suspending cells in 10% dimethyl sulfoxide, decreasing the temperature of the suspension gradually to —50°C followed by storage at —196°C. The extent to which antibody-producing cells are damaged by certain other freezing procedures was evaluated.

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