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1. Jeffries, C. D., J. Bacteriol. **86**, 1358 (1963).
2. Beck, L. V. and Linkenheimer, W., Proc. Soc. Exptl. Biol. Med. **81**, 291 (1952).
3. Grunert, R. R. and Phillips, P. H., Am. J. Physiol. **165**, 574 (1952).
4. Jocelyn, P. C., Clin. Chim. Acta **3**, 401 (1958).
5. Barron, E. S., Advan. Enzymol. **11**, 201 (1951).
6. Booth, J., Boyland, E., and Sims, P., Biochem. J. **79**, 516 (1961).
7. Goldstein, J. and Combes, B., J. Lab. Clin. Med. **67**, 863 (1966).
8. Ribi, E., Kelsey, M., and Perrine, T., J. Immunol. **82**, 75 (1959).
9. Holden, H. F., Biochem. J. **19**, 727 (1925).
10. Thompson, J. W. and Voegtlin, C., J. Biol. Chem. **70**, 793 (1926).
11. Henneman, D. H., Altschule, M. D., and Goncz, R. M., *in* "Glutathione" (S. Colowick, A. Lazarow, E. Racker, D. R. Schwartz, E. Stadtman, and H. Waelsch, eds.), p. 304. Academic Press, New York (1954).
12. Clark, W. G. and Geissman, T. A., J. Pharmacol. Exptl. Therap. **95**, 363 (1949).
13. Hořejsí, J. and Stěrba, O., Folia Haematol. **86**, 220 (1966).

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Freezing Human Peripheral Lymphocytes and a Technique for Culture in Monolayers (33471)

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Peripheral lymphocytes grown in culture undergo a blast-like transformation in the presence of specific antigens and substances such as phytohemagglutinin (PHA) and poke weed mitogen (1). Increasing understanding of the mechanism of this phenomenon is leading to new insights concerning the immune response and delayed hypersensitivity (2). Clinical and epidemiologic applications have been limited, however, because techniques are difficult and cumbersome (3).

Our laboratory has been involved in a series of experiments to facilitate the handling and culturing of lymphocytes. We present a relatively simple and inexpensive method for freezing lymphocytes to be cultured at a later date and for growing lymphocytes (which ordinarily attach poorly to smooth surfaces) in adherent monolayer cultures. By freezing lymphocytes we have greater flexibility in timing experiments, in repeating results, and most important in storing white blood cells from patients with rare and fatal diseases. The method of culturing cells in an adherent monolayer enables us to change fluids in the culture without centrifugation, to observe the cells in growth, and to harvest directly on the petri plates or cover slips minimizing manipulation of the fragile lymphocytes and eliminating the technical difficulties of preparation of slides with even distribution of cells.

In the experiments described below we used PHA, PPD, and vaccinia and measured stimulation by autoradiography employing tritiated thymidine.

Methods and Results. General. Peripheral blood was collected for all experiments in plastic disposable syringes which were prewet with 0.5 to 1.0 ml of sodium heparin. The syringe was placed upright from 60 to 90 min at 37° to permit the red cells to settle and the white cell-rich plasma was removed by the method of Mellman (4). The cell-rich plasma was then spun for 15 min at 1000 rpm and the supernatant plasma was removed and stored. The cells were resuspended in media and counted.

The medium used in all experiments was MEM[1] to which were added 2% glutamine and antibiotics. In most experiments the autologous plasma was used at a concentration of approximately 16%. When we did not use autologous plasma uninactivated fetal calf serum was substituted at a concentration of 20%. The total volume of all cultures was 3 ml containing 1,500,000 mononuclear cells. Except in the monolayer cultures described below the cultures were grown in screw cap tubes 16 × 125 mm which were maintained at 37.5° at a 15° angle in a humidified incubator with 5% CO_2. The day before harvesting (usually day 3 for PHA and day 7 for vaccinia and PPD) 0.1 ml of a solution containing 3 μCi of tritiated thymidine and 0.1 ml of a stock solution of polystyrene particles were added. To harvest the cultures the cells were agitated and those adhering to the sides were scraped free with a Pasteur pipette. A small amount of media was added (1 or 2 ml) and the culture tube spun at 800 rpm for 15 min. The supernatant was then removed carefully not to disturb the cell button. Slides were prewashed carefully with alcohol and subsequently coated with a 0.5% gelatin suspension by dipping at room temperature and air drying in an upright position. Using a Pasteur pipette the cell button was transferred to the slides, each culture making approximately two slides. The drop was smeared immediately with a bacterial wire loop. The cells were air dried and fixed in methanol for 3 min after which they could be stored at room temperature until processing.

To detect the autoradiographic tagging the following procedures were used. Coplin jars containing 25 ml of distilled water at 37° and 25 ml of emulsion were mixed in total darkness. Slides were dipped for 5 sec and allowed to dry in an upright position and then placed in a box with a dehydrating agent and wrapped in gauze. The box was then wrapped with tin foil so it was completely light free and kept in a refrigerator at approximately 4° for 6 days. Slides were then developed and fixed in complete darkness and subsequently were dehydrated and stained with azure-A Giemsa stain. Counts were made of labeled cells per 1000 mononuclear cells which did not contain polystyrene.

Freezing experiments. After centrifugation and removal of plasma as described above, the cells were resuspended in 3 ml of MEM which after gentle agitation was divided into 2 equal 1.5-ml aloquots. One aloquot was cultured immediately while the second was frozen for 3 hr and subsequently thawed and cultured. Techniques for freezing lymphocytes for purposes other than antigen transformation studies have been described (5–7). Our method did not differ greatly from those previously reported except that no automatic time controlling or recording devices were used. The cell suspension was carefully mixed to insure homogeneity and then 0.5 ml of the suspension was put into each of three 1.85-ml vials[2] which were then placed in a 50-ml beaker containing wet ice. After the vials were chilled 0.5 ml of a chilled 22% suspension of dimethylsulfoxide (DMSO) was added giving a final concentration of 11% DMSO. (Chilling limits the exothermic effect of DMSO.) The vials were then capped loosely, carefully dried, placed in a biological freezer[3] and inserted into a liquid nitrogen refrigerator[4]. The rate of freezing was controlled by adjusting the depth of the vials suspended in the vapor phase of the refrigerator. A formula for the optimum depth and duration for the specific number of vials to be frozen is provided by the manufacturer.[5] At the appropriate time the lymphocytes were completely immersed in the liquid nitrogen refrigerator for storage or transferred to a super freezer at —72°.

In order to limit the exothermic effect of DMSO in thawing the following technique was utilized. A 20-ml syringe was filled with MEM at 37°. One vial at a time was thawed; the vial was placed in a 50-ml beaker con-

[1] MEM, Flow Laboratories, 12601 Twinbrook Parkway, Rockville, Maryland 20852.

[2] Greener Scientific Corporation, New York.

[3] Linde BF-5, Linde Division, Union Carbide Corp. New York.

[4] Linde LR-35-9, Linde Division, Union Carbide Corp. New York.

[5] Linde BF-5-Operating Instructions

TABLE I. Comparison of Transformation in Cultures of Fresh and Frozen–Thawed Lymphocytes.

	PHA		Vaccinia		PPD		Control	
Donor	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
J.N.	31.0[a]	39.0	0.6	1.1	0.2	0.9	0.2	0.4
	10.3	24.8	0.6	0.1	0.0	0.1	0.0	0.6
M.H.	10.7	19.7	44.0	9.3			0.1	0.0
	32.0	43.0	5.6	6.4			0.0	0.0
	9.2	9.0	9.4	7.9			0.1	0.4
	17.0	16.0	10.0	1.5			0.1	0.2
	6.0	14.0	21.0	12.0			0.0	0.5
J.B.	59.0	32.0			3.0	16.0	0.1	0.6
	41.1	41.1			1.5	2.5	0.1	0.3
	6.0	21.0			0.5	1.7	0.0	0.5

[a] Percentage of tagged cells.

taining approximately 0.5 in. of water in a 37° water bath. As soon as thawing commenced, the liquid was aspirated back and forth into the syringe until all the cells were in suspension. If it was desirable to use the other vials they were treated in a similar fashion with the same syringe already containing the lymphocytes from the first vial. The material was then transferred to a 40-ml conical centrifuge tube and spun at 800 rpm for 15 min. The supernatant was removed quickly and the cells were resuspended in growth media. (We observed that cells tended to clump unless we resuspended quickly.) The cells were then counted and appropriate cultures were set up in parallel to the cultures prepared from the unfrozen portion of the specimen. With practice and careful attention to the above procedures we were able to recover virtually all the white cells.

In order to compare rates of transformation paired cultures of fresh and frozen–thawed lymphocytes were tested in parallel on the same day using PHA, vaccinia, PPD, and control cultures containing media alone. A total of 10 blood specimens were collected from 3 individuals on different days over a 4-month period and were tested on the day collected. As shown in Table I the rate of transformation of lymphocytes from a given individual varied more on different occasions than did the fresh, frozen–thawed pair inoculated on the same day. We do not know if the day by day variation we observed is a reflection of our own techniques or altered responsiveness on the part of the host. The phenomenon of fluctuating responses of a given individual's lymphocytes when repeatedly tested with the same antigen over a period of time has been reported by Oppenheim (8) who postulates an "adjuvant effect" as causing the variations.

In unstimulated control cultures significant tagging was not encountered. Highest rates of nonspecific transformation were noted in the frozen–thawed material but this was never more than 0.6% and did not influence the interpretation of results. In the PHA stimulated pairs the percentage of tagged cells among frozen–thawed lymphocyte cultures exceeded that found in the paired fresh cultures on 5 occasions, was identical once, and was less in 4 instances.

In vaccinia cultures the 2 specimens from J. N. were essentially negative both with fresh and frozen–thawed material. In one frozen–thawed culture 1.1% of cells transformed which approached significance since the control culture on the same day had only 0.4% transformation. All 5 culture pairs from M. H. contained significant tagging. Once, however, with frozen–thawed material only 1.5% of cells were tagged while 10.0% of the fresh cells reacted. Both J. N. and M. H. have been vaccinated in the past. We do not know why one should be consistently posi-

tive while the other negative; nor do we know if the response itself is to vaccinia viruses or to calf lymph or other additives in the product. This is currently under investigation in our laboratory.

J. N. is tuberculin negative while J. B. is tuberculin positive. In the PPD cultures of the former the results were negative on 2 occasions with fresh and frozen–thawed cells. In the 3 cultures of the latter the frozen–thawed material reacted more strongly than did the paired fresh cells. Once cells from a fresh culture had only 0.5% transformation. Since J. B. is tuberculin positive this result constitutes a false negative.

Growth of lymphocytes in monolayers. A method for growth of lymphocytes in monolayers was developed using petri dishes[6] or glass cover slips placed in petri dishes. Initially the petri dish or cover slip was coated by dipping briefly into a 0.5% suspension of gelatin in MEM with antibiotics. The surfaces were dried either at room temperature or in a 37° incubator. Techniques for preparing the cultures, the number of cells, amounts of additives and total volume were identical with those described above for tube cultures. (Recently we have modified the technique slightly to eliminate all centrifugation by placing 0.5 ml of the cell-rich plasma directly in the culture.) The cells settled gradually onto the bottom of the petri dish or cover slip placed in an ungelatinized petri dish and within 24 hr almost 100% of cells were firmly adherent. Microscopically they did not appear to be imbedded in the gelatin but were lying along the surface. After 24 hr the media was poured or aspirated off and the cells washed with little or no cell loss. It was our impression that the cells settled less well in the absence of autologous plasma since in several experiments in which the cultures were prepared with only fetal calf serum the cells did not precipitate but appeared to be agglutinated in the media. Therefore, we grew the cells in autologous plasma for the first 24 hr and then removed the plasma and added fresh media with 20% fetal calf serum and whatever virus, antigen, or blastogenic stimulant we were testing. This procedure was of particular value with PHA because the cells were evenly dispersed before they come into contact with PHA which has lymphocyte agglutinating properties. Therefore, we did not encounter large clumps of transformed cells which were difficult to count accurately in routine slides made from tube cultures. After several days some debris and dead cells were noted in the media above the monolayer. The viable cells however remained adherent through as many as 12 washings over an 8-day period.

The petri plate cultures in our humidified incubator tended to become dehydrated in 6–8 days. In addition, fungal contamination has been a problem. Recently we have added nystatin to our solutions and wrapped the petri plates in tin foil. These procedures have been effective in minimizing contamination and fluid loss.

Tritiated thymidine and polystyrene were added exactly as in tube cultures. The monolayers were harvested by simply removing the fluids and using the petri dish or cover slip in the same manner as the slides described above.

In order to compare rates of transformation in culture tubes and on gelatin treated petri plates a series of parallel tests were conducted. Typical results are shown on Table II with PHA, vaccinia stimulation, and control cultures. The range of transformation was similar by the two techniques.

Discussion. Our method for freezing peripheral lymphocytes using relatively inexpensive equipment contributes to the existing knowledge about lymphocytes by demonstrating that after thawing the cells are capable of transformation in the presence of specific antigens. Freezing of lymphocytes adds greatly to the ease and convenience in conducting experiments and should have immediate application to studies where banking of white blood cells from people with specific diseases or histocompatibility antigens is desired. Results were not identical between cultures from frozen–thawed or unfrozen lymphocytes, but the overall response rates were at least as high using frozen–thawed material. Most important was the fact that there were

[6] Falcon Plastics time culture dish, 35 × 10 ml.

TABLE II. Comparison of Transformation of Lymphocytes Grown in Culture Tubes and as Monolayers in Petri Plates.

	Phytohemagglutinin		Vaccinia		Control	
Test	Culture tube	Petri plates	Culture tube	Petri plates	Culture tube	Petri plates
1	36[a]	32			0.05	0.2
2			1.9	2.2	1.3	0.1
3	13	27	14.7	14.2	0.3	0.1

[a] Percentage of tagged cells.

no false negatives among cultures from frozen–thawed lymphocytes and in every instance in which a positive transformation response was anticipated it was encountered. It is probable that careful attention to minimize the exothermic effect of DMSO permitted our results with frozen–thawed lymphocytes to be more reproducible than those reported by some investigators (9).

The technique for growing lymphocytes in adherent monolayers greatly simplifies the preparation and harvesting of cells and permits us to treat the lymphocytes more gently since we totally avoid centrifugation. The advantage of being able to manipulate the cultures on different days with different products, and hopefully, to observe the responses as with ordinary tissue cultures may provide new methods for measuring and quantitating the lymphocyte transformation response. We have already had some success growing viruses in lymphocytes and detecting their presence by standard hemadsorption techniques. The similarity between the results in tube cultures and petri plates calls into serious question the concept that cell concentration and surface area are critical in achieving maximum response (10). The area on which the lymphocytes grew in petri plates was approximately 804.2 mm^2 while in our culture tubes it was approximately 29 mm^2.

Summary. A successful, relatively inexpensive method is described for freezing peripheral lymphocytes. These lymphocytes when thawed and grown in the presence of antigens transformed at approximately the same rate as unfrozen control cultures from the same individuals. A method is also described for successfully culturing lymphocytes which adhere to the surfaces of cover slips or petri plates previously treated with 0.5% gelatin. Rates of lymphocyte transformation in these cultures were similar to those encountered in culture tubes.

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1. Robbins, J. H., Science **146**, 1648 (1964).
2. Lawrence, H. S., Federation Proc. **27**, 3 (1968).
3. Coulson, A. S. and Chalmers, D. G., Nature **209**, 378 (1966).
4. Mellman, W. J., "Human Chromosome Methodology," Academic Press, New York (1965).
5. Albright, J. F., Makinodan, T., and Mazur, P., Proc. Soc. Exptl. Biol. Med. **114**, 489 (1963).
6. Flynn, R., Troup, G. M., and Walford, R. L., Interno. Arch. Allergy **29**, 478 (1966).
7. Perry, V. P., Kerby, C. C., and Gresham, R. B., Ann. N.Y. Acad. Sci. **114**, 651 (1964).
8. Oppenheim, J. J. Federation Proc. **27**, 21 (1968).
9. Kasakura, S. and Lowenstein, L., Proc. Soc. Exptl. Biol. Med. **125**, 355 (1967).
10. Moorhead, J. F., Connolly, J. J. and McFarland, W., J. Immunol. **99**, 413 (1967).

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