THE IN VITRO TRANSFORMATION OF FROZEN-STORED LYMPHOCYTES IN THE MIXED LYMPHOCYTE REACTION AND IN CULTURE WITH PHYTOHEMAGGLUTININ AND SPECIFIC ANTIGENS

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The in vitro transformation of human lymphocytes in the mixed lymphocyte reaction (MLR)\(^1\) and in culture with phytohemagglutinin (PHA) or specific antigen is a phenomena of increasing scientific interest and medical usefulness.

The transformation response of lymphocytes from patients with chronic lymphatic leukemia (1-9), acute leukemia (10, 11), chronic myelogenous leukemia (12), Hodgkin's disease (4,13, 14), lymphoma (13, 14), ataxia telangiectasia (15, 16), autoimmune diseases (17, 18), myasthenia gravis (19), renal failure (20, 21), and infectious mononucleosis (22) have all been evaluated. The MLR is being evaluated for the detection of tumor-specific antigens (23, 24). Alteration in the MLR following homograft rejection has been described (25-27). In many of these cases, it would be interesting to measure the response of lymphocytes before, during, and after the institution of therapy and/or the correction of the altered immune status.

Unfortunately, the lymphocyte culture systems are dependent on many variables (28, 29), and it is difficult to compare the results of one experiment to those obtained several days later. Ideally, one would like to have at the same moment, several samples of lymphocytes obtained at different times during the course of an illness or a procedure. These samples could then be placed in culture at the same time and the multiple variables could thus be controlled.

In order to develop such a system, we have evaluated the transformation of lymphocytes that have been frozen and stored at low temperatures. Lymphocytes frozen with a cryoprophylactic agent and stored at low temperatures will retain the ability to make antibodies (30) and they will maintain their antigenicity as measured by leukogglutination (31).

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\(^1\) The following abbreviations are used in this paper: DMSO, dimethyl sulfoxide; MLR, mixed lymphocyte reaction; PHA, phytohemagglutinin; R.P.M.I., Roswell Park Memorial Institute.
We have investigated the ability of lymphocytes that have been frozen and stored at low temperatures to respond to PHA, to specific antigen, and to react in the two-way MLR, and the one-way MLR as well as their antigenic stability as donors in the one-way mixed lymphocyte reaction.

**Methods and Materials**

**Freezing the Cells.**—Heparanized blood (1000 units per 60 ml of whole blood) was drawn by ventipuncture from normal human volunteers and was allowed to sediment in an inverted syringe at room temperature for approximately 2 hr. The leukocyte-rich plasma was aspirated and white blood cell counts were performed with a Model B Coulter counter using Cetrimide diluent (Fisher Scientific Company, Pittsburgh, Pa.). This plasma was then diluted with R.P.M.I. media 1640 containing 20% dimethyl sulfoxide (DMSO) to reach a final concentration of 50% plasma and 10% DMSO. The cells were then transferred to 150 ml plastic blood transfusion bags (FENWAL, Fenwal Laboratories, Morton Grove, Ill.). The bags were sealed and immediately placed in a Linde BF4-2 Biological Freezer (Union Carbide Corp., Linde Division, New York) slow freeze apparatus and frozen at a rate of −1°C per min to −60°C. The cells were then stored in the vapor phase of a liquid nitrogen freezer for various periods of time.

**Defrosting the Cells.**—The plastic bags were removed from the liquid nitrogen freezer and were immediately immersed in a 37°C water bath and thawed by gentle agitation. A volume of R.P.M.I. 1640 media equal to that volume already present was added to each bag, and after thorough mixing, the cells were centrifuged at 1000 rpm 0°C for 10 min. The supernate was decanted and the cells were resuspended in 50 ml of 1640 media with 15% pooled human serum. The cells were counted and the concentration was adjusted to 3 × 10^6 cells/cc in R.P.M.I. 1640 media with 15% human serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 300 μg/ml glutamine. The final DMSO concentration ranged from 0.05 to 0.2%.

**Preparation of PHA and Specific Antigens.**—5 ml of sterile saline was added to PHA-M (Difco, Inc., Detroit, Mich.) vial. This solution was then diluted 1:19 with R.P.M.I. 1640 media to obtain a 1:100 PHA-M solution. Tetanus toxoid solution (Wyeth Laboratories, Inc., Marietta, Pa.) was diluted 1:100 with R.P.M.I. media 1640. Second strength purified protein derivative (Parke, Davis & Co., Detroit, Mich.) tablets were dissolved with the manufacturer's diluent to a concentration of 0.05 mg/ml. This solution was then diluted with R.P.M.I. media 1640 to reach a final concentration of 2 μg/ml.

**Irradiating the Cells.**—The one-way MLR's were performed with 1.5 × 10^6 irradiated cells and 1.5 × 10^6 responding cells. The cells that were to be irradiated were given 1000 rads of ionizing radiation with a Theratron 80, cobalt 60 therapy unit (Atomic Energy of Canada Ltd., Ottawa, Canada) with a half-value layer of 10.2 mm of lead, an air exposure rate of 240 R per min at a treatment distance of 52 cm from the source.

**Cell Culture System.**—1 ml of cells was pipetted into quadruplicate sets of 16 X 150 mm sterile test tubes with Morton stainless steel caps. The final volume was raised to 1.5 ml by the addition of 0.5 ml of either R.P.M.I. media 1640, 1:100 PHA-M solution, 1:100 tetanus toxoid, or 2 μg/ml of PPD solution. The tubes were incubated at 5°C from the horizontal in a 5% CO₂, 95% air humid atmosphere at 37°C.

**Assay Procedures.**—After the appropriate culture period has elapsed, 0.5 ml of R.P.M.I 1640 media containing 2 μCi of tritiated thymidine (specific activity 1.9 Ci/mmmole [Schwarz Bio Research, Inc., Orangeburg, N. Y.]) was added to each tube. After 4 additional hr of culture, 8 ml of ice cold saline was added to three tubes of each set. These tubes were then centrifuged at 1500 rpm at 5°C for 15 min. The supernate was decanted and the pellets were frozen until they could be assayed for radioactivity.
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Slide preparations were made of the cells in the fourth tube of each set using the Shandon Cytocentrifuge (Shandon Scientific Co. Ltd., London, England). These slides were stained with Wright-Giemsa stain (Fisher Scientific Company, Pittsburg, Pa.). Cell differentials were performed on 700 cells of each sample. All mitosis, blast-like cells and plasma cells were classified as transformed cells. The 95% confidence limit of a differential count of 700 cells is 1.00–3.34% for as low as 2% of transformed cells.

The triplicate specimens were later defrosted, washed twice with 5 ml of 5% trichloroacetic acid, washed twice with 5 ml of absolute methanol, and dissolved in 1 ml of Hyamine (Packard Instrument Co., Inc. Downers Grove, Ill.). 14 ml of scintillation fluid (Liquiflour, New England Nuclear Corp. Boston, Mass.) was added to each tube and the radioactivity was assayed in a Model 4233 Packard Tri-Carb Scintillation Counter. A quench calibration curve derived from external standardization, was used to convert counts per minute to disintegrations per minute.

A background count per minute was subtracted from each sample. All of the triplicated samples were averaged and the standard error of the means was computed.

Results are reported as disintegrations per minute (dpm) ± standard error. Any two means greater than three average standard errors apart are significantly different at the 95% confidence level.

Experimental Design.—The white blood cells of normal human volunteers were frozen and stored weekly for 3 consecutive wk. The cells were all defrosted at the same time (2 wk after the last collection) and were placed in culture, along with cells freshly prepared on the day of the experiment.

The cells are numbered according to the week in which they were frozen. Thus, A1 was frozen wk 1 and stored for 4 wk, A2 was frozen on wk 2 and stored for 3 wk, and A3 was frozen wk 3 and stored for 2 wk. The fresh cells drawn on the day of the experiment (wk 5) were designated AF.

RESULTS

Cell Recovery.—After the cells were defrosted and washed, 52–97% of the cells originally frozen were recovered (Table I). The trypan blue viability of these cells ranged from 66 to 94%. There was no statistical difference in cell yield or viability between samples frozen at different times and stored for various periods. Most of the cells lost during the process were neutrophils.

The Mixed Lymphocyte Reaction.—Thawed cells were cultured with fresh cells in the one-way and two-way MLR. The sequence of thymidine incorporation of frozen cells in the MLR and in the PHA culture is shown in Fig. 1. The days of maximum activity (day 3–4 in PHA culture and 7 in the MLR) are the same as those usually observed in cultures of fresh lymphocytes. Thus, the PHA samples were assayed on the 4th day and the MLR and the specific antigen experiments on the 7th day.

When the frozen-stored cells were reacted with irradiated fresh cells in the one-way MLR they retained their ability to transform in each of 27 experiments. In every case the increase in thymidine incorporation was greater than 100 times the control value. The reproducibility of this reaction is demonstrated in Table II. The cells of three subjects, labeled A, B, and C were frozen once a week for 3 wk, defrosted on the 5th wk, and placed in culture with the irradiated
fresh cells of three other individuals labeled Xx, Yx, and Zx. Each population is compared to the other two frozen populations of the same donor as well as to freshly drawn cells labeled AF, BF, and CF.

**TABLE I**

*Cell Viability, Recovery and Differential Count*

|            | No. of Cells frozen (X 10^8) | No. of Cells recovered (X 10^4) | Trypan blue viability (post-thaw) | Differential cell count* |
|------------|-----------------------------|--------------------------------|-----------------------------------|--------------------------|
|            |                             |                                | %                                | Poly-morphs  | Lymphocytes  | Eosinophils  | Monocytes  |
| AF†        | 7.1                         | 3.3                            | 89.3                             | 32           | 12           | 1            |            |
| A1§        | 5.3                         | 4.6                            | 86.8                             | 5            | 77           | 17           | 1          |
| A2§        | 6.5                         | 6.4                            | 97.7                             | 8            | 71           | 19           | 2          |
| BF         |                            |                                |                                  |              |              |              |            |
| B1         | 5.9                         | 5.5                            | 92.4                             | 4            | 89           | 5            | 3          |
| B2         | 7.7                         | 4.7                            | 61.0                             | 4            | 84           | 7            | 5          |
| B3         | 6.6                         | 5.2                            | 78.0                             | 5            | 88           | 5            | 2          |
| CF         |                            |                                |                                  |              |              |              |            |
| C1         | 6.2                         | 4.2                            | 67.8                             | 11           | 82           | 6            | 1          |
| C2         | 5.3                         | 4.0                            | 75.5                             | 5            | 88           | 5            | 1          |
| C3         | 6.8                         | 4.9                            | 71.3                             | 6            | 85           | 7            | 2          |
| DF         |                            |                                |                                  |              |              |              |            |
| D1         | 4.5                         | 3.3                            | 73.3                             | 3            | 88           | 6            | 3          |
| D2         | 4.0                         | 2.6                            | 65.0                             | 7            | 87           | 5            | 1          |
| D3         | 4.6                         | 3.1                            | 67.4                             | 5            | 88           | 6            | 1          |
| EF         |                            |                                |                                  |              |              |              |            |
| E1         | 8.6                         | 4.5                            | 52.3                             | 8            | 86           | 5            | 1          |
| E2         | 9.9                         | 6.5                            | 64.8                             | 12           | 82           | 5            | 1          |
| FF         |                            |                                |                                  |              |              |              |            |
| F1         | 10.5                        | 6.8                            | 64.8                             | 11           | 86           | 1            | 2          |
| F2         | 16.0                        | 10.0                           | 62.5                             | 16           | 82           | 1            | 1          |

* Differential cell count, 700 cells.
† AF, freshly drawn cells.
§ A1–A3, cells frozen at different times, stored in liquid nitrogen and defrosted on day of experiment.

It can be seen that in every case, the degree of transformation of each of the three frozen populations of an individual subject was not statistically different. In seven of nine cases the degree of thymidine incorporation was also not statistically different. It should be further noted that although there was good correlation of the degree of transformation of the frozen to that of the fresh
cells, in almost every case the frozen cells incorporated more thymidine than the fresh cells.

In order to determine the stability of the antigenicity of frozen-stored cells, each of the thawed populations was irradiated and then cultured with unirradiated fresh cells (Table III). In all of these cases the degree of transformation induced by the irradiated frozen-stored cells from each individual was not

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**TABLE II**

*Ability of frozen cells to transform in the one-way MLR*

| Sample | Cells cultured alone 3 X 10⁶ cells | One-way MLR 3 X 10⁶ cells |
|--------|-----------------------------------|--------------------------|
|        | DPM ± #  | Trans. cells  | X*  | DPM ± #  | Trans. cells  | Y*  | DPM ± #  | Trans. cells  | Z*  | DPM ± #  | Trans. cells  |
|        |         |               |     |          |               |     |          |               |     |          |               |
| AF     |         |               |     |          |               |     |          |               |     |          |               |
| 118 ± 60 | <3    | 13,973 ± 4,600 | 54  | 171,140 ± 14,215 | 56  | 21,193 ± 2,661 | 50  |
| A11    | 218 ± 6 | <3    | 33,079 ± 4,268 | 70  | 131,425 ± 5,589 | 63  | 33,377 ± 3,546 | 46  |
| A12    | 390 ± 43| <3    | 37,200 ± 1,406 | 66  | 125,813 ± 12,843 | 59  | 32,190 ± 4,141 | 53  |
| A13    | 126 ± 74| <3    | 58,377 ± 1,876 | 67  | 75,563 ± 5,697  | 66  | 30,341 ± 1,900 | 49  |
| B1     | 301 ± 30| <3    | 52,721 ± 8,527 | 65  | 49,569 ± 3,629  | 65  | 19,501 ± 4,973  | 63  |
| B2     | 163 ± 202| <3    | 92,298 ± 1,840 | 71  | 116,774 ± 9,226 | 65  | 60,173 ± 11,470 | 59  |
| B3     | 494 ± 114| <3    | 80,286 ± 1,747 | 68  | 119,314 ± 10,966 | 69  | 49,198 ± 6,558  | 59  |
| 677 ± 59 | <3    | 114,729 ± 1,207 | 68  | 142,098 ± 19,203 | 68  | 52,405 ± 1,415  | 61  |
| C1     | 681 ± 380| <3    | 36,332 ± 4,878 | 33  | 463,616 ± 11,698 | 63  | 34,418 ± 2,826  | 44  |
| C2     | 204 ± 54 | <3    | 38,492 ± 5,160 | 34  | 400,099 ± 13,655 | 61  | 145,856 ± 6,044 | 41  |
| C3     | 242 ± 27 | <3    | 38,790 ± 8,501 | 36  | 487,083 ± 13,247 | 62  | 172,334 ± 19,830 | 49  |
| C4     | 166 ± 29 | <3    | 51,830 ± 1,090 | 39  | 523,469 ± 20,695 | 64  | 148,493 ± 14,086 | 45  |

| Cells cultured alone 3 X 10⁶ cells | 106 ± 76 | <3    | 110 ± 11 | <3    | 95 ± 23 | <3  |

* X*, Y*, Z*: irradiated cells of three different donors. (Drawn freshly on day of experiment.)

† AF, freshly drawn cells.

§ A1-A3, cells frozen at different times, stored in liquid nitrogen and defrosted on day of experiment.

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**Fig. 1.** The response of frozen-stored lymphocytes cultured with phytohemagglutinin and in the one-way MLR. AFro are cells that have been frozen and stored for one month. Bx are homologous cells that are freshly obtained and have received 1000 rads of ionizing radiation.
statistically different. Although the frozen cells induced the same amount of 
transformation that the fresh cells did, there was increased thymidine incorpora-
tion in the cultures containing the irradiated frozen cells.

A similar experiment was performed on the cells of three other subjects 
labeled D, E, and F that were cultured with three fresh populations in two-way 
MLR's (Table IV). In seven of nine two-way MLR's the degree of thymidine 
incorporation was not statistically different for each group of frozen cells. In all 

| Sample | Cells cultured alone 3 X 10^6 cells | One-way MLR 3 X 10^6 cells |
|--------|-----------------------------------|---------------------------|
|        | DPM ± SE | Trans. cells | DPM ± SE | Trans. cells | DPM ± SE | Trans. cells |
|        | %        |             | %        |             | %        |             |
| AFx    | 54 ± 20  | <3          | 55       | 1,903      | 46       | 1,945       |
| A1x§   | 55 ± 5   | <3          | 58       | 6,717      | 45       | 2,804       |
| A2x§   | 44 ± 7   | <3          | 34       | 5,181      | 40       | 7,588       |
| A3x§   | 41 ± 7   | <3          | 58       | 6,057      | 41       | 3,003       |
| BFx    | 58 ± 13  | <3          | 34,449   | 8,781      | 64       | 10,533      |
| B1x    | 49 ± 5   | <3          | 41,043   | 1,590      | 60       | 14,498      |
| B2x    | 33 ± 12  | <3          | 32,359   | 6,533      | 64       | 2,449       |
| B3x    | 69 ± 34  | <3          | 32,449   | 8,403      | 63       | 16,057      |
| CFx    | 95 ± 46  | <3          | 54,307   | 8,794      | 34       | 16,057      |
| Clx    | 20 ± 7   | <3          | 39,025   | 5,615      | 35       | 17,345      |
| C2x    | 29 ± 13  | <3          | 76,020   | 15,443     | 37       | 26,786      |
| C3x    | 44 ± 11  | <3          | 68,718   | 8,972      | 35       | 26,786      |

* X, Y, Z: freshly drawn cells of three different donors.
* AFx, freshly drawn irradiated cells.
* A1x-A3x, cells drawn at different times, frozen and stored, defrosted and irradiated on day of experiment.

experiments the degree of lymphocyte transformation was also not different 
within each group. Once again, the cultures containing frozen cells incorporated 
more thymidine than the fresh cell cultures, while both cultures showed the 
same degree of lymphocyte transformation.

It should be emphasized that all of the above MLR's contained one frozen 
population and one fresh population. When frozen lymphocytes are used as 
both partners in either one-way or two-way MLR's the reaction is greatly 
diminished (Table V).

Response to PHA.—The cells of 16 different individuals were cultured with 
PHA for 4 days (Table VI). In all cases the frozen-stored cells responded to 
PHA with a thymidine incorporation at least 100 times the control cultures.
The percentage of cells transformed is not statistically different for each population. In five of six cases the incorporation of thymidine is also not statistically different for each of the groups. It is again observed that in five of the six groups, the frozen-stored cells incorporate more thymidine than the fresh populations.

Response to Specific Antigen.—The cells of three individuals, known to be sensitized to PPD and tetanus toxoid, were used to test the reactivity of frozen cells to specific antigens (Table VII). It will be noted that in contrast to the previous experiments, the frozen-stored cells reacted very slightly or not at all while the fresh populations showed marked transformation and increased thymidine incorporation in the presence of specific antigens. It should be noted that when these frozen cells were cultured simultaneously with PHA and in the MLR, they transformed and incorporated increased thymidine in a manner similar to the cells in Tables II and IV.

Upon summarizing our data to this point, we were able to draw certain promising conclusions and we observed some interesting points that necessitated further experimental elucidation.

We observed that frozen cells respond reproducibly when cultured with...
PHA, as either responding cell or irradiated donor in the one-way MLR, or as one partner in the two-way MLR. The response of frozen cells cultured with specific antigens or used as both populations in the MLR was minimal.

We further observed that while fresh and frozen samples of lymphocytes cultured with PHA or placed in the MLR transformed to the same degree, the frozen cells usually incorporated more thymidine than the fresh cells.

In order to evaluate the marked inability of frozen cells to respond to specific antigen and as both partners in the MLR, the difference between the frozen cells and populations of fresh cells were examined. Aside from the unknown effect of the freezing process on the lymphocyte itself, there are two major differences between our samples of fresh and frozen cells. First, the frozen cells did have a small residual amount of DMSO (0.05-0.2%) in the supernate.

Secondly, the freezing process has long been known to effectively destroy neutrophils as measured by trypan blue viability and phagocytosis (32, 33). Indeed our postthaw cell differentials show a diminished percentage of neutrophils and a corresponding increased proportion of lymphocytes (Table I). Thus, our frozen populations have an increased percentage of lymphocytes and a diminished number of neutrophils with little or no functional activity left.

The Effect of DMSO and Transformation.—Freshly obtained cells were cultured in DMSO in concentrations of from 0.01 to 3.0%. As seen in Fig. 2, increasing concentrations of DMSO caused increasing inhibition of PHA stimulation. Over the range of DMSO present in our frozen cultures (0.05-0.02%) this inhibition was approximately 5-25%. DMSO had a similar effect on the MLR and response to specific antigen.

The Effect of Cell Differential.—Because of our frozen cell populations contain a high percentage of lymphocytes and because of the known deleterious effect

| TABLE V |
| MLR—Frozen Cells one Population vs. Frozen Cells Both Populations |
| Frozen cells one partner* | Frozen cells both partners | Cells cultured alone |
| Two-way MLR | Two-way MLR | A 801 ± 125 |
| A$_{Fro}$ + B 61,090 ± 3,899 | A$_{Fro}$ + B$_{Fro}$ 8,540 ± 880 | A$_{Fro}$ 313 ± 88 |
| A + B$_{Fro}$ 43,629 ± 8,284 | | B 538 ± 53 |
| One-way MLR (reactant and irradiated donor) | One-way MLR (reactant and irradiated donor) | |
| A$_{Fro}$ + B 25,267 ± 3,389 | A$_{Fro}$ + B$_{Fro}$ 975 ± 207 | A$_{Fro}$ 132 ± 18 |
| B$_{Fro}$ + A 39,222 ± 1,415 | B$_{Fro}$ + A$_{Fro}$ 8,340 ± 1,021 | A$_{Fro}$ 115 ± 29 |
| One-way MLR (irradiated donor) | | B$_{Fro}$ 122 ± 5 |
| A + A$_{Fro}$ 27,497 ± 1,427 | | B$_{Fro}$ 121 ± 8 |
| B + A$_{Fro}$ 66,090 ± 7,054 | | |

* A$_{Fro}$, frozen-stored cells; A, fresh cells; A$_{irr}$, irradiated cells.
of freezing on the neutrophil, we hypothesized that our frozen samples were reacting like purified populations of lymphocytes. We therefore examined the kinetics of cell cultures containing various proportions of lymphocytes. Since the reactivity of purified lymphocytes in culture with specific antigen and as

| Sample | Cells cultured alone 3 x 10⁶ cells | Cells plus PHA 3 x 10⁶ cells |
|--------|----------------------------------|-----------------------------|
|        | DPM ± se | Transformed cells | DPM ± se | Transformed cells |
| AF*    | 224 ± 33 <3 | 36,598 ± 4,135 | 67 |
| A1‡    | 151 ± 10 <3 | 221,822 ± 13,615 | 69 |
| A2‡    | 228 ± 7 <3 | 172,837 ± 5,067 | 72 |
| A3‡    | 234 ± 25 <3 | 160,886 ± 3,253 | 66 |
| BF     | 347 ± 18 <3 | 117,740 ± 6,176 | 66 |
| B1     | 291 ± 15 <3 | 173,435 ± 9,887 | 68 |
| B2     | 380 ± 51 <3 | 200,954 ± 15,726 | 64 |
| B3     | 255 ± 33 <3 | 208,570 ± 16,600 | 63 |
| CF     | 323 ± 21 <3 | 44,106 ± 6,952 | 59 |
| C1     | 194 ± 9 <3 | 104,385 ± 9,631 | 63 |
| C2     | 232 ± 45 <3 | 110,838 ± 9,060 | 62 |
| C3     | 157 ± 18 <3 | 122,268 ± 8,394 | 63 |
| DF     | 367 ± 122 <3 | 67,070 ± 12,820 | 85 |
| D1     | 366 ± 47 <3 | 407,140 ± 8,279 | 80 |
| D2     | 379 ± 44 <3 | 298,350 ± 45,000 | 83 |
| D3     | 272 ± 10 <3 | 326,197 ± 19,982 | 84 |
| EF     | 358 ± 117 <3 | 116,058 ± 8,253 | 91 |
| E1     | 321 ± 31 <3 | 369,401 ± 31,942 | 86 |
| E2     | 209 ± 31 <3 | 347,277 ± 32,478 | 87 |
| FF     | 692 ± 91 <3 | 282,939 ± 6,239 | 85 |
| F1     | 455 ± 53 <3 | 235,548 ± 5,523 | 82 |
| F2     | 229 ± 35 <3 | 167,350 ± 16,692 | 81 |

* AF, freshly drawn cells (plus PHA).
† A1–A3, cells frozen on different days, stored and defrosted on day of experiment. (Plus PHA.)

both populations of the MLR have been described (34, 35), we concentrated our analysis on the relationship between the percentage of transformed cells and the thymidine incorporation, as well as the effect of the manipulation of only one partner of the MLR.
TABLE VII  
Response of Frozen Cells to Specific Antigen

|       | Cells cultured alone | Cells plus tetanus toxoid | Cells plus tubercle bacillus purified protein derivative |
|-------|-----------------------|---------------------------|--------------------------------------------------------|
|       | DPM ± se | Trans. cells | DPM ± se | Trans. cells | DPM ± se | Trans. cells |
| D1*   | 349 ± 111 | <3         | 308 ± 199 | <3         | 208 ± 113 | <3         |
| D2*   | 288 ± 178 | <3         | 174 ± 63  | <3         | 219 ± 126 | <3         |
| D3*   | 213 ± 49  | <3         | 193 ± 19  | <3         | 172 ± 59  | <3         |
| DF†   | 809 ± 351 | <3         | 122,032 ± 16,891 | 44 | 39,135 ± 1,310 | 49 |
| E1    | 327 ± 156 | <3         | 183 ± 130 | <3         | 3,345 ± 145 | 15         |
| E2    | 298 ± 86  | <3         | 130 ± 5   | <3         | 1,580 ± 105 | 11         |
| E3    | 245 ± 89  | <3         | 134 ± 16  | <3         | 442 ± 246  | <3         |
| EF    | 1,113 ± 301| <3         | 8,745 ± 1,015 | 45 | 266,913 ± 39,725 | 57 |

F1    | 213 ± 11  | <3         | 112 ± 14  | <3         | 309 ± 107  | <3         |
| F2    | 273 ± 113 | <3         | 211 ± 98  | <3         | 222 ± 138  | <3         |
| F3    | 347 ± 207 | <3         | 176 ± 35  | <3         | 481 ± 45   | <3         |
| FF    | 509 ± 25  | <3         | 578,386 ± 16,178 | 59 | 587,769 ± 56,824 | 58 |

*= D 1, 2, 3: cells frozen at different times, stored in liquid nitrogen, and defrosted on day of experiment.
†DF, freshly drawn cells (donor sensitized to PPD and tetanus toxoid).

Fig. 2. The effect of dimethyl sulfoxide (DMSO) on the response of lymphocytes to phytohemagglutinin.
In order to manipulate cell population differential count, relatively pure populations of lymphocytes were obtained as follows:

40 ml of heparinized normal human blood was placed in 40 ml test tubes and centrifuged at 400 rpm (50 g) at 10°C for 15 min. The supernate was removed with a 10 ml pipette to the junction of the plasma and erythrocytes. This plasma consistently contained 95-99% pure lymphocytes. These lymphocytes were adjusted to a concentration of $3 \times 10^6$ cells per ml and mixed in various ratios with an equal concentration of cells from the same donor that were obtained by the sedimentation method described above. The different samples were then cultured simultaneously with PHA and in the MLR.

TABLE VIII

The Effect of Increasing the Percentage of Lymphocytes in the Irradiated Population of the One-way MLR

| Lymphocytes in reactant population | Lymphocytes in irradiated population | MLR | Transformed lymphocytes |
|-----------------------------------|--------------------------------------|-----|-------------------------|
| %                                 | %                                    | DPM ± SE | %                       |
| Experiment 1                      |                                      |       |                         |
| 41                                | 30                                   | 72,068 ± 9,353 | 25                     |
| 41                                | 58                                   | 158,845 ± 3,345 | 27                     |
| 41                                | 74                                   | 30,711 ± 4,139 | 26                     |
| 41                                | 95                                   | 20,402 ± 1,897 | 22                     |
| Experiment 2                      |                                      |       |                         |
| 26                                | 28                                   | 154,031 ± 26,205 | 56                     |
| 26                                | 60                                   | 275,220 ± 29,895 | 54                     |
| 26                                | 87                                   | 217,901 ± 18,500 | 51                     |
| 26                                | 98                                   | 208,346 ± 16,742 | 47                     |
When the percentage of lymphocytes was increased in PHA-stimulated cultures, the thymidine incorporation increased while the percentage of lymphocytes transformed remained statistically the same (Fig. 3). Thus it appears that the PHA is stimulating a certain constant percentage of lymphocytes and that since the purified cultures contain more lymphocytes there is a greater absolute number of transforming lymphocytes in these cultures and hence a greater over-all incorporation of thymidine. Similar results were obtained when purified lymphocytes were cultured with nonpurified irradiated cells in the one-way MLR.

Of additional interest is the observation that when the percentage of irradiated lymphocytes in a one-way MLR is increased, the unirradiated non-purified cells will incorporate more thymidine (Table VIII). This response reaches a maximum when the percentage of irradiated lymphocytes is approximately twice the usual value and may then reach a plateau or actually decline.

DISCUSSION

Transformation of lymphocytes is a multifaceted technique applicable to many aspects of immunology. The ability to store cells and to preserve their ability to transform does overcome some of the limitations of the in vitro method and opens new paths of investigation.

One of the major stumbling blocks to the practical application of lymphocyte transformation has been the numerous variables associated with the culture system. Small alterations in the number of lymphocytes, the concentration of serum, the pH of media, the oxygen and carbon dioxide content of the atmosphere, the temperature and humidity of the incubator, the concentration of activity of the isotope, and slight variations of the assay procedure can all effect the final result (28, 29). Thus, it has been extremely difficult to compare the results obtained in one experiment to those of another.

By freezing lymphocytes and storing them until all populations needed for comparison are obtained, one can place all of the cells in culture at the same time and thus control many of the variables.

Our evaluation of this approach does appear to present some promising results. Frozen-stored lymphocytes transform remarkably consistently when cultured with PHA or as one partner in the one-way or the two-way MLR. They also induce reproducible transformation when irradiated and cultured with fresh cells. The amount of thymidine incorporated by the stimulated frozen cells is not as reproducible as the percentage of transformation. (Of 31 triplicate groups of MLR's and PHA cultures, one of the values in the group was statistically different from the other two values in eight groups. In no triplicates were all three values statistically different from each other.) The percentage of transformed lymphocytes of a frozen population may be reliably compared to an autologous fresh population.
The disparity between the thymidine incorporation of fresh and frozen cells and the inability of the frozen cells to react to specific antigen or as both partners in the MLR deserve further comment. The small concentration of DMSO present in the thawed cell cultures may cause some inhibitions of reactivity, but we have been unable to show either enhancement or complete inhibition at these levels. Both of these phenomena are more probably related to the increased number of lymphocytes in our frozen-stored culture and the concurrent decrease in the number and loss of function of polymuclear cells and macrophages.

Oppenheimer et al. (34, 35) noted that column separated lymphocyte pure populations transformed the same as unpurified cells to optimum doses of PHA, but that the lymphocyte response to specific antigen and the MLR was diminished. However, Gordon (36) noted that the diminished reaction of purified lymphocytes in the one-way MLR could be restored by the addition of mitomycin treated donor macrophages.

Of additional interest is the finding (Fig. 3) that the over-all thymidine incorporation of PHA-stimulated and MLR culture increases as the percentage of reactant lymphocytes is increased. Since the percentage of transformed lymphocytes remains constant, it seems that there are just more cells reacting in cultures with a higher proportion of lymphocytes and thus there is greater over-all DNA synthesis.

Apparently, neutrophils or macrophages perform an important function in the MLR and the response to specific antigens (possibly the processing of antigen). The freezing process effectively destroys this ability but the frozen cells, like purified lymphocytes, can use neutrophils of irradiated donors in the MLR to restore this reactivity. Once this activity is restored by the presence of just a few neutrophils the over-all activity of a culture may be related to the number of lymphocytes present.

Our finding that more pure populations of irradiated lymphocytes induce more thymidine incorporation in the one-way MLR is similar to those findings communicated in the reports of Bach and coworkers (37, 38) in which increasing numbers of mitomycin-treated cells were added to a constant number of responding cells. Those investigators observed increasing activity as the number of stimulating cells was increased. Although the response was variable, this increased activity most often reached a peak when the proportion of responding cells to stimulating cells was about 1:2 or 1:3. This ratio is approximately that noted in our experiments with irradiated lymphocytes (Table VIII) and also is approximately the proportion of lymphocytes in the frozen, compared to the fresh, populations (Table 1).

The practical applications of transformation of frozen lymphocytes are numerous. Alterations in the ability to respond in the MLR or to PHA may reflect alterations in the immune status and may be a useful index of the status
of a disease or the evaluation of immunosuppressive therapy. Frozen cells are currently being used for histocompatibility testing with cytotoxicity assays (39). The adaption of the MLR of frozen cells may enhance the value of this assay of histocompatibility. Lymphocytes of potential recipients and/or donors could be frozen and stored in a central typing bank for future cross-matching. Changes in the reactivity of serially frozen lymphocytes may help predict impending homograft rejection.

Our evaluation of the transformation of frozen-stored human lymphocytes indicates that this is a practical technique and that it probably warrants further investigation and practical application.

SUMMARY

We have investigated the in vitro transformation of lymphocytes that have been frozen and stored at low temperatures. Cells frozen at different times and stored for various times do transform in a reproducible manner when placed in culture with PHA or as one population of the two-way or the one-way MLR.

When frozen-stored lymphocytes are cultured with specific antigen or as both partners in the MLR the response is minimal.

The freezing process destroys neutrophils, and the remaining population transforms in a manner similar to cultures of purified lymphocytes.

Practical aspects of the technique are discussed and we have suggested possible practical applications for future investigation.

BIBLIOGRAPHY

1. Astaldi, G., G. Costa, R. Airo, and N. Duarte. 1965. Lymphocytes from leukemic blood cultured with phytohemagglutinin. *Eur. J. Cancer*. 1:259.
2. Bernard, C., A. Geraldes, and M. Boiron. 1964. Effects of phytohaemagglutinin on blood cultures of chronic lymphocytic leukaemias. *Lancet*. 1:667.
3. Bouroncle, B. A., K. P. Clausen, and J. F. Aschenbrand. 1969. Studies of the delayed response of phytohemagglutinin (PHA) stimulated lymphocytes in 25 chronic lymphatic leukemia patients before and during therapy. *Blood J. Hematol.* 34:166.
4. Holm, G., P. Perlmann, and B. Johansson. 1967. Impaired phytohaemagglutinin-induced cytotoxicity in vitro of lymphocytes from patients with Hodgkin's disease or chronic lymphatic leukaemia. *Clin. Exp. Immunol.* 2:351.
5. Oppenheim, J. J., J. Whang, and E. Frei, III. 1965. Immunologic and cytogenic studies of chronic lymphocytic leukemic cells. *Blood J. Hematol.* 26:121.
6. Schrek, R. and Y. Rabinowitz. 1963. Effects of phytohemagglutinin on rat and normal and leukemic human blood cells. *Proc. Soc. Exp. Biol. Med.* 113:191.
7. Sharman, C., P. E. Crossen, and P. H. Fitzgerald. 1966. Lymphocyte number and response to phytohaemagglutinin in chronic lymphocytic leukaemia. *Scand. J. Haematol.* 2:375.
8. Thomson, A. E. R., M. A. Robinson, and G. Wetherley-Mein. 1966. Heterogeneity of lymphocytes in chronic lymphocytic leukaemia. *Lancet*. 2:200.
9. Winter, G. C. B., D. G. Osmond, J. M. Yoffey, and D. J. Mahy. 1964. Leucocyte cultures with phytohaemagglutinin in chronic lymphatic leukaemia. *Lancet.* 2: 563.
10. Astaldi, G., L. Massimo, R. Airo, and P. G. Mori. 1966. Phytohaemagglutinin and lymphocytes from acute lymphocytic leukemia. *Lancet.* 1:1265.
11. Kourilsky, F. M., L. Lovric, and A. Levacher. 1966. Phytohaemagglutinin and lymphocytes from acute leukaemia. *Lancet.* 2:856.
12. Elves, M. W., and J. F. Wilkinson. 1963. The effects of phytohaemagglutinin on normal and leukaemic leucocytes when cultured in vitro. *Exp. Cell Res.* 30:200.
13. Elves, M. W. 1968. The effect of PHA on lymphocytes from subjects with leukaemias and other diseases of reticulo-endothelial system. In *Biological Effects of Phytohemagglutinin.* M. W. Elves, editor. W. F. Crane, Ltd., Oswestry, England. 169.
14. Hersh, E. M., and J. J. Oppenheim. 1965. Impaired in vitro lymphocyte transformation in Hodgkin's disease. *N. Engl. J. Med.* 273:1006.
15. Leikin, S. L., M. Bazelon, and K. H. Park. 1966. In vitro lymphocyte transformation in ataxia telangiectasia. *J. Pediat.* 68:477.
16. Oppenheim, J. J., M. Barlow, T. A. Waldman, and J. B. Block. 1966. Impaired in vitro lymphocyte transformation in patients with ataxia telangiectasia. *Brit. Med. J.* 2:330.
17. Hashem, N., and D. H. Carr. 1963. Mitogenic stimulation of peripheral lymphocyte cultures by autologous lymphocyte extracts in autoimmune diseases. *Lancet.* 2:1030.
18. LaPolla, J. J., and T. F. Thurmon, III. 1966. Lymphocyte transformation in autoimmune disorders: A preliminary study. *J. Pediat.* 69:1129.
19. Housley, J., and J. J. Oppenheim. 1967. Lymphocyte transformation in thymectomized and nonthymectomized patients with myestenia gravis. *Brit. Med. J.* 2:569.
20. Elves, M. W., M. C. G. Israels, and M. Collinge. 1966. An assessment of the mixed leukocyte reaction in renal failure. *Lancet.* 1:682.
21. Kasakura, S., and L. Lowenstein. 1967. The effect of uremic blood on mixed leukocyte reactions and on cultures of leukocytes with phytohemagglutinin. *Transplantation.* 5:283.
22. Epstein, L. B., and G. Brecher. 1965. DNA and RNA synthesis of circulating atypical lymphocytes in infectious mononucleosis. *Blood J. Hematol.* 25:197.
23. Fridman, W. H., and F. M. Kourilsky. 1969. Stimulation of lymphocytes by autologous leukemic cells in acute leukemia. *Nature* 224:277.
24. Kanner, S. P., R. J. Mangi, and M. R. Mardiney, Jr. 1970. The mixed lymphocyte reaction as a method of determining antigenic differences between host and tumor cells. *Proc. Amer. Ass. Cancer Res.* 11:42.
25. Hirschhorn, K., F. Bach, F. T. Rapaport, J. M. Converse, and H. S. Lawrence. 1964. The relationship of in vitro lymphocyte compatibility to homograft sensitivity in man. *Ann. N.Y. Acad. Sci.* 120:303.
26. Oppenheim, J. J., J. Whang, and E. Frei, 3rd. 1965. The effect of skin homograft rejection on recipient and donor mixed leukocyte cultures. *J. Exp. Med.* 122:651.
27. Wilson, D. B., W. K. Silvers, and P. C. Nowell. 1967. Quantitative studies on the mixed lymphocyte interaction in rats (II). *J. Exp. Med.* 126:655.

28. Moorhead, J. F., J. J. Connolly, and W. McFarland. 1967. Factors affecting the reactivity of human lymphocytes in vitro. *J. Exp. Zool.* 169:161.

29. Wilson, D. B. 1966. Analysis of some of the variables associated with the proliferative response of human lymphoid cells in culture. *J. Exp. Zool.* 162:161.

30. Stoner, R. D., and V. P. Bond. 1963. Antibody formation by transplanted bone marrow, spleen, thymus, and lymph node cells in isologous, homologous, and heterologous recipients. *Blood J. Haematol.* 21:772.

31. Cohen, E., and A. W. Rowe. 1965. Detection of leukocyte with dimethylsulfoxide (DMSO) protected leukocytes frozen with liquid nitrogen. *Vox Sang.* 10:543.

32. Rowe, A. W., and E. Cohen. 1965. Phagocytic activity and antigenic integrity of leukocytes preserved with dimethyl sulfoxide at a cryogenic temperature (−196°C). *Vox Sang.* 10:382.

33. Bouroncle, B. A. 1967. Preservation of human normal and leukemic cells with dimethyl sulfoxide at −80°C. *Cryobiology.* 3:445.

34. Oppenheim, J. J., E. M. Hersh, and J. B. Block. 1968. The response to various stimuli of human peripheral lymphocytes which have been purified through glass bead columns. In *Biological Effects of Phytohemagglutinin.* M. W. Elves, editor. W. F. Crane, Ltd., Oswestry, England. 183.

35. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. *J. Immunol.* 101:262.

36. Gordon, J. 1968. Role of monocytes in the mixed leukocyte culture reaction. *Proc. Soc. Exp. Biol. Med.* 127:30.

37. Albertini, R. J., and F. H. Bach. 1968. Quantitative assay of antigenic disparity at HL-A—The major histocompatibility locus in man. *J. Exp. Med.* 128:639.

38. Bach, F. H., and N. K. Voynow. 1966. The one-way stimulation in mixed leukocyte cultures. *Science.* 153:545.

39. Terasaki, P. I., D. L. Vredevoe, and J. D. McClelland. 1965. Serotyping for homotransplantation VI use of cryogenically stored cells for purification of antisera. In *Histocompatibility Testing.* Munksgaard, Copenhagen. 267.