The Acridine Orange Viability Test Applied to Bone Marrow Cells. Correlation with Trypan Blue and Eosin Dye Exclusion and Tissue Culture Transformation

By William E. Hathaway, Louis A. Newby and John H. Githens

Many methods have been used to assay the viability of hematopoietic cells during the course of procurement, preservation, and transplantation of animal and human tissue. Tritiated thymidine is frequently used as a cell marker in the study of hematopoietic proliferation. In vitro DNA synthesis has been reported as an index of viability. Growth and transformation of living cells in tissue culture also indicate viability. Determination of cell motility is used to detect living cells. The resistance of viable cells to staining with trypan blue and eosin is probably the most frequently used technic. The problems and limitations encountered in determining cell viability by these and other in vitro methods have recently been reviewed. In the field of bone marrow transplantation the most precise measurement of viability is the ability of the tissue to transplant, proliferate, and protect a lethally irradiated host. Because this in vivo method is not feasible prior to transplantation with preserved human tissue, there is a need for a rapid and simple in vitro estimation of cell viability. Following the suggestion of Pegg that the acridine orange (A-O) staining reaction may be correlated with the viability of bone marrow cells after preservation by freezing, the following studies were done in order to establish an “A-O viability test of marrow cells.”

Methods

Unless otherwise indicated, the following conditions were carefully observed throughout the experiment.

Preparation of Marrow

The marrow was obtained from the femurs of freshly killed Balb/c mice and suspended in Puck’s tissue culture media (N-16 40 percent, saline F 30 percent, fetal calf serum 30 percent) as single cells at an approximate concentration of 40–50 x 10⁶ per ml. This suspension was observed immediately after preparation and is called “fresh marrow.” All other cell suspensions were at a similar concentration.

Frozen marrow samples were prepared by suspending marrow cells in Puck’s tissue culture media after passage through a 25 gauge needle to reduce particles to single cells at a concentration of approximately 50 x 10⁶ per ml. Glycerol or dimethylsulfoxide (DMSO) was added prior to freezing in sealed 2 ml. glass ampules. The samples were frozen in a CO₂-95 percent ethanol bath by slowly reducing the temperature 1°C per minute to −40°C and then rapidly to −79°C. The vials were then immersed in liquid nitrogen.

This work was supported by American Cancer Society Grants T1385 and T255.

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Submitted Sept. 4, 1963; accepted for publication Oct. 29, 1963.

Blood, Vol. 23, No. 4 (April), 1964
Methods for Producing Injury to Cells

Aliquots of "fresh marrow" were treated in the following ways in order to note the effects of various injurious agents on the staining characteristics with acridine orange.

a. pH—the pH of the cell suspensions were changed to 8.9 by addition of 0.1N sodium hydroxide and to 6.0 by addition of 0.1N hydrochloric acid. After incubation at 37 C. for 40 minutes, the cells were resuspended in fresh Puck's media and observed.

b. Heat—Cell suspensions were heated to 60 C. for 30 minutes and observed.

c. Rapid freezing—Cell suspensions were frozen quickly by placing them in a freezer at −20 C. without the use of a protective agent such as DMSO or glycerol. The cells were then rapidly thawed and observed.

d. Aging of cells without tissue culture media or serum—Bone marrow cells were allowed to stand in a balanced salt solution (saline F) for 6 hours at room temperature, and periodic observations were made. The depletion of serum or tissue culture media from the suspending fluid for long periods decreases the protective ability of bone marrow cells being prepared for transplantation.

e. Poison—0.25 ml. of a 700 mg. per cent solution of sodium cyanide was added to 1.5 ml. of marrow cell suspension for 1 hour at 37 C.

Viability Test

a. Acridine orange: One drop of a murine marrow suspension was placed on a clean microscope slide and mixed with a second drop of acridine orange suspension. A number 0 coverslip was then applied with firm pressure to produce a layer of single cell thickness. The edges were sealed with petrolatum or Kronig's cement. The acridine orange dye (National Aniline Lot 1411p) was prepared fresh every 2 weeks as a 9 mg./100 ml. suspension in Puck's media buffered with tris(hydroxymethyl)-amino methane to pH 7.4. The slide-cover slip preparation was observed with fluorescent microscopy at 25–27 C. in a partly darkened room and the necessary differential count or photograph made within 1 to 2 minutes of fluorescence per field.

The microscope was a Leitz Ortholux with the Osram HBO 200 mercury vapor lamp as the light source. Two (UGI) exciter filters, one 4 mm., the other 2 mm., were used with a Blau-absorption filter (Leitz). Differential cell counts of the supravital preparations were done with 430X and 970X magnification. Color transparencies were produced with 35 mm. Ektachrome, type B, color film with an exposure index of tungsten 100 and exposure times of 30 to 60 seconds.

b. Eosin and trypan blue: Differential counts were done in the same manner (one drop of cell suspension plus one drop of dye) under light microscopy with 1 per cent and 0.1 per cent eosin Y and 0.05 per cent and 0.02 per cent trypan blue (in Puck's media at pH 7.4). The percentage of unstained cells (viable cells) was determined. The preparations with eosin Y were examined within 5 minutes in order to avoid possible photodynamic cytotoxicity.

c. Tissue culture transformation (TCT): A semiquantitative estimation of growth in tissue culture was devised following the suggestion of Porterfield and Ashwood-Smith. Aliquots of each specimen were plated on cover slips in Petri dishes at cell concentration of 12 x 10⁶ per ml. When frozen specimens were used, the DMSO was reduced to 2½ per cent. Puck's culture media and a 5 per cent CO₂—95 per cent air incubator (37 C.) were used. After 4 days, the cover slips were removed, air-dried, and stained with Wright's stain. The average number of "fibroblastic" or large "mononuclear" cells per h.p.f. (average of 25 random fields) was recorded. The control was fresh murine marrow in Puck's media or 2½ per cent DMSO in Puck's media at the same cell concentration (see figure 1).
ACRIDINE ORANGE TEST AND BONE MARROW CELLS

Fig. 1.—Above, photograph of Wright's stained cover slip demonstrating the tissue culture transformation into "fibroblasts" and "mononuclear cells" of fresh murine bone marrow cultured for 4 days. Below, photograph demonstrating poor tissue culture transformation of bone marrow cells treated with sodium cyanide prior to 4-day culture.
RESULTS

With the factors of dye concentration, cell concentration, pH of the suspending media, length of exposure to ultraviolet light, and thickness of the preparation carefully controlled, a uniformly reproducible appearance of fresh murine bone marrow cells was obtained. The fresh, "viable," supravitally stained nucleated cell displayed a bright, apple-green nucleus, and pale, dull-green cytoplasm with varying amount of bright orange cytoplasmic granulations present. With experience, a conventional differential count based on the usual morphologic characteristics could be done even at 430X magnification. In general, the supravital staining properties of mouse bone marrow\textsuperscript{17} cells are the same as for human marrow,\textsuperscript{18} but decidedly different from fixed preparations\textsuperscript{19,20} of either mouse or human marrow.

By comparing the acridine orange staining characteristics with the degree of viability demonstrated by the various marrow preparations in tissue culture, it was possible to recognize several staining patterns which indicated cell death or injury. It became evident that the typical "injured" or "dead" cell nucleus and cytoplasm stained diffusely bright red, and could be readily distinguished following injury by pH change, NaCN poisoning, and aging without culture media. In addition, certain cells displayed an increase in red granulation of the cytoplasm and an orange hue to the nucleus. These cells were considered to be injured or damaged to a lesser degree than the "dead" cells.

A second type of "dead" cell staining reaction was observed in the cells killed by rapid freezing or heating. The nuclei of cells disrupted by quick freezing stained a deep, dull-green. They could be distinguished from fresh cells by the absence of intact cytoplasm and total loss of red granulations. In a similar manner, the bare nuclei produced by mechanical trauma in preparation of the suspensions could be distinguished by a dull-green stain and absence of cytoplasmic granulations. Heated cells showed a diffuse, dull-green staining reaction. Figure 2 illustrates the difference in the above designated A-O staining properties of "live," "injured," and "dead" cells.

The results of the experiments designed to produce injured or "dead" cells are summarized and compared with the eosin and trypan blue dye exclusion test and tissue culture transformation in table 1.

a. pH—Change of pH to 8.9 revealed decreased numbers of viable cells by the A-O method and TCT which was not indicated by eosin or trypan. However, pH 6.0 effected the A-O test but not the TCT or dye exclusion tests.

b. Heat—All methods indicated death of most of the cells. The A-O stained cells were diffusely dull-green.

c. Quick freezing without protective agent—The A-O cells were stained a dull-green, easily differentiated from "live" cells. Most of the cells were stained with eosin and trypan blue and showed little growth in tissue culture.

d. Aging "without" serum or tissue culture media—The TCT indicated decreased viability of cells suspended for 6 hours in saline F. This observation correlated well with the A-O test but poorly with the dye exclusion tests.

e. Sodium cyanide—A-O cells were diffusely red and orange. The TCT also
ACRIDINE ORANGE TEST AND BONE MARROW CELLS I

Fig. 2.—1. Fresh marrow suspension, X430. 2. Fresh marrow suspension, X900 (oil immersion). Note cluster of “dead” cells in center. 3. Fresh marrow, X900, predominately granulocytic elements. 4. Fresh marrow, X900. Note megakaryocyte. 5. Non-viable cells, X900. 6. “Injured” cells, mixed with two “live” cells, X900.

Varying amounts of protective agent (DMSO)—Reduction of DMSO concentration below 10 per cent gave evidence of decreased viability by all the tests employed.

The results of the application of the A-O viability test to fresh and freshly thawed specimens of murine marrow preserved by freezing in DMSO or glycerol, and the comparison with eosin and trypan dyes is shown in table 2. With A-O, the cells staining diffusely red or red-orange and those with bare
Table 1.—Percentage of Viable Cells as Determined by Supravital Staining and Tissue Culture Transformation

<table>
<thead>
<tr>
<th>Treatment of Marrow</th>
<th>Staining Methods</th>
<th>Tissue Culture</th>
<th>Modality used</th>
<th>% Viable cells</th>
<th>Average number of live cells per h.p.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-O</td>
<td>1.0% Eosin</td>
<td>0.1% Eosin</td>
<td>.06% T.B.</td>
<td>.02% T.B.</td>
</tr>
<tr>
<td>Control (fresh marrow)</td>
<td>97</td>
<td>90</td>
<td>93</td>
<td>98</td>
<td>94</td>
</tr>
<tr>
<td>Heat (60 C.—30 min.)</td>
<td>0</td>
<td>0–1</td>
<td>0</td>
<td>0–1</td>
<td>0–1</td>
</tr>
<tr>
<td>Rapid freezing</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>pH 6.0 (40 min.)</td>
<td>62</td>
<td>98</td>
<td>92</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td>pH 8.9 (40 min.)</td>
<td>10</td>
<td>93</td>
<td>97</td>
<td>99.5</td>
<td>98</td>
</tr>
<tr>
<td>NaCN (1 hr.)</td>
<td>11</td>
<td>54</td>
<td>82</td>
<td>80</td>
<td>92</td>
</tr>
<tr>
<td>Aging without serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6 hr.)</td>
<td>30</td>
<td>92</td>
<td>99</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>Freezing—2¼% DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(14 days)</td>
<td>48</td>
<td>5</td>
<td>83</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>Freezing—5% DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(14 days)</td>
<td>74</td>
<td>65</td>
<td>90</td>
<td>73</td>
<td>81</td>
</tr>
<tr>
<td>Freezing—10% DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(14 days)</td>
<td>80</td>
<td>82</td>
<td>97</td>
<td>80</td>
<td>91</td>
</tr>
<tr>
<td>Freezing—12¼% DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(14 days)</td>
<td>82</td>
<td>65</td>
<td>97</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>Freezing—15% DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(14 days)</td>
<td>80</td>
<td>68</td>
<td>95</td>
<td>79</td>
<td>92</td>
</tr>
</tbody>
</table>

*T.B. = Trypan blue.

green nuclei were considered non-viable in the differential count. In general, the A-O stain gave a consistent result from specimen to specimen and correlated well with the dye exclusion tests. One per cent (1 per cent) eosin occasionally gave markedly different values in comparison to the other stains.

**DISCUSSION**

Our findings regarding the A-O staining reaction of healthy and damaged marrow cells are in accord with the studies of Wolf and Aronson for other tissues. They have described the fluorescence and metachromasy of fibroblasts from chick embryo hearts, rabbit lens epithelium and choroidal melanocytes cultured in the presence of acridine orange. They have demonstrated that relatively healthy cells stained orthochromatically (deoxyribonucleic acid = hues of green; ribonucleic acid = hues of red); more diffuse metachromatic staining (red and orange) accompanied cell injury; and complete cytoplasmic metachromasy accompanied irreversible injury (red). In accord also, is the finding of diffuse green staining (loss of metachromasy) at an early stage of degeneration. This finding was demonstrated in our study by the staining reaction of the cells damaged by heat. Wolf and Aronson have emphasized that the “A-O viability test” must be interpreted along with a study of morphology and history of the cells being tested.

Emphasis should be placed on factors which need to be carefully controlled in order to have reproducible results when employing the A-O viability test. (1) The cell concentration:stain concentration ratio should be kept constant. The number of “dead” cells at the usual concentration of 40–50 x 10⁶ cells per
Table 2.—Comparison of A-O Staining and Dye Exclusion to Indicate Viability of Murine Marrow Preserved by Freezing for Varying Periods of Time

<table>
<thead>
<tr>
<th>Protective Agent</th>
<th>Length of Storage (days)</th>
<th>A-O</th>
<th>Per Cent Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eosin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1%</td>
</tr>
<tr>
<td>Fresh (average 5 samples)</td>
<td></td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>7</td>
<td>76</td>
<td>42</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>14</td>
<td>83</td>
<td>74</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>22</td>
<td>81</td>
<td>76</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>31</td>
<td>90</td>
<td>84</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>48</td>
<td>77</td>
<td>79</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>78</td>
<td>83</td>
<td>77</td>
</tr>
<tr>
<td>15% DMSO</td>
<td>180</td>
<td>89</td>
<td>44</td>
</tr>
<tr>
<td>15% Glycerol</td>
<td>90</td>
<td>51</td>
<td>48</td>
</tr>
</tbody>
</table>

ml. and 9 mg. per cent A-O stain is appreciably fewer than would be noted when the cell concentration is 10-20 x 10⁶ cells per ml. (2) The pH of the suspending media is critical and easily controlled by use of the tris buffer. (3) The cell preparations should be promptly examined under the ultraviolet microscope with as little U-V radiation per field as possible in order to avoid photodynamic effect on cell viability. Khodas has emphasized the importance of these factors in studies of acridine orange staining of peripheral blood cells after X-irradiation.

The results of the present study indicate that the A-O test correlated well with the TCT for all types of marrow cell injury, although the A-O staining results suggested greater injury with pH change than was apparent from the tissue culture growth. The A-O test compared favorably with the 0.1 per cent eosin and 0.02 per cent trypan blue dye exclusion tests in determining the viability of fresh hemic cells and those damaged by heat, and by rapid or slow freezing. The 1 per cent eosin and 0.05 per cent trypan blue occasionally gave results that correlated much less well with the other methods than the 0.1 per cent eosin and 0.02 per cent trypan blue.

Furthermore, cell damage due to injury of metabolic processes associated with change of pH, depletion of serum, and treatment with sodium cyanide was indicated by the acridine orange test and confirmed by tissue culture, but was not indicated by the eosin or trypan blue dye exclusion tests. Eaton et al. have shown that the tumor-producing capacity of Ehrlich ascites cells injured with NaCN or viruses tends to diminish at a somewhat greater rate than cellular respiration or the ability to exclude eosin or trypan blue dyes. Cells which have been frozen and preserved at low temperatures for long periods of time probably undergo biochemical denaturation.

Although in the present study eosin and trypan blue results correlated well with the A-O and TCT in revealing cell death of marrow frozen up to 180 days, it has been shown that the eosin exclusion test does not correlate with the ability of long-term frozen cells to transplant. Tullis has shown that the eosin exclusion test is not sensitive in determining viability when compared to amoeboid or phagocytic activity of preserved leukocytes. Whether the
A-O test is any more sensitive in indicating damaged or dead cells after long-term freezing, or in indicating marrow which will not transplant, remains to be shown.

An additional advantage of the acridine orange test is that the viability of specific types of cells can be noted by performing cell type differential counts simultaneously with the “live—dead” determination. Cell differentiation is not possible with the eosin and trypan blue dyes.

**SUMMARY**

Suspensions of murine bone marrow cells were stained with acridine orange (A-O) and observed under fluorescent microscopy after treatment with various injurious agents in order to establish the staining characteristics of “live” and “dead” cells. The percentage of viable cells demonstrated by the “A-O viability test” were correlated with eosin and trypan blue dye exclusion and tissue culture transformation viability tests. In general, the A-O test demonstrated the viability of cells preserved by freezing as effectively as the other in vitro tests. In addition, the A-O test may be more sensitive in determining the viability of cells where metabolic processes have been injured by poisons or change in pH.

**SUMMARIO IN INTERLINGUA**

Suspensiones de murin cellulas de medulla ossee esseva tincturate con orange acridinic (O-A) e observate per microscopia fluorescente post tractamento con varie agentes nocive, con le objectivo de establir le caracteristicas tincturatori de cellulas “vive” e “morte.” Le procentaje de cellulas viabile demonstrate per le “test de viabilitate a O-A” esseva correlacionate con tests de viabilitate a exclusion de eosina e trypan blau e a transformation de cultura de tissu. A generalmente parlar, le test a O-A demonstrava le viabilitate de cellulas preservate per congelation tanto efficacemente como le altere tests in vitro. In plus, le test a O-A es possibilemente plus sensibile in determinar le viabilitate de cellulas in casos in que le processos metabolic es lesionate per venenos o per alterationes del pH.

**REFERENCES**

The Acridine Orange Viability Test Applied to Bone Marrow Cells I.
Correlation with Trypan Blue and Eosin Dye Exclusion and Tissue Culture Transformation

WILLIAM E. HATHAWAY, LOUIS A. NEWBY and JOHN H. GITHENS