THE PRESERVATION OF HUMAN BONE MARROW AT -79°C .

A TEMPERATURE-CONTROLLED METHOD OF TWO-STAGE COOLING

BY

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Lorenz, Uphoff, Reid, and Shelton (1951) showed that mice which had received lethal doses of ionizing radiation could recover if injected intravenously with viable bone marrow cells. Barnes and Loutit (1955) showed that erythropoietic cells of the infant mouse spleen, frozen by the technique of Polge and Lovelock (1952) and stored at -79°C . for periods up to 83 days, would also protect mice against lethal doses of x radiation. Ferrebee, Billen, Urso, Wan Ching Lu, Thomas, and Congdon (1957) demonstrated that mouse bone marrow suspensions could be preserved in this manner, and in a series of careful experiments obtained maximum survival of irradiated mice by suspending the marrow cells in tissue culture fluid with 15% glycerol, and freezing them at not more than 1°C . per minute to -15°C . and thereafter at not more than 10°C . per minute. The suspensions were prepared for injection by rapid thawing followed by reduction of the glycerol concentration with hypertonic glucose (Lovelock, 1952; Sloviter, 1956). It had been shown by Barnes and his co-workers (Barnes, Corp, Loutit, and Neal, 1956) that murine leukaemia could be treated by total body radiation followed by bone marrow transfusions, and in 1957 Thomas, Lochte, Wan Ching Lu, and Ferrebee reported a human case of chronic lymphatic leukaemia treated with total body radiation to a tissue dose of 250 r. followed by infusion of isologous stored marrow prepared by Ferrebee's technique. Good evidence was presented that the grafted marrow functioned for at least 43 days.

It was clear that for clinical work in the human being a reliable apparatus was required to freeze and store large volumes of marrow cell suspension in this manner. It was considered desirable that the machine should be compact and portable,

fully automatic and yet simple to construct and operate. A degree of flexibility would also be useful should further experiments show different cooling rates to be preferable.

The Apparatus

Initially a replica of the apparatus of Polge and Lovelock (1952) based on a 1-litre polythene beaker was constructed, but, as Barnes and Loutit found, this failed to produce a satisfactory cooling curve (Fig. 1). It should, however, be noted that, whereas Polge pre-cooled his apparatus to between 2° and 5°C . before immersion in the -79°C . bath, we wished to start at room temperature. The addition of a heating coil to slow down the initial fall in temperature (Barnes and Loutit, 1955) produced a much improved gradient, but for clinical use the apparatus had several disadvantages. In the first

Fig. 1.—Cooling curve of Polge apparatus.

FIG. 2.—Dimensions of cooling vessel.

place the temperature in the inner beaker was not uniform; this was rectified by fitting a thick insulating lid of tufnol and incorporating a small stirrer to circulate the spirit thoroughly. More serious was the fact that the thermocouple measuring the temperature inside one of the ampoules had to be read at 30-second intervals to estimate the heating current required, while the inertia of the system made a smooth fall in temperature very difficult to obtain. The failure of the apparatus to produce cooling in excess of 2°C. per minute below -15°C. was also considered to be a serious fault in view of the evidence presented by Smith (1954) that the temperature zone between -15°C. and -25°C. should be passed in two minutes or less. Thus the minimum cooling rate of the second stage had to be 5°C. per minute. Experiments showed that rapid cooling below -15°C. could be obtained only if the insulation of the cooling vessel was radically reduced, although this naturally made slow initial cooling more difficult. However, a satisfactory compromise was provided by insulating the lower part of the beaker only; partial immersion in the -79°C. bath then produced slow cooling while complete immersion produced much more rapid cooling. It was found that suitable insulation could be provided by a layer

of histological embedding wax (m.p. 60°C.) about 1 cm. thick placed inside the lower third of a 1-litre stainless steel beaker and held in place by a thin (1.5 mm.) polythene container. The precise dimensions are shown in Fig. 2. It was found that a very close approximation to the desired two-stage curve could be produced if the beaker was first immersed to a depth of 5 cm. and then, when the contents had reached -15°C. , lowered to a depth of 12 cm. (Fig. 3). In order to obtain reproducible results it was essential to standardize the temperature of the vessel before cooling started (22°C.), the volume of methylated spirit it contained (520 ml.), and the size, content, and number of ampoules (19 10-ml. ampoules each containing 7 ml. of cell suspension in 15% glycerol). When less than 19 ampoules were being handled the total was made up with dummy ampoules containing 15% glycerol.

The temperature in the beaker is recorded automatically by a platinum resistance thermometer incorporating a 3 in. (7.7 cm.) pen recorder (Record Electrical Ltd.). The platinum resistance used has a fundamental interval of 180 ohms, but it is probable that a standard commercial model with suitable amplification would be satisfactory. A simple D.C. Wheatstone network has proved quite adequate. The amplifier is a three-stage balanced D.C. transistor circuit feeding a 1 m/a f.s.d. meter. The recorder employs a chart speed of 4 in. (10.2 cm.) per hour and is calibrated to read from $+30^{\circ}\text{C.}$ to -70°C. (Fig. 4).

Automatic lowering of the beaker from a depth of 5 cm. to the 12 cm. level is achieved by a thermostatically operated circuit (Fig. 5). The beaker is suspended from one end of a counterpoised arm. This is balanced so that the beaker will drop slowly when the solenoid which supports the arm is

FIG. 3.—Cooling curve of modified apparatus.

FIG. 4.—Circuit diagram of recording thermometer. The O.C.71 transistors are mounted in a large brass heat sink to minimize thermal effects.

FIG. 5.—Circuit diagram of power supplies for recorder, blower, stirrers and solenoid control circuit. All components between points X—X are mounted remotely from the chassis. PLA: 12-pin outlet socket; PLB: 3-pin outlet socket for thermometer chassis only. W: warning light mounted on solenoid to indicate that microswitch has operated. To reset, Sw. 6 must be switched off and on again.

FIG. 6.—Modified apparatus (A) cooling at 1° C. per minute and (B) cooling at 5° C. per minute. (a) Solenoid (b) Microswitch assembly (c) Cooling vessel (d) Dewar flask of "cardice" and methylated spirit (e) Adjustable clamp for stop (f) Stirrer (g) Stop (h) Blower

energized. The thermostat controlling the solenoid is set to close at -15° C. and is immersed in the beaker with the ampoules. As the counterpoised arm drops it actuates a microswitch which breaks the solenoid control circuit. The arm is arrested by a stop placed 7 cm. below the solenoid (Fig. 6). Excessive frothing during the lowering of the beaker is prevented by a small blower mounted at the edge of the -79° C. bath.

A rack holding 19 ampoules was constructed to fit inside the beaker and rest on top of the wax lining. It is arranged that the stirrer, thermometer, and thermostat, which are attached to the lid, take up their correct positions without disturbing the ampoules (Fig. 7). Fig. 8 shows the complete apparatus in use.

FIG. 7.—Cut-away view of cooling vessel. A, Ampoule. B, Platinum resistance thermometer. C, Stirrer. D, Stirrer motor. E, Thermostat. F, Thermostat setting screw. G, Insulating jacket. H, Ampoule rack. J, Tufnol cap.

It was found that when the temperature of an ampoule had fallen to -63° C. it could be immersed directly in spirit at -79° C. without exceeding the cooling rate of 10° C. per minute. It has therefore been our practice to transfer the ampoules to the storage flasks when they have reached -63° C. The ampoules are stored in quart Dewar flasks containing industrial spirit and solid carbon dioxide. The flasks are placed in a large insulated cabinet and surrounded by pieces of solid carbon dioxide.

The apparatus has been used to set up a bone marrow bank using ribs removed at thoracotomy for benign conditions. Schwartz and Tocantins' (1958) method is used to extract the marrow cells. The ribs are cut into 1 cm. fragments and each piece is crushed before being placed in a 250-ml. conical flask containing 30 ml. of heparinized tissue culture fluid. The flask is agitated gently for 20 minutes. The cell suspension is then freed from fat and filtered as described elsewhere for aspirated marrow (Newton, Humble, Wilson, Pegg, and Skinner, 1959).

The stored marrow has been administered with some success to two patients suffering from chronic lymphatic leukaemia treated by half-body irradiation, and to one patient who received large doses of HN_2 in connexion with surgery for carcinoma of the stomach. This had produced marked leucopenia and marrow hypoplasia, but following the marrow infusion the white cell count rose rapidly (Humble, Newton, and Kemp, 1958). Isologous stored marrow has also been administered to one patient suffering from aplastic anaemia due to chloramphenicol: no radiation or chemotherapy was given in this case and there was no evidence that the infused marrow survived.

Autologous infusion of stored marrow has also been practised in patients receiving wide-field, high-dose radiotherapy (Newton *et al.*, 1959) and in another series of patients who had received intensive chemotherapy for widespread malignant disease (Westbury, Humble, Newton, Skinner, and Pegg, 1959). It is noteworthy that stored marrow has been administered both intravenously and into the abdominal aorta without any evidence of harmful effect (Humble and Newton, 1958).

It would appear that treatment by marrow cell infusion should be considered in the type of case referred to above. It is also the logical treatment for accidental exposure to intense radiation, and in this connexion it is well to be prepared with large stocks of stored marrow. The possibility that human leukaemia may be successfully treated by total body radiation and marrow replacement remains and is being intensively studied here and elsewhere (Thomas *et al.*, 1957; Meighan and Bean, 1958; Reinhard, Brittingham, Moore, Holtz, Chaplin, Harrington, Loeb, Lessner, and Banson, 1958; Tocantins, 1958; Thomas, Lochte, and Ferrebee, 1959; Atkinson, Mahoney, Schwartz, and Hesch, 1959). Other interesting applications will undoubtedly present themselves as experience is gained with the method. Among these may be mentioned the possibility of organ grafting after irradiation and marrow replacement (Ferrebee and Merrill, 1957) and the modification of the severe homozygous states in the inherited haemolytic anaemias (Russell, Smith, and Lawson, 1956; Dameshek, 1957).

Summary and Conclusions

Viable bone marrow may be preserved at -79°C . in 15% glycerol. The cooling rate is restricted to 1°C . per minute to -15°C ., after which a minimum of 5°C . per minute is required.

FIG. 8.—Complete apparatus.

An apparatus is described whereby cell suspensions may be cooled in this manner.

Possible clinical uses for stored human bone marrow are briefly discussed.

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