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Use of an Adaptable Cell Culture Kit for Performing Lymphocyte and Monocyte Cell Cultures in Microgravity

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Abstract The results of experiments performed in recent years on board facilities such as the Space Shuttle/Spacelab have demonstrated that many cell systems, ranging from simple bacteria to mammalian cells, are sensitive to the microgravity environment, suggesting gravity affects fundamental cellular processes. However, performing well-controlled experiments aboard spacecraft offers unique challenges to the cell biologist. Although systems such as the European 'Biorack' provide generic experiment facilities including an incubator, on-board 1-g reference centrifuge, and contained area for manipulations, the experimenter must still establish a system for performing cell culture experiments that is compatible with the constraints of spaceflight. Two different cell culture kits developed by the French Space Agency, CNES, were recently used to perform a series of experiments during four flights of the 'Biorack' facility aboard the Space Shuttle. The first unit, Generic Cell Activation Kit 1 (GCAK-1), contains six separate culture units per cassette, each consisting of a culture chamber, activator chamber, filtration system (permitting separation of cells from supernatent in-flight), injection port, and supernatent collection chamber. The second unit (GCAK-2) also contains six separate culture units, including a culture, activator, and fixation chambers. Both hardware units permit relatively complex cell culture manipulations without extensive use of spacecraft resources (crew time, volume, mass, power), or the need for excessive safety measures. Possible operations include stimulation of cultures with activators, separation of cells from supernatent, fixation/lysis, manipulation of radiolabelled reagents, and medium exchange. Investigations performed aboard the Space Shuttle in six different experiments used Jurkat, purified T-cells or U937 cells, the results of which are reported separately. We report here the behaviour of Jurkat and U937 cells in the GCAK hardware in ground-based investigations simulating the conditions expected in the flight experiment. Several parameters including cell concentration, time between cell loading and activation, and storage temperature on cell survival were examined to characterise cell response and optimise the experiments to be flown aboard the Space Shuttle. Results indicate that the objectives of the experiments could be met with delays up to 5 days between cell loading into the hardware and initial in flight experiment activation, without the need for medium exchange. Experiment hardware of this kind, which is adaptable to a wide range of cell types and can be easily interfaced to different spacecraft facilities, offers the possibility for a wide range of experimenters successfully and easily to utilise future flight opportunities. J. Cell. Biochem. 70:252–267, 1998. © 1998 Wiley-Liss, Inc.

Key words: lymphocyte; monocyte; cell line; cell culture; microgravity; experiment development

Experiments aboard orbiting spacecraft in the last three decades, particularly aboard the U.S. Space Shuttle, strongly suggest that mammalian cell function is altered in microgravity [reviewed in Cogoli and Gmünder, 1991; Moore and Cogoli, 1996; Hatton et al., 1997]. Observed alterations in cells exposed to microgravity compared to cells under terrestrial 1.*g* conditions include decreased proliferation and sensitivity to mitogens in peripheral human lymphocytes [Cogoli et al., 1984, 1993], reduced cytokine synthesis in human T-cell and monocyte cell

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lines [Limouse et al., 1991], reduced early immediate gene expression in a human epidermal carcinoma cell line [De Groot et al., 1991], and a gravidependant localisation of Protein Kinase C in a human monocyte and T-cell line [Schmitt et al., 1996].

Despite significant progress in this field in recent years, performing cell culture experiments in space still poses unique problems. An experiment on board an orbiting spacecraft is subject to a number of constraints, which requires the design of specific hardware to perform the experiment. The mass, volume, and power requirements of the experiment are often limited, requiring miniaturisation of the hardware, which may limit experiment sample volume and/or number of replicates. On Space Shuttle missions, the experiment is generally only a small component of the payload. Hence both preflight crew training time and in-flight crew time available to perform experiment manipulations are usually limited. Therefore, the experiment must be designed for simple operation, with the possibility of incorrect operation minimised. Additionally, safety is a critical issue on any human space flight, so the design of experiments must ensure that the contents of the hardware will not escape into the spacecraft cabin under any circumstances. This is usually achieved by two or three levels of containment, where the contents of the experiment are isolated from the crew by successive containers or enclosures such as a glovebox.

The general requirements of many different mammalian cell biology experiments performed aboard orbiting spacecraft are similar. Common requirements include a controlled temperature (i.e., 37°C±0.5°C), electrical power or data connections, and a contained area for experiment manipulations. Additionally it is desirable to have an on-board centrifuge to provide an in-flight 1.*g* control, so that the effects of microgravity can be differentiated from other spaceflight factors, such as launch acceleration, cosmic radiation, and spacecraft environmental conditions.

Many of these generic requirements can be provided by multiuser, multipurpose experiment facilities. A very successful example of such a system is the European Space Agency 'Biorack' [Genzel and Mesland, 1988]. This is a single rack unit that can interface with the Spacelab or Spacehab pressurised modules, which are accommodated in the Space Shuttle payload bay. The Biorack consists of a number of different elements, including two incubators (usually running at 37°C and 22°C, respectively), a glovebox, and a 4°C cooler and a -20°C freezer. Individual experiments are designed to be accommodated in either of two types of container, which provide one level of containment and an interface to the facility. The smaller container (known as a Type-I) has dimensions of 20.4x40.4x83 mm, whereas the larger Type-II container is 63.2x63.2x87 mm. A modified Type-I container with electrical connections permits automated experiment operation and in-situ data acquisition. Each incubator contains a rack for Type I containers (microgravity experiments) and two independent centrifuges (1*g* control experiments) with slots for Type I containers. Additionally there are slots for Type II containers, although it is not possible to accommodate the larger containers on the centrifuges. A glovebox provides containment during crew manipulations of the experiment. Biorack has made six flights; three in the Spacelab module (D-1, IML-1, IML-2) and three in the Spacehab module during Shuttle-Mir docking flights (S/MM-03, -05, -06). A total of 81 different experiments were performed in Biorack during these flights. A number of simplified derivatives of the system based on the incubator/centrifuge unit exist or are under development including the automated 'Biobox' for Biocosmos [Demets, 1993] missions and the 'BioPack' incubator for the Shuttle middeck [Jack Van Loon, unpublished communications].

Although the availability of generic spacecraft experiment facilities solves some of the general problems of experimental control, the experimenter generally has to develop experiment specific hardware to interface with the facility [Briarty, 1989]. The hardware must be capable of performing the experimental investigation successfully, within the operational and safety constraints of the mission. As a result, space experiment preparation is costly and time-consuming, since materials specifications and construction requirements are strict and the prototype hardware must pass a number of validation tests. A further problem is ensuring that the experiment hardware is biocomptible with the experimental material, requiring extensive testing and preparation experiments.

We present here the results of ground studies performed in preparation for a series of experiments flown aboard in the Biorack facility dur-

ing four Space Shuttle flights using specially designed experiment specific hardware known as the Generic Cell Activation Kit (GCAK). These experiments used Jurkat cells, purified peripheral blood T cells, and U937 cells, all of which are types of human leukocytes. The overall objective of the Shuttle experiments was to examine the effect of microgravity on cell function, particularly signal transduction processes, cytoskeleton dynamics, and growth activation. The results of several of these experiments are published elsewhere [Schmitt et al., 1996; Lewis et al., 1998). The objectives of each experiment, cell types, and general protocol relevant to the ground based data presented here are listed for the six Shuttle experiments in Table I.

The objectives of the ground-based studies presented here were to determine how the viability and responsiveness to activation of the cells varied under environmental conditions likely to be encountered in the course of the space experiments listed in Table I. Of primary concern was the effect of culturing in a novel multipurpose culture cassette and the effect of

TABLE I. Spaceflight Experiments Using GCAK Cassettes for Mammalian Cell Culture Experiments in the ESA Biorack Facility

Experiment name and flight	Objectives	Cell types	Protocol	Parameters examined
Cytokine IML-2 (6/94) Schmitt et al., 1996	Examine cytokine synthesis in response to phorbol ester stimulation.	Jurkat, U937	1 day storage at ambient temperature; 8 h at 37°C before stimulation with phorbol esters, cells frozen 15 h later.	IL-1β and TNF-α in U937 cells. IL-2 in Jurkat, D-Glucose utilisation.
Phorbol IML-2 (6/94) Schmitt et al., 1996	Examine subcellular localisation and amount of Protein Kinase C (PKC).	Jurkat, U937	1 day storage at ambient temperature; 8 h at 37°C before stimulation with radiolabelled phorbol ester, samples frozen 1 h later.	Subcellular localisation of PKC with radiolabelled phorbol ester DNA concentration.
Phorbol S/MM-03 (3/96) Hatton et al. (in prep.)	Examine translocation and amount of PKC isoforms.	U937, T cells	4-day storage at ambient temperature; 15 h at 37°C before stimulation with radiolabelled phorbol ester (U937). Samples frozen at 0, 10, and 60 min after stimulation.	U937 cells: Subcellular localisation of PKC with radiolabelled phorbol ester DNA concentration, T-cells: PKC isoforms, DNA concentration.
T-Cell S/MM-03 (3/96) Lewis et al. (1998)	Examine cytoskeleton organisation.	Jurkat	36 h storage at ambient temperature, then 15 h at 37°C. Cells stimulated with serum and samples fixed at 4 h and 48 h after stimulation.	Microtubule organisation. Cell number.
Isozyme S/MM-05 (1/97)	Examine PKC isoform translocation in response to a range of agonists.	U937, T cells	36 h storage at ambient temperature, then 15 h at 37°C. Cells stimulated and samples frozen at 0, 10, and 60 min after stimulation.	PKC isoforms in subcellular fractions. DNA concentration.
Cytokine S/MM-06 (5/97)	Examine early immediate gene expression, cytokine synthesis, and PKC/cytoskeleton colocalisation.	U937, T cells	3-day storage at ambient temperature, then 15 h at 37°C. Cells stimulated and fixed 1 h or 8 h later.	U937 cells: various mRNAs, cytokines. T cells: microtubule and PKC localisation by fluorescence microscopy

a lengthy prelaunch storage at a temperature other than 37°C. The results of this study should provide a useful example of the solutions to the many problems inherent in performing cell biology experiments on board spacecraft.

MATERIALS AND METHODS

Generic Cell Activation Kit '1' and '2' Cassettes (GCAK-1 and GCAK-2)

The GCAK cassettes are compact units for performing short-term experiments involving activation of cells in liquid culture aboard manned spacecraft. Both units were originally designed for use in the Biorack experiment system and are enclosed in a Biorack Type I container (Fig. 1). The cassettes were constructed by Comat (Toulouse, France) under a contract with the French Space Agency, CNES. Both GCAK cassettes consist of a polycarbonate block with drilled-out chambers for internal components. Each cassette comprises six independent culture chambers, which are enclosed by a silicon piston assembly. The culture chamber can be connected or isolated from daughter chambers (containing activator or fixative) by a stainless steel vane that has internal conduit. Experiment manipulations are performed by turning vanes to connect or isolate the individual chambers and by pushing the pistons to displace fluid from chamber to chamber. A special key and tool are used for these operations, enabling simultaneous or individual operation of the culture units. Piston ports and vanes are colour coded to indicate the sequence of experiment manipulations. The simplicity of cassette operations ensures that experiment manipulations can be performed rapidly with minimal risk of error and without the requirement for excessive resources.

The GCAK-2 cassette was used in four spaceflight experiments and consists of a cell culture chamber (500 μl initial volume) and two daughter activator/fixative chambers (150 μl). The GCAK-1 cassette was also used in four experiments and permits filtration of cells from supernatent. The cassette consists of two blocks, the first of which contains a 500 μl initial volume culture chamber and a 120 μl activator chamber separated by a vane. Filtration is performed by depressing the culture chamber piston forcing the culture medium through a low shear filter unit in between the two blocks. Supernatent is received in an empty chamber in the second block, whereas cells collect in the filter unit. A modified commercially available multi-injector unit is used to inject liquid into the cassette enabling resuspension of cells in the culture unit (Socorex, Lausanne, Switzerland). In the IML-2 Cytokine experiment a cell lysing solution was injected, whereas later experiments involved sequential injections of fixative, cell filtration, and wash buffer. Additionally, it is possible to use the same system for in-flight medium exchange (liquid can be withdrawn from the same port using a flight qualified blood draw kit) or introduction of experimental samples, such as blood samples. Both cassettes are designed to be autoclaved at 121°C and can be frozen at -30°C for in-flight preservation of sensitive biochemical samples.

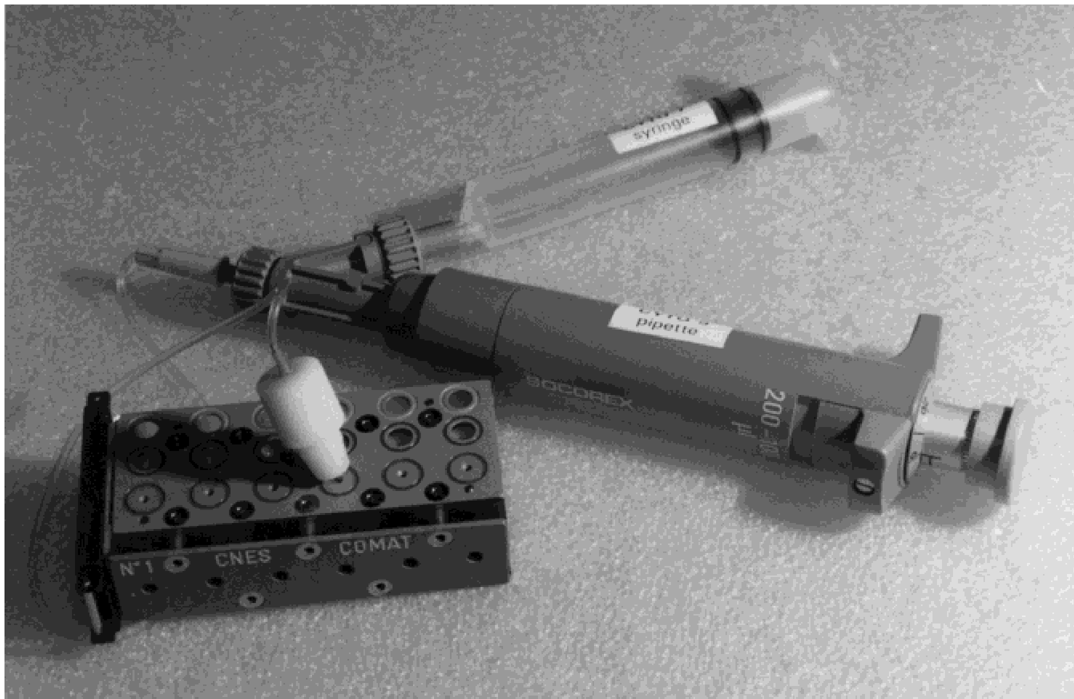
Culture Media

All cell lines were routinely sub cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% v/v fetal bovine serum (Gibco, Paisley, UK), 1% v/v L-glutamine, and 1% v/v penicillin, streptomycin, neomycin (PSN) antibiotic mix (all supplements from Gibco). Biorack does not provide a 5% CO_2 atmosphere; therefore, for experiments in the GCAK cassettes, the RPMI-1640 mix supplemented with 25 mM hepes, 12 mM sodium bicarbonate, and 1 mM sodium pyruvate, all from Sigma. These supplements replace the requirement for atmospheric CO_2 by buffering the medium and providing a source of soluble bicarbonate. For some experiments, sodium pyruvate was omitted as indicated in the experimental protocols detailed below. The S/MM-03 T-cell flight and preparation experiments with Jurkat cells used a modified medium composition that consisted of RPMI-1640, supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1 ml/100 ml of 100x nonessential amino acids, 100 units penicillin, 100 mg/ml streptomycin, and 12.5 mM hepes buffer. Cells were prepared and loaded into the cassettes in this medium supplemented by 2% fetal bovine serum. Additional serum was added to the cells at transfer to 37°C as indicated in the experimental protocol to increase the serum concentration to 10%. All reagents were obtained from Sigma unless otherwise stated.

Cells

The cell lines Jurkat JE 6.1, a human leukemic lymphocyte line and the human leukemic monocyte line, U937, were used in this study.

a



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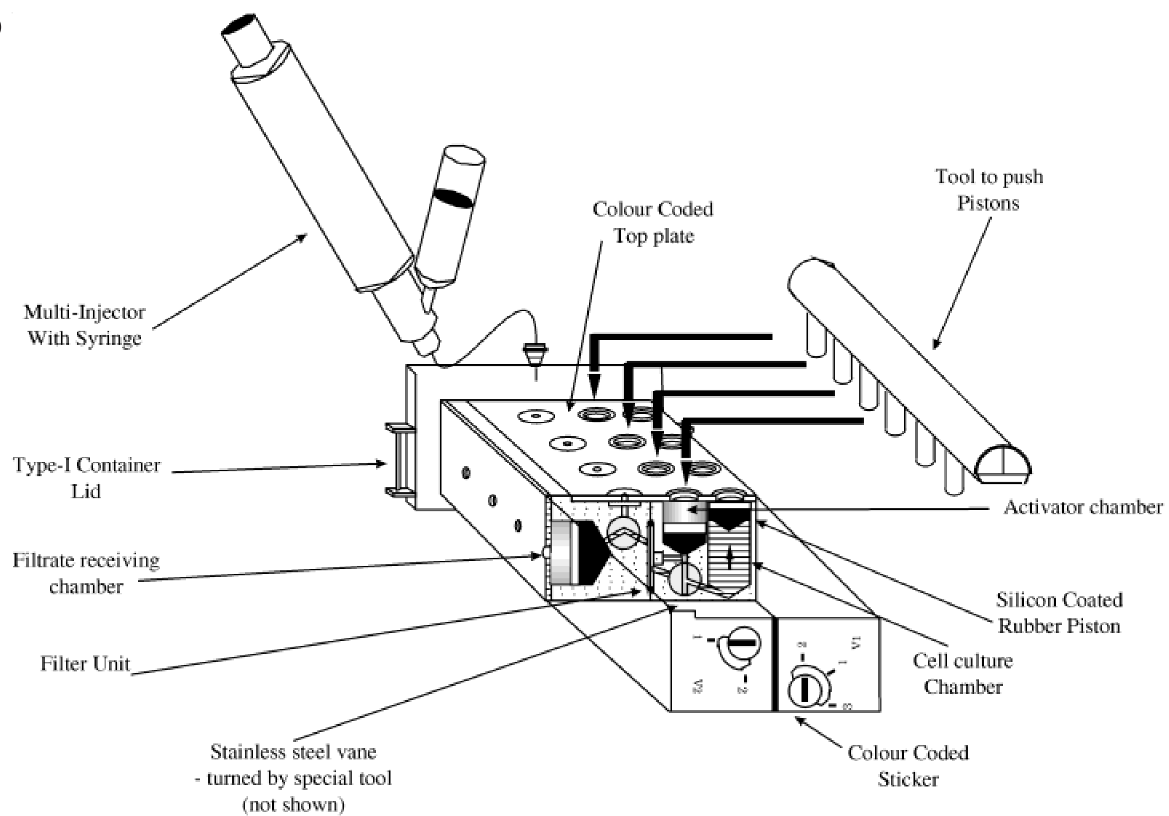
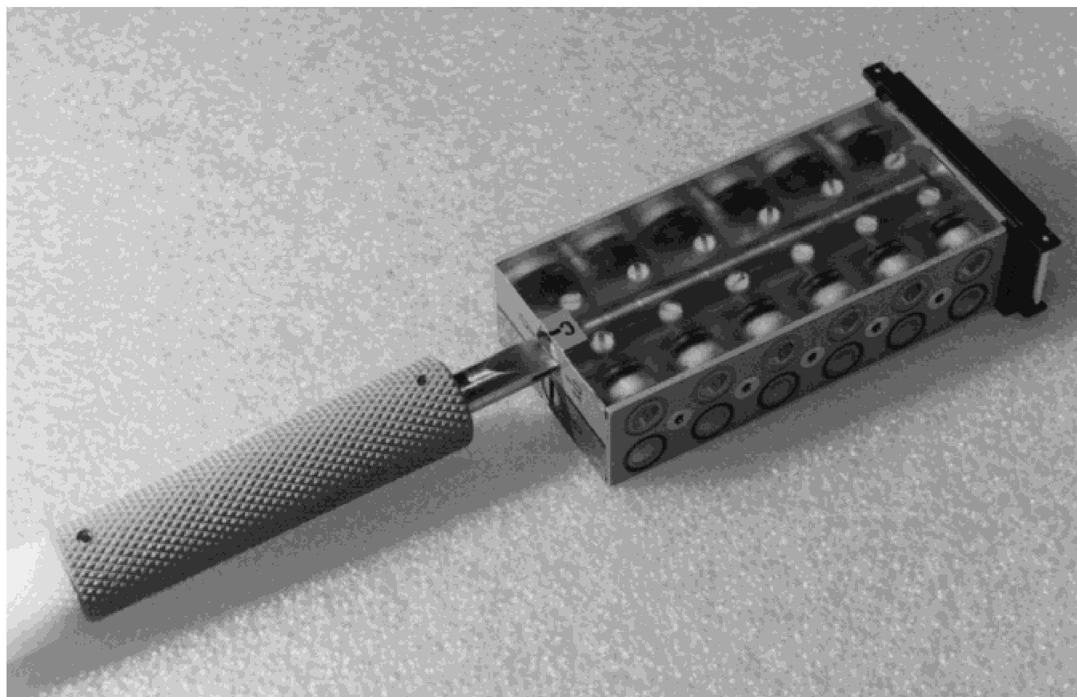


Fig. 1. GCAK experiment cassettes. (**a**) The GCAK-1 cassette. An overall view of an individual cassette with the multi-injector unit. (**b**) A cross section of the GCAK-1 cassette, showing culture chamber, activator chamber, filter, injection port, supernatent receiving chambers, and vanes. (**c**) The GCAK-2 cassette, with tool to turn vane. (**d**) Cross section of the GCAK-2 cassette, showing the culture, activator, and fixative chambers, along with vanes.

c



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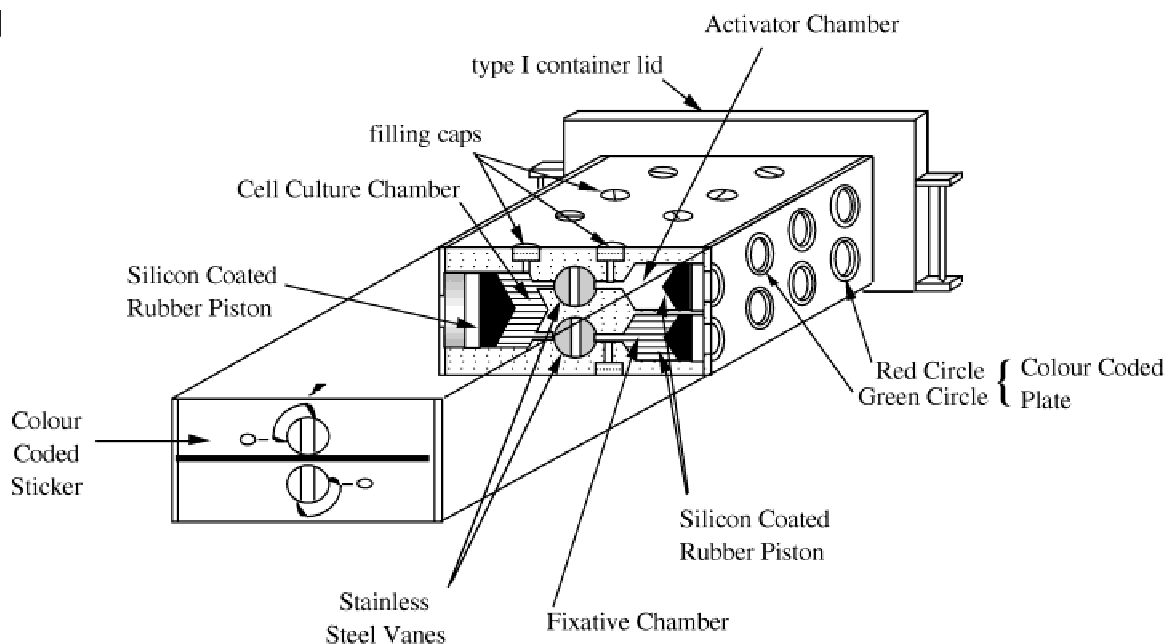

Figure. 1. (Continued.)

Both cell types were unstimulated and undifferentiated unless otherwise stated in the text. The cell lines were obtained from the American Cell Type Collection and were regularly checked for mycoplasma contamination by a polymerase chain reaction assay.

Peripheral blood T cells were purified from human peripheral blood using a ficoll gradient centrifugation (Lymphocyte Separation Medium, Gibco). After separation, the lymphocytes were washed twice in the supplemented RPMI-1640 medium before being mixed with magnetic beads coated with anti-CD14, CD-19, and CD-56 antibodies (Dynal France, Compiègne, France) for 2x 30 min at 4°C with rotation to remove monocytes, B cells, and natural

killer cells. After purification, purified T cells were resuspended at the concentration required for the experiment.

Experimental Investigation

Experiments were performed in both the GCAK-1 and GCAK-2 units. The culture chamber volume and material composition of both cassette types are identical, so cell culture data from one cassette type can be taken as representative for both cassette types. Time/temperature profiles were those projected for the planned flight experiments. Several different time/temperature profiles and experimental protocols were tested in the preparation of the flight experiments, corresponding to those of each individual mission. Test conditions were examined in triplicate unless otherwise indicated. Each cassette chamber was loaded with 0.5 ml of cell suspension using an eppendorf multi-injector and a 21G needle. The cassettes were then placed in Type-I containers and removed only for experimental manipulations or sample recovery.

The following investigations were performed. (1) Cell viability during storage in the cassettes at 4ºC and 24ºC. These experiments simulated the storage conditions in refrigerated (4ºC) and ambient stowage (24ºC) on the Space Shuttle middeck. Cells were loaded into GCAK-1 cassettes at initial concentrations of $1x10^6$, $2x10^6$, or $4x10^6$ cells per ml. Triplicate samples were recovered from the cassettes at 24 h intervals following transfer of the cassettes to the incubator, up to 144 h after transfer. Each of the triplicate samples was taken from a different cassette and was subject to viable and total cell counts (described below).

(2) Cell viability before and after transfer to 37ºC for various mission time/temperature profiles. An initial test of the S/MM-03 PHORBOL experiment temperature/time profile was made with U937 cells in the GCAK-1. Cells were loaded into the cassettes at an initial concentrations of $1x10^6$ cells /ml and stored for 96 h at 4ºC or 24ºC before transfer to 37ºC for 24 h. Total and viable cell counts were made immediately before transfer to 37ºC and 15 h after transfer. Additionally, 15 h after transfer to 37ºC, the cells were stimulated with 100 ng/ml Phorbol-12,13-Dibutyrate (PDBu) during 6 h to induce tumour necrosis factor alpha (TNF-α) synthesis and secretion. After 6 h of stimulation with PDBu, cell suspensions were harvested from the cassette, centrifuge, and the supernatent recovered for ELISA analysis of TNF-α.

Further experiments were performed with U937 cells in different temperature time profiles simulating three different mission profiles in the GCAK-2 cassettes; 24 h storage at 24ºC (simulated IML-2 mission profile), 72 h at 24ºC (simulated S/MM-06 mission profile), and 96 h at 24ºC (simulated S/MM-03 PHORBOL experiment profile). Cassettes were to 37ºC for 15 h immediately after 24ºC storage. Cells were loaded into the cassettes at an initial concentration of $2x10^6$ cells /ml in the RPMI-1640 medium specified above, except that sodium pyruvate was not added to the medium. Total and viable cell counts were performed at the end of 24ºC storage and 15 h after transfer to 37ºC. Cytofluorimetry was used to assess cell size and shape at the same times as cell counts.

(3) S/MM-03 T-CELL experiment preparation with Jurkat cells.

The viability and glucose utilisation of Jurkat cells in GCAK-1 cassettes was examined in preparation for the S/MM-03 T-CELL experiment. An initial assessment of biocompatability of the cassettes with the cells was performed by comparing total and viable cell number in the cassettes at 37ºC with cultures in multiwells at 24 h, 48 h, and 72 h after cell loading. Similar measurements, as well as an assessment of D-glucose utilisation, were examined following a protocol simulating that of the flight experiment. Jurkat cells were incubated at 24ºC for 36 h and then transferred to 37ºC for 48 h. Cells were stored up to transfer to 37ºC in the RPMI-1640 medium mix specified in the reagents list with 2% serum. At transfer to 37ºC, serum was added to the culture medium to bring the concentration up to 10%. Samples were taken for total and viable cell counts and glucose measurements at 4 h and 48 h after transfer to 37ºC.

Experiments to Determine Effect of Pyruvate on Cell Viability

The effect of sodium pyruvate on U937 cell viability was examined in a series of time/temperature profile simulating typical conditions for an experiment on the Space Shuttle, using 2 ml sterile disposable syringes (Becton Dickinson France, Le Pont de Claix, France) as simple analogues of the culture conditions in the cassette. U937 cells (0.5 ml) at $5x10^5$ or $2x10^6$ cells/ml were loaded into each syringe. Cells were prepared in RPMI-1640 medium as

specified above for experiments in the GCAK cassettes, with or without 1 mM sodium pyruvate. Syringes loaded with cells were stored in the dark for 0, 1, 2, 3 days at 24°C and then transferred to 37°C for 18 h at the end of the 24°C storage period. Total cell number and cell viability were assessed by trypan blue exclusion, as described below, both before transfer to 37°C and at the end of 37°C incubation.

Cell Counts and Cytofluorimetric Analysis of Cell Size and Shape

Viable and total cell counts were determined from Trypan Blue exclusion counts under the microscope using a heamocytometer. Two counts of at least 200 cells were performed for each sample in the cassettes. The initial viability of the cells was determined before loading of the cells into the cassettes. For some experiments with the cell lines, size and morphology measurements for individual cells were assessed by a fluorescent antibody cell sorter system (FACscan or FACSort, Becton Dickinson, San Jose, CA). At least 10,000 cells were acquired in the cytofluorimeter for each sample well.

Cytokine Immunoassays and D-Glucose Enzymatic Assay

An enzyme-linked, immunosorbent immunoassay kit from Immunotech (Marseille, France) was used to determine TNF-α concentrations in the U937 cell sample supernatents. The glucose concentration in culture medium from which the cells were filtered was evaluated with a Glucose Analyzer 2 (Beckman Instruments, Brea, CA) using the reagents and protocol accompanying the Beckman glucose reagent analysis kit. Data were expressed as the mean of triplicate glucose analyser readings for medium samples taken from each of the six replicate cell culture chambers for each cassette.

^{3}H-Phorbol Ester Labelling of Protein Kinase C and PKC Translocation Kinetics

The ^{3}H labelled form of the phorbol ester Phorbol-12,13-Dibutyrate (3H-PDBu, Amersham International, Little Chalfont, UK) was used to both label and activate Protein kinase C in U937 cells. 3H-PDBu (125 µl) of 500 ng/ml (2 µCi) was loaded into each activator chamber in the GCAK-2 cassette, giving a final ^{3}H-PDBu concentration of 100 ng/ml with the cells. Samples were incubated in the cassettes either for 15 h at 37°C or 96 h at 24°C, before transfer to 37°C for 15 h, simulating the longest anticipated storage duration for an experiment aboard a Shuttle Mir docking mission. After these incubation periods, cells were stimulated with ^{3}H-PDBu for 0, 10, 20, 50, and 60 min in the cassette at 37°C. Cell activation was stopped by injection of an inhibitor solution that permeabilises the cells, permitting examination of PKC localisation in cytosolic and particulate fractions of the cells (0.5 mg/ml digitonin, 50 mM MOPS, PH7.2, 180 mM NaCl, 5 mM NaF, 5 mM EDTA, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na3MoO4, 10 µM β-methylaspartic acid, 10 µg/ml soybean trypsin inhibitor, all reagents obtained from Sigma). Samples were frozen until subsequent analysis. Particulate and cytosolic fractions were separated by ultracentrifugation. Unbound ^{3}H-PDBU was removed by washing through GF/B glass fibre filters (Whatman, Maidstone, UK). Radioactivity in each fraction was quantified by liquid scintillation counting (Packard 1900 TR liquid scintillation analyser, Packard Instruments, Meridian, CT).

RESULTS

Cell Viability, Cell Shape, and Size Measurements

Cell viability during 24°C and 4°C storage. Cell viability for both U937 and Jurkat cells was examined during ambient (24°C) or cold (4°C) storage from between 1 and 6 days after loading of cells into the hardware. For both cell lines, the viability decreased gradually with increasing storage time (Figs. 2 and 3). No significant differences could be seen in viability between U937 cell samples at 1 and $2x10^6$ cells/ml concentrations at 24°C, with viability remaining 80% up to 4 days of storage. However, viability rapidly decreased after this time, dropping to 65% by the 6th day of storage (Fig. 2a). At a U937 cell concentration of 4×10^6 cells/ml, he decrease in viability with time was greatly accelerated compared to lower cell concentrations. In contrast, the rate of decrease in viability for all U937 cell concentrations at 4°C was slower than at 24°C. At both 1 and $2x10^6$ cells/ml viability remained in excess of 80% up to the 6th day of storage (Fig. 2b). A similar pattern was observed for Jurkat cells at 1 and $2x10^6$ cells/ml at 24°C (Fig. 3).

Cell viability at 37°C following transfer from ambient or cold storage. *Experiments in syringes to determine the effect of pyruvate on U937 cell viability.* Pyruvate is known to

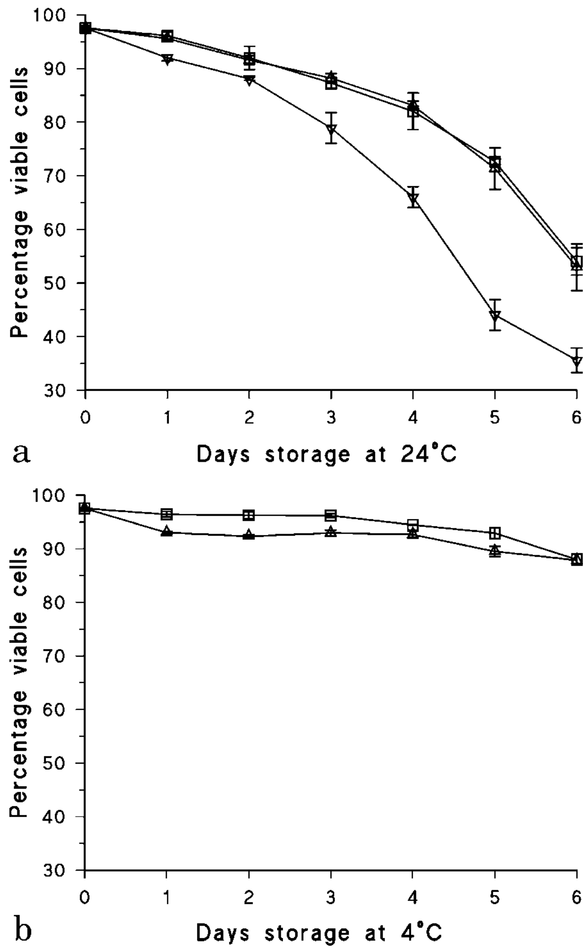

Fig. 2. Viability of U937 cells stored in GCAK-1 measured at intervals through storage period. (a) 24°C storage. (b) 4°C storage. Cells were loaded at 1 (■), 2 (▲), or 4x10⁶ cells /ml (▼). For each time point n=3 and error bars are standard error of the mean (SEM).

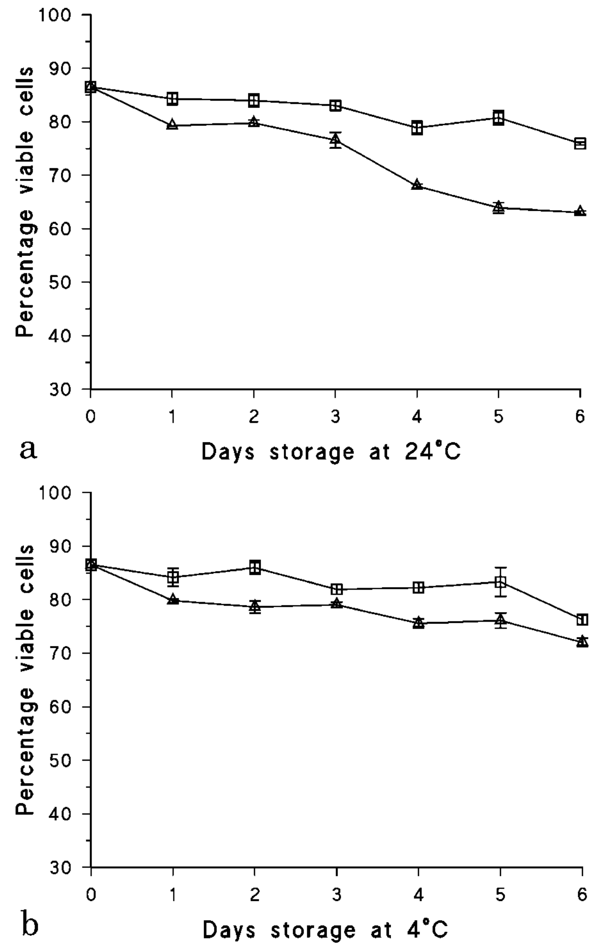

Fig. 3. Viability of Jurkat cells stored in GCAK-1 measured at intervals through storage period. (a) 24°C storage. (b) 4°C storage. Cells were loaded at $1x10^6$ cells/ml (■), or $2x10^6$ cells /ml (▲) . For each time point n=3 and error bars are standard error of the mean (SEM).

provide a substrate that promotes endogenous production of CO_2 by cells [Freshney, 1987] and has been implicating in modulating cell viability and function of mammalian cells in culture [Kroll et al., 1986; Borle and Stanko, 1996]. Therefore, we examined the effect of the presence or absence of 1 mM pyruvate in the culture medium of U937 cells cultured in 2 ml syringes according to typical Shuttle experiment temperature profiles. No significant difference was noted in U937 cell viability between cells cultured in the syringes with medium containing sodium pyruvate and with medium without pyruvate at either $5x10^5$ or $2x10^6$ cells /ml (Fig. 4). Only $2x10^6$ cells /ml data is presented for clarity.

Experiments with U937 cells in GCAK cassettes. The viability of U937 cells in GCAK-2 cassettes was examined in a time/temperature profile simulating the S/MM-03 PHORBOL experiment. Cells were loaded into the cassettes at an initial concentration of $1x10^6$ cells/ml . Viability was examined both directly after 4 days storage at 24°C or 4°C and after subsequent transfer to 37°C for 15 h. For samples stored at 24°C, the viability immediately after ambient storage was 75%, but dropped to 51% after 15 h at 37°C (Table II). Whereas the initial viability of cells stored at 4°C was slightly higher (79%), almost no viable cells were detected following transfer to 37°C . Similarly, T-cells at an initial concentration of $2x10^6$ cells/ml exposed to the same S/MM-03 PHORBOL experiment time/temperature profile showed higher viabilities after 24°C storage than 4°C storage (Table II).

An experiment was performed with U937 cells in the GCAK-2 cassettes in RPMI-1640

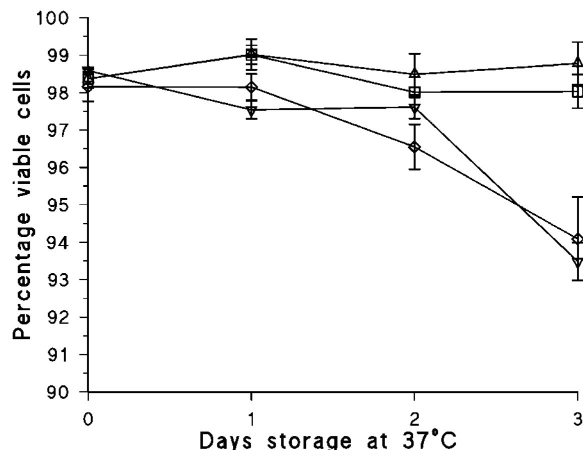

Fig. 4. Viability of U937 cells stored in 2 ml syringes in RPMI-1640 medium with or without 1 mM pyruvate. Cells were loaded at $2x10^6$ cells /ml. Viability immediately after 24°C storage in medium either with pyruvate (■) or without (▲). Viability after 18h at 37°C following transfer from 24°C, with (▼) or without (◆) pyruvate. For each data point n=4.

medium without pyruvate in temperature time profiles simulating the IML-2, S/MM-03 PHORBOL, and S/MM-06 time temperature profiles (Fig. 5). The decrease in viability with time was slower than in previous experiments, due to the use of a different strain of U937 cells. Little loss of viability was noted on transfer from 24°C to 37°C. The size and shape of cells were assessed using cytofluorimetry in this experiment. This showed that the forward scatter (cell size) decreased with increased storage time at 24°C. After transfer to 37°C, side scatter (cell shape) increased compared to 24°C stored samples (Fig. 6).

Experiments with Jurkat cells. Jurkat cells cultured in the GCAK-1 cassettes for up to 72 h at 37°C from an initial concentration of $5x10^5$ cells/ml grew faster than corresponding samples cultured in multiwells (Fig. 7a). In the same experiment cell viabilities were comparable in the cassettes and multi-well, being at least 80% after 72 h of culture (Fig. 7b). In a simulation of the T-cell experiment, profile cell numbers increased compared to the initial cell concentrations of $5x10^5$ and $1x10^6$ cells when measured 4 h and 48 h after transfer to 37°C. At both time intervals mean viabilities remained in excess of 90% (Fig. 8a). Likewise, D-glucose utilisation increased after transfer to 37°C and addition of fetal calf serum, indicating growth activation (Fig. 8b).

Cellular Activation in the Experiment Cassettes

Cytokine synthesis following phorbol ester stimulation. The ability of cells to produce cytokines following phorbol ester stimulation was used as a measure of cell growth activation following storage in the experiment cassette. U937 cells were subject to the S/MM-03 PHORBOL experiment temperature profile (4 d at 24°C, 15h at 37°C), as detailed in Materials and Methods, before stimulation with 100 ng/ml PDBu. TNF-α is a cytokine secreted in response to phorbol ester stimulation. Significant quantities of TNF-α were secreted by the cells, compared to unactivated cultures, indicating that the cells were still sensitive to stimuli after storage under a simulated Space Shuttle mission profile (Table III).

Protein kinase C translocation. Protein kinase C (PKC) is a serine/threonine kinase that mediates signal transduction from the membrane to the cell nucleus. When PKC is activated, it transiently translocates to the particulate (membrane/cytoskeleton) fraction of the cell from the cytosol, so this phenomena can be used as a measure of cellular activation. Since PKC is directly activated by phorbol esters, we examined PKC translocation in U937 cells following ^{3}H-PDBu stimulation in the GCAK-2 cassette. The translocation kinetic of PKC following the S/MM-03 PHORBOL experiment temperature profile (4 d at 24°C, then 15 h at 37°C) was compared to an identical sample stored for 15 h at 37°C (Fig. 9). Although the magnitude of the kinetic is diminished in the S/MM-03 temperature profile samples compared to the control sample, the pattern of translocation appears to be unchanged. This result demonstrates that the cells are still capable of responding to stimulation following storage in a temperature profile comparable to that of a Shuttle-Mir docking mission.

DISCUSSION

The aim of our ground experiments presented here was to determine if the objectives of a series of experiments proposed for flight aboard the Space Shuttle in the ESA Biorack facility could be met within the operational constraints of the mission. Our results show the feasibility of performing experiments with the cell lines examined in the GCAK cassettes under temperature and time profiles likely to be encountered in the course of an experiment aboard the Space Shuttle. Unlike cell culture in

TABLE II. U937 and Purified Human T-Cell Viability Under Simulated Shuttle Mir (S/MM) 03 Phorbol Experiment Flight Profile

Cell type	4°C		24°C	
Treatment	4-day storage	4-day storage, then 15 h at 37°C	4-day storage	4-day storage, then 15 h at 37°C
U937 cells	79.9% ± 2.9%	No viable cells	75.5% ± 0.2%	51.2% ± 3.7%
T cells	—	75.9% ± 4.4%	—	91.7% ± 2.0%

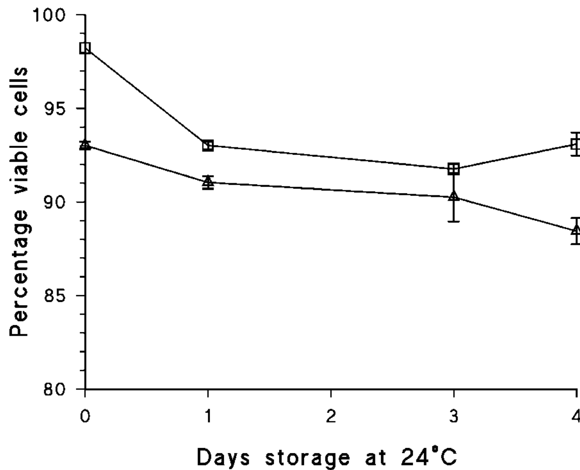

Fig. 5. Viability of U937 cells stored in GCAK-2 cassettes at 24°C before and after transfer to 37°C. Cells were subject to storage temperature time profiles that simulated three different flight experiment protocols; IML-2 Cytokine experiment (1 day storage), S/MM-06 Cytokine (3 days storage), and S/MM-03 Phorbol experiment (4 days storage). (■) Viability measured at the end of the 24°C storage period immediately before transfer to 37°C. (▲) Viability measured after 15 h incubation at 37°C, following 24°C storage. For each time point n=3 and error bars are standard error of the mean (SEM).

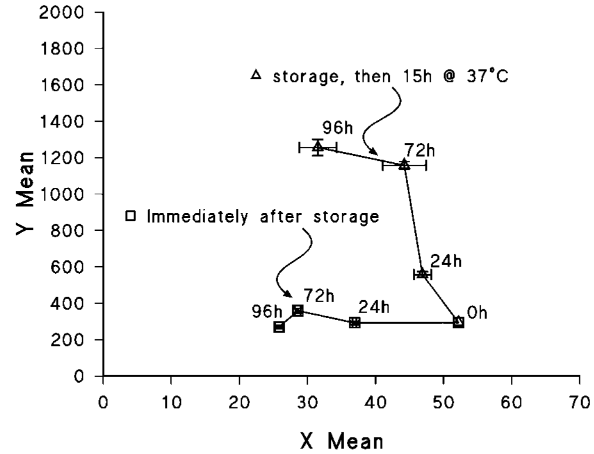

Fig. 6. Forward scatter and side scatter measurements in a cytofluorimeter of U937 cells cultured in GCAK-2 cassettes in the same experiment shown in Figure 5. Forward scatter values are proportional to cell size, whereas side scatter is a measure of the irregularity of cell shape. 2x10^6 cells/ml. (■) measurements obtained at the end of the 24°C storage period immediately before transfer to 37°C. (▲) Viability measured after 15 h incubation at 37°C, following 24°C storage. Storage time in hours is indicated next to individual data points. Each time is the mean of three sample wells in a cassette and error bars are standard error of the mean (SEM).

a terrestrial laboratory, the experiment must be performed in sealed culture systems, packaged to permit the experiment to be performed with minimum manipulations by crew members and satisfy stringent safety requirements. We developed the GCAK cassette system, which incorporates these requirements and permits several different cell culture manipulations during Shuttle flight missions.

An initial concern was the biocompatability of the experiment cassette with the cell cultures. Routine laboratory cell culture typically uses disposable flasks, usually made of just one material, typically polystyrene. In contrast, in the GCAK cassette uses several different materials, including polycarbonate, rubber, silicone coatings, and stainless steel. Additionally, the cassettes are designed to be reusable, which introduces the possibility of degradation of materials and the release of cytotoxic substances. Initial experiments showed that the silicone-coated rubber pistons in the culture chambers released toxic products into the culture medium as a result of degradation of the material during autoclaving (data not shown). This problem was resolved by inserting fresh γ-irradiated sterile pistons from disposable syringes into the experiment hardware after autoclaving of the hardware. The results of culture experiments with both U937 and Jurkat cells in the cassettes indicate that cell growth and viability are not adversely affected in the cassette and indeed in the case of Jurkat cells cell growth may even be enhanced in the cassette for specific culture conditions (Figs. 5 and 7).

The Biorack does not provide a standard laboratory incubator atmosphere with 5% CO_2. Therefore, the requirement for atmospheric CO_2, which buffers the medium and provides a source of hydrogen bicarbonate, was replaced with hepes, sodium bicarbonate, and sodium pyruvate [Freshney, 1987]. Sodium pyruvate

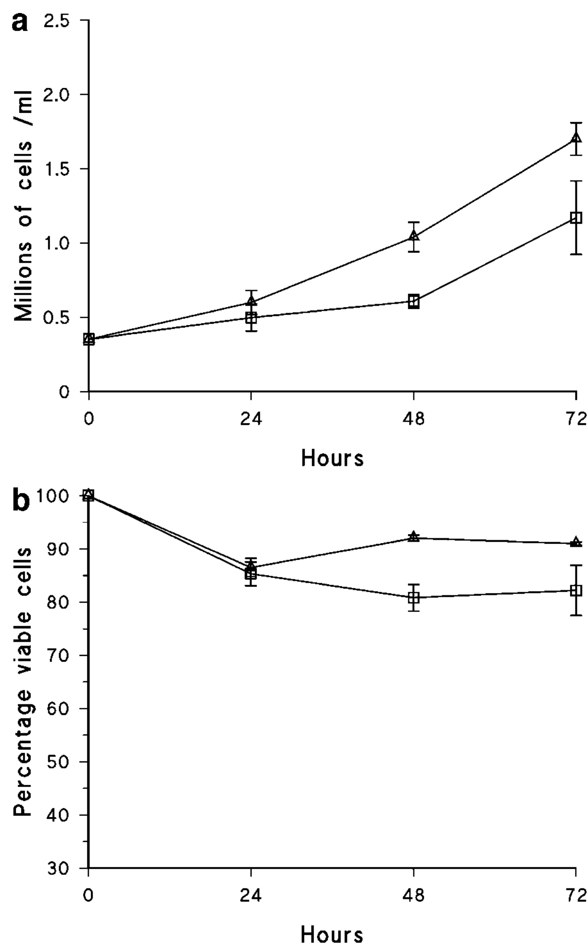

Fig. 7. Biocompatability of Jurkat cells with GCAK-1 cassettes. (**a**) Growth of Jurkat cells at 37°C up to 72 h after initial loading of cells into the cassette at 4×10^5 cells /ml (**b**) Percentage of viable cells in hardware in same experiment. (■) number of cells in cassettes, (▲) number of cells in multi-well controls. For each time point n=6 and error bars are standard error of the mean (SEM).

provides a substrate that promotes endogenous production of CO_2 by the cells and may modulate cell viability and proliferation [Kroll et al., 1986; Borle and Stanko, 1996]. This may be necessary in the total absence of CO_2, since HCO_3- may be totally converted to H_2CO_3 and gaseous CO_2 under these conditions and thus be unavailable to the cells. However, our experiments showed that the U937 cell viability was unaffected by the presence or absence of pyruvate (Fig. 4). The reason for this is most likely at the cell concentrations used for these experiments the cells were producing adequate quantities of CO_2 even in the absence of pyruvate. However, for other cell types, especially those that are slow growing and at low concentrations, pyruvate may be necessary to ensure the maintenance of adequate pH and bicarbonate concentrations.

The operational constraints of a typical Space Shuttle mission often result in the experiment being exposed to temperatures that are not normally optimal for mammalian cell growth. The ambient temperature of the Shuttle cabin is generally around 24°C. Some mammalian cell cultures experiments have successfully been performed at the ambient temperature of the Space Shuttle cabin, e.g., a series of experiments with the mouse cell line LM929 [Woods and Chapes, 1994]. However, these experiments may not reflect the behaviour of the cell under physiological conditions, and some cell types may fail to function normally at a temperature other than 37°C. Biorack provided a 37°C±0.5°C incubator for our experiments. However, prior to Biorack activation the experiments were stored in a locker in the Shuttle middeck.

Since no power was available for active temperature control in the middeck lockers on Biorack missions, the samples had to be stored at either ambient temperature (between 21°C and 24°C) or at 4°C in a Passive Thermal Conditioning Unit (PTCU). Due to operational reasons, the latest that an experiment could be installed aboard the Shuttle was 18 h before launch, with the time between launch and cassette loading into the Biorack incubator varied between 7 h and 3 days. Due to the possible detrimental effect of this lengthy storage time at a temperature other than 37°C on cell viability and function, we examined the viability of U937 and Jurkat cells at 24°C and 4°C for up to 6 days of storage. As expected, viability was found to decrease with time, although at a greater rate in 24°C stored cultures. Decrease in cell viability was also accelerated at high cell concentrations, as would be expected due to increased nutrient utilisation in the medium. However, on transfer to 37°C, the viability of U937 cells decreased markedly and almost all cells stored at 4°C died. Therefore, ambient stowage conditions were chosen for all experiments performed with U937 and Jurkat cells in the GCAK cassettes. For U937 cells, a modification in the culture medium composition improved viability in storage and after transfer to 37°C, as indicated above. Similarly, Jurkat cells subject to a temperature/ time simulating that of the flight experiment profile maintained good viability. The cytofluorimetry data shows that the U937

cells became more granular and larger, suggesting the cells are becoming apoptotic. This is to be expected since the cells consume nutrients in the medium during storage in the cassettes and so will be subject to increased environmental stress as time progresses. U937 cells remained responsive to phorbol ester stimulation, as indicated by cytokine synthesis and PKC translocation, although the magnitude of response was decreased compared to freshly prepared cells. However, we cannot tell from these data if the response is from the whole cell population, or a subset of the cell population. These data indicated that it was feasible to perform the flight experiments according to the time/temperature protocol proposed for each mission. However, different cell types showed different sensitivities to storage conditions. Whereas U937 cells could withstand the worse case storage conditions of the S/MM-03 Phorbol experiment, Jurkat cells did not synthesis IL-2 in response to phorbol esters after this time/temperature profile (data not shown). Therefore, this shows the importance of choosing a suitable cell type that can tolerate the storage conditions of a proposed flight experiment. However, the ground experiments presented here and results of flight experiments [Schmitt et al., 1996; Lewis et al., 1998] demonstrate that certain cell types can be stored at ambient temperature during several days before transfer to 37°C, without a serious impact on the functionality of the cells.

The experimental constraints associated with performing the series of experiments on the Space Shuttle presented here are typical for many different experiments using mammalian cell types. Our solution to these challenges was to develop the GCAK cassette system, which permits short-term cell culture, with the possibility to add fixative and activators to cells in suspension, as well as separation of cells from supernatent by filtration in the GCAK-1 unit. The principle advantages of this cassette system is that up to six replicate samples can be manipulated simultaneously in each cassette, the unit is simple to operate, and it is possible to perform many of the general manipulations required for cell biology experiments in microgravity.

During the six flights of Biorack, a total of 81 experiments were performed, 49 of these involving some form of cell culture (including microrganisms and plant protoplasts). Thirty-six of these experiments required addition of liquids to a cell culture; 16 experiments required sample removal, medium exchange, or filtration of the cell culture. Many of the experiments flown in Biorack used multiuser cassettes that fulfilled these general requirements, including the 'Cytos' cassette (8 experiments), the 'Blood' hardware (6 experiments), and 'Eggs' hardware (7 experiments) [Planel et al., 1981; Ubbels et al., 1990; Pippia et al., 1996]. Equally the GCAK cassette system could be easily adapted to many cell biology investigations feasible in Biorack. In fact, aside from the experiments with leukocytes discussed above, the GCAK cassette has been adapted for use in an experiment with bacteria (B. Pyle, pers. comm.) and two experiments with sea urchin sperm (J. Tash., pers. comm.). Several other facilities and experiment hardware, which are not compatible with Biorack, have been developed to perform mammalian cell culture experiments on the Space Shuttle that permit similar manipulations to those possible with the GCAK cassette. These include the Materials Dispersion Apparatus (MDA) minilab, which contains cells and reagents in small wells in separate polycarbonate blocks [Lewis et al., 1994; Hughes-Fulford and Lewis, 1996]. Addition of reagents to cell cultures is effected by sliding the polycarbonate blocks past each other. The Bioprocessing Module (BPM) is a system of four syringes interconnected through a valve [M.L. Lewis, unpublished communications], whereas the Fluid Processing Apparatus (FPA) consists of a glass cylinder partitioned with septa to create 2-3 chambers with the contents of the chambers being mixed by pushing a plunger [Klement and Spooner, 1994; Spooner et al., 1994]. The MDA, BPM, and FPA typically use a Commercial Incubator Refrigerator Module (CRIM) for temperature control, which is usually accommodated in a single Shuttle middeck locker. Therefore, these facilities potentially offer regular access to microgravity, since the CRIM only takes up a small space on the Shuttle middeck and so can be flown as a secondary payload on many missions. However, unlike Biorack, there is no 1.*g* centrifuge and freezer/cooler storage of samples may require the use of a separate facility.

Another facility that is accommodated in a middeck locker is the Space Tissue Loss (STL)/Cell Culture Module (CCM), an automated facility with active temperature control that uses

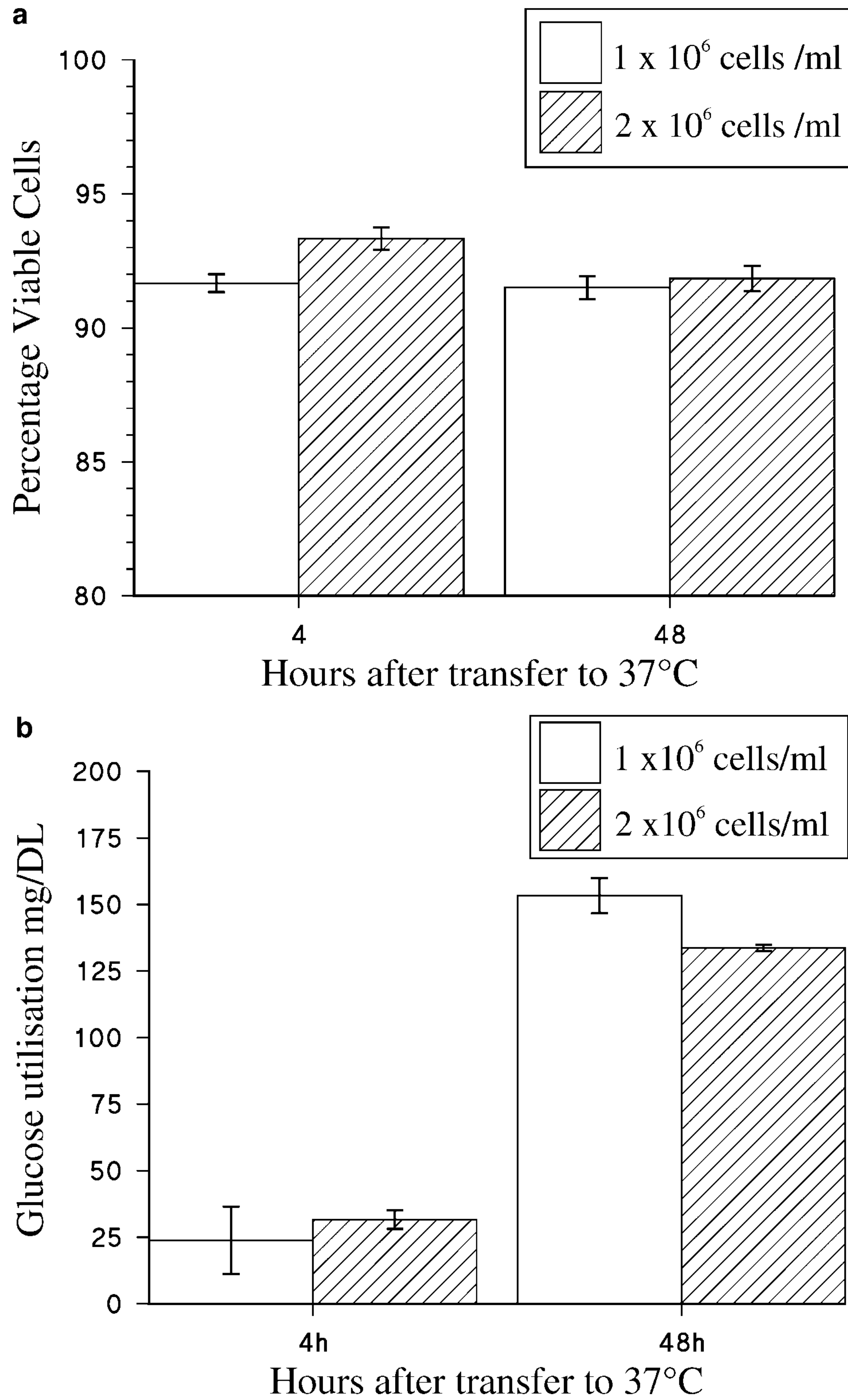

Fig. 8. Viability and glucose utilization by Jurkat cells in a simulated temperature/time profile for the S/MM-03 T-cell experiment. Cells were maintained at 24°C for 36 h before transfer to 37°C, when the culture medium serum concentration was also raised from 2% to 10% (**a**) Viability measurements taken at 4 and 48 h after transfer to 37°C. (**b**) D-Glucose utilization at 4 h and 48 h after transfer. (■) 1x10^6 cells/ml (▲) 2x10^6 cells/ml. For each time point n=3 and error bars are standard error of the mean (SEM).

TABLE III. TNF-alpha Synthesis by U937 Cells in GCAK-2 Cassettes for a Simulated S/MM-03 PHORBOL Temperature Profile

TNF-α concentration in supernatent, pg/ml	
Unstimulated samples	Stimulated samples
72.2 ± 10.1	246.4 ± 24.6

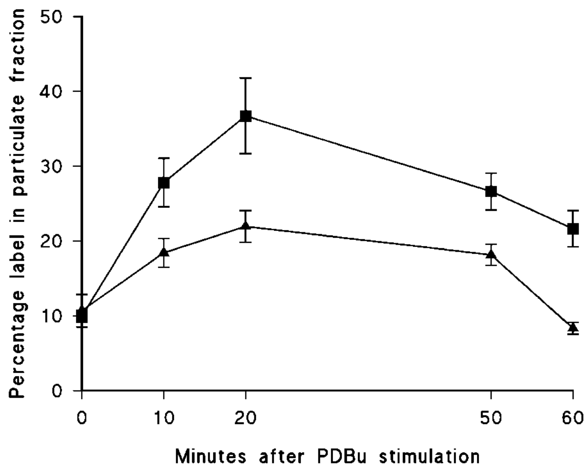

Fig. 9. Protein kinase C translocation in U937 cells in response to phorbol ester stimulation. U937 cells were loaded into GCAK-2 cassettes at $1x10^6$ cells/ml and incubated for either 15 h at 37°C (■) or 4 days at 24°C, before transfer to 37°C for 15 h (▲). PKC translocation to the particulate fraction is expressed as percentage of total labelling with the PKC marker ^{3}H-PDBu. For each time point n=6 and error bars are standard error of the mean (SEM).

commercially available disposable hollow fibre bioreactors [Kulesh, 1994; Davis et al., 1996]. Unlike the culture systems discussed above, the STL/CCM can continuously perfuse the cells with actively oxygenated medium, as well as add reagents to the culture medium. Additionally, sophisticated experiment manipulations are possible including real time measurements of medium pH, periodic sample collection, and examination of the cell culture using a microscope. Such measurements are important to more fully understand the mechanism by which microgravity acts on the cell, particularly to differentiate between direct effects of microgravity on intracellular processes and indirect effects on the intracellular environment. Clearly, there are many different solutions to the challenges associated with performing mammalian cell culture experiments aboard the Space Shuttle, each of which has advantages or disadvantages compared with other systems.

The international space station (ISS) will soon be assembled in orbit. The ISS will provide enhanced research capabilities and longer duration microgravity in comparison with existing research facilities aboard the Space Shuttle, although it also presents new technical challenges for cell biology experimentation since there are several months between resupply flights. Dedicated cell biology experiment facilities are planned for the station, but experimenters will still need to develop experiment specific hardware to perform experiments in these facilities. Experience with Space Shuttle facilities suggests that many different experiments could be performed with appropriately designed multiuser experiment hardware. Experience gained from prior experiments with apparatus such as the GCAK cassette system, along with ground-based tests to determine the effect of mission and technical constraints will be invaluable in designing new hardware to take advantage of the ISS facilities.

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REFERENCES

Borle AB, Stanko RT (1996): Pyruvate reduces anoxic injury and free radical formation in perfused rat hepatocytes. Am J Physiol 270: G535-540.

Briarty LG (1989): 'Biology in Microgravity: A Guide for Experimenters.' Paris: European Space Agency.

Cogoli A, Tschopp A, Fuchs-Bislin P (1984): Cell sensitivity to gravity. Science 225:228-230.

Cogoli A, BechlerB, Cogoli-Greuter M, Criswell SB, Joller H, Joller P, Hunzinger E, Müller O (1993): Mitogenic signal transduction in T lymphocytes in microgravity. J Leukoc Biol 53:569-575.

Cogoli A, Gmünder FK (1991): Gravity effects on single cells: Techniques, findings and theory. Adv Space Biol Med 1:183-248.

Davis TA, Wiesmann W, Kidwell W, Cannon T, Kerns L, Serke C, Delaplaine T, Pranger A, Lee KP (1996): Effects of spaceflight on human stem cell hematopoiesis: Suppression of erthropoiesis and myelopoiesis. J Leukoc Biol 60:69-76.

Demets R (1993): BIOBOX: ESA's programmable flight incubator. Microgravity news from ESA 6:27-28.

De Groot RP, Rijken PJ, Den Hertog J, Boonstra J, Verkleij AJ, De Laat SW, Kruijer W (1991): Nuclear responses to protein kinase C signal transduction are sensitive to gravity changes. Exp Cell Res 197:87-90.

Freshney RI (1987): ‘Culture of Animal Cells.‘ New York: Alan R. Liss.

Genzel P, Mesland D (1988): The ESA Biorack facility. In Hunt J (ed): ‘3rd European Symposium on Life Sciences Research in Space,‘ Vol. ESA SP-271. Paris: European Space Agency, pp 21-26.

Hatton JP, Cazenave J-P, Schmitt DA (1997): Protein kinase C mediated signal transduction is sensitive to gravity. In Sato A (ed): ‘Frontiers of Biological Science in Space.‘ Tokyo: Taiyo Print, pp 82-103.

Hatton JP, Gaubert F, Darsel Y, Ohlmann P, Cazenave J-P, Schmitt DA: Protein kinase C quantity in U937 cells is increased in microgravity (in prep.).

Hughes-Fulford M, Lewis ML (1996): Effects of microgravity on osteoblast growth activation. Exp Cell Res 224:103-109.

Klement B, Spooner B (1994): Pre-metatarsal skeletal development in tissue culture at unit- and microgravity. J Exp Zool 269:230-241.

Kroll W, Postius S, Schneider F (1986): Proliferation kinetics and metabolic features of in vitro grown Ehrlich ascites tumour cells in the prescence of exogenous pyruvate. Z Naturforsch 41:787-794.

Kulesh DA, Anderson LH, Wilson B, Otis EJ, Elgin DM, Barker MJ, Mehm WJ, Kearney GP (1994): Space Shuttle flight (STS-45) of L8 myoblast cells results in the isolation of a nonfusing cell line variant. J Cell Biochem 55:530-544.

Lewis ML (1994): Space: A new ‘laboratory‘ for cell biology research. Earth Space Rev 3:22-28.

Lewis ML, Piepmeier EH, Hatton JP, Reynolds JL, Cubano LA, DeSales Lawless B (1998): Spaceflight alters microtubule structure and increases apoptosis in human lymphocytes (Jurkat). FASEB (in press).

Limouse M, Manie S, Konstantinova I, Ferrua B, Schaffar L (1991): Inhibition of phorbol ester-induced cell activation in microgravity. Exp Cell Res 197:82-86.

Moore D, Cogoli A (1996): Gravitational and space biology. In Moore D, Bie P, Oser H (eds): ‘Biological and Medical Research in Space.‘ Berlin: Springer-Verlag, pp 1-107.

Planel H, Tixador R, Nefedov Y, Gretchko G, Richoilley G, Bassler R, Monrozies E (1981): Spaceflight effects on Paramecium tetraurelia flown aboard Salyut 6 in the Cytos I and Cytos M experiments. Adv Space Res 1:95-100.

Pippia P, Sciola L, Cogoli-Greuter M, Meloni MA, Spano A, Cogoli A (1996): Activation signals of T lymphocytes in microgravity. J Biotech 47:215-222.

Schmitt DA, Hatton JP, Emond C, Chaput D, Paris H, Levade T, Cazenave J-P, Schaffar L (1996): The distribution of protein kinase C in human leukocytes is altered in microgravity. FASEB J 10: 1627-1634.

Spooner BS, Hardmann P, Paulsen A (1994): Gravity in mammalian organ development: Differentiation of cultured lung and pancreas rudiments during spaceflight. J Exp Zool 269:212-222.

Ubbels GA, Berendsen W, Kerkvliet S, Narraway J (1990): Fertilisation of Xenopus eggs in space. In David P (ed): ‘Proceedings of the IVth European Symposium on Life Sciences Research in Space, ESA SP-307.‘ Paris: European Space Agency, pp 249-254.

Van Loon J: Presentation of Biopack facility at European Low Gravity Association (ELGRA) Biennial meeting, Paris, 17-19 March 1997 (pers. comm.).

Woods KM, Chapes SK (1994): Abrogation of TNF-mediated cytotoxicity by space flight involves protein kinase C. Exp Cell Res 211: 171-174.