Suppressed PHA Activation of T Lymphocytes in Simulated Microgravity is Restored by Direct Activation of Protein Kinase C with Phorbol Ester

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Abstract

Various aspects of spaceflight, including microgravity, cosmic radiation, and physiological stress, may perturb immune function. We sought to understand the impact of microgravity alone on the cellular mechanisms critical to immunity. We utilized clinostatic RWV bioreactors that simulate aspects of microgravity to analyze the response of human PBMC to polyclonal activation. PHA responsiveness in the RWV was almost completely diminished. IL-2 and IFN-γ secretion was reduced whereas IL-1β and IL-6 secretion was increased, suggesting that monocytes may not be as adversely affected by simulated microgravity as T cells. Activation marker expression (CD25, CD69, CD71) was significantly reduced in RWV cultures. Furthermore, addition of exogenous IL-2 to these cultures did not restore proliferation. Reduced cell-cell and cell-substratum interactions may play a role in the loss of PHA responsiveness. However, PHA activation in Teflon culture bags that limit cell-substratum interactions did not suppress PHA activation. Furthermore, increasing cell density and, therefore, cell-cell interactions in the RWV cultures did not help restore PHA activation. However, placing PBMC within small collagen beads did partially restore PHA responsiveness. Activation of both PBMC and purified T cells with PMA and ionomycin was unaffected by RWV culture, indicating that signaling mechanisms downstream of PKC activation and calcium flux are not sensitive to simulated microgravity. Furthermore, sub-mitogenic doses of PMA alone but not ionomycin alone restored PHA responsiveness of PBMC in RWV culture. Thus, our data indicate that during polyclonal activation the signaling pathways upstream of PKC activation are sensitive to simulated microgravity.
Introduction

Several physiological systems are altered by the environment of spaceflight (1). Studies over the past two decades show spaceflight to alter immune performance (2). Psychological and physical stress, cosmic radiation, as well as microgravity are all potentially responsible for the adverse effects of spaceflight (3). Any of these perturbations may affect immune performance directly or indirectly. Interpretation of spaceflight studies is confounded by the inability to isolate the direct effect of microgravity on cells from the other factors associated with spaceflight. Since immune performance is complex and reflects the influence of multiple organ systems within the host, we sought to understand the potential impact of microgravity alone on the cellular mechanisms critical to immunity. We achieved this by isolating immune cells \textit{ex vivo} and challenging them with simulated microgravity within an experimental setting.

We utilized Rotating Wall Vessel (RWV)$^3$ bioreactors developed at NASA's Johnson Space Center that simulate aspects of microgravity (4, 5). The RWV bioreactors are based on a previous clinostat design (6). The RWV consists of a zero head-space cylindrical culture vessel that rotates at a slow speed around a horizontal axis. These conditions create a solid fluid body that can suspend cells or small particles in a perpetual free fall condition simulating conditions of microgravity for cell cultures (7). The system provides us with the ability to assess immune function at a cellular level, without the confounding influences of psychoneuroendocrine factors. Cells can also be analyzed while they are experiencing simulated microgravity rather than after they have been exposed. The RWV was used to demonstrate suppression of lymphocyte locomotion through collagen by simulated microgravity that correlated with similar orbital flight studies (8). The RWV bioreactor provides for a unique opportunity to study the response of cells to microgravity without the limitations of flight experiments.

Many orbital experiments have analyzed the immune system's response to microgravity and spaceflight. Astronauts post-flight of long-duration missions have an
increase in leukocyte count, a decrease in T cell count, decreased mitogen induced IL-2 production, and decreased response to PHA (9). Astronauts post-flight of short-duration missions have a decrease in lymphocyte count, an increase in neutrophil count, and a decreased response to PHA (10). Astronauts in-flight of both long and short-duration missions have a reduced delayed-type hypersensitivity skin test response (9-11). Furthermore, *in vitro* experiments performed in both true and simulated microgravity demonstrate a suppressed response of lymphocytes to mitogens (12-17).

In our current study, we used the RWV to further characterize the suppression of PHA responsiveness by simulated microgravity. Cytokine secretion was measured to determine the activity of lymphocytes and accessory monocytes in this system. We found monocyte associated cytokines, IL-1β and IL-6, to be elevated while T cell associated cytokines, IL-2 and IFN-γ, were reduced. Activation marker expression was also reduced in RWV cultures. Early markers, CD69 and CD25, were slightly upregulated in RWV cultures but the late marker CD71 was not induced. Several experiments were designed to address the possible reduced cell-cell and cell-substratum interactions that microgravity could impart. Lymphocytes were activated with PHA in Teflon culture bags to assess the role of the limitation of cell-substratum interactions on activation. Proliferation was unchanged in the Teflon culture bags. Cell densities in the RWV's were increased to enhance cell-cell interactions and facilitate activation. We found that increasing cell densities to high levels did not aid activation in simulated microgravity. However, partial restoration of PHA responsiveness in the RWV was achieved by placing PBMC within small collagen beads prior to activation in the RWV. Several experiments addressed the status of signaling messengers in PHA activated lymphocytes in the RWV. Both PBMC and column purified T cells could be activated with PMA and ionomycin in the RWV. Furthermore, sub-mitogenic doses of PMA could restore PHA activation in the RWV. Ionomycin alone had no effect on PHA activation. These experiments suggest that the calcium signaling pathway may be
intact during PHA stimulation in the RWV and that signal transduction mechanisms upstream of protein kinase C (PKC) activation are sensitive to simulated microgravity.
Material and Methods

Cells and Media

Normal human blood buffy coats were obtained from the Gulf Coast Regional Blood Center (Houston, TX). The PBMC were isolated from the buffy coats on a Ficoll-Hypaque gradient (Pharmacia LKB, Piscataway, PA), washed three times in HBSS, and resuspended in complete RPMI 1640 (GibcoBRL, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Hyclone Labs, Logan, UT) and penicillin(100 U/ml)-streptomycin(100 µg/ml, GibcoBRL). Purified T cells were isolated from PBMC's cleared of erythrocytes (Erythrocyte Lysing Kit, R&D Systems, Minneapolis, MN) on T cell enrichment columns (R&D Systems).

RWV bioreactor

The RWV bioreactor (Synthecon, Houston, TX) is a cylindrical culture vessel with zero headspace and a silicon membrane for direct gas exchange with the culture medium. For each experiment, a sterile 55 ml RWV was filled completely with complete medium and bubbles were removed through the syringe ports. The vessel was conditioned for at least one hour by rotating the vessel at 14 rpm in a humidified 37°C incubator with approximately 1 L/min 5% CO₂, 95% air pumped passed the oxygenation membrane. After the conditioning period, cells and reagents were introduced to the RWV through the syringe ports or the main port. The vessel was then incubated as described above. The vessels must remain completely filled throughout the experiment. Therefore, cells and media that were sampled were replaced by fresh media. Typically, 5 ml samples were taken by pumping 5 ml of fresh media into one syringe port while simultaneously withdrawing 5 ml from the other syringe port. Cells were then set up in proliferation assays and supernatant was frozen at -20°C for later analysis. Due to the limited number of available RWV bioreactors and the limited number of total cells obtainable
from a single donor buffy coat, most experiments were repeated independently at least three times. The data shown are of one representative experiment.

**PHA stimulation and proliferation assays**

PBMC (1 x 10^6 cells/ml) were stimulated with 5 µg/ml phytohemagglutinin (PHA-M, Sigma, St. Louis, MO) in standard T-75 tissue culture flasks, 55 ml RWV's, or 100 ml Teflon culture bags (American Fluoroseal Corp., Columbia, MD). For the IL-2 study, recombinant human IL-2 (GibcoBRL) was also added at 10, 50, and 100 U/ml. For the PMA/ionomycin study, PMA (0.5 - 5 ng/ml) and ionomycin (50 - 500 ng/ml, both from Sigma) were added to the PHA cultures. Rotation of the RWV was started immediately except for the static pre-incubation period studies where several RWV's were held stationary for 30 - 180 min and thereafter rotated. RWV's and T-flasks were incubated as described above. Proliferation was determined for sampled cells by [³H]thymidine incorporation. Sampled PBMC (2 x 10^6 cells/well) were labeled with [methyl-³H]thymidine (1 µCi/well, 5 Ci/mmol, Amersham Life Sciences, Arlington Heights, IL) in triplicate for 18 hours in standard 96 well plates, harvested onto glass filter paper, and analyzed by standard liquid scintillation techniques. The data are presented as Δ cpm (experimental cpm minus background cpm).

**Cytokine measurement**

PBMC (2 x 10^6 cells/ml) were stimulated with 5 µg/ml PHA in both a 55 ml RWV and a T-75 T-flask. At the time points shown, culture media samples were collected, cleared of cells and particles by centrifugation, and frozen at -20°C for later analysis. Later, the supernatants were thawed, diluted as needed, and run on BioSource Cytoscreen ELISA kits for IL-1β, IL-2, IL-6, and IFN-γ (Camarillo, CA) as described in their protocols. The media was diluted 2, 10, and 20-fold to keep results within the range of standard curves. The plates were read on a Dynatech MRS plate reader and analyzed with Dynatech BioLinx software (Chantilly, VA).
Flow cytometry

mAbs: CD25-Spectral Red (Tu69, Southern Biotechnology Associates, Birmingham, AL), CD69-R-Phycoerythrin (Leu23, Becton-Dickerson, San Jose, CA), and CD71-FITC (BerT9, Dako, Carpinteria, CA). Sampled PBMC (1 x 10^6 cells/reaction) were stained with 10 µl of each labeled antibody in 100 µl PBS with 2% FBS (staining buffer) for 30 minutes on ice and in the dark. The cells were washed twice in staining buffer and then resuspended in 500 µl 1% paraformaldehyde. Antibody binding was analyzed on a Coulter flow cytometer.

Collagen bead assay

PBMC were incorporated into small collagen beads. Collagen solutions were prepared as described previously (18, 19). Briefly, a stock type I collagen solution (2 mg/ml) was prepared from frozen rat tail tendons (Pel-Freeze, Rogers, AK). A concentrated complete medium solution was prepared by combining 20 ml 10X RPMI-1640 (GibcoBRL), 20 ml FBS (Hyclone), 10 ml 7.5% NaHCO_3 (GibcoBRL), and 2 ml 100X penicillin/streptomycin (GibcoBRL). To create a collagen polymerization solution, 6 ml of stock collagen solution was combined with 1 ml concentrated complete medium solution, 300 µl 7.5% NaHCO_3, and 250 µl 0.34N NaOH on ice. PBMC (3 x 10^7 cells/ml) were suspended in the collagen polymerization solution on ice. Beads were produced by pipetting 25 µl of cell-collagen solution into sterile wells of Terasaki microtiter plates (Nunc, Intermountain Scientific, Bountiful, UT) yielding 7.5 x 10^5 cells/bead. The beads were then polymerized by incubating the plates at 37°C for 2-3 minutes. The beads were removed from the plates and put into either a 55 ml RWV or T-75 T-flask containing complete RPMI with 5 µg/ml PHA at a concentration of approximately 100 beads per vessel. The RWV and T-flask were incubated as described above.
At the time points shown, beads were sampled from the vessels, collected and washed briefly with HBSS on Falcon nylon cell strainers (Fisher, Houston, TX). The collagen beads were then digested with a collagenase cocktail containing 0.7 mg/ml collagenase type III, 0.5 mg/ml collagenase type IV, 0.1 mg/ml DNAse (all from Worthington, Freehold, NJ), 25 mM HEPES, 5% FBS, in PBS for 30 min in a 37°C shaking water bath. The cells were collected by centrifugation and washed 2 times in complete RPMI. The collected PBMC were set up in a proliferation assay as described above.

**PMA and ionomycin activation assay**

PBMC or column purified T cells (2 x 10^6 cells/ml) were stimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 4 hours in both a RWV and T-75 T-flask. To minimize the toxic effects of PMA and ionomycin, the cells were isolated by centrifugation after 4 hours and reconstituted in fresh media without PMA and ionomycin. The cells were then cultured in a new RWV or T-flask for three days. After three days, sampled cells were set up in a proliferation assay as described above.
Results

Proliferation in the RWV bioreactor

Several immunological functions have been assessed in astronauts during and post spaceflight. The response of PBMC to polyclonal activation by PHA is a basic lymphocytic function. PHA stimulates T cells by crosslinking the TCR/CD3 complex as well as other cell membrane glycoproteins (20-22). There is a requirement for monocytes in the culture to provide costimulation through both physical contact and soluble factors (23). The response to PHA of lymphocytes from astronauts is suppressed after spaceflight (9, 10). Furthermore, the PHA responsiveness of lymphocytes cultured in clinostatic rotation and true microgravity is suppressed (12-17). The RWV bioreactors are an adaptation of clinostatic technology and therefore, we were interested in analyzing the response of PBMC to PHA in our system. PBMC were cultured for three days with PHA in the RWV bioreactors. We found proliferation to be almost completely suppressed in the RWV bioreactor at all time points measured (Fig. 1). Lymphocytes in the RWV cultures do aggregate and remain viable but do not form blasts. We concluded that the RWV bioreactor is an appropriate system for studying the suppression of lymphocyte activation by simulated microgravity.

Cytokine secretion in the RWV bioreactor

Within a PBMC population, cytokines secreted from both T cells and accessory monocytes are important for the proliferative response of T cells to PHA. IL-2 is a key autocrine cytokine in the activation and differentiation process of T cells. IL-1β and IL-6 are important costimulatory cytokines secreted by monocytes (24-28). The activity of lymphocytes and monocytes within the PBMC population stimulated with PHA in the RWV bioreactor was analyzed by their cytokine secretion profiles. We analyzed the
supernatants from cultures of PBMC activated with PHA in the RWV bioreactor for the presence of IL-2, IFN-γ, IL-1β and IL-6 (Fig. 2). The secretion of T cell associated cytokines, IL-2 and IFN-γ, was suppressed in the RWV bioreactor. IL-2 was undetectable throughout the experiment. Interestingly, IFN-γ secretion was initially suppressed but returned to normal levels at day three. Rather than being suppressed, the secretion of costimulatory cytokines, IL-1β and IL-6, was dramatically enhanced in the RWV bioreactor. The secretion profiles of these cultures demonstrate that monocytes and lymphocytes may respond differently to simulated microgravity. Thus, RWV culture suppressed lymphokine secretion whereas it enhanced monokine secretion.

Activation marker expression in the RWV bioreactor

PHA stimulation induces the expression of numerous cell surface molecules some of whose expression is required for complete activation (29). To gauge the extent of activation of our RWV cultures we analyzed the expression of three activation markers: CD25 (inducible α-chain of the IL-2 receptor) (30), CD69 (EA-1, Leu23, AIM) (31, 32), and CD71 (transferrin receptor) (33). PBMC from PHA cultures were analyzed by flow cytometry for both percentage stained cells and mean fluorescence intensity (MFI). While the late marker, CD71, failed to be induced in the RWV cultures, the induction of the early markers, CD25 and CD69, was suppressed by over 50% (Fig. 3A). Furthermore, of those cells that were positive for CD25 and CD69 in the RWV bioreactor, the MFI was significantly reduced (Fig. 3B). Thus, some early activation marker expression was seen in the RWV cultures but the number of positive cells and the MFI of those cells were significantly reduced.

Addition of exogenous IL-2 to PHA-RVV cultures
IL-2 is a key growth factor for T cells during activation. The interaction of IL-2 with its high affinity receptor on the T cell represents a committed step toward proliferation (34). Since IL-2 levels were undetectable in the PHA-RWV cultures, we added exogenous recombinant IL-2 to our PHA-RWV cultures to attempt to reconstitute proliferation. Various concentrations of recombinant IL-2 were added along with PHA to PBMC cultures in the RWV bioreactor. No amount of IL-2, even as high as 100 U/ml, could significantly restore lymphocyte proliferation to the control values (Fig. 4). Interestingly, the RWV culture actually suppressed some of the background proliferation caused by IL-2 alone.

Role of cell-cell and cell-substratum interactions on activation in the RWV bioreactor

It is important to consider characteristics of microgravity and rotational culture other than those directly related to gravitational effects. Cultures may be affected by the lack of sedimentation that occurs in space as well as in clinostatic bioreactors. There is a potential for cell-cell and cell-substratum interactions to be reduced under these conditions. We designed three experiments to address these concerns. PBMC were stimulated with PHA in Teflon culture bags to mimic the potential reduced cell-substratum interactions of the RWV bioreactor. These Teflon culture bags greatly impair the ability of cells to bind to and interact with the bag surface and are primarily used to force monocytes to grow in suspension (35). PBMC activated with PHA in Teflon culture bags proliferated to the same levels as those activated in standard T-flasks (Fig. 1). The reduced cell-substratum interactions of these conditions did not effect the ability of the cells to respond to PHA. Therefore, reduced cell-substratum interactions in the RWV bioreactor may not be important to the suppression of PHA activation.

Reduced cell-cell interactions may play a role in the suppressed response of PBMC to PHA in simulated microgravity. PBMC were stimulated with PHA in the RWV
bioreactor at higher than usual cell densities to augment cell-cell interactions. Two separate donor buffy coats had to be used for each higher cell density due to the limited total cell number obtainable from a single buffy coat unit and the volume demands of the RWV bioreactor. Increasing the cell density to $5 \times 10^6$ and $1 \times 10^7$ cells/ml did not help restore the proliferation of the PBMC (Fig. 5A, 5B). Therefore, the possible reduced cell-cell interactions in the RWV bioreactor may not be important to the suppression of PHA activation.

However, to further augment cell-cell and cell-substratum interactions in the RWV bioreactor, PBMC were polymerized within small collagen beads at a high cell population density. Within the beads the cells could interact with each other and the collagen matrix. The beads were suspended in the RWV bioreactor and stimulated with PHA. Later, cells were isolated from the collagen and analyzed. The proliferation of PBMC within collagen beads stimulated with PHA in the RWV bioreactor was restored by approximately 50% (Fig. 6). Thus, providing a stable support for the cells helped them resist the adverse effects of simulated microgravity.

**Effect of non-rotated pre-incubation period on activation in the RWV bioreactor**

Simulated microgravity could interfere with the T cell activation process at several points. We were interested in localizing points along the T cell activation signal transduction pathway that were sensitive to microgravity. Therefore, we provided PHA cultures with "head-starts" by allowing them to pre-incubate in the presence of normal gravity before they were exposed to simulated microgravity in the RWV bioreactors. PBMC were allowed to pre-incubate with PHA in non-rotating (non-microgravity simulating) RWV bioreactors for several periods of time. After the pre-incubation period, the RWV bioreactor's rotation was started (microgravity simulating) at the normal speed and continued rotating for three days until cells were sampled for analysis. One RWV bioreactor remained stationary for the entire experiment (72 hr). The longer the RWV cultures were allowed to pre-incubate at normal gravity the more...
proliferation was restored (Fig. 7). Some proliferation was restored after as short as 30 minutes at normal gravity. This suggests that once cells are allowed to start their activation process in normal gravity, they can continue on to proliferation even in simulated microgravity. Furthermore, the cells that remained in the non-rotated RWV bioreactor for the entire experiment (72 hr) proliferated to the control levels. This demonstrates that the suppression of proliferation seen in the RWV bioreactors is due to the rotation (microgravity simulation) and not other conditions of the vessels.

**PMA and ionomycin activation in the RWV bioreactor**

Two of the main activation signals of PHA activation are sent through the PKC and the calcium secondary messenger systems (36). To locate signaling mechanisms of the T cell activation process that are sensitive to simulated microgravity, we activated PBMC and column purified T cells with PMA and ionomycin. The direct activation of PKC by PMA and the addition of a calcium ionophore sufficiently drives T cells to proliferate without the need for monocyte costimulation or crosslinking of molecules on the cell surface. Proliferation of PMA/ionomycin activated PBMC and column purified T cells was not significantly affected by RWV culture (Fig. 8A, 8B). We therefore conclude that activation mechanisms downstream of PKC activation and calcium flux are not sensitive to simulated microgravity. Once cells are advanced to this stage of the T cell activation process, they are resistant to the suppressive effects of simulated microgravity. This may account for the proliferation seen in cells allowed to pre-incubate in normal gravity (Fig. 7).

Both the PKC and calcium signals are required for PHA to drive T cells to proliferate. One or both of these signals may be defective in the activation of T cells in the RWV bioreactor. To address this we added PMA and ionomycin, alone and together, to PHA cultures of PBMC in the RWV bioreactors. If either of the two signals are defective, the PMA or ionomycin should be able to complement the system and allow proliferation. Sub-mitogenic concentrations of PMA alone, but not ionomycin alone, was able to restore some proliferation in the PHA-RWV cultures. PMA and
Ionomycin together also restored proliferation but no more than PMA alone. These concentrations of PMA and ionomycin were shown to be sub-mitogenic since a culture of both in a T-flask without PHA produced no proliferation (Fig. 9A). Several mitogenic concentrations of PMA were effective at restoring proliferation in the PHA-RWV cultures (Fig. 9B). No concentration of ionomycin from 50 ng/ml to 500 ng/ml significantly restored proliferation in the PHA-RWV cultures (Fig. 9C). These results suggest that the calcium signal is functioning in the PHA-RWV cultures but the PKC signal is not. Therefore, the signaling pathways upstream of PKC activation are sensitive to simulated microgravity.
Discussion

All the biological systems on our planet have evolved in a gravity environment with a constant accelerational force of 9.8 m/s². How cellular systems will respond to the loss of this physical force is poorly understood. We focused on the immune system and specifically the activation and proliferation of lymphocytes which is critical to immunity. The suppression of the lymphocytic PHA response has been observed in both the lymphocytes of astronauts after spaceflight and in *in vitro* experiments in true and simulated microgravity (9, 10, 12-17). Using the RWV bioreactors in our lab to simulate microgravity, the direct effects of gravity changes on isolated lymphoid cell populations were demonstrated by the dramatic reduction of PHA responsiveness (Fig. 1). The present study focused on the mechanism of this suppression. We have isolated the location of the impairment in the T cell activation process to pathways upstream of PKC activation.

The stimulation of T cells to proliferate by PHA is a complex process involving both T cells and accessory cells (monocytes and B cells in a PBMC population). PHA, a lectin, binds to and crosslinks cell surface glycoproteins. Specifically on T cells, PHA can crosslink CD2, CD3, and the TCR complex, as well as other glycoproteins, and provide for receptor capping to initiate a sufficient activation signal into the cell (20-22). Costimulation is required and is provided by accessory monocytes through soluble factors such as IL-1β, IL-6, and TNF-α, as well as possibly by cell-cell ligand interaction (24-28). We found the cultures activated with PHA in simulated microgravity to have sufficient costimulation in the form of IL-1β and IL-6 (Fig. 2). In fact, the levels of these cytokines were increased. Increased secretion of these cytokines has been seen previously in cells exposed to microgravity in orbit (37, 38) and may reflect a cellular response to stress. The increased levels of IL-1β and IL-6 in the PHA-RWV cultures could also indicate reduced uptake of these cytokines by receptors on the T cells. The expression of cytokine receptors can significantly remove cytokines from the cultures through receptor binding and internalization. This can be seen as early as two days for
IL-2 in the T-flask cultures (Fig. 2). Therefore, the increased IL-1\(\beta\) and IL-6 levels may indicate a reduced IL-1\(\beta\) receptor and IL-6 receptor expression. This may explain the failure of the T cells to respond to the costimulatory cytokines. The key response to costimulation, the expression of the T cell autocrine growth factor IL-2, was completely absent in the PHA-RWV cultures. Previous flight studies by Cogoli et al. have measured both reduced (15) and normal (12) levels of IL-1 in cultures activated with Con A in microgravity. Our results support the latest findings that IL-1 secretion is not adversely affected in microgravity. IL-1 is a strong costimulatory signal for T cells and can provide the second signal required to initiate IL-2 production (26). In the PHA-RWV cultures, the IL-1\(\beta\) signal was strong but the T cells failed to initiate IL-2 production. This suggests there may be a lack of appropriate cytokine receptor expression or that there is a block in the costimulation signal transduction mechanism. Furthermore, the induction, production, and secretion of cytokines reflect a complex interplay of cytokine networks. The interference of these networks may result in unusual, and not easily explained, results such as the delayed secretion of IFN-\(\gamma\), a key T cell effector cytokine.

The expression of activation markers indicates that while the T cells were not completely unresponsive to the PHA signal, their activation state was weak at best. CD25 and CD69, two early markers, were upregulated on about 40% of the cells (Fig 3A). However, the MFI was very low compared to control levels (Fig. 3B). These results suggest that the PHA signal was not completely shut down at the surface of the T cell but rather the signal was not sufficient for full activation. The T cells did not advance to the later stage of activation as indicated by the lack of CD71 expression. While the monocytes were active, the T cells failed to respond to the costimulation, to produce IL-2, and to commit to proliferate. Furthermore, the failure to proliferate was not simply due to the lack of IL-2 since exogenous IL-2 could not restore proliferation (Fig. 4). This was not surprising considering the low amount of high affinity IL-2 receptor on the cell surface. Similar flight studies have also failed to restore mitogen activated proliferation with both exogenous IL-1 and IL-2 (12). These results suggest
that both reduced cytokine and cytokine receptor expression may be critical to the suppression of T cell activation in microgravity.

Although some studies suggest there may be a microgravity induced PKC defect (39, 40), we were able to fully activate T cells to proliferate in simulated microgravity with PMA and ionomycin (Fig. 8A, B). The pharmacological agents PMA, a phorbol ester, and ionomycin, a calcium ionophore, act synergistically to activate T cells to proliferate. These agents bypass the mechanisms at the cell surface and impart their effect inside the cell: PMA by mimicking 1,2-diacylglycerol (DG) and activating the serine/threonine kinase PKC; ionomycin by increasing the cytoplasmic free calcium ion concentrations (36). The ability to activate T cells in the RWV with PMA and ionomycin implies that all the cellular mechanisms involved in T cell activation downstream of PKC activation and calcium ion flux are intact in simulated microgravity. These results provide insight on why PHA responsiveness could be partially restored by pre-incubating PHA cultures at normal gravity before exposing them to simulated microgravity (Fig. 7). Cells that achieved PKC activation and calcium flux could continue on to proliferate even when they were exposed to simulated microgravity. Since PKC activation and calcium flux occur quickly after receptor triggering, one might expect full activation to be restored by pre-incubating the PHA cultures. Although these events do occur within minutes, sustained receptor triggering of up to 48 hours is required for optimal lectin activation (41). Once the pre-incubated cultures were exposed to simulated microgravity the signal from the receptor was no longer sustained due to the microgravity induced lesion somewhere upstream of PKC activation.

The signaling pathways upstream of PKC and not upstream of calcium are sensitive to simulated microgravity. This conclusion is based on the observation that PMA alone could restore PHA responsiveness, while ionomycin alone could not (Fig. 9A, B, C). Since the direct activation of PKC alone could restore PHA responsiveness, the calcium pathway is probably intact in the PHA-RWV cultures. The main activating molecules of PKC and calcium flux, DG and inositol trisphosphate (IP3), respectively, come from the cleavage of the same precursor, phosphatidylinositol 4,5-bisphosphate (PIP2) (42). Therefore, if calcium flux is intact, the cleavage of PIP2 is likely intact and
some signaling from the TCR/CD3 complex is present. We speculate that the interaction of DG with PKC is aberrant in simulated microgravity. However, the levels of intracellular calcium, IP$_3$, and DG have not been determined for PHA cultures in simulated microgravity. Secondly, the data suggest that the T cells are not receiving or processing the costimulation signal. PKC function is critical to costimulatory signals being transferred from the cell membrane to the nucleus. Activation of PKC with phorbol esters can mimic costimulation (43, 44). Therefore, the addition of PMA to the PHA-RWV cultures could possibly fulfill the lacking costimulation signal needed to upregulated IL-2 and initiate proliferation.

It is important to consider indirect effects that simulated microgravity has on cell culture conditions and what role they play in the suppression of T cell activation. Since cells remain dispersed throughout the media in the RWV, the interactions of cells with both substratum and other cells may be altered. There is evidence that PHA activation of PBMC requires cell contact between monocytes and T cells (45). Despite cells remaining dispersed in RWV cultures, there is evidence that normal cell-cell interactions do take place in cell cultures exposed to true and simulated microgravity. Aggregation of cells occurs in mitogen activated PBMC in microgravity (16, 46) and was observed in the PHA-RWV cultures. A suspension of cells will adhere to microcarriers in the RWV (8, 47). Furthermore, in the current study, IL-1β secretion in the PHA-RWV cultures was present. There is evidence that cell contact between T cells and monocytes through LFA-1 and CD69 is required for monocytes to produce IL-1 (48, 49). Nonetheless, cell-cell and cell-substratum interactions may be weaker in the RWV. However, when we tried to increase the amount of cell-cell contact by increasing cell densities in the RWV, proliferation was not restored (Fig 5A, B). Although some studies suggest that cell-substratum interaction is important to T cell activation (50), we found that PBMC activated with PHA in Teflon culture bags was unaffected (Fig 1). The ability of cells to interact with substratum may not be as important as the ability to interact with other cells. However, when PBMC were secured inside collagen beads, some proliferation was restored. Similar results were seen in an orbital flight study by
Cogoli et al. (15, 17). In their study, PBMC adhered to microcarriers and exposed to microgravity in earth orbit had an increased proliferative response to Con A than those in suspension or even those on microcarriers at normal gravity. How providing for adherence helped the PBMC resist the effects of RWV culture is not clear. Some studies suggest that the cytoskeleton is a gravity receptor for eucaryotic cells (51-53). This hypothesis proposes that cells exposed to microgravity are altered through changes in the cytoskeleton. Furthermore, lymphocytes exposed to microgravity undergo cytoskeleton-independent cell shape changes (54). It is possible that once cells adhere to collagen through adhesion molecules, which connects to the cytoskeleton via focal adhesion points, cells might be resistant to cell shape changes and cytoskeleton alterations caused by microgravity. Again, this is speculative at this point but certainly the response of the cytoskeleton to microgravity is an important area of study in relation to suppressed T cell activation. This is especially true considering the potential importance of both cell shape (55) and cytoskeletal elements (56) to T cell activation.

The suppression of PHA activation of T cells by simulated microgravity may be caused by several factors. The activation signals from both the primary TCR/CD3 signal and the costimulatory signals may be blocked at the activation of PKC. Adhering PBMC to substratum may help cells resist some of the adverse effects of simulated microgravity by helping them resist cell shape and cytoskeleton alterations. Further analysis of microgravity induced T cell unresponsiveness should provide insight on the roles of costimulation, the cytoskeleton, cell shape, cell contact, and cell-substratum interactions in polyclonal activation of T cells under normal conditions. The RWV bioreactor is only a simulation of microgravity and results found from these studies should be repeated in true microgravity. However, orbital flight studies are expensive, limited, and logistically tedious. Therefore, results from RWV bioreactor experiments are extremely valuable in directing the design of future flight studies by allowing the testing of hypotheses on earth.
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References


Footnotes:

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3 Abbreviations used in this paper: RWV, rotating wall vessel; PKC, protein kinase C; MFI, mean fluorescence intensity; DG, 1,2-diacylglycerol; IP3, inositol 1,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate.
FIGURE 1. PHA activation in the RWV bioreactor and Teflon culture bags. PBMC were stimulated with 5 \( \mu \)g/ml PHA for three days in three 55 ml RWV's, three Teflon culture bags, and three T-75 T-flasks. At each time point, 5 ml of culture was sampled from the vessels and 2 x 10⁵ cells/well were set up in an 18 hour \([^3]H\)thymidine assay in triplicate. Data are expressed as mean ± SEM of triplicate cultures.

FIGURE 2. Cytokine secretion of PHA activated PBMC in the RWV bioreactor. PBMC were stimulated with 5 \( \mu \)g/ml PHA for three days in a 55 ml RWV and a T-75 T-flask. At each time point, 5 ml of culture was sampled and the supernatant was frozen for later analysis. Culture supernatants were analyzed for cytokines by ELISA. The data are representative of three independent experiments.

FIGURE 3. Activation marker expression of PHA activated PBMC in the RWV bioreactor. PBMC were stimulated with 5 \( \mu \)g/ml PHA for three days in a 55 ml RWV and a T-75 T-flask. At each time point, 5 ml of culture was sampled and 1 x 10⁶ cells were tripled stained for CD25, CD69, and CD71. Results are expressed as percentage positive cells (A) or mean fluorescence intensity (B). The data are representative of three independent experiments.

FIGURE 4. The effect of exogenous IL-2 on PHA activation in the RWV bioreactor. PBMC were stimulated with 5 \( \mu \)g/ml PHA with and without the presence of IL-2 for three days in a 55 ml RWV (open bars) and a T-75 T-flask (closed bars). PBMC were also stimulated with IL-2 alone in a T-75 T-flask (hatched bars). Sampled PBMC from each vessel were set up in an 18 hour \([^3]H\)thymidine assay in triplicate (2 x 10⁵ cells/well). The data are representative of three independent experiments.

FIGURE 5. Effect of cell density on PHA activation in the RWV bioreactor. PBMC were stimulated with 5 \( \mu \)g/ml PHA at standard cell densities (1 x 10⁶ cells/ml) and higher cell densities: A: 5 x 10⁵ cells/ml; B: 1 x 10⁷ cells/ml in a 55 ml RWV and a T-75 T-flask for three days. At each time point, 5 ml of culture was sampled from the vessels and 2 x 10⁵ cells/well were set up in an 18 hour \([^3]H\)thymidine assay in triplicate. The data are representative of three independent experiments.
FIGURE 6. PHA activation within collagen beads in the RWV bioreactor. PBMC were polymerized within 25 µl of type I collagen. The cells/beads were stimulated with 5 µg/ml PHA in a 55 ml RWV and a T-75 T-flask for three days. At each time point, beads were sampled from the vessels and digested with a collagenase cocktail. Isolated PBMC were set up in an 18 hour [³H]thymidine assay in triplicate (2 x 10⁵ cells/well). The data are representative of three independent experiments.

FIGURE 7. Effect of a static (non-rotating) pre-incubation period on PHA activation in the RWV bioreactor. PBMC were stimulated with 5 µg/ml PHA for three days in 55 ml RWV's and a T-75 T-flask. Several RWV's were initially allowed to pre-incubated with cells and PHA in a non-rotating (non-microgravity simulating) condition for several lengths of time. After each pre-incubation period the RWV was rotated at its usual speed except for the 72 hr RWV which remained non-rotated for the entire experiment. Sampled PBMC from each vessel were set up in an 18 hour [³H]thymidine assay in triplicate (2 x 10⁵ cells/well). The data are representative of three independent experiments.

FIGURE 8. PMA/ionomycin activation in the RWV bioreactor. PBMC (A) and column purified T cells (B) were stimulated with PMA and ionomycin for 4 hours in a 55 ml RWV and a T-75 T-flask. After 4 hours the cells were placed into fresh PMA and ionomycin free medium and appropriate vessels (RWV or T-flask) and cultured for three days. Sampled cells from each vessel was set up in an 18 hour [³H]thymidine assay in triplicate (2 x 10⁵ cells/well). The data are representative of three independent experiments.

FIGURE 9. Addition of PMA and ionomycin to PHA activation in the RWV bioreactor. A, PMA (0.5 ng/ml), ionomycin (50 ng/ml), or both were added to PBMC cultures stimulated with PHA (5 µg/ml) in a 55 ml RWV and a T-75 T-flask. B, Several concentrations of PMA were added to PBMC cultures stimulated with PHA (5 µg/ml) in a 55 ml RWV and a T-75 T-flask. C, Several concentrations of ionomycin were added to PBMC cultures stimulated with PHA (5 µg/ml) in a 55 ml RWV and a T-75 T-flask. Sampled PBMC from each vessel were set up in an 18 hour [³H]thymidine assay in triplicate (2 x 10⁵ cells/well). The data are representative of three independent experiments.
Figure 2.

![Graph showing IL-2, IFN-γ, IL-1β, and IL-6 levels over time](image)

**IL-2**
- T-Flask: Peaks at 24 hr, decreases thereafter.
- RWV: Stays constant.

**IFN-γ**
- Increases over time, reaching a peak at 72 hr.

**IL-1β**
- Peaks at 24 hr, decreases thereafter.
- RWV: Stays constant.

**IL-6**
- Increases over time, reaching a peak at 72 hr.

**Time (hr):** 0, 24, 48, 72
Figure 3.

A

T-Flask

Percentage Stained Cells

RWV

- CD25
- CD69
- CD71

Time (hr)

B

T-Flask

Mean Fluorescence Intensity

RWV

- CD25
- CD69
- CD71

Time (hr)
Figure 4.
Figure 6.
Figure 7.
Figure 8.
Figure 9

A

- PMA/ionomycin
- Ionomycin
- PMA
- PHA-PMA/ionomycin
- PHA-ionomycin
- PHA-PMA
- PHA

B

- PMA

C

- Ionomycin

\[ \Delta \text{CPM} \times 10^{-3} \]