Simulated microgravity inhibits the genetic expression of interleukin-2 and its receptor in mitogen-activated T lymphocytes

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Abstract Experiments conducted in space in the last two decades have shown that T lymphocyte activation in vitro is remarkably reduced in microgravity. The data indicate that a failure of the expression of the interleukin-2 receptor (measured as protein secreted in the supernatant) is responsible of the loss of activity. To test such hypothesis we have studied the genetic expression of interleukin-2 and of its receptor in concanavalin A-activated lymphocytes with the RT-PCR technology. Microgravity conditions were simulated in the fast rotating clinostat and in the random positioning machine. The latter is an instrument introduced recently to study gravitational effects on single cells. Our data clearly show that the expression of both IL-2 and IL-2Rα genes is significantly inhibited in simulated $0\times g$. Thus full activation is prevented.

Key words: T lymphocyte; Gravitational biology; Signal transduction; Interleukin-2; Interleukin-2 receptor

1. Introduction

Several in vitro studies conducted in space and in simulated microgravity on ground have shown unexpected alterations of T lymphocyte function, reviewed in [1,2]. In fact, in an experiment conducted in Spacelab 1 in 1983 we observed a near total (97%) loss of activation in peripheral blood leukocytes exposed to concanavalin A (Con A) compared to identical ground controls [3]. Such finding was confirmed later in several Spacelab flights as D-1 in 1985, SLS-1 in 1991, IML-2 in 1994 whereby controls were incubated in an in-flight $1\times g$ reference centrifuge. A systematic approach to understand the causes of the loss of activation was conducted in space, in sounding rockets, and in microgravity simulations in the fast rotating clinostat (FRC). Our findings can be summarized as follows: Mitogenic activation, measured as the mitotic index after 3 days of incubation, is reduced between 98–50% at $0\times g$ when compared to $1\times g$ controls either in space or on the ground [1,2]; while the binding of Con A to membrane glycoproteins is unchanged, patching and capping are slightly retarded at $0\times g$ [4]; cell-cell interactions as well as autonomous cell movements are occurring at $0\times g$, although in slight lesser extent than at $1\times g$ [5]; surprisingly, the production of interleukin-1 (IL-1) by monocytes as accessory cells is enhanced at $0\times g$ [6]; changes of the structure of microfilaments (vimentin) occur already 30 s after exposure to microgravity [4]; the amount of interleukin-2 receptor (IL-2R) measured as protein secreted in the medium is strongly depressed at $0\times g$ [7]; the secretion of interleukin-2 (IL-2) in the medium is also reduced at $0\times g$ [7]. Such findings support the hypothesis that an alteration of the IL-2/IL-2R function may be the cause of the effects.

The behavior of lymphocytes in microgravity has been studied also by other investigators. In particular, in an experiment with human leukocytes activated with phorbol esters conducted by Schmitt et al. [8] in Spacelab IML-2 it was shown that the distribution of PKC, a key element of the signal transduction of T cells, is altered in microgravity. Recently, Cooper and Pellis reported that the loss of activation of T cells in microgravity, simulated in a device called rotating wall vessel, was restored by direct activation of PKC with phorbol myristate [9]. However, such restoration was only partial under optimum activation conditions. We also disagree with Cooper and Pellis' interpretation that lack of cell-cell contacts contribute significantly to the loss of activity at $0\times g$.

A depression of mitogenic activation, compared to the preflight (14 days before launch) and post-flight baseline (7 days after landing) has also been measured ex vivo in PBL drawn from space crew members during (3rd day in orbit) and immediately (1 h) after flight, whereby the in-flight cultures were incubated in centrifuge at $1\times g$. However, our and others' studies clearly indicate that the effects observed in vitro are real gravitational effects occurring at the cellular level, whereas the ex vivo effects are caused by the physical and psychological stress of space flight on the immune system via the neuroendocrine system, reviewed in [10–12].

In vitro studies in microgravity have provided a useful tool towards the understanding of the complex activation mechanism of T lymphocytes. In fact, microgravity can be used as a non-invasive inhibitor of cell activation.

IL-2 and IL-2R play a crucial role as third signal in T cell activation whereby the first signal is delivered either by the antigen-presenting cell, or by a mitogen such as Con A, or by anti-CD3; the second signal is either anti-CD28 or IL-1. Only upon the interaction between IL-2 and IL-2R the full activation of T lymphocytes is triggered. Cells start to divide and two populations, the effector and the memory cells, are generated.

In this context, there is strong interest today in the study of the genetic expression of the IL-2R in human tumor cells as

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Abbreviations: Con A, concanavalin A; FRC, fast rotating clinostat; IL-1, interleukin-1; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; PBL, peripheral blood leukocytes; PKC, protein kinase C; RPM, Random positioning machine; rpm, rotation per minute; RT-PCR, reverse transcriptase-polymerase chain reaction

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IL-2 may be used in cancer therapy to inhibit tumor cellular proliferation [13].

The objective of the work described here was to test the IL-2/IL-2R dysfunction hypothesis by studying the effect of simulated microgravity on the genetic expression of IL-2 and of its receptor. Two instruments were used to simulate microgravity. One is the FRC proposed long ago by Muller [14], and developed by Briegleb [15] on the base of the calculations of Silver [16]; the other is the random positioning machine (RPM), called also three-dimensional clinostat. The RPM has been developed by Hoson at the Osaka City University [17,18] and has been used for the study of plant gravitropism. This is the first report on the use of the RPM with single cells. The main advantages of the RPM compared to the FRC are the larger number and volume of cultures that can be processed in one experiment cycle and the three-dimensional versus the uni-dimensional rotation.

2. Materials and methods

2.1. Microgravity simulations

The FRC has been manufactured in our workshop as described earlier [19]. It consists of a tube of 0.8 cm diameter and 100 cm length rotating around its longitudinal axis at speed of 90 rpm. Cell cultures were sealed in tubes made of Teflon of 0.45 cm diameter and 10 cm length.

The RPM was developed by Fokker Space, Leiden, The Netherlands. In principle, probes are fixed as close as possible to the center of a frame. This frame is rotating within a second rotating frame. Both frames are driven by separate motors. Rotation of each frame is random and autonomous and regulated by computer software. The rotation velocity of the frames was 60° s^{-1}. The RPM was located in a 37°C room. A box containing the cell cultures sealed in Eppendorf tubes was installed on the center of the inner frame of the RPM. Although real microgravity is attained only in orbital laboratories such machine offers an inexpensive and suitable instrument for simulation in the ground laboratory (Schwarzenberg et al., submitted for publication). Control (static) cultures were installed on the basement of the RPM.

2.2. Lymphocyte isolation

Peripheral blood lymphocytes were purified from buffy-coat preparations (Blood bank, Red Cross, Zurich or Sassari) diluted 1:10 with Hanks buffer by gradient centrifugation on Ficoll-Hypaque. Cell viability was assessed by fluorescein-diacetate-positive test (0.002% FDA in PBS). The cells were stored at ambient temperature for at least 4 h prior to activation. This allows the cells to recover from the stress of the isolation. In fact, centrifugation steps lasting 10–30 min at $200\times g$ or more may activate certain genes and thus cause artefacts due to hypergravity stress.

2.3. Lymphocyte activation

The cells were resuspended at a concentration of 10^7 ml^{-1} in RPMI 1640 (Sigma) supplemented with 40 mM HEPES, 5 mM sodium bicarbonate, gentamycine (50 μg ml^{-1}), and 10% heat-inactivated fetal calf serum (mycoplasma-free, Gibco). T cells were activated by addition of Con A at a final concentration of 10 μg ml^{-1}. To avoid the presence of large air bubbles, which could lead to shear force damages of the cells on the RPM, the 2 ml Eppendorf tubes were completely filled. The cells were activated for periods between 0 and 12 h as shown in the figures.

2.4. mRNA extraction and RT-PCR

After incubation, the cells were pelleted by short centrifugation, the supernatant was discarded and the cells were immediately lysed by addition of the lysing buffer. The semi-quantitative RT-PCR analyses were performed either with (a) total cellular RNA (IL-2Rα, β-actin); or (b) mRNA (IL-1, IL-2, IL-2Rα, IL-2Rβ, β-actin).

(a) Total cellular RNA was isolated by a method developed by M. Hughes-Fulford (personal communication) based on guanidine thiocyanate and adapted to the working conditions particular of space laboratories. The RNA concentrations were measured spectrophotometrically. Typically, 800 ng of the total RNA were reverse-transcribed with random hexamers or oligo dT for priming and the *Gene Amp RNA PCR kit* (Perkin Elmer, N808-0143). A fourth of the RT reaction was used for PCR amplification in a total volume of 50 μl. IL-2Rα and β-actin transcripts were amplified with specific primers following the supplier's instruction (Stratagene). The PCR products were separated by electrophoresis with EtBr, the gels were photographed and digitalized by densitometry (*Imaging Densitometer GS-700*, BioRad). Analysis of the band intensity was performed with the software *Molecular Analyst* 2.1.1 (BioRad) The results of the IL-2Rα expression were standardized by comparison with the expression of the constitutively expressed β-actin.

(b) Cellular RNA was first isolated with a modified guanidine thiocyanate method (guanidine thiocyanate 8 M/Triton 1%), then mRNA was captured by a silica-based resin and resuspended in water (Nurex, Sassari, Italy). The totality of the mRNA was reverse-transcribed with oligo dT and a commercial kit. cDNA was synthesized during 1 h incubation at 37°C. A fifth of the reaction was used for each specific amplification (IL-1, IL-2, IL-2Rα, IL-2Rβ, and β-actin). PCR amplification was performed with 1 U of Taq DNA polymerase (Life Technologies) in a final volume of 25 μl. Primer sequences and annealing temperatures were as already described [20]. Amplified DNA was cross-linked to prevent cross-contamination and stained in a stable manner by DNA Block and Stain (Nurex, Sassari, Italy). Fluorescent stained DNA was separated by agarose gel electrophoresis and gel intensity was determined by densitometric measurement of the specific bands.

The optimal number of amplification cycles for each primer set was chosen in the exponential phase of amplification, which was determined experimentally by measuring the amount of specific products after every two cycles between 20 and 35.

3. Results and discussion

In this study five independent experiments, i.e. with the blood of five donors, were performed either on the RPM or in the FRC. The data obtained with both machines are consistent. We have shown that the results obtained with the FRC [1] and with the RPM (Schwarzenberg et al., manuscript submitted for publication) are in agreement with those obtained in space. In addition, we have used two different methods to purify and process the RNA. Both methods gave also consistent results.

3.1. IL-2 expression

In an experiment conducted earlier in space we found after a 46 h incubation at $0\times g$ a lower level of IL-2 (600 pg ml^{-1}) than in $1\times g$ controls, either in a $1\times g$ reference centrifuge in-flight (920 pg ml^{-1}) or on ground (800 pg ml^{-1}, [7]). After

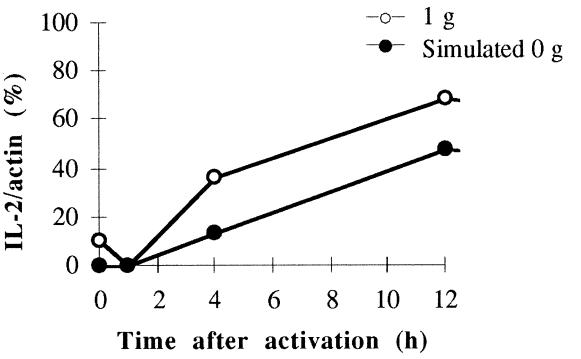

Fig. 1. Effect of simulated microgravity on the genetic expression of IL-2. The data shown here are from one experiment in the FRC and are consistent with another in the FRC and with three analogous experiments on the RPM.

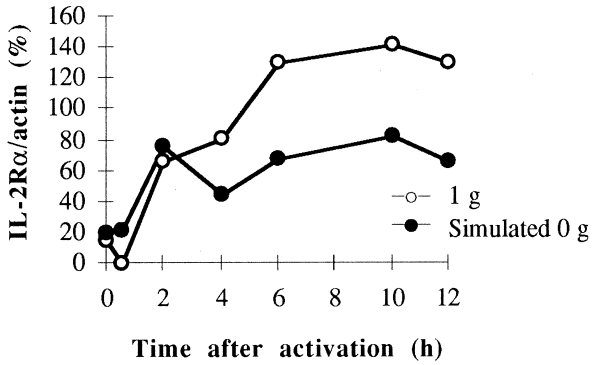

Fig. 2. Effect of simulated microgravity on the genetic expression of IL-2Rα. The data shown here are from one experiment on the RPM and are consistent with four analogous experiments on the RPM.

65 h, however, the amount of IL-2 remained unchanged at $0\times g$, whereas it was reduced to nearly 0 in both $1\times g$ controls. The activation of the T cells, measured as tritiated thymidine incorporated into DNA, was reduced by 85% after 46 h at $0\times g$ and by 50% after 65 h compared to the $1\times g$ controls. These data indicated, first, that there was a reduced synthesis/secretion of IL-2 at $0\times g$; second that activated cells (at $1\times g$) bound all IL-2 available in the culture medium, whereas the IL-2 was not taken up at $0\times g$. Moreover it was seen in two space flights that the amount of IL-2R in the supernatant of the $0\times g$ samples was reduced by 95 and 74%, respectively, compared to the $1\times g$ in-flight and ground controls [6,7]. Such depression observed in T cells at $0\times g$ could be due either to a reduction of genetic expression, or of secretion, or of uptake/insertion in the membrane of IL-2 and IL-2R.

The data in Fig. 1 show that the onset of genetic expression of IL-2 in Con A-activated cells begins approximately 1 h after exposure to the mitogen at $1\times g$ as well as in simulated microgravity conditions; however, there is a clear inhibition at $0\times g$, ranging after 6 h from 20 to 50% in the five experiments performed. We believe now that this inhibition contributes to the decrease of T cell response at $0\times g$.

The regulation of the transcription of the IL-2 gene is based on complex and still obscure mechanisms involving transcription factors such as NFAT, AP-1, NF-κB and other and it is today object of extensive studies, reviewed recently in [21,22]. In addition, the T cell response may differ depending on the nature of the first and the second (co-stimulatory) signals as recently shown by Kalli et al. with either anti-CD3-IL-1 or anti-CD3-anti-CD28 as activators [23]. The question now is what causes the reduction of genetic expression of IL-2. This can be due to the alterations of the PKC seen by Schmitt et al. [8] in space; or to a disturbance of signal transduction upstream of the PKC regulation as described by Cooper and Pellis [9] in experiments conducted in a rotating wall vessel; or, finally, either to a dysfunction of the regulation of the transcription of the IL-2 gene or of the posttranscriptional stabilization of IL-2 mRNA as discussed by Kalli et al. [23] and by Lindsten et al. [24].

3.2. IL-2R expression

The IL-2 receptor consists of three protein chains. The α-subunit is responsible, in association with the β- and γ-chains, of the high affinity of the receptor toward IL-2, but only the β-chain and γ-chain are involved in the intracellular signal transduction [25]. Also the regulation of the IL-2α expression is a complex and not yet completely understood process [26]. In our investigation of the IL-2R, we focused our attention on the α- and β-chains only, because the γ-chain is constitutively expressed. The expression of the α-chain is also significantly inhibited (Fig. 2), though the range of the percentage (15–70% after 6 h) of inhibition in the five experiments performed is larger than that of IL-2. The reason of such variation is not clear, but it might be due to a higher instability of the IL-2Rα specific mRNA. It is important to notice that, in contrast to IL-2, IL-2R expression is not under PKC regulation [26].

Interestingly enough, the expression of the β-chain is not influenced by simulated microgravity (Fig. 3). Therefore, the intracellular signal mediated by the β-chain is not the influencing factor in the depression of the lymphocyte activation in microgravity. This is similar to what has been shown by Suminami et al. [13]: the growth inhibition of carcinoma cell lines by exogenous IL-2 was not due to different forms of the IL-2R β-chain.

3.3. IL-1 expression

Interleukin-1 is important in the mediation of the second signal in the activation of lymphocytes. In a previous experiment in space, we found that its production was increased at $0\times g$ [6]. Such finding was confirmed by Cooper and Pellis in the rotating wall vessel [9]. Also the results obtained at the

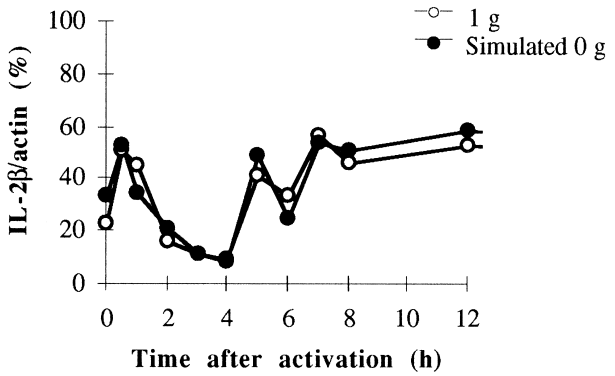

Fig. 3. Effect of simulated microgravity on the genetic expression of IL-2Rβ. The data shown here are from one experiment on the RPM and are consistent with another analogous experiment in the FRC.

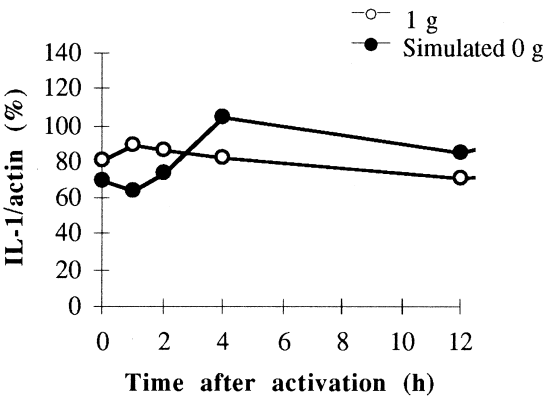

Fig. 4. Effect of simulated microgravity on the genetic expression of IL-1. The data shown here are from one experiment on the RPM and are consistent with another analogous experiment in the FRC.

molecular level (Fig. 4) show that the IL-1 expression is enhanced on the RPM in comparison to the $1\times g$ control. This excludes also the possibility that the reduction in the expression of the other genes was due to a mechanical damage of the cells on the RPM or in the FRC.

4. Conclusions

The data presented here together with our previous studies permit us to conclude that either at $0\times g$ or under simulated microgravity conditions: (i) the binding of Con A is unaffected as seen in sounding rockets, however the process of patching and capping is slightly retarded [4]; (ii) the secretion and the genetic expression of IL-2 is inhibited [6,7]; (iii) the secretion and the genetic expression of IL-1 is enhanced [6]; (iv) the secretion of the IL-2 receptor is reduced at $0\times g$ [7]; (v) the activation of IL-2Rα gene is significantly depressed in simulated $0\times g$ whereas that of the IL-2Rβ is not affected. Based on such findings and those of Cooper and Pellis we suggest that different events contribute synergistically to the loss of activity of T cells in microgravity. The most important are the dysfunction of the transcription of the IL-2R gene and of the signal transduction upstream of PKC.

This work helped to clarify an important aspect of the inhibition of signal transduction in T cells in microgravity. We believe that this approach may also contribute to the further understanding of the complex activation mechanism. Moreover, the RPM has proven to be a useful tool to simulate microgravity in the ground laboratory and to prepare space investigation. In fact, we will continue our study of the genetic expression during T lymphocyte activation with an experiment selected with high priority by NASA and ESA for a flight scheduled in the year 2000.

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