APOPTOSIS, CELL CYCLE AND DELAYED LUMINESCENCE OF HUMAN LEUKEMIA JURKAT T-CELLS UNDER PROTON-IRRADIATION AND OXIDATIVE STRESS CONDITIONS

I. Baran[a,*], C. Ganea[a], A. Scordino[b,c], V. Barresi[d], F. Musumeci[b,c], S. Tudisco[b,c], S. Privitera[b,c], R. Grasso[b,c], D. F. Condorelli[d], I. Ursu[e], V. Baran[e], E. Katona[a], M. M. Mocanu[a], R. Ungureanu[a], N. Musso[d], M. Gulino[b,c], G. A. Pablo Cirrone[b], G. Cuttone[b], L. M. Valastro[b]

a) "Carol Davila" University of Medicine and Pharmacy, Dept. of Biophysics, 8 Eroii Sanitari, 050474 Bucharest, Romania
b) Istituto Nazionale di Fisica Nucleare – Laboratori Nazionali del Sud, 62 S. Sofia, Catania, Italy
c) Dip. di Metodologie Fisiche e Chimiche per l'Ingegneria, Università di Catania, I-95125 Catania, Italy
d) Sezione di Biochimica e Biologia Molecolare, Dip. di Scienze Chimiche, Università di Catania, 6 A. Doria, I-95125 Catania, Italy
e) IFIN-HH, 407 Atomistilor Str., 077125 Magurele, Bucharest, Romania
f) "Victor Babes" National Institute, Dept. of Immunology, 99-101 Spl. Independentei, Bucharest, Romania
**Corresponding author, e-mail: baran@ifin.nipne.ro*

Abstract

We investigated the relation between apoptosis and delayed luminescence (DL) in human leukemia Jurkat T-cells undergoing various treatments. We used menadione, hydrogen peroxide and quercetin to induce oxidative stress conditions under different doses and treatment times. We irradiated Jurkat cells by using modulated beams of accelerated protons with energies up to 62 MeV, under a dose of 10 Gy distributed uniformly inside the cell suspension. We assessed cell proliferation, clonogenic survival and delayed luminescence of treated cells. Apoptosis and cell cycle distributions were measured by flow-cytometry. Irradiation with protons produced a modest increase in the apoptotic rate, but blocked the cell cycle at the G_2/M phase for at least 48 h after irradiation, suggesting the presence of severe DNA damage. A 34% reduction of the DL quantum yield was observed in a specific DL time interval, 1-10 ms after the laser excitation of the cell samples after 1 h from irradiation, whereas the DL quantum yield exhibited an increase of 27% in the DL time interval 0.1-1 ms in cell samples probed 24 h after irradiation. The treatments using menadione, hydrogen peroxide and quercetin as oxidant agents potently induced apoptosis of Jurkat cells in a dose-dependent manner and consistently decreased the intensity of delayed photoemission. We obtained a strong anti-correlation between apoptosis of human leukemia Jurkat cells and UV-induced delayed photoemission on a specific time interval ranging from 100 μs to 1 ms after UV-excitation of the cell samples.

INTRODUCTION

Menadione (vitamin K_3) is a clinically important chemotherapeutic agent used in the treatment of leukemia and other cancer types [1], which produces intracellularly large amounts of superoxide anion ($O_2^{-\bullet}$). Quercetin (QC) and epigallocatechin gallate (EGCG) are two well-investigated natural flavonoids which are known to specifically inhibit cell proliferation and induce apoptosis in different cancer cell types [2-4]. Menadione, hydrogen peroxide and quercetin can activate the apoptotic program via a Ca^{2+}-dependent mitochondrial pathway, by promoting elevation of cytosolic Ca^{2+} levels, mPTP opening, collapse of the mitochondrial membrane potential ($\Delta\psi_m$) and release of cytochrome *c* from mitochondria [2,5-7].

The sensitivity to radiation of Jurkat cells is relatively high [8,9] and high doses (≥10 Gy) of radiation can induce significant apoptosis in a time- and dose-dependent manner [8,10]. Failure to eliminate cells that have been exposed to mutagenic agents by apoptosis has been associated with the development of cancer and resistance to anticancer therapy.

We have investigated the correlation between apoptosis and delayed luminescence (DL) under oxidative stress and irradiation conditions. Quantification of these aspects can help answering the question on the origin of DL, which at the moment is still a matter of debate. DL represents a very weak, long-time scale light emission following exposure to pulsed light or UV radiation. It is generally thought that DL in biological systems may arise from a variety of possible reactions and sources, such as direct emitters like flavins, carbonyl derivatives and aromatic compounds, molecular oxygen and its species, the DNA, as well as collective molecular interactions, e.g. triplet-triplet annihilation, collective hydrolysis, charge recombination within the mitochondrial/chloroplast electron transport system, or the cytoskeleton [11-15]. In this work we present direct evidence for a connection between apoptosis and DL.

MATERIALS AND METHODS

Cell cultures

Human Human leukemia Jurkat T cell lymphoblasts were cultured in suspension in MegaCell RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM

L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified incubator with a 5% CO_2 atmosphere. We used hydrogen peroxide 30% solution and stock solutions of menadione sodium bisulphite dissolved in phosphate buffer saline (PBS), or dihydrated quercetin and epigallocatehin gallate dissolved in dimethyl sulfoxide (DMSO). DMSO was 0.1% (v/v) in all cultures. After the treatment, cells were washed twice with PBS and resuspended in PBS (for DL samples, ~40 × 10^6 cells/ml) or in complete medium for apoptosis assessment (~0.2 × 10^6 cells/ml). DL samples were analyzed immediately by DL spectroscopy. Cell density, viability and morphology were examined with a CCD camera Logitech QuickCam Pro 4000, connected to an Olympus CK30 phase contrast microscope. For cell density assessment, 25 μl- aliquots of the DL samples were diluted in PBS, stained with 0.4% trypan blue solution and ~1500-2000 cells were imaged on a Burker haemocytometer at the time of the DL assay. Cell count evaluation was performed both during DL experiments, directly by visual inspection under the microscope, and later on, by analyzing the recorded photographs with the ImageJ software.

Irradiation conditions

Cell suspensions (7 ml) were irradiated in 50 ml-centrifuge tubes in vertical position. Clinical proton beams accelerated by the superconducting cyclotron at LNS-INFN, Catania (Italy) were used for proton-irradiation at a dose rate of 11.76 Gy/min. The proton beams were modulated to give uniform distribution of the absorbed dose in the entire cell suspension. The maximal proton energies were 62 MeV at the entrance, and 15 MeV at the exit from the cell sample. A plane-parallel advanced PTW 34045 Markus ionization chamber was adopted as a reference dosimeter. The dose measurements were performed in a water phantom, according to International Atomic Energy Agency (IAEA) TRS 398. The absorbed dose to water per monitor unit (cGy/M.U.) was measured at isocenter, at the depth corresponding to the middle of the modulated beam, with the reference circular collimator (diameter = 25 mm).

Clonogenic survival assay

After treatment, cells were harvested and plated in 96 well plates at a plating density of 4 cells/well in 100 μl of complete medium per well. After 4 weeks of incubation, the plates were inspected by microscopy and the wells containing clones with >50 cells were counted. The plating efficiency was calculated as ln[96/(no. of negative wells)]/(plating density) × 100. Clonogenic survival was calculated as the ratio between the plating efficiency of treated and control cells, respectively.

Flow cytometry

24 and 48 h after the treatment, samples containing 10^6 cells were fixed in 70% ethanol and frozen at -20°C. For flow-cytometer determinations, the ethanol-fixed samples were washed with PBS, incubated with a propidium iodide PI/RNAse staining buffer (PHARMINGEN 550825) for 30 min. at 37ºC in the dark and analyzed with a Becton Dickinson FACS Calibur flow-cytometer. For data acquisition and analysis we used the CellQuest, WinMDI 2.8 and Cylchred software. Apoptosis was evaluated as the fraction of hypodiploid cell fragments (the sub-G_0/G_1 cell fraction). The G_0/G_1, S and G_2/M cell fractions were calculated for the non-apoptotic cell population, by excluding the hypodiploid events from cell cycle analysis. For each treatment, the 24/48-h data were used collectively to calculate the mean and the standard error of the mean (s.e.m.).

Delayed Luminescence Spectroscopy

We used an improved version of the ARETUSA set-up [14], a highly sensitive equipment able to detect single photons. The cell samples were excited by a Nitrogen Laser source (Laser Photonics LN 230C; wavelength 337 nm, pulse-width 5 ns, energy 100 ± 5 μJ/pulse). A multi-alkali photomultiplier tube (Hamamatsu R-7602-1/Q) was used as a detector for photoemission signals with wavelengths in the range 400-800 nm, in single photon counting mode. The detected signals were acquired by a Multi-channel Scaler (Ortec MCS PCI) with a minimum dwell-time of 200 ns. DL measurements were done on at least 3 different drops from each cell sample (drop volume 15-25 μl) at room temperature (20 ± 1ºC). PBS luminescence was subtracted from all the recordings. Photoemission was recorded between 11 μs and 10 ms after laser-excitation. DL intensity (I) was obtained as the number of photons recorded within a certain time interval divided to that time interval and to the number of living cells in the drop. The quantum yield was calculated in three time-domains of the DL emission: 11-100 μs (DL-I), 100 μs - 1 ms (DL-II) and 1-10 ms (DL-III), as the ratio between the I-integral and the energy of the laser.

RESULTS AND DISCUSSION

Irradiation with 10 Gy of protons produced a modest increase in the apoptotic rate, but blocked the cell cycle at the G_2/M phase for at least 48 h after irradiation, suggesting the presence of severe DNA damage (Fig. 1). Clonogenic survival after irradiation was below the resolution of our assay (0.12%), indicating that 10 Gy of protons induced massive necrosis in this cell system, in agreement with other reports [9,10]. Trypan blue exclusion tests confirmed high necrotic rates of 18.4% and 46.6% at 24 h and 48 h after irradiation, respectively. Delayed light emission of the living cells exhibited different characteristics when probed at 1 h or 24 h after irradiation. A 34% reduction of the DL-III relative quantum yield was observed after 1 h from irradiation, whereas the DL-II relative quantum yield exhibited an increase of 27% in cell samples probed 24 h after irradiation.

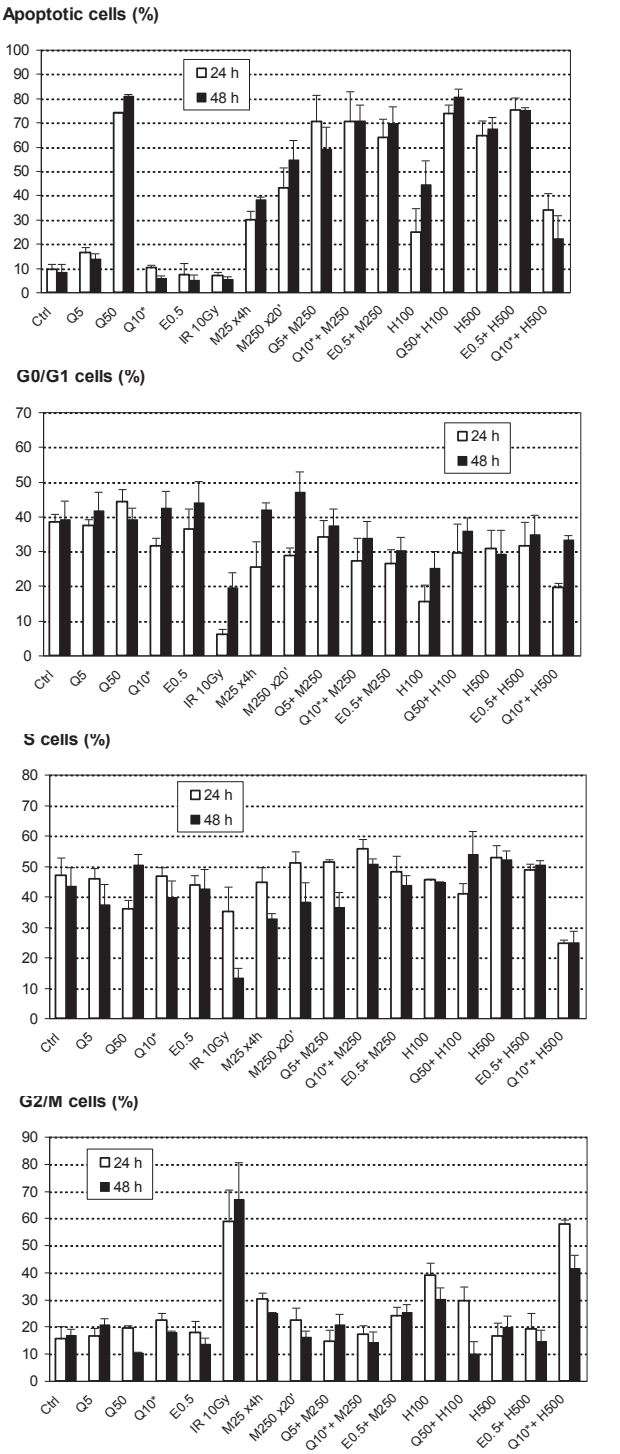

Figure 1: Apoptosis and cell-cycle distributions after treatment of Jurkat cells with 0.5, 5 or 50 μM quercetin for 24 h (Q0.5, Q5, Q50), 10 μM quercetin for 1 h (Q10*), 0.5 μM EGCG for 24 h (E0.5), menadione (M25: 25 μM for 20 min. or 4 h as indicated, M: 250 μM for 20 min.), 100 or 500 μM H_2O_2 for 20 min. (H100 or H500), or after combined treatments (quercetin or EGCG pre-incubation followed by addition of 250 μM menadione or 100/500 μM H_2O_2 for 20 min.).

QC, MD and H_2O_2 induced apoptosis in Jurkat cells in a dose- and time-dependent manner (Fig. 1). The lower dose of H_2O_2 (100 μM for 20 min.) produced 34.8 ± 9.9% apoptotic cells and a G_2/M ratio of 34.6 ± 4.5% 24-48 h after the treatment, whereas the highest dose used (500 μM for 20 min.) only slightly increased the S-phase cell fraction (Fig. 1). H_2O_2 can induce cell cycle arrest at G_2/M by expression of $p21^{Cip1}$, a cyclin-dependent kinase inhibitor [16]. It is possible that in our experiments, Jurkat cells were unable to cope with extensive oxidative damage and thus could not complete the current cell-cycle phase in less than 48 h, since damage repair following a milder stress appeared to be quite slow (as discussed above, the G_2/M blockade persisted for >48 h in both treatments with 100 μM H_2O_2 and 500 μM H_2O_2 + 10 μM QC-preincubation). In addition, haemocytometer counts indicated that there was no net growth in cell cultures 24-48 h after the most severe oxidative treatments (namely "M" and "H"). Together, these results suggest that under excessive oxidative stress induced by menadione or hydrogen peroxide, Jurkat cells accumulate hardly repairable damage and therefore are stopped in all the phases of the cell cycle. Under our experimental conditions, a large part of the arrested cells initiated the apoptotic program; however, it appeared that the surviving cells eventually managed to repair the damage and continued to proliferate after >48 h from exposure (we obtained clonogenic survival 41.2 ± 2.9 % for the treatment with 250 μM MD). Finally, it is worth mentioning that trypan-blue viability tests indicated that necrosis was low (<15%) under all oxidative treatments, which is consistent with other reports [17] and with the clonogenic survival data.

At the moment, the current available data on the effects of quercetin on apoptosis induced by menadione and hydrogen peroxide in Jurkat T-cells are extremely limited. In human normal lymphocytes quercetin can produce noticeable quantities of $O_2^{-\bullet}$ and $OH^{\bullet}$ at levels ≥50 μM within 30 min. of treatment [4]. After intake, quercetin and EGCG are rapidly metabolized and normal levels in the plasma remain below 10 μM [3,18], but can increase considerably in human tissues, in particular at the inflammatory sites. In this work, a 24-hour treatment with physiological levels (0.5 - 5 μM) of quercetin and EGCG enhanced apoptosis induced by menadione in human leukemia Jurkat T cells, whereas a short-term treatment with 10 μM QC reduced apoptosis induced by hydrogen peroxide. In addition, preincubation with a very low level (0.5 μM) of EGCG significantly increased the G2/M cell fraction after the 250 μM MD-treatment. While long-term administration of quercetin or EGCG could improve significantly the menadione-based treatment of leukemia, it is important that normal cells remain unexposed to noxious levels of the flavonoids used.

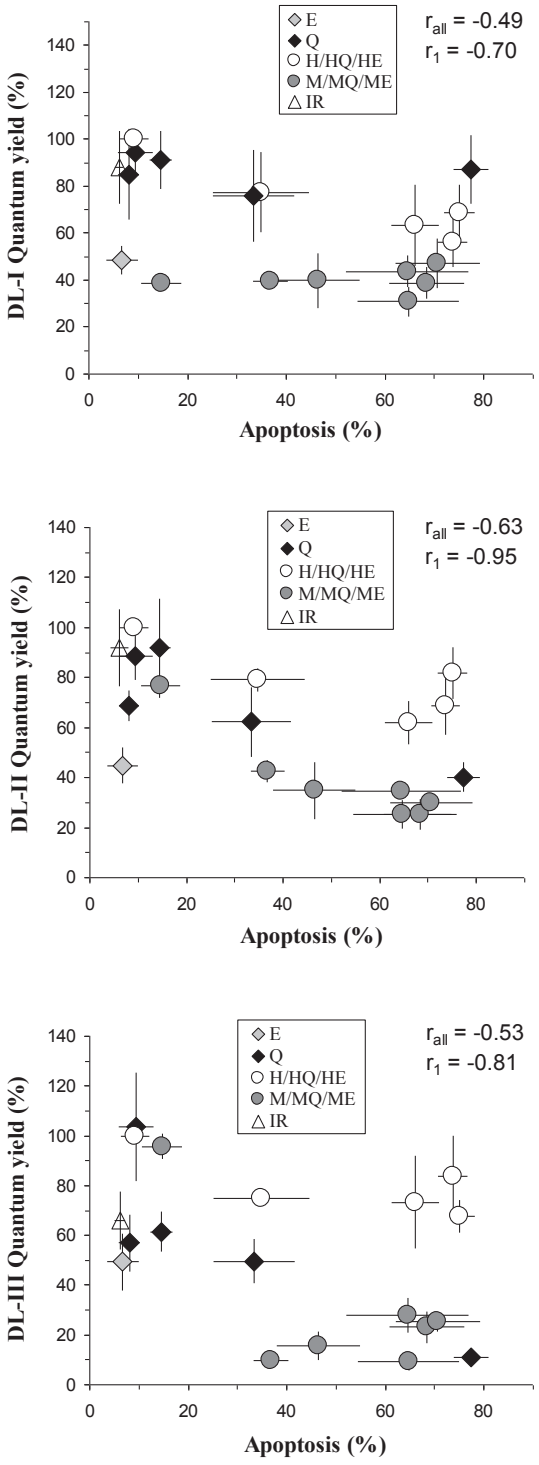

Figure 2: DL-quantum yield (relative to control) and its correlation to the apoptotic cell fraction under the treatments indicated in Fig. 1. Q, E, M/MQ/ME, H/HQ/HE and IR denote single QC- or EGCG-treatments, MD-treatments with or without QC-or EGCG-preincubation, H_2O_2-treatments with or without QC- or EGCG-preincubation, and 10 Gy proton-irradiation, respectively. The Pearson correlation coefficients are shown for all treatments (r_{all}) and for M/MQ/ME/IR treatments (r_1).

Both quercetin and menadione inhibited DL in a dose-dependent manner. Surprisingly, we found that QC and MD at high doses exhibited virtually identical effects on DL over a wide time-interval, from 100 μs to 10 ms after laser-excitation. Having in view the molecular interactions that are common to both chemicals, it is most likely that their similar effects on DL were caused by superoxide production and inhibition of Complex I of the mitochondrial respiratory chain.

Our studies provide new insights into the relation between the cell status and delayed luminescence. With treatments of varying time and dosage of three different pro-apoptotic agents (menadione, hydrogen peroxide and quercetin) we obtained a significant anti-correlation (r = -0.63) between apoptosis of human leukemia Jurkat cells and UV-induced delayed photoemission on a specific time interval ranging from 100 μs to 1 ms after UV-excitation of the cell samples (Fig. 2). More specifically, the M/MQ/ME/IR treatments were associated with a strong anti-correlation between DL-I, DL-II and -III and apoptosis (r_1 = -0.70, -0.95 and -0.81, respectively), suggesting that proton-irradiation increases the intracellular superoxide rather than the H_2O_2 level. Indeed, the effects of H_2O_2 on DL emission were relatively reduced even when very high levels of this oxidant agent were used (Fig. 2). A potential application of DL spectroscopy can thus be envisioned as a rapid and accurate method to assess the pro-apoptotic capacity of certain treatments for acute leukemia.

ACKNOWLEDGEMENTS

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PLASMA LASER ENERGETIC ION ACCELERATION & DIAGNOSTICS (PLEIADI): PROJECT UPGRADE 2010

L. Torrisi[1,2], S. Gammino[1], L. Giuffrida[1,2], L. Andò[1], F. Caridi[3], D. Mascali[1], F. Di Bartolo[2] and A. Baglione[2]

[1]*INFN Laboratori Nazionali del Sud, Catania, Italy*
[2]*Dipartimento di Fisica, Università di Messina, Italy*
[3]*Facoltà di Scienze MFN, Università di Messina, Italy*

Abstract
The 2010 upgrade of the PLEIADI (Plasma Laser Energetic Ion Acceleration & Diagnostics) project is presented and discussed. The research activities concern the improvements of the laser ion source (LIS), of the ion extraction chamber, of the post ion acceleration system (PIAS) and of the ion diagnostics.
Ion collectors and ion energy analyzers, such as a Thomson parabola, were employed for the ion beam diagnostics of the charged particles emitted from the LIS and from the PIAS.
Multi-energetic and multi species ion beams can be accelerated from 0 to 30 kV in order to produce ion implantation in different substrates.

INTRODUCTION

PLEIADI (Plasma Laser Energetic Ion Acceleration & Diagnostics) is a INFN Project devoted to investigate on the realization of a Laser Ion Sources (LIS) and a Post-Ion Accelerated System (PIAS).
The project uses a Nd:Yag laser operating at a fundamental wavelength of 1064 nm, at intensities of the order of 10^{10} W/cm^2, 0.9 J pulse, 9 ns pulse duration, 0.1-30 Hz repetition rate, in order to generate non-equilibrium plasmas, in high vacuum, by interacting with solid matter. Plasma is produced with high directionality along the normal to the target surface [1]. The ion temperature of obtained plasma is of the order of 100 eV, the plasma density of the order of 10^{17}/cm^3, the charge states can reach 10+ for heavy elements and the kinetic ion energies are proportional to the ion charge state and may reach about 6 keV [1]. The ion acceleration is due to the non-uniformity of the spatial charge distribution in the plasma, due to the faster electrons mobility with respect to ions, which induces formation of a high electric field directed along the normal to the irradiated target surface. The calculation of the electric field is possible by knowing the equivalent ion acceleration and the electronic temperature and density of the plasma; its value is of the order of 10 MV/cm [2].
Laser-plasma accelerated ions show "Coulomb-Boltzmann-Shifted" (CBS) distributions, which are shifted towards high energy increasing the ion charge state [3].
Thank to the high ablation yield, obtainable by focusing the laser beam on the target surface, of the order of 10^{17} ats/pulse for fundamental wavelength ablating metals, and to the high laser repetition rate, the LIS, using the ablation of a roto-translating thick target, permits to extract a constant high current density, of the order of tens mA/cm^2 [4]. The low energy ions emitted from the plasma can be extracted, focused and submitted to a post-acceleration, due to an external high electric field, in order to generate a multi-energetic ion beam with more interesting energies of employment. Actually PLEIADI uses 30 kV post acceleration but a work is in progress to employ 60 kV voltage and to reach high ion dose-rates. Focalization systems, using electrical and/or magnetic deflections, can be employed in order to increase the ion current towards the acceleration axe or to increase the implantable ion rate.
The post accelerated ion beams can be employed in different scientific fields, such as the ion implantation technique, in order to modify the physical and chemical properties of different substrates, the ion sputtering processes to modify the surface morphology of different polymers, and it can be employed also to plasma diagnostic and to test different detectors dedicated to the range of radiations at low energy levels.

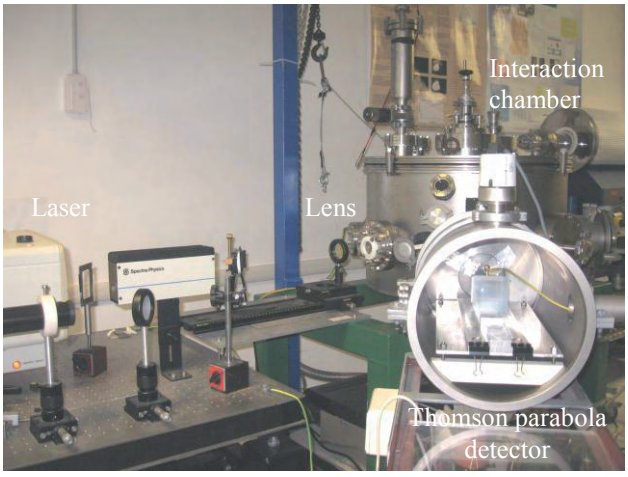

Fig. 1: Photo of the experimental set up.