

QUALITY OF LIFE AND MANAGEMENT OF LIVING RESOURCES

**Final Report**

**Immunological Health and Adaptation Following Chronic Exposure to  
Environmental Ultraviolet Radiation**

ACRONYM:

**IHA-UV**

**KEY ACTION 4: ENVIRONMENT AND HEALTH**

## **Table of contents**

<b>1.</b>	<b>Objectives and expected achievements</b>	<b>3</b>
<b>2.</b>	<b>Project workplan</b>	<b>4</b>
<b>2.1.</b>	<b>Introduction</b>	<b>4</b>
<b>2.2.</b>	<b>Project structure, planning, and timetable</b>	<b>7</b>
<b>2.3.</b>	<b>Description of workpackages and progress made</b>	<b>13</b>
<b>3.</b>	<b>Role of participants</b>	<b>120</b>
<b>4.</b>	<b>Project management and Co-ordination</b>	<b>125</b>
<b>5.</b>	<b>Exploitation and Dissemination Activities</b>	<b>127</b>
<b>6.</b>	<b>Ethical Aspects and Safety Provisions</b>	<b>127</b>

## **1. OBJECTIVES AND EXPECTED ACHIEVEMENTS.**

It is well established that UV radiation has several adverse effects on human health. One of the best known of these is the role of UV as a carcinogen in skin cancer. UV can also induce immunosuppression, known to be an important factor in the oncogenic process. In addition, down-regulation of immunity to a wide variety of antigens including chemical allergens and microorganisms has been demonstrated. However the majority of studies which illustrate the immunomodulating effects of UV radiation involve protocols where laboratory animals or human subjects are subjected to a single or a limited number of UV doses from artificial sources over a short time period, frequently causing erythema (acute exposure). In real life, people are generally exposed to solar UV over longer periods of time (chronic exposure) as would occur during the summer months, with perhaps shorter times of peak exposures, such as around noon on fine days in the summer. The consequences of this type of irradiation for immune responses are not known and are the main objective of the present grant proposal. It is possible that adaptation may take place so that immunomodulation is no longer induced and the mechanism of such a change, if it is found, will also be examined in the project.

Such an investigation is considered timely and important as changes in lifestyles within Europe regarding sun exposure have begun to impact on human health issues. There is, for example, more leisure time generally, and frequent sunshine holidays are being enjoyed by many people, together with an increased use of artificial sunbeds to induce tanning, an activity which is promoted as being healthy and desirable. Global warming may further increase the number of hours spent outdoors. Although it is unlikely that the present hole in the ozone layer will affect the amount of UV reaching populated regions of Europe to any marked extent in the foreseeable future, other areas of the world, such as Chile, may not be so fortunate. Therefore insights into the effects of chronic UV radiation on the immune system and the contribution of adaptation to this progress are urgently required.

Specific questions to be addressed in the project include:

1. Is chronic UV exposure a hazard to the immune system, and what are adverse health outcomes?
2. Does chronic UV exposure produce adaptation regarding the immunological effects?
3. If such an adaptation is found, what mechanisms are involved and do they relate to the non-immune responses (tanning and epidermal thickening) of the skin to chronic UV?
4. If the UV is given chronically or chronically with peak exposures, is the immunomodulation affected? Does the area of the body irradiated under these circumstances influence the immunological outcome?
5. What is the individual (genetic) variability with respect to immunomodulation and adaptation following chronic UV exposures?

To this end, experiments will be carried out in humans and in mice that will be experimentally exposed to solar simulating UV-radiation (Philips Cleo Natural lamps), and effects on innate and adaptive aspects of immunity will be established.

Once answers to these important questions have been provided, then it will be possible to formulate advice and recommend protective measures as are necessary to the general public concerning UV exposure and behaviour. A major objective of the project is therefore to disseminate this type of information to national and EU health authorities.

## **2. PROJECT WORKPLAN**

### **2.1.1. Introduction**

O project will represents a major attempt to collect pertinent data concerning the influence of different UV-exposure profiles on susceptibility to UV-induced immunological and genomic insults. To make the data as applicable as possible to health promotion policy decisions, solar simulating radiation will be used throughout, the patterns of exposure are chosen to mimic real-life human behaviour, special attention is paid to long-term, repeated UV exposures, and the protocols will enable the measurement of adaptive, perhaps protective events.

In the project, real-life situations will be imitated to the greatest possible extent in order to generate data which are applicable to decision making for health promotion policies. For UV exposures, solar simulating UV-radiation (SSR) will be used throughout the study, and exposure schemes are chosen to imitate human sun-related behaviour. For this purpose, we will utilise the new Philips Cleo Natural lamps which emit the solar UV spectrum with regard to the UVB and UVA wavebands. In addition, these lamps will provide a standardised source for the four centres involved in the collaboration. To mimic an intense sun burning of a limited skin area, such as the face or the back of the neck, a dose of 3 minimal erythematous doses (MED) on a limited skin area (of the lower back) will be employed. To imitate moderate sun overdosing, we will use whole-body exposures to 0.7 MED, which corresponds to a minimally perceptible erythema dose (MPED). To mimic the types of UV irradiation experienced on a weekend, a sun resort holiday and a summer vacation, the whole body dose will be repeated for 2, 10 and 30 consecutive days. Similar protocols will be followed for the mice, but the irradiation will be prolonged to 90 consecutive days.

In preliminary experiments, we have established that a single acute exposure of mice to the Cleo Natural lamp induces a local suppression of contact hypersensitivity. The major innovative aspect of the proposed project is to UV-irradiate human subjects and mice over an extended period of time as described above which will allow skin adaptation to take place and to test the function of the immune system throughout this period using carefully selected parameters. In both human subjects and in mice a contact sensitizer will be applied to the irradiated site with subsequent measurement of contact hypersensitivity which will allow an assessment of T cell activity.

Dendritic cells (DC) are required for the initiation of the immune response and also determine the nature of that response. They are likely to play a crucial role in modulating immunity following UV exposure. Therefore, dendritic cell function as a measure of antigen presentation will be examined during the UV irradiation procedures, and correlated, for the first time, with innate aspects of immunity including granulocyte, mast cell and natural killer cell activities. Any immunomodulation will then be related to adaptation in the skin as assessed by pigmentation, epidermal thickening and extent of keratinocyte proliferation.

Gene polymorphisms comprise a major contributing factor in an individual's susceptibility to various diseases including infectious, autoimmune and allergic diseases. An increasing number of human polymorphisms are being identified at present and their significance established. International collaborations have been devised to collect all the known polymorphisms into a single database. Already a database has been created for cytokine gene polymorphisms and their relationship to disease. An association of a promoter polymorphism of TNF  $\alpha$  with subacute cutaneous lupus erythematosus and distinct photoregulation of transcription has been described. To our knowledge, no other gene polymorphisms have been identified thus far that are related to susceptibility to UV, with regard to DNA damage or immunosuppression in humans. In this project the human volunteers will be further investigated for various genetic polymorphisms to understand the basis of any chronic UV effects on immunity. We will restrict ourselves to measurement of polymorphisms that play a role in the innate immune system and/or contact hypersensitivity. Cytokine gene polymorphisms have been shown already to be important in determining immunity and are therefore chosen for study. They include polymorphisms of IL-1 alpha, IL-1 beta, IL-12, IL-18, IL-10, and TNF-alpha. In addition, the role of the skin phototype in relation to chronic irradiation and its impact on immune responses will be evaluated for the first time. Mice are divided into those strains which are susceptible to the immunosuppressive effects of acute UV and those which are more resistant. Again this will be monitored for the first time in the context of chronic UV irradiation by using C3H/HeN mice which are UV-susceptible in comparison with BALB/c mice which are UV-resistant.

The main immunotoxicological parameters of the study meet the following criteria: they are influenced by UV exposure, the four partners in the collaboration are experienced in their use, and they represent different aspects of cell mediated immunity, i.e. innate and adaptive immunity, and afferent and efferent responses. In addition, other parameters, e.g. related to DC subsets, will be employed as innovative aspects. The genotoxic insults will be quantified by measuring UV-specific DNA damage, such as pyrimidine dimers and 6-4 photoproducts.

The studies will be carried out in both species. Whereas the questions of chronic versus acute UV immune effects and adaptation to immunosuppression by UV can be addressed well in humans, the advantage of the mice studies is that the duration of the exposure can be relatively longer. In addition, questions regarding the impact of the surface area that is exposed in relation to dose, and the duration of exposure in relation to dose, require many different experimental groups, and for practical reasons are best answered in

mice. As such, the experiments in humans and in mice are complementary to one another. An additional advantage of related investigation both in humans and in mice is to allow a comparison of the results that will enable the large amount of data obtained already in mice to be evaluated in terms of human health.

## 2.2. Project Structure, planning, and timetable.

**Table 1. Detailed project description broken down in workpackages**

WPI.	Workpackage list
------	------------------

Work-package No	Workpackage title	Responsible participant No	Start month	End month	Deliverable(s) No
WP 1	Co-ordination	1			
	• Brochure	1	1	4	1
	• Kick off meeting Bilthoven	1	1	1	2
	• Progress meeting Turku	2	7	9	3
	• Midterm review meeting Bilthoven	1	16	18	4
	• Progress meeting Lodz	3	25	27	5
	• Progress Meeting Edinburgh	4	33	35	6
	• International Symposium Bilthoven	1	33	36	7
2	Chronic effects of UV exposure on innate immune parameters in humans	2	1	18	8
3	Adaptation to effects of UV exposure on innate immune parameters in humans	2	15	34	9
4	Chronic effects of UV exposure acquired immune parameters in humans	3	1	18	10
5	Adaptation to effects of acquired immune parameters in humans	3	15	34	11
6	Chronic effects of UV exposure on innate immune parameters in mice	4	1	18	12
7	Adaptation to effects of UV exposure on	4	15	34	13

	innate immune parameters in mice				
8	Chronic effects of UV exposure on acquired immune parameters in mice	1	1	18	14
9	Adaptation to effects of UV exposure on acquired immune parameters in mice	1	15	34	15
10	Integration of the results	1	34	36	16

<b>DL. Deliverables list</b>
------------------------------

<b>Deliverable No</b>	<b>Deliverable title</b>	<b>Delivery date</b>	<b>WP</b>	
<b>BROCHURE</b>				
1	Preparation of Project Brochure	Month 4	1	Done, circulated
<b>MEETINGS</b>				
2	Kick off meeting in Bilthoven	Month 1	1	Done, meeting report sent
3	Progress Meeting in Turku	Month 9	1	Done, meeting report sent
4	Midterm Review in Lodz	Month 16	1	Done, meeting report and reviewers report sent
5	Progress Meeting in Edinburgh	Month 27	1	Done, meeting report sent
6	Progress and Preparatory Meeting in Bilthoven	Month 35	1	Done, meeting report sent

7	International Symposium in Bilthoven	Month 36	1	Meeting held; abstracts sent to commission
<b>REPORTS</b>				
8	Report on effects of chronic exposure to UV on human innate immune parameters	Month 18	2	In press, see after WP2 and 3
9	Report on adaptation to chronic UV exposure induced effects on human innate immune parameters	Month 32	3	In press, see after WP 2 and 3
10	Report on effects of chronic exposure to UV on human acquired immune parameters	Month 18	4	In press, see after WP 4 and 5
11	Report on adaptation to chronic UV exposure induced effects on human acquired immune parameters	Month 32	5	In press, see after WP 4 and 5
12	Report on effects of chronic exposure to UV on murine innate immune parameters	Month 18	6	In press and submitted, see after WP 6 and 7
13	Report on adaptation to chronic UV exposure induced effects on murine innate immune parameters	Month 32	7	In press and submitted, see after WP 6 and 7
14	Report on effects of chronic exposure to UV on murine acquired immune parameters	Month 18	8	Submitted, see after WP 8 and 9
15	Report on adaptation to chronic UV exposure induced effects on murine acquired immune parameters	Month 32	9	Submitted, see after WP 8 and 9
16	Integrated report on effects of and adaptation to chronic environmental UV exposure on immunological health	Month 36	10	In preparation, see WP 10

In addition to these specific deliverables, periodic reports to the Commission will be made annually by the co-ordinator, assisted by the participants.



innate immune  
parameters in mice

\*\*\*\*\*

Establishment of  
chronic UV effects  
on acquired immune  
parameters in mice

\*\*\*\*\*

Establishment of  
adaptation to UV  
effects on acquired  
immune parameters in mice

\*\*\*\*\*

Integration: immunological  
health and adaptation  
following chronic  
environmental UV-exposure.  
Recommendations for sun  
exposure behaviour and  
protection

\*\*\*\*\*

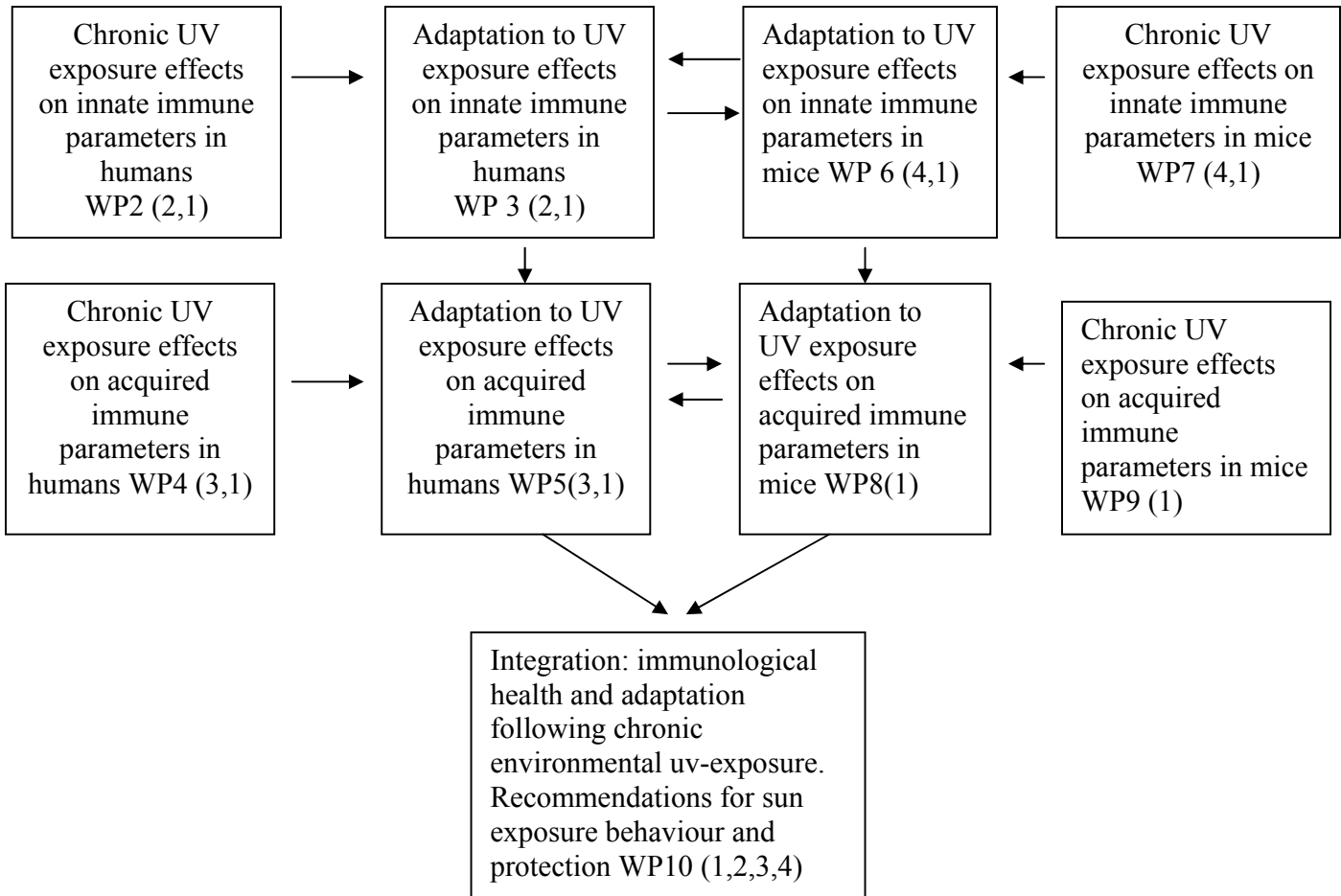
Progress meeting  
Edinburgh

\*

International meeting  
Bilthoven

\*

RELATIONSHIP BETWEEN SCIENTIFIC MILESTONES AND PARTICIPANTS  
(NUMBERS IN BRACKETS)



## DESCRIPTION OF THE WORKPACKAGES AND PROGRESS MADE

### WP.1 Co-ordination

**Workpackage number:** 1

**Start date of starting event:** month 1

**Completion date:** month 36

**N° of the partner responsible:** 1, 2, 3, 4

**Person-months per partner:** CO1:5.7; CR2:1.5; CR3:1.5 ; CR4: 1.5

**Total person months:** 10.2

### Objectives

To ensure the proper flow, outcome, and dissemination of results of the project

### Methodology

To ensure the proper flow, outcome, and dissemination of results of the project, a kick off meeting and progress meetings will be organised with the co-ordinator, all participants, represented by the principle investigators and at least one co-worker per participant will be organised. Representatives from the Commission will be invited. The aim of these PI meetings will be to review progress (including milestone achievements and deliverables), plan future work, and prepare annual progress reports to the Commission. Decisions will be taken on the basis of consensus; in case of disagreement the co-ordinator will make the final decision. During the progress meetings, and during other forms of communication, any deviations from the plans written in the proposal will receive extra attention. Those deviations from the original proposals that have a fundamental impact on achieving the goals as laid out in the proposal will be discussed with the EU officer who is responsible for the key action Environment and Health. To facilitate all processes, the co-ordinator will, in addition to meeting in person, bilaterally or multilaterally communicate with the participants by E-mail, telephone and/or conference calls, as may be required.

In addition to these meetings, a midterm review meeting will be organised, for which reviewers will be invited after consulting the Commission.

Dissemination of the results of the project and stimulating public awareness will be ensured along three different avenues:

1. A brochure of describing the project will be made, and an international symposium to which national and EU health authorities will be invited will be organised toward the end of the project;
2. Reports will be published in the open literature, and results will be presented in international scientific meetings. It is envisaged that publications will appear in different types of journals, i.e. those focused on photobiology, dermatology and immunology, those focused on epidemiology, and those focused on risk assessment, in order to further ensure adequate external scientific feed back and dissemination of our work. In addition, we will ensure the dissemination of these publications to the relevant (inter)national health authorities. In such cases, presentation of such documents will be accompanied by executive summaries, detailing the impact on health policies.
3. Results will be presented through links of the individual participants with several (inter)national bodies that are concerned with adverse health effects of UV radiation, such as WHO, the Dutch Health Council,

UNEP, the Dutch Ministry of Welfare, Health, and Sports, the British Government Advisory Group on Non-ionising Radiation, International Commission on Non-ionising Radiation Protection of ILO, WHO, and EU, and the UV committee of the Finnish Dermatology Society; 4. An international symposium to which (inter)national health authorities will be invited to discuss the outcome of the project will be organised at the end of the project .

#### **Deliverables**

A brochure describing the project will be made. In addition, a kick off meeting in Bilthoven; a progress meeting in Turku, a midterm meeting in Bilthoven, progress meetings in Lodz and Edinburgh, and an international symposium in Bilthoven will be organised.

#### **Milestones and expected results**

The proper flow, and adequate dissemination of results of the project

#### **Progress report year 2002 WP 1**

To ensure the proper conduct of the project and to discuss collaborative aspects and dissemination of the results, a kick-off meeting was held in Bilthoven, the Netherlands, on 18 and 19 January 2002 and a progress meeting was held in Turku, Finland, on 14 and 15 June 2002. At both meetings, all the participants presented their work within the project and the initial results were discussed. Minutes were made and sent subsequently to the participants and the Commission.

A brochure describing the project has been designed and copies given to the participants. The web-site is finished but problems have been encountered in entering the host company which have not yet been resolved.

A midterm review meeting will take place on 4 and 5 April 2003 in Lodz, Poland. Professor A. Young, London, UK, and Dr S. Pavel, Leiden, the Netherlands, have been selected by the Commission as reviewers, based on suggestions made by the partners in the project.

#### **Progress report year 2003 WP 1**

The midterm review proces has taken plave, in cinjunction with a progress meeting in Lodz, in April 2003. The report has been submitted, and suggestions made by the reviewers are being followed.

The website of the project has become operational.

Dr. M. Zak-Prelich, the principal investigator at Lodz, has left her position, and dr. J. Narbutt has taken her place.

#### **Progress report year 2004 WP 1**

A progress report was organized in Bilthoven, December 10 and 11, 2004. Progress was evaluated, and minutes of the meeting sent to the Commission.

Much co-ordination time has been spent on organizing the international symposium, that will take place in Bilthoven March 31, 2005.

#### **Progress report year 2005 WP 1**

An international symposium was held March 31, 2005 in Bilthoven, the Netherlands. The meeting was attended by 30 participants.

**Doc. Version of the flyer used:**

Dear potential participant of the IHA-UV workshop **“What is the immunological risk associated with UVB exposure?”**

You are hereby cordially invited to the above workshop that will take place at the RIVM in Bilthoven, The Netherlands, Thursday 31 March 2005

The aim of the workshop is on one hand to present results from the EU 5<sup>th</sup> Framework programme projects “Immunological health and adaptation following chronic exposure to environmental ultraviolet radiation”. In addition, the workshop will give the state of the art of the immunological risk and health impact of UV exposure.

A further objective of the meeting will be to address how the information available from the project may be used in UVB exposure policy context.

We will invite an audience at this workshop that not only consist of scientists in the field of UV-radiation, but also stakeholders within industry, health and policy.

We do hope that you will find the workshop program interesting and that you will come to Bilthoven and attend the meeting.

To register for this workshop please fill in the enclosed registration form, preferable by the 20<sup>th</sup> of January 2005. Please note that we are only able to accommodate a maximum of 90 participants!

**Tentative programm:**

History of research on immunologic effects of UV  
UV carcinogenesis – an immunological angle"  
Mechanisms of UV effects on the immune system  
Immune effects of UV in human subjects  
Effects of UV on infections  
Epidemiology of UV effects on infections  
Prevention of UV effects  
Sun screens  
Adaptation to UV effects on the immune system  
UV: is there an immunologic risk?

Jan van der Leun, Bunnik, NL  
Frank de Gruijl, Leiden, NL  
Marcel Teunissen, A'dam, NL  
Joanna Narbutt.Lodz, Poland  
Mary Norval Edinburg, UK  
Fabian Termorshuizen A'dam, NL  
Peter Steerenberg, Bilthoven, NL  
Anthony Young, London, UK  
Christer Jansen. Turku, Finland  
Henk van Loveren, Bilthoven , NL

Your sincerely,

Henk van Loveren  
Peter Steerenberg

**WP.2 Chronic effects of UV exposure on innate immune parameters in humans**

**Workpackage number:** 2  
**Start date of starting event:** month 1  
**Completion date:** month 18  
**N° of the partner responsible:** 1, 2  
**Person-months per partner:** CO1:2; CR2:18  
**Total person months:** 20

**Objectives**

To establish the effect of chronic UV exposure on innate immune parameters in humans

**Description of work**

Groups of 50 individuals will be exposed (whole body irradiation) to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for 30 consecutive days. Care will be taken to have an equal distribution of skin types and gender in the study group, and in addition that possible influences on the immune system other than UV will be ruled out (infections, immunosuppressive drugs, drug abuse). For this reason healthy volunteers will be included only. Prior to exposure, and at 2, 10, and 30 days after exposure, blood will be drawn to test for innate immune parameters: natural killer activity (by radioactive chromium release), neutrophil phagocytic activity (by uptake of fluorescent particles and surface complement receptor analysis), dendritic cell type differentiation (by FACS analysis). Dendritic cell functions will be measured (by measuring antigen presentation, i.e. induction of lymphocyte proliferation and cytokine production).

In addition, biopsies of irradiated skin will be obtained for dendritic cell type characterisation and evaluation of skin mast cell degranulation (by histochemical techniques).

In order to compare immune effects with non immune effects of chronic UV exposure, non-immune parameters will be assessed as well: skin type and pigmentation (by visual evaluation and measurement by the UV optimise 555 device), skin thickening (by laser technique), MED values after exposure, DNA damage, and keratinocyte proliferation in the biopsies (by histochemical techniques).

We will analyse cytokine polymorphism that the study subjects display (by PCR).

MED values will be determined using the CLEO natural lamps that will also be used for the experimental exposure. For MED determination, the skin will be shielded, except smaller spots on which the evaluation will be performed visually.

**Deliverables**

Report on effects of chronic exposure to UV on human innate immune parameters

**Milestones and expected results**

We will have established whether effects of chronic UV exposure that resembles that of outdoor exposure on innate immune parameters differ quantitatively from acute exposure or not, and how such immune effects compare to non-immune effects on the skin. In addition, information will be provided on interindividual variability.

## **Progress report year 2002 WP 2 and 3**

### **Amendments to original plan**

It was collectively decided in the Progress meeting in Turku in June 2002 that the original workpackages for human volunteer studies be modified in certain aspects. First, a separate pilot study with nine volunteers should be performed in order to find an immunologically effective dose for whole-body irradiation with CLEO Natural tubes. This is because initial tests had shown that these tubes produce very little erythema which could be used as a measure of a biologically effective dose. A new indicator would be the cytotoxic activity of peripheral blood natural killer (NK) cells. The minimal immunosuppressive dose (MID) would be determined as a dose of solar-simulating UV irradiation that induces a 50% suppression of NK cell activity. All subsequent whole-body irradiation doses in the different volunteer adaptation protocols in Workpackages 2 to 5 would then be set at 0.7 MID determined in the pilot experiment. Second, volunteers to be invited to attend should have skin type 2 only. The skin type should be determined with standard protocols using a UVB device. Third, the single local (10 x 10 cm are at the lower back) irradiation dose of 3 MED required in WP3 should be given with an established UVB source instead of CLEO Natural. In Turku this is the same device as the one used in MED testing. Approval for the changed volunteer protocol was applied and received from the Ethical Committee of the Hospital District of Varsinais-Suomi. It was also later decided to study the number of dendritic cells (DC) in blood and their surface antigen intensities and not to do functional testing of isolated DC, because the lengthy isolation procedure is likely to affect both cell number and function, and at least ten times larger blood sample volumes (around 100 ml) would be required.

### **Description of work done in 2002**

The whole-body cabin for CLEO Natural irradiation was set up with the help by electrical engineers, and an examination room for the study was kindly provided by the Turku University Hospital. Irradiation intensities in the cabin were measured using a Solar Light radiation meter identical to and calibrated with those purchased by the other contractors. The starting irradiation doses to be required in the pilot experiment were estimated by calculation against UVB irradiance and immunosuppression data of known lamp types. Dr. Laura Huurto (Radiation and Nuclear Safety, Helsinki) has helped during the year in irradiation protocols and measurement issues. Two identical UV Optimize 555 devices were received by Turku and Lodz from Denmark to be used to record skin erythema and pigmentation during the volunteer irradiation protocols. The K562 target cell line to be used in NK cell assays was donated by Dr. Jaakko Uksila (Dept. Medical Microbiology, Univ. Turku, Turku). Novel protocols for NK activity (modified protocol for a kit from Perkin-Elmer/Wallac, time-resolved fluorescence measurement of an  $\text{Eu}^{3+}$  chelate-labelled target cells) and neutrophil phagocytosis activity (FACS analysis on uptake of FITC-labelled zymosan particles) were tested and optimised using cells isolated from buffy coats and fresh blood samples, and the tested protocols were then delivered to other partners. Analysis protocol for blood DC was tested using both isolated mononuclear cells and whole blood to adopt a protocol where fresh blood samples could be used with minimal manipulation. We have also tested several antibody combinations

and concentrations to find optimal conditions for DC detection by flow cytometry. Results from DC optimisation were delivered to Lodz and other partners.

Irradiation studies with human subjects cannot be performed in summer, so we had to wait until August to begin volunteer recruitment (July is the main holiday season in Finland). In the meantime, protocol schemes and optimisation tests (such as those explained above) were performed, volunteer timetables and recruitment Web pages created. Volunteer recruitment was started by launching a new Web site ([www.uvtutkimus.net](http://www.uvtutkimus.net), in Finnish only) and by informing the PR and Press Office of the University of Turku. Within a few weeks, about 310 people contacted through e-mail, mail, or phone to enter the study. The contacts were promptly responded by a reply mail with a questionnaire about sun sensitivity and sunbathing behaviour, and an outline of the study. The questionnaires were formulated so that it would be easy to pick out those most probably representing skin type 2. People with skin diseases or on internal medication were strictly excluded. We had 260 questionnaires returned, out of which suitable persons were selected to get an invitation for further testing. Having obtained a written informed consent from the invited volunteers, minimal erythemal dose (MED) threshold testing was performed with a conventional TL12-type UVB device used routinely in the Turku University Hospital. During the autumn 2002, 72 individuals were tested for MED. People that showed skin type 2 erythema response were finally invited to enter one of the four experiment groups.

The first accepted volunteers, however, were included in the pilot group of nine individuals divided into three subgroups. In the pilot experiment, blood was drawn before the first irradiation and 24 hours after each daily whole-body irradiation with CLEO Natural to determine NK cell activity only. The first pilot group received a dose of 1 standard erythemal dose (SED). A decrease in NK cell activity took place after two irradiation doses, but irradiation was still continued with another two daily doses to make sure the observation was real. The suppressed NK activities remained low even after the last irradiation. No erythema was seen at any time point. Because the 1 SED dose seemed to produce the desired effect so soon but one volunteer was omitted because of low initial NK activity, the same protocol was performed with a second group. Again one out of the three volunteers showed very low initial NK activities and was omitted. Combined data from the remaining four volunteers in the first two pilot groups showed that NK cell activity could be suppressed below a mean of 50% after two consecutive daily doses of 1 SED with CLEO Natural. The third pilot group received 0.75 SED daily irradiation, but indication of suppression remained elusive partly due to methodological problems. The exact MID value (50% suppression) calculated from data of the two pilot groups was 1.7 SED, and the 0.7 MID dose to be used in adaptation protocols thus equals 1.2 SED. We proposed and it was approved by others that the routine daily irradiation dose for human volunteers be 1.2 SED. In our whole-body irradiation cabin this corresponds to irradiation for 7 min 12 s, which is a reasonable time for a volunteer to spend in the cabin.

Having agreed on the irradiation dose, the irradiation protocols of WP2 and WP3 were started. WP2 and WP3 were merged into a single protocol for four different groups (A to D) of 25 volunteers each (see Table below), because the same parameters were to be

determined at each time point in both workpackages. Volunteers were included in all different groups to avoid possible influence of seasonal variation. Three additional individuals were recruited for a 30-day follow-up of blood DC numbers without irradiation. The pilot study was completed between Sept 9 and Sept 27, 2002, and the four partial groups in WP2 and WP3 were analysed during Sept 30 – Nov 22. It was also decided to have blood samples taken at day 2 from volunteers in the 10-day irradiation group (C) and at day 10 in the 30-day irradiation group (D) to collect more data on the blood cell responses.

Group	Number of volunteers (2002)	MED testing	Whole-body irradiation	Local irradiation
Pilot	9	Yes	1 or 0.75 SED for 4 days	None
		<b>WP3</b>		
		<u>WP2</u>		
A	7	Yes	none	3 MED
B	5	Yes	0.7 MID for 2 days	3 MED
C	4	yes	0.7 MID for 10 days	3 MED
D	3	yes	0.7 MID for 30 days	3 MED
DC follow-up	3	yes	none	None
<b>Total</b>	<b>31</b>			

In all groups, blood samples and skin biopsies were taken from the accepted individuals before and after whole-body irradiation (WP2) and after the local 3 MED irradiation dose (WP3). Skin biopsies were snap frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until shipment to Bilthoven. From isolated cells in all blood samples, analyses were promptly performed on neutrophil phagocytosis activity and expression of surface complement receptors, NK cell cytotoxic activity, and DC number and surface antigen expression. Blood spots were collected on blood collection cards for interleukin polymorphism determination in Bilthoven. Erythema and pigmentation of the skin was measured at the lateral upper arm during each visit to the clinic.

Analysis of groups B and C showed that the 0.7 MID daily dose of whole-body irradiation suppresses the NK cytotoxic activity at day 2 (in 7 out of 9 individuals), thus confirming the observations in the pilot experiment and justifying the choice of the MID value. In group A (no whole-body irradiation) or at later time points (10 or 30 days after start of irradiation protocol), no suppression of NK activity was observed, which may be an indication of immunological adaptation in the latter groups. The phagocytosis activity of polymorphonuclear neutrophils tended to be suppressed at day 2 but elevated at day 10. No significant changes could be seen in the number of CD1c+/HLA-DR+ or BDCA-2+/CD123+ DC of either non-irradiated (DC follow-up group) or irradiated (group D) volunteers. These data are very preliminary, however, because of the small number of

volunteers per group studied so far. The effect of the local UVB dose remains to be analysed in the data later.

### Prospects for year 2003

- WP2 and WP3 will be proceeding side by side until both are completed in 2004
- Volunteer protocols will continue on Jan 13. We plan to take 30–40 volunteers in the spring (by end of Apr) and about 30 in the autumn (Sept–Dec)
- One full-time scientist will work for 12 months in the project
- Protocols may be slightly modified according to results from optimisation tests performed during or after volunteer period in autumn 2002. It will remain possible, however, to compare results to earlier data.

### Progress report year 2003

#### Spring 2003/WP2 and WP3

Experiments were started in volunteer groups with similar lay-out as in 2002. The Cleo Natural whole-body irradiation dose was 1.2 SED. The volunteers were selected to represent skin type 2. Groups A (receiving no whole-body irradiation), B, C, and D (whole-body irradiation for 2, 10, and 30 days, respectively), and a control group K (no irradiation) were studied in a mixed order. One day after the last irradiation, a local 3 MED dose was given to the lower back skin (about 10 x 10 cm area) with a TL12-type UVB device. A summary of volunteer groups is shown in Table 1.

**Table 1.**  
Spring 2003

Group	Number of volunteers	Whole-body irradiation	Light source	Local UVB
Pilot	2	1.2 SED for 10 days	Cleo	3 MED
A	12	none	-	3 MED
B	7	1.2 SED for 2 days	Cleo	3 MED
C	13	1.2 SED for 10 days	Cleo	3 MED
D	12	1.2 SED for 30 days	Cleo	3 MED
K	13	none	-	None
<b>Total</b>	<b>59</b>			

Blood and skin biopsy samples were collected and assayed as before. The phagocytosis activity of peripheral blood neutrophils remained unchanged except on day 10 both in 10- (C) and 30-day (D) irradiation groups where a reduction of about 19 % and 12 % was seen (non-significant). No reduction was seen in groups A, B, or K. Data for the complement receptor expression in neutrophils remains to be analysed. NK cell activity was tested but the data could not be calculated because of technical problems in the

assay. The reason for the problems that suddenly appeared in late autumn 2002 couldn't be resolved although the assay has now performed excellently after summer 2003. The BDCA2+ dendritic cells in the blood did not respond to these SSR or local UVB irradiation doses. A selection of skin biopsies was sent to Bilthoven for further analyses.

### **Autumn 2003/Pilot experiments**

As discussed in the Midterm Meeting in Lodz in April, it should be considered whether it might be advantageous to change the lamp type if the Cleo lamps do not induce consistent down-regulation in NK cell activity. Therefore, in October 2003, a pilot study was performed to evaluate the efficiency of UV6 whole-body irradiation in comparison to Cleo Natural in modulating systemic NK cell and neutrophil activities, and to estimate the irradiation dose needed in further studies.

We had our Solar Light UV meter calibrated against Cleo Natural and UV6 tubes by Radiation and Nuclear Safety Authority (Laura Huurto, Helsinki) to be able to measure equal SED values for the Cleo (22 x 100 W) and UV6 (40 x 100 W) cabins available in Turku University Central Hospital.

Two pilot experiments with five MED-tested skin type 2 volunteers were performed;

Pilot 1 2 volunteers, **1.2 SED** Cleo Natural

3 volunteers, **1.2 SED** UV6

Pilot 2 3 volunteers, **2.0 SED** Cleo Natural

2 volunteers, **2.0 SED** UV6.

Whole-body irradiation was given daily for 9 (Pilot 2) or 10 (Pilot 1) consecutive days. No local irradiation was given. Blood samples were drawn immediately before the first irradiation (base line), then on days 1, 2, 3, 4, 7, and 9 or 10, each time about 24 hours after the previous irradiation. NK cell and neutrophil activity was assayed in each sample, pursuing to minimise any day-to-day variation in the protocols or timing. NK cell analyses were done as two parallel assays using two different batches of K562 target cells (one received from Turku, the other purchased from ATCC). Dendritic cell were not analysed.

In the Pilot 1 group (1.2 SED), NK cell activities went down to their lowest point on day 4. After this time point, a recovery response beyond the base line levels was seen, but no difference between the irradiation regimen was observed. Neutrophil phagocytosis was also at its lowest level on day 4, then recovering higher than the initial levels. This irradiation dose did not produce visible erythema except mildly in one volunteer after three days of UV6 irradiation. In the Pilot 2 group (2.0 SED), NK cell activities decreased on day 4 by almost 50 %. We saw a small recovery phase also with this dose, but the activities in the end of the experiment remained low. Comparison between Cleo and UV6 was complicated by unequal initial mean base line levels in the two groups. Neutrophil phagocytosis activity showed a profile closely resembling the NK results in Pilot 2. UV6 and Cleo produced similar phagocytosis effects, and also erythema was observed in both irradiation regimen. In conclusion, the pilot experiments showed that the lamp types do not differ in producing effects on these parameters. Rather, the effects

depend on the irradiation dose. In subsequent experiments under WP2 and WP3, we decided to use the larger dose of 2 SED. On the other hand, with a dose bigger than 2 SED, erythema could become a problem. The MID (minimal immunosuppressive dose, 50% suppression) value could not be defined from the pilot data.

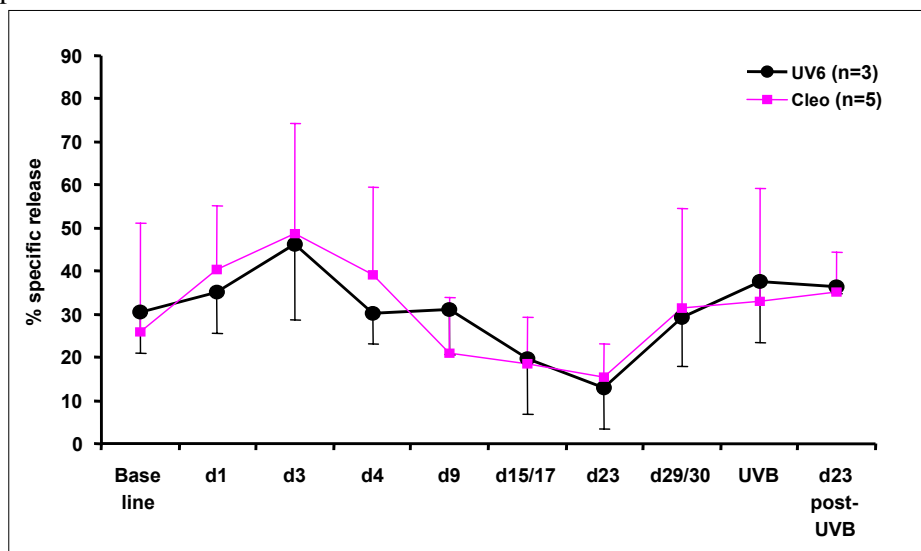
### **Autumn 2003/WP2 and WP3**

The pilot studies performed in the early autumn 2003 indicated that the individual doses of the repeated UV exposures were to be increased to 2 SED. In studies initiated thereafter, the 2 SED dose was thus applied, and two different lamps studied in parallel, i.e. Cleo Natural and UV6. A call for volunteers resulted, in comparison to earlier calls, in a smaller number of eligible applicants (MED-confirmed type-2 skin; no recent UV exposure or other exclusion factors). Thus, only 10- and 30-day exposure series were initiated. Due to practical personnel situation in Turku, we had an active time period of six weeks for volunteer recruiting/laboratory work starting on October 13. To utilise this time as efficiently as possible, we constructed a time table for two groups D (irradiation for 30 days) and for two groups C (irradiation for 10 days) with intermediate sampling. All the four groups was planned to hold five volunteers each, including two volunteers for Cleo and three for UV6, and vice versa. Irradiation dose was 2 SED. In the end of each irradiation period, local UVB (TL12) was given according to the Workpackages. Analyses and sampling was performed as earlier. A summary of the groups is shown in Table 2.

**Table 2.**  
Autumn 2003

Group	Number of volunteers	Whole-body irradiation	Light source	Local UVB	CHS (DPCP)
Pilots	2	1.2 SED for 8 days	Cleo	None	None
	3	1.2 SED for 8 (1) or 10 (2) days	UV6	None	None
	3	2.0 SED for 9 days	Cleo	None	Yes (2)
	2	2.0 SED for 8 (1) or 9 (1) days	UV6	None	Yes
C	3	2.0 SED for 10 days	Cleo	3 MED	None
	5	2.0 SED for 10 days	UV6	3 MED	Yes (4)
D	4	2.0 SED for 30 days	Cleo	3 MED	Yes (2)
	4	2.0 SED for 30 days	UV6	3 MED	Yes (2)
<b>Total</b>	<b>26</b>				

From volunteers irradiated for 30 consecutive days, blood samples were taken at days 1, 3, 4, 9, 15, 23, 30, 31 (after local UVB), and 53 (23 days after last irradiation). NK cell activities had first a transient increase (days 1-3), then they were suppressed gradually but clearly (close to 50 % level from base line) in group D as long as until day 23. In the end of the 30-day irradiation, activities were again close to initial base line levels and remained unchanged when assayed three weeks thereafter. UV6 and Cleo irradiation produced a similar effect (Fig. 1). In neutrophils, the phagocytosis activity also decreased from day 4 at least up to day 23 independent of the light source. Several assayed parameters remain to be calculated.



**Figure 1.** NK cell activity in volunteers given a daily dose of 2 SED UV6

and Cleo Natural whole-body irradiation.

Flow cytometer analysis of peripheral blood dendritic cells gave results much more clearly divergent in the UV6 and Cleo groups than what was seen in NK or neutrophil parameters. In the 30-day group, all DC subtypes, pDC2 (BDCA-2+CD123+ cells), CD33+ (CD3-CD16-) DC, pDC1 (BDCA-1+ CD11c+ HLA-DR+ cells), and BDCA-3+ HLA-DR+ cells, had a minimum on day 4 and a maximum on day 15/17 in their relative cell number, most clearly in the UV6 irradiation group. When the ratio pDC2/pDC1 was calculated, it showed a profile almost reciprocal to NK cell activity in the UV6 group. These observations need to be confirmed in a larger volunteer series. The results suggest that the studied innate immune parameters in blood of humans respond to chronic whole-body irradiation in a time scale of a few days to at least four weeks. Induction, suppression and restoration of those parameters may take place within one month, but the relation of the parameters needs to be defined in more detail. Equal erythemally weighted doses of solar-simulating UV or UV6 irradiation yield generally similar responses if the dose is large enough.

Measurement of skin erythema showed that the erythema response induced by acute local 3 MED UVB dose at the lower back skin site was the milder the more times (2 to 30 days) the skin had been exposed to whole-body irradiation. Solar-simulating UV (Cleo) was more efficient than UV6 irradiation in adapting the skin against acute UVB erythema induction. No erythema induction with 1.2 SED dose of Cleo irradiation could be observed as measured in the upper arm. However, both lamps resulted in gradual induction of erythema with the 2 SED whole-body dose throughout the 30-day time period with equal slopes in induction, substantiating the calibration of the UV meter against the lamp types and calculation of the erythemogenic dose.

Most volunteer participating in the irradiation groups gave their consent to continue in a contact sensitisation experiment. The study lay-out included (1) Whole body irradiation with 2 SED for either 10 or 30 consecutive days, (2) Local irradiation on UV-exposed upper buttock skin, with 3 MED of UVB, (3) Sensitisation, in the same skin area, with 40 µg of DPCP, (4) Observation of the induction site for possible primary allergic reaction, and (5) Patch testing, on a shielded site of the inner upper arm, with dilutions of DPCP. The acquired data, presented in Table 3, indicates that 10 or 30 consecutive whole-body irradiations of 2 SED with the Cleo Natural lamp may, in some people, induce a resistance towards immune suppression by a later applied, erythemogenic dose of UVB radiation. In contrast, immune adaptation was not recorded in the volunteers receiving similar series of whole-body irradiation with the UV6 light source. These preliminary results need to be validated in a larger group of volunteers.

**Table 3.**

Initials	Whole-body irradiation (2 SED)		PAR	6.4 µg	3.2 µg	1.6µg	0.8 µg	0.4 µg	Interpretation
	Cabin	Days							
LJ	UV6	10	No	-	-	-	-	-	Ineffective
KT	UV6	10	No	-	-	-	-	-	Ineffective
HJ	UV6	10	No	-	-	-	-	-	Ineffective
RE	UV6	30	No	-	-	-	-	-	Ineffective
VV	UV6	30	No	-	-	-	-	-	Ineffective
RL	Cleo	10	++	ND	ND	+	+	+	<u>Effective</u>
LA	Cleo	10	No	-	-	-	-	-	Ineffective
PJ	Cleo	10	No	-	-	-	-	-	Ineffective
T-HJ	Cleo	30	No	-	-	-	-	-	Ineffective
VP	Cleo	30	No	-	-	-	-	-	Ineffective
IA-L	Cleo	30	+	+	+	-	-	-	<u>Effective</u>
KH	Cleo	30	+	-	-	-	-	-	<u>Effective</u>

### Prospects for year 2004

- WP2 and WP3 will be proceeding side by side until both are completed in 2004.
- Volunteer protocols can be continued in January until March. Because the laboratory has to move into another premises, it is uncertain to which extent it is possible do analyses after March.
- One full-time scientist will work for at least 9 months in the project.

### Progress report year 2004

Experiments were continued to complete the series started in autumn 2003 through 2004. The Cleo Natural or UV6 whole-body irradiation doses of 2.0 SED were given to volunteers of skin type 2 as before. A major change in the study groups was that only group D (daily whole-body irradiation for 30 days followed by a single 3 MED dose of UVB given to the back skin ; n=12) and a group of unirradiated volunteers (n=10) of the same skin type was studied. Blood samples were drawn on seven occasions during the irradiation period and one sample about three weeks after the last irradiation. The unirradiated control group gave also eight blood samples at the same time points. No skin

biopsies were taken. All volunteers were sensitised with DPCP after the irradiation period.

At most sampling time points, the cell number in all blood dendritic cell (DC) subtypes (BDCA-2+ CD123+, BDCA-1+ CD11c+ HLA-DR+, and BDCA-3+ HLA-DR+ cell) increased during whole-body irradiation. The increase was significant and appeared elevated between 14 and 30 days from beginning of irradiation. The Cleo Natural and UV6 irradiation produced similar effects. Subjects in the control group did not show such an increase.

It was earlier agreed that control experiments should be done on the NK cell assay. We assessed the NK cell activity with two different assays in single blood samples from ten unirradiated subjects that had participated in the study several months earlier. The traditional <sup>51</sup>Cr 18-h release assay in most cases gave higher specific release as expected in comparison with the BADTA-Eu time-resolved 4-h release assay. Variation in both assays was very small, and also the BADTA-Eu assay showed good response to effector-to-target cell ratio. In our hands, the BADTA-Eu assay has been the only practical alternative, considering the fact that all the assays in our analysis palette have to be performed on the same day from fresh blood samples. NK cell activities did not change during irradiation, and they remained constant also in the unirradiated control group.

The phagocytosis activity of peripheral blood neutrophils remained unchanged in all subjects. Calculations on neutrophil surface antigens are underway, but no significant changes are expected. It is possible that suppression of phagocytosis activity may take place as an acute response, already before the second sampling time point on day 3.

Skin urocanic acid (UCA) concentrations were measured 24 h after the last whole-body irradiation at various time points. The mean percentage of the *cis*-UCA concentrations were around 6% at the baseline, increased significantly to about 60% from total UCA at the first (3 d) sampling point, remained at the same level during the 30-d irradiation period, and were close to initial baseline levels when measured three weeks after the last irradiation. Again, both Cleo Natural and UV6 irradiation sources produced identical results. The total UCA content decreased during the 30-d period.

DPCP contact sensitisation assessment was done on all group D and control subjects participating in the study in 2004. The assessment followed strictly the protocol of the previous experiments. Irradiated subjects showed no DPCP sensitisation in 8/12 subjects, and sensitisation to DPCP in four. In unirradiated control subjects, 3/10 did not get sensitised, while seven showed a positive reaction. It is inferred that 30-day whole-body irradiation of 2 SED with Cleo Natural or UV6 predisposes to resistance against immunosuppression by a later applied, erythemotogenic dose of UVB radiation.

In 2004, a series of experiments not specifically included in our Workpackages were performed on serum C-reactive protein (CRP), an acute-phase inflammatory factor and a general marker of inflammation and infection in various organs. CRP was measured in serum samples drawn simultaneously with blood sampling for the other experiments. We

hypothesized that local inflammation induced by UVB irradiation on a small skin area could elevate serum CRP levels. A novel separation-free high-sensitivity assay for CRP, analysed in collaboration with the Laboratory of Biophysics, Institute of Biomedicine, University of Turku, revealed an increase by 42% ( $p=0.046$ ) in CRP concentrations at 24 h after a local 3 MED UVB dose. In volunteers subjected to whole-body SSR (1.2 SED) for 10 or 30 consecutive days, no increase in CRP could be measured following the UVB dose. The results suggest that possibly photoadaptation by repeating suberythemal doses of SSR reduced the signs of systemic inflammation. This conclusion is further supported by observations on skin erythema measurements, representing the local inflammation aspect. The UVB-induced skin erythema was partially but significantly attenuated by 30 preceding days of SSR ( $p=0.00066$ ). An unexpected finding was that the mean baseline CRP concentrations ( $0.24 \pm 0.21$  mg/l) declined by 35% ( $p=0.018$ ) after 10 daily SSR doses (no local UVB). The samples were so selected as to reject all volunteers from the analysis whose samples showed an exceptionally high CRP concentration at any time point, indicating a possible unknown inflammatory state. The results in this section were collected into a paper that has been resubmitted as a revised manuscript.

All data from 2004 will be merged with data from previous similar experiments.

A total of 137 volunteers (109 F, 28 M) participated in the studies of the Turku group in 2002-2004.

### **Progress report year 2005 WP 2, 3**

A manuscript considering the possible photoadaptation by repeating suberythemal doses of SSR in systemic C-reactive protein (CRP) and skin erythema readings was revised and resubmitted. It was accepted for publication in February 2005 (Laihia et al, in press). No laboratory work on the workpackages was done in January-March 2005, and no full-time personnel was employed.

### **OVERALL EVALUATION AND CONCLUSIONS, WP.2 AND WP.3**

As a basis for the evaluation of the results, it must be appreciated that the workpackages concerning studies with human subjects were collectively modified in the first Progress meeting in Turku in June 2002. First, the dose for whole-body solar-simulating UV irradiation with CLEO Natural tubes was decided to be determined by an immunological measure instead of a personal MED, based on suppression of peripheral blood NK cell activity. A second change was to investigate only individuals with skin type II. The third change was to use TL-12-type UVB source for the single local irradiation.

The human volunteers who participated in the study in Turku in 2002-2004, a total of 137 individuals, comprised a good sample of the fair-skinned population. Only healthy subjects representing skin type II were accepted. Individuals with skin diseases or on

internal medication were strictly excluded. It was not possible to select an even distribution by gender (109 women, 28 men).

A critical point in the beginning of the project was the determination of a minimal immunosuppressive dose (MID) of irradiation that would induce a 50% suppression of NK cell activity in humans. Because the Cleo Natural lamps induce very little erythema, it was difficult to find a biologically or immunologically relevant dose for the study. The determination of MID was performed in a couple of small pilot experiments, and the results seemed elusive. However, all subsequent whole-body irradiation doses in the different volunteer adaptation protocols in Workpackages 2 to 5 were dependent on the dose-finding pilot experiments. Considering larger sets of data on NK cell activity acquired later, the initial dose of whole-body irradiation (1.2 SED) determined in the pilot experiments was not relevant in terms of suppression of NK cell activity.

In later studies with primary effects of the whole-body irradiation on certain physical non-immune parameters in the skin, both significant photoisomerisation of *trans*-urocanic acid (*trans*-UCA) to *cis*-UCA and DNA damage was recorded, with slight epidermal thickening and minimal pigmentation, signifying that the doses do induce physical/chemical processes that are known to have further biological and immunological consequences.

In innate immune parameters, the number of various peripheral blood dendritic cell (DC) subtypes increased significantly during whole-body irradiation at most sampling time points, whereas phagocytosis activity of peripheral blood neutrophils remained unchanged. NK cell activity was not suppressed.

The question of the ability of the repeating whole-body solar-simulating UV irradiation to produce adaptation to a subsequent exposure of UVB was approached by applying a 3 MED dose of UVB on a small skin area that had been exposed to preceding daily doses of solar-simulating UV irradiation. No protective effect was noted in formation of DNA lesions. Other non-immune parameters (UCA photoisomerisation, epidermal thickening, pigmentation) were not determined. However, we recorded significant attenuation of UVB-induced skin erythema after 30 daily doses of solar-simulating UV irradiation. This was accompanied by significantly lower induction of the C-reactive protein (CRP) in serum samples from the same group of individuals. These two parameters suggest that adaptation exist. Innate immune parameters could not be evaluated in respect to adaptation, because the local UVB dose did not produce recordable responses in the first place.

All analyses comparing the solar-simulating UV irradiation (CLEO Natural) and UV6-type irradiation show that there is no difference in the biological response, suggesting that similar doses in SED units (weighed according to human skin erythema formation) produce similar local and systemic effects also in other parameters than skin erythema.

**WP.3      Adaptation to effects of UV exposure on innate immune parameters in humans**

**Workpackage number:** 3

**Start date of starting event:** month 18

**Completion date:** month 32

**N° of the partner responsible:** 1, 2

**Person-months per partner:** CO1:2, CR2:18

**Total person months:** 20

**Objectives**

To establish the adaptive activity of UV exposure on subsequent exposures of UV that suppress innate immune responses in humans

**Description of work**

Groups of 50 individuals will be exposed (whole body) to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for either 2, 10, or 30 consecutive days. Subsequent to exposure, the exposed individuals and matched controls will be exposed to a single immunosuppressive exposure of 3 personal MED (values determined at the beginning of the experiment) on areas of 10 x 10 cm at the lower back. Innate immune parameters as well as non-immune parameters as indicated in workpackage 1 will be evaluated

**Deliverables**

Report on adaptation to chronic UV exposure induced human innate immune parameters

**Milestones and expected results**

The results of this workpackage will yield information on the adaptation activity of UV exposure to subsequent immunosuppressive UV exposures on innate immune parameters, and on how this compares to such adaptation regarding non-immune parameters. In addition, information will become available on interindividual differences with regard to adaptation to the suppressive effects of UV.

**Progress report year 2002 WP 3:** See WP 2

**Progress report year 2003 WP 3:** See WP 2

**Progress report year 2004 WP 3:** See WP 2

**Progress report year 2005 WP 3:** See WP 2

**Conclusions, see WP.2**

**WP.4 Chronic effects of UV exposure on acquired immune parameter in humans**

**Workpackage number:** 4  
**Start date of starting event:** month 1  
**Completion date:** month 18  
**N° of the partner responsible:** 1, 3  
**Person-months per partner:** CO1:2; CR3:18  
**Total person months:** 20

**Objectives**

To establish the effect of chronic UV exposure on acquired immune parameters in humans

**Description of work**

Groups of 50 individuals will be exposed (whole body) to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for 2, 10, or 30 consecutive days. Subsequently, exposed and non-exposed individuals will be sensitised to diphenyl cyclopropanone (DPCP) on the irradiated skin, followed by DPCP exposure as a challenge on a UV shielded site at the upper arm. The DPCP challenge reaction will be evaluated visually. In addition, biopsies of irradiated and sensitised skin will be obtained for dendritic cell type characterisation and evaluation of skin mast cell degranulation (by histochemical techniques).

Non-immune parameters will be assessed as well: skin type and pigmentation (by visual evaluation), skin thickening (by laser technique), DNA damage, and keratinocyte proliferation in the biopsies (by histochemical techniques). In addition, dendritic cell type characterisation will be performed in peripheral blood by FACS analysis. We will analyse cytokine polymorphism that the study subjects display (by PCR).

**Deliverables**

Report on effects of chronic exposure to UV on human acquired immune parameters

**Milestones and expected results**

The expected result is that we will have insight in the effects of chronic UV exposure that resembles that of outdoor exposure on acquired immune parameters, and how such immune effects relate to non-immune effects on the skin. In addition, information will be provided on interindividual variability.

## **Progress report year 2002 WP 4 and 5**

### MED Testing with Cleo Lamps

Initially MED testing using the Cleo lamps was undertaken. In total 18 people, mainly with skin type II, were tested on inner area of the forearm. However, even after 30 minutes of irradiation, no erythema was observed. Therefore it was decided to use 0.7 minimal immunosuppressive dose (MID) in all the human experiments instead of 0.7 MED, as stated in the original protocol. The MED was established for all the volunteers on their first visit, using a UV21 Philips lamp. The mean MEDs for the control and irradiated groups were not significantly different ( $p>0.05$ ).

### Recruitment of volunteers

Recruitment of volunteers was accomplished by an announcement at the Medical University of Lodz and by advertising in local newspapers. The response resulted in over 700 phone calls or personal visits. After preliminary interviews, 150 healthy people without any skin or other diseases and not taking any medicines were included in the study. All of them had blood count and urinalysis performed according to the Local Ethical Committee advice and underwent thorough physical examination. After obtaining informed consent, the study procedures were started.

### Protocols for exposed and non-exposed individuals

The volunteers were divided into a non-exposed (control) group or groups exposed to the Cleo lamps for 2, 10 or 30 consecutive days. Before irradiation the following procedures were performed: MED was established, a blood sample for gene polymorphism analysis, differential cell count and dendritic cell phenotyping was collected, the pigmentation on the outer arm skin was measured and a biopsy from the buttock skin was taken. Then 24 hours after 2, 10 or 30 days of irradiation a blood sample for dendritic cell phenotyping and a second biopsy from the buttock were collected, erythema was measured and the DPCP sensitization disks were taped on the un-biopsied buttock. Three weeks later, the antigen challenge with DPCP was performed and, after 48 hours, erythema was measured and a biopsy from the challenge site was collected.

### Irradiation with Cleo Lamps

The MID was demonstrated by the Turku team for natural killer cell activity and 0.7 MID corresponded to 1.2 standard erythema dose (SED). The SED was established using the output of the Cleo lamps, calibration of the irradiance solar light 3D meter, and the properties of Cleo lamps. For our lamps the 1.2 SED irradiation time was 10 min 30 sec.

### Blood dendritic cell phenotypic analysis by FACS

Peripheral blood mononuclear cells (PBMC) were purified from whole blood. The immunophenotype of the dendritic cells (DC) in the PBMC population was determined using a set of monoclonal antibodies (MoAbs), directed against following antigens: CD11c, CD32, CD64, CD123, CD80, CD86, BDCA-1, BDCA-2, BDCA-3, HLA-DR (all from Caltag). As a control, the respective mouse IgG1 MoAbs were used (Caltag). The immunophenotype was assessed by dual-colour/triple colour cytometry

using a FACScan (Becton-Dickinson, San Jose, CA, USA) and analysed with green (FL1), orange (FL2) or red (FL3) standard emission filters.

The configuration of the antibodies was as follows

1. BDCA-1 CD11c CD64
2. BDCA-1 CD123 HLA-DR
3. BDCA-2 CD123 CD64
4. BDCA-2 CD86 CD80
5. BDCA-2 CD32 CD83

The results were counted as % positive/100 000 events

The results from first 10 volunteers before and after 2 days of irradiation were as follows

(a - before irradiation, b - 24 hours after 2 days of irradiation, con. -configuration):

Volunteers	Con.1 a	Con.1 b	Con.2. a	Con.2 b	Con.3a	Con.3b	Con.4a	Con.4b	Con.5a	Con.5b
1	0,33	0,35	0,03	0	0,57	0,31	0,04	0,41	0,06	0,1
2	0,3	0,85	0,02	0,12	0,26	0,68	0,04	0,08	0,02	0,17
3	0,29	0,36	0,02	0,02	0,9	1,26	0,04	0,03	0,04	0,03
4	0,2	0,34	0,04	0,01	0,58	0,81	0,05	0,09	0,14	0,02
5	0,12	0,17	0,03	0,02	0,42	0,99	0,18	0,22	0,02	0
6	0,19	0,37	0,02	0,04	0,49	0,97	0,07	0,02	0,07	0,03
7	0,38	0,53	0,02	0,04	0,41	1,25	0,04	0,02	0,03	0,22
8	0,27	0,42	0,02	0,03	0,59	0,67	0,01	0,04	0,03	0,04
9	0,49	0,61	0,02	0,06	0,14	0,64	0,05	0,03	0,03	0,06
10	0,18	0,49	0,06	0,02	0,62	0,66	0	0,05	0,02	0

**Statistically significant differences:**

Configuration 1.

BDCA1/11C

	n	Mean	Median	Min	Max	SED
Before	10	0,275	0,280	0,120	0,490	0,109
After	10	0,449	0,395	0,170	0,850	0,185

Before-after:  $t=-3,560$   $p=0,006122^*$

t-Student test

Configuration 3.

BDCA2/CD123

	n	Mean	Median	Min	Max	SED
Before	10	0,498	0,530	0,140	0,900	0,210
After	10	0,824	0,745	0,310	1,260	0,296

Before-after:  $t=-3,291$   $p=0,009350^*$

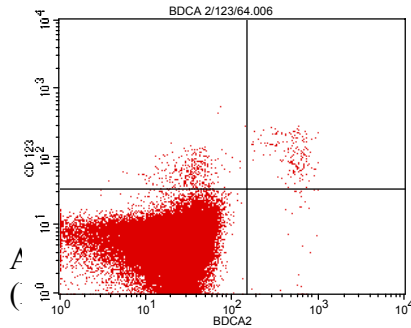
t-Student test

It seems that, after 2 days of irradiation with 1.2 SED from Cleo lamps, the percentage of DC positive for the antigens in configuration 1 and 3 nearly doubled.

**Results from volunteer 5 and 6 as an example:**

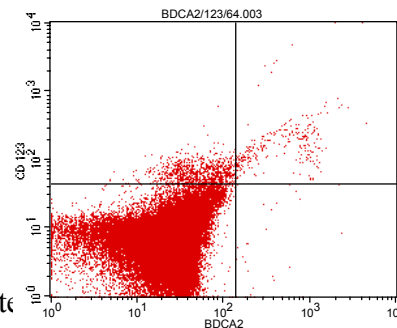
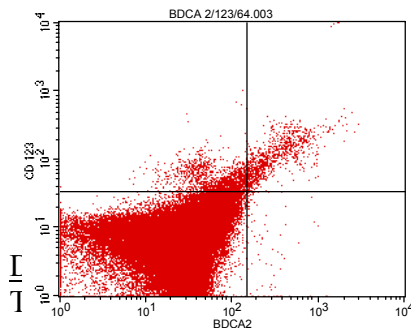
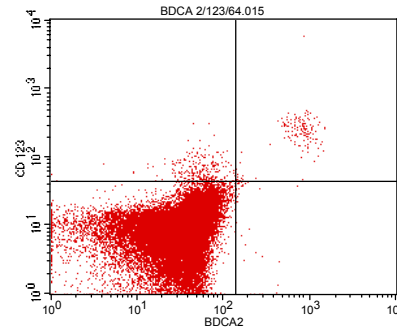
Before irradiation

(BDCA-2/CD123/CD64)



Before irradiation

(BDCA-2/CD123/CD64)



the left irradiated and the final exposure using two petrolatum-backed 7 mm filter disks, each soaked in 20 µl of 1 mg of diphenylcyclopropenone (DPCP) in 1 ml of acetone (20 µg/20µl). The disks were mounted inside 8 mm aluminium Finn chambers and the 2 chambers were taped to the skin and left in place for 48 hours. The primary allergic response (PAR) was recorded during the next 3 weeks. Three weeks after sensitization the subjects received an antigenic challenge on the unirradiated, right upper inner arm with a titration series of DPCP (0.4 µg, 0.8 µg, 1.6µg, 3.2µg, 6.4µg) and acetone control. All the disks remained in place for 6 hours. The sites were evaluated 48 hours later by eye using the scoring recommended by the International Contact Dermatitis Research Group and using the skin erythema/pigmentation meter UV Optimize. A punch biopsy was taken from the sensitized 3.2 µg site and immediately frozen in liquid nitrogen, then stored in at -80°C. The mean values of the erythema measured with UV Optimize as the percentage of redness on the first 30 volunteers were 29.48% compared with 16.80% (p<0.0002) on the skin beside the site of antigenic challenge.

**Progress WP 4 and 5, 2003**

**I. Study performed in autumn 2002 - spring 2003**

**Characteristics of the study**

140 volunteers completed the study. They were divided into a non-exposed (control) group and groups exposed to the Cleo lamps for 2, 10 or 30 consecutive days. Before irradiation the following procedures were performed: MED was established, blood samples for gene polymorphism analysis, differential cell count and dendritic cell

phenotyping were collected, the pigmentation on the outer arm skin was measured and a biopsy from the buttock skin was taken. Then 24 hours after 2, 10 or 30 days of irradiation a second blood sample for dendritic cell phenotyping and a second biopsy from the buttock were collected, erythema was measured and the DPCP sensitization discs were taped on the un-biopsied buttock. Three weeks later, the antigen challenge with DPCP was performed and, after 48 hours, erythema was measured and a biopsy from the challenge site of 3.2 µg DPCP was collected.

Characteristics of 40 individuals from the control group and 100 individuals irradiated with CLEO lamps is shown in Table I

Clinical characteristics of the healthy volunteers				
Features	Control group (n=40)	Group 1 (n=33)	Group 2 (n=34)	Group 3 (n=33)
Gender F/M	17 F 23 M	17 F 16 M	13 F 21 M	15 F 18 M
Mean age	24,9	25,4	24,2	26.75
Phototype**	50% II 50% III	55% II 45% III	65% II 35 % III	67% II 33%III
Mean MED(J/cm2)	0,17	0,16	0,16	0,15
Mean MED with Regard to phototype II/III	0.15/0.18	0.14/0.17	0.14/0.18	0.14/0.17

### 1. Contact hypersensitivity response analysis

The intensity of contact hypersensitivity (CHS) reaction was assessed using visual scoring scale (no reaction - 0 points, macular erythema -1 point, erythema + infiltration -2 points, erythema + infiltration + papules or vesicles -3 points, bullous reaction - 4 points) as well as using UV optimize 555 device. There were no statistically significant differences between the control group and 2, 10 and 30 days irradiation group in relation to the intensity of CHS measured both ways. Moreover, no differences in the time point when PAR (primary allergic response) appeared and how long it lasted were observed. As the analysis of all the parameters indicated no differences in CHS response in non-exposed and exposed groups, new study design was proposed and accepted during the Progress Meeting (Lodz, April 2003).

### Analysis of skin biopsies taken from DPCP elicitation site from CLEO irradiated individuals

Immunohistochemical examination was performed in Lodz with the use of mouse monoclonal anti-CD1a and HLA-DR antibodies ( Novacastra Laboratories Ltd.). So far 20 skin biopsies taken from DPCP elicitation site (left inner arm skin) from unirradiated and CLEO irradiated individuals as well 10 biopsies from unchallenged inner arm skin (control) have been analysed. The preliminary results show that HLA –DR positive cells were present in all sensitised groups - (over 50% of cellular infiltrate in control and 2-day irradiations group, 50% of cellular infiltrate in 10-day irradiations group and less than

50% of cellular infiltrate in 30-day irradiations group) compared to the control (less than 10% of cellular infiltrate).

The immunohistochemical studies are being further performed.

## **2. Analysis of DNA damage**

Biopsies from 3 volunteers taken before and after irradiation were sent to Bilthoven (autumn 2003) in order to assess DNA damage.

## **3. Blood dendritic cell phenotype analysis**

The analysis of blood dendritic cells (DCs) phenotype was performed in 94 SSR exposed volunteers aged 19-51 years old, with either II or III skin phototype. Blood samples were taken twice: before irradiation and 24 hours after final exposure. Dendritic cells were detected in peripheral blood mononuclear cells (PBMC) population. The three main subpopulations of DCs were distinguished by their expression profile: two myeloid types of DCs - BDCA-1+ /CD1c+ /HLA-DR+ (MDC1) and BDCA-3+/CD32-/CD64-/HLA-DR+ (MDC2), and the plasmacytoid subset (PDCs) - BDCA-2+/CD123+/HLA-DR+. The percentages and absolute numbers of the DCs and subsets were calculated for each subject. The immunophenotype of DCs was determined using the panel of MoAbs directed against several DCs-defined antigens. There were: FITC-conjugated BDCA-1, BDCA-2, and BDCA-3 (Miltenyi Biotec), PE-conjugated CD11c, CD32 (Caltag Laboratories) and CD123 (Miltenyi Biotec), as well as TC-conjugated HLA-DR (Caltag Laboratories). In preliminary study combinations of another MoAbs, against CD64, CD14 and CD19 antigens, as well as CD80 and CD86 co-stimulatory molecules (all Caltag Laboratories) were also tested. As a control, the respective isotype mouse IgG1 MoAbs were used (all Caltag Laboratories). The immunophenotype was assessed by triple-color cytometry. Cell fluorescence was measured by flow cytometer (FACScan, Becton-Dickinson, San Jose, CA, USA) and analysed with green (FL1), orange (FL2) or red (FL3) standard emission filters. Based on SC vs. FS distribution of DCs has been gated from whole PBMC population.

The DCs represent 0.81% of the PBMC and the median of total DC number was 46.7/ $\mu$ l. We found a weak, but statistically significant inverse correlation between the total DC count and age ( $R=-0.20$ ;  $p=0.049$ ). Moderate positive correlation of total DC count with MED values ( $R=0.21$ ;  $p=0.038$ ) was also found.

The median percentage of myeloid MDC1 and MDC2 types of DCs was 0.20 and 0.10, respectively. The median number of MDC1s was nearly twice higher than MDC2s (10.3/ $\mu$ l and 5.9/ $\mu$ l; respectively). The median PDC count was 26.7/ $\mu$ l, and these cells represented 0.45% of the PBMC. There was an inverse correlation between the PDC count and age ( $R=-0.21$ ;  $p=0.049$ ) and a correlation between PDC count and MED ( $R=0.29$ ;  $p=0.004$ ). There were no statistical differences between gender or II and III phototype and particular DC subsets.

### *DC subtypes after UV irradiation*

The percentage of DCs was continuously elevated in all three groups, reaching a 1.27-fold increase ( $p=0.050$ ) after 2 days of irradiation, and a 1.28-fold increase after 30 days ( $p=0.045$ ), when compared with the percentage of DCs before UV exposure. A particularly high increase in the percentage of MDC1 was observed after 10 days and 30 days of UV radiation (2-fold increase;  $p=0.010$  and 4-fold increase;  $p=0.00003$ ,

respectively). The other myeloid DC subtype, MDC2, showed a 1.20-fold increase after 10 days of UV exposure ( $p=0.047$ ), and a subsequent decrease after 30 days to 60% of the initial value ( $p=0.016$ ). The PDCs were increased after UV irradiation in all three groups, but these changes did not reach statistical significance

No statistically significant relationship between phototype and the response of the DC subsets to UV irradiation was found in any of the three groups. There was a significantly negative correlation between MED value and response of DCs to UVR after 30 days of exposure ( $R=-0.41$ ;  $p=0.029$ ).

The highest increase in BDCs following SSR is due mainly to the MDC1 and MDC2 myeloid subtypes of BDCs. A raised percentage of MDC1 was noted after 10 (2-fold) and 30 (4-fold) days of UV exposure. The percentage of MDC2 also increased after 10 days (1.2 –fold), then decreased after 30 days of irradiation to 60% of the pre-exposure value. The drop may be an indication of adaptation of the MDC2 subsets to the effects of UVR on BDCs or to a different response in this subset compared with the other DC subsets.

### **Pilot study**

After irradiation with 1.2 SED dose of CLEO lamps we obtained no immunosuppression, so that according to Progress Meeting held in April 2003 in Lodz, we decided to run pilot experiments. 4 healthy volunteers (age 27.8; 5 F) were irradiated with a dose of 1 personal MED Cleo for 2 consecutive days. After 24 hours they were DPCP sensitised with subsequent elicitation (after 3 weeks). The second pilot study included other 5 healthy volunteers (age 29.5, 1 M, 3 F) irradiated with a dose of 0.7 personal MED Cleo for 2 consecutive days with subsequent DPCP sensitization and elicitation. The mean dose of personal CLEO MED in both groups was 7.65 J/cm<sup>2</sup> (with distance 10 cm, intensity approx. 5mJ/cm<sup>2</sup>). In 1 person irradiated with a dose of 1.0 MED and in 1 – irradiated with 0.7 MED we found no CHS. Because of the lack of immunosuppression after CLEO irradiation we decided to change our protocols, according to suggestions during the Progress Meeting.

## **II. Study performed in autumn 2003 - spring 2004**

### **1. Recruitment of volunteers**

Recruitment of volunteers was accomplished by an announcement at the Medical University of Lodz and by advertising in local newspapers. The response resulted in over 600 phone calls or personal visits. After preliminary interviews, 120 healthy people without any skin or other diseases and not taking any medicines were included in the study. All of them had blood count and urinalysis performed according to the Local Ethical Committee advice and underwent thorough physical examination. After obtaining informed consent, the study procedures were started.

### **2. MED assessment**

Phototesting (UVB radiation) was made with Waldmann Medizintechnik UV 109 device with incremental doses series on six squares (1cm x 1 cm), on inner surface of forearm skin. MED was defined as a perceptible erythema at 24 hours and was determined for each volunteer.

### **3. New protocols**

The volunteers were divided into 4 groups (30 individuals by each group) exposed to irradiation in the following way:

- 1/ irradiation with UVB lamps 0.7 personal MED (whole body, 10 days) followed by 3 MED (buttock skin), followed by DPCP sensitization and elicitation
- 2/ irradiation with 3 MED with UVB (buttock skin 10x10 cm) followed by DPCP sensitization
- 3/ irradiation with UVB lamps 0.7 personal MED (whole body, 10 days) followed by DPCP sensitization and elicitation
- 4/ irradiation with CLEO (10 days, 1.2 SED) followed by UVB 3 MED (buttock skin) and DPCP sensitization

#### **Group 1- 30 individuals – preliminary results**

##### **Study protocol**

Before irradiation the following procedures were performed: UVB MED was established, blood samples for gene polymorphism analysis, differential cell count and dendritic cell phenotyping were collected, the pigmentation/erythema on the right buttock skin was measured. 24 hours after the final exposure of 10 days irradiation blood samples for differential cell count and dendritic cell phenotyping were collected, the pigmentation/erythema on the right buttock skin was measured, skin biopsy (buttock) was taken and a single exposure of 3 MED on the right buttock (10x10 cm) was performed. Next day blood samples for differential cell count and dendritic cell phenotyping were collected, the pigmentation/erythema on the right buttock skin was measured, skin biopsy (buttock) was taken and the DPCP sensitization discs were taped on the irradiated buttock. Three weeks later, the antigen challenge with DPCP was performed and, after 48 hours, erythema was measured and a biopsy from the challenge site was collected.

##### **Contact hypersensitivity analysis (CHS)**

Preliminary results (Group 1) based on the visual scoring scale indicate suppression of CHS response when compared to CLEO irradiated groups. In 16 out of 30 volunteers we found no visual reaction after the antigen challenge. In 4 out of 30 individuals only slight erythema was observed, in 4 - erythema and infiltration was found and in 5 - infiltration and papules or vesicles were noted. Only in one volunteer bullous reaction was observed. Statistical analysis and immunohistochemical analyses will be performed.

##### **Blood dendritic cell phenotype analysis**

Blood dendritic cell phenotype analysis is performed in the same way as previously.

The configuration of the antibodies is as follows:

BDCA-2+/CD123+/HLA-DR+

BDCA-1+/CD1c+/HLA-DR+

BDCA3+/CD86+/HLA-DR+

##### **Preliminary results (Group 1)**

The preliminary results from Group 1 are available. Before irradiation, the median percentage of particular DC subsets was as following: MDC1s- 0.34%, MDC2s –0.1%, PDCs-0.57% of PBMC. Percentages of DCs before and after irradiation are shown in Fig. 1.

After 10 days of 0.7 MED whole body irradiation with a subsequent irradiation of a single dose of 3 MED (buttock skin) a slight increase in MDC1 subtype was noted (median-0.39 and median-0.4, respectively), however not statistically significant. A statistically significant drop of percentage of MDC2s was found after 10 days of irradiation (median-0.08) and after 3 MED (median-0.05). PDC rate was also decreased both after 10 days and 3 MED irradiation (median-0.52 and median-0.47; respectively), but the differences were not statistically significant.

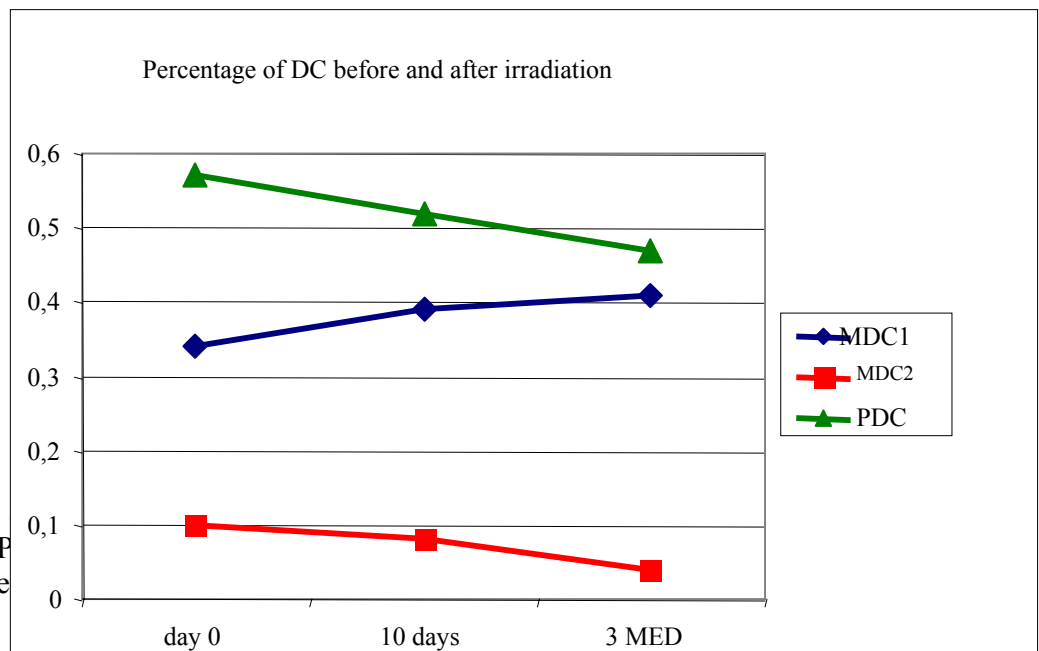
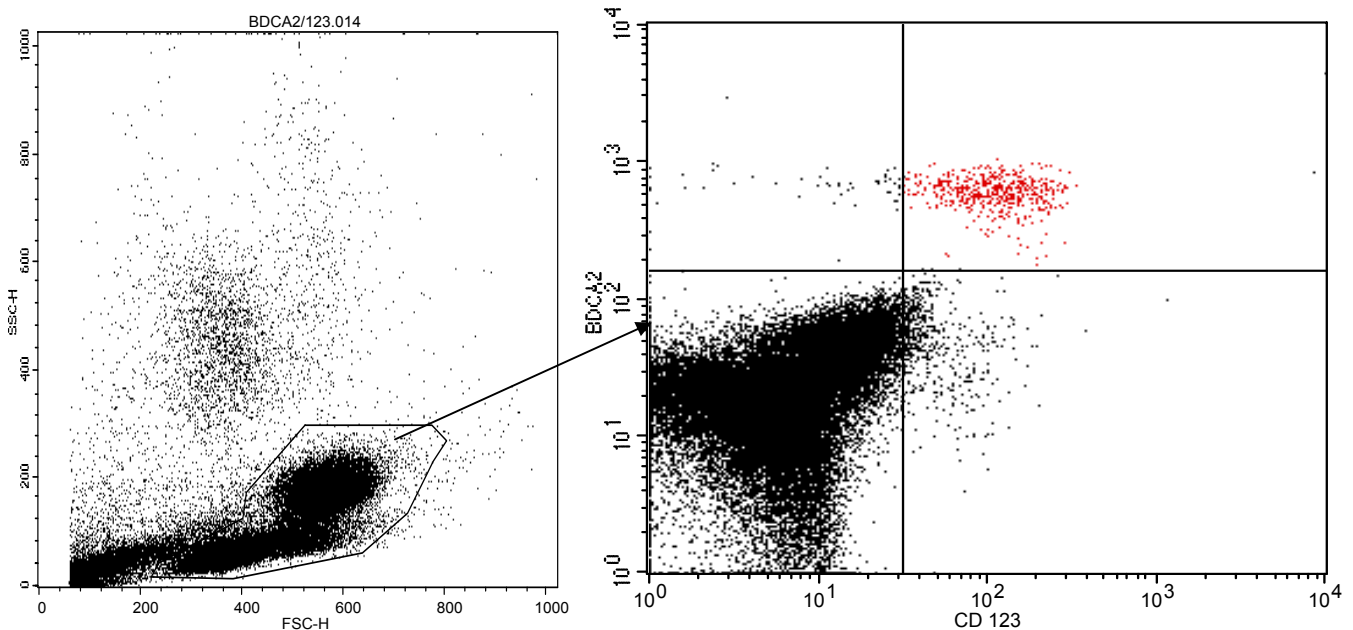


Figure 2 shows  
been made on P  
DCs (PDCs) we



In 2004 a detailed statistical analysis of contact hypersensitivity reaction after irradiation with SSR was performed in a group volunteers recruited in autumn 2002- spring 2003. The study group consisted of 165 healthy volunteers with either II or III skin phototype.

A hundred individuals were irradiated (whole body irradiation in two half-walls cabinets) in three groups, group 2 consisting of 33 people (17 women, 16 men; mean age 26.1 years) irradiated for 2 consecutive days, group 3 consisting of 34 (13 women, 21 men; mean age 24.3 years) irradiated for 10 consecutive days, and group 4 consisting of 33 (16 women, 17 men; mean age 26.7 years) irradiated for 30 consecutive days with a dose of 1.2 SED on each occasion.

In addition, 40 non-irradiated individuals (17 women, 23 men; mean age 25.0) were assessed as a control group (group 1). All the subjects from group 1 to 4 were sensitized using DPCP by the protocol described in progress 2002 and 2003.

For histological analysis, another 25 individuals (12 women, 13 men, mean age 25.4) who were neither irradiated nor sensitised acted as negative controls for the histology study. Their skin samples were taken from the same body site as in the other groups.

The CHS response was evaluated after 48 h by using a reflectance device, by histology and by a subjective visual scoring system where 0-no reaction, 1-macular erythema, 2-erythema with infiltration, 3-erythema with infiltration and papules or vesicles, and 4-bullous reaction.

Erythema and pigmentation were quantified using the UV Optimise 555 device. Each site of elicitation plus the control site was measured three times (groups 1-4). In addition measurements were taken before and after irradiation on the buttock skin in the UV-exposed groups (groups 2-4). The mean values of the three readings were calculated.

### Histological examination

*A 3mm-punch skin biopsy was taken from the 3.2 µg DPCP site in each subject 48 h after elicitation. Additionally, 3-mm punch skin biopsies were taken from the same skin site of the 25 non-sensitised, non-irradiated volunteers.*

In each group of the volunteers, the histological analysis included an assessment of the following parameters: total thickness of the epidermis (mean value obtained from the 10 serial sections), the presence of the intraepidermal vesicles and intensity of spongiosis in the 10 serial sections graded as 0-no spongiosis; 1-slight oedema, no intraepidermal vesicles; 2-oedema and single intraepidermal vesicles; and 3-severe oedema and multiple intraepidermal vesicles. The results obtained from the sensitized, irradiated individuals were compared with the sensitised, unirradiated individuals. The same parameters were examined in non-sensitised, non-irradiated volunteers.

### Statistical analysis

The statistical analysis of the data was performed using the Mann-Whitney test, Wilcoxon pair test,  $\chi^2$  test and non parametric correlation of Spearman, where necessary. A p value of less than 0.05 was considered as statistically significant.

## RESULTS

Statistical analysis of the erythema values measured with the UV Optimize 555 device revealed no significant differences before and after the final irradiation within each group or between the groups ( $p > 0.05$ ). Analysis of the pigmentation values before and after the last irradiation gave significant differences within each of the groups ( $p = 0.004$  for group 2,  $p = 0.003$  for group 3 and  $p = 0.001$  for group 4), but there were no differences between the groups ( $p > 0.05$ ).

PARs were detectable between 3-19 days after sensitisation. The response ranged from erythema with definite borders to strong erythema with oedema and blistering. The diameter varied between 11-25 mm. There were no statistically significant differences between groups 1-4 regarding the time point at which PAR first appeared and how long it lasted ( $p > 0.05$ ). A statistical analysis of the visual assessment of the CHS revealed no difference between any of the groups at any of the DPCP concentrations. The sum clinical score obtained from adding together the CHS response in each of the 4 groups at all 4 DPCP concentrations also showed no difference ( $p > 0.05$ ). However, when the CHS score was simplified by treating it as a binomial trait where 0 represents no reaction and 1 represent a response, differences between the groups were found. After using Spearman statistics, a significant negative correlation ( $R = -0.2$ ;  $p = 0.015$ ) was seen between the CHS score and the number of days of irradiation when 0.4 µg DPCP was used as the challenge dose. Although similar trends were found for the other three DPCP concentrations used, the differences between the groups were not statistically significant.

### CHS assessed by erythema and pigmentation

There were higher erythema values in response to the elicitation doses of 0.4, 0.8, 1.6 and 3.2 µg DPCP compared with the acetone site in groups 2 and 3 ( $p < 0.05$ ) and in response to 0.8, 1.6 and 3.2 µg DPCP compared with the acetone site in group 4. However when the erythema values following elicitation were compared between groups 1-4, no statistically significant differences at any DPCP concentration were found. Similarly no

significant differences between the groups regarding pigmentation were revealed ( $p > 0.05$ ).

CHS assessed by histology

The mean value for the thickness of the epidermis in the 25 healthy subjects who had not been UV-irradiated or sensitised was 0.061 mm. In groups 1-4, the mean thickness was 0.207, 0.166, 0.163 and 0.108 mm respectively following elicitation with a dose of DPCP of 3.2  $\mu\text{g}$ . There was a statistically significant difference in epidermal thickness between group 1 and each of groups 2-4 ( $p < 0.05$ ). The mean thickness of the epidermis correlated positively with the sum score of the CHS response to all four of the DPCP concentrations, as assessed visually, in all 4 groups

The highest intensity of spongiosis was observed in the group 1 subjects (unirradiated and sensitised). When the number of days of SSR and the spongiosis score were treated as continuous variables, a negative correlation between these 2 factors was revealed ( $R = -0.28$ ,  $p < 0.001$ ). In addition a significant correlation between the intensity of spongiosis and the clinical score for the CHS in response to 3.2  $\mu\text{g}$  DPCP was found ( $p < 0.000001$ ), and between the intensity of spongiosis and the sum clinical score for all the DPCP concentration was observed ( $p < 0.00004$ ). When the spongiosis score was treated as a binomial trait, where 0=no spongiosis and 1=any reaction, differences were seen between the number of individuals with no reaction and the others. A significant negative correlation was found between the spongiosis intensity (0 or 1) and the number of days of SSR ( $R = -0.2$ ,  $p = 0.01$ ).

Based on the results we conclude that small daily doses of SSR induce suppression of CHS in human subjects and the effect is cumulative, indicating that there is no adaptation to the immunomodulating effects of UVR, at least over the test period of 30 days.

### **Study performed in volunteers recruited in autumn 2003**

Recruitment of volunteers was accomplished by an announcement at the Medical University of Lodz and by advertising in local newspapers. The response resulted in over 600 phone calls or personal visits. After preliminary interviews, 120 healthy people without any skin or other diseases and not taking any medicines were included in the study. All of them had blood count and urinalysis performed according to the Local Ethical Committee advice and underwent thorough physical examination. After obtaining informed consent, the study procedures were started.

#### **Study protocol**

Before irradiation the following procedures were performed: UVB MED was established, blood samples for gene polymorphism analysis, differential cell count and dendritic cell phenotyping and for assessment of serum concentration of selected cytokines were collected, the pigmentation/erythema on the right buttock skin was measured, skin samples (buttock) were taken. 24 hours after the final irradiation blood samples for differential cell count and dendritic cell phenotyping as well as for assessment of serum concentration of chosen cytokines were collected, the pigmentation/erythema on the right buttock skin was measured, skin biopsy (buttock) was taken and a single exposure of 3 MED on the right buttock (10x10 cm) was performed. Next day blood samples for differential cell count and dendritic cell phenotyping and for cytokines' concentrations were collected, the pigmentation/erythema on the right buttock

skin was measured, skin biopsy (buttock) was taken and the DPCP sensitization discs were taped on the irradiated buttock. Three weeks later, the antigen challenge with DPCP was performed and, after 48 hours, erythema was measured and a biopsy from the challenge site was collected.

The volunteers were divided into 5 groups exposed to irradiation in the following way:

1/ irradiation with UVB lamps 0.7 personal MED (whole body, 10 days) followed by 3 MED (buttock skin), followed by DPCP sensitization and elicitation – Group A

2/ irradiation with UVB lamps 0.7 personal MED (whole body, 10 days) followed by DPCP sensitization and elicitation – Group B

3/ irradiation with CLEO Natural lamps (10 days, 1.2 SED) followed by UVB 3 MED (buttock skin), followed by DPCP sensitization and elicitation – Group C

4/ irradiation with 3 MED with UVB (buttock skin 10x10 cm) followed by DPCP sensitization and elicitation – Group D

5/irradiation with 4 MED of UVB (buttock skin 10 x 10 cm) followed by DPCP sensitization and elicitation – Group E

Clinical characteristics of all the volunteers recruited in 2003/2004 is shown in Table 1

Group	No of subjects	Gender F/M	Mean age	Phototype II/III	Mean MED (J/cm2)
A	30	16/14	27.8	18/12	0.15
B	30	19/11	30.0	14/16	0.16
C	30	17/13	24.7	15/15	0.16
D	30	16/14	29.4	11/19	0.15
E	10	4/6	30.6	5/5	0.15

### 1. Contact hypersensitivity analysis (CHS)

The study group consisted of 165 healthy volunteers with either II or III skin phototype. DPCP sensitization and elicitation was performed exactly the same as described above. The CHS response was evaluated after 48 h by using a reflectance device (UV Optimise 555 device), by histology and by a subjective visual scoring system where 0-no reaction, 1-macular erythema, 2-erythema with infiltration, 3-erythema with infiltration and papules or vesicles, and 4-bullous reaction.

Statistical analysis concerning intensity of CHS response in all the groups when compared to the controls (Table 7) was performed using chi2 test with Yeats correction (df=1) or Fischer's exact test. In statistical analysis CHS response was treated by

binominal trait. In all the examined groups we found statistically significant differences. Detailed results of CHS response are shown in Tables 2-7.:

Table 2. Group A 0.7 MED of UVB (10 days) + 3 MED UVB (single)

CHS Score	Titration of DPCP			
	0.4	0.8	1.6	3.2
0	21/30 (73.3%)	17/30 (56.6%)	16/30 (53.3%)	16/30 (53.3%)
1	2/30 (6.6%)	4/30 (13.3%)	5/30 (16.6%)	4/30 (13.3%)
2	6/30 (20%)	5/30 (16.6%)	5/30 (16.6%)	4/30 (13.3%)
3	1/30 (3.3%)	3/30 (10%)	3/30 (10%)	5/30 (16.6%)
4	0/30 (0%)	1/30 (3.3%)	1/30 (3.3%)	1/30 (3.3%)
Statistical analysis	p=0.0002	p=0.0002	p=0.0002	p=0.0002

Table 3. Group B 0.7 MED of UVB (10 days)

CHS Score	Titration of DPCP			
	0.4	0.8	1.6	3.2
0	20/30 (66.6%)	19/30 (63.3%)	18/30 (60%)	12/30 (40%)
1	8/30 (26.6%)	4/30 (13.3%)	2/30 (6.6%)	6/30 (20%)
2	2/30 (6.6%)	4/30 (13.3%)	4/30 (13.3%)	4/30 (13.3%)
3	0/30 (0%)	3/30 (10%)	6/30 (20%)	6/30 (20%)
4	0/30 (0%)	0/30 (0%)	0/30 (0%)	2/30 (6.6%)
Statistical analysis	p=0.0065	p<10 <sup>-5</sup>	p<10 <sup>-5</sup>	p=0.0076

Table 4. Group C 1.2 SED SSR (10 days) + 3 MED UVB

CHS Score	Titration of DPCP			
	0.4	0.8	1.6	3.2
0	22/30 (73.3%)	20/30 (66.6%)	16/30 (53.30%)	15/30 (50%)
1	2/30 (6.6%)	2/30 (6.6%)	5/30 (16.6%)	4/30 (13.3%)
2	5/30 (16.6%)	6/30 (20%)	3/30 (10%)	5/30 (16.6%)
3	1/30 (3.3%)	2/30 (6.6%)	6/30 (20%)	5/30 (16.6%)
4	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)
Statistical analysis	p=0.0001	p<10 <sup>-5</sup>	p=0.0002	p=0.0006

Table 5. Group D 3 MED UVB

CHS Score	Titration of DPCP			
	0.4	0.8	1.6	3.2
0	20/30 (80%)	18/30 (60%)	16/30 (53.3%)	15/30 (50%)
1	1/30 (3.3%)	5/30 (16.6%)	4/30 (13.3%)	1/30 (3.3%)
2	2/30 (6.6%)	3/30 (10%)	5/30 (16.6%)	7/30 (23.3%)
3	2/30 (6.6%)	4/30 (13.3%)	4/30 (13.3%)	6/30 (20%)
4	1/30 (3.3%)	0/30 (0%)	1/30 (3.3%)	1/30 (3.3%)
Statistical analysis	p=0.0005	p=0.0001	p=0.0008	p=0.0006

Table 6. Group E 4 MED UVB

CHS Score	Titration of DPCP			
	0.4	0.8	1.6	3.2
0	9/10 (90%)	9/10 (90%)	7/10 (70%)	7/10 (70%)
1	1/10 (10%)	1/10 (10%)	1/10 (10%)	1/10 (10%)
2	0/10 (0%)	0/10 (0%)	1/10 (10%)	1/10 (10%)
3	0/10 (0%)	0/10 (0%)	1/10 (10%)	1/10 (10%)
4	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
Statistical analysis	p=0.0002	p<10 <sup>-5</sup>	p=0.0003	p=0.0003

Table 7. Controls n=40

CHS Score	Titration of DPCP			
	0.4	0.8	1.6	3.2
0	9/40 (22.5% )	5/40 (12.5%)	4/40 (10%)	4/40 (10% )
1	20/40 (50%)	10/40 (25%)	3/40 (7.5% )	2/40 (5%)
2	5/40 ( 12.5%)	19/40 ( 47.5 %)	16/40 (40% )	10/40 (25%)
3	5/40 ( 12.5%)	5/40 (12.5%)	12/40 (30%)	18/40 (45% )
4	1/40 (2.5% )	1/40 (2.5% )	5/40 (12.5% )	6/40 (15% )

**CONCLUSIONS:**

- 1/ Irradiation with low chronic doses of UVB (whole body) causes suppression of CHS response to DPCP (in all concentrations)
- 2/ single local exposure to 3 MED and 4 MED of UVB causes suppression of CHS response to DPCP (in all concentrations), more distinct in 4 MED group
- 3/ Irradiation with low chronic doses of UVB (whole body) gives no adaptation (CHS response in group A is almost the same as in group B and D)
- 4/ Irradiation with low chronic doses of SSR (whole body, 10 days) does not adapt human organisms against acute doses of UVB- (CHS response in group C is almost the

same as in group A and group D). It is consistent with our previous data in which 10-day irradiation with 1.2 SED of SSR did not have immunosuppressive effect.

Histological analysis and analysis of erythema and pigmentations values are being performed.

## **2. Blood dendritic cells**

The study included 130 healthy volunteers aged 18-55, with either II or III skin phototype from groups A-E (described above).

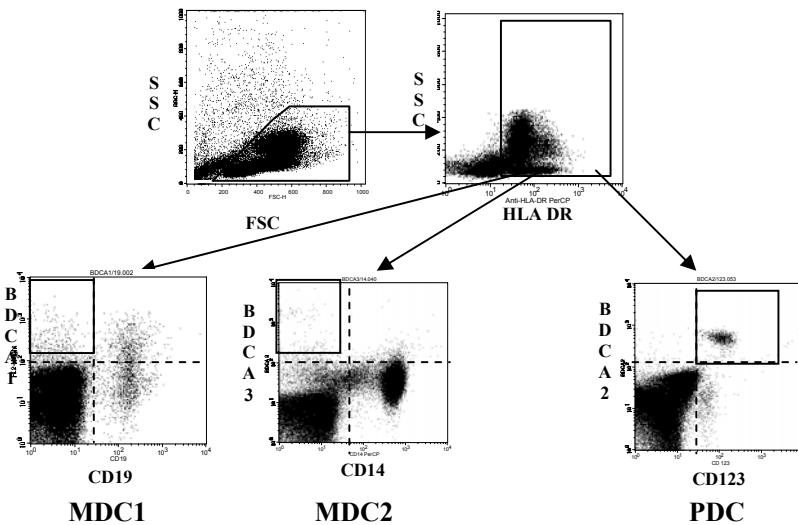
### **Blood collection**

In all the individuals from group A and C blood samples were taken three times (before the phototesting, 24 hours after 10<sup>th</sup> exposure to UVB or SSR and 24 hours after local 3 MED UVB irradiation). In the subjects from group B, D and E blood samples were taken twice (before phototesting, 24 hours after 10<sup>th</sup> exposure in group B, and 24 hours after local 3 MED UVB irradiation in group D and E).

### **Immunophenotyping**

This was determined using a panel of monoclonal antibodies (MoAbs) directed against several DC-defined antigens: FITC-conjugated BDCA-1, BDCA-2, and BDCA-3 (Miltenyi Biotec, Bergish Gladback, Germany), PE-conjugated CD11c, CD32 (Caltag Laboratories, Burlingame, CA, USA) and CD123 (Miltenyi Biotec, Bergish Gladback, Germany), as well as TC-conjugated HLA-DR (Caltag Laboratories, Burlingame, CA, USA). In parallel, MoAbs against lineage antigens CD14 and CD19 (Becton-Dickinson, San Jose, CA, USA) were also used. As an isotype control, the respective mouse IgG1 MoAbs were used (all Caltag Laboratories, Burlingame, CA, USA). The immunophenotype was assessed by triple-color flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA) and analysed with green (FL1), orange (FL2) or red (FL3) standard emission filters. From each sample 100.000 events were acquired.

Based on side scatter (SC) vs. forward scatter (FS), and then appropriate antigens pattern BDC were gated from the whole PBMC population (Fig. 1). The percentage of BDC was determined by flow cytometry. The three main subpopulations of DC were distinguished by their expression profile: two myeloid types of DC: MDC1 (BDCA-1+/CD11c+/HLA-DR+/CD14-) and MDC2 (BDCA-3+/CD32-/HLA-DR+/CD19-). The third, plasmacytoid supopulation, PDC, was discriminated based on BDCA-2+/CD123+/HLA-DR+ expression. Details of the identification of the BDC types by flow cytometry are shown in Fig. 1.



S  
S  
C

FIG. 1

### Statistics

For the statistical analysis, the medians and the range of values (min-max) were calculated. The nonparametric Wilcoxon pair test was used for comparison of the results before and after irradiation. The correlation between features was evaluated using the Spearman R test. Comparisons and correlations were considered significant when  $p < 0.05$ .

### RESULTS

Analyzing all BDC in volunteers irradiated chronically with various sources of UV we observed an increase in their median amounts, however, differences were not statistically significant. After local 3 MED irradiation we observed no changes in BDC rate. Higher dose applied locally (4 MED), however, caused a 1.17-fold increase of BDC rate (statistically insignificant,  $p = 0.386$ ). In groups A and C, after additional irradiation with acute dose of UVB a drop in median BDC rate was observed (decrease to 0.90- and 0.89-fold, respectively).

MDC1 subtype also was both elevated after 10 days irradiation, irrelevant from UV source (1.20-fold in group A, 1.14-fold in group B and 1.24-fold in group C) and after high local 4 MED (1.11-fold increase) (all  $p > 0.5$  in pair test). After a single 3 MED irradiation MDC1 number did not change and after the same dose applied subsequently after chronic irradiation (group A and C) their median rate decreased from 0.77 to 0.74% in group A and from 0.47% to 0.36% in group C ( $p > 0.5$  in pair test).

MDC2 subtype showed a tendency to rise after chronic irradiation, in group A and B an increase was statistically significant ( $p = 0.036$  and  $0.002$ , respectively). The median rate of MDC2 did not change under local high doses, neither under 3 MED nor 4 MED. Additional exposure to a dose of 3 MED, applied 24 hours after a final low-dose irradiation caused a drop in MDC2 subtype (for group A and C,  $p = 0.063$ ,  $p = 0.241$ , respectively), however these values were not statistically significant.

PDC number alterations were not statistically significant as well but the tendency to increase was observed. After chronic irradiation with either UVB or SSR the median rates of PDC were moderately increased from 0.74% to 0.77% in group A and from 0.52% to 0.69% in group B ( $p = 0.076$  and  $p = 0.067$  in pair comparison, respectively). In

contrast to the results given above, PDC were also elevated after a single exposure of 3 MED (1.16-fold increase) and 4 MED (1.42-fold increase) (for both  $p > 0.5$  in pair test). Irradiation with 3 MED after chronic SSR exposure (group C) caused a decrease in median PDC rate from 0.70% to 0.66% ( $p > 0.5$  in pair test). In group A (chronic UVB) PDC number was not altered by additional acute UVB dose.

### 3. Serum cytokine concentrations – before and after irradiation

Thus, the aim of our study was to compare serum concentration of IL-1  $\beta$ , IL-6, IL-8, IL-10 and TNF- $\alpha$  in human healthy volunteers before and after exposure to different schedules of UV irradiation, mainly low, chronic doses. Our experiment was also designed to show whether repeated exposure to low, everyday doses of UV exerts an adaptative effect for consecutive single high (immunosuppressive) dose of UVB.

The study included healthy 115 volunteers from Groups A-E (described above). Clinical characteristics is presented in Table:

Group	No of subjects	Gender F/M	Mean age	Phototype II/III	Mean MED (J/cm <sup>2</sup> )
A	30	16/14	27.7	8/22	0.15
B	25	12/13	31	12/13	0.16
C	25	13/12	25	14/11	0.14
D	25	13/12	29.5	16/9	0.14
E	10	3/7	30.6	5/5	0.15

In all the individuals from group A and C blood samples were taken three times (before the phototesting, 24 hours after 10<sup>th</sup> exposure to UVB or SSR and 24 hours after local 3 MED UVB irradiation). In the subjects from group B, D and E blood samples were taken on two occasions (before phototesting, 24 hours after 10<sup>th</sup> exposure in group B, and 24 hours after local 3 and 4 MED UVB irradiation in group D and E). After centrifugation all serum samples were stored at -70°C until analysed.

Serum samples were analysed for IL-1  $\beta$ , IL-6, IL-8, IL-10 TNF- $\alpha$  concentration with chemiluminescence assay (Diagnostic Products Corporation, Los Angeles, USA) according to manufacturer's instructions. Sensitivity and reference range for IL-1 beta, IL-6, IL-8, IL-10, TNF- $\alpha$  were: 5.0 pg/ml, 0-5 pg/ml; 2.0 pg/ml, 0-9.7 pg/ml; 5.0 pg/ml, 0-62 pg/ml; 1.5 pg/ml, 0-9.1 pg/ml; 4.0 pg/ml, 0-8.1 pg/ml, respectively.

For the statistical analysis, the medians and the range of values (min-max) were calculated. The ANOVA Friedman test and nonparametric Wilcoxon pair test were used for comparison of the results before and after irradiation. The correlation between features was evaluated using the Spearman R test. Comparisons and correlations were considered significant when  $p < 0.05$ .

IL-1  $\beta$  concentrations were below detectable levels in all (115/115) volunteers, both before and after UVB or SSR irradiation.

IL-6 was detectable in 20 out of 115 individuals before irradiation and its concentration increased slightly after UV exposure (in each assessed group). However, no statistical correlation between UV exposure and IL-6 level was found.

IL-8 levels were detectable before irradiation in 36 out of 115 volunteers. Only in group A and B we found statistically significant ( $p < 0.05$ ) differences in IL-8 level before and after 10 days of UVB irradiation. Local acute UVB irradiation which was additionally applied in group A did not cause any significant changes in this cytokine level. No changes in IL-8 level were observed in groups D and E in which only local acute doses of UVB were applied. In opposite to chronic low doses of UVB, repeated irradiation with SSR (group C) did not lead to any alterations in IL-8 serum concentration.

In group A IL-10 was elevated before UVB irradiation (15.5 pg/ml), only in one volunteer but its level was much higher after 10 day UVB exposure (29.8 pg/ml) and undetectable after the additional 3 MED irradiation. The same tendency was observed in group B also in one individual before irradiation the level was 12.0 pg/ml and after 10 days repeated exposure - 14.5 pg/ml. In group C two subjects had elevated IL-10 levels before irradiation, however a 10-day SSR exposure did not cause any increase of the interleukin level. In group D and E no influence of an acute single dose of UVB on serum level of this cytokine was observed.

TNF- $\alpha$  serum level was detected in 97 out of 115 volunteers. Only in group A and B a slight tendency to its increase after 10 days irradiation was observed ( $p = 0.05$ ,  $p > 0.05$ ; respectively). Local acute UVB doses and repeated SSR irradiation did not change this cytokine serum concentration.

In our study no significant alterations in serum cytokines' levels, apart from IL-8 level, were found. Based on our results, obtained with commercially available chemiluminescence assay, we suggest that those schedules of irradiation have no impact on examined cytokines' serum levels.

#### **4. Assessment of IL-1 beta, IL-6, IL-8, IL-10 and TNF-alpha mRNA levels in UV-irradiated human skin in vivo by relative quantitative reverse transcription PCR assays of mRNA**

Skin biopsies for assessment of mRNA of selected cytokines were taken from:

- 10 healthy individuals (unirradiated, unsensitised) – controls
- Group A – 15 individuals (biopsy taken from buttock skin 24 h after 10 days of 0.7 MED of UVB irradiation and 24 h after 3 MED)
- Group C – 15 individuals (biopsy taken from buttock skin 24 h after 10 days of 1.2 SED SSR irradiation and 24 h after 3 MED)
- Group D – 15 individuals (biopsy from buttock skin taken before and 24 h after 3 MED. Irradiation)
- Group 3 - 15 individuals irradiated for 30 days with SSR (1.2 SED daily) (biopsy from buttock skin taken before and 24 h after final irradiation)

#### **Relative quantitative reverse transcription PCR assays of mRNA**

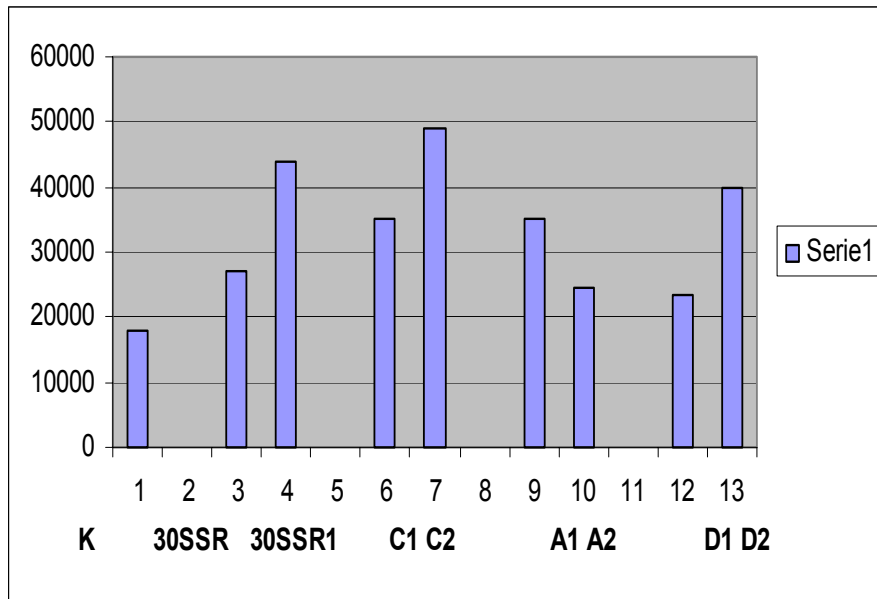
Samples (50 mg) of skin tissue were dissolved in the Fenzol reagent and used to purify total mRNA by a total RNA Prep Plus Kit (A&A Biotechnology). The first strand of cDNA was synthesized according to the manufacturer's instruction using (dT)20 primer. The reverse transcription was carried out for 60 min at 30 °C and stopped at 0 °C.

Then, mRNAs for TNF $\alpha$ , IL-10, IL-6, IL-8, and IL-1 $\beta$  were determined by relative quantitative RT PCR using actin mRNA as an internal standard RT PCR. The following oligodeoxyribonucleotide primers were used: 5'gtagcccatgtttagcaaacc3' and 5'gaggacctgggagtagatgagg3', 5'gagaacagctgcaccacttcc3' and 5'ctgggtcttgggttctcagcttgg3', 5'acctgaaccttccaaagatgg3' and 5'gactgcaggaactccttaaagc3', 5'tctgcagctctgttgaagg3' and 5'cttctccacaacctctgc3', 5'ctcgcagtgaaatgatgg3' and 5'gcatttctcagcttgc3', respectively. RT PCR assay conditions were as follows: 5  $\mu$ l cDNA sample, 10x Taq polymerase buffer (Epicentre Technologies), 2 mM MgCl<sub>2</sub>, 1x PCR enhancer, dNTP mix, 25 pmol of each primer and 1.25 U of Taq DNA polymerase (Epicentre Technologies) in 50  $\mu$ l reaction volume. The samples were denatured at 95 °C for 5 min, then cooled on ice and, then the enzyme was added. The reaction was performed in twenty five to thirty extension cycles consisting of: 30 s denaturation step at 94 °C, a 30 s annealing step at 60 °C, and a 40 s polymerase extension step at 72 °C. Finally, each reaction was terminated with a 10 min elongation step at 72 °C. To detect actin mRNA, the same protocol was used with oligodeoxyribonucleotide primers 5'cagcagattcaagcagctatgg3' and 5'gtctgtggtgctgatctcatcc3'. The final products, TNF $\alpha$ , IL-10, IL-6, IL-8, and IL-1 $\beta$  mRNA and actin mRNA, were separated by electrophoresis in 7% polyacrylamide gels in TAE buffer using the genetic size marker 100 bp DNA Ladder (Promega). Bands were visualized by UV light, the results were recorded photographically and analyzed densitometrically using LKB Ultrascan XL Enhanced Laser Densitometr. Concentrations of mRNAs for tested cytokines were normalized in each sample in relation to actin mRNA.

Analysis of mRNA expression of IL-8 and IL-1 $\beta$  is being performed.

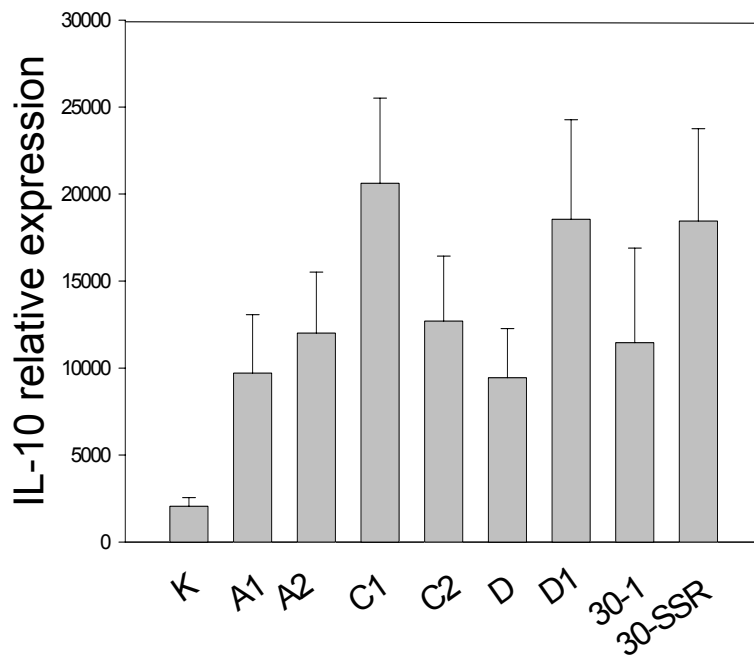
#### **TNF-alpha relative expression**

We found an increase in the expression of mRNA of TNF-alpha in all groups before and after irradiation (for each group differences were statistically significant, p<0.05). Both chronic and low doses of UVB or SSR and acute dose 3 MED of UVB cause statistically significant changes in skin expression of this cytokine  
3 MED applied after 10 days of 0.7 MED of UVB caused a decrease in TNF-alpha mRNA expression (p<0.05)



**IL-10 relative expression**

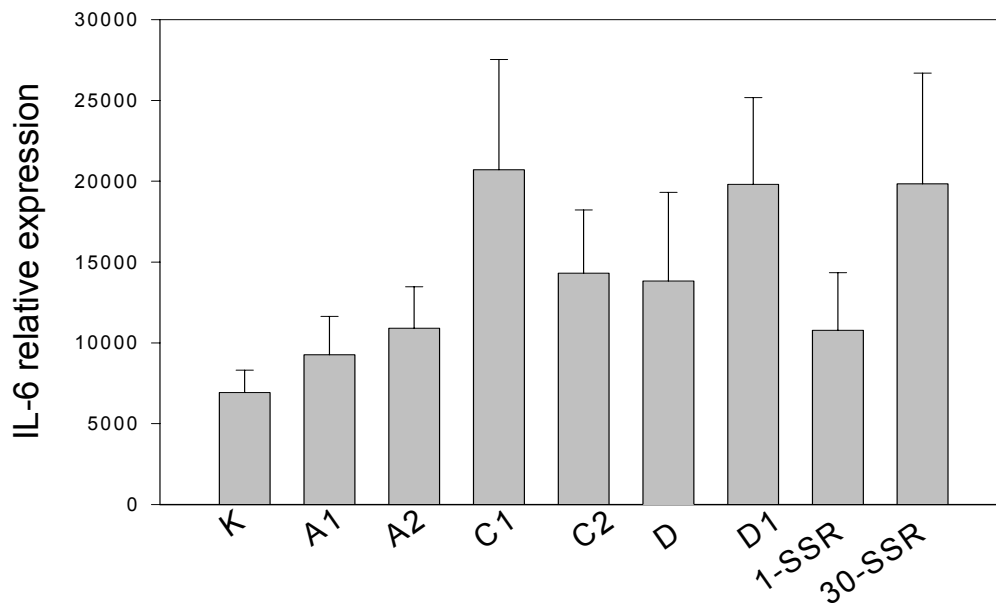
We found an increase in the expression of mRNA of IL-10 in all groups before and after treatment with sublethal doses of UVB or this cytokine, assessed 7 days of 1.2 SED of SSR



### IL-6 relative expression

An increase in the expression of mRNA of IL-6 in all groups before and after irradiation was found ( $p < 0.05$ ).

A dose of 3 MED applied after 10 days of irradiation with 1.2 SED of SSR caused a decrease in IL-6 mRNA expression ( $p < 0.05$ )



These preliminary results confirm the lack of adaptation to immunomodulating effects mediated by high doses of UVR.

### 5. Gene polymorphism: TNF-alpha and IL-1 beta

Immunogenetic study comprised 234 volunteers (blood samples taken before irradiation) and 150 healthy unrelated subjects (controls).

Three known dimorphic sites within *IL-1B* gene: T→C (1903, AluI) within the promotor region, G→A (5810, ItaI) within intron 4 and C→T (5887, TaqI) within exon 5 and polymorphic site within *TNFA* gene: G→A (at position -308) were analyzed using RFLP-PCR technique

Statistical analysis of *TNFA* and *IL-1B* polymorphic sites in volunteers and controls were compared using chi2 (df=2) with Yates' correction. Values of  $p < 0.05$  were considered statistically significant.

We analysed correlation between any polymorphism and phototype/photosensitivity (MED)

No significant differences in -308 *TNFA* polymorphism distribution between the volunteers and controls were observed and no correlation with photosensitivity. Significant differences were found in TagI polymorphism (C → T) within *IL-1B* gene.

Genotype TT was statistically more frequent in individuals with phototype II ( $p=0.019$ ) than with individuals with phototype III.

Allele T carriers were more frequent in the individuals with phototype II ( $p=0.02$ ) and in those with lower MED value ( $p=0.000009$ )

CT genotype and TT genotype were statistically more frequent within individuals with lower MED values ( $p=0.0002$  and  $p=0.0139$ , respectively)

## **Progress report 2005 WP 4 and 5**

### **1. Contact hypersensitivity response analysis**

#### **A/ SSR-irradiated groups**

In the present study, healthy individuals, divided into four groups each consisting of approximately 34 subjects, were whole-body irradiated with 1.2 standard erythema doses of SSR for 2, 10 or 30 consecutive days, or were unirradiated. They were sensitised with DPCP on one exposed body site 24 h after the final UVR. The occurrence and severity of the primary allergic response were noted, and both parameters were shown to be significantly lowered in the group irradiated for 30 days compared with the unirradiated group. Elicitation of CHS was undertaken 3 weeks after the sensitisation, using a range of concentrations of DPCP on a UV-protected body site. The extent of the CHS at 48 h was assessed by the clinical score, by an erythema meter and by histological examination of a biopsy taken from the site challenged with one selected concentration of DPCP (3.2  $\mu\text{g}$ ). Although erythema and pigmentation did not differ between the groups, a significant negative correlation was found between the clinical CHS score and the number of days of UV exposure, at the lowest challenge dose of DPCP. In addition a significant negative correlation was revealed between the intensity of spongiosis (intraepidermal oedema and vesicles, as evaluated by histology) and the number of days of UV exposure. Thus small daily doses of SSR induce suppression of CHS in human subjects and the effect is cumulative, indicating that there is no adaptation to the immunomodulating effects of UVR, at least over the test period of 30 days.

#### **B/ UVB-irradiated groups**

The study group consisted of 165 healthy volunteers with either II or III skin phototype. DPCP sensitization and elicitation was performed exactly the same as described above. The CHS response was evaluated after 48 h by a subjective visual scoring system where 0-no reaction, 1-macular erythema, 2-erythema with infiltration, 3-erythema with infiltration and papules or vesicles, and 4-bullous reaction.

Statistical analysis concerning intensity of CHS response in all the groups when compared to the controls was performed using  $\chi^2$  test with Yeats correction ( $df=1$ ) or Fischer's exact test. In statistical analysis CHS response was treated by binominal trait. In all the examined groups we found statistically significant differences ( $p<0.001$ ).

We found that irradiation both with low chronic doses of UVB (whole body, 0.7 MED) and with a single local 3 MED and 4 MED of UVB cause suppression of CHS response to DPCP in all concentrations (for all  $p < 0.001$ ), however the most distinct in 4 MED group.

Repeated irradiation with low doses of UVB gives no adaptation.

Irradiation with low chronic doses of SSR (whole body, 10 days) does not adapt human organisms against acute doses of UVB. It is consistent with our previous data in which 10-day irradiation with 1.2 SED of SSR did not cause immunosuppression.

## **2. Blood dendritic cell phenotype analysis**

### **A/ Healthy population**

The aim of the study was to assess a normal profile of BDCs in non cultured human blood of Polish healthy volunteers. BDCs were detected among peripheral blood mononuclear cells (PBMC) from 99 healthy persons, aged 18-56 years. Based on the panel of novel anti-BDCA1, BDCA2 and BDCA3 monoclonal antibodies (MoAbs) three main subpopulations of BDCs were distinguished: two myeloid types of BDCs - MDC1 (BDCA-1+/CD11c+/HLA-DR+) or MDC2 (BDCA-3+/CD32-/CD64-/HLA-DR+) and plasmacytoid subtype, PDC (BDCA-2+/CD123+/HLA-DR+). The number and percentage of BDCs were correlated with age, gender, photosensitivity (phototype, minimal erythema dose-MED) and morphological parameters of healthy volunteers. BDCs represented 0.83% of the PBMC and the median total BDCs number was 44.0 cell/ $\mu$ l. Total BDCs number correlated with WBC count ( $\rho = 0.40$ ;  $p < 0.001$ ) as well as lymphocyte and monocyte counts ( $\rho = 0.20$ ;  $p = 0.045$  and  $\rho = 0.26$ ;  $p = 0.009$ , respectively). The median percentage of MDC1 count (0.20%) was twice higher than MDC2 count (0.10%). The median PDC count was 28.2 cell/ $\mu$ l, and these cells represented 0.50% of the PBMC. There was a positive correlation between PDC and the skin photosensitivity ( $\rho = 0.28$ ;  $p = 0.005$ ). An inverse correlation between PDC count and age of examined volunteers was also found ( $\rho = -0.22$ ;  $p = 0.029$ ). In conclusion, this was the first study on absolute number of BDC subsets in healthy Polish population, revealed by flow cytometry, and defined by the novel anti-BDCA MoAbs panel. Our study provides the first referential data on normal rates and counts of BDCs and their subpopulations, assessed by the new panel of anti-BDCA MoAbs, in healthy Polish subjects. The method used in the study allowed determining BDC and their subsets number in relatively small blood volume. The knowledge of normal values of BDCs may be helpful in detecting any immune disturbances in young people and creating normal range of BDCs in healthy subjects.

### **B/ SSR-irradiated groups**

Healthy volunteers (n=94) were irradiated with a dose of 1.2 SED (standard erythema dose) of SSR for 2, 10 or 30 consecutive days. Blood samples were taken before the first exposure and 24 hours after final exposure. The three main subsets of BDCs were distinguished by flow cytometry: BDCA-2+/CD123+/HLA-DR+ (plasmacytoid, PDCs), and two myeloid subtypes: BDCA-1+/CD11c+/HLA-DR+ (MDC1s) and BDCA3+/CD32-/HLA-DR+ (MDC2s). The percentages and absolute numbers of DCs and their subsets were calculated.

The percentage of total DCs was elevated in all groups by the UV exposure, and was increased 1.27 fold after 2 days of irradiation and 1.28-fold after 30 days ( $p=0.006$  and  $0.018$ , respectively). A particularly distinct, approximately 2-fold increase was observed in the percentage of the MDC1s after 2 and 30 days ( $p=0.022$  and  $p<0.0001$ , respectively). The MDC2s showed an increase after 10 days (1.20-fold;  $p=0.135$ ), and a subsequent significant decrease after 30 days irradiation (0.6-fold;  $p=0.031$ ). PDC subset increased after 2 days of SSR ( $p=0,0006$ ) and later came back to baseline values (after 30 days). In conclusion, we found that exposure to SSR induced an increase in the percentage of BDCs in healthy human individuals, especially apparent in the MDC1 subtype.

### **C/ UVB-irradiated groups**

All the BDC and the 3 subsets, PBC, MDC1 and 2, were analysed in the blood before and after the 10 days of suberythemal UVB whole-body exposures and after the local 3 MED UVB exposure. It can be seen that, generally, the UVB did not induce significant changes in the percentages of circulating BDC or the subsets. The one exception was for the MDC2 subset where there was a significant increase following the 10 days of chronic UV-B irradiation. It should be noted that the acute local UVB dose did not lead to a change in this subset. The chronic UVB exposure also tended to increase the percentage of PDC in 10-day irradiated groups although this was not statistically significant ( $p=0.067$  and  $p=0.076$  in pair comparison respectively).

### **3. Blood dendritic cells in skin**

Immunofluorescent staining of skin samples for BDCA-1, 2, 3 and 4 revealed only a few positive cells. The BDC were located both in the epidermis and dermis. Myeloid subtypes (BDCA-1 and -3) were observed mainly in the middle layers of the epidermis and it was estimated that there were 5-7 cells in the epidermis (one specimen); single positive cells were also found in the upper part of the dermis. PDC (BDCA-2 and BDCA-4) were detected mainly in the dermis with the number being about 3-4 cells in whole specimen, although single BDCA-2 cells were present in the epidermis on occasion. No difference in the number and distribution of any of the subsets was revealed when sections taken before and after UVR were compared, irrespective of whether the UV-B was repeatedly suberythemal or given as a single erythemal dose.

### **3. Serum cytokine concentration**

The study included 105 healthy volunteers, aged between 18 and 55 years with either II or III skin phototype.

In the subjects from group irradiated for 10 days with UVB and in a group exposed to a single 3 MED UVB dose blood samples were taken on two occasions (before phototesting and 24 h after final exposure). In other individuals blood samples were taken three times (before the phototesting, 24 hours after 10<sup>th</sup> exposure to UVB or SSR and 24 hours after 3 MED irradiation).

Serum samples were analysed for IL-1  $\beta$ , IL-6, IL-8, IL-10 TNF- $\alpha$  concentration with chemiluminescence assay (Diagnostic Products Corporation, Los Angeles, USA) according to manufacturer's instructions. Sensitivity and reference ranges for IL-1  $\beta$ , IL-

6, IL-8, IL-10, TNF- $\alpha$  were: 5.0 pg/ml, 0-5 pg/ml; 2.0 pg/ml, 0-9.7 pg/ml; 5.0 pg/ml, 0-62 pg/ml; 1.5 pg/ml, 0-9.1 pg/ml; 4.0 pg/ml, 0-8.1 pg/ml, respectively.

IL-1  $\beta$  concentrations were below detectable levels in all (105/105 ) volunteers, both before and after UVB or SSR irradiation. IL-6 was detectable in 17 out of 105 individuals before irradiation and its concentration increased slightly after UV exposure (in each assessed group). However, no statistical correlation between UV exposure and IL-6 level was found. IL-8 levels were detectable before irradiation in 29 out of 105 volunteers. We found a statistically significant increase ( $p < 0.05$ ) in IL-8 serum level after 10 days of UVB irradiation. Local acute UVB irradiation which was additionally applied after 10-day irradiation in one group did not cause any significant changes in this cytokine level. No changes in IL-8 level were observed in group in which only local acute dose of UVB was applied. In contrast to chronic low doses of UVB, repeated, 10 days, irradiation with SSR did not lead to any alterations in IL-8 serum concentration.

In group irradiated with repeated UVB, IL-10 was over reference range (0-9.1 pg/ml) before UVB irradiation (15.5 pg/ml) only in one volunteer but its level was much higher after 10 day UVB exposure (29.8 pg/ml) and undetectable after the additional 3 MED irradiation. In group irradiated for 10 days with SSR two subjects had elevated IL-10 levels before irradiation, however 10-day SSR exposure did not cause any increase in the interleukin level. In group exposed to 3 MED no effect of an acute single dose of UVB on serum level of this cytokine was observed.

Before irradiation TNF- $\alpha$  serum level was detected in 90 out of 105 volunteers (86% %). In 76 out of 90 its concentration was within reference ranges and only in 14 cases it was over expected values (8.2 -24.3 pg/ml). In groups irradiated with repeated UVB, slight, statistically significant, increase in serum level was observed after 10 days of irradiation ( $p < 0.05$ ).

Local acute UVB doses and repeated SSR irradiation did not change the concentration of this cytokine further.

In our study no significant alterations in cytokine levels in serum, apart from IL-8 and TNF- $\alpha$  were found. Until now not much was known about the influence of suberythemal doses of UVB and/or SSR irradiation on serum profile of selected cytokines, nor had we any information whether local irradiation with acute UVB doses could influence these parameters. Our results indicate warrant further studies to determine the threshold for different sources and different outputs of UV which could cause impairment of systemic immunity.

#### **4. mRNAs expression for selected cytokines**

The aim of the study was to examine the effect of low and repeated irradiation with UVB on expression of IL-1 beta, IL-6, IL-10, and TNF- alpha mRNAs in healthy human volunteers. We also tried to find whether repeated exposure to UVB causes adaptation to acute UVB irradiation.

Our study included 45 healthy volunteers who were exposed to UVB: 15 subjects (whole-body, low doses of UVB for 10 days with subsequent irradiation with 3 MED UVB), 15 subjects (locally, 3 MED UVB), 15 subjects (whole-body, low doses of UVB for 10 days UVB). Additionally 10 individuals served as controls. Skin samples were taken twice or three times before and 24 h after final exposure. IL-1beta, IL-6, IL-10 and TNFalpha

mRNAs were determined in each specimen by relative quantitative RT PCR. Concentrations of mRNAs for tested cytokines were normalized in each sample in relation to beta actin mRNA.

We found a statistically significant increase ( $p < 0.001$ ) in the expression of IL-1, IL-6, IL-10, and TNF-alpha mRNAs in all volunteers after irradiation with repeated, low doses of UVB. In group exposed only to a single acute dose of UVB, the same significant tendency was observed ( $p < 0.001$ ). Additional irradiation of 3 MED UVB after repeated exposure to UVB caused a statistically significant increase in mRNA expression of IL-1beta, IL-6, IL-10, however, a statistically decrease in TNF-alpha mRNA expression. Our results indicate that UVB radiation (both repeated as well as a single dose) regulate mRNA expression of the examined cytokines. A 10-day exposure of humans to suberythemal doses of UVB causes no clinical symptoms, however it induces alterations in skin immunity at the molecular level. Single exposure to erythemal dose of UVB, applied 24 h after repeated UVB irradiation, down-regulates TNF-alpha mRNA, what is probably at least partially responsible for lowering intensity of inflammatory response, as observed by clinical symptoms (erythema, oedema).

## 5. Gene polymorphism: TNF-alpha and IL-1 beta

Immunogenetic study comprised 234 volunteers (blood samples taken before irradiation) and 150 healthy unrelated subjects (controls).

Three known dimorphic sites within *IL-1B* gene: T→C (1903, AluI) within the promotor region, G→A (5810, ItAI) within intron 4 and C→T (5887, TaqI) within exon 5 and polymorphic site within *TNFA* gene: G→A (at position -308) were analyzed using RFLP-PCR technique.

Statistical analysis of *TNFA* and *IL-1B* polymorphic sites in volunteers and controls were compared using chi2 ( $df=2$ ) with Yates' correction. Values of  $p < 0.05$  were considered statistically significant.

We analysed correlation between any polymorphism and phototype/photosensitivity (MED)

No significant differences in -308 *TNFA* polymorphism distribution between the volunteers and controls were observed and no correlation with photosensitivity.

Significant differences were found in TagI polymorphism (C → T) within *IL-1B* gene.

Genotype TT was statistically more frequent in individuals with phototype II ( $p = 0.019$ ) than with individuals with phototype III.

Allele T carriers were more frequent in the individuals with phototype II ( $p = 0.02$ ) and in those with lower MED value ( $p = 0.00009$ )

CT genotype and TT genotype were statistically more frequent within individuals with lower MED values ( $p = 0.0002$  and  $p = 0.0139$ , respectively)

## 6. Expression of cyclooxygenases (COX-1 and COX-2) in the epidermis and DNA damage after repeated SSR or UVB irradiation

Immunohistochemical examinations assessing intensity of COX-1 and COX-2 expression and DNA damage were performed in all groups of irradiated results (SSR and UVB).

We assessed COXs expression based on intensity of colour in a following way.

In general we found that the highest expression of COX-1 and COX-2 was found in skin biopsies from volunteers irradiated with high, acute dose of 3 MED and the lowest one - in groups irradiated for 30 days with low doses (1.2 SED) of SSR. These results suggest that repeated radiation with SSR has anti-inflammatory properties. Besides, the obtained results confirm the lack of adaptation of chronic UVB and/or SSR irradiations to immunomodulating effects mediated by high doses of UVR.

Intensity of DNA damage was assessed by determining the numbers of cells in epidermis which gave positive staining.

We found the highest number of cells with thymine dimmers in group irradiated with acute dose of UVB (3 MED) .

In groups irradiated with UVB intensity of dimmers in specimens was significantly higher than in group irradiated for 2- or 10 days with SSR. In group irradiated for 30 days of SSR the number of cells with thymine dimmers was comparable with those observed in UVB groups . The obtained results testify that repeated irradiation with low doses of SSR causes DNA damage and has no adaptation.

Between January and March 2005 we performed additional studies assessing distribution of blood dendritic cells in skin, IL-1beta mRNA expression, expression of cyclooxygenases and DNA damage in skin biopsies obtained from selected volunteers irradiated with SSR or UVB.

## **1. DISTRIBUTION OF BLOOD DENDRITIC CELLS IN EPIDERMIS AND SKIN BEFORE AND AFTER IRRADIATION**

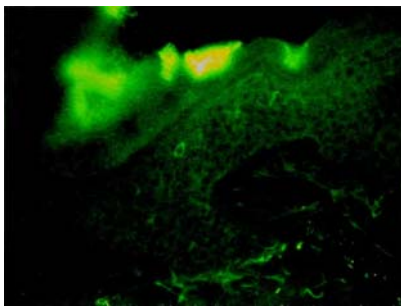
### **Skin samples and immunofluorescence**

3 mm punch biopsies were taken from the buttock skin of seven individuals from each group: In the group A subjects, samples were collected before phototesting and 24 h after the tenth exposure to 0.7 MED UVB; in the group B subjects, samples were collected before phototesting, 24 h after the tenth exposure to UVB (before the 3 MED local UVB), and 24 h after the local UVB. In the group C subjects, skin samples were collected before and 24 h after the local UVB. After taking they were immediately put in liquid nitrogen and stored at -80°C until analysis. Immunofluorescent staining of cryostat sections was performed using monoclonal mouse IgG1 antibodies directed against BDCA-1, BDCA-2, BDCA-3, all labelled with FITC, and BDC-4, labelled with PE (all Miltenyi Biotec, Bergish Gladback, Germany). Skin samples were incubated with a 1:9 dilution of the antibodies for 60 min at room temperature in the dark. After washing in phosphate buffered saline, the tissue specimens were dried and mounted in glycerol. Sections were analyzed with a fluorescent microscope (Olympus Bx40 Olympus Optical Co., LTD, Tokyo, Japan) coupled to a digital camera (Camedia, Olympus, Japan) and DPX Olympus Software programme. Three sections from each biopsy were examined. The skin specimens were evaluated in 400x high power fields (in five sequences) and count of each BDC subtype was assessed

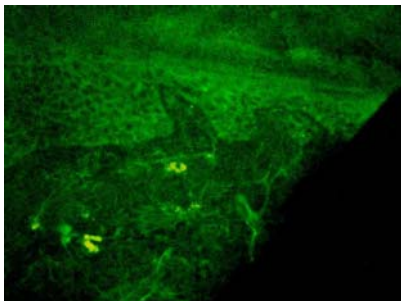
### **Results:**

Immunofluorescent staining of skin samples for BDCA-1, 2, 3 and 4 revealed only a few positive cells. The BDC were located both in the epidermis and dermis. Myeloid subtypes (BDCA-1 and -3) were observed mainly in the middle layers of the epidermis (Figure 1) and it was estimated that there were 5-7 cells in the epidermis (one specimen); single positive cells were also found in the upper part of the dermis. PDC (BDCA-2 and BDCA-4) were detected mainly in the dermis (Figure 2) with the number being about 3-4 cells in whole specimen, although single BDCA-2 cells were present in the epidermis on occasion.

No difference in the number and distribution of any of the subsets was revealed when sections taken before and after UVR were compared, irrespective of whether the UVB was repeatedly suberythemal or given as a single erythemal dose.



**Figure 1.** Myeloid blood dendritic cell stained with antibodies directed against BDCA-3 antigen (direct immunofluorescence test, mag. x400)



**Figure 2.** Plasmacytoid blood dendritic cell stained with antibodies directed against BDCA-2 antigen (direct immunofluorescence test, mag. x400)

## **2. ASSESSMENT OF IL-1 BETA, mRNA LEVELS IN UV-IRRADIATED HUMAN SKIN IN VIVO BY RELATIVE QUANTITATIVE REVERSE TRANSCRIPTION PCR ASSAYS OF mRNA**

Skin biopsies for assessment of mRNA of selected cytokines were taken from:

- 10 healthy individuals (unirradiated, unsensitised) – controls
- Group A – 15 individuals (biopsy taken from buttock skin 24 h after 10 days of 0.7 MED of UVB irradiation and 24 h after 3 MED)
- Group C – 15 individuals (biopsy taken from buttock skin 24 h after 10 days of 1.2 SED SSR irradiation and 24 h after 3 MED)
- Group D – 15 individuals (biopsy from buttock skin taken before and 24 h after 3 MED. Irradiation)

- Group 3 - 15 individuals irradiated for 30 days with SSR (1.2 SED daily) (biopsy from buttock skin taken before and 24 h after final irradiation)

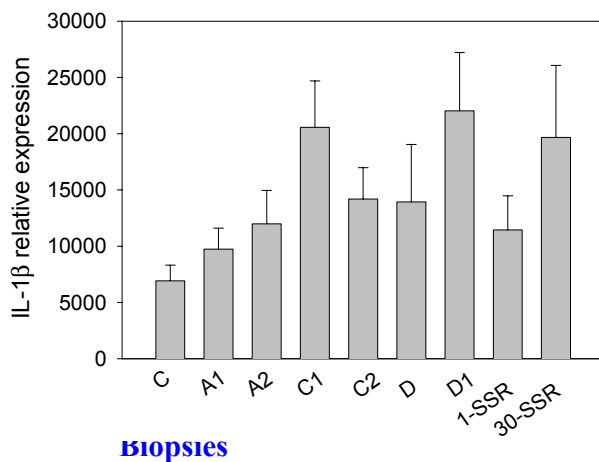
### Relative quantitative reverse transcription PCR assays of mRNA

Samples (50 mg) of skin tissue were dissolved in the Fenzol reagent and used to purify total mRNA by a total RNA Prep Plus Kit (A&A Biotechnology). The first strand of cDNA was synthesized according to the manufacturer's instruction using (dT)20 primer. The reverse transcription was carried out for 60 min at 30 °C and stopped at 0 °C. Then, mRNA for IL-1 $\beta$  was determined by relative quantitative RT PCR using actin mRNA as an internal standard RT PCR. The following oligodeoxyribonucleotide primers were used: 5'ctgccagtgaaatgatgg3' and 5'gcattctctcagctgtgcc3. RT PCR assay conditions were as follows: 5  $\mu$ l cDNA sample, 10x Taq polymerase buffer (Epicentre Technologies), 2 mM MgCl<sub>2</sub>, 1x PCR enhancer, dNTP mix, 25 pmol of each primer and 1.25 U of Taq DNA polymerase (Epicentre Technologies) in 50  $\mu$ l reaction volume. The samples were denatured at 95 °C for 5 min, then cooled on ice and, then the enzyme was added. The reaction was performed in twenty five to thirty extension cycles consisting of: 30 s denaturation step at 94 °C, a 30 s annealing step at 60 °C, and a 40 s polymerase extension step at 72 °C. Finally, each reaction was terminated with a 10 min elongation step at 72 °C. To detect actin mRNA, the same protocol was used with oligodeoxyribonucleotide primers 5'cagcagattcaagcagctatgg3' and 5'gtctgtggtgctgatctcatcc3'. The final products, IL-10 mRNA and actin mRNA, were separated by electrophoresis in 7% polyacrylamide gels in TAE buffer using the genetic size marker 100 bp DNA Ladder (Promega). Bands were visualized by UV light, the results were recorded photographically and analyzed densitometrically using LKB Ultrascan XL Enhanced Laser Densitometr. Concentrations of mRNAs for tested cytokine was normalized in each sample in relation to actin mRNA.

### Results:

An increase in the expression of mRNA of IL-1beta in all groups before and after irradiation.was found (p<0.05).

A dose of 3 MED applied after 10 days of irradiation with 1.2 SED of SSR caused a decrease in IL-1beta mRNA expression (p<0.05)



SES: COX-1 AND COX-2 AND

Immunohistochemical examinations assessing intensity of COX-1 and COX-2 expression and DNA damage were performed in all groups of irradiated results (SSR and UVB). 63 biopsies a 3 mm biopsies were taken from buttock skin from the following volunteers: 7 biopsies from non-irradiated controls (group 1), 7 biopsies were taken after 24 h after 2-day irradiation with SSR (group 2), 7 biopsies – 24 h after 10 days of irradiation with SSR (group 3) and 7 biopsies – 24h after 30 days of irradiation with SSR (group 4). Fourteen biopsies were taken from 7 volunteers from group A (7 ones collected 24 h after tenth irradiation with 0.7 MED and 7 ones - 24 h after exposure with 3 MED UVB), 14 biopsies were taken from 7 volunteers from group C (7 ones collected 24 h after 10<sup>th</sup> irradiation with 1.2 SED of SSR and 24 h after exposure to 3 MED) and 7 biopsies from subjects from group D (biopsies collectes 24 h after 3 MED irradiation).

## **METHODS:**

### **COXs**

Cryostat section for immunocytochemistry studies were fixed in acetone for 10 minutes and stored at -20° C until use. The immunohistochemical detection was performed on frozen 4 µm-thick biopsy sections. Sections were collected onto Superfrost Plus slides (BDH) and air dried. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. Slides were washed in phosphate buffered saline (PBS, pH 7.4 ) and incubated with normal horse serum in Tris-HCl-phosphate-buffered saline (TPS) and 1% BSA. After rinsing the section were incubated overnight at 4°C with the primary antibody. The following primary antibodies were used: COX-1 (dilution: 1:40; Novocastra Laboratories Ltd; NCL-COX-1; Clone 12E12) and COX-2 (dilution: 1:50; Novocastra Laboratories Ltd; NCL-COX-2; Clone 4H12). Detection was performed with LSAB+ System-HRP (Dako Cytomation). Color was developed with diaminobenzidine (DAB+; Dako Cytomation). The slides were counterstained with hematoxyline, dehydrated and coverslipped. Negative controls were performed by omitting the primary antibody.

### **Thymine dimmers**

The immunohistochemical detection were performed on frozen 4 µm-thick biopsy sections. Sections were collected onto Superfrost Plus slides (BDH), air dried and fixed 1h in 4% buffered formalin in room temperature. The sections were treated by 10 minutes of microwave oven heating in Target Retrieval Solution (citrate buffer, pH 6.0; Dako Cytomation), at 800 W and then transferred to distilled water. Endogenous peroxidase was blocked by incubation with 0,3% hydrogen peroxide in methanol. Slides were washed in phosphate buffered saline (PBS, pH 7.4 ) and incubated with 10% normal rat serum in Tris-HCl-phosphate-buffered saline (TPS) and 1% BSA. After rinsing the section were incubated overnight at 4°C with the primary antibody-Anti-Thymine Dimer (Clone KTM53; Kamiya Biomedical Company,USA) Detection was performed with LSAB+ System-HRP (Dako Cytomation). Color was developed with diaminobenzidine (DAB+; Dako Cytomation). The slides were counterstained with hematoxyline, dehydrated and coverslipped. Negative controls were performed by omitting the primary antibody.

### RESULTS ON COXs EXPRESSION

We assessed COXs expression based on intensity of colour in a following way:

- 0- no expression
- 1- moderate expression
- 2- strong expression

All detailed results are shown in Table:

GROUP	COX-1	COX-2
CONTROL	0/1	0/1
2 DAY SSR	1	1
10 DAY SSR	1	0
30 DAY SSR	0/1	0
10 DAY 0.7 MED UVB	1	1
10 DAY 0.7 MED UVB + 3 MED UVB	1	1/2
10 DAY SSR + 3 MED UVB	1/2	1
3 MED UVB	2	2

Results from non-irradiated (control) group are shown in figure 3 a and 3 b.

Concluding we found that the highest expression of COX-1 and COX-2 was found in skin biopsies from volunteers irradiated with high, acute dose of 3 MED (figure 4 a and b) and the lowest one - in groups irradiated for 30 days with low doses (1.2 SED) of SSR. These results suggest that repeated radiation with SSR has anti-inflammatory properties. Besides the obtained results confirm the lack of adaptation of chronic UVB and/or SSR irradiations to immunomodulating effects mediated by high doses of UVR.

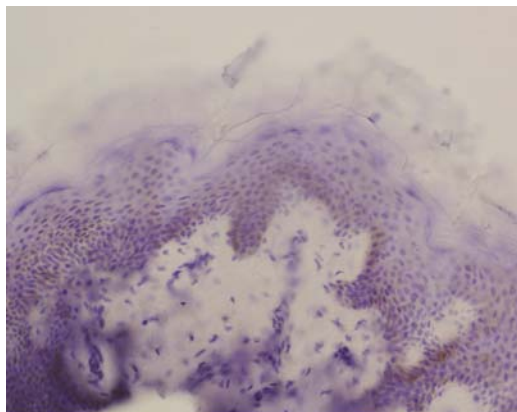


Fig. 3 a Control – COX-1

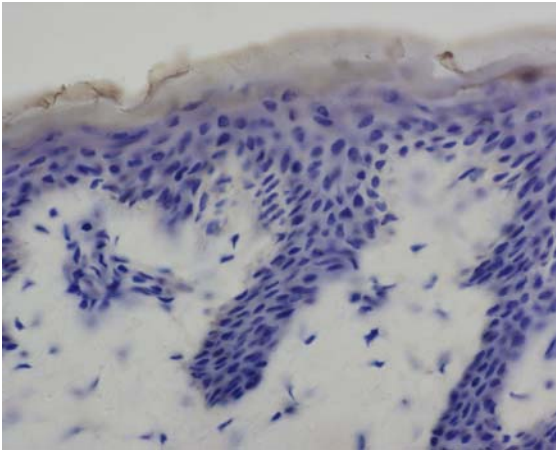


Fig. 3 b Control – COX-2

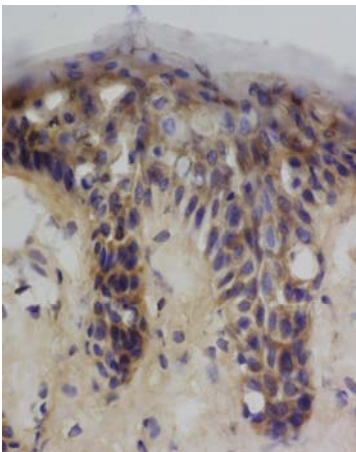


Fig. 4 a. COX-2 expression in biopsy after irradiation with 3 MED

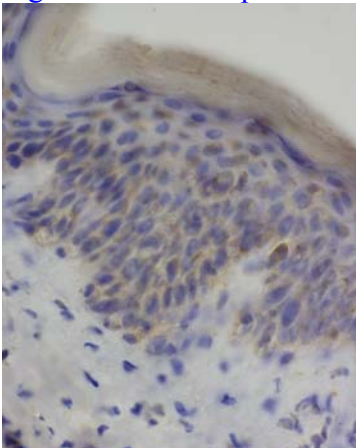


Fig. 4 b COX-1 expression in biopsy after irradiation with 3 MED

### **RESULTS ON DNA DAMAGE**

Intensity of DNA damage was assessed in a following way :

0-no dimmers

- 1- dimers found in up to 25% of cells in epidermis
- 2- dimers found in 25% - 50% of cells in epidermis
- 3- dimers found in over 50% of cells in epidermis

Results from non-irradiated (control) group are shown in figure 5.

We found the highest number of cells with thymine dimers in group irradiated with acute dose of UVB (3 MED) – figure 6.

In groups irradiated with UVB intensity of dimers in specimens was significantly higher than in group irradiated for 2- or 10 days with SSR. In group irradiated for 30 days of SSR the number of cells with thymine dimers was comparable with those observed in UVB groups (figure 7). The obtained results testify that repeated irradiation with low doses of SSR causes DNA damage and has no adaptation.

GROUP	THYMINE DIMERS INTENSITY
CONTROL	0
2 DAY SSR	1
10 DAY SSR	1; single cases 1/2
30 DAY SSR	2/3
10 DAY 0.7 MED UVB	1
10 DAY 0.7 MED UVB + 3 MED UVB	2
10 DAY SSR + 3 MED UVB	2/3
3 MED UVB	3

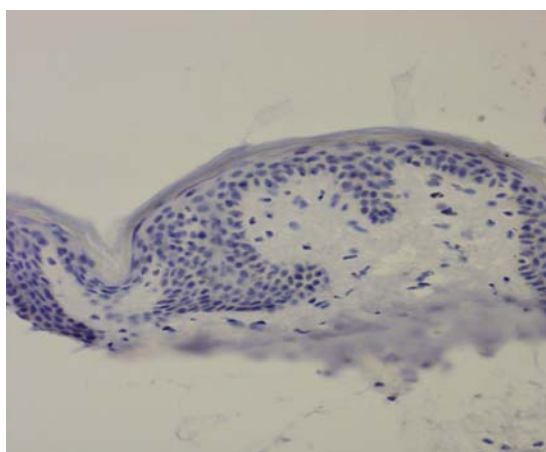


Fig. 5 Control – DNA damage

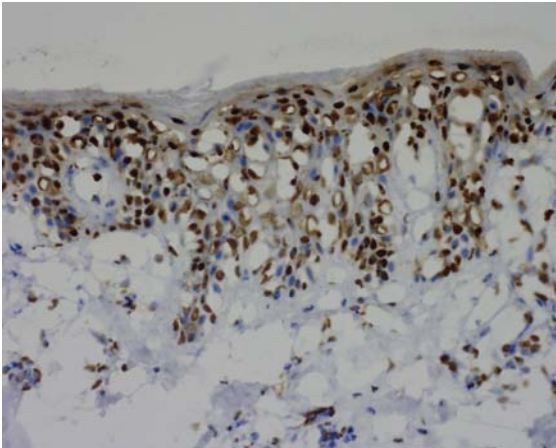


Fig. 6 – 3 MED UVB – DNA damage

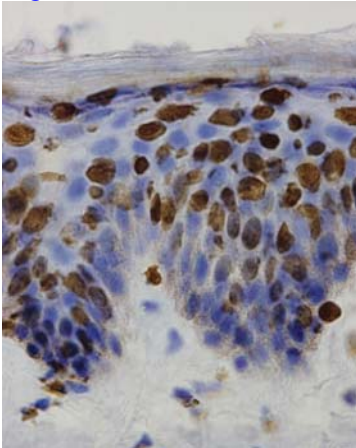


Fig. 7 – 30 days of SSR – DNA damage

### **OVERALL EVALUATION AND CONCLUSIONS**

Small daily doses of SSR induce suppression of CHS and related markers in human subjects and the effect is cumulative, indicating that there is no adaptation to the immunomodulating effects of UVR, at least over the test period of 30 days.

### **DELIVERABLES**

Narbutt J, Skibinska M, Lesiak A, Wozniacka A, Sysa-Jedrzejowska A, Cebula B, Robak T, Smolewski P. Exposure to low doses of solar-simulated radiation induces an increase in the myeloid subtype of blood dendritic cells. *Scand J Immunol.* 2004 ,60, 429-35.

Joanna Narbutt, Aleksandra Lesiak, Malgorzata Skibinska, Anna Wozniacka, Henk van Loveren, Anna Sysa-Jedrzejowska, Iwona Lewy-Trenda, Aleksandra Omulecka, Mary Norval. Suppression of contact hypersensitivity after repeated exposures of humans to low doses of solar simulated radiation. In press.

**WP.5      Adaptation to effects of UV exposure on acquired immune parameters in humans**

**Workpackage number:** 5  
**Start date of starting event:** month 18  
**Completion date:** month 32  
**N° of the partner responsible:** 1, 3  
**N°s of other partners involved:** 1,3,5  
**Person-months per partner:** CO1:2, CR3:18  
**Total person months:** 20

**Objectives**

To establish the adaptive activity of UV exposure on subsequent exposures of UV that suppress acquired immune responses in humans

**Description of work**

Groups of 50 individuals will be exposed (whole body) to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for 2, 10, or 30 consecutive days. Then, the exposed individuals and controls will be exposed to a single immunosuppressive exposure of 3 personal MED on areas of 10 x 10 cm at the lower back.

Subsequently, pre-exposed and non-pre-exposed, as well as non-exposed individuals will be sensitised to DPCP on the irradiated skin. Immune as well as non-immune parameters as indicated in workpackage 3 will be evaluated.

**Deliverables**

Report on adaptation to chronic UV exposure induced effects on human acquired immune parameters.

**Milestones and expected results**

The results of this workpackage will yield information on the adaptation activity of UV exposure to subsequent immunosuppressive UV exposures on acquired immune parameters, and on how they compare to such adaptation regarding non-immune parameters. In addition information will become available on interindividual differences with regard to adaptation to the suppressive effects of UV.

**Progress report year 2002 WP 5: See WP 4**

**Progress report year 2003 WP 5: See WP 4**

**Progress report year 2004 WP 5: See WP 4**

**Progress report year 2005 WP 5: See WP 4**

**Conclusions WP 5: See WP4**

**WP.6 Chronic effects of UV exposure on innate immune parameters in mice**

**Workpackage number:** 6  
**Start date of starting event:** month 1  
**Completion date:** month 18  
**N° of the partner responsible:** 1, 4  
**N°s of other partners involved:** 1,4,  
**Person-months per partner:** CO1:1; CR4:16.5  
**Total person months:** 17.5

**Objectives**

To establish the effect of chronic UV exposure on innate immune parameters in mice

**Description of work**

Groups of shaved BALB/c (UV resistant) and C3H (UV sensitive) mice will be exposed to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for 2, 10, 30, or 90 consecutive days. Subsequent to exposure, spleens will be taken to test for innate immune parameters: natural killer activity (by radioactive chromium release), phagocytic activity (by uptake of fluorescent particles), and dendritic cell type differentiation (by FACS analysis) and dendritic cell function. In addition, biopsies of irradiated skin will be obtained for dendritic cell type characterisation and evaluation of skin mast cell degranulation (by histochemical techniques).

In order to compare immune effects with non-immune effects of chronic UV exposure, non-immune parameters will be assessed as well: skin oedema (by measuring skin fold thickness), MED, DNA damage, and as keratinocyte proliferation in the biopsies (by histochemical techniques).

In addition to these experiments, other experiments will be carried out in groups of mice of both strains in which both duration and dose of UV exposure are varied in such a fashion that the total irradiation that the mice will receive remains constant. Different combinations of duration and dose will be tested, in which the total dose will be equal. For this purpose, the same parameters as indicated above will be studied.

Finally, experiments will be carried out in which the total dose that the animals receive will be similar, but the skin surface that will be exposed is varied. For this purpose, the same parameters as indicated above will be studied.

**Deliverables**

Report on effects of chronic expose to UV on murine innate immune parameters and the duration x dose and dose x surface relationship of UV induced suppression of innate immune parameters.

**Milestones and expected results**

This workpackage will establish effects of chronic UV exposure on innate immune responses in mice, and how such effects compare to effects on non-immune parameter. Also, the impact of different genetic background will be established. As such, the information yielded by this workpackage may confirm and extend the data gained from the experiments in humans. In addition, the relation between duration and dose of UV exposure, which cannot readily be assessed in

humans, will be established.

## Progress report year 2002 WP 6 and 7

Preliminary experiments were required to evaluate the biological properties of the Cleo lamps. Four were set up in parallel and they were found to emit 720mJ/cm<sup>2</sup> UVB and 9900 mJ/cm<sup>2</sup> UVA in 60 minutes at a distance of 20 cm. A dose of 1080mJ/cm<sup>2</sup> UVB on each of three consecutive days was not sufficient to induce erythema as judged by increased vascularisation of the inner dorsal skin. Therefore we decided to establish the approximate minimal dose of UV radiation required to affect innate immunity, and to use that dose for the chronic exposure followed by three times that dose to test adaptation.

Four parameters of innate immunity have been tested following exposure of C3H/HeN mice to the Cleo lamps: number of Langerhans cells (LCs), macrophage activity, natural killer (NK) cell activity and dendritic cell (DC) numbers and phenotype in lymph nodes draining irradiated sites.

### LC numbers

The mice were irradiated with various single doses from the Cleo lamps and LCs were counted in the upper epidermis of the ear using an ATPase stain 24h later. A significant reduction in Langerhans cell numbers from 531 per mm<sup>3</sup> to 339 per mm<sup>3</sup> was found after a dose of 240 mJ/cm<sup>2</sup> UVB, while a dose of 120 mJ/cm<sup>2</sup> UVB did not result in a loss. A similar reduction was seen after 360, 1080, 1440 and 2160 mJ/cm<sup>2</sup> UVB as after 240 mJ/cm<sup>2</sup> UVB.

For the chronic exposure and adaptation experiments, we decided to use a dose of 252 mJ/cm<sup>2</sup> on each of 2, 10 or 30 days, with one group receiving an additional immunosuppressive dose (ID) of 504mJ/cm<sup>2</sup> UVB on days 2, 10 or 30. LC numbers were counted on the day following the last exposure.

	<u>Number per mm<sup>3</sup></u>	<u>% reduction</u>	<u>p value *</u>
Unirradiated	549		
Chronic irradiation, 2 days	425	22.5%	0.02
Chronic irradiation 2 days + ID	351	36%	0.005
Chronic irradiation 10 days	460	16%	0.008
Chronic irradiation 10 days + ID	497	9%	0.03
Chronic irradiation 30 days	471	14%	0.04
Chronic irradiation 30 days +ID	501	9%	0.08

\* The p values are based on comparisons between irradiated and unirradiated groups

The results show that in all irradiation protocols, except for the 30-day chronic irradiation + ID, LC numbers were significantly reduced in the epidermis of C3H mice 24h after UV. The results also show that there was no significant difference between LC numbers in mice irradiated chronically for 2, 10 or 30 days. There was however, a significant difference in LC numbers between mice receiving the immunosuppressive dose on day 2 and those receiving the immunosuppressive dose on day 10 (p=0.012) and on day 30 (p=0.008).

Thus we conclude that LCs were consistently reduced throughout the prolonged chronic irradiation but that some adaptation did occur.

### Macrophage activity

Mice were irradiated with various single doses from the Cleo lamps and the phagocytic activity of peritoneal macrophages (assessed by the ingestion of red blood cells (RBCs)) was measured 24h later. A dose of 360mJ/cm<sup>2</sup> UVB did not suppress phagocytic activity whereas a dose of 1080mJ/cm<sup>2</sup> UVB demonstrated a slight suppression of phagocytosis. 1440mJ/cm<sup>2</sup> UVB reduced the number of RBCs from 3.8 to 3.1 per macrophage and 2160mJ/cm<sup>2</sup> UVB reduced the number of RBCs from 3.8 to 3.0 per macrophage.

For the chronic exposure and adaptation experiments we decided to use a dose of 1008 mJ/cm<sup>2</sup> UVB on each of 2, 10 or 30 days, with one group receiving an additional immunosuppressive dose (ID) of 2016mJ/cm<sup>2</sup> UVB on days 2, 10 or 30. Phagocytic activity was measured on the day following the last exposure.

	<u>No. of RBCs/ Macrophage</u>	<u>% reduction</u>	<u>p value*</u>
Unirradiated control 2 days	3.0		
Chronic irradiation 2 days	1.8	40%	0.000015
<u>Chronic irradiation 2days + ID</u>	<u>1.9</u>	<u>37%</u>	<u>0.00005</u>
Unirradiated control 10 days	2.1		
Chronic irradiation 10 days	1.8	14%	0.278
<u>Chronic irradiation 10 days + ID</u>	<u>1.5</u>	<u>28%</u>	<u>0.0078</u>
Unirradiated control 30 days	2.5		
Chronic irradiation 30 days	2.0	20%	0.078
<u>Chronic irradiation 30 days +ID</u>	<u>2.1</u>	<u>16%</u>	<u>0.069</u>

\* The p values are based on comparisons between irradiated and unirradiated control groups.

Mice receiving the chronic irradiation protocol for 2 days plus the immunosuppressive dose on day 2 showed a significant reduction of phagocytic activity of peritoneal macrophages. Those mice given the chronic irradiation for 10 days did not show a significant suppression of phagocytic activity, but when the immunosuppressive dose was administered on day 10 there was a significant suppression of phagocytic activity.

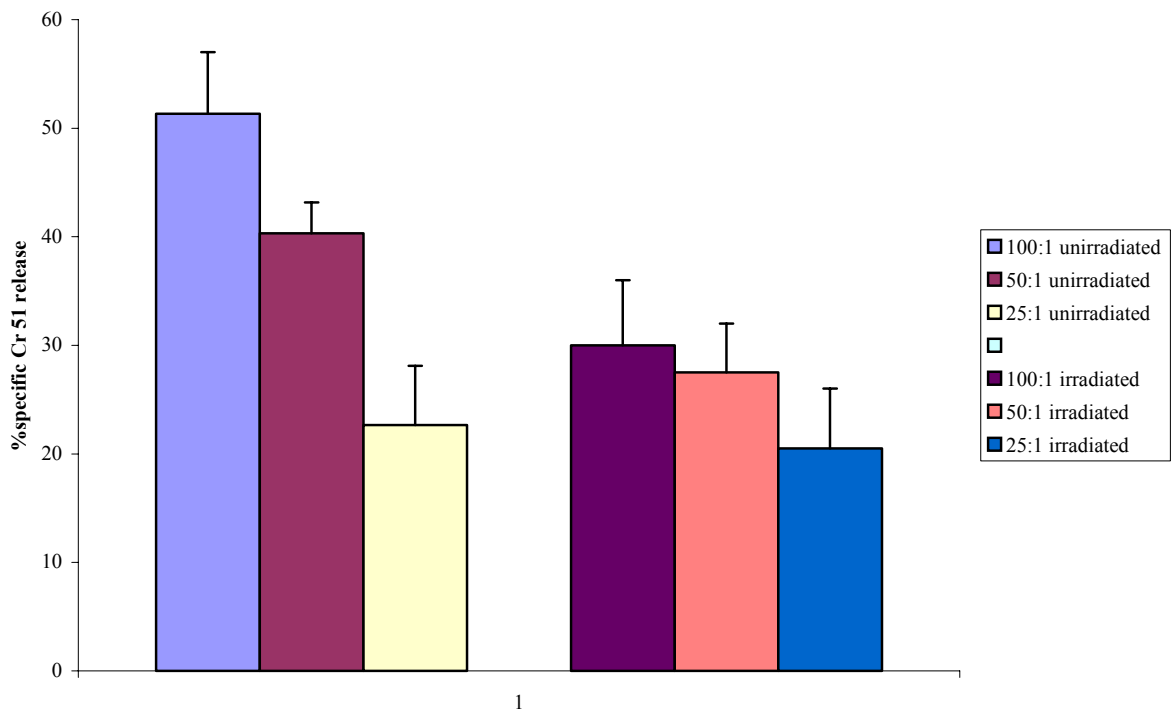
Mice receiving the chronic irradiation protocol for 30 days or the chronic protocol plus the immunosuppressive dose on day 30 did not show a significant reduction in phagocytic activity.

Thus we conclude that adaptation did occur following chronic irradiation in this study.

### NK cell activity

Mice were irradiated with  $2160\text{mJ}/\text{cm}^2$  UVB Cleo (3hours) and 24h later NK cell activity was measured in the auricular lymph nodes by radioactive chromium release from labelled YAC cells as targets. Irradiated mice showed a reduced NK cell response as can be seen in the graph below.

**The effects of 3h CLEO on NK cell activity**



Where the effector: target cell ratio was 100:1 there was a 40% reduction in NK cell activity, at 50:1 a 31% reduction and at 25:1 a 9% reduction. Current experiments aim to establish the minimal suppressive dose for NK cell activity and then to carry out the chronic exposure and adaptation study with regard to this parameter.

#### DC numbers and Phenotype

Analysis of the cell surface molecules, MHC II and B7-2, on DCs in lymph nodes draining irradiated sites of C3H mice following  $2160\text{mJ}/\text{cm}^2$  UVB was carried out. No change in the expression of these markers was observed. Analysis of DC numbers has so far indicated an increase in DC numbers in the draining lymph nodes of mice irradiated with  $2160\text{mJ}/\text{cm}^2$  of Cleo natural irradiation (3hours). As indicated for the NK cell study above, further experiments will be carried out to establish the minimal suppressive dose for alteration in DC numbers, and then to carry out the chronic exposure and adaptation study with regard to this parameter.

### **Progress Report year 2003 WP6 and 7**

In this research the chronic effects of UV exposure and immunological adaptation to the effects of UV exposure on innate immune parameters in C3HHeN mice have been studied. The UV source used was 4 Cleo natural sunlamps, set up in parallel. They were found to emit 720mJ/cm<sup>2</sup> UVB and 9900mJ/cm<sup>2</sup> UVA in 60 minutes at a distance of 20cm.

Parameters of innate immunity that have been tested include the number of Langerhans cells in the epidermis, the phenotype and activity of peritoneal macrophages, natural killer (NK) cell activity, the numbers and phenotype of dendritic cells in lymph nodes draining irradiated sites, dermal mast cell numbers, the viability of bone marrow cells and the phagocytic activity of blood neutrophils.

#### **Number of Langerhans cells in the epidermis**

For the chronic exposure and adaptation experiments, a dose of 252mJ/cm<sup>2</sup> was used daily for 2, 10, 30 or 60 days, followed by an immunosuppressive dose (ID) of 504mJ/cm<sup>2</sup>. The results are given below, which represent the mean of three separate experiments. We conclude that LCs are consistently reduced throughout the prolonged chronic irradiation protocol, and that immunological adaptation of this parameter does not occur.

<u>Irradiation protocol</u>	<u>% reduction in LC numbers</u>	<u>p value</u>
Chronic 2 day	18%	0.04
Chronic + ID 2 day	27%	0.02
Chronic 10 day	21%	0.014
Chronic + ID 10 day	24%	0.04
Chronic 30 day	26%	0.04
Chronic 30 day + ID	31%	0.02
Chronic 60 day	36%	0.009
Chronic 60 day + ID	28%	0.007

It was observed that LCs became elongated following chronic exposure. LC length was therefore measured by image analysis after 30 days of chronic irradiation and after 30 days of chronic irradiation plus an immunosuppressive dose of 504mJ/cm<sup>2</sup>. The results demonstrated that LCs increased from an average mean length of 36µm to 54µm

( $p=0.03$ ) following the chronic irradiation protocol and to  $50\mu\text{m}$  ( $p=0.01$ ) following the chronic irradiation protocol plus the immunosuppressive dose.

We have also analysed the chronic effects of the Cleo natural lamps on LC numbers in mice irradiated from the age of 4 days. They received a daily dose of  $252\text{mJ}/\text{cm}^2$  for 20 days. LC numbers were reduced by 40% ( $p=0.003$ ) in comparison with non-irradiated littermates.

### Macrophage activity

Chronic exposure has been undertaken for 2, 10 and 30 days. A daily dose of  $1008\text{mJ}/\text{cm}^2$  was used throughout the chronic experiments and an immunosuppressive dose of  $2016\text{mJ}/\text{cm}^2$  was given on days 2, 10 or 30. Macrophage activity was measured by counting the ingestion of opsonised sheep red blood cells. Three experiments have been completed and the results are shown below. The statistical analysis is currently being undertaken.

#### Irradiation protocol    % reduction in SRBCs/mac

Chronic 2 days	40%
Chronic + ID 2 days	48%
Chronic 10 days	18%
Chronic + ID 10 days	21%
Chronic 30 days	7%
Chronic + ID 30 days	8%

We conclude that adaptation to the suppressive effect of UV radiation on macrophage activity occurred following chronic exposure.

We also analysed the acute effects of the Cleo natural lamps on the ability of peritoneal macrophages from C3HHeN mice to phagocytose *Staphylococcus aureus* bacteria. Doses of  $1440\text{mJ}/\text{cm}^2$  UVB and  $2160\text{mJ}/\text{cm}^2$  UVB suppressed the phagocytic activity of peritoneal macrophages 24h after irradiation by 35% ( $p=0.05$ ) and 49% ( $p=0.01$ ) respectively. By comparison  $300\text{mJ}/\text{cm}^2$  and  $450\text{mJ}/\text{cm}^2$  UVB TL-12 suppressed the phagocytic activity of peritoneal macrophages 24h after irradiation by 24% ( $p=0.15$ ) and 48% ( $p=0.05$ ) respectively.

The expression of MHC II, B7.2, CD11b and FcR has been analysed on peritoneal macrophages following irradiation of C3HHeN mice with a single dose of  $2160\text{mJ}/\text{cm}^2$  by flow cytometry. No significant change in any of these markers was observed 24h after irradiation.

Supernatants from cultured macrophages collected from the peritoneum of non irradiated mice and mice irradiated with a single dose of 2160mJ/cm<sup>2</sup> UVB, and stimulated with lipopolysaccharide for for 48h *in vitro* have been collected, and will be analysed for IL-12, IL-10 and TNF- $\alpha$  by ELISA.

#### Natural Killer (NK) cell activity

The mice were irradiated with various single doses from the Cleo lamps, and NK cell activity was measured in cells prepared from the auricular lymph nodes by radioactive chromium release from labelled YAC cells as targets. UVB doses of 2160mJ/cm<sup>2</sup>, 1440mJ/cm<sup>2</sup> and 720mJ/cm<sup>2</sup> reduced NK cell activity, but 360mJ/cm<sup>2</sup> did not. For the chronic exposure and adaptation experiments we used a daily dose of 756mJ/cm<sup>2</sup>, for 2, 10 or 30 days. An immunosuppressive dose of 1512mJ/cm<sup>2</sup> was given on days 2, 10 or 30.

<u>Irradiation protocol</u>	<u>% reduction in NK cell activity</u>	<u>%stimulation in NK cell activity</u>
Chronic 2 days (E: T 100:1) (n=3)	65%	0%
Chronic + ID 2 days (E: T 100:1) (n=3)	0%	10%
Chronic 10 days (E: T 100:1) (n=1)	50%	0%
Chronic + ID 10 days (E: T 100:1) (n=1)	0%	55%
Chronic 30 days (E: T 100:1) (n=1)	0%	27%
Chronic + ID 30days (E: T 100:1) (n=1)	0%	15%

Our preliminary conclusions are that (1) adaptation occurs following 30 days of chronic Cleo irradiation and (2) stimulation of NK cell activity occurs when the immunomodulatory dose is administered after 2 and 10 days of UV exposure. Further experiments are in progress to confirm these results.

#### The numbers and phenotype of dendritic cells (DCs)

Analysis of the cell surface molecules, MHC II and B7.2 on DCs enriched from auricular lymph nodes draining irradiated sites of C3H mice 42h after irradiation with 2160 mJ/cm<sup>2</sup>, 1440mJ/cm<sup>2</sup> and 720mJ/cm<sup>2</sup> UVB has been carried out. No change in the expression of these markers was observed.

Analysis of DC numbers has so far indicated an increase in the draining lymph nodes of mice 42h after irradiation with a single dose of 2160mJ/cm<sup>2</sup> or 1440mJ/cm<sup>2</sup>. DC numbers in the auricular lymph nodes of mice irradiated with 252mJ/cm<sup>2</sup> daily for 60days and mice irradiated with 252mJ/cm<sup>2</sup> daily for 60 days plus an immunosuppressive dose of 504mJ/cm<sup>2</sup> have been analysed. A 2-3-fold increase in DC numbers was counted following both irradiation protocols as shown in the table below.

	mean total DC numbers (4 auricular lymph nodes) (n=3)	% DCs (auricular lymph nodes) (n=2)
non irradiated	10, 588	0.11%
irradiated chronic 60 d	21, 850	0.21%
irradiated chronic 60d + ID	34, 000	0.28%

We conclude that chronic irradiation with the above irradiation protocols increases both the number and percentage of dendritic cells in the auricular lymph nodes.

Four-day-old mice were irradiated with 252mJ/cm<sup>2</sup> for 20 days and DC numbers were counted in the auricular, axillary, brachial and inguinal lymph nodes. The percentage and number of DCs was lower in the irradiated group (0.14%) in comparison to the non-irradiated group (0.23%). Results are shown in the table below.

	mean total DC numbers (16 lymph nodes) (n=2)	% DCs
non irradiated	134,500	0.23%
Irradiated	64,500	0.14%

We conclude that irradiating 4-day-old mice chronically for 20 days results in a reduction in total DC numbers and percentage of DCs in the lymph nodes.

### Mast cells

Mast cell numbers have been counted in skin sections from mice that were irradiated with 756mJ/cm<sup>2</sup> for 2 days and in mice that also received an immunosuppressive dose of 1512 mJ/cm<sup>2</sup> on day 2. There was no significant difference in mast cell numbers in mice that had received the chronic irradiation dose for 2 days and non irradiated mice, in mice that received the immunosuppressive dose on day 2 there was a 27% (p=0.01) increase in mast cell numbers. The results are shown in the table below.

cells/field	<u>average no. of mast cells per field</u> <u>(approx. 15 fields per section)</u>			mean no. of mast
	<u>section 1</u>	<u>section 2</u>	<u>section 3</u>	
Non Irradiated	15	15	14	15
Chronic 2 day	11	13	14	13
Chronic 2 day + ID 2d	20	20	19	20

We conclude that mast cell numbers are increased in the dermis following irradiation

with 2 daily doses of 756mJ/cm<sup>2</sup> UVB plus an additional dose of 1512mJ/cm<sup>2</sup> UVB on day 2.

### Bone Marrow Cells

We have observed that bone marrow cells taken from the femur of Cleo irradiated mice grew better in culture in the presence of GM-CSF and IL-4 in comparison to bone marrow cells from non irradiated mice. This effect has been observed with both acute and chronic irradiation protocols. The number and phenotype of these bone marrow cells following 60 days of Cleo irradiation with 252mJ/cm<sup>2</sup> and in mice that received an immunosuppressive dose of 504mJ/cm<sup>2</sup> on day 60 have been examined. The number of bone marrow cells was counted 24h after the final irradiation in all groups. Bone marrow cells were then cultured overnight for 24h and the non-adherent cells were taken and counted. The results are shown in the table below.

	<u>total bone marrow cell count</u> <u>(day 0) 2 mice (n=2)</u>	<u>non adherent cell count</u> <u>(day 1) (n=2)</u>
Non-irradiated	10.0 x 10 <sup>6</sup>	5.1 x 10 <sup>6</sup>
Chronic 60 day	8.4 x 10 <sup>6</sup>	9.56 x 10 <sup>6</sup>
Chronic 60 day + ID	9 x 10 <sup>6</sup>	9.4 x 10 <sup>6</sup>

In the non irradiated group the non adherent cell count (day 1) was lower than the total cell count (day 0), but was higher than the total cell count in the 60 day UV irradiated group and in the group receiving the 60 day chronic irradiation plus the immunosuppressive dose. We conclude that UV helps bone marrow cells survive *in vitro*. Phenotypic analysis of bone marrow cells by flow cytometry is being undertaken.

### Phagocytic activity of blood neutrophils

We have attempted to set up a protocol to analyse the effects of the Cleo natural lamps on the phagocytic activity of blood neutrophils in mice using opsonised zymosan particles. However attempts have been unsuccessful. The main problem was that there was no difference in fluorescence between neutrophils incubated in the presence of opsonised zymosan particles at 4 °C or at 37°C. This suggests that it was not possible to distinguish between zymosan particles on the outside of the cells and those that had been internalised. The zymosan particles also clumped together making it difficult to distinguish between clumps of zymosan particles and neutrophils.

### Progress Report year 2004 WP6 and 7

In this research the chronic effects of UV exposure and adaptation to the effects of UV exposure on non-immune parameters and innate immune parameters in C3H/HeN mice

have been studied. The UV source used was 4 Cleo natural sunlamps set up in parallel. They were found to emit  $720\text{mJ}/\text{cm}^2$  UVB and  $9900\text{mJ}/\text{cm}^2$  UVA in 60 minutes at a distance of 20cm.

Non –immune parameters analysed were erythema, epidermal thickness and DNA damage. Parameters of innate immunity that have been tested include the number of Langerhans cells in the epidermis, the numbers and phenotype of dendritic cells in lymph nodes draining irradiated sites, the phenotype and activity of peritoneal macrophages, natural killer (NK) cell activity, dermal mast cell numbers, quantitative measurement of cytokine levels in the skin, the viability of bone marrow cells and the phagocytic activity of blood neutrophils.

### Erythema

Erythema has been analysed 24h after irradiation of mice with an acute dose of  $2016\text{mJ}/\text{cm}^2$  UVB and following irradiation of mice with  $756\text{mJ}/\text{cm}^2$  UVB daily for 2 or 30 days. Groups of mice also received an additional dose of  $1512\text{mJ}/\text{cm}^2$  UVB on days 2 or 30.

Erythema was analysed by visualisation of vascularisation in the inner dorsal skin and in some experiments using an erythema meter. For vascularisation analysis, photographs were taken to compare irradiated with non irradiated skin. An increase in vascularisation was observed 24h after irradiation of mice with all irradiation protocols. Visualising the vascularisation on the inner dorsal skin is not an accurate way to quantify erythema, we are therefore not able to say whether adaptation has occurred, but only that erythema is still present following 30 days of irradiation and that it appeared to be exacerbated when the additional dose was administered on day 30.

The erythema meter measures the amounts of two principle skin chromophores; haemoglobin and melanin, takes the ratio of these and calculates the Erythema Index. The results are shown in the table below.

<u>Irradiation Protocol</u>	<u>Mean Erythema Index <math>\pm</math> SEM</u>	<u>pvalue</u>
Non Irradiated	$25 \pm 2$	
$2016 \text{ mJ}/\text{cm}^2$	$36 \pm 3$	0.0007
<b>Non Irradiated</b>	<b><math>21 \pm 2</math></b>	
2 days	$28 \pm 2$	0.01
2 days + AD	$36 \pm 3$	0.0001

The results obtained from the erythema meter readings confirmed the findings from the analysis of vascularisation that erythema was present following a single acute irradiation with  $2016\text{mJ}/\text{cm}^2$  UVB, following 2 days of irradiation with  $756\text{mJ}/\text{cm}^2$  UVB and following 2 days of irradiation with  $756\text{mJ}/\text{cm}^2$  UVB plus an additional dose of

1512mJ/cm<sup>2</sup> UVB on day 2. The increase in erythema index detected following irradiation was small, probably because the mice were pigmented, but statistically significant differences were obtained. Because the increase in erythema index was small we decided that this was not an ideal way to detect erythema in the C3HHeN mice, we therefore did not use the meter in the remainder of the study.

### Epidermal Thickness

Mice were irradiated with a daily dose of 756 mJ/cm<sup>2</sup> UVB for 2, 30 and 60 days. An additional dose of 1512 mJ/cm<sup>2</sup> was given on days 2, 10 and 30 in some groups of animals. Epidermal thickness was measured from haematoxylin stained paraffin embedded ear skin sections (dorsal surface). Photographs were taken of each section and viewed by image analysis. The thickness of the epidermis was measured 30 times for each sample and the results are shown below.

<b>Irradiation protocol</b>	<b>Mean thickness of epidermis (µm) ± SEM</b>	<b>Significance compared with non-irradiated</b>
Non-irradiated	10.9 ± 0.5	
2 days	12.9 ± 0.5	p=0.005
2 days + AD	13.0 ± 0.7	p=0.01
Non-irradiated	10.9 ± 0.6	
30 days	12.4 ± 0.6	p=0.08
30 days + AD	15.5 ± 0.8	p<0.001
Non-irradiated	12.6 ± 2.6	
60 days	15.0 ± 2.7	p=0.0008

We conclude that a small but significant increase in epidermal thickness occurred as a result of the irradiation protocol.

### DNA Damage

Mice were irradiated with a daily dose of 756mJ/cm<sup>2</sup> UVB for 2, 10 or 30 days. An additional dose of 1512mJ/cm<sup>2</sup> UVB was given on days 2, 10 or 30. 5mm punch skin biopsy samples were taken and DNA damage was analysed by immunohistochemistry using an antibody detecting thymine dimmers. The sections were given a score between 0-10, 0 being no DNA damage (no positive nuclei in the epidermis) and 10 being maximal DNA damage (all nuclei in the epidermis positive).

<b>Irradiation Protocol</b>	<b>mean DNA damage score (scale of 0-10)</b>
<b>Non-irradiated</b>	<b>0</b>
2016mJ/cm <sup>2</sup>	10

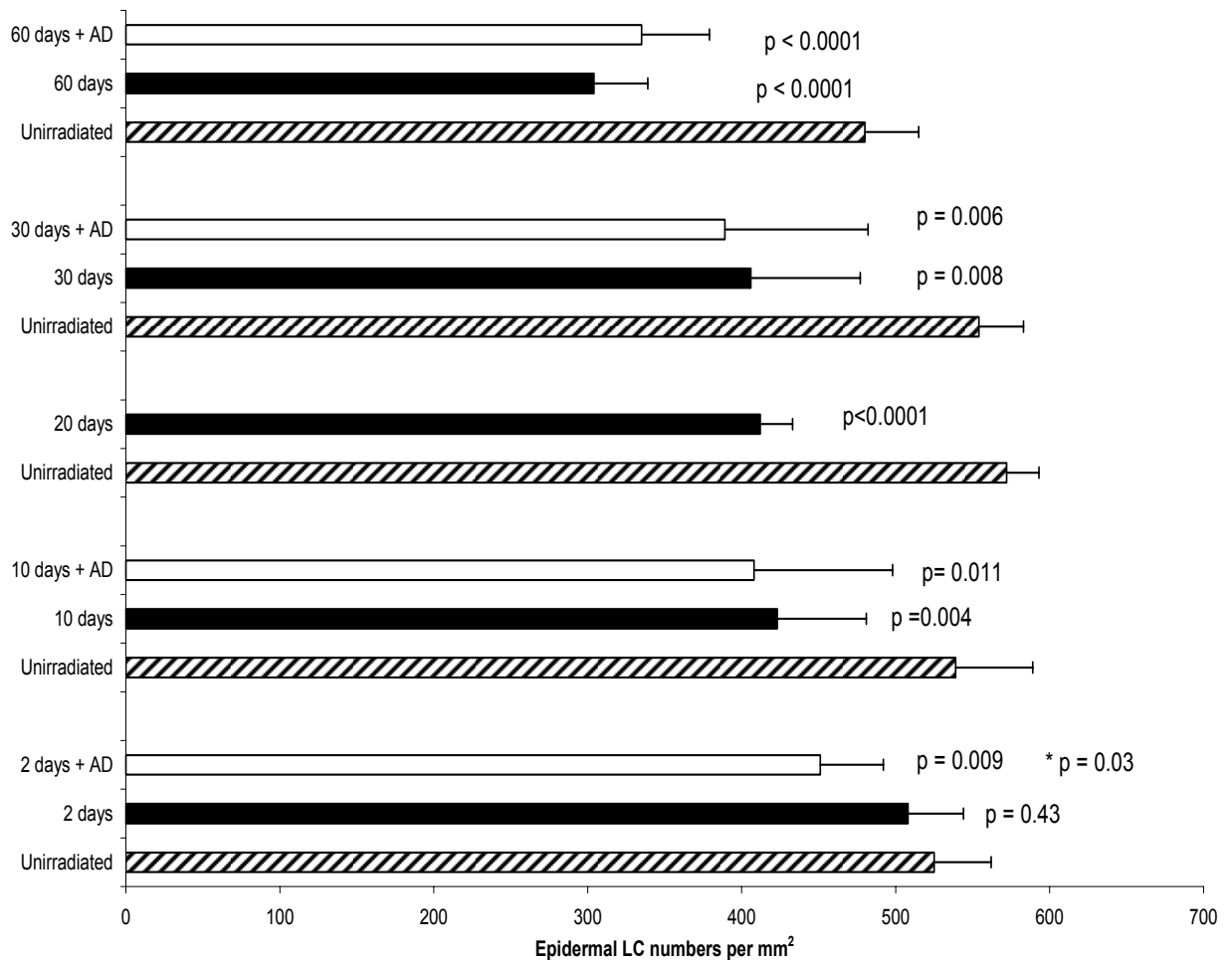
Non-irradiated	0
2 days	9
2 days + AD	7
Non-irradiated	0
10 days	8
10 days + AD	10
Non-irradiated	0
30 days	9
30 days + AD	8

DNA damage was at its maximum or close to the maximum (on the scale of 0-10 used) throughout the chronic irradiation study. Because of this it was not possible to detect small protective changes that may have occurred. DNA damage was still maximal after 30 days of irradiation; we therefore conclude that there was no apparent photoadaptive response.

Number of Langerhans (LCs) cells in the epidermis

For the chronic exposure and adaptation experiments, a dose of 252mJ/cm<sup>2</sup> UVB was used daily for 2, 10, 20, 30 or 60 days. Groups of mice also received an additional dose (AD) of 504mJ/cm<sup>2</sup> UVB on days 2, 10, 30 or 60 to test for adaptation. LCs were counted in epidermal sheets prepared from the ears after staining with ATPase. The results are given below.

Effect of exposure to SSR (3.7 J/cm<sup>2</sup>, 252 mJ/cm<sup>2</sup> UVB) daily for 2, 10, 30 or 60 days followed in some cases by a final additional dose (AD) of 7.4 J/cm<sup>2</sup>, on the number of epidermal Langerhans cells (LCs) in adult mice. P values show the statistical significance of the difference between irradiated and unirradiated mice (\* comparison between 2 day and 2 day + AD).



As the LC numbers were consistently reduced by the chronic irradiation protocol, we conclude that probably no adaptation or photoprotection developed. However there was a possible indication of a small protective effect of the UVR as, after 2 days of exposure, there was a significant reduction in the LC numbers in the mice receiving the AD compared with those not receiving the AD, while at all further time points, the numbers in these two groups did not differ significantly.

It was observed that LCs became elongated following chronic exposure. LC length was therefore measured by image analysis after 30 days of chronic irradiation and after 30 days of chronic irradiation plus an additional dose of 504mJ/cm<sup>2</sup> UVB. The results demonstrated that LCs increased from an average mean length of 36µm to 54µm (p=0.03) following the chronic irradiation protocol and to 50µm (p=0.01) following the chronic irradiation protocol plus the additional dose.

LC numbers were counted in the epidermal sheets from 4 day old mice that had been irradiated for 20 days with 252mJ/cm<sup>2</sup> UVB and in 4-day old mice that were not irradiated. Comparisons were made with adult mice that had been irradiated with the same irradiation protocol.

<u>Irradiation Protocol</u>	<u>Mean no. of LCs</u>	<u>% reduction LC numbers</u>	<u>p-value</u>	<u>95%CI</u>
Non Irradiated	509			
20 days (4-day-old mice)	336	34%	0.002	-113 (-266 to -81)

LC numbers were significantly decreased in mice irradiated from the age of 4 days old for 20 days in comparison to unirradiated control mice. The reduction in 4-day-old mice (34%) was greater than the reduction seen in adult mice (28%) that were also irradiated for 20 days, but this difference was not statistically significant. The difference in means of the number of LCs in irradiated adult mice and irradiated 4 day old mice was -76 (95% CI: -166 to 14),  $p = 0.085$  (unequal variances two-sample t-test).

The numbers and phenotype of dendritic cells (DCs)

DC numbers in the auricular lymph nodes of mice irradiated with  $252\text{mJ}/\text{cm}^2$  UVB daily for 2 or 60 days plus an additional dose of  $504\text{mJ}/\text{cm}^2$  UVB on days 2 or 60 have been analysed. DC numbers in the auricular, brachial, axillary and inguinal lymph nodes of mice irradiated with  $252\text{mJ}/\text{cm}^2$  UVB daily for 20 days have also been analysed. The results are shown below.

Effect of exposure to SSR ( $3.7\text{ J}/\text{m}^2$ ,  $252\text{ mJ}/\text{cm}^2$  UVB) daily for 2, 20 or 60 days, followed in some cases by a final additional dose (AD) of  $7.4\text{ J}/\text{m}^2$ , on the number of dendritic cells (DCs) in lymph nodes draining irradiated sites.

Irradiation Protocol	Mean DC number per lymph node †	SD	p-value (compared with unirradiated)
Unirradiated	2000	464	
2 days	2987	1105	0.35
2 days + AD	3658	1186	0.13
Unirradiated	3568	540	
20 days	7795	696	0.002
Unirradiated	2645	1025	
60 days	5462	1337	0.044
60 days + AD	5750		0.04

† Arithmetic mean of 3 experiments except for the 60 days + AD where the mean of 2 experiments is shown.

We conclude that chronic irradiation with the above irradiation protocol increases the number of DCs in the auricular lymph nodes and that no adaptation of this response occurs. No change in the expression of MHC II or B7.2 was observed at anytime point throughout the chronic irradiation protocol, as assessed by flow cytometric analysis.

### Macrophage Activity

For acute exposure mice were irradiated with single doses ranging from 360mJ/cm<sup>2</sup> to 2016mJ/cm<sup>2</sup>. Macrophage activity was measured by counting the ingestion of opsonised sheep red blood cells (SRBCs). The minimal immunosuppressive dose was found to be 2016mJ/cm<sup>2</sup> which induced a mean reduction in macrophage phagocytic activity of 35% in comparison to unirradiated mice.

Chronic exposure was undertaken for 2, 10 and 30 days. A daily dose of 1008mJ/cm<sup>2</sup> was used throughout the chronic experiments and an immunosuppressive dose (ID) of 2016mJ/cm<sup>2</sup> was given on days 2, 10 or 30.

<b>Irradiation protocol</b>	<b>Mean % reduction in SRBC per macrophage</b>	<b>Significance compared with non-irradiated</b>
2 days	32%	p<0.001
2 days + ID	39%	p<0.001
10 days	18%	p=0.009
10 days + ID	21%	p=0.007
30 days	4%	p=0.9
30 days + ID	7%	p=1.0

Statistical analysis was carried out using the students 2 sample t-test. Statistically significant reductions in macrophage phagocytic activity (p<0.05) were obtained in all experiments (n=3) when mice were irradiated with a single acute dose of 2016mJ/cm<sup>2</sup> UVB, irradiated for 2 days with 1008mJ/cm<sup>2</sup> UVB and irradiated for 2 days with 1008mJ/cm<sup>2</sup> UVB plus an additional dose of 2016mJ/cm<sup>2</sup> UVB.

In mice irradiated for 10 days with 1008mJ/cm<sup>2</sup> UVB and in mice irradiated for 10 days plus an additional dose of 2016mJ/cm<sup>2</sup> UVB a statistically significant reduction in macrophage activity was not obtained in all experiments (n=3). In mice irradiated for 30 days with 1008mJ/cm<sup>2</sup> UVB and in mice irradiated for 30 days with 1008mJ/cm<sup>2</sup> UVB plus an additional dose of 2016mJ/cm<sup>2</sup> UVB there was no significant reduction in macrophage phagocytic activity in any experiment (n=3)

We conclude that adaptation to the suppressive effect of UV radiation on macrophage activity occurred following chronic exposure.

We also analysed the acute effects of the Cleo Natural lamps on the ability of peritoneal macrophages from C3H/HeN mice to phagocytose *Staphylococcus aureus* bacteria. Doses of 1440mJ/cm<sup>2</sup> UVB and 2016mJ/cm<sup>2</sup> UVB suppressed the phagocytic activity of peritoneal macrophages 24h after irradiation by 35% (p=0.05) and 49% (p=0.01) respectively. By comparison 300mJ/cm<sup>2</sup> and 450mJ/cm<sup>2</sup> UVB from the TL-12 lamps suppressed the phagocytic activity of peritoneal macrophages 24h after irradiation by 24% (p=0.15) and 48% (p=0.05) respectively.

The expression of MHC II, FcRII/III, B7.2 and CD11b has been analysed on peritoneal macrophages by flow cytometry, following irradiation of C3H/HeN mice with a single dose of 2016mJ/cm<sup>2</sup> UVB from the Cleo Natural lamps. No significant change in any of these markers was observed 24h after irradiation; the results are shown below.

<b>Irradiation Protocol</b>	<b>% of cells within gated population expressing MHC II*</b>	<b>% of cells within gated population expressing FcRII/III †</b>	<b>% of cells within gated population expressing B7.2 †</b>	<b>% of CD11b †</b>
<b>Non irradiated</b>	<b>74 ± 4</b>	<b>82±7</b>	<b>95±3</b>	<b>93±4</b>
2016mJ/cm <sup>2</sup>	74 ± 3	81±3	92±0	95±3

\* Expressed as arithmetic mean of three experiments ± SEM

† Expressed as arithmetic mean of two experiments ± SEM

Supernatants from cultured macrophages collected from the peritoneum of non-irradiated and mice irradiated with a single dose of 2016mJ/cm<sup>2</sup> UVB, and stimulated with lipopolysaccharide for 48h *in vitro*, have been analysed for IL-12p40 and TNF-α.

<u>Irradiation Protocol</u>	<u>IL-12p40 levels pg/ml mean of 3 experiments</u>	<u>p-value</u>
<b>Non Irradiated</b>	<b>32</b>	
2016mJ/cm <sup>2</sup>	49	p=0.056

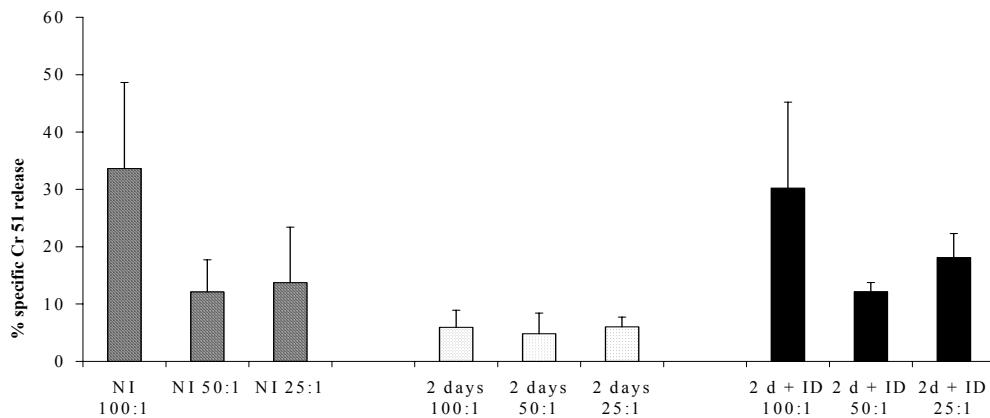
<u>Irradiation protocol</u>	<u>TNF-α levels pg/ml mean of 3 experiments</u>	<u>p-value</u>
<b>Non Irradiated</b>	<b>4600</b>	
2016mJ/cm <sup>2</sup>	6133	p=0.3

IL-10 and IL-12p40 levels were increased in the supernatant of macrophages from mice irradiated with 2016mJ/cm<sup>2</sup> UVB. However this increase was not statistically significant.

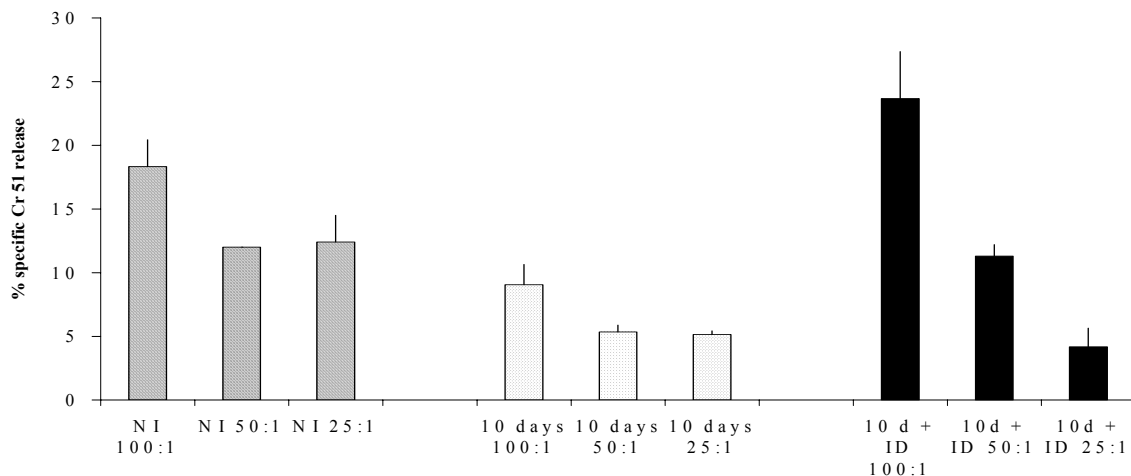
Natural Killer (NK) cell activity

Mice were irradiated with various single doses from the Cleo lamps, NK cell activity was measured in cells prepared from the auricular lymph nodes by radioactive chromium release from labelled YAC cells as targets. UVB doses of 2016mJ/cm<sup>2</sup>, 1440mJ/cm<sup>2</sup> and 720mJ/cm<sup>2</sup> reduced NK cell activity, but 360mJ/cm<sup>2</sup> did not.

For the chronic exposure and adaptation experiments we used a daily dose of 756mJ/cm<sup>2</sup> UVB for 2, 10 or 30 days. An additional immunosuppressive dose of 1512mJ/cm<sup>2</sup> UVB was given on days 2, 10 or 30.

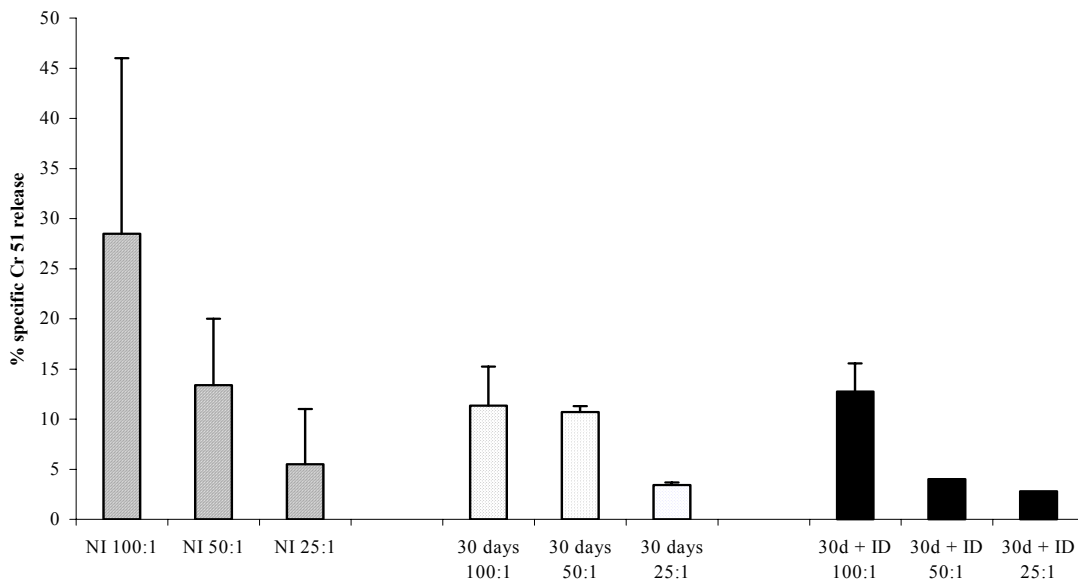


**Figure 1:** NK cell activity in non-irradiated mice (NI) (diagonal bars), in mice irradiated for 2 days with 756mJ/cm<sup>2</sup> UVB (dotted bars) and in mice irradiated for 2 days plus an immunosuppressive dose (ID) of 1512mJ/cm<sup>2</sup> UVB (solid bars) at effector: target cell ratios of 100:1, 50:1 and 25:1. The graph depicts the mean ± SEM of three experiments.



**Figure 2:** NK cell activity in non-irradiated mice (NI) (diagonal bars), in mice irradiated for 10 days with 756mJ/cm<sup>2</sup> UVB (dotted bars) and in mice irradiated for 10 days plus an

immunosuppressive dose (ID) of 1512mJ/cm<sup>2</sup> UVB (solid bars) at effector: target cell ratios of 100:1, 50:1 and 25:1. The graph depicts the mean ± SEM of two experiments.



**Figure 3:** NK cell activity in non-irradiated mice (NI) (diagonal bars), in mice irradiated for 30 days with 756mJ/cm<sup>2</sup> UVB (dotted bars) and in mice irradiated for 30 days plus an immunosuppressive dose (ID) of 1512mJ/cm<sup>2</sup> UVB (solid bars) at effector: target cell ratios of 100:1, 50:1 and 25:1. The graph depicts the mean ± SEM of two experiments.

NK cell activity varied considerably between experiments and the standard errors of the mean were large. However notable trends were present suggesting that irradiation of mice with 756mJ/cm<sup>2</sup> UVB daily for 2 or 10 days suppressed NK cell activity and that stimulation of NK cell activity occurred when the additional dose was administered on days 2 or 10. NK cell activity in mice that have been irradiated with 756mJ/cm<sup>2</sup> UVB daily for 30 days and in mice that received an additional dose on day 30, showed a trend towards suppression of NK cell activity. Whilst there was stimulation of NK cell activity when the additional dose was administered on days 2 and 10, this was not the case when the additional dose was administered on day 30. We therefore conclude that chronic irradiation for 30 days does not induce adaptation.

### Mast Cells

Mast cell numbers have been counted in skin sections from mice that were irradiated with 756mJ/cm<sup>2</sup> UVB for 2, 10 or 30 days and in mice that received an additional dose of

1512mJ/cm<sup>2</sup> UVB on days 2, 10 or 30. Mast cell numbers have also been counted in skin sections from mice irradiated with an acute dose of 2016mJ/cm<sup>2</sup> UVB.

<u>Irradiation Protocol</u>	<u>Mean number of mast cells per field</u>	<u>value</u>
<b>Non-Irradiated</b>	<b>15.8</b>	
Acute 2016mJ/cm <sup>2</sup>	14.0	
Non-Irradiated	15.3	
2 days	13.6	
2 days + AD	18.4	0.09
Non-Irradiated	14	
10 days	14.4	
10 days + AD	14.3	
Non -Irradiated	14.3	
30 days	15.6	
30 days + AD	14.4	
Non-irradiated (4 day old mice)	15.8	
Irradiated 20 days (4 day old mice)	14.6	

A small increase in mast cell numbers was observed when the additional dose was administered on day 2, but this was not statistically significant (p= 0.09), no change in mast cell numbers was observed at any other time.

### **Quantitative measurement of cytokine levels in the skin**

Quantitative analysis of cytokine levels (IL-10, IL-12p40, TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ ) in the skin of mice has been carried out following an acute irradiation of 2016mJ/cm<sup>2</sup> UVB Cleo and following a chronic irradiation protocol of 756mJ/cm<sup>2</sup> UVB for 2, 10 or 30 days. Groups of mice also received an additional dose of 1512mJ/cm<sup>2</sup> UVB on days 2, 10 or 30.

Cytokine levels were measured by ELISA in the supernatant of 5mm homogenised skin biopsies.

The results demonstrated that TNF- $\alpha$  and IL-10 levels were significantly increased following 10 days of irradiation with 756mJ/cm<sup>2</sup> UVB; the quantities of these cytokines are shown in the table below.

<u>Irradiation Protocol</u>	<u>TNF -<math>\alpha</math> levels pg/g wet weight</u>	<u>IL-10 levels pg/g wet weight</u>
-----------------------------	--	-------------------------------------

<b>Non irradiated</b>	<b>2300</b>	<b>10,000</b>
10 days	5800 (p < 0.05)	36,000 (p<0.05)

There was no statistically significant increase in TNF- $\alpha$  and IL-10 levels at any other time in the study. There was no statistically significant increase in IL-1 $\beta$ , IFN- $\gamma$  or IL-12p40 at any time point throughout the study.

#### Bone Marrow Cells

We have observed that bone marrow cells taken from the femur of Cleo irradiated mice grew better in the presence of GM-CSF and IL-4 in comparison to bone marrow cells from non irradiated mice. This effect has been seen with both acute and chronic irradiation protocols. The number and phenotype of these bone marrow cells following 60 days of Cleo irradiation with 252mJ/cm<sup>2</sup> UVB and in mice that received an additional dose of 504mJ/cm<sup>2</sup> UVB on day 60 have been examined. The number of bone marrow cells in four femurs was counted 24h after the final irradiation in all groups. Bone marrow cells were then cultured overnight for 24h and the non-adherent cells were counted. The results are shown in the table below.

<u>Irradiation Protocol</u> <u>count</u>	<u>Total bone marrow cell count</u> <u>(day 0) (4 femurs)</u>	<u>Non adherent cell</u> <u>after culture for 24h</u>
Non-irradiated	10.0 x 10 <sup>6</sup>	5.1 x 10 <sup>6</sup>
60 days	8.4 x 10 <sup>6</sup>	9.56 x 10 <sup>6</sup>
60 days + AD	9 x 10 <sup>6</sup>	9.4 x 10 <sup>6</sup>

In the non irradiated group the non adherent cell count (24h) was lower than the total cell count (day 0). In the 60 day UV irradiated group and in the group receiving the 60 day chronic irradiation plus the additional dose, the non adherent cell count (24h) was higher than the total cell count. We conclude that UV irradiation of mice promotes the survival of bone marrow cells *in vitro*.

#### Phagocytic activity of blood neutrophils

We have attempted unsuccessfully to develop a protocol to analyse the effects of the Cleo Natural lamps on the phagocytic activity of blood neutrophils in mice using opsonised zymosan particles. The main problem was that there was no difference in fluorescence between neutrophils incubated in the presence of opsonised zymosan particles at 4°C or 37°C. This suggests that it was not possible to distinguish between zymosan particles on the outside of the cells and those that had been internalised. The zymosan particles also clumped together making it difficult to distinguish between clumps of zymosan particles and neutrophils. No further progress has been made in this area.

### **Progress report year 2005 WP 6 and 7**

In 2005 the experiments done earlier were completed and statistical analysis was finalized. Where this led to additional information, tables and figures that were reported earlier were replaced (see above in blue).

### **Overall evaluation and conclusions WP6 and 7.**

In conclusion, from the results obtained in Workpackages 6 and 7, repeated exposure to small doses of solar simulated radiation consistently suppressed innate immune responses in mice, with no evidence for photoadaptation or for the development of photoprotection. The only exception was for the phagocytic activity of peritoneal macrophages which was initially suppressed by the UV exposures but which recovered to the pre-irradiation level as the exposures continued: the mechanism of this photoadaptation was not discovered.

### **Deliverables:**

Macve JC, McKenzie RC and Norval M. (2004) Exposure to multiple doses of UVB radiation reduces the numbers of epidermal Langerhans cells and lymph node dendritic cells in mice. *Photochemical and Photobiological Sciences* 3, 91-95.

McLoone P and Norval M. Adaptation to the UV-induced suppression of phagocytic activity in murine peritoneal macrophages following chronic exposure to solar simulated radiation. Submitted to *Photochemical and Photobiological Sciences*.

McLoone P, Woods GM and Norval M. Decrease in Langerhans cells and increase in lymph node dendritic cells following chronic exposure of mice to suberythemal doses of solar simulated radiation. Submitted to *Photochemistry and Photobiology*.

Norval M and McLoone P. The chronic effects of ultraviolet radiation on immune parameters. Manuscript in preparation.

**WP.7      Adaptation to effects of UV exposure on innate immune parameters in mice**

**Workpackage number:** 7  
**Start date of starting event:** month 18  
**Completion date:** month 32  
**N° of the partner responsible:** 1, 4  
**Person-months per partner:** CO1; CR4:16.5  
**Total person months:** 17.5

**Objectives**

To establish the adaptive activity of UV exposure on subsequent exposures of UV that suppress innate immune responses in mice

**Description of work**

Groups of shaved BALB/c (UV sensitive) and C3H (UV resistant) mice will be exposed to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for 2, 10, or 30 consecutive days. At these time points, these mice and controls will receive a single suppressive dose of 3 MED. Subsequent to exposure, spleens will be taken and tests for innate immune parameters as mentioned in workpackage 5 will be performed. In order to compare immune effects with non-immune effects of chronic UV exposure, the non-immune parameters as indicate in workpackage 5 will be assessed as well.

In addition to these experiments, other experiments will be carried out in both strains of mice in which both duration and dose of the UV pre- exposure are varied in such a fashion that the total irradiation that the mice will receive remains constant. For this purpose, the same parameters as indicated above will be studied.

**Deliverables**

Report on adaptation to UV effects on innate immune parameters in the mouse, and the duration times dose relationship of UV induced adaptation to suppression of innate immune parameters.

**Milestones and expected results**

This workpackage will the potency of chronic UV exposure to induce adaptation to subsequent exposure to suppressive doses of UV on innate immune responses in mice, and how such adaptation compares to adaptation to non-immune effects of UV. Also, the impact of different genetic background will be established. As such, the information yielded by this workpackage may confirm and extend the data gained from the experiments in humans. In addition, the relation between duration and dose of UV pre-exposure, which cannot readily be assessed in humans, will be established.

**Progress report year 2002 WP 7: See WP 6**

**Progress report year 2003 WP 7: See WP 6**

**Progress report year 2004 WP 7: See WP 6**

**Progress report year 2005 WP 7: See WP 6**

**Overall conclusion WP7: See WP6**

**WP.8 Chronic effects of UV exposure on acquired immune parameters in mice**

**Workpackage number:** 8  
**Start date of starting event:** month 18  
**Completion date:** month 18  
**N° of the partner responsible:** 1  
**Person-months per partner:** CO1:20  
**Total person months:** 20

**Objectives**

To establish the effect of chronic UV exposure on acquired immune parameters in mice

**Description of work**

Groups of shaved BALB/c (UV resistant) and C3H (UV sensitive) mice will be exposed to UV emitted from CLEO natural lamps, at 0.7 individual MED doses for 2, 10, 30, or 90 consecutive days. Subsequent to exposure, the mice and control mice will be sensitised with DPCP on the irradiated skin, and the contact hypersensitivity response to DPCP challenge of the UV shielded ears will be measured. These experiments will test the effects of UV exposure on primary immune responses.

In other experiments, mice will be sensitised first, and then exposed to UV. After termination of exposure, *ex vivo/in vitro* antigen specific proliferative responses of spleen lymphocytes will be assayed, to investigate the effects of UV on secondary immune responses.

In addition to these experiments, other experiments will be carried out in which both the duration and dose of UV exposure are varied in such a fashion that the total irradiation that the mice will receive remains constant.

In both sets of experiments, dendritic cell type characterisation will be performed in skin biopsies of sensitised and irradiated skin (histochemistry), FACS analysis of lymph node cells, and functional capacity of dendritic cells in lymph nodes (by *in vitro* antigen-presentation, measured as induction of lymphocyte proliferation and production of cytokines in culture supernatants).

Finally, experiments will be carried out in which the total dose that the animals receive will be similar, but the skin surface that will be exposed is varied. For this purpose, the same parameters as indicated above will be studied.

**Deliverables**

Report on effects of chronic exposure to UV exposure on murine acquired immune parameters

**Milestones and expected results**

The expected result is that we will have insight in the effects of chronic UV exposure that resembles that of outdoor exposure on acquired immune parameters, and how such immune effects relate to non-immune effects on the skin. In addition, information will be provided on interindividual variability. Duration x dose and dose x surface relationships of UV induced suppression of responses will become clear. The results may confirm and extend the information on chronic effects on acquired immune responses in humans, in that besides effects on primary immune responses also effects on secondary immune responses will become clear.

## **Progress report year 2002 WP 8 and 9**

### *Pilot study*

Within our department effects of ultraviolet B exposure on hepatitis B vaccination responses have been investigated in the past four years. Hepatitis B vaccination was used as a model of the immune response to an infectious agent, and effects of UVB exposure on these vaccination responses have been determined in mice and in human volunteers. The overall conclusion was that UVB exposure prior to hepatitis B vaccination could suppress the vaccination responses in two different mouse strains (BALB/c and C57Bl/6). In human volunteers the picture was a bit more complicated; effects were noted but suppressed hepatitis B vaccination responses after UVB exposure were only observed in subpopulations of the human volunteers (i.e. depending on IL-1 $\beta$  polymorphism).

Within this project we investigated whether adaptation to UVB could explain the interspecies difference between the results from the mouse and the human hepatitis B vaccination model. We investigated this in BALB/c mice (3 weeks of age) that were adapted to UVB for 6 consecutive weeks with 1/3 MED UVB per day, after which they received an immunosuppressive protocol of 5 consecutive days with 3/4 MED UVB per day. After this irradiation protocol, mice were either vaccinated against hepatitis B, or sensitised on unirradiated skin with a contact-allergen, picryl chloride. Delayed type hypersensitivity (DTH) responses to hepatitis B antigen or contact hypersensitivity (CHS) responses to picryl chloride were assessed. It was demonstrated that our adaptation protocol of 6 weeks with 1/3 MED UVB per day could prevent the UVB-induced suppression of DTH responses to hepatitis B antigen. In addition, the UVB-induced suppression of antigen-specific lymphocyte proliferation responses was also prevented. In contrast, the used adaptation protocol could not prevent the UVB-induced systemic suppression of CHS responses to picryl chloride. This disparity in results may stem from mechanistic differences in UV-induced suppression of the different immune responses. Alternatively, the vigour of the immune responses due to hepatitis B vaccination and picryl chloride are difficult to compare, and differences in the strength of the responses may result in differences in sensitivity to immunosuppression by UV.

### *Cleo Natural lamps*

Earlier studies on UV effects and UV-caused adaptation have used artificial UV emitters, such as the TL12 UVB lamp. However, radiation from this lamp is not similar to radiation emitted by natural sunlight. Cleo natural lamps are designed to approximate natural sunlight as close as possible, and within this project it was decided to use this lamp in order to investigate chronic and adaptive responses. For the experiments in mice, Cleo natural lamps with a length of 1.20 meters were made as a special order by Philips, Eindhoven, the Netherlands. The Cleo natural lamps did not work in the animal exposure setting built for the FS40 and TL12 lamps at RIVM. The electronics required adaptation which resulted in a delay in the onset of the experiments. The lamps are now in operation.

### *Solar Light 3D meters*

To measure the intensity of the Cleo lamps all participants are using the Solar Light 3D UV meter. These devices were ordered by the participants in Turku, and sent to us so we

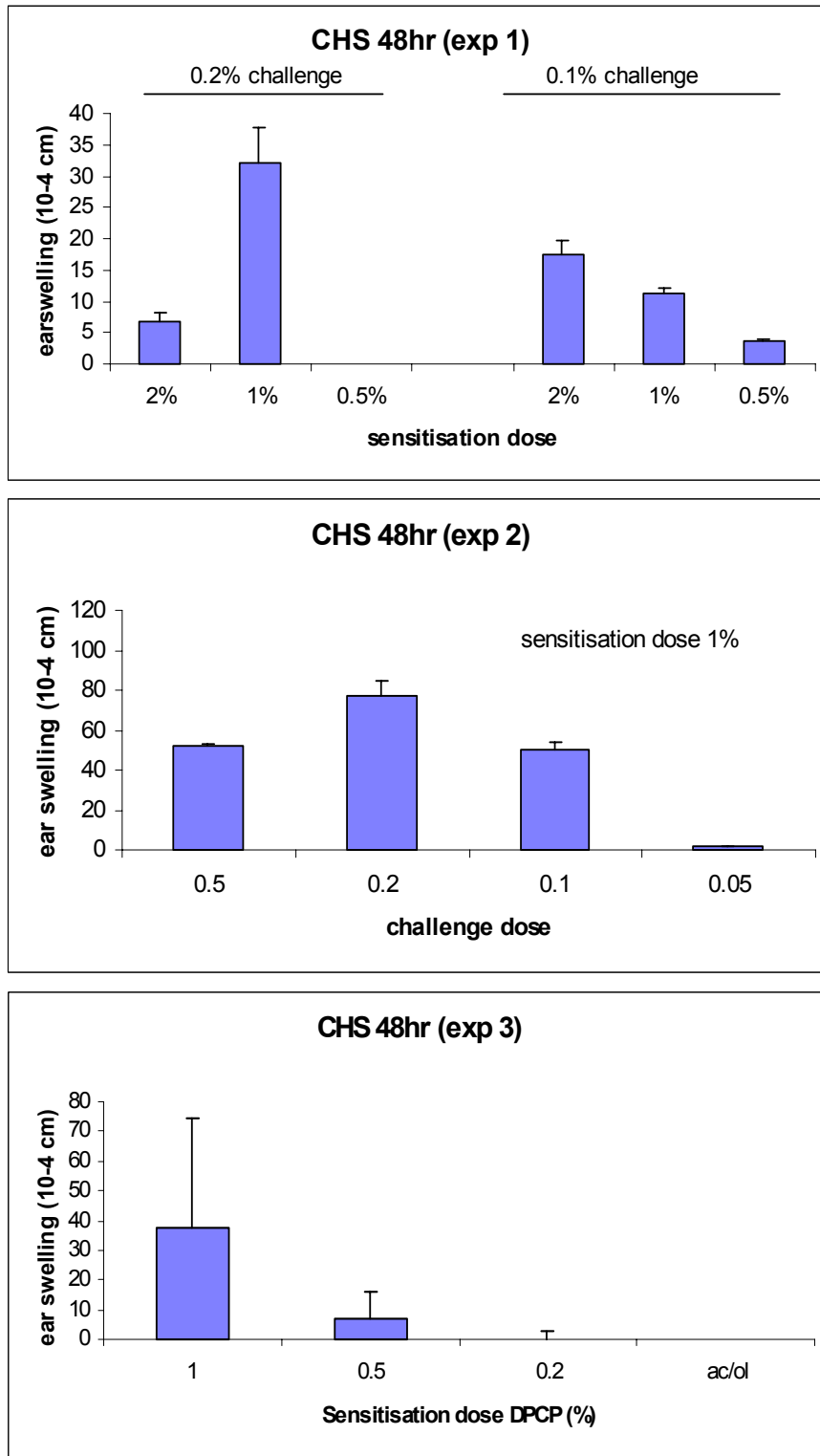
could arrange the calibration of all four meters. One of the meters was calibrated by Huib van Weelden (University Medical Center Utrecht) using 10 Cleo Natural lamps of 70 W at a distance of 50 cm and an Optronics Spectrophotometer. We then compared the calibrated meter with the other three meters using 4 Cleo Natural lamps of 35W at a distance of 20 cm. The correction factors between the meters were calculated, and every participant then received a table stating the output of their own meter.

*MED testing with the Cleo Natural lamps.*

Experiments were performed to determine the MED in mice. BALB/c mice were irradiated for 0, 15, 30, 45, 60, 75, 90, 105 or 120 minutes with the Cleo Natural lamps. Ear swelling and redness/oedema were measured 24 and 48 h later, and the back skin examined for vascularisation at 48 h. No signs of MED were detected. The output of the Cleo lamps was compared with that of the broadband TL12 lamp, knowing that 2000 J/m<sup>2</sup> UVB (280-320 nm) from the TL12 source is sufficient to induce a MED in BALB/c mice. After 60 minutes irradiation with the Cleo Natural lamp the mice had received a dose of approximately 2000 J/m<sup>2</sup> UVB (280-320 nm) and 28500 J/m<sup>2</sup> UVA (320-380 nm). Therefore, we expected that this UVB dose would have been sufficient to cause oedema. However it failed to do so, and the design of the mouse experiments will require further consideration as a result (see below).

*Analysis of optimal sensitisation and challenge doses of diphenylcyclopropenone (DPCP)*

To enable the mice and human studies to be compared, DPCP will be used as a sensitiser. As data on sensitisation and challenge doses of DPCP in mice have not been published, experiments were performed to determine the optimal doses (See figure 1). Mice were sensitised on shaved back skin with 150 µl DPCP in acetone/olive oil (4:1). Challenge was performed 4 days later on the ears with one drop (27-gauge needle) of DPCP in acetone/olive oil (4:1). Ear swelling was measured 24, 48 and 96 hours after challenge. The first experiment showed that 1% DPCP was the optimal sensitisation dose. The challenge doses, however, needed to be further defined. In the second experiment the mice were sensitised with 1% DPCP, and challenged with various doses. This experiment showed that the optimal challenge dose was 0.2% DPCP. However, in this experiment the back skin of the mice was damaged by the sensitisation, and some of the sensitised mice died. For this reason, we wanted to perform an additional experiment, that was also required by the animal ethical committee of our institute. In this third experiment we found that the sensitisation (1%) and challenge (0.2%) dose were optimal, and within this experiment no skin lesions or pathological changes were observed within the animals, besides a slight increase in draining lymph nodes. The reason for the lesions in the earlier experiment could not be explained, and must be characterized as an anomaly in the experiment.

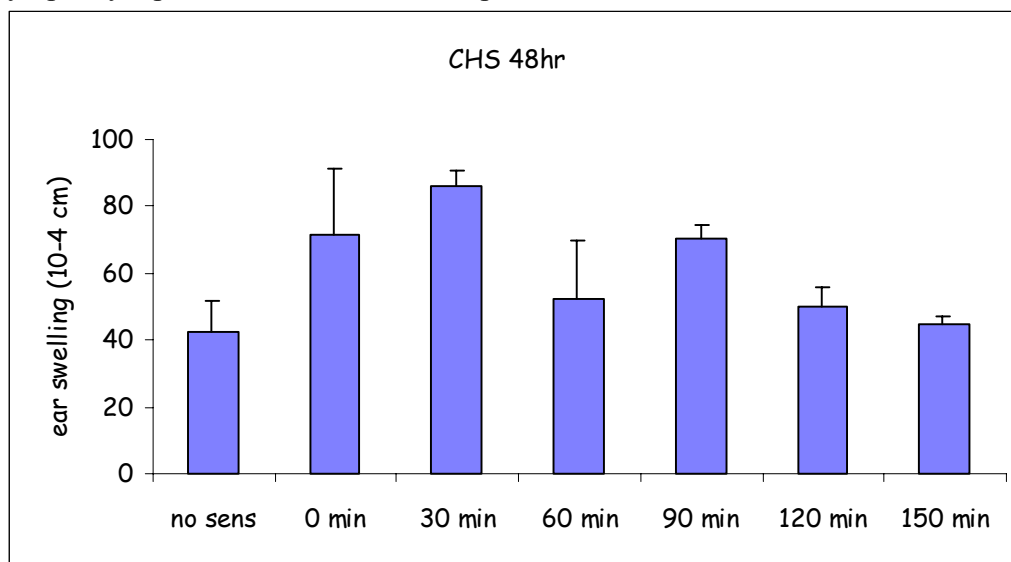


**Figure 1.** Optimisation of sensitisation and challenge doses of diphenylcyclopropenone (DPCP). Vertical axis shows the net ear swelling 48 h after challenge with DPCP.

Horizontal axis shows varying sensitisation doses (experiment 1 and 3), or varying challenge doses (exp 2).

*Minimal immunosuppressive dose of Cleo Natural lamp*

In both the human and mouse studies done to date using the Cleo lamps, there are problems in obtaining a MED. Therefore it was decided by all the participants at the progress meeting in June at Turku that the mice will be irradiated chronically with 0.7 of the minimal immunosuppressive dose (MID) of the Cleo Natural lamp (in stead of 0.7 MED), after which they receive a 3 MID dose to determine adaptation. RIVM attempted to determine the MID dose for suppression of contact hypersensitivity parameters. Within this context one experiment has been performed, in which BALB/c mice were irradiated once with the Cleo Natural lamp for 30, 60, 90, 120 or 150 minutes. The following day, mice were sensitised with DPCP and four days later the ears were challenged. Irradiation with Cleo Natural lamps did not induce significant suppression of CHS responses (figure 2) In addition lymphocyte proliferation responses were performed with spleen cells, but no significant effects were detectable on mitogenic responses, and antigen-specific lymphocyte proliferation needs to be optimised.



**Figure 2.** Contact hypersensitivity responses after irradiation with Cleo Natural for 0, 30, 60, 90, 120 or 150 minutes (horizontal axis)

*Future experiments*

Reasons for the fact that we did not observe significant suppression of CHS responses after irradiation with the Cleo Natural lamps for up to 21/2 h could be that we exposed the mice to one single dose, and sensitised them the following day, as opposed to protocols used earlier on, in which immunosuppressive (TL12 lamps) doses were given at 5 consecutive days, followed by sensitisation 3 days after the last UV exposure. Therefore, currently an experiment is being performed in which BALB/c mice are exposed 5 consecutive days to different doses of the Cleo lamps. Sensitisation will be

performed three days later. Within this experiments draining lymph nodes will be collected in stead of spleen cells to perform lymphocyte proliferation tests.

Preliminary studies using splenic lymphoid cells from DPCP sensitised mice have been carried out. So far, no in vitro specific proliferative responses to co-culture with the hapten were observed. New experiments will be performed, in which various conditions (i.e. the concentration of DPCP in vitro, the use of carriers to which DPCP are conjugated, etc.) will be investigated. In addition, additional experiments are set up, to optimise isolation of dendritic cells form draining lymph nodes and characterisation of dendritic cell subtypes.

### *Concerns*

In the Netherlands the rules to perform animal experiments have recently been changed. One of the rules is that experiments that can be performed in humans are in principle not allowed to be performed in animals. One of the goals of this project was that parallel studies are performed in mice and human volunteers to make extrapolation from the mice to the human situation possible, to increase the risk estimation of the health consequences of ultraviolet exposure for humans. Obviously, studies performed in mice and humans are not fully identical, yet, this legislation hampers us from makes it for us very difficult to obtain permission from our animal ethical committee, and causes a delay in the performance of the experiments.

### **Progress report year 2003 WP 8 and 9**

In the model using the contact hypersensitivity (CHS) responses after sensitization with diphenylcyclopropenone (DCP) we were not able to find reproducible ear swelling responses. Moreover, lymphocyte proliferation responses performed with spleen cells did not show sufficient stimulation after sensitization with DCP and under these conditions no immunosuppression was detected. Studies were carried out to optimize antigen-specific lymphocyte responses.

A number of experiments have been performed to find immunosuppression induced by the Cleo natural lamps. Since DCP did not induce a CHS response on the ears of mice, a more potent sensitized was tested: picryl chloride (PCI). In Table 1 the experimental design of the induction of immunosuppression caused by short-time radiation is presented. Cleo natural lamp radiation was carried out during 5 days for 30 to 120 min. to induce immunosuppression. (during 60 min. radiation with Cleo lamps mice received a dose of 2000J/m<sup>2</sup> UVB (280-320nm) and 28500 J/m<sup>2</sup> UVA (320-380nm)). As a positive control for immunosuppression, mice were irradiated with the TL12 lamps for 20 min. (3/4 MED, 1500 J/m<sup>2</sup>). Three days after the last radiation mice were sensitized to PCL (5%) on back and feet and challenged (0.8%) 4 days later on both ears.

group (n=4)	UV min.	5% PCL (dag 0)	Chal 0.8% PCL (dag 4)
1	0	-	+
2	0	+	+
3	Cleo 30	+	+
4	Cleo 60	+	+
5	Cleo 90	+	+
6	Cleo 120	+	+
7	TL12 20	+	+

Table 1. Effect of Cleo and TL12 on the CHS of PLC: experimental design.

In Figure 1 the results of the CHS immunosuppression are presented. PCI induced a high CHS response (control). During the Cleo exposure from 30 to 120 min. an increasing immunosuppression was noticed, which was statistically significant after 120 min of radiation. The immunosuppression due to the TL12 lamp for 20 min was much more pronounced.

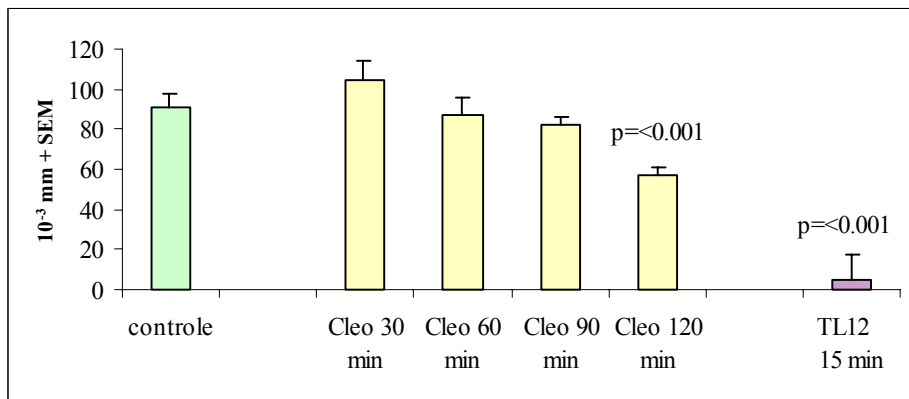


Figure 1 Effect of Cleo and TL12 radiation on CHS to PLC

Since CHS ear swelling responses were not sufficiently induced by DCP, the lymphoproliferative responses of draining lymph nodes was used to measure DCP responsiveness. The experimental design of the induction of immunosuppression caused by short-time radiation was as follows: Cleo natural lamp radiation was carried out during 5 days for 30 to 120 min. to induce immunosuppression. (during 60 min. radiation with Cleo lamps mice received a dose of 2000J/m<sup>2</sup> UVB (280-320nm) and 28500 J/m<sup>2</sup> UVA (320-380nm). similar to the study design used for immunosuppression of PCL. Three days after the last radiation mice were sensitized to DCP (1%) on back and feet and challenged (0.2%) 4 days later on both ears. The next day, axillary and inguinal lymph

nodes were isolated, and cell suspensions were prepared and tested in vitro for proliferation by incorporation of labeled tritium thymidine ( $^3\text{H-Thy}$ ).

In Figure 2 it is shown that the proliferation after challenge is high compared to mice not sensitized. The immunosuppression of mice irradiated with the TL12 lamp was very strong. Irradiation with the Cleo Natural lamps caused suppression after daily irradiation from 60 to 120 min. ( $p < 0.05$ ). Irradiation during 120 min. daily was comparable with the immunosuppression caused by the TL12 lamps.

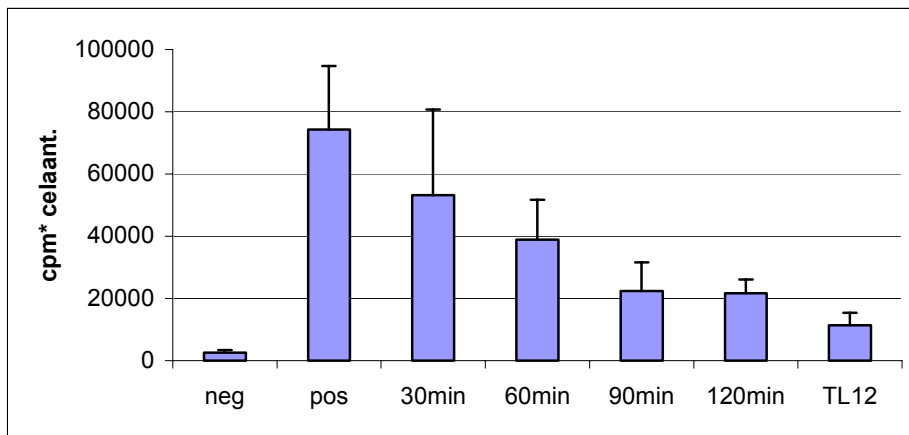


Figure 2: Immunosuppression by Cleo and TL12 measured by effects on lymphocyte proliferation

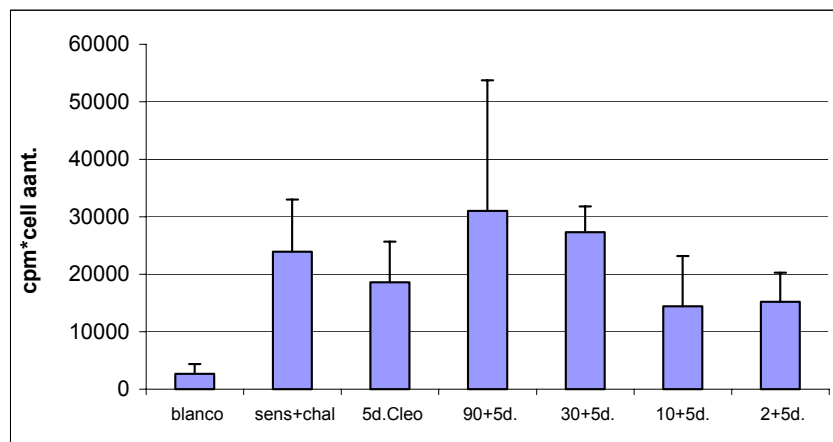
From the results described above (Figure 2), a study was initiated to evaluate the potential adaptation potency of the Cleo Natural lamps reducing immunosuppression. To induce adaptation, mice were irradiated (75 min) with the Cleo Natural lamps for 90, 30, 10 and 2 days. Thereafter, the mice were irradiated again with 75-min. Cleo Natural lamps for 5 days to induce immunosuppression. After three days without irradiation, mice were sensitized and challenged with DCP during 5 days and axillary and inguinal lymph nodes were isolated after sacrifice of the mice (Table 2).

Table 2: induction of adaptation of Cleo induced immunosuppression: experimental design

group	Cleo 75 min	Cleo 75 min 5 days	Sensitization 1%DCP	Challenge 0,2% DCP
1	-	-	-	-
2	-	-	+	+
3	-	+	+	+
4	90 days	+	+	+
5	30 days	+	+	+
6	10 days	+	+	+
7	2 days	+	+	+

In Figure 3 it is shown that sensitization and challenge with DPC induces proliferation of cells, however the immunosuppression by Cleo natural lamps (5d.Cleo) was less pronounced compared to the previous study (Figure 2). However, adaptation during 5 or 10 days show also a tendency of immunosuppression while 30 days and 90 days of adaptation showed a tendency of immunostimulation. The reason that in this experiment the immunosuppression was less pronounced may be a result of the daily radiation of 75 min during the induction of immunosuppression. The experiment is now being repeated with immunosuppression induced by 120 min. Cleo exposure.

Figure 3. Induction of adaptation of Cleo induced immunosuppression in DCP immune mice



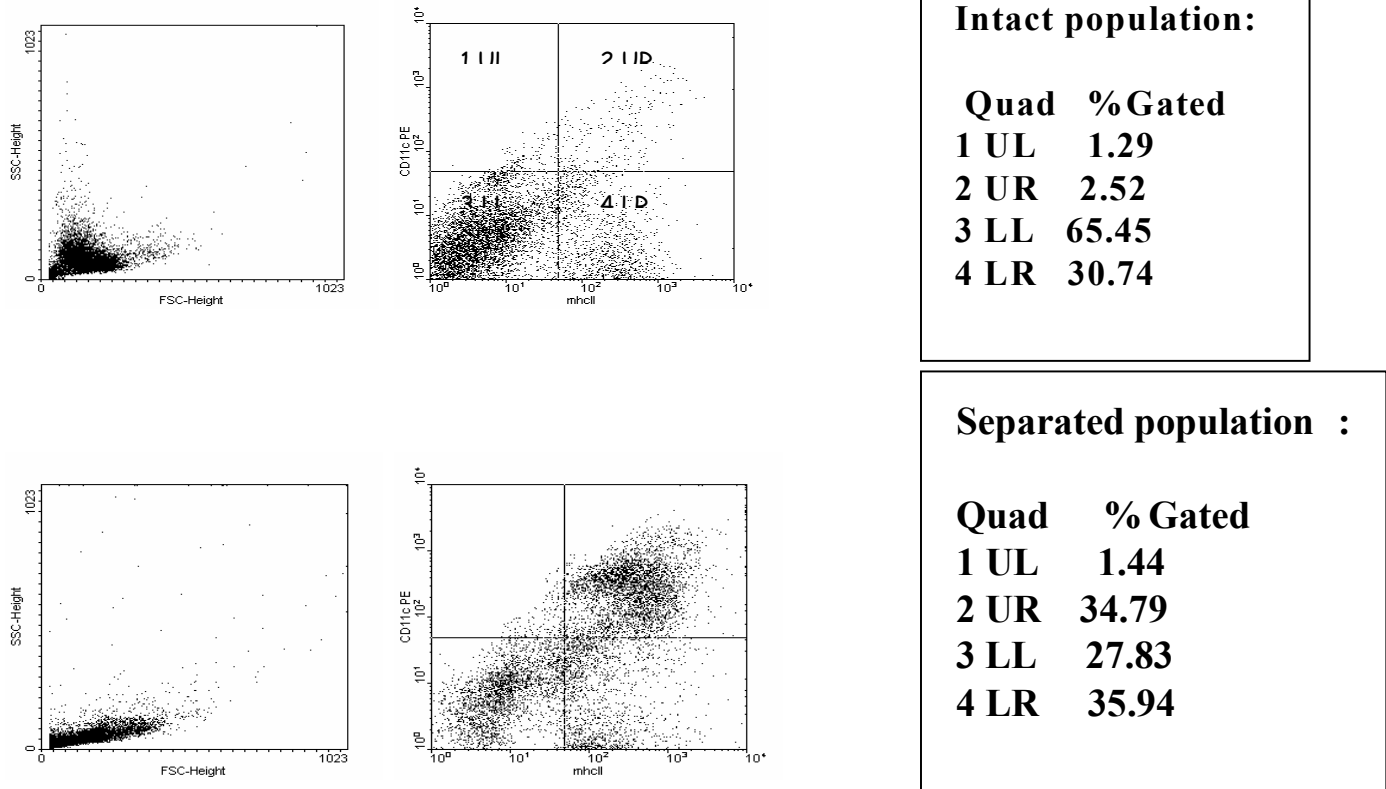
### Role of dendritic cell (DC) in the induced immunosuppression or adaptation

#### Experimental design.

To study the role of the DC in the induction of immunosuppression and adaptation, lymph node cells of DCP immune mice are studied. Mice are sensitized and challenged on ear, back and feet to collect a high number DCP immune cells. To study the role of DC's, free DCP, DC (from immune irradiated and non-irradiated mice) and DC pulsed

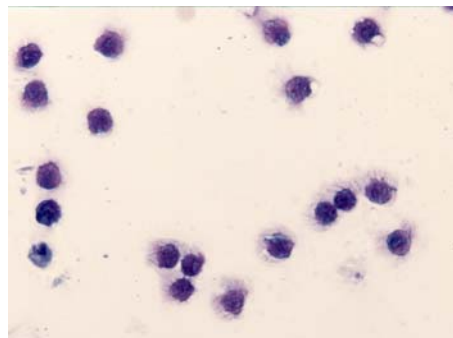


**Figure 3 Results of separation DC: FACS analysis**



Using only the first step of enrichment, from Figure 5 it is shown that DC can be enriched from 2.5 to 34.7%. To increase the DC population more, cells were incubated (one hour 37<sup>0</sup>C) on a plastic surface to allow adherence of the cells. After washing carefully the plastic surface the remaining cells adhere to the surface (Figure 6).

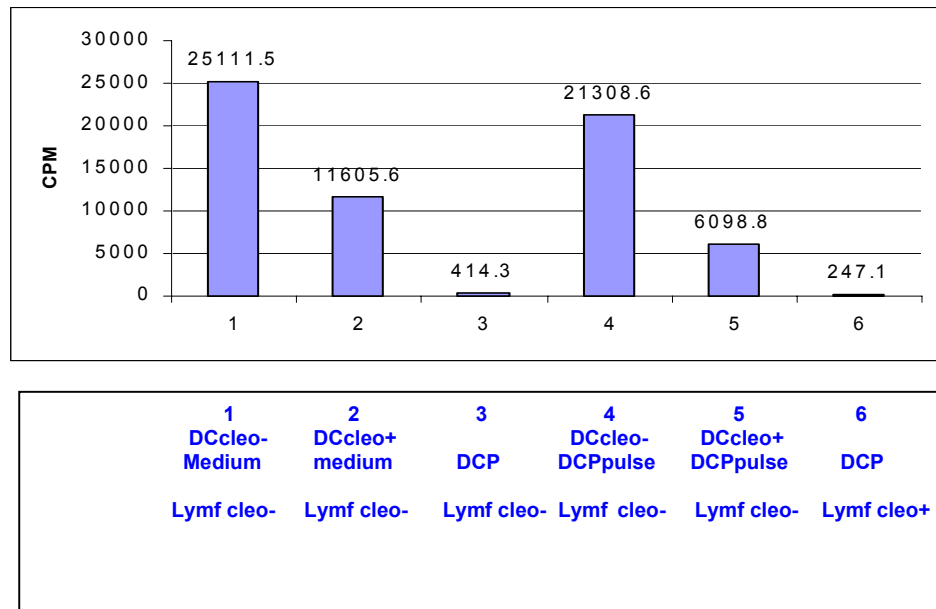
**Figure 6: Adherent DC**



**Effect of enriched DC (after adherence) from Cleo irradiated and non-irradiated lymph nodes on the in vitro stimulation of DCP-immune lymph node cells.**

To study the effect of UV radiation on the function of enriched DC population, mice were irradiated 5 days with Cleo (120min/day) and after 3 days they were sensitised and challenged with DCP. One group was not irradiated. Lymph node cells were isolated and a DC enriched population was prepared (irradiated and non-irradiated). The isolated DC enriched population was cultured for one hour to adhere to the surface of the 96 wells, washed and pulsed with DCP for another one hour. To avoid free DCP, cells were washed again and incubated with the intact non irradiated population of DCP immune lymph node cells for 4 days. Proliferation was measured by <sup>3</sup>HTd uptake at day 4.

Figure 7



From Figure 7 it is shown that adherent DC stimulate the proliferative response during 4 days. Addition of DC from non-irradiated mice stimulate more actively than do DC from irradiated mice. There was no additional effect of pulsing DC with DCP. Free DCP added to the lymph node culture evoked only a very low proliferative response as compared to Figure 3. From Figure 7 it can be concluded that DC from irradiated lymph nodes do not stimulate an intact lymph node culture for 4 days as well as DC isolated from non-irradiated lymph nodes. The reproducibility of this study will be tested. If the results can be reproduced, DC will also tested after adaptation by prolonged radiation by Cleo lamps (after 30 and 90 days).

**DNA damage**

To test DNA damage after irradiation with Cleo lamps in human tissues from studies carried out in Finland and in Turku, monoclonal antibodies to thymidine dimers were investigated. To test these monoclonal antibodies, nude mice were irradiated with a high

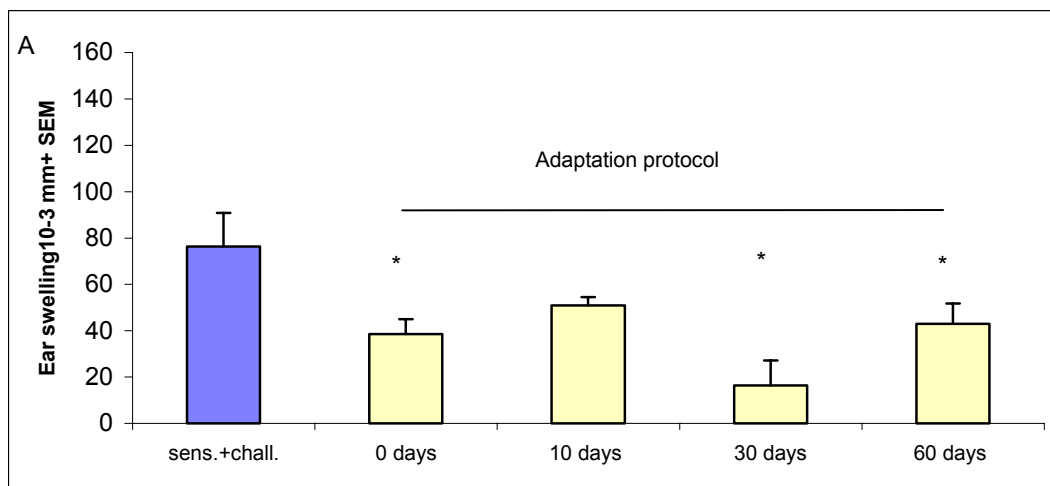
dose of UV. So far, with monoclonal antibodies available at RIVM, that were obtained from dr. Len Rosa no thymidine dimers were found. This antibody is no longer available, and in fact is no longer being used for this type of studies. The alternative monoclonal antibodies produced by Kamiya (Seattle, USA) has been ordered, and in fact been paid for, and has been sent with only after much delay. Now it has been tested, and it appears to work fine; samples will be analysed shortly.

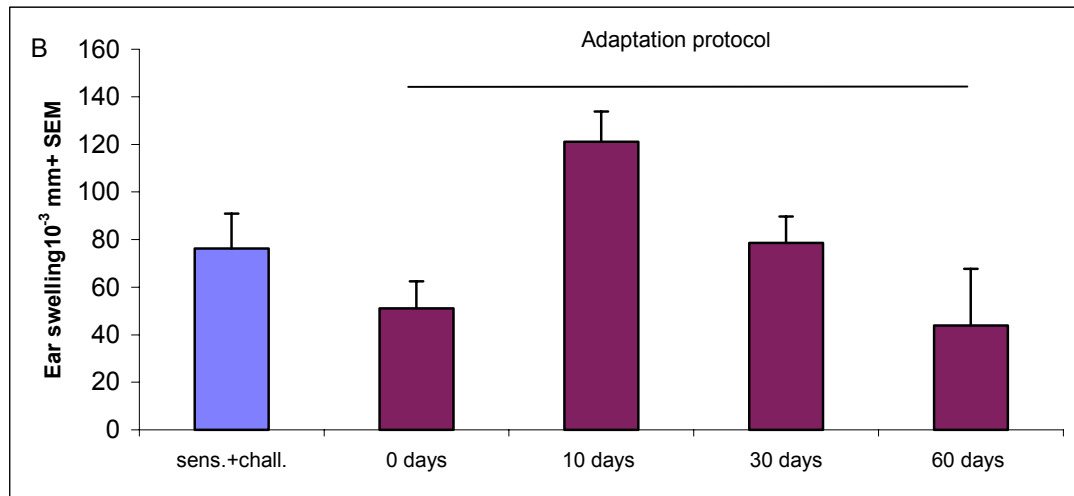
### **Progress report year WP 8 and 9, 2004**

#### **Effect of chronic exposure of Cleo Natural Lamps on suppression of CHS induced by TL12 or Cleo**

Studies with picryl chloride (PCI) were continued. Two experiments were carried out to show the effect of adaptation after chronically irradiation with Cleo natural lamps.

To study the adaptation properties of Cleo lamps, mice were chronically irradiated with Cleo lamps during 75 min (3/4 of the erythema dose) for 10, 30 and 60 days. After irradiation to induce suppression (TL12, 15 min or Cleo lamps, 120 min), mice were sensitized with PCI. Figures 1a,b show after sensitization a strong ear swelling response which was statistically significantly suppressed after irradiation with the TL12 lamp (Figure 1a). However, irradiation with the Cleo lamp (120 min) did not result in immunosuppression (Figure 1b). Therefore only the results in the groups exposed to TL12 were evaluated. In these groups it was shown that immunosuppression was still present after adaptation for 10, 30 and 60 days. These experiments were repeated and similar results were obtained.



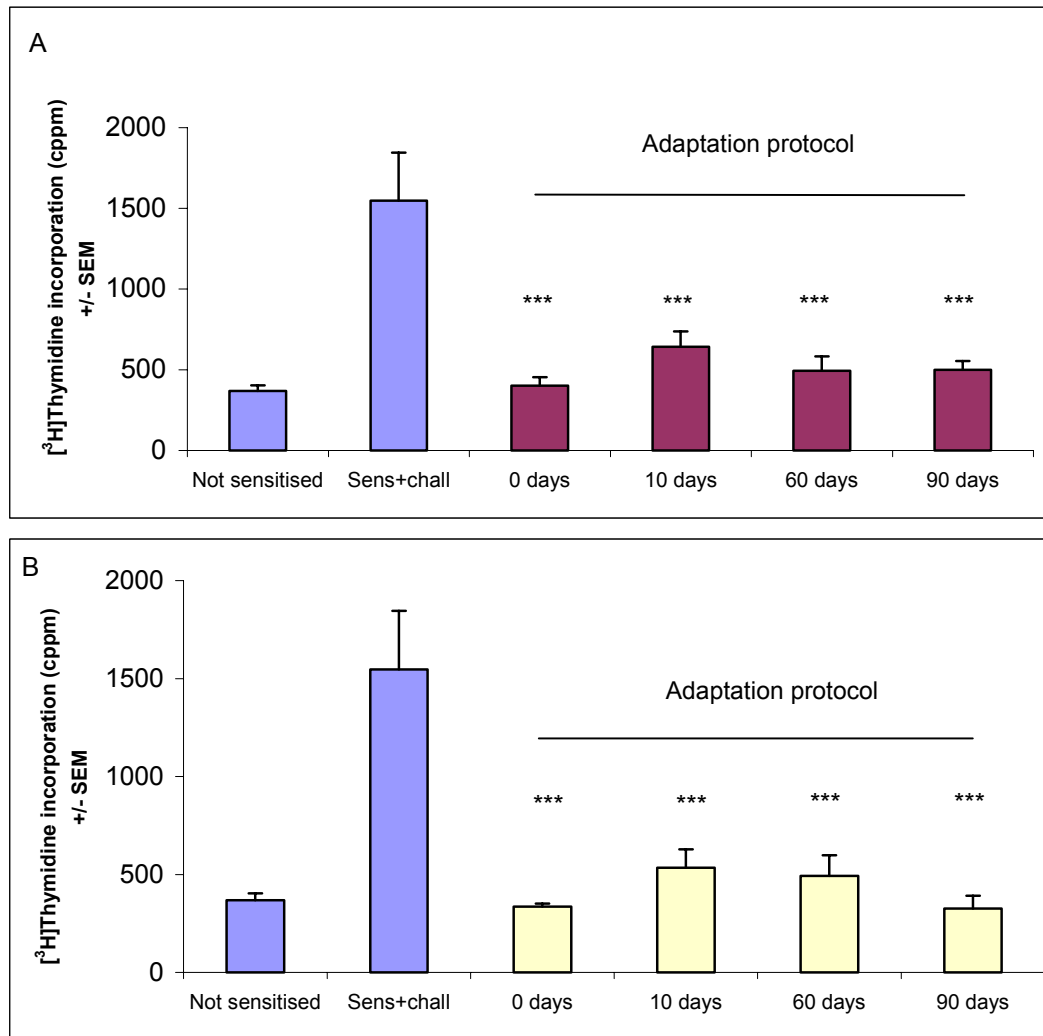


**Figure 1 a, b. Effect of chronic exposure of Cleo Natural lamps on the induction of immunosuppression induced by Cleo Natural or TL-12 lamps measured for PCI induced CHS.** Mice (n=5) were chronically irradiated during 10, 60 and 90 days with cleo natural lamps (75 min/day) followed by A) immunosuppressive dose of cleo natural lamps (during 5 days, 120 min/day or B) TL12 lamps (during 5 days, 15 min/day). Mice show a significant suppression of the proliferative response ( $p < 0.05$ ) compared to mice which were sensitized and challenged with PCI but not irradiated.

#### ***Effect of chronic exposure of Cleo Natural lamps***

Since CHS ear swelling responses were not sufficiently induced by DPCP in mice, the lymphoproliferative responses of draining lymph nodes was used to measure DPCP responsiveness and the immunosuppression by TL12 and Cleo (see progress report 2003). To study adaptation, mice were irradiated (75 min) with the Cleo Natural lamps for 10, 60 and 90 d. Thereafter, the mice were irradiated again with with TL-12 lamps (15 min) or with Cleo Natural lamps (120-min.) for 5 days to induce immunosuppression. After three days without irradiation, mice were sensitized and challenged with DPCP during 5 days and axillary and inguinal lymph nodes were isolated after sacrifice of the mice.

In Figures 2a,b it is shown that sensitization and challenge with DPCP induces increased proliferation of cells compared to the non sensitised mice. The proliferative response of the lymph node cells of mice irradiated with the immunosuppression protocol by TL12 lamps or Cleo Natural only, was comparable with the non sensitised mice. However the immunosuppression by TL-12 lamps or Cleo Natural was still present whether the adaptation time was 10, 60 or 90 minutes. Studies were repeated three times with similar results.



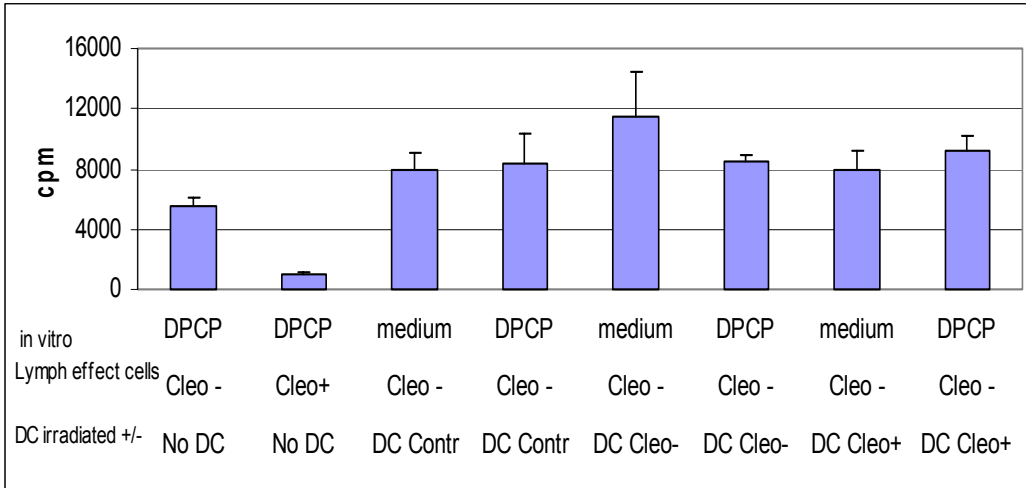
**Figure 2. Effect of chronic exposure of Cleo Natural lamps on the induction of immunosuppression induced by TL-12 or Cleo Natural lamps measured on lymph node cell proliferation in DPCP-sensitized and challenged mice.** Mice (n=5) were chronically irradiated during 10, 60 and 90 days with cleo natural lamps (75 min/day) followed by a immunosuppreve dose of cleo natural lamps (during 5 days, 120 min/day or b) TL12 lamps (during 5 days, 15 min/day). Mice show a significant suppression of the proliferative response ( $p < 0.001$ ) compared to mice which were sensitized and challenged with DPCP but not irradiated.

### Dendritic cell activity (DC)

The first two bars of Figure 3 show that lymphocyte proliferation is reduced after irradiation. To study the cell activity of DC after the immunosuppressive protocol, DC of local lymph nodes were isolated by MACS separation and added to a culture of local lymph node cells of mice sensitized and challenged with DPCP. In Figure 3 it is shown that adding DC increased (from 5000-8000 cpm) the proliferation significantly. However, there was no differences in proliferation after adding extra DC from control mice or mice sensitized with DPCP, either irradiated or not irradiated. Compared to the previous study

with dendritic cells (progress report 2003) it was concluded that the quality of the DC were still optimal, regardless of the irradiation. Therefore, it was concluded that the DC were not accountable for the suppression of the proliferative responses seen in figure 2a and b. No further studies were carried out on this subject.

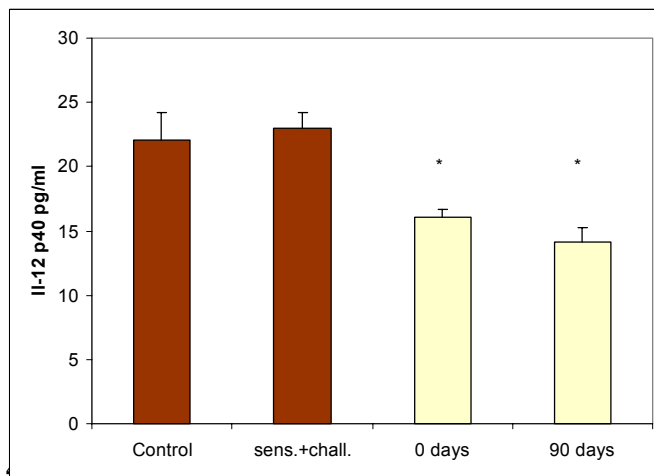
Figure 3



**Figure 3. Effect of addition of DC (control, DPCP-immune or irradiated-immune) to lymph node cultures**

***Cytokine alteration by TL12 immunosuppressive protocol***

Cytokine activation by LPS of spleen cells was tested to measure possible adaptation activity after chonical irradiation with Cleo natural for 90 days (75 min/daily). In Figure 4 IL12 p40 production after stimulation of LPS and IFNg is statistically significant suppressed by TL12. When mice were chronically exposed to Cleo natural lamps for 90 day (75 min daily) no change was observed in this reduction of cytokine release. For IL12p70, IL10 and TNF-a no statistical significant alterations were observed.



**Figure 4. Natural lamps on the induction of immunosuppression induced by TL-12 lamps and measured for**

**IL12 p40 production in spleen after stimulation with LPS and IFNg.** Controle no treatment; sens.+chall.: mice sensitized and challenged with DPCP; 0 days: mice irradiated with TL12 (immunosuppressive protocol) and sensitized and challenged with DPCP. 90 days: mice chronically irradiated (90 days with Cleo natural lamp for 75 min. daily, and irradiated with TL12 (immunosuppressive protocol) and sensitized and challenged with DPCP.

**DNA damage:**

To test DNA damage after irradiation with Cleo lamps in human or mouse tissues from studies carried out in Bilthoven, Finland, Poland and Edinburgh, monoclonal antibodies to thymine dimmers were investigated. The monoclonal antibodies produced by Kamiya (Seattle, USA) was found to be very useful to stain these thymine dimmers (Photo1, negative and Photo 2 positive). In studies with adaptation in the Dutch experiments no DNA damage was found. This was due to the sensitization and challenge period of 8 days, in which the repair of DNA was completed. In a preliminary study, we found DNA damage 24 h after the last irradiation and with a small number of mice, we did not show adaptation after 60 and 90 days of chronically exposure. (This will be confirmed in a larger group). Moreover, similar results were found in the tissue received from Edinburgh, in which adaptation did not result in a reduction of the DNA lesions (Table 1). In addition the tissue collected in Finland and Poland confirmed the mice studies, showing that adaptation did not resulted in a reduction of the DNA-damage (Table 2).

Taken all together, it is clearly shown that chronic exposure to Cleo (10 to 90 days) does not induce adaptation for the UV-induced immunosuppression based on lymphocyte proliferation, CHS, IL-12 p40 production and DNA damage.

**Table 1.**  
*Effect of UV adaptation on DNA damage ( skin human tissueTurku)*

<b>Number of volunteers</b>	<b>Adaptation Cleo1.2 sed</b>	<b>Suppression Local TL12</b>	<b>Results</b>
8	-	+	8x10
8	10 days	+	8x10
8	30 days	+	3x10;2x7;8
4	30 days*	+	4x10

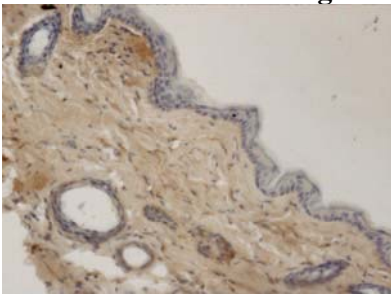
\*adaptation 2.0 SED for 30 days

Local TL-12= 3 MED single dose on 10x10 area

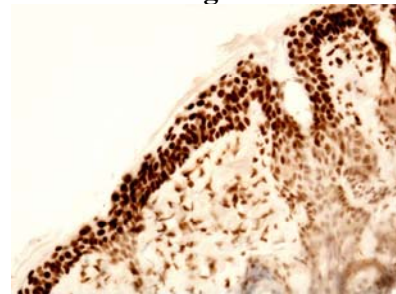
**Table 2. Effect of UV adaptation on DNA damage ( skin mouse tissue Edinburgh)**

<b>Number of animals</b>	<b>Adaptation protocol 756mJ/cm2 Cleo</b>	<b>Immunosuppressive protocol 2016 mJ/cm2 Cleo</b>	<b>Results</b>
13	-	-	0
3	-	+	10, 10, 9
2	+, 2 days	-	8, 9
4	+, 10 days	-	8, 5, 10, 10
4	+, 30 days	-	10, 8, ND, ND
2	+, 2 days	+	5, 8
4	+, 10 days	+	9, 10, 10, 10
4	+, 30 days	+	8, 8, ND, ND

**Photo 1: No DNA damage Score : 0**



**Photo 2: DNA damage score: 10**



**Progress report WP 8 and 9, 2005.**

**Cytokine production after adaptation with Cleo and immunosuppression with TL12**

The cytokine levels in spleen and lymph node cell cultures of mice irradiated with Cleo Natural lamps for 60 or 90 days followed by an immunosuppressive dose of TL12 irradiation were assessed (Figure 1)..

Cytokine production of spleen cells

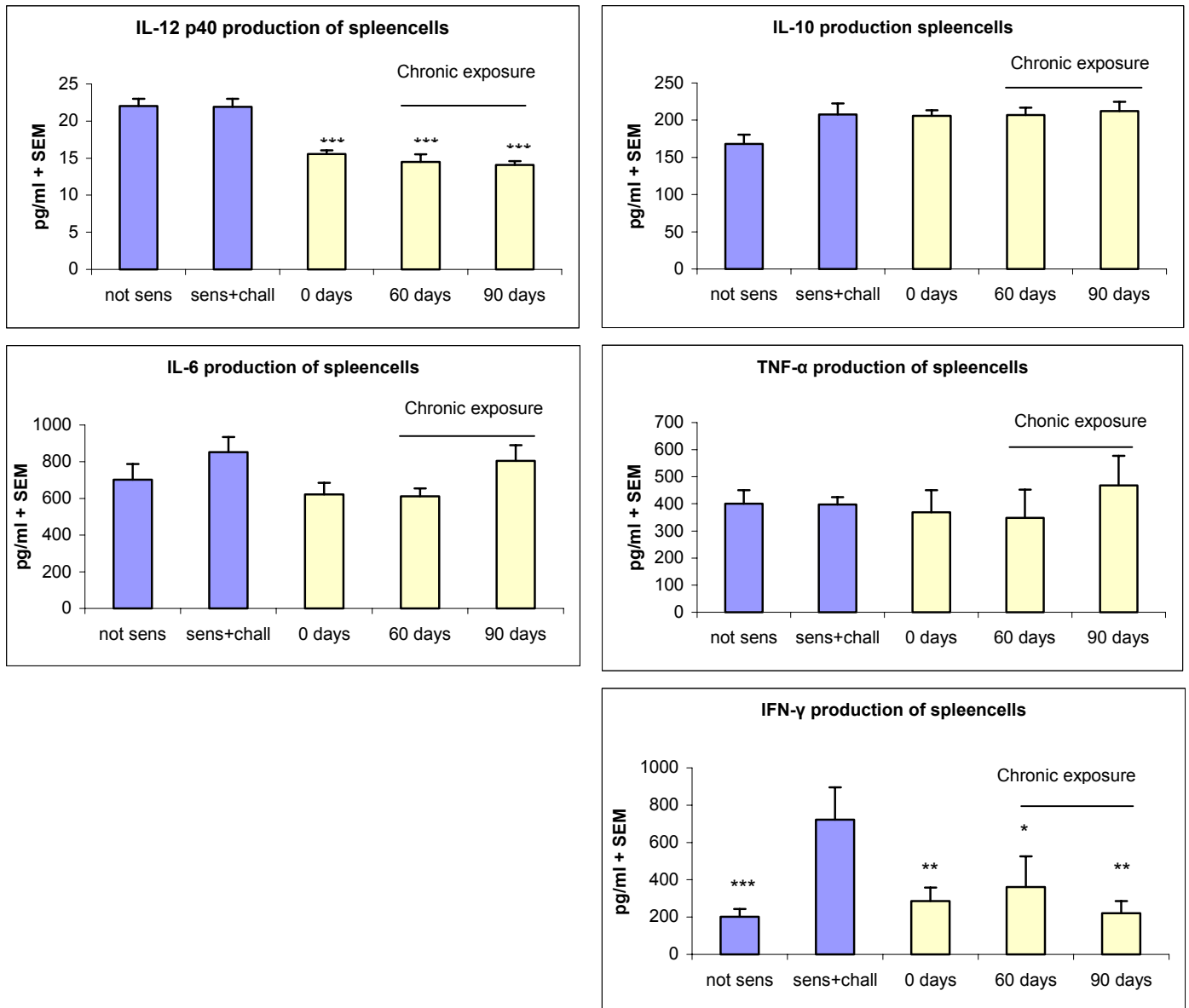


Figure 1. IL-12 p40, IL-10, TNF- $\alpha$ , IL-6 and IFN- $\gamma$  levels in spleen cell culture supernatants from mice (n = 5) chronic irradiated with Cleo (75 minutes per day) and irradiated with an immunosuppressive dose of TL12 (15 minutes per day for five days)

followed by sensitization and challenge with DPCP (n = 5, for the 60 days group n = 3). Mice in the “not sens” group did not receive any treatment. Spleen cells were cultured for 24 hours with LPS. The significant difference compared to the sens+chall group: \*\*\* = P< 0.001.

Figure 1 shows that the IL-12 p40 production is significant suppressed by TL12 irradiation (“0 days” group). When mice were chronically exposed to Cleo natural lamps for 60 and 90 days there is still a suppression observed in the IL-12 p40 cytokine level. The IFN- $\gamma$  production of the spleen cells was significant increased after sensitization and challenge. The mice which received UV radiation showed however a significant decrease in IFN- $\gamma$  production of the spleen cells. This decrease was seen in the group which received only the immunosuppressive dose of TL12 (“0 days” group) and also in the groups that received chronic irradiation. There was no significant difference observed in IL-10, TNF- $\alpha$  and IL-6 levels after UV radiation compared to the group that did not receive UV radiation. IL-12 p70 was not detectable in the spleen cell culture supernatants.

This experiment was repeated twice, in one experiment PCL was used for sensitization and challenge and in the other experiment DPCP. In the repetition experiment where the animals were sensitized and challenged with PCL there was also a reduction observed in IL-12 p40 production after UV radiation. This was the case for the group that only received the immunosuppressive dose of TL12 irradiation (“0 days“ group) and for the groups that received chronic irradiation for 60 or 90 days with Cleo (results not shown). This reduction in IL-12 p40 production was however not significant. In the second repetition experiment there were also no significant differences in IL-12 p40 and IFN- $\gamma$  production, although the same tendency was observed as in figure 22. In both repetition experiments no significant differences were observed in IL-10, IL-6 and TNF- $\alpha$  level after UV radiation compared to the group that did not receive UV (results not shown).

Cytokine production of lymph node cells

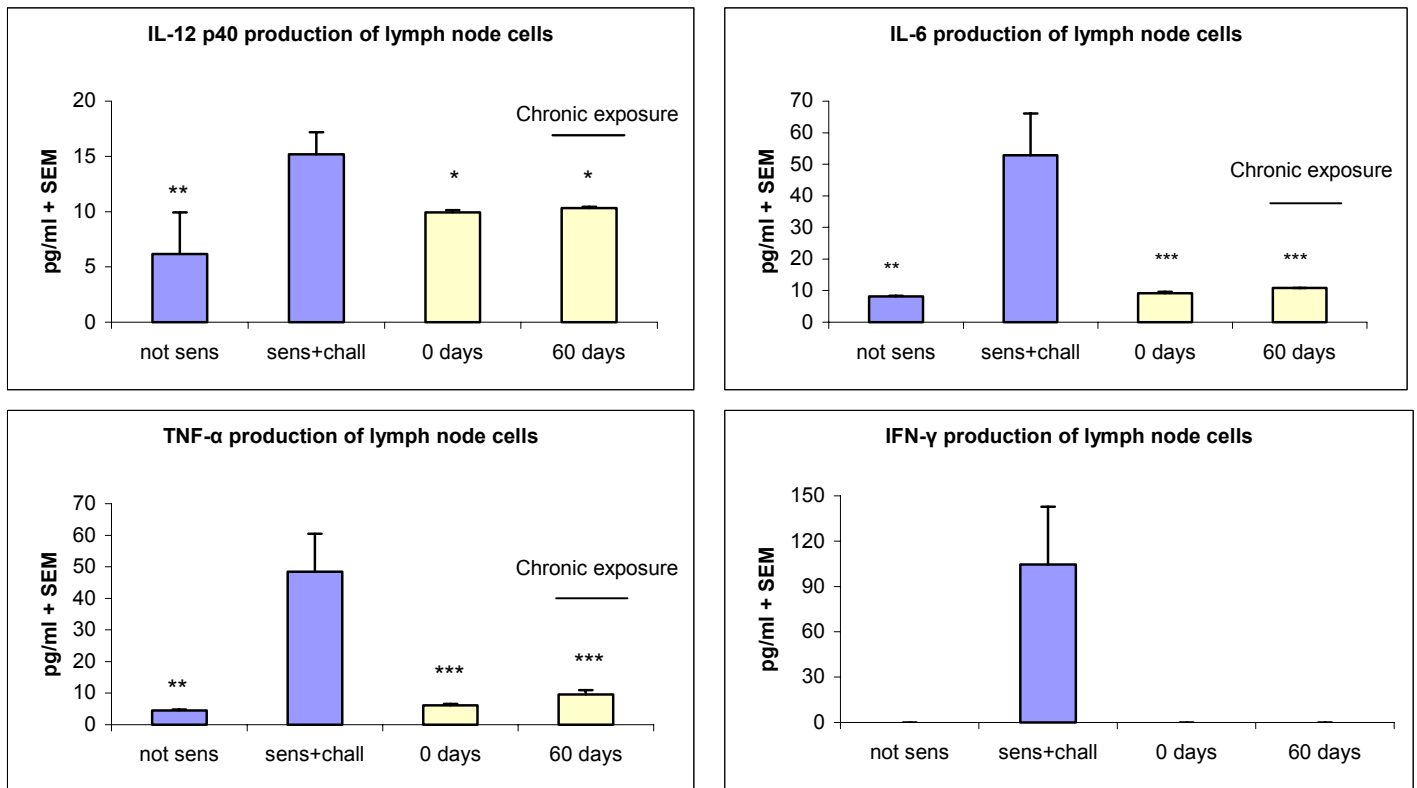


Figure 2. IL-12 p40, TNF- $\alpha$ , IL-6 and IFN- $\gamma$  levels in lymph node cell culture supernatants of mice chronically irradiated with Cleo (75 minutes per day for 60 days) and irradiated with an immunosuppressive dose of TL12 (15 minutes per day for five days) followed by sensitization and challenge with DPCP (n = 6, in the “not sens” group n = 3). The mice in the “not sens” and in the “sens+chall” group did not receive UV radiation. Lymph node cells were cultured for 72 hours with LPS. The significant difference compared to the “sens+chall” group: \* = P < 0.05, \*\* = P < 0.01, \* \*\* = P < 0.001).

IL-12 p70 and IL-10 were not detectable in the lymph node cell culture supernatants. The lymph node cells did produce IL-12 p40, TNF- $\alpha$ , IL-6 and IFN- $\gamma$ . There was a significant increase in the production of these cytokines after sensitization and challenge. When the mice were irradiated with an immunosuppressive dose of TL12 irradiation (“0 days” group) the production was significantly suppressed. When mice were chronically exposed to Cleo natural lamps for 60 days there was still a suppression observed in IL-12 p40, TNF- $\alpha$ , IL-6 and IFN- $\gamma$  production.

In another experiment the mice were sensitized and challenged with PCL and the IL-12 p40, TNF- $\alpha$ , IL-6 and IL-10 levels in the lymph node cell culture supernatants were assessed (results not shown). There was also a significant reduction (P < 0.001) in IL-12 p40 level observed after an immunosuppressive dose of TL12 irradiation. This significant reduction was still present in the groups that received chronic irradiation for 60 (P < 0.01) or 90 days (P < 0.001). There was also a significant suppression of the TNF- $\alpha$  production

( $P < 0.01$ ) observed after irradiation with an immunosuppressive dose of TL12. The mice that received chronic irradiation with Cleo for 60 or 90 days still had lower TNF- $\alpha$  levels, but this was however not significantly different from the group that received no UV radiation (“sens+chall” group). The IL-6 levels showed the same tendency as in **figure 2**. The mice which received UV radiation (0, 60 or 90 days of chronic irradiation) had lower levels of IL-6 production compared to the group that was not irradiated (“sens+chall” group), this difference was however not significant. In this experiment was, in contrast to the results of **figure 2**, IL-10 detectable in the lymph node cell culture supernatant (12 to 98 pg/ml). After UV radiation a reduction in the IL-10 level was observed, the longer the chronic irradiation period, the lower the IL-10 level. This decrease in IL-10 level was however not significant.

### Effect of ultraviolet radiation on the DNA in the skin of mice

To assess the effect of DNA damage (cyclobutane dimers) in the skin after UV radiation biopsies were taken at different time points. The biopsies were taken from the ear of a mouse 24 hours after the chronic irradiation period (**figure 4**) followed by a second biopsy taken from the ear of the same mouse 24 hours after the immunosuppressive dose, five days later (**figure 5**). The last biopsy was taken eight days after the last immunosuppressive irradiation (**figure 6**). With immunohistochemistry of the skin biopsies the DNA damage was demonstrated. Cells without thymine dimers show blue nuclei, while cells with thymine dimers show dark (brown, black) nuclei.

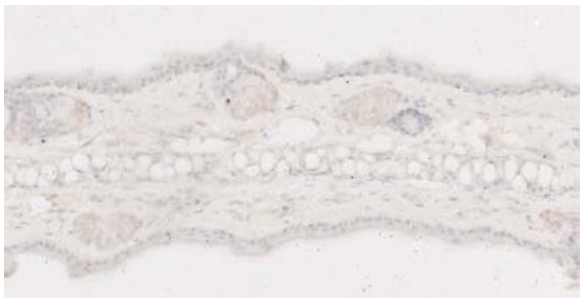


Figure 3. Negative control; sensitized and challenged with PCL, but not irradiated.

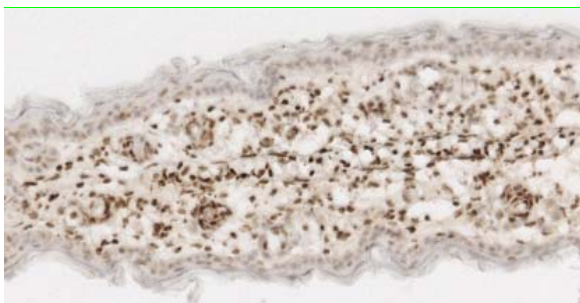


Figure 4. Mouse irradiated for 60 days with Cleo natural lamps (75 minutes per day).

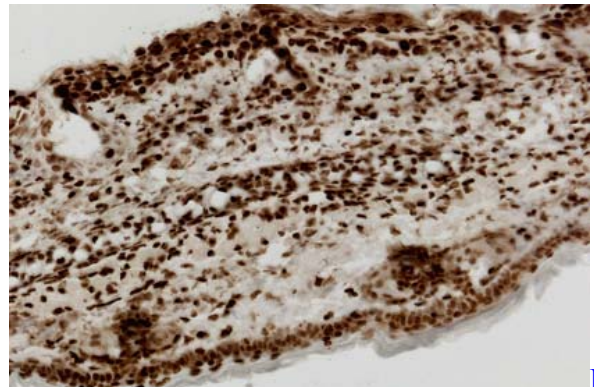


Figure 5. Mouse irradiated for 60 days with Cleo natural lamps (75 minutes per day) and for five days with TL12 (15 minutes per day).

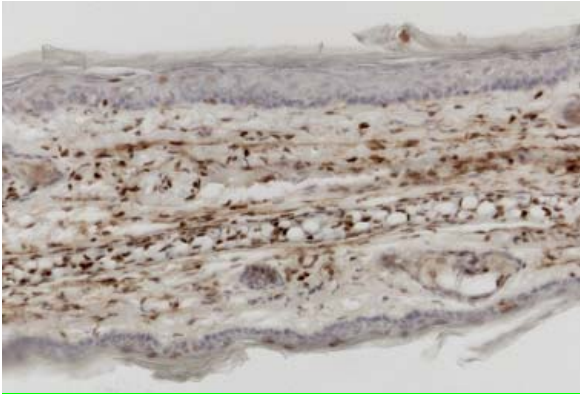


Figure 6. Eight days after the mouse received the last irradiation with TL12. The mouse was irradiated for 60 days with Cleo natural lamps (75 minutes per day) and for five days with TL12 (15 minutes per day).

### Development of the DNA damage

Mice were chronic irradiated for 0 or 60 days with Cleo followed by an immunosuppressive dose of TL12 irradiation (15 minutes per day for five days). Table 1 shows the DNA damage (thymine dimers) in the epidermis and table 2 shows the DNA damage in the stromal tissue (dermis, subcutis and cartilage).

Table 1. Mean DNA damage (thymine dimers) in the epidermis (n = 6) 24 hours after chronic irradiation (75 minutes per day) with Cleo and 24 hours after an immunosuppressive dose of TL12 irradiation(15 minutes per day for five days) ± SEM. Significant difference compared to the previous DNA score time-point \*\*\* = P < 0.001

Chronic irradiation for:	DNA damage in the epidermis (mean score ± SEM)		
	After chronic irradiation	After immunosuppressive dose	Eight days after the last irradiation
0 days	0	10 ± 0	2 ± 0.52 ***
60 days	2.7 ± 0.42	9.3 ± 0.33***	2 ± 0.37***

Table 2. Mean DNA damage (thymine dimers) in the stromal tissue (dermis, subcutis and cartilage) (n = 6) 24 hours after chronic irradiation with Cleo (75 minutes per day) and 24 hours after an immunosuppressive dose of TL12 irradiation (15 minutes per day for five days) ± SEM. Significant difference compared to the previous DNA score time-point: \*\*\* = P < 0.001. Significant difference between the group receiving 60 days chronic irradiation and the group receiving no (0 days) chronic irradiation: ## = P < 0.001.

Chronic irradiation for:	DNA damage in the stromal tissue (mean score ± SEM)		
	After chronic irradiation	After immunosuppressive dose	Eight days after the last irradiation
0 days	0	8 ± 0.63	4,3 ± 0.56***
60 days	8.7 ± 0.33	8.7 ± 0.33	7,5 ± 0.43####

Table 1 shows that after 60 days of chronic irradiation minimal to slight DNA damage was observed in the epidermis. When the mice were subsequently irradiated with an immunosuppressive dose of TL12 irradiation the DNA damage in the epidermis significantly increased. This severe DNA damage was also observed in the mice that only received the immunosuppressive dose of TL12 (“0 days” group). Eight days after the last irradiation the DNA damage in the epidermis was significantly decreased again to a minimal level.

Table 2 shows that the DNA damage after chronic irradiation with Cleo Natural lamps was greater in the stromal tissue than in the epidermis. At this time point the mice which received 60 days of chronic irradiation showed marked to severe DNA damage in the stromal tissue. When the mice were subsequently irradiated with an immunosuppressive dose of TL12 irradiation the DNA damage in the stromal tissue did not increase. The mice which received only the immunosuppressive dose of TL12 irradiation (“0 days” group) showed at this time-point also marked DNA damage in the stromal tissue. Eight days after the last irradiation the DNA damage in the epidermis was decreased. Mice which received only the immunosuppressive dose of TL12 irradiation showed a significant decrease in DNA damage in the stromal tissue.

The mice which received 60 days of chronic irradiation followed by an immunosuppressive dose of TL12 also showed a decrease in DNA damage, however this was a small (not significant) decrease. So eight days after the last irradiation there is a significant difference in DNA damage in the stromal tissue between the mice which received chronic irradiation and the mice which received no chronic irradiation.

#### DNA damage eight days after immunosuppression with TL12 or Cleo

Eight days after the last irradiation ear biopsies were taken from mice chronic irradiated for 0, 10, 60 or 90 days with Cleo (75 minutes per day) and irradiated with an immunosuppressive dose of TL12 (15 minutes per day for 5 days) or Cleo (120 minutes per day for five days). At this time-point the DNA damage in the epidermis was for all mice absent to minimal (score  $\leq 2$ ). In the dermis, subcutis and cartilage however thymine dimmers were found (**figure 8**).

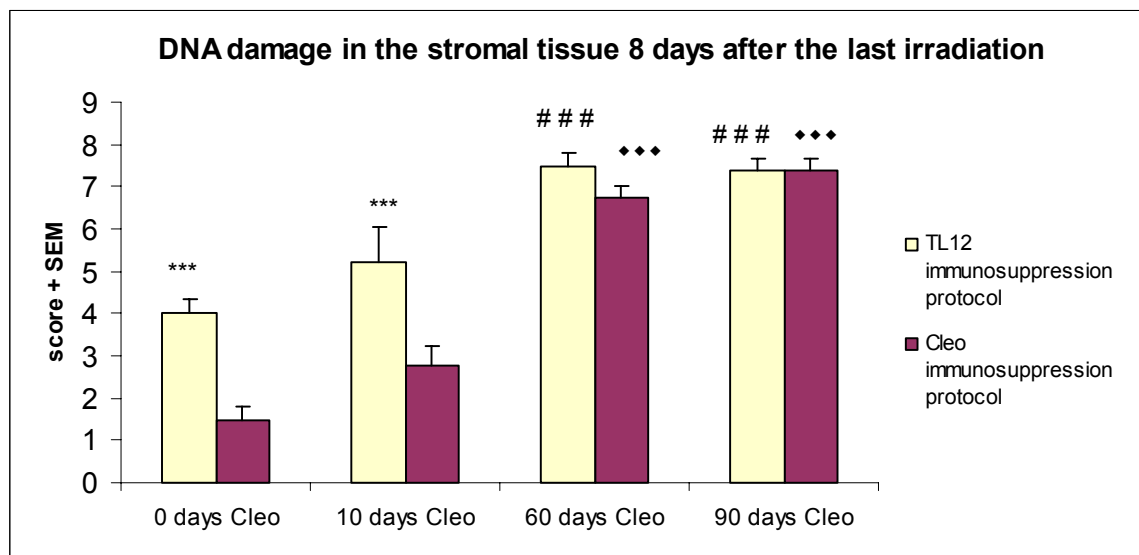


Figure 8. DNA damage in the dermis, subcutis and cartilage eight days after the last irradiation. Mice received chronic irradiation with Cleo (75 minutes per day) for 10, 60 or 90 days and an immunosuppressive dose of Cleo (120 minutes per day for five days) or TL12 (15 minutes per day for five days) followed by sensitization and challenge with PCL (n = 5). Significant difference between the groups receiving the same chronic irradiation period but a different source of immunosuppressive radiation (Cleo or TL12): \*\*\* =  $P < 0.001$ . Significant difference compared to the mice receiving only an immunosuppressive dose of TL12 irradiation ("0 days Cleo" group): # # #:  $P < 0.001$ . Significant difference compared to the mice receiving only an immunosuppressive dose of Cleo irradiation ("0 days Cleo" group): ◆◆◆ =  $P < 0.001$ .

**Figure 8** shows that mice which received after a chronic irradiation period of 0, 10 or 60 days an immunosuppressive dose of TL12 irradiation had more DNA damage in the skin than mice which received an immunosuppressive dose of Cleo irradiation. This difference was significant ( $P < 0.001$ ) for mice irradiated for a chronic irradiation period of 0 or 10 days. There was no significant difference between a TL12 or Cleo immunosuppressive dose after a chronic irradiation period of 60 or 90 days. For these mice which received a chronic irradiation period for 60 or 90 days there was however

a significant increase ( $P < 0.001$ ) in DNA damage compared to the mice which didn't receive the chronic irradiation ("0 days Cleo" group).

**Overall conclusion WP 8 and 9**

The data do not indicate that adaptation to suppression by UV of acquired immune responsiveness occurs in the mouse.

**Deliverables**

P.A. Steerenberg, F. Daamen, E. Weesendorp and H. van Loveren. Effect of long term Cleo natural lamp exposure on the UV induced immunosuppression of acquired immunity to diphenylcyclopropanone and picryl chloride in mice. Submitted

**WP.9      Adaptation to effects of UV exposure on acquired immune parameters in the mouse**

**Workpackage number:** 9  
**Start date of starting event:** month 18  
**Completion date:** month 32  
**N<sup>o</sup> of the partner responsible:** 1  
**Person-months per partner:** CO1:20  
**Total person months:** 20

**Objectives**

To establish the adaptive activity of UV exposure on subsequent exposures of UV that suppress acquired immune responses in mice

**Description of work**

Groups of shaved BALB/c (UV sensitive) and C3H (UV resistant) mice will be exposed to UV emitted from CLEO natural lamps, at 0.3 individual MED doses for 2, 10, 30, or 90 consecutive days. Then the mice and control will receive a single suppressive dose of 3 MED. Subsequent to exposure, the mice will be exposed sensitised to DPCP on the irradiated skin, and ear swelling reactions to DPCP challenge of the UV shielded ears will be measured. These experiments will address adaptation to effects of UV exposure on primary immune responses. In other experiments, pre-exposed mice will be sensitised first, and then exposed to the suppressive dose of UV. Finally, experiments will be carried out in which both duration and dose of the UV pre-exposure are varied in such a fashion that the total irradiation that the mice will receive remains constant. The immune parameters as indicated in workpackage 7 will be studied.

**Deliverables**

Report on adaptation to chronic UV exposure induced effects on acquired immune parameters in mice.

**Milestones and expected results**

This workpackage will address the potency of chronic UV exposure to induce adaptation to subsequent exposure to suppressive doses of UV on primary and secondary acquired immune responses in mice, and how such adaptation compares to adaptation to non-immune effects of UV. Also, the impact of different genetic background will be established. In addition, the relation between duration and dose of UV pre-exposure, which cannot readily be assessed in humans, will be established. As such, the information yielded by this workpackage may confirm and extend the data gained from the experiments in humans.

**Progress report year 2002 WP 9: See WP 8**

**Progress report year 2003 WP 9: See WP 8**

**Progress report year 2004 WP 9: See WP 8**

**Progress report year 2005 WP 9: See WP 8**

**Overall conclusions WP 9: See WP 8**

## **WP.10 Integration of the results**

**Workpackage number:** 10

**Start date of starting event:** month 33

**Completion date:** month 36

**N° of the partner responsible:** 1, 2, 3, 4

**Person-months per partner:** CO1:2.2; CR2:1.5; CR3:1.5; CR4:1.5

**Total person months:** 6.7

### **Objectives**

To integrate all data from work packages 1-8, and on the basis of this formulate recommendations for sun exposure behaviour and protection from adverse effects.

### **Description of work**

Writing a report addressed at policy makers at national and EU authority levels. Evaluation of UV index and adaptation by prior to sun vacation UV exposure.

### **Deliverables**

Integrated report on effects of and adaptation to chronic environmental UV exposure on immunological health. This report will form the basis for the discussions in the international symposium to be organised (deliverable G).

### **Milestones and expected results**

The integration of all results from this project will provide crucial insight on the effects of chronic exposure to innate and acquired immunity, that adaptation to such effects, and the similarities or differences of immune health effects to non-immune effects such as pigmentation and skin thickening. Such information will be of great value for providing advice to the general population regarding sun exposure behaviour, and protection from exposure. It is especially this milestone that will be the basis for the discussions in the international symposium to be organised (deliverable G).

### **Progress report 2005: WP 10**

The project has yielded important new information in that it is clear that UV does appear to have chronic immunosuppressive effects. Some effects in particular systemic effects on macrophages show adaptation, whereas other innate immune parameters, such as NK cells and antigen presenting Langerhans cells and dendritic cells do not. In addition, acquired immune responses do not.

The results have been presented in an international symposium where the impact of the data were discussed.

## Publications

McLoone P and Norval M (2003). The effects of UV irradiation with Cleo natural sunlamps on innate immune parameters in mice. Abstract, 10th Congress of the European Society for Photobiology.

Kim TH, Moodycliffe AM, Yarosh DB, Norval M, Kripke ML and Ullrich SE. (2003). Viability of the antigen determines whether DNA or urocanic acid act as initiator molecules for UV- induced suppression of delayed type hypersensitivity. *Photochem Photobiol* 78, 228-234.

De Gruijl FR, Longstreth J, Norval M, Cullen AP, Slaper HI, Kripke ML, Takizawa Y and van der Leun JC (2003). Health effects from stratospheric ozone depletion and interactions with climate change. *Photochem Photobiol Sci* 2, 16-28.

Norval M (2003). The consequences of sunlight exposure for human viral infections. *Appl Envir Sci Pub Health I*, 23-32.

Sleijffers A, Yucesoy B, Kashon M, Garssen J, De Gruijl FR, Boland J, Van Hattum J, Luster MI, Van Loveren H. (2003). Cytokine polymorphisms play a role in susceptibility to ultraviolet B-induced modulation of immune responses after Hepatitis B vaccination. *J. Immunol.*, 170, 3423-3428

Sleijffers A, Herreilers M, Van Loveren H, Garssen J. (2003). Ultraviolet B radiation induces upregulation of calcitonin gene-related peptide levels in human Finn chamber skin samples. *J. Photochem. Photobiol B: Biol.* 69, 149-152.

Termorshuizen F, Hogewoning AA, Bouwes-Bavinck JN, Goettsch WG, De Fijter JW, Van Loveren H. (2003). Skin infections in renal transplant patients and the relation with ultraviolet radiation. *Clinical Transpl.* 17, 522-527

Sleijffers A, Kammeyer A, de Gruijl FR, Boland GJ, Van Hattum J, Van Vloten WA, Van Loveren H, Teunissen MBM, Garssen J. Epidermal cis-urocanic acid levels correlate with lower specific cellular immune responses after hepatitis B vaccination of ultraviolet B-exposed humans. *Photochem. Photobiol.* 2003, 77, 271-275.

Sleijffers A, Garssen J, Vos JG, Van Loveren H. Ultraviolet light and resistance to infections. *J. Immunotoxicol.* 1,1-12, 2004.

Termorshuizen F, Feltkamp MCW, Struijk L, De Gruijl FR, Bouwes-Bavinck JN, Van Loveren H. Sunlight exposure and (sero)prevalence of epidermodysplasia verruciformis-associated human papilloma virus. *J. Invest. Dermatol.*, 122, 1456-1462, 2004

McLoone P, Man I, Yule S, Fluitman A, van Loveren H, Norval M, Gibbs NK. Whole-body (TL-01) or UVA-1 irradiation does not alter immunomodulatory cytokines in the serum of human volunteers. *Photodermatol. Photoimmunol. Photomed.* 2004, 20, 76-80.

Kammeyer A, Garssen J, Sleijffers A, Van Loveren H, Eggelte TA, Bos JD, Teunissen MBM. Suppression of different phases of contact hypersensitivity by urocanic acid oxidation products. *Photochem. Photobiol.* 2004, 80, 72-77.

Termorshuizen F, Wijga A, Gerritsen J, Nijens HJ, Van Loveren H. Exposure to solar ultraviolet radiation and respiratory tract symptoms in 1-year old children. *Photodermatol Photoimmunol Photomed.* 2004, 20, 270-271

Narbutt J, Skibinska M, Lesiak A, Wozniacka A, Sysa-Jedrzejowska A, Cebula B, Robak T, Smolewski P. Exposure to low doses of solar-simulated radiation induces an increase in the myeloid subtype of blood dendritic cells. *Scand J Immunol.* 2004 ,60, 429-35.

Macve JC, McKenzie RC and Norval M. Exposure to multiple doses of UVB radiation reduces the numbers of epidermal Langerhans cells and lymph node dendritic cells in mice. *Photochemical and Photobiological Sciences* 2004. 3, 91-95.

Laihia JK, Koskinen JO, Waris ME, Jansen CT. Adaptation of the human skin by chronic solar-simulating ultraviolet irradiation prevents ultraviolet-B irradiation-induced rise in serum C-reactive protein levels. *Photochem Photobiol*, in press.

Joanna Narbutt, Aleksandra Lesiak, Malgorzata Skibinska, Anna Wozniacka, Henk van Loveren, Anna Sysa-Jedrzejowska, Iwona Lewy-Trenda, Aleksandra Omulecka, Mary Norval. Suppression of contact hypersensitivity after repeated exposures of humans to low doses of solar simulated radiation. *J. Invest. Dermatol*, In press.

### **3. ROLE OF THE PARTICIPANTS**

Four institutions sited in the Netherlands, Scotland, Finland, and Poland will carry out the project:

#### **C01**

DR HENK VAN LOVEREN

Head, Section of Immunobiology and Haematology

Laboratory for Pathology and Immunobiology

National Institute of Public Health and the Environment

PO Box 1, 3720 BA Bilthoven, the Netherlands

Tel 31-30-2742476 Fax 31-2744437 Email H. van.loveren@rivm.nl

#### **CR2**

PROFESSOR CHRISTER JANSÉN

Chairman, Department of Dermatology

University of Turku, TYKS,

FIN-20520 Turku, Finland

Tel +358-2-261 2600

Fax 358-2-261 1610 Email cjansen@utu.fi

Research group home page <http://www.utu.fi/med/ihotauti/uvttiimi/>

#### **CR3**

DR JOANNA NARBUTT

Consultant in Dermatology and Allergology

Department of Dermatology, Medical University, Krzemieniecka 5, 94-017 Lodz, Poland

Tel 48 42 686 79 81 Fax 48 42 688 45 65 Email: gosia@sunlib.p.lodz.pl

#### **CR4**

PROFESSOR MARY NORVAL

Professor, Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.

Tel 44 131 650 3167 Fax 44 131 650 6531 Email M.Norval@ed.ac.uk

None of these participants are contractually linked.

#### **OVERVIEW OF ACTIVITIES**

Participant 1 will be responsible for the co-ordination of the project, and hence for the workpackage 1 and 10. In addition, he will be responsible for the execution of the workpackages 8, and 9, in which the effects of the UV exposures on the acquired immune responses is investigated in mice. This participant will also perform analysis of interleukin polymorphisms in the packages 2-5.

Participant 2 will be responsible for the study of UV effects in on innate immune parameters in human volunteers, workpackages 2 and 3. In addition, he will contribute to the workpackages 1 (co-ordination, organisation of meetings), and 10 (integration of the results).

Participant 3 will be responsible for the study of UV effects in on acquired immune parameters in human volunteers, workpackages 4 and 5. In addition, he will contribute to the workpackages 1 (co-ordination, organisation of meetings), and 10: (integration of the results).

Finally, participant 4 will be responsible for the study of UV effects in on innate parameters in mice, workpackages 6 and 7. In addition, he will contribute to the workpackages 1 (co-ordination, organisation of meetings), and 10: (integration of the results).

Activity	Participant	Person months
WP 1		
Co-ordination	RIVM UTURKU ULODZ UEDIN	5.7 1.5 1.5 1.5
WP 2		
Chronic exposure and controls, 75 subjects, innate immune parameters	UTURKU	18
Histopathology, polymorphisms, 75 subjects	RIVM	2
WP 3		
Chronic and acute exposure and controls, innate immune parameters, 75 subjects	UTURKU	18
Histopathology, polymorphisms. 75 subjects	RIVM	2
WP 4		
Chronic exposure and controls, acquired immune parameters, 75 subjects	ULODZ	18
Histopathology, polymorphisms, 75 subjects	RIVM	2
WP 5		
Chronic and acute exposure and controls, acquired immune parameters, 75 subjects	ULODZ	18
Histopathology, polymorphisms. 75 subjects	RIVM	2
WP 6		
Chronic exposure and controls, 250 mice, innate immune parameters	UEDIN	16.5
Histopathology, 100 mice	RIVM	1
WP 7		
Chronic and acute exposure and controls, 250 mice, innate immune parameters	UEDIN	16.5
Histopathology, 100 mice	RIVM	1
WP 8		
Chronic exposure and controls, 250 mice, acquired immune parameters, histopathology, 100 mice	RIVM	20
WP9		
Chronic and acute exposure and controls, 200 mice, acquired immune parameters, histopathology 100 mice	RIVM	20
WP10		
Integral report	RIVM	2.2

	UTURKU	1.5
	ULODZ	1.5
	UEDIN	1.5

<b>DL. Deliverables list by participant</b>
---

<b>Deliverable No</b>	<b>Deliverable title</b>	<b>Delivery date</b>	<b>Participant</b>	
<b>BROCHURE</b>				
1	Preparation of Project Brochure	Month 4	1	Done, circulated
<b>MEETINGS</b>				
2	Kick off meeting in Bilthoven	Month 1	1	Done, report sent to Commission
3	Progress Meeting in Turku	Month 9	2	Done, report sent to Commission
4	Midterm Review in Lodz	Month 18	1	Done, report and review sent to Commission
5	Progress Meeting in Edinburgh	Month 27	3	Done, report sent to Commission
6	Progress Meeting in Bilthoven	Month 35	4	Done, report sent to Commission
7	International Symposium in Bilthoven	Month 36	1	Done, abstracts sent to Commission
<b>REPORTS</b>				
8	Report on effects of chronic exposure to UV on human innate immune parameters	Month 18	2	In press, see WP 2 and 3
9	Report on adaptation to chronic UV exposure induced effects on human innate immune parameters	Month 32	2	In press, see WP 2 and 3
10	Report on effects of chronic exposure to UV on human acquired immune parameters	Month 18	3	In press, see report 4 and 5

11	Report on adaptation to chronic UV exposure induced effects on human acquired immune parameters	Month 32	3	In press, see report 4 and 5
12	Report on effects of chronic exposure to UV on murine innate immune parameters	Month 18	4	In press and submitted, see report 6 and 7
13	Report on adaptation to chronic UV exposure induced effects on murine innate immune parameters	Month 32	4	In press and submitted, see report 6 and 7
14	Report on effects of chronic exposure to UV on murine acquired immune parameters	Month 18	1	Submitted, see report 8 and 9
15	Report on adaptation to chronic UV exposure induced effects on murine acquired immune parameters	Month 32	1	Submitted, see report 8 and 9
16	Integrated report on effects of and adaptation to chronic environmental UV exposure on immunological health	Month 36	1	In preparation

#### 4. PROJECT MANAGEMENT AND CO-ORDINATION

##### Administration

The co-ordinator will execute administrative and scientific duties aimed at a proper flow of the project and achieving the goals set. The co-ordination will be executed in close communication with administrative officials from the Commission where this appears necessary.

These duties pertain to:

1. delivering periodic reports every 12 months, in which the progress of the work and the resources devoted to the project will be outlined. These reports will be prepared in close communication with all partners. Progress meetings will be used for this purpose, but in addition, interim communications may be necessary. Any deviations from the plans as outlined in the technical annex will be discussed among the participants, and with the Scientific Officer of the Commission, and these deviations will also be outlined in the periodic reports. Where this will be necessary, the co-ordinator will personally meet with the Scientific Officer from the Commission in Brussels.

Reporting will be done according to the Guidelines available on CORDIS "Quality of Life" webpage at the following address:

<http://www.cordis.lu/life/src/projmgmt.htm>

2. delivering a final report, presenting all the work, objectives, results, and conclusions drawn by the project.
3. delivering a technical implementation plan (TIP), a draft version around half way in the project and a final version together with the final report. The further

development of this plan will take shape during the project, and during the international symposium that will be organised at the end of the project.

4. periodic cost statements. The participants will have every 12 months and at the end of the project their individual cost statements made with their financial departments; and will send them at the time to the co-ordinator. He will check them, and prepare the collated cost statements for the entire consortium, to be filed with the Commission. The financial department of RIVM will be involved in this process, and any difficulties that need to be resolved will be communicated to the commission.

### **Scientific co-ordination**

Three major meetings, in which all partners shall participate have been and will be organised at places and dates agreed with the EC scientific officer:

- Kick-off meeting (at the start of the project)
- Mid-term meeting (around half way in the project)
- Final meeting (near the end of the project)

The EC scientific officer will receive in due course the minutes of the technical meetings involving all partners.

The kick off meeting was organised in Bilthoven, and a progress meetings has been organised in Turku. A midterm review meeting, for which the Commission has selected Drs. A. Young and S. Pavel to act as reviewers, is being organised to take place April 4 and 5 in Lodz. The purpose of this meeting will be to report on the progress to date and to redefine (if necessary) the Project Programme for the remaining part of the contract. Procedures for managing future exploitation of results will be discussed and assessed. Further progress meetings will be organised in Edinburgh and Bilthoven respectively by the co-ordinator and the local participant.

Planning of all meetings will start at least 2 months in advance. The aim of the progress meetings will be to review progress (including milestone achievements and deliverables), plan future work, and prepare annual progress reports to the Commission. Decisions will be taken on the basis of consensus; in case of disagreement the co-ordinator will make the final decision. During the progress meetings, and during other forms of communication, any deviations from the plans written in the proposal will receive extra attention. Those deviations from the original proposals that have a fundamental impact on achieving the goals as laid out in the proposal will be discussed with the responsible EU officer. To facilitate all processes, the co-ordinator will, in addition to meeting in person, bilaterally or multilaterally communicate with the participants by E-mail, telephone and/or conference calls, or in person, as may be required.

An international symposium to which (inter)national health authorities will be invited will be organise by the co-ordinator in Bilthoven, at the end of the project.

All principal investigators will peer-review the reports and publications as they arise from the project in an open collegiate discussion. In addition, depending on the authors of the manuscript, relevant in-house reviewing system will be applied. Further quality assurance is carried out according to the laboratory, clinical, and epidemiological routines available in the participating institutions. The results will be published in international journals, which receive extensive peer-review by anonymous independent experts. It is envisaged that publications will appear in different types of journals, i.e. those focused on photobiology, dermatology and

immunology, those focused on epidemiology, and those focused on risk assessment, in order to further ensure adequate external scientific feed back and dissemination of our work. In addition, we will ensure the dissemination of these publications to the relevant (inter)national health authorities. In such cases, presentation of such documents will be accompanied by executive summaries, detailing the impact on health policies.

## **5 Exploitation and Dissemination activities**

It is likely that the results of our project will provide further knowledge on the health impact of UV exposure as can and will be encountered by the general population, and will have an influence on protection policies, and eventually on producers of sun tanning devices, sunscreens and other forms of sun protection, such as sunspectacles and clothing. The results are not suitable for commercialisation.

The results will be published in high-impact international journals. It is envisaged that publications will appear in different types of journals, i.e. those focused on photobiology, dermatology and immunology, those focused on epidemiology, and those focused on risk assessment, in order to further ensure adequate external dissemination in the scientific world. In addition, we will ensure the dissemination of these publications to the relevant (inter)national health authorities. In such cases, presentation of such documents will be accompanied by executive summaries, detailing the impact on health policies.

We have made and will further make contributions to the public awareness of the topic by serving as members of various bodies. Dr van Loveren is joint head of the WHO Collaborative Centre of Immunotoxicology and Allergic Hypersensitivity, and in this capacity the outcome of the activities in the field of UV research have been channelled to WHO. He has also served as an advisor for the Dutch Health Council concerning the effects of UV, and has acted as reviewer for UNEP with regard to their assessment of UV associated risks. RIVM is part of the Dutch Ministry of Welfare, Health, and Sports. The research at RIVM on adverse health effects of UV is partly carried out at the account of and for that Ministry, and the results form the basis for Ministerial advice to the general public. Professor Norval is a member of the British Government Advisory Group on Non-ionising Radiation, a member of UNEP panel on ozone depletion, and a consulting member of the International Commission on Non-ionising Radiation Protection of ILO, WHO, and EU. Dr. Koulu is a member of the UV committee of the Finnish Dermatology Society.

## **6. Ethical Aspects and Safety Provisions**

The proposed project involves the use of both human subjects and laboratory animals. The experiments fulfil all the legal and ethical requirements of the member states of the European Union as explained below.

### Human subjects

A total of 500 adult healthy human volunteers will be investigated in Finland and Poland following UV irradiation with solar simulated light. The subjects will be drawn from students, personnel in the local hospital, and volunteers. We will aim at an even gender distribution. The studies have received local ethical permission. All

study subjects will be informed about the nature of the studies, the nature of the assays performed, and will be asked for their consent. In some cases tests of immune function will involve contact sensitisation and elicitation at a later date. Such assays cannot be mimicked *in vitro* and it is critical to the success of the project to obtain as much data as possible in human subjects, but using relatively non-invasive methodologies. These methods are in regular clinical use for studying the immune status of dermatological patients. In addition to these cutaneous tests, small quantities of venous blood will be collected to assess effects on circulating immune cells and cytokine polymorphisms, and small skin biopsies will be taken to study keratinocytes and dendritic cells. Genetic polymorphisms of the Finnish and Polish study subjects are carried characterised at RIVM in the Netherlands. Confidentiality of the data will be ensured according the rules that RIVM applies for storing data.

All individuals involved as study subject in the project will receive the final report. In this report the outcome of the studies will not be traceable to individuals.

#### Laboratory animals

The mouse models will be studied in Bilthoven and Edinburgh. Permission has been granted from the Veterinary Inspectorate and Home Office respectively, and appropriate animal licences issued, together with permission from the local ethical committees (animal ethical committee). All this work will involve studies using adult mice with fully developed immune systems. Such experiments will yield valuable information regarding the chronic effects of UV on immunity, adaptation to UV irradiation, effects on memory responses and genetic factors regarding susceptibility to UV-induced immunosuppression. These *in vivo* systems cannot be mimicked *in vitro*. New experiments need to be evaluated by the ethical animal committees. Discussions concerning experiments that duplicate experiments in humans have been a matter of debate at RIVM. This has delayed some of the activities. The committee agrees that the animal experiments are not identical to the experiments in humans, and will yield additional information. Yet, the committee has decided to evaluate every new experiment to be done individually.

The laboratory assays performed in the context of this project do not include working with especially hazardous agents such as carcinogens or infectious microorganisms. Yet, in all participating institutions provisions are made regarding safety such as the mandatory use of personal protection, strict hygiene measures, detailed working instructions, and plans in case of accidents. Local and national authorities evaluate these safety measures.