IN VITRO ANALYSIS OF THE PRO-SENSITISING POTENTIAL ON HUMAN DENDRITIC CELLS

CUSTOMER	**Chase Life Extension Foundation Ltd 64 Stapleford Cres, Browns Bay 0630 Auckland - New Zealand**
SAMPLE	**TA Serum 818 Lotto/Batch: F7NCT/200114**
REPORT DATE	**02/13/2014**
REPORT N.	**REL/0207/2014/ALTOX/ELB**

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SUMMARY

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Preliminary

This report contains the experimental data compiled during the *in vitro* safety evaluation studies of the test product.
The test results are presented in a concise table format for easy interpretation.
The first part provides information regarding sponsor and test product identifications, assay type(s), entrusted laboratory, study initiation and completion dates and supervisory personnel.
The second part describes the study design, including materials and procedures.
The test results are presented in the third and last part of the report.
A copy of this report is kept on file at ABICH S.r.l.
The raw data that support the results and conclusions are kept at the San Raffaele Scientific Institute.

Note:

The results reported in the present brochure refer only to the tested sample/samples and to the particular experimental conditions hereby described. This report or parts of it can be reproduced only with the experimenters' agreement.

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1 FIRST PART –General facts

1.1 Customer

Chase Life Extension Foundation Ltd
64 Stapleford Cres, Browns Bay
0630 Auckland () - New Zealand

1.2 Test sample

TA Serum 818; Batch: F7NCT/200114, internal code 0824/14-02

1.3 Sample receipt date

01/27/2014

1.4 Executed tests

- Preliminary evaluation of cytotoxicity on human dendritic cells through MTT assay.
- Evaluation of stimulating activity on human dendritic cells through FACS analysis.

1.5 Entrusted laboratory

San Raffaele Scientific Institute, via Olgettina 60, Milano –Italy, Cell and molecular Immunology Laboratory

1.6 Test ending date

02/10/2014

1.7 Main investigator

Dr. Samuele Burastero, MD, Allergology and Clinical Immunology Specialist, Researcher at Scientific Institute San Raffaele Hospital

1.8 /Study director

Dr. Elena Bocchietto, Biotechnology specialist ABICH S.r.l. - Via 42 Martiri– 28924 -Verbania -Italy tel +39 (0)323 586239

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2 SECOND PART - Study description

2.1 Aim

The aim of the test is to evaluate that the tested product does not cause pro-sensitising effects on the involved cell model. For every product intended to come into direct and prolonged contact with the skin, it is important to consider, beside the irritating potential, even the sensitising potential in order to predict the general safety of the finished formula to avoid risks for the consumers.
In the test we used a cell model of dendritic cells to evaluate to immunological reactivity of typical immunity cells, specialised as antigen presenting cells, exposed for prolonged time (48h) to the tested product at different concentrations. In the skin, the dendritic cells specialized for the first contact as antigen presenting cells, are named *Langerhans Cells*.
It is necessary to premise that toxicological data from cell models cannot totally replace in vivo tests, but a wide scientific literature is available to support their reliability. Furthermore, the VIth and VIIth amendment at the EC Dir. 76/768 plans the abandon of animal testing for cosmetic products, and as sensitisation testing on human volunteers is ethically problematic, the development of alternative in vitro method to predict skin sensitisation is a forced route to evaluate the safety of employ of cosmetics (1-11). Furthermore, the use of in vitro tests, instead of in vivo models, is strongly recommended by the UNI/EN 10993 rules (12).
In the present study, we used monocytes-derived immature dendritic cells from human healthy blood volunteers, as prototypic blood-derived immunologically active cells. These cells play a central role in the skin immune response towards topical products . Human primary dendritic cells are an *in vitro* model of Langerhans cells. On these cells we checked out the expression of two co-stimulatory molecules, CD80 (B7.1) and CD86 (B7.2) using bacterial lipopolysaccharides (LPS, generic stimulators of immune response) and Nickel sulphate as two positive controls, Nickel being a well known contact sensitising agents, both in in vitro and in vivo models.
When the lymphocytes T' TCR recognize the antigen (signal 1) on the Antigen-Presenting Cell (APC), additional molecules (called co-stimulatory) on the APC's membrane are necessary to obtain a complete functional immune response (signal 2).
The signal 2 is very important to define the kind of immune response that is going to be activated (umoral, cellular, etc.)
The costimulatory molecules CD80 and CD86 (also called B7.1 and B7.2) are necessary to obtain an efficient antigen presentation by the T cell receptor (TCR) and hence to obtain a correct immune response. Both these molecules are membrane glycoprotein expressed on the surface of different antigen-presenting cells (dendritic cells, Langerhans cells, monocytes/macrophages, keratinocytes) and they recognise a further molecule, a glycoprotein called CD28 on the T lymphocyte membrane.
The switching on of the ligand/receptor system CD28/B7 avoids the T cell apoptosis and sustains their proliferation and differentiation and the production of many cytokines.

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In the first phase of the physiological immune response, B7.2 is expressed as default and it modulates both the Th1 and Th2 responses. As the immune response goes on, also B7.1 is up-regulated and the costimulatory signal increase, with an expansion of T cells and the production of different cytokines. B7.1 is also preferentially up-regulated during the acute phase of auto- immune response (13-25).
The increasing level of expression of CD80 and CD86 on the dendritic cells is a signal of activation of the immune response derived from the exposition to a potentially sensitising contact antigen. Functionally, the expression of co-stimulatory molecules on the dendritic cells means activation of the immunological response in terms of capability to present the antigen in the typical tissues (skin in our case), where, *in vivo*, the immune protective response is triggered.

2.2 Samples description

Name	Description
TA Serum 818 Batch: F7NCT/200114 Internal code: 0824/14-02	opaque light yellow gel
LPS	White powder
Nickel sulfate	Crystals dissolved in PBS, positive control as a sensitizer

2.3 Sample preparation

The sample was dissolved in ethanol and then diluted in the cell culture medium at different concentrations. The product underwent a preliminary cytotoxicity screening on the cells to decide the best concentration to test it without cytotoxic effects on the cells, in order to avoid false results. Cell medium exposed to the same experimental conditions were used as a negative control.

2.4 Cell model

The test is carried out on a primary cell line of dendritic cells derived from human monocytes of healthy volunteers peripheral blood . Cells are kept in RPMI 1640, FCS (10%), GM-CSF (50 ng/ml) e IL-4 (1000 IU/ml) added.

2.5 MTT preliminar assay execution

The MTT assay is simple, accurate and yields reproducible results. This method has been developed originally by Mossman (27). The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. This product is of yellowish colour in solution. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, leading to the formation of purple crystals which are insoluble in aqueous solutions. The crystals are re-dissolved in acidified isopropanol and the resulting purple solution is measured spectrophotometrically. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

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MTT-medium is prepared as described (27). After exposure of the cells to the test material, the cells are washed with PBS and exposed to the MTT-medium at 37℃.

At the end of the incubation period, the MTT-medium is removed and the cells receive the MTT solubilization solution. The plate is shaken on a rotatory plate shaker for 20-30 minutes, ensuring that all the crystals have dissolved from the cells and have formed a homogeneous solution.
The absorbance is measured as described with background elimination.
The results are expressed in terms of viability:

$$\% \text{ Viability} = \frac{\text{OD treated cultures} \times 100}{\text{OD untreated control cultures}}$$

2.6 Treatment and Exposure

Following the preliminary cytototoxicity investigation, the optimal testing concentrations were defined. The sample was diluted in the cell medium at the wished final concentrations and put in contact with the dendritic cells *in vitro*. The exposure has been carried out for 48 h at 37°C with 5% CO_2.

2.7 Sensitisation assay execution

After the incubation with the tested substance and the controls, cells are collected, checked under the microscope for their vitality by staining with Trypan Blue dye and counting in a cell counter chamber, washed in PBS and then marked with a fluoresceinated anti-B7.1 or B7.2 antibody.
After washing, to eliminate the excess antibody, the MFI (Mean Fluorescence Intensity) linked to the cells was evaluated by means of a flux cytofluorimeter (FACS, Fluorescence Activated Cell Sorter, Becton Dickinson, Mountain View, CA).
This value is proportional to the expression of costimulatory molecules.
The MFI of the non-treated cells and of cells after reaction with a monoclonal isotype-matched antibody was used as an internal control (basal fluorescence).

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3 THIRD PART - Results and conclusions

3.1 Results

In table 1 are reported the results expressed as co-stimulatory molecules expression after the exposition of the primary cell line for 48h to the investigated and control substances, subtracted of the negative controls (non treated cells basal values).

Table 1

Samples	CD80 (MFI*)	CD86 (MFI*)
Nickel Sulfate 20 µg/ml	26,66	42,86
Nickel Sulfate 10 µg/ml	21,14	32,52
Nickel Sulfate 4 µg/ml	13,53	14,76
LPS 1 µg/ml	29,41	78,78
TA Serum 818	0,89	-0,25
TA Serum 818	-0,04	-0,22

* MFI = Mean Fluorescence Intensity – it is the geometric average of the fluorescence intensity of the cells decorated with the fluoresceinated antibody and it is proportional to the number of stained molecules per cell.

In figure 1 are plotted the values of expression of CD80 and CD86 found for the tested sample and its relative controls, subtracted of the negative control (value of the sample treated with a fluoresceinated antibody of unrelevant specificity).

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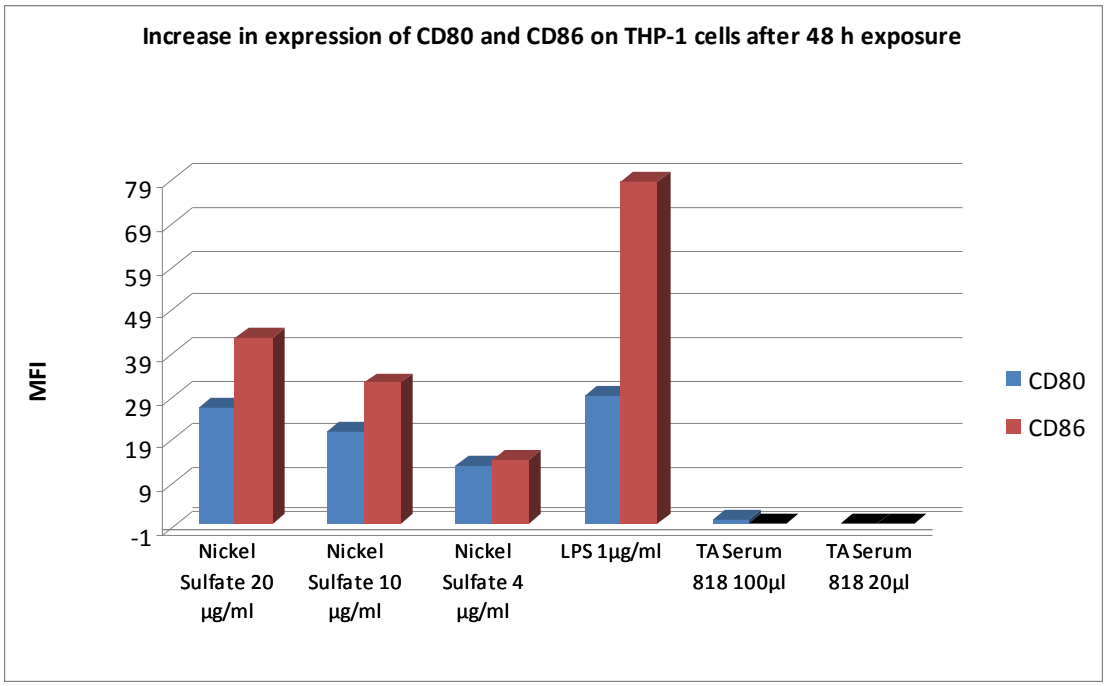
Figure 1



Showing the behaviour of Nickel, a prototypic sensitising substance, we can see how this is characterised by a) high increase of both the markers, with a predominance of CD80; b) direct correlation between concentration and intensity of the response; c) relevant effects even at very low doses. Also LPS increases the expression of both the investigated markers.
The tested dose of 4 µg/ml of Nickel Sulphate ($NiSO_4 \cdot 6H_2O$) corresponds to more or less 1 ppm of Nickel, dosage that is around the minimal sensitising threshold in already sensitised individuals with irritated skin (28, 29). From above reported data, we can notice how a so low dose is already able to cause an increase from 2 times upwards of the investigated molecules (data not shown). The concentration that is able to cause an allergic reaction in most of the sensitive subjects is anyway around higher value, over the 100 ppm of Nickel Sulphate·$6H_2O$ in contact with safe and intact skin .

The sample did not show any increase in the expression of both the investigated markers.

The sample did not show any cytotoxic effect on the cells used for the test (IC_{50}<200 mg/ml).

We did not observed any signal related to apoptosis on the treated cells.

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3.2 Conclusions

In the above experimental conditions, the sample:

TA Serum 818

Batch: F7NCT/200114

doesn't affect in this in vitro model the investigated markers expression in immunocompetent cells and hence *it doesn't show any stimulating potential on the immune cellular response.*

Date: 02/13/2014

Il Study Director
Dr. Elena Bocchietto

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