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Investigation Report

Test for Sensitization with Mischgewebe Zink-Lyocell

1.	**Customer:**	TITK e. V. Rudolstadt
2.	**Order Number:**	A281-12/08
3.	**Address:**	Breitscheider Str. 97 07407 Rudolstadt
4.	**Object of Investigation:**	**textile material Zink-Lyocell**
5.	**Date of Receipt:**	December 09, 2008
6.	**Investigation:**	Tests for irritation and delayed-type hypersensitivity (Local Lymph Node Assay - LLNA) DIN EN ISO 10993-10: 2007 (ISO 10993-10: 2002 + Amendment 1: 2006)
	Test Facility: **Address:**	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg
	Test Item:	Mischgewebe Zink-Lyocell
	Report:	084742
	Pages:	15
7.	**Report sends to customer:**	
	Date:	February 20, 2009
	Form:	sent by mail
	Recipient:	Ms. Bauer

8. **Place and Date:**
Aachen, February 20, 2009

9. **Study Director:**

Dr.-Ing. Ute Müller
Head of Laboratory
BMP GmbH

BIOSERVICE

SCIENTIFIC LABORATORIES GmbH

Test for Sensitisation

(Local Lymph Node Assay - LLNA)

with

Mischgewebe Zink-Lyocell

Report

BSL BIOSERVICE Project No.: 084742

Sponsor

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Preface

General

Sponsor:	BMP Labor für medizinische Materialprüfung GmbH Pauwelsstraße 19 52074 Aachen Germany
Study Monitor:	Dr.-Ing. Ute Müller
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstrasse 6/8 82152 Planegg Germany
BSL BIOSERVICE- Project No.:	084742
Test Item:	Mischgewebe Zink-Lyocell
Title:	Test for Sensitisation (Local Lymph Node Assay - LLNA) with Mischgewebe Zink-Lyocell

Project Staff

Study Director:	Dr. Daniela Stelter
Deputy Study Director:	Dr. Patricia Löffler
Head of Quality Assurance Unit:	Dipl. Biol. Uwe Hamann

Schedule

Arrival of Test Item:	11 December 2008
Start of Study:	17 December 2008
Start of Experimental Phase:	25 January 2009
End of Experimental Phase:	05 February 2009
Date of Report:	12 February 2009

Project Staff Signatures

Study Director: Dr. Daniela Stelter

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Date: 12 Feb 2009

Quality Assurance

This study was performed in conformity with internal Quality Assurance regulations, on the basis of GLP regulations.

The test facility BSL BIOSERVICE Scientific Laboratories GmbH is certified according to the Principles of Good Laboratory Practice and accredited according to 93/42/EWG, 90/385/EWG and DIN EN ISO/IEC 17025:2000.

Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, number 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted: 24th April 2002).

Commission Regulation (EC) No 440/2008, L 142, Annex Part B, 30 May 2008

ISO 10993-1: 2003 "Evaluation and testing"

DIN EN ISO 10993-10: 2007 (ISO 10993-10: 2002 + Amendment 1: 2006) "Tests for irritation and delayed-type hypersensitivity"

ISO 10993-12: 2007 "Sample preparation and reference materials"

Archiving

All original data generated during the conduct of the study (raw data, copy of report) will be stored in the Scientific Archives of BSL BIOSERVICE Scientific Laboratories GmbH for 12 years after issue of the report.

Summary

The test item extract was assayed at three concentrations of 100%, 50% and 25% (v/v). The 100% extract concentration corresponds to an extraction ratio of 120 cm^2 / 20 mL.

The negative control was AOO (3+1 (v/v) Acetone/Olive Oil).

Each mouse was treated by topical application of the test item extract to the entire dorsal surface of each ear once daily over three consecutive days.

Five days after the first topical application all mice were injected intravenously with ^{3}H-methyl thymidine.

Approximately 5 hours after ^{3}H-methyl thymidine-injection all mice were sacrificed and the draining "auricular lymph nodes" were excised in order to prepare a single cell suspension of the lymph node cells for each animal.

The ^{3}H-methyl thymidine – incorporation was measured in a ß-counter and expressed as the number of disintegrations (Zerfall) per minute (DPM). Determination of radioactivity was performed individually for each animal.

The proliferative response of lymph node cells was calculated as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test item / negative control, was calculated for each concentration.

The stimulation index at an extract concentration of 25%	was	**1.2**
The stimulation index at an extract concentration of 50%	was	**0.8**
The stimulation index at an extract concentration of 100%	was	**1.0**

The EC3 value (derived by linear interpolation) could not be stated, as all measure points were below the stimulation index of three.

Conclusions

Considering the reported data of this sensitisation test it can be stated that the test item Mischgewebe Zink-Lyocell causes no reactions identified as sensitisation, as the stimulation index was below 3.0 for each concentration tested.

Introduction

The LLNA has been developed as an alternative method for the identification of skin sensitizing test items and measures the proliferation of lymphocytes isolated from lymph nodes (auricular lymph nodes) draining the site of exposure (dorsal aspect of the ears) in mice.

Lymphocyte proliferation is measured by determining the incorporation of ^{3}H-methyl thymidine.

No validated *in vitro* method is available for assessing sensitisation potential.

Materials and Methods

Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor.

Name:	Mischgewebe Zink-Lyocell
Sample No.:	S0855-12/08
Order No.:	UA281-12/08
Safety Precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety

Extraction of the Test Item

Extract preparation was performed according to guideline ISO 10993-12 and in consideration of OECD 429.

Extraction conditions: 37 ± 1 °C for 72 ± 2h.

In total a ratio of 120 cm^2 of sample to 20 mL of extraction medium was used.

Extraction vehicle was AOO (3+1 (v/v) Acetone/Olive Oil).

(Acetone; lot K39156414, Merck; olive oil highly refined, lot 058K0684, Sigma).

Up to the administration within the same day, the extracts were stored at room temperature.

Preparation of the Test Item

The test item was extracted as described above. The extract of the test item was diluted to gain the following concentrations:

Concentration I: 100% (undiluted); concentration II and III were 50% and 25%. Dilution vehicle was AOO (3+1 (v/v) Acetone/Olive Oil).

The preparations were made immediately prior to each dosing.

Control

The vehicle (AOO) served as negative control and was incubated at 37 ± 1 °C for 72 ± 2 h.

Other Materials

^{3}H-methyl thymidine (TRK 300, 25 Ci/mmol; Lot B276; Amersham), diluted to a working concentration of 80 µCi/mL.

NaCl 0.9%, B. Braun Melsungen, lot 8221A121

Trichloroacetic acid (TCA), Sigma, lot 046K0734.

Phosphate buffered saline (PBS), BSL BIOSERVICE, lot 081216 and lot 090127.

Test Animals

Mice, CBA/J Rj, female, age 8 – 12 weeks, 5 mice per test group

The animals were derived from a controlled full barrier maintained breeding system (SPF).

Source: Janvier, F-53940 Le Genest-Saint-Isle.

According to Art. 9.2, No.7 of the German Act on Animal Welfare the animals are bred for experimental purposes.

Animal Husbandry

The animals were barrier maintained (semi-barrier) in an air-conditioned room

- Temperature: 22 ± 3 °C
- Rel. humidity: 55 ± 10%
- Artificial light, sequence being 12 hours light, 12 hours dark
- Air change: at least 10 x / hour
- Feeding ad libitum, Altromin 1324 maintenance diet for rats and mice
- Free access to tap water, sulphur acidified to a pH value to approx. 2.8 (drinking water, municipal residue control, microbiol. controlled periodically)
- The animals were kept in IVC cages, type II L, Polysulphone cages on Altromin saw fiber bedding.
- Certificates of food, water and bedding are filed at BSL Bioservice
- Adequate acclimatisation period (at least 5 days)

Preparation of the Animals

The animals were randomly selected.

Identification was ensured by cage number and individual marking (tail).

Clinical Observation

Prior to the application and once a day thereafter all animals were observed in order to detect special clinical signs or reactions to treatment.

Weight Assessment

The animals were weighed prior to the application and at the end of the test period.

Dose Groups

3 Test Groups (3 different concentrations) and 1 Negative Control Group (vehicle) were tested.

Test Regime

Topical Application

Each mouse was treated by topical application of 25µL of the selected solution to the entire dorsal surface of each ear.

Topical applications were performed once daily over three consecutive days.

Administration of ^{3}H-methyl thymidine

Five days after the first topical application all mice were dosed with 20 µCi ^{3}H-methyl thymidine by intravenous injection (tail vein) of 250 µL of ^{3}H-methyl thymidine, diluted to a working concentration of 80 µCi/mL.

Preparation of cell suspension

Approximately 5 hours after ^{3}H-methyl thymidine-injection all mice were sacrificed. The draining "auricular lymph nodes" were excised, pooled for each animal (2 lymph nodes per animal, if technically possible) and collected in PBS. A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through polyamide gauze (200 mesh size). After washing the gauze with PBS the cell suspension was pelleted in a centrifuge. The supernatant was discarded and the pellets were resuspended with PBS. This washing procedure was repeated.

After the final wash each pellet was resuspended in approx. 1 mL 5% TCA at approx. 4 °C overnight for precipitation of macromolecules. Each precipitate was once washed again, resuspended in 1 mL 5% TCA and 5 mL scintillation fluid was added. Then this solution was transferred into scintillation vials and stored at room temperature overnight.

Determination of incorporated ^{3}H-methyl thymidine

The ^{3}H-methyl thymidine – incorporation was measured in a ß-counter and expressed as the number of disintegrations per minute (DPM). Similarly, background ^{3}H-methyl thymidine levels were also measured (5% TCA). Determination of radioactivity was performed individually for each animal.

Evaluation of Results

The proliferative response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (DPM/NODE) and as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals (STIMULATION INDEX). Before DPM/NODE values were determined, background values were subtracted.

EC3 values, calculated concentrations which induce stimulation indices of three, are determined by linear interpolation {EC3 = c + [(3-d) / (b-d)] x (a-c)}, between two points of the stimulation indices axis, one above (a,b) and one below (c,d) the stimulation index of three. If all measured points are above or below the stimulation index of three, no EC3 value can be stated.

A substance is regarded as a 'sensitizer' in the LLNA if at least one concentration of the test item results in a 3 fold or greater increase in ^{3}H-methyl thymidine - incorporation into lymph node cells of the lymph nodes of the test group animals, relative to that recorded for the lymph nodes of control group animals **(Stimulation Index equal to or greater than 3.0).**

Results

None of the three tested concentrations of the test item induced the stimulation index of 3.0.

The ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals, relative to that recorded for control group animals (stimulation index) for the test item extract was

at a concentration of	25%	**1.2**
at a concentration of	50%	**0.8**
at a concentration of	100%	**1.0**

All animals showed the expected weight development which includes a weight loss of up to 2 g throughout the study.

At the daily clinical observation the animals did not show any visible clinical symptoms.

For individual data see the following tables.

Conclusions

Considering the reported data of this sensitisation test it can be stated that the test item Mischgewebe Zink-Lyocell causes no reactions identified as sensitisation, as the stimulation index was below 3.0 for each concentration tested.

Table 1: Weight Gain (g)

Group	*Animal No.*	*Start of study*	*End of study*	*Weight gain*
extract of	1	24	23	-1
Mischgewebe	2	22	21	-1
Zink-Lyocell	3	25	24	-1
	4	23	22	-1
25% in AOO	5	23	22	-1
extract of	6	24	23	-1
Mischgewebe	7	22	21	-1
Zink-Lyocell	8	21	20	-1
	9	24	22	-2
50% in AOO	10	24	23	-1
extract of	11	23	22	-1
Mischgewebe	12	22	21	-1
Zink-Lyocell	13	23	23	0
	14	23	22	-1
100%	15	23	21	-2
Negative	16	22	21	-1
control	17	23	22	-1
AOO	18	22	22	0
	19	23	23	0
	20	22	21	-1

Table 2: Radioactive determination of the test substance groups.

POS	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/ Node	Stimu-lation Index
18	359.0	Negative		16	779.0	763.2	381.6	
19	358.0	Control		17	786.0	770.2	385.1	
20	266.0			18	580.0	564.2	282.1	
21	233.0			19	511.0	495.2	247.6	
22	257.0			20	561.0	545.2	272.6	
MV	**294.6**			**MV**	**643.4**	**627.6**	**313.8**	**1.0**
SD	**53.3**			**SD**	**115.8**	**115.8**	**57.9**	
97	343.0	**Extract of**	25	1	753.0	737.2	368.6	1.2
98	318.0	**Mischgewebe**		2	688.0	672.2	336.1	1.1
99	319.0	**Zink-Lyocell**		3	694.0	678.2	339.1	1.1
100	357.0			4	773.0	757.2	378.6	1.2
101	423.0			5	913.0	897.2	448.6	1.4
MV	**352.0**			**MV**	**764.2**	**748.4**	**374.2**	**1.2**
SD	**38.4**			**SD**	**81.3**	**81.3**	**40.7**	**0.1**
102	288.0	**Extract of**	50	6	627.0	611.2	305.6	1.0
103	247.0	**Mischgewebe**		7	537.0	521.2	260.6	0.8
104	257.0	**Zink-Lyocell**		8	567.0	551.2	275.6	0.9
105	898.0*			9	1943.0*			
106	173.0			10	376.0	360.2	180.1	0.6
MV	**241.3**			**MV**	**526.8**	**511.0**	**255.5**	**0.8**
SD	**42.2**			**SD**	**92.9**	**92.9**	**46.4**	**0.1**
109	700.0*	**Extract of**	100	11	1524.0*			
110	309.0	**Mischgewebe**		12	668.0	652.2	326.1	1.0
111	206.0	**Zink-Lyocell**		13	452.0	436.2	218.1	0.7
112	305.0			14	660.0	644.2	322.1	1.0
113	343.0			15	741.0	725.2	362.6	1.2
MV	**290.8**			**MV**	**630.3**	**614.5**	**307.2**	**1.0**
SD	**51.1**			**SD**	**107.6**	**107.6**	**53.8**	**0.2**
121	7.0	**Background**			16.0			
122	7.0	**Szinti and**			15.0			
123	8.0	**TCA**			18.0			
124	5.0				12.0			
125	8.0				18.0			
MV	**7.0**			**MV**	**15.8**	**0.0**	**0.0**	**0.0**
SD	**1.1**			**SD**	**2.2**			

* outlier; failed Nalimov, Grubbs and Dixon

If not noted individually, the results include both lymph nodes of an animal.

CPM = counts per minute; DPM = disintegrations per minute; MV = mean value;
SD = standard deviation; Szinti = scintillation fluid; TCA = trichloroacetic acid

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