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REPORT

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Test of Titernal W for Skin Sensitization in Guinea Pigs

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I, the undersigned, hereby declare that the work described in this report was performed under my supervision, as Study Director, and that the report provides a true and accurate record of the results obtained.

This is a translation of the original report, No. 203101041-005, written in Japanese.

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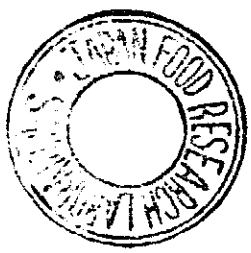

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1. Abstract

The test sample, Titernal W, was tested for skin sensitization by the guinea pig maximization test.

For the first stage of induction, the test sample was intradermally injected to 10 animals. At the second stage of induction, the test sample was applied occlusively for 48 hours on the next week. For the challenge, the test sample and 10 and 1 w/v% solution of the test sample in water for injection were each applied occlusively on the skin. As a result, none of the animals developed any skin reaction in any of 48 and 72 hours after the application.

Consequently, we concluded that the test sample caused no skin sensitization in guinea pigs in the present experiment.

2. Test sample

Titernal W

Character: Opalescent liquid with whitishsediment

3. Test period

From November 4 to December 26, 2003

4. Experimental animals

Female guinea pigs of the Hartley-strain, purchased from Japan SLC Inc., were used. They were obtained at an age of five weeks and acclimated for about one week. Six and 20 animals, showing no abnormality in the skin, were used for the preliminary test and the main test, respectively. They were housed in FRP cages (five animals per cage) under the standard laboratory conditions (temperature: 22 ℃±2 ℃, light-dark cycle: 12/12 hours) and given Labo G Standard diet [Nihon Nosankogyo K.K.] and tap water *ad libitum*.

5. Preliminary test

1) Procedures

① Dose-finding study for the intradermal injection (Preliminary test 1)

Each test sample and 50, 25, 10, 5 and 1 w/v% solution of the test sample in physiological saline were intradermally injected at a dose of 0.1 mL on the clipped and shaved flank of each of two guinea pigs. The injected sites were examined in 24, 48 and 72 hours and 7 days after injection to find the highest concentration, which causes no ulcer, represented by peeling off and/or losing the tissue.

② Dose-finding study for the topical induction (Preliminary test 2)

A 0.1-mL portion of the test sample spread on a 2-cm square of filter paper was applied on the clipped and shaved flank of each of two animals by the closed-patch-exposure method. Next, each 0.01-mL portion of 50 and 25 w/v% solutions of the test sample in acetone were applied directly on the same animal. Twenty-four hours after application, the application sites were wiped off with absorbent cotton with 70 % ethanol to remove the test sample still remaining. The skin reactions were examined in 48 and 72 hours after application to find the highest concentration which causes no severe irritation response.

③ Dose-finding study for the challenge (Preliminary test 3)

E-FCA* was intradermally injected to two animals. Twenty-one days after injection, 0.1-mL portion of the test sample and 50 and 25 w/v% solutions of the test sample in water for injection spread on a 2 cm square of filter paper were applied occlusively on the clipped and shaved flank of each of them. Twenty-four hours after application, the application sites were wiped with absorbent cotton wet with 70 % ethanol to remove the remnant of the test sample. The skin reactions were examined in 48 and 72 hours after application to find the highest non-irritating concentration.

2) Results

① Preliminary test 1

Erythema was observed at the concentrations of 5 w/v% and above. Severe damage such as ulcer, represented by peeling off and/or losing the tissue, was not found at any injection site. Therefore, the test sample was employed for the intradermal injection.

② Preliminary test 2

No skin reaction was found at any concentration. Therefore, the test sample was employed for the topical induction, and the occlusive application was adopted.

③ Preliminary test 3

No skin reaction was found at any concentration. Therefore, the test sample and solution of the test sample in water for injection was employed for the challenge.

* Physiological saline emulsified with an equal volume of Freund's Complete Adjuvant (FCA: Composed of liquid paraffin, a surface-active agent, and tuberculosis germs [Difco Laboratories](water-in-oil type). The treatment with FCA may lower the threshold level of skin irritation. The extract should therefore be applied to the FCA-treated animals in the preliminary test for the challenge accompanied with no false-positive response.

6. Main test

1) Grouping of animals

Ten animals were used for the experimental group, and five each for the negative and positive control groups (the known-sensitizer-treated group). The body weights of the animals before treatment ranged from 342 to 397 g.

2) Procedures

① Induction 1 (Intradermal injection)

The test animals were clipped in the mid-dorsal region near the scapulae with an electric hair clipper. Three pairs of symmetrical intradermal injections (0.1 mL each) were given as shown in Figure 1.

Experimental group
A: E-FCA
B: the test sample
C: the test sample with an equal volume of FCA

Negative control group
A: E-FCA
B: physiological saline
C: E-FCA

Positive control group
A: E-FCA
B: a 0.1 w/v% solution of DNCB*1 in olive oil
C: a 0.2 w/v% solution of DNCB in FCA emulsified with an equal volume of physiological saline

② Induction 2 (48-hour closed patch)

One week after the intradermal injection, the injection area of the experimental animal was clipped, shaved and treated with 10 % sodium lauryl sulfate in petrolatum. In 24 hours after the treatment, petrolatum was wiped on the area with absorbent cotton wet with 70 % ethanol. Then, 0.2 mL of the test sample spread on a 2 cm×4 cm square of filter paper was applied on the injection site of each experimental animal for 48 hours by the closed-patch-exposure method. Petrolatum alone and a 0.1 % mixture of DNCB in petrolatum were also applied to the negative and positive control animals, respectively, in the same manner as described above. In 48 hours after the treatment, all application sites were wiped with absorbent cotton wet with 70 % ethanol.

③ Challenge and reading the skin reactions

The animals were challenged two weeks after the induction 2.

In the experimental group, 0.1 mL each of the test sample and 10 and 1 w/v% solution of the test sample in water for injection was applied occlusively on the clipped and shaved site on the flank of each experimental animal. Water for injection alone and 0.1 % mixture of DNCB in petrolatum were applied in the negative and positive control groups, respectively, in the same manner as described above. Furthermore, in the negative control group, the test sample and 10 and 1 w/v% solution of the test sample in water for injection was simultaneously applied*2. In 24 hours after the application, all application sites were wiped with absorbent cotton wet with 70 % ethanol.

The skin reactions were examined in 48 and 72 hours after the challenge according to the criteria of the Draize test shown in Table 2, and the mean scores*3 were calculated. The sensitization rate (%) at each observation time was obtained by dividing the positive number of animals by the total number of animals and multiplying by 100.

The animals were weighed at the completion of the test.

*1 2,4-dinitrochlorobenzene [Wako Pure Chemical Industries, Ltd.].
*2 The negative control animals should be exposed to the same substance as in the experimental group to detect any false positive response.
*3 Mean score＝Sum of scores／Total number of animals

3) Results and conclusion (Tables 3 to 8)

In the experimental group, no skin reaction was detected at the test sample and 10 and 1 w/v% solution of the test sample in water for injection in any of 48 or 72 hours after the application. The sensitization rates were 0 % in any of 48 and 72 hours (all mean scores: 0).

In the negative control group, water for injection caused no skin reaction in any of 48 or 72 hours after the application. The sensitization rate was 0 % in any of 48 and 72 hours (all mean scores: 0). No skin reaction was detected at the test sample and 10 and 1 w/v% solution of the test sample in water for injection in any of 48 or 72 hours after the application. The sensitization rates were 0 % in any of 48 and 72 hours (all mean scores: 0).

In the positive control group, necrosis and eschar formation (both score 4) and edema (score 1) were observed in 48 hours. Eschar formation was observed in 72 hours. The sensitization rates were 100 % in any of 48 and 72 hours (mean scores: 4.2 and 4.0, respectively).

Abnormalities were not observed in the general condition although body weight reduction was seen in one animals of the positive control group. In the other experimental animals, abnormalities were not observed in the body weight change and the general condition during the experimental period.

Consequently, we concluded that the test sample caused no skin sensitization in guinea pigs.

7. References

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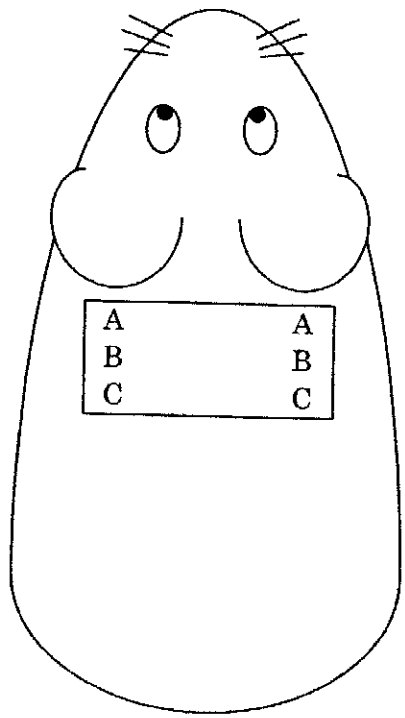

Figure 1. Location of the induction sites

A, B and C: Intradermal injection sites
□: Application area (2 cm×4 cm)

Table 2. Classification system for skin reaction

Erythema and eschar formation

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4*

* Bleeding, ulcer and necrosis will be classified in score 4 for injuries in depth.

Edema formation

No edema	0
Very slight edema (barely perceptible)	1
Well-defined edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4

Total possible score for irritation 8

Table 3. Summary of results of sensitization

Group	Number of animals	Application concentration (w/v%)	Observation time (hours)	Sensitization rate (%)	Mean score
Experimental	10	100*1	48	0	0
			72	0	0
		10	48	0	0
			72	0	0
		1	48	0	0
			72	0	0
Negative control	5	0*2	48	0	0
			72	0	0
		100*1, *3	48	0	0
			72	0	0
		10*3	48	0	0
			72	0	0
		1*3	48	0	0
			72	0	0
Positive control	5	0.1*4	48	100	4.2
			72	100	4.0

*1 Test sample
*2 Water for injection
*3 Detection of any false positive response
*4 Mixture of DNCB in petrolatum (%)

Table 4. Results of challenge in the experimental group

Application concentration (w/v%)	Observation time (hours)	Individual scores*1										SR*3	MS*4
		1*2	2	3	4	5	6	7	8	9	10		
100*5	48	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0
10	48	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0
1	48	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0

1 Erythema and eschar formation/Edema formation
2 Animal number
3 Sensitization rate (%)
4 Mean score
5 Test sample

Table 5. Results of challenge in the negative control group

Application substance	Observation time (hours)	Individual scores*1					SR*3	MS*4
		1*2	2	3	4	5		
Water for injection	48	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0	0

*1 Erythema and eschar formation/Edema formation
*2 Animal number
*3 Sensitization rate (%)
*4 Mean score

Table 6. Results of application of the test sample for detection of any false positive response in the negative control group

Application concentration (w/v%)	Observation time (hours)	Individual scores*1					SR*3	MS*4
		1*2	2	3	4	5		
100*5	48	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0	0
10	48	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0	0
1	48	0/0	0/0	0/0	0/0	0/0	0	0
	72	0/0	0/0	0/0	0/0	0/0	0	0

*1 Erythema and eschar formation/Edema formation
*2 Animal number
*3 Sensitization rate (%)
*4 Mean score
*5 Test sample

Table 7. Results of challenge in the positive control group

Application concentration (%)	Observation time (hours)	Individual scores*1					SR*3	MS*4
		1*2	2	3	4	5		
0.1*5	48	4/0	4/0	4/1	4/0	4/0	100	4.2
	72	4/0	4/0	4/0	4/0	4/0	100	4.0

*1 Erythema and eschar formation/Edema formation
*2 Animal number
*3 Sensitization rate (%)
*4 Mean score
*5 Mixture of DNCB in petrolatum

Table 8. Body-weight changes

Group	Animal number	Body-weight (g)	
		Before treatment	Completion of the test
Experimental	1	383	481
	2	371	410
	3	397	437
	4	342	431
	5	381	467
	6	376	429
	7	388	425
	8	392	476
	9	390	408
	10	396	420
Negative control	1	391	428
	2	348	378
	3	369	386
	4	381	398
	5	355	397
Positive control	1	355	375
	2	366	390
	3	363	382
	4	387	373
	5	365	374