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INITIAL SUBMISSION: PRELIMINARY REPORT ON PILOT EXPERIMENT USING HUMAN SKIN XENOGRAFTED TO ATHYMIC NUDE MICE WITH COVER LETTER DATED 01/26/95		
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January 26, 1995

Dear FYI Coordinator:

In accordance with API's policy of providing the federal government with copies of research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause a risk of injury to health or the environment, we are enclosing a copy of the following preliminary report:

**(Identification no: FYI not assigned) Preliminary Report on Pilot Experiment Using Human Skin Xenografted to Athymic Nude Mice--December 15, 1994**

Please note that this information is provided in accordance with the full disclosure policy of API and does not constitute a formal submission as required by a test rule. This document does not contain confidential information. If you have any questions, please communicate with me.

Sincerely,

Robert T. Drew, Ph.D.

**Preliminary report on pilot experiment using human skin xenografted to athymic nude mice - December 18, 1984**

The initial design of the experiment was outlined in a letter to Dr. DiGiovanni (attached), prepared prior to his visit to UBTL on 10/4/84. During his visit it was decided to modify the protocol further to eliminate biopsies at 48 hr post-final dosing. This decision was made to maximize the number of biopsies that could be acquired at the most important time points, i.e. 6 and 24 hr post-final dosing. In addition, it was decided to select two mice (one acetone-treated and one TPA-treated) for a terminal sacrifice at 6 hr post-final dosing; thus allowing acquisition of the maximum number of 6 mm dia. biopsies at the time of presumed maximum ODC induction. The modified treatment/biopsy schedule is shown in Table 1.

**Table 1 Treatment/biopsy schedule**

Treatment	Animal No.	Biopsy for histopath (3 mm)		Biopsy for ODC, 6 hr	
		6 hr	24 hr	4 mm	6 mm
Acetone, 50 $\mu$ L	235081/7	X			X
	235083/3		X	X	
	235082/2		X	X	
TPA, 4.25 nmole/50 $\mu$ L	235084/4	X			X
	235086/6		X	X	
	235085/5		X	X	

Table 2 provides additional information on the timing of biopsies from individual mice. The 3 mm dia. biopsies were used for evaluation of histopathology, epidermal thickness, and labeling (BrdUrd) index; while the 4 and 6 mm dia. biopsies were utilized for assay of epidermal ODC activity. The histologic, histomorphologic, and immunohistochemical evaluations were performed by Claudio Conti, D.V.M., Ph.D. and the ODC assays were performed in the laboratory of John DiGiovanni, Ph.D.

**Histopathology**

The preliminary histologic evaluation is summarized in the attached report. Xenografts from two mice (Nos. 235082/2 and 235084/4) exhibited marked necrosis and/or inflammatory infiltrates. Although it was initially assumed that the histopathology was indicative of graft rejection and the histopathology report so states, toxic effects of TPA cannot be excluded. Significant TPA-induced epidermal hyperplasia was not observed in either the xenografted skin or the skin of athymic nude mice located adjacent to the xenograft and presumably exposed to TPA; consequently, histomorphologic measurements of epidermal thickness were not warranted. It should be noted that TPA was applied dermally at a concentration of 4.25 nmole/50  $\mu$ L, i.e. a dose higher than the highest dose (3.4 nmole/50  $\mu$ L) used in the previous study (Dermigen Protocol No. 810301) with CD-1 mice. As expected, viable human skin xenografts exhibited a thick papillary epidermis.

### Labeling (BrdUrd) Index

The determination of labeling index in skin specimens collected 24 hr post-final dosing is summarized in Table 3. In the case of specimens from two mice (No. 235081/7 and 235084/4) no BrdUrd-labeled cells were detected in the xenograft or untreated mouse skin. The staining was repeated twice with the same results. Since other specimens processed at the same time exhibited positive staining, a BrdUrd dosing problem is indicated. Xenografts from mice Nos. 235082/2 and 235084/4 were not evaluated because of extensive necrosis and inflammatory infiltration. From the limited observations available, no TPA-induced increase in the labeling of basal epidermal cells by BrdUrd was observed. In fact, the labeling index of one human skin xenograft (No. 235085/8) was actually lower than the acetone-treated control.

### Epidermal ODC activity

The assay of ODC activity in skin biopsies collected 6 hr post-final dosing is summarized in Table 4. Dermal application of TPA (4.25 nmole/50  $\mu$ L) did not induce ODC activity in the human skin xenografts. In one mouse (No. 235084/4) TPA produced a 5.8-fold increase in ODC activity of nude mouse skin. This observation was not repeated in mouse No. 235081/7; but uneven exposure to the test article in nude mouse skin adjacent to the xenografts cannot be excluded. It should be noted that the ODC activities of human skin xenografts from two mice (Nos. 235082/2 and 235084/4) have not been calculated, because these xenografts exhibited clear histopathology.

### Discussion

The absence of TPA-induced epidermal hyperplasia or ODC activity in the human skin xenografts was unexpected, since a relatively high dose of TPA was utilized, i.e. 4.25 nmole/50  $\mu$ L. Comparing doses on the basis of surface area exposure, 4.25 nmole TPA/50  $\mu$ L is equivalent to 17 nmole TPA/200  $\mu$ L. The highest dose of TPA used with CD1 mice in Dermigen Protocol No. 910901 was 13.6 nmole/200  $\mu$ L. The higher TPA dose was chosen based on the resistance of human skin xenografts to TPA promotion of chemically initiated human skin xenografts (1, 2). Dr. Slaga was aware of an experiment in which TPA was administered to intact human skin (single dermal application of TPA in the dose range of 16-65 nmole/50  $\mu$ L) and his recollection is that the epidermal hyperplasiogenic effect, as measured by effects on epidermal thickness, was less than that for mouse skin. On the other hand, Krueger and Shelby (3) reported that a single dermal application of low doses (0.016-0.16 nmole/50  $\mu$ L) of TPA induced epidermal hyperplasia in human skin xenografts, as measured by labeling ( $^3$ H-thymidine) index. The relevance of this observation to the present study is questionable, since Krueger and Shelby applied TPA in acetone:DMSO [1:1 v/v]

Like mouse skin, TPA most likely possesses a dose-dependent hyperplasiogenic effect on human skin. Krueger and Shelby (3) state that under their conditions of dermal application [single application in 50  $\mu$ L acetone:DMSO, 1:1 (v/v)] a dose-dependent effect on labeling index is observed in the range 0.016-1.6 nmole TPA/50  $\mu$ L, with maximal enhancement occurring at 1.6 nmole. At TPA concentrations higher than 1.6 nmole/50  $\mu$ L the epidermal labeling index decreased until maximal inhibition was reached at 161 nmole/50  $\mu$ L. Inhibition of epidermal

Hyperplasia in human skin xenografts by high doses of TPA is also supported by unpublished experiments by Dr. Krueger. Dermal application (Zwick for 2 wk) of TPA at doses of 81 nmole - 8.1  $\mu$ mole/50  $\mu$ L acetone yielded no epidermal hyperplasia, as measured by epidermal thickness, suggesting that these doses of TPA were inhibitory.

Given the limited data available from the pilot experiment and the conflicting observations from published and unpublished experiments on TPA-induced epidermal hyperplasia in human skin or human skin xenografts, Dermigen is unable to ascertain with any certainty if the TPA dose (4.25 nmole/50  $\mu$ L) utilized in the pilot experiment was too high or too low. In addition, the use of large circular xenografts introduces some additional variables that may compromise the results, including poorer xenograft viability and separations at the xenograft/nude skin junction because of xenograft shrinkage.

### Recommendations

It is essential that an additional pilot experiment be undertaken to identify the dose range of TPA that produces an epidermal hyperplasiogenic effect in human skin xenografts. Dermigen recommends that such an experiment (outlined below) be performed using a wide range of TPA concentrations and that this additional pilot experiment substitute for Subprotocol 1 specified in Dermigen Protocol No. 910828. This second xenograft pilot experiment should also be designed to address the following technical problems identified in the first xenograft pilot experiment:

#### 1. Shrinkage of the xenografts during healing.

The circular (2.5 cm dia) xenografts used in the first xenograft pilot experiment exhibited more shrinkage than anticipated. The xenografts were more susceptible to shrinkage in the anterior/posterior direction, as shown by the measurements in Table 2. The surface area of the graft decreased by approx. 60% during healing. In addition to reducing the surface area available for biopsy, stretching of the xenograft at its interface with the adjacent nude mouse skin in some cases caused physical separation at the junction. Access of the vehicle (acetone) to the underlying dermis at sites of separation can be anticipated to produce an irritative effect and this may have contributed to the observed histopathology of xenografts used in the first xenograft pilot experiment. Cognizant of this shrinkage problem, and at the request of Dr. Walborg, Dr. Krueger investigated the use of a rectangular graft (approx 3x2cm) with its long axis placed in the dorsal/ventral direction. Although shrinkage remained approx. the same, problems with separations at the junction of xenograft with nude mouse skin were reduced.

#### 2. Inconsistent labeling of epidermal cells by BrdUrd

Although accidental injection of the BrdUrd into the lumen of the gut can occur, this is a rare event. The fact that no BrdUrd label was found in two of the five animals examined indicates another type of delivery problem. Increasing the volume of the solution containing the BrdUrd may minimize the dosing problem.

#### 3. Evaluation of epidermal hyperplasia in nude mouse skin.

Evaluation of treated nude mouse skin was limited to that located adjacent to the xenograft. Since the 50  $\mu$ L dose volume allowed coverage of the xenograft, but minimized spillover to adjacent nude mouse skin, acquisition of consistently dosed nude mouse skin was difficult.

- Dermigen recommends that an additional pilot experiment be performed using a minimum of 5 mice to test an expanded range of TPA doses and that the experiment be designed to address problems encountered in the first xenograft pilot experiment.

A protocol to accomplish this is outlined below:

1. Dr. Krueger's laboratory shall supply a minimum of 5 mice bearing human skin xenografts, using skin obtained from abdominoplasties or organ donors. Grafts shall be rectangular (approx. 3x2 cm) with their long axis placed in the dorsal/ventral direction. These grafts shall be allowed to heal for a period of time deemed adequate by Dr. Krueger. Only mice bearing viable xenografts of suitable quality as judged by Dr. Krueger will be used and such evaluation shall be made within two days prior to their transfer to UBTL. Although these modifications cannot preclude significant shrinkage of the xenograft, it should provide grafts of better quality.
2. Dosing and tissue collection - xenograft. TPA shall be tested over a 4 log dose range: 10, 100, 1000, or 10,000 ng/50  $\mu$ L acetone. The dosing schedule shall be that described in Dermigen Protocol No. 910828, i.e. a total of 4 dermal dosings, 2 applications/week. One animal shall serve as the vehicle (acetone) control. At 6 hr post-final dosing, three 4 mm dia biopsies will be taken from one end of the xenograft. These biopsies, which will be used for assay of epidermal ODC activity, will be snap frozen and stored at -70°C. At 23.5 hr post-final dosing 0.5 mL (note larger volume) of a solution containing 100  $\mu$ g BrdUrd/g body weight will be administered by i.p. injection. At 24 hr post-final dosing the mice shall be sacrificed and the entire xenograft with 1 cm of surrounding nude mouse skin will be excised and placed in 10% buffered formalin. This specimen will be used to assess histopathology, epidermal thickness, and labeling index.
3. Dosing and tissue collection - nude mouse skin. TPA shall be tested on nude mouse skin contralateral to the xenograft at the same doses and schedule mentioned in No. 2 above. At 6 hr post-final dosing, three 4 mm dia biopsies will be taken from the treated nude mouse skin. These biopsies, which will be used for assay of epidermal ODC activity, will be snap frozen and stored at -70°C. Epidermal hyperplasia will be evaluated as a function of epidermal thickness and labeling (BrdUrd) index. Use of treated nude mouse skin contralateral to the xenograft will allow unequivocal evaluation of TPA-induced epidermal hyperplasia in nude mouse skin.
4. Analysis of skin specimens. Histopathologic, histomorphologic, and immunohistochemical (labeling index) evaluations will be performed by Dr. Claudio Conti using procedures outlined in Dermigen Protocol No. 910828, except where modifications are stipulated above.

The induction of epidermal ODC activity will be determined by Dr. DiGiovanni using methods specified in Dermigen Protocol No. 910828.

**References**

1. Yuspa, S.H. Maintenance of human skin on nude mice for studies of chemical carcinogenesis. *Cancer Lett.*, 6: 301-310, 1979.
2. Green, N. epidermal changes following application of 7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoyl-13-acetate to human skin transplanted to nude mice studies with histological species markers. *Cancer Res.*, 49: 275-284, 1989.
3. Krueger, G.G. and Shelby, J. Biology of human skin transplanted to the nude mouse: 1. Response to agents which modify epidermal proliferation. *J. Invest. Dermatol.*, 76: 505-510, 1981.

**Table 2 Size and siting of biopsies**

Animal no., treatment, biopsy site <sup>a</sup>	Size of trans- plant, cm <sup>b</sup>	No./size/timing of biopsies			
		6 hr			24 hr
		6 mm	4 mm	3 mm	3 mm
235081/7	1.50 x 1.50				
Acetone					
THS		2		2	
THS/N				2	
TN		3		3	
UN		3		2	
235083/3	1.25 x 1.50				
Acetone					
THS			3		1
THS/N					2
TN					3
UN					2
235082/2	0.75 x 2.00				
Acetone					
THS			2		1
THS/N					2
TN					3
UN					2
235084/4	1.00 x 2.00				
TPA					
THS		2		2	
THS/N				2	
TN		3		3	
UN		3		2	
235086/6	0.75 x 2.00				
TPA					
THS			2		strip <sup>c</sup>
THS/N					2
TN					3
UN					2
235085/8	0.75 x 2.00				
TPA					
THS			2		1
THS/N					2
TN					3
UN					2

<sup>a</sup>Treatment/site code:

THS = treated human skin

THS/N = junction of xenograft with host nude mouse skin

TN = treated nude mouse skin, i.e. nude mouse skin adjacent to xenograft

UN = untreated nude mouse skin, i.e. remote from treatment area.

<sup>b</sup>First measurement was made in anterior/posterior direction and second measurement in dorsal/ventral direction.

<sup>c</sup>Instead of a biopsy, a strip of tissue was excised.

**Histology report**

**Animal No. 235081/7**

1. Acetone, THS, 6 hr. Skin transplant of normal human histology with some inflammatory infiltrates, probably of murine origin, present in dermis. Differentiation of epithelium was normal, papillary and rete are marked.
2. Acetone, THS/N, 6 hr. Clear junction of human and mouse skin. Human skin similar to No. 1 above. Nude mouse skin characterized by hyperplastic epidermis, presence of anagen-like hair follicles and well developed sebaceous glands.
3. Acetone, TN, 6 hr. Nude mouse skin, nothing remarkable.
4. Acetone, UN, 6 hr. Nude mouse skin, nothing remarkable.

**Animal No. 235083/3**

1. Acetone, THS, 24 hr. Human epidermis similar to that of animal No. 235081/7.
2. Acetone, THS/N, 24 hr. An area of human epidermis and an area of reepithelialized epidermis, probably of mouse origin.
3. Acetone, TNS, 24 hr. Nude mouse skin, nothing remarkable.

**Animal No. 235082/2**

1. Acetone, THS, 24 hr. Coagulative necrosis of human epidermis with large subcorneal abscess.
2. Acetone, THS/N, 24 hr. Areas of human epidermis of relatively normal histology, but showing clear signs of rejection, some areas of nonepithelialized dermis.
3. Acetone, TNS, 24 hr. Nude mouse skin, nothing remarkable.
4. Acetone, UN, 24 hr. Nude mouse skin, nothing remarkable.

**Animal No. 235084/4**

1. TPA, THS, 6 hr. Necrotic human skin with heavy inflammatory infiltration.
2. TPA THS/N, 6 hr. Epithelium infiltrated with inflammatory elements and signs of degeneration. Subcorneal microabscess. No clear demarkation between mouse and human skin; in fact, in one specimen no normal murine skin visible.
3. TPA, TNS, 6 hr. Nude mouse skin, nothing remarkable.

**Animal No. 235086/6**

1. TPA, THS, 24 hr. Epidermis of poor morphology, human or murine origin unclear. A deepithelialized area was visible.
2. TPA, THS/N, 24 hr. Normal murine epithelium with an area in which murine epidermis has reepithelialized adjacent tissue, also areas of deepithelization.
3. TPA TNS, 24 hr. Nude mouse skin, probably some hyperplasia.
4. TPA UNS, 24 hr. Nude mouse skin, probably some hyperplasia of hair follicles.

Histology report (cont.)

Animal No. 235065/8

1. TPA, THS, 24 hr. Human skin with reasonable histology, but some signs of rejection. Specimen needs to be recent.
2. TPA, THS/N, 24 hr. No clear junction of human and murine skin. Normal murine epithelium and an area in which the murine epidermis has reepithelialized adjacent tissues.
3. TPA, TNS, 24 hr. Nude mouse skin, probably some hyperplasia.
4. TPA, UNS, 24 hr. Nude mouse skin, probably some hyperplasia of hair follicles.

**Table 3 Labeling (BrdUrd) index of skin epidermis**

Treatment	Animal no.	Biopsy site <sup>a</sup>	Labeling index
Acetone, 50 $\mu$ L	235081/7	THS	No label present
	235081/7	TN	No label present
	235081/7	UN	No label present
TPA, 4.25 nmole/50 $\mu$ L	235083/3	THS	3.3%
	235084/4	TN	No label present
	235084/4	UN	No label present
	235086/6	THS	3.6%
	235085/8	THS	1.0%

<sup>a</sup>Treatment/site code:

THS = treated human skin

THS/N = junction of xenograft with host nude mouse skin

TN = treated nude mouse skin, i.e. nude mouse skin adjacent to xenograft

UN = untreated nude mouse skin, i.e. remote from treatment area.

<sup>b</sup>Not calculated, since xenograft exhibited significant histopathology

**Table 4 Assay of epidermal ODC activity**

Animal No.	Treatment	Biopsy site <sup>a</sup>	Biopsy size, dilution	mm <sup>2</sup> /0.1 mL	nmol CO <sub>2</sub> /mm <sup>2</sup> /hrX10 <sup>4</sup>
235081/7	Acetone	THS	2 X 6mm/1.0 mL	5.65	38.0
	Acetone	TN	3 X 6mm/1.0 mL	8.48	103.0
	Acetone	UN	3 X 6mm/1.0 mL	9.49	92.0
235083/3	Acetone	THS	3 X 4mm/0.5 mL	7.54	40.6
235082/2	Acetone	THS	3 X 4mm/0.5 mL	7.54	N.C. <sup>b</sup>
235084/4	TPA	THS	2 X 6mm/1.0 mL	5.65	N.C. <sup>b</sup>
	TPA	TN	3 X 6mm/1.0 mL	8.48	193.2
	TPA	UN	3 X 6mm/1.0 mL	8.48	32.8
235086/6	TPA	THS	2 X 4mm/0.5 mL	5.03	18.2
235085/8	TPA	THS	3 X 4mm/0.5 mL	7.54	20.6

<sup>a</sup>Treatment/site code:

THS = treated human skin

THS/N = junction of xenograft with host nude mouse skin

TN = treated nude mouse skin, i.e. nude mouse skin adjacent to xenograft

UN = untreated nude mouse skin, i.e. remote from treatment area.

<sup>b</sup>Not calculated, since xenograft exhibited significant histopathology



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