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A COMPARISON OF THE SKIN IRRITANCY AND SKIN SENSITISATION POTENTIALS OF THE AGENTS CS AND CR IN ANIMALS

by

N.H. Creasey, J.A. Fletcher, and D.V. Sinkinson

Technical Paper No. 109

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Chemical Defence Establishment,
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A COMPARISON OF THE SKIN IRRITANCY AND SKIN SENSITISATION POTENTIALS OF THE AGENTS CS AND CR IN ANIMALS

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SUMMARY

Using the term 'Chemical Irritant' to mean a substance capable of causing on superficial contact a non-allergic inflammation of the skin, and 'Skin Sensitiser' to mean a substance capable of inducing on superficial contact a state of allergic sensitivity to itself, then:

CS is a chemical irritant in the rabbit; in the guineapig it is both a chemical irritant and a skin sensitiser;

CR is not a chemical irritant in the rabbit; in the guineapig it is neither a chemical irritant nor a sensitiser.

The histopathology of the various skin effects is described.

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INTRODUCTION

In the concentrations used for riot control, CS (o-chlorbenzylidene malononitrile) smoke produces temporary incapacitation by virtue of its irritant and lachrymatory effects on the eyes and nasopharynx. Recovery is complete and without after effects within about 15 minutes of the end of the exposure (1). In hot and humid conditions prolonged exposure of the skin to high concentrations of CS smoke or to heavy contamination with CS powder or aqueous suspensions of CS causes a dermatitis which can vary from mild erythema to vesication; allergic sensitisation has also occasionally been observed, (2,3).

The present study, carried out on rabbits and guineapigs, was undertaken to see whether CR (dibenz (b,f)-1,4-oxazepine), an irritant with riot control potential similar to that of CS, might be safer in use than CS through being free from the tendency to produce dermatitis.

In the living animal the pathological response of the skin to contact with an irritant chemical substance may be, excluding carcinogenesis, (a) a Chemical Irritant Response (CIR) where the substance produces a cytotoxic, non-allergic inflammation, and, in extreme cases, chemical burning, of the skin of all individuals provided that it is allowed to act long enough and in sufficient concentration; (b) a Delayed Allergic Skin Response (DASR) which

occurs only when individuals sensitised by previous contact are exposed to concentrations of the substance to which they were previously insensitive, or (c) a mixture of the two responses produced by exposure of sensitised individuals to an irritant concentration of a contact allergen (4, 4a, 5, 6).

In external appearance the CIR and the DASR are similar but may often be distinguished from one another by their appearance in histological section (6-9).

In the present investigation the CIRs to CS and to CR were compared in the rabbit and in the guineapig, and the DASRs were compared in the guineapig; these experiments confirmed the irritant and sensitisation potential of CS and showed that CR was harmless in both respects.

METHODS

Preliminary experiments with dinitrofluorobenzene (DNFB) for validation of method of distinguishing between CIR & DASR. The CIR to DNFB was elicited by applying 20µl of DNFB (8%) in acetone: olive oil (4:1) solvent cutaneously to a clipped guineapig. This provided even coverage of an area *ca* 2 cm in diameter and a contamination density of *ca* 0.5 mg/cm^2. The DASR was elicited by the cutaneous application of 20µl of DNFB (0.25%) in acetone: olive oil (4:1) solvent (= *ca* 0.016 mg/cm^2) to guineapigs sensitised to it by applying 50µl DNFB (10%) in acetone: olive oil (1:1) to one ear 2 weeks previously (10).

The screening of CS or CR for a CIR was performed by applying a 1cm diameter disc of the finely powdered material under clear adhesive polythene tape (Lassotape 16, T.J. Smith and Nephew Ltd) to the skin of rabbits which had been depilated 1 week previously (11). A dressing was prepared which consisted of a 2.5cm square of filter paper with a 1.5cm diameter hole in the middle stuck to the centre of a piece of adhesive tape 5 x 25cm. The hole was then applied to the mouth (also 1.5cm diameter) of the bottle

containing the powdered irritant and the bottle shaken. This resulted in about 0.5-1.0 mg of the irritant being picked up by the exposed tape. The dressing was then secured around the thorax so as to bring the irritant into contact with a depilated area of skin in the middle of the animal's back. After 24 hours the skin was observed through the tape, then the tape was removed and the treated area rubbed with the tip of the finger to see whether the epidermis had been loosened. This was supplemented by taking samples of other treated areas for histological examination.

Skin Sensitisation Potential was investigated in guineapigs by both Rothberg's (12) and by Magnusson and Kligman's (7) methods.

In Rothberg's (12) method sensitisation was attempted by applying 0.1ml of a 1% solution (=1mg) of the compound in diethyl ether daily 5 times per week for 3 weeks to the clipped flanks; the animals were rested for 2 weeks and challenged with 0.1ml 1% or 0.1% solution of the compound in diethyl either applied to the opposite clipped flank; the proportion of responders in the group was noted and samples of the lesions taken for histological examination. Evaluation of the response was made at 24 and 48 hours; positive responses varied from faint pinkening of the skin to erythema, oedema and crust formation.

In Magnusson and Kligman's "Maximisation Procedure" (7) sensitisation of groups of guineapigs was attempted as follows. In each animal a row of three intradermal injections was made on each side of the mid-line of the back in the scapular region: 0.1ml CR (10%) or CS (0.5%) in Freund's complete adjuvant (Difco), 0.1ml CR (10%) or CS (0.5%) in propylene glycol (concentrations of CS and of CR chosen so that nothing more than a mild lesion was produced) and 0.1ml Freund's complete adjuvant alone. One week later the same area of the CR animals was treated for 48 hours with sodium lauryl sulphate (10%) in vaseline. This treatment enhances sensitisation by non-irritant substances by provoking a inflammatory reaction (7); it was omitted in the case of CS which produced its own inflammatory reaction. 24 hours later a suspension of finely powdered CS (0.5%) or CR (25%) in vaseline was applied to the

same area for 24 hours under an occlusive dressing. The animals were rested for 2 weeks and then challenged by applying CS (0.25%) or CR (25%) in vaseline to the flank, again under an occlusive dressing. 24 hours later the dressing was removed, the site cleaned with ether, shaved with an electric shaver and inspected about 2 hours later, and after a further 24 hours i.e. 48 hours after commencing the challenge. The minimum criterion of a positive response in this test is erythema; a just detectable pinkening of the skin which was observed in some animals treated with vaseline alone was recorded but was neglected in the scoring. The significant parameter is the frequency of the response in groups of 10 or 25 animals and not its intensity in individuals. Samples of the treated skin were taken for histological examination at the stages in the investigation indicated in the tables.

The rating of sensitisation potential (allergenicity) in Magnusson and Kligman's Procedure (7) was based on the percentage of animals which showed erythema after the challenge: 0-8% = I or weak, 9-28% = II or mild, 29-64% = III or moderate, 65-80% = IV or strong, and 81-100% = V or extreme.

Histological examination: Samples of the treated areas or lesions were fixed in Bouin's fixative and embedded in paraffin. 10μ sections were cut perpendicular to the surface and generally parallel to the direction of hair growth; they were stained with Lillie's haematoxylin-eosin-azur stain (13). To obtain the proportion of monocytes (MNC), i.e. free non-granular cells, the 1/6" high power field was divided into 80 squares by means of an eye-piece graticule. The total number of cells (fibroblasts, histiocytes and endothelial cells as well as mononuclear cells was counted in 2-3 rows of squares parallel to and between the hair follicles. The number of mononuclear cells in the area was then expressed as a percentage of the total.

RESULTS

Validation of method for distinguishing between the CIR and the DASR

The cutaneous application of a chemical irritant to a guineapig not already allergically sensitised to it produces a macroscopic

inflammatory lesion, the Chemical Irritant Response (CIR) within 12 hours. Depending on the substance and the amount applied the appearance may vary from mild erythema, with or without oedema, to complete necrosis and destruction of the tissue by chemical burning.

If, however, the animal has, by previous skin contact, acquired an allergic sensitivity to the substance, the cutaneous application of a much smaller dose than that required to produce even the mildest CIR will produce, over the slightly longer period of 12-24 hours, a marked erythematous reaction known as the Delayed Allergic Skin Response because of the longer period required for it to appear. In the human subject the DASR may take up to 48 hours or longer to develop (6). At this lower dose the proportion of animals which respond is much higher in a sensitised group than in an unsensitised or 'naive' group where the number of responders may be zero.

A further difference between the CIR and the DASR may often be seen in the histopathogenesis of the two lesions. Essentially the allergic lesion shows no damage to the epidermis prior to its invasion by mononuclear cells whereas in the CIR the epidermis is damaged by the irritant and then the upper dermis is invaded by predominantly neutrophilic polymorphonuclear cells (neutrophils) (6, 7, 14).

These differences were well illustrated during the course of preliminary experiments with cutaneously applied DNFB in the guinea-pig where, like dinitrochlorophenol (14), it is both an irritant and a sensitiser (17). For example, by applying 20µl of 8% DNFB in acetone: olive oil (4:1) cutaneously a CIR in the form of an erythematous wheal was produced in 3-6 hours. Histological sections of the lesion at 24 hours showed the entire epidermal cytoplasm to be eosinophilic with large numbers of perinuclear vacuoles, regions of separation at the dermo-epidermal junction and heavy invasion of the upper dermis by neutrophils (Fig 1 & 2). There was also a considerable accumulation of serous fluid beneath the adipose layer (Fig 1). The cutaneous application of a similar volume of 1% DNFB

in acetone: olive oil produced a much milder CIR. At 24 hours this appeared as slight erythema of the treated area; in histological section the epidermal changes were similar to those observed with 8% DNFB but the dermis was much less oedematous and contained scarcely any leucocytes (Figs 3 & 4).

The DASR was produced by the cutaneous application of 20µl of 0.25% DNFB in the acetone:olive oil solvent to sensitised guineapigs (see Methods). The response at 24 hours consisted of a non-raised red area; in histological section both epidermis and dermis were invaded by mononuclear cells, the epidermal cytoplasm was basophilic with many perinuclear vacuoles and intercellular oedema (spongiosis) (Fig 5 & 6). The application of this dose of DNFB to unsensitised guineapigs produced only a slight pinkening of the clipped skin, and histological sections of the treated areas were indistinguishable from the normal; a few mononuclear cells were present in the dermis (Fig 7 & 8). When the concentration of DNFB was reduced to 0.125% 5/5 sensitised guineapigs responded compared with 1/5 naive guineapigs.

These results confirm the reported differences between the allergic and irritant responses in the guineapig (6, 14); in the following paragraphs the results of attempts to apply these criteria to the effects produced in the skin of rabbits or guineapigs by the cutaneous application of CS or CR will be described.

Effect of cutaneous application of powdered CS or CR to unsensitised rabbits (CIR)

Because their skin develops a visible lesion after exposure to CS under occlusion rabbits as well as guineapigs were used for comparing the skin irritancy of CS and of CR. In confirmation of Mershon's (11) findings 24 hours' treatment of depilated rabbit skin with powdered CS under an airtight waterproof dressing damaged the epidermis so much that it could be rubbed off with the tip of the finger; the underlying dermis showed a marked inflammatory reaction consisting of extensive invasion by neutrophils (Figs 9 & 10). These histological changes were similar in character to those observed in the CIR to DNFB in the guineapig (Figs 1 & 2)

The maximum response to CR when tested in the same way was a "just detectable pinkening" of the skin; histological sections of the treated areas were normal in appearance (Fig 11).

Effect of cutaneous application of suspensions of CS or of CR in vaseline to unsensitised guineapigs (CIR).

The objectives of this group of experiments were to demonstrate the CIR to CS and to CR in guineapigs and to determine, within a factor of approximatley 2, the highest concentration of the agents in vaseline that could be applied to clipped guineapigs without causing erythema in the treated area. This concentration would then be used as a challenge to other guineapigs which had been subjected to the Magnusson & Kligman (7) sensitisation procedure (see below).

Suspensions of CS (24, 12, 6, 3, 1.5, 0.5, and 0.25%) in vaseline were applied under an occlusive dressing to the skin of clipped guineapigs, using one animal for each concentration. 24 hours later all sites treated with concentrations of more than 0.25% CS had become erythematous, the intensity being approximately proportional to the concentration applied.

Histological sections (Figs 12 & 13) of the 24 hour lesion caused by CS (12%) in vaseline showed the cellular damage to be present almost entirely in the epidermis; for example the epidermis was widely separated from the dermis over most of the lesion, the gap being filled in places by serous fluid. The epidermal cytoplasm was eosinophilic and the nuclei pyknotic. Cellular damage in the upper dermis was slight and consisted of a few pyknotic nuclei immediately beneath the basal layer of the epidermis. There was a mild influx of leukocytes, mainly neutrophils, into the dermis.

The site treated with CS (0.25%) showed a just detectable pinkening of the skin; the response to this concentration of CS was, therefore, investigated in a larger group of 15 naive guineapigs.

24 hours after applying the CS suspension 10/15 animals had developed a "just detectable pinkening" of the treated areas. Histological examination of the treated sites from two responders selected at random showed small localised regions of the epidermis to be slightly increased in thickness and in the granularity of the granular layer (Fig 14); this was a very mild response and was occasionally noticed after the application of vaseline alone.

Similar experiments performed with CR showed that 3/10 guinea-pigs responded to the cutaneous application of CR (25%) in vaseline with no more than a "just detectable pinkening" of the treated area. Histological examination of the treated sites (Fig 15 & 16) showed slight thickening of the epidermis and in the upper dermis the accumulation of a few mononuclear cells and generally increased cellularity; sections of skin treated with vaseline alone were normal in appearance.

Effect of cutaneous application of CS or CR to "sensitised" guineapigs (DASR)

Guineapigs subjected to Rothberg's (12) sensitisation procedure with up to 38 daily applications of 0.1ml CS (1%) in diethyl ether became mildly sensitised in that 5/7 responded to subsequent applications of CS (0.1%) in ether with slight erythema whereas naive guineapigs did not react to this treatment. Histological sections of the 1 day old response in the sensitised animals (Fig 17) and in similarly treated naive animals (Fig 18) were normal in appearance.

Magnusson & Kligman's Maximisation Procedure (7) was also used to test the sensitisation potential of CS in the guineapig: CS (0.25%) in vaseline was used as the challenge and, for the assessment of the group response, only those animals which responded with at least redness of the treated area were scored as positive, i.e. the just detectable pinkening of the skin referred to earlier was neglected as this was also observed in some animals treated with vaseline alone. The type of response obtained to CS (0.25%) and the proportions of sensitised and naive animals showing it are

summarised in Table 1. 24 hours after beginning the challenge none of the naive animals showed more than a "just detectable pinkening" of the skin whereas 50% of the sensitised animals developed obvious reddening of the treated area (Table 1). On Magnusson and Kligman's scale (7) CS would, therefore, be rated as a Class III, or "Moderate" sensitiser.

Two challenged skin areas taken at random from the CS sensitised responders showed an allergic type of response with spongiosis and vacuolisation of the epidermis and invasion of the upper dermis by mononuclear cells, (Fig 19 & 20). Naive animals treated with the same concentration of CS in vaseline produced either no response visible to the naked eye or a just detectable pinkening of the skin; histological sections of the treated skin from the naive animals showed a very mild non-allergic irritation of the epidermis and no invasion of either dermis or epidermis by inflammatory cells (Fig 14). Even with CS (12%) very few inflammatory cells were present at 24 hours (Fig 12 & 13).

It was not possible to sensitise guineapigs to CR by Rothberg's (12) procedure, i.e. there were no responders either in the naive or in the sensitised groups and all the treated sites, whether from naive or from sensitised animals appeared normal in histological section. In the far more provocative Maximisation Procedure of Magnusson & Kligman (7), in which both the sensitisation and the challenge involved the application of CR (25%) in vaseline for 24 hours under an occlusive dressing, neither the naive nor the sensitised groups of guineapigs responded to the challenge with reddening of the skin; in the sensitised group however a higher proportion of the animals than in the naive group developed a "just detectable pinkening" of the treated area (Table 2). On Magnusson and Kligman's scale (7) CR would, therefore be rated as a Class I or "Weak" sensitiser. The response of either group as seen in histological sections of the treated areas was slight, but there were consistent differences between the two. In both groups the epidermis was 2-3 times its usual thickness (Figs 15 & 21), presumably a response to slight irritation, but in the naive group the upper

dermis was mildly invaded by mononuclear cells only (Fig 16) whereas in the "sensitised" group the mononuclear cells were accompanied by neutrophils and eosinophils (Fig 22). In the naive animals there was no significant difference between the proportions of mononuclear cells (as a percentage of all cells present) in the normal and treated skin sites, whereas in the "sensitised" group there was a significantly higher proportion of mononuclear cells in the treated sites (67%) than in the untreated sites (35%), (Table 3). These observations may indicate a different, although still subclinical, reaction to CR in the "sensitised" animals.

After 85 daily cutaneous applications to the same site of 0.1ml CR (1.0%) in diethyl ether to a group of 3 guineapigs there was no external lesion and the skin had a normal appearance in histological section (Figs 23 & 24).

DISCUSSION

The present investigation shows that CS is both a Chemical Irritant and a Sensitiser whereas CR is not a Chemical Irritant and is most likely not a Sensitiser.

The Chemical Irritant Response (CIR) of the skin results from the penetration of a non-corrosive amount of a cytotoxic substance through the stratum corneum and its interaction with the cells of the epidermis, and possibly the dermis, to produce effects which vary from mild reddening (detergents, soaps, solvents) to vesication (mustard gas, cantharidin). "Corrosives" such as concentrated acids or alkalies cause rapid full thickness destruction, or chemical burning, of the skin and are "irritant" in low concentrations only.

Lesions similar in gross appearance to the CIR may result from the application of relatively small amounts of a chemical substance to the skin of an individual who has become sensitive to it by a previous contact, which itself may have produced no visible lesion. The mature reaction may take 12-48 hours or more to develop and is consequently known as the Delayed Allergic Skin Response (DASR) (6, 16, 17). It is a cell mediated immunological reaction to the antigenic hapten-protein conjugate formed between the sensitising chemical and epidermal protein, and may vary in appearance from

redness to vesication over the entire area of contact and beyond.

In histological sections of a typical DASR, the epidermis retains its normal appearance until it is invaded and destroyed by mononuclear cells. By contrast, sections of a typical CIR show that the epidermis and possibly also the dermis is first injured or killed by the cytotoxic chemical and only then is the skin invaded by inflammatory cells, mostly neutrophils, as a "cleaning-up" operation.

In Magnusson & Kligman's Maximisation Procedure (7) for detecting skin sensitisers the various factors which promote sensitisation are optimised. For example the attempted sensitisation is carried out in the presence of Freund's complete adjuvant (16) and on inflamed skin (7) with the highest tolerable concentration of the agent. The conditions for the challenge are equally severe, namely the highest concentration of agent which produces no effect under occlusion in naive animals, so that even weak sensitisers have a good chance of being detected. The essential parameter is the percentage of guineapigs in a sensitised group which respond to the cutaneous application of the highest concentration of sensitiser which produces no response in a naive group. Any doubt about whether the response is allergic or "irritant" is resolved by examining histological sections of the treated sites.

The Magnusson and Kligman sensitisation procedure is thus more provocative than Rothberg's (12) procedure and is claimed to predict the human response to the agent in question.

The test is not strictly a biological assay of allergenicity since there is no attempt to relate either the sensitising or the challenge dose of agent to the frequency or to the intensity of the allergic response. It merely establishes whether a particular substance has the ability to sentitise guineapigs. Experience has shown that compounds having a positive reaction in this test are potential sensitisers in the human, although when used as intended the concentration of the compound should be so low that this potential

may never be realised. When none of the guineapigs becomes sensitised this indicates an allergic potential so low that no imaginable human exposure is likely to be attended by a significant incidence of sensitisation.

Both Rothberg's (12) and Magnusson and Kligman's (7) tests showed CS to be a sensitiser; on Magnusson and Kligman's scale it would rank as a Class III or "Moderate" sensitiser, i.e. capable of sensitising 29-64% of the guineapigs tested. CS was also a potent irritant, comparable with mustard oil (allyl isothiocyanate) for example, both when applied as a powder under occlusion in the rabbit by Mershon's technique (11) and when applied as a suspension of 0.5% or more in vaseline to naive guineapigs by the Magnusson and Kligman method (7). The histopathology of the chemical irritant lesions was similar to that reported by Weigand et al (19) for the dog and the rabbit.

CR on the other hand was not a Chemical Irritant, and by Magnusson and Kligman's scoring was only a Class I or "Weak" sensitiser. It could be applied to the skin as powder or as a 25% suspension in vaseline without causing any pathological effects either in normal animals or in those which had been subjected to the sensitisation procedure, whereas the cutaneous application of 0.5% CS in vaseline caused an inflammatory lesion, i.e. at 1/50 of the tolerable concentration of CR.

Although the guineapigs did not become sensitised to CR the presence in the treated skin of a higher proportion of mononuclear cells than in the treated skin of unsensitised animals may indicate a different type of reponse in the sensitised group.

The difference in sensitisation potential between CS and CR may be associated with differences in their chemical reactivity. CS readily alkylates protein SH groups to form a covalently bonded hapten-protein conjugate (20). CR on the other hand forms only weak, probably hydrophobic, links with proteins which are readily broken by dialysis (21). Such weak bonds are known to confer little if any sensitisation potential (22).

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TABLE 1

Skin sensitisation potential of CS in the guineapig as determined by Magnusson & Kligman's (7) Maximisation Procedure

CS (0.25%) in vaseline was applied under an occlusive dressing to the clipped backs of naive and sensitised guineapigs. The appearance of the treated sites was recorded 24 hours after beginning the exposure.

	Type of Response and proportion of animals showing it				Proportion of responders with those showing "Just detectable pinkening" ignored
	Nil	Just detectable pinkening	Reddening	Reddening + oedema	
Naive, 24h	5/15	10/15	0/15	0/15	0/15
Sensitised 24h	2/14	5/14	6/14 (1)	1/14 (1)	7/14 (50%)
48h	0/14	9/12	3/12	0	3/12

50% of animals responding = Class III or "Moderate" sensitiser;

( ) = number of animals taken for histology at 24 hours.

Table 2

Skin sensitisation potential of CR in the guineapig as determined by Magnusson & Kligman's (7) Maximisation Procedure

CR (25%) in vaseline was applied under an occlusive dressing to the clipped backs of naive and sensitised guineapigs. The appearance of the treated sites was recorded 24 and 48 hours after beginning the exposure

		Type of Response and the proportion of animals showing it				Proportion of responders with those showing "Just detectable pinkening" ignored
		Nil	Just detectable pinkening	Reddening	Reddening + oedema	
Naive:	24h	7/10	3/10 (2)	0/10	0/10	0/10
	48h	8/8 (8)	0/8	0/8	0/8	0/8
Sensitised:	24h	2/24	22/24 (4)	0/24	0/24	0/24
	48h	15/20 (15)	5/20 (5)	0/20	0/20	0/20

( ) = number of animals taken for histology.

None of the sensitised animals showed reddening of the skin; this is equivalent to a Class I or "weak" sensitiser.

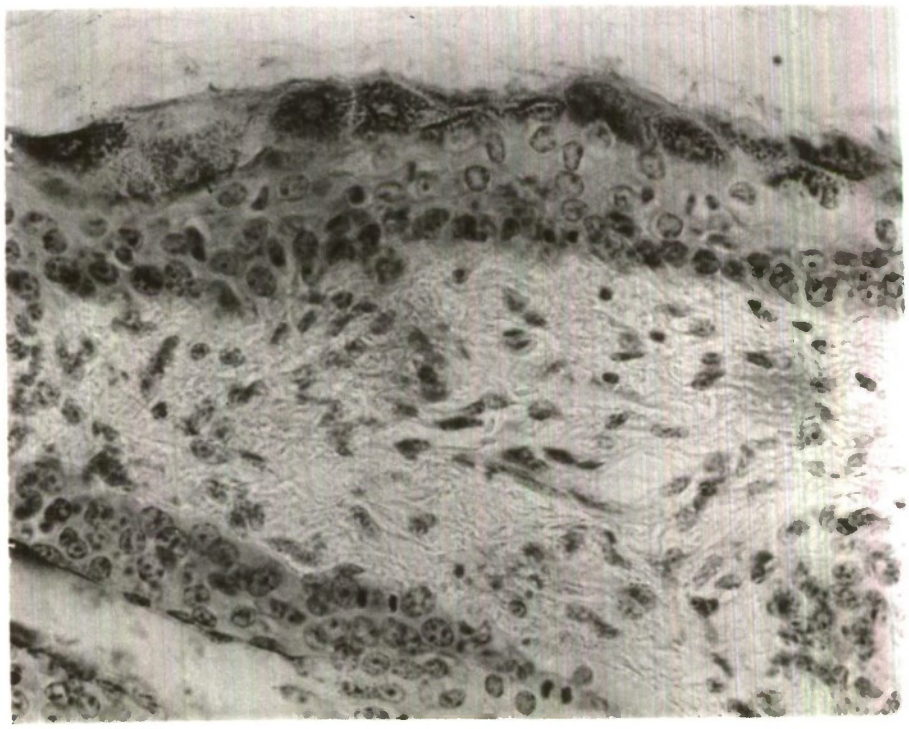

FIG. 14.

FIG. 15 CIR in the naive guineapig to CR (25%) in vaseline applied under occlusion to the clipped flank 24 hours previously. (x 110).

FIG. 16. Outlined area of Fig. 15. (x 320).

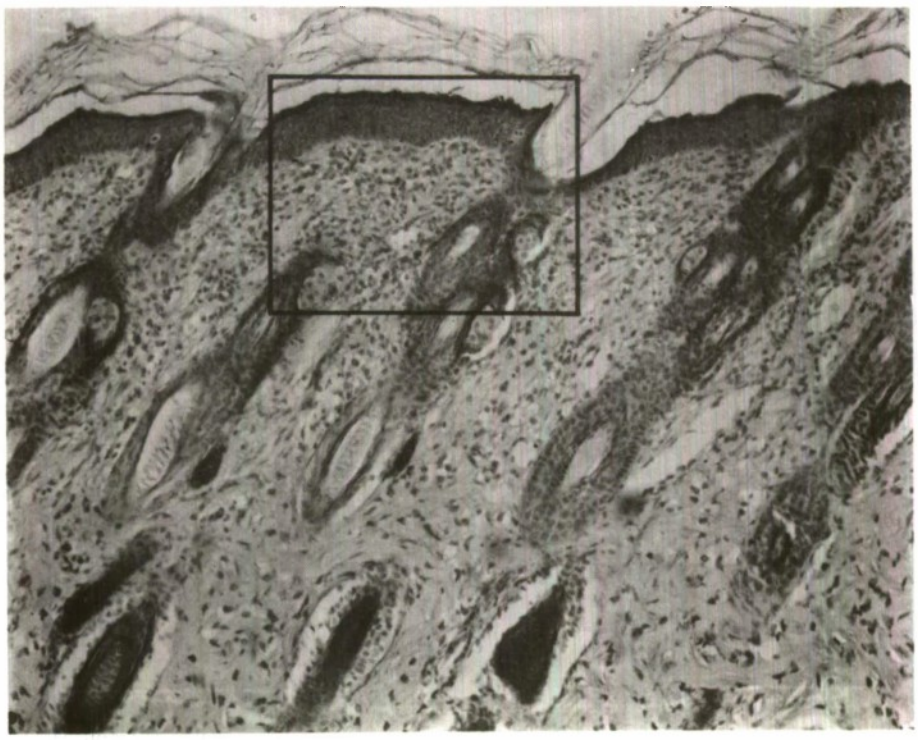

FIG. 15.

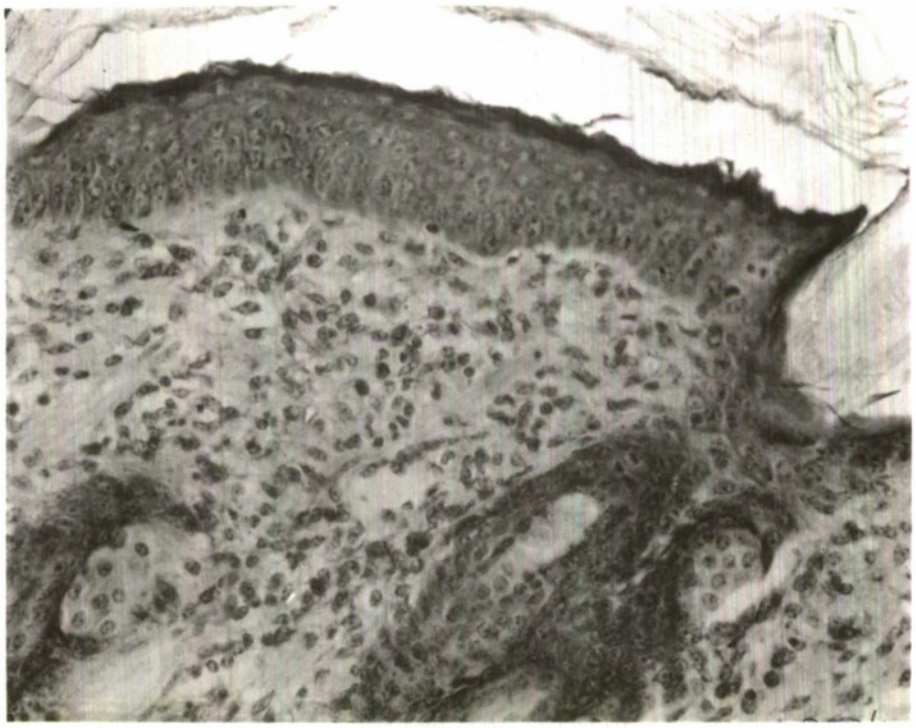

FIG. 16.

FIG. 17. DASR at 24 hours in guineapigs sensitised to CS by Rothberg's technique (see Methods) and challenged by applying 0.1 ml CS (1%) in diethyl ether to the clipped flank. (x 330).

FIG. 18. CIR in naive guineapig to 0.1 ml CS (1%) in diethyl ether applied to the clipped flank 24 hours previously. (x 315).

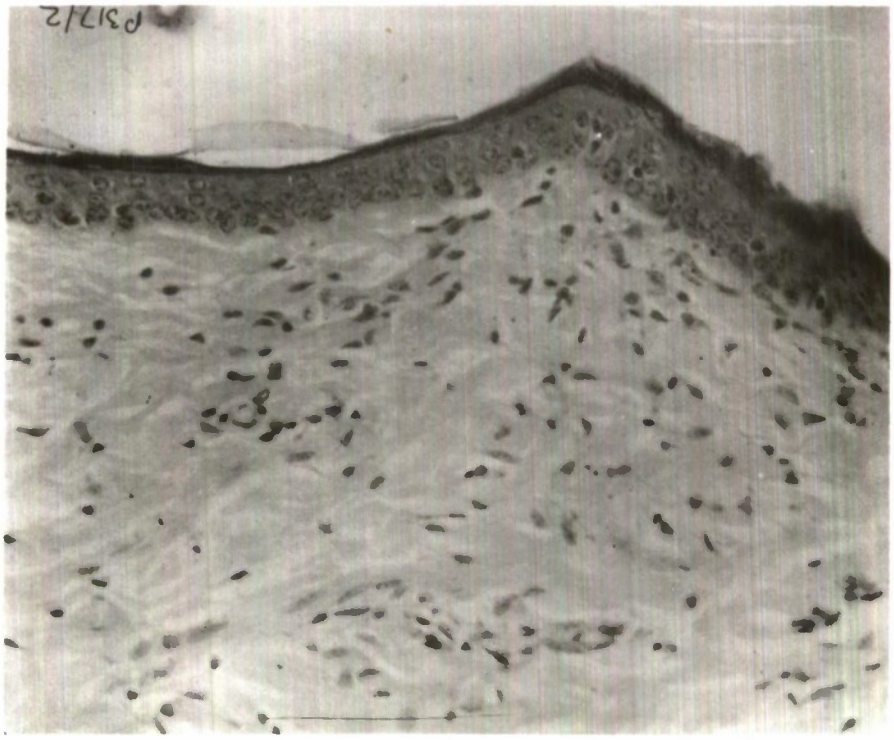

FIG. 17.

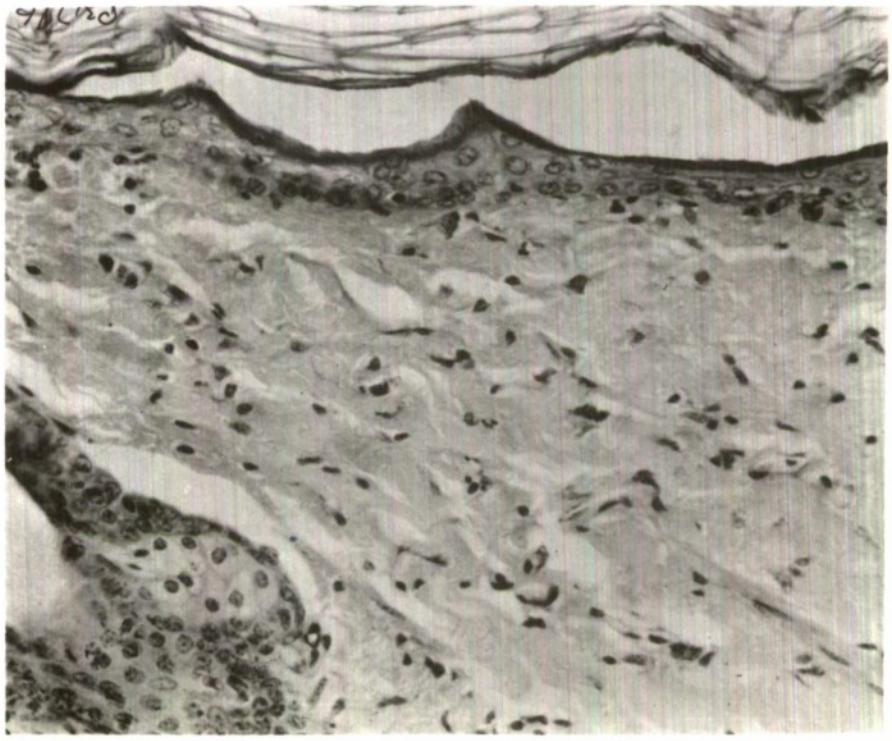

FIG. 18.

FIG. 19. DASR at 24 hours in guineapig sensitised to CS by Magnusson and Kligman's technique (see Methods) and challenged by applying CS (0.25%) in vaseline under occlusion to the clipped flank. (x 195).

FIG. 20. Outlined area of Fig. 19. (x 690).

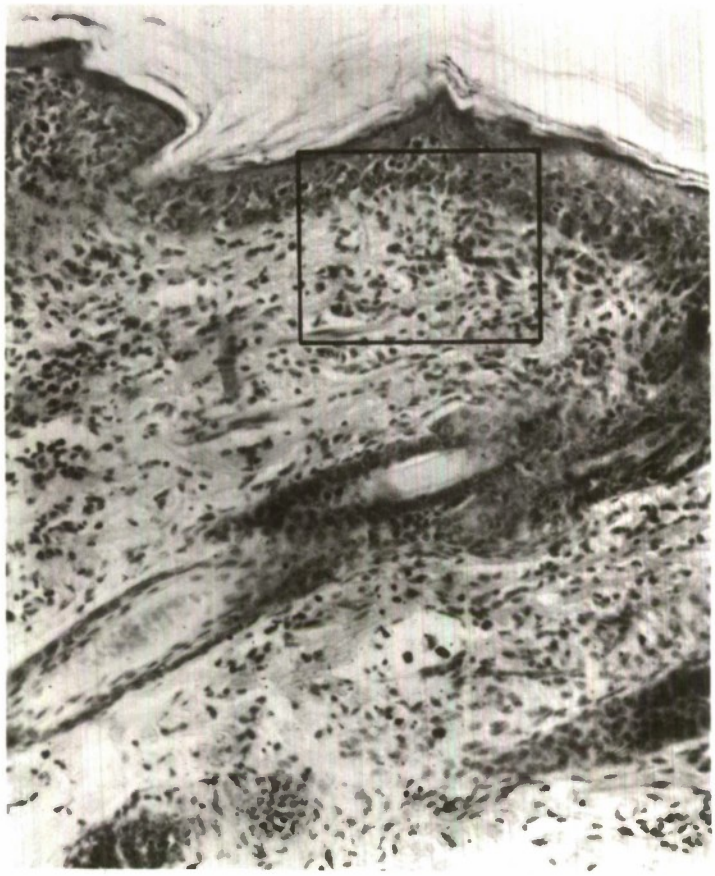

FIG. 19.

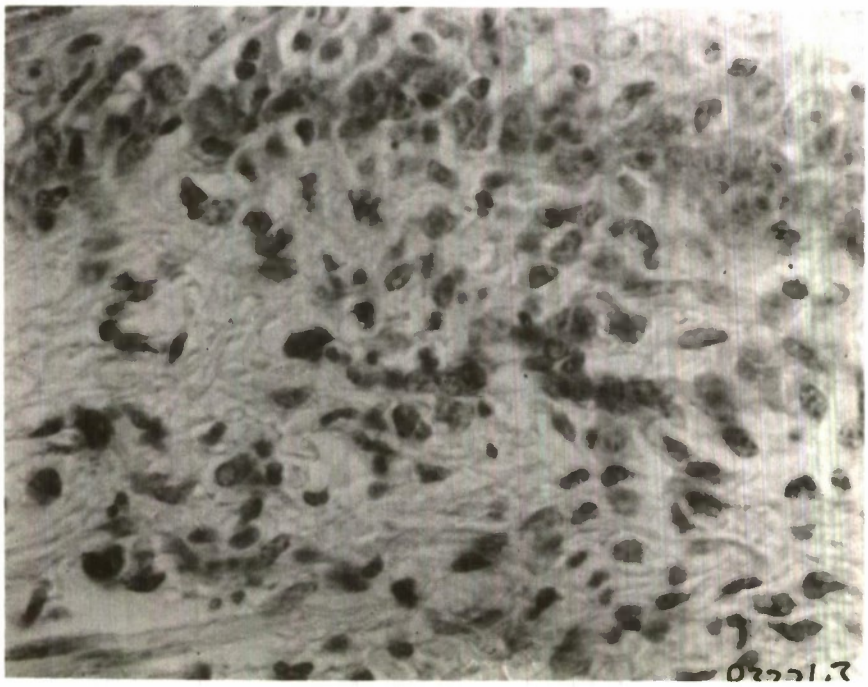

FIG. 20.

FIG. 21. DASR at 24 hours in guineapig sensitised to CR by Magnusson and Kligman's technique (see Methods) and challenged by applying CR (25%) in vaseline under occlusion to the clipped flank. (x 170).

FIG. 22. Outlined area of Fig 21. (x 680).

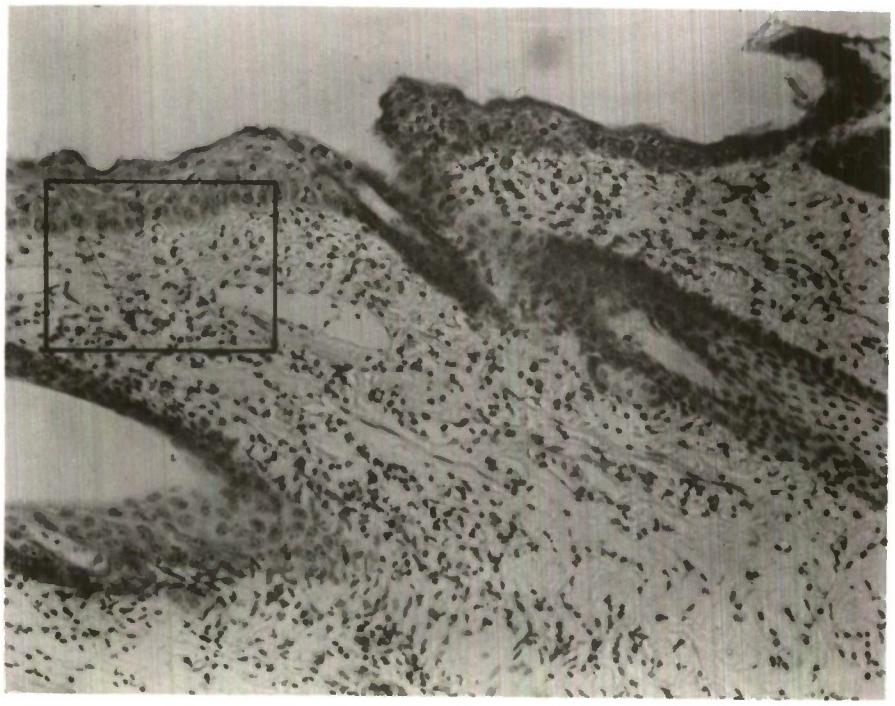

FIG. 21.

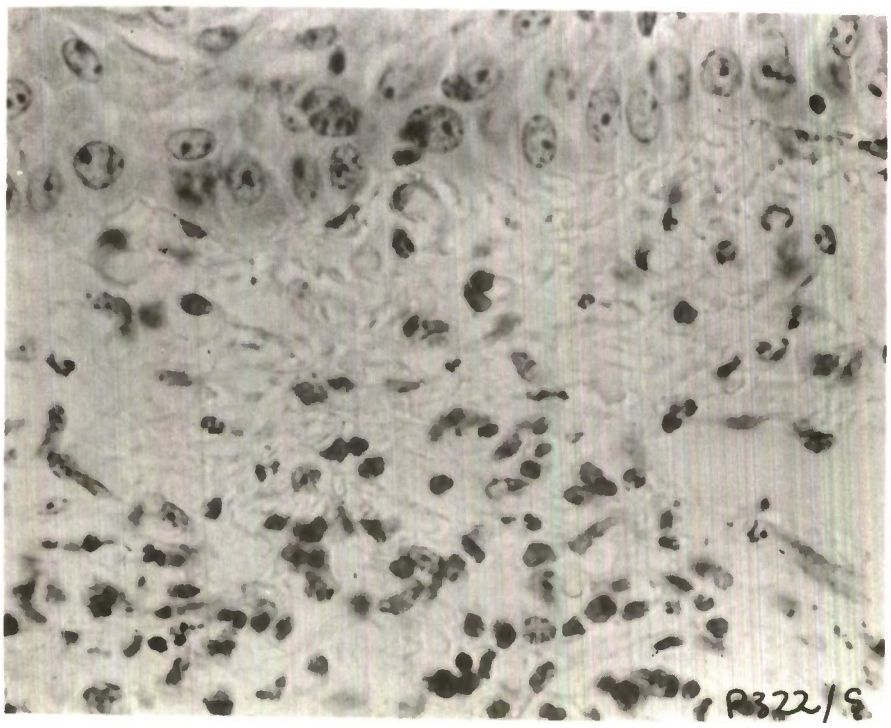

FIG. 22.

FIG. 23. Guineapig skin after 85 daily applications to the clipped flank of 0.1 ml CR (1%) in diethyl ether. (x 110).

FIG. 24. Outlined area of Fig. 23. (x 440).

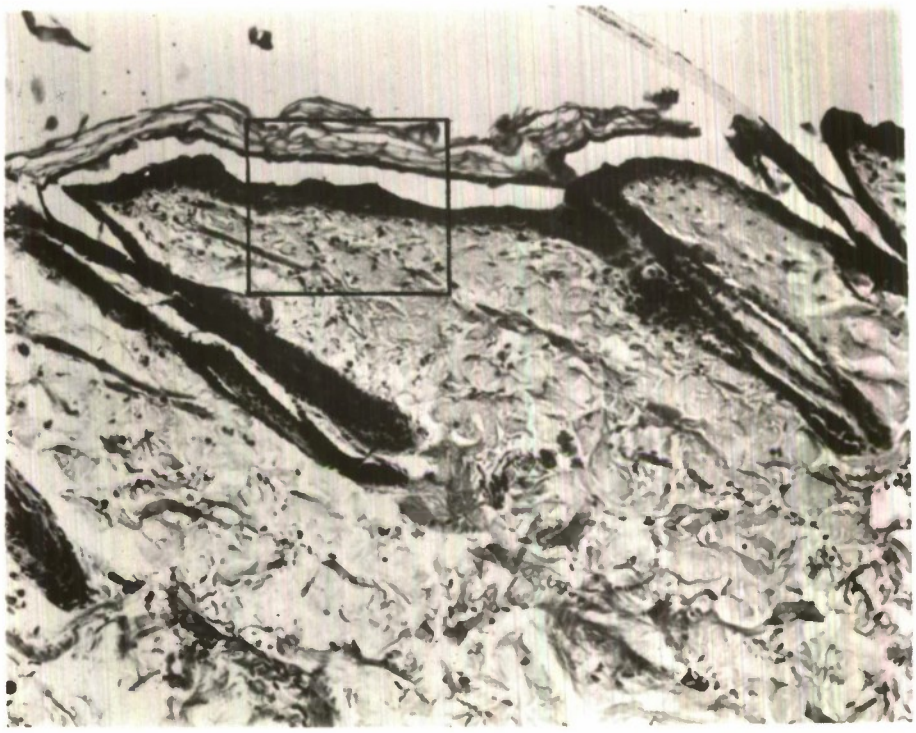

FIG. 23.

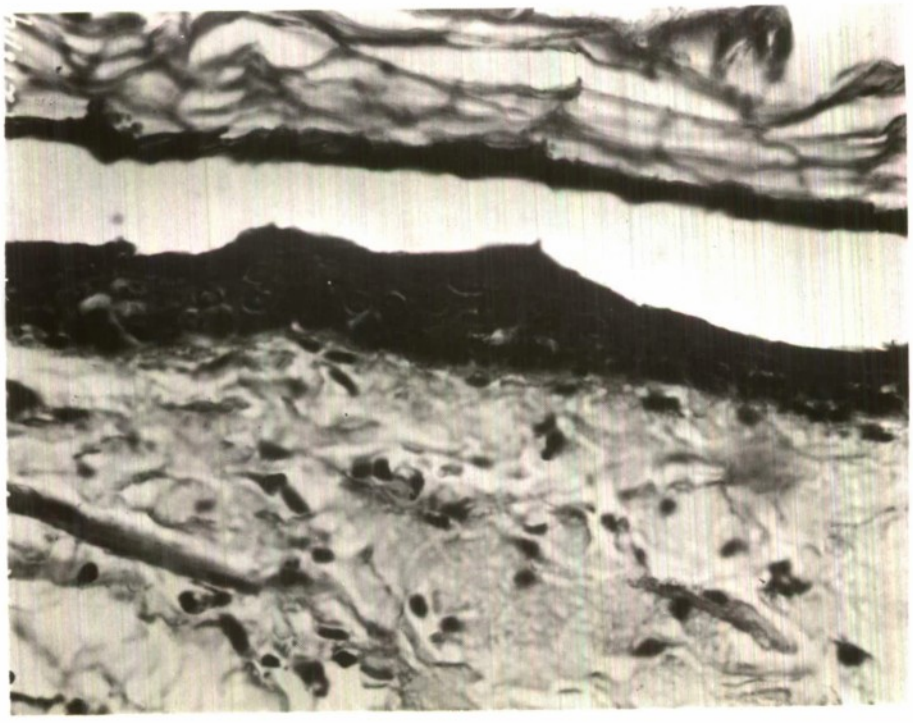

FIG. 24.

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