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LOCAL ACTION OF SOME ANTIBACTERIAL SUBSTANCES AGAINST *CORYNEBACTERIUM OVIS* IN GUINEA-PIG SKIN

BY

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When a suspension of living *Corynebacterium ovis* was injected intradermally in guinea-pigs, a lesion of roughly circular outline developed within 24 hr. Lesions of smaller diameter arose if benzylpenicillin, dequalinium, hedaquinium, cetrimide, or oxytetracycline were injected at the identical site, either before, with, or after *C. ovis*. Evidence has been obtained that such reductions of lesion diameter are due to direct action of drugs on bacteria and not to antitoxic or anti-inflammatory actions. Lesion diameters became less as drug dosage increased up to a limit, and these reductions provided a measure of local antibacterial action *in vivo*. Intradermal injection of higher concentrations of antibacterials, without *C. ovis*, produced comparable but somewhat flatter lesions, diameters of which increased with increasing concentration of drug and provided a measure of intradermal toxicity.

In clinical practice, bacterial infections of skin and mucous membranes are often treated by local application of antibacterial substances. To evaluate the topical efficiency of new antibacterials, we have attempted to develop a method in guinea-pigs, based on the immunological work of Miles (1949), in which bacteria and drug are injected intradermally at the same site.

Miles (1956) and Miles, Miles, and Burke (1957) have shown that several species of bacteria produce measurable lesions when injected intradermally in guinea-pigs. For the work described in this paper, we chose *Corynebacterium ovis* for several reasons. First, the lesions produced were large, regular and clearly marked. Second, this species was not only sensitive to the drugs which we wished to evaluate, but to our reference compound penicillin, which has been much used topically in clinical practice. Third, Miles had shown that *C. ovis* lesions in guinea-pigs were particularly easy to reduce by the systemic administration of penicillin. This suggested that local chemotherapeutic effects might also be expected.

MATERIALS AND METHODS

Drugs

We used dequalinium chloride (decamethylenebis-(4-aminoquinaldinium chloride), Dequadin), hedaquinium chloride (hexadecamethylenebis-(2-*iso*quinolinium chloride), Teoquil), benzylpenicillin (potassium salt) B.P., procaine penicillin B.P., oxytetracycline hydrochloride B.P., cetrimide B.P. and cortisone acetate B.P. Weights of drugs are expressed throughout as salts.

Inocula

C. ovis (B1/50) was maintained on Löffler slopes. In preparing inocula for *in vitro* tests, cultures were grown at 37° for 18 hr. in 1% peptone-water containing 10% normal horse serum (serum-peptone), diluted in the same medium to contain *circa* 10^7 organisms/ml. (opacity 2, Brown's tube), and then by 1/100. For intradermal injection, the 18 hr. growth from a Löffler slope was suspended in 0.9% NaCl in water (saline) and diluted in saline to opacity 2. The *in vivo* inoculum was prepared by diluting this suspension as required.

In some *in vivo* experiments inocula were treated either by exposure to boiling water for 30 sec. or by incubation with potassium penicillin at a concentration of 10 μg./ml. for 18 hr. at 37°. The action of penicillin was terminated by adding penicillinase from *Bacillus subtilis* and standing for 30 min. at room temperature. Destruction of penicillin by penicillinase was confirmed by plate assay. Sterility tests on treated cultures were performed in serum-peptone.

Experiments in vitro

An inoculum of 0.02 ml. was added to each 5 ml. aliquot of series of geometrically decreasing concentrations of each antibacterial in serum-peptone. Tubes were incubated at 37° and examined visually for growth at 1 and 5 days. The minimal inhibitory concentration (MIC) was taken as the lowest concentration of drug inhibiting visible growth for each period.

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Experiments in vivo

Before intradermal injection, guinea-pigs were shaved with an electric razor and then depilated chemically (Sleek, Elizabeth Arden). Intradermal injections were carried out with short bevel, 0.45 mm. needles. In order to make a second injection along the same needle path as the first (superinjection), the needle tip was dipped in Indian ink before being inserted the first time (Miles, 1949). Doses are expressed throughout as quantities injected/site. Drugs were administered, in geometrically decreasing concentrations, either dissolved or suspended in saline.

Generally 4 guinea-pigs were used in each test; and in all experiments, where appropriate, drugs, bacteria and/or saline were injected intradermally in each guinea-pig at 16 sites, doses being arranged in a 4 × 4 Latin square. All lesions were roughly circular in outline. An ink line was drawn around the edge of the lesion and two diameters at right angles measured with spring-bow dividers against a Vernier scale. The arithmetic mean of these diameters was taken as a measure of response at each site and is referred to below as lesion diameter.

With C. ovis.—In tests with *C. ovis*, guinea-pigs weighing 200 to 250 g. were used. In some tests (simultaneous), equal volumes of drug and of inoculum were mixed and immediately afterwards 0.1 ml. of the mixture was injected at each site. In prophylactic tests, 0.05 ml. of the drug preparation was injected and later 0.05 ml. of the inoculum superinjected. This order of administration was reversed in therapeutic tests. For control purposes, *C. ovis* without drug was given routinely, either in a single injection of 0.1 ml. or in 0.05 ml. preceded or followed by 0.05 ml. saline. As an additional control, saline was given either in 0.1 ml. or in 0.05+0.05 ml. injections.

Diphtheria Toxin.—Schick test toxin B.P., diluted 1/6, was mixed with an equal volume of saline, or of dequalinium or penicillin in saline, and 0.1 ml. aliquots were injected intradermally in guinea-pigs weighing 200 to 250 g. Schick control B.P. was similarly diluted, mixed with saline and injected in the control sites.

Tuberculin.—About 5 weeks before giving purified protein derivative of old tuberculin B.P., guinea-pigs weighing 350 to 400 g. were sensitized by subcutaneous injection of 0.5 ml. of suspension containing 2 mg. of killed *Mycobacterium tuberculosis*. In some experiments, three successive daily subcutaneous doses each of 1 mg. cortisone in 0.5 ml. saline were given and, 4 hr. after the last dose, 0.2 ml. of tuberculin in saline was injected intradermally. In other experiments, guinea-pigs received intradermal doses of cortisone, dequalinium or hedaquinium, and, 30 min. later, 0.1 ml. of tuberculin in saline was superinjected.

RESULTS

Experiments in vitro

The geometric mean MIC values obtained with the antibacterial substances used are given in Table 1.

TABLE I

IN VITRO ACTIVITIES OF SOME ANTIBACTERIAL SUBSTANCES AGAINST *CORYNEBACTERIUM OVIS* IN SERUM-PEPTONE WATER AT 37°

MIC refers to minimal inhibitory concentration.

Drug	Geometric Mean MIC (μg./ml.)	
	1 Day	5 Days
Dequalinium chloride ..	0·110	0·312
Hedaquinium chloride ..	0·221	0·221
Benzylpenicillin	0·039	0·055
Oxytetracycline hydrochloride	0·276	3·125
Cetrimide	4·416	4·416
Procaine penicillin	0·110	0·156

It will be seen that all drugs readily inhibited *C. ovis* in serum-peptone.

Saline *Intradermal Experiments*

Twenty-four hr. after saline had been given at one site, either in a single injection of 0.1 ml. or in two of 0.05 ml., we regularly observed a very small flat lesion, presumably due to mechanical damage. This mark may just be discerned in Fig. 1. Throughout the whole series of tests, the diameter of this

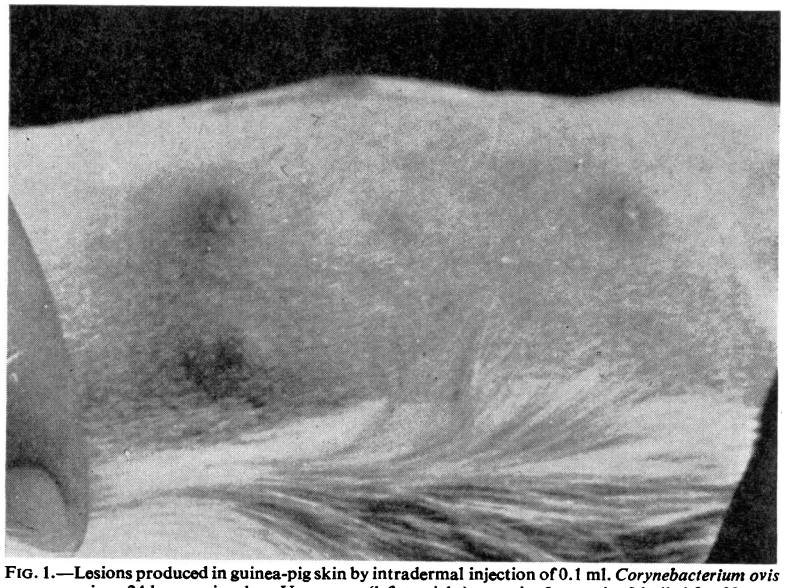

FIG. 1.—Lesions produced in guinea-pig skin by intradermal injection of 0.1 ml. *Corynebacterium ovis* suspensions 24 hr. previously. Upper row (left to right): opacity 2; opacity 2 boiled for 30 sec.; opacity 2/100. Lower row (left to right): opacity 2/10; no injection; 0.1 ml. 0.9% NaCl in water.

lesion did not diverge appreciably from the overall mean of 1.925 mm., whether the saline was given in single or divided doses.

C. ovis *without Drugs*

Living Bacteria.—Injection with *C. ovis* produced at 24 hr. a bright red conical lesion (Fig. 1), the outline of which was clear in the living animal. To examine the effects of inoculum density and time of measurement, 4 guinea-pigs were injected with *C. ovis* suspensions at opacities 2, 2/10, and 2/100. At the control sites, one animal received saline, a second boiled inoculum at opacity 2, and the other

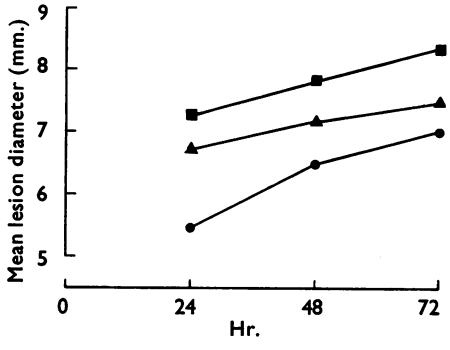

FIG. 2.—Effects of inoculum density and duration of infection on diameter of lesions due to intradermal injection of 0.1 ml. *Corynebacterium ovis* in guinea-pigs. Points at 24 hr. are based on 4 sites in each of 4 animals, subsequent points on 4 sites in each of 3 animals. ■, opacity 2 (Brown's tube); ▲, opacity 2/10; ●, opacity 2/100.

two boiled inoculum at opacity 2/10. The guinea-pig receiving saline died between 24 and 48 hr. after inoculation. All lesion diameters in all animals were measured at 24 hr. and in the survivors at 48 and 72 hr. The mean lesion diameters for the 16 and later 12 sites inoculated with living *C. ovis* are plotted as the ordinate against time in Fig. 2. It will be seen that lesion diameter increased with inoculum density and with time.

As by 48 hr. one pig had died and lesion edges in survivors were harder to distinguish, we chose 24 hr. as the time of measurement in subsequent experiments. In these we used an inoculum corresponding to 0.1 ml. at opacity 2/10 (0.05 ml. at opacity 2/5). At this inoculum density, no deaths occurred within 24 hr., confirming the general finding of Miles *et al.* (1957).

Bacteria Exposed to Heat or Benzylpenicillin.—In the experiments described above, boiled inocula at opacity 2 or 2/10 produced no larger lesion at 24 hr. than did saline (see Fig. 1).

In other experiments, cultures exposed to penicillin failed to produce within 24 hr. lesions larger than those produced by saline or by penicillin+penicillinase without bacteria.

Sterility tests showed that no bacteria survived being boiled for 30 sec.; but, after exposure to penicillin, some growth occurred after 4 days in serum-peptone, presumably due to a few surviving bacteria. The guinea-pigs inoculated with penicillin-treated cultures were kept and small lesions also appeared after 4 days.

C. ovis *with Drugs*

Antibacterials.—Injecting any of the antibacterial substances in suitable doses before, with or after *C. ovis* at the same site produced lesions smaller in diameter than those arising from *C. ovis* alone. This result is exemplified by the experiment illustrated in Fig. 3, in which dequalinium solutions were superinjected 30 min. after *C. ovis*.

In order to examine the effect upon lesion diameter of the time interval between injections of bacteria

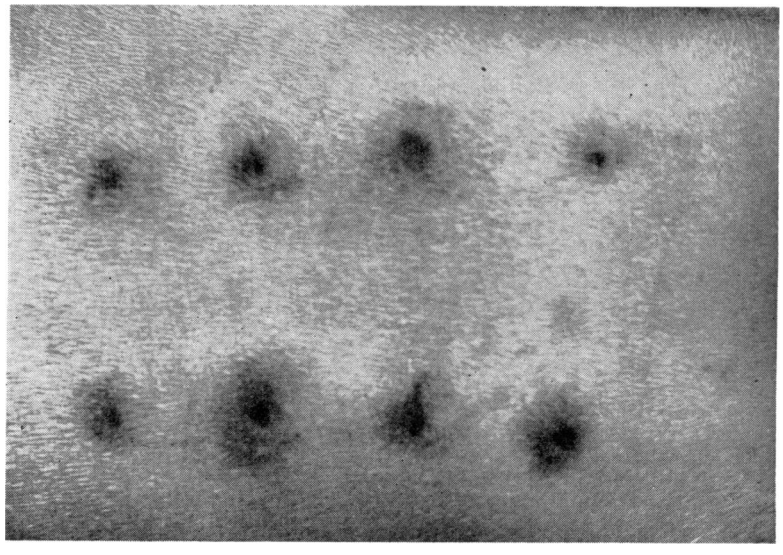

FIG. 3.—Lesions produced in guinea-pig skin by intradermal injection of *C. ovis* suspension followed by dequalinium solution 30 min. later along the same needle path. Upper row (left to right): *C. ovis* +0.2 μg. dequalinium; *C. ovis* +0.05 μg. dequalinium; *C. ovis* alone; *C. ovis*+0.8 μg. dequalinium. Lower row (left to right): *C. ovis*+0.05 μg. dequalinium; *C. ovis* alone; *C. ovis* + 0.8 μg. dequalinium; *C. ovis*+0.2 μg. dequalinium.

TABLE II

EFFECT ON LESION DIAMETER OF TIME INTERVAL BETWEEN INTRADERMAL INJECTIONS OF *C. OVIS* AND OF BENZYLPENICILLIN ALONG THE SAME NEEDLE PATH IN GUINEA-PIGS

S.E. refers to standard error of the mean based on diameters of 16 lesions.

Expt. No.	Treatment	Mean Lesion Diameter ±S.E. (mm.)
1	*C. ovis* alone	5·638±0·105
	Drug 2 hr. before *C. ovis*	4·730±0·079
	,, 1 ,, ,, ,,	4·670±0·057
	,, 0·5 ,, ,, ,,	4·544±0·066
2	*C. ovis* alone	5·972±0·141
	Drug 0·5 hr. after *C. ovis*	5·020±0·083
	,, 1 ,, ,, ,,	5·124±0·100
	,, 2 ,, ,, ,,	5·075±0·108

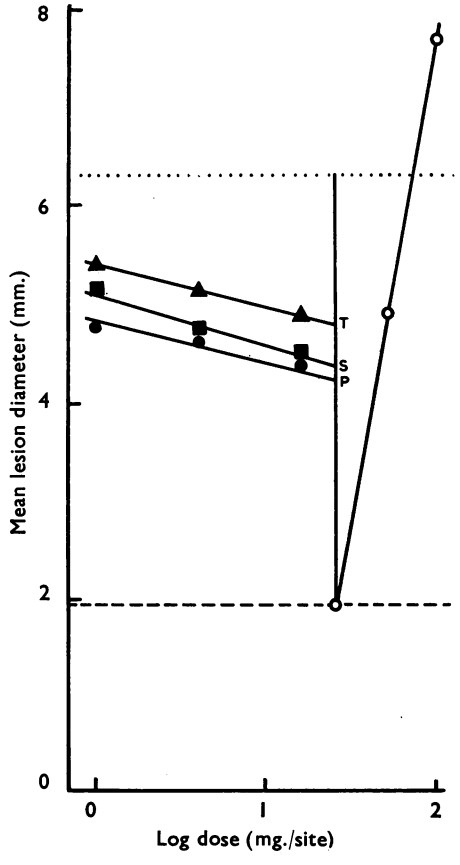

FIG. 4.—Relation of intradermal dose of benzylpenicillin in guinea-pigs to lesion diameter in the presence and absence of *C. ovis*. In antibacterial tests, 0.05 ml. benzylpenicillin solution + 0.05 ml. *C. ovis* suspension were injected at the same site. In toxicity tests 0.1 ml. benzylpenicillin solution was injected. ●, Benzylpenicillin 30 min. before *C. ovis*; ■, benzylpenicillin with *C. ovis*; ▲, benzylpenicillin 30 min. after *C. ovis*; O, benzylpenicillin alone; ...., diameter of lesion produced by *C. ovis* alone; ---, diameter of lesion produced by 0.05+0.05 ml. 0.9% NaCl in water. For explanation of P, S, and T, see discussion.

and of drug, benzylpenicillin was given at various times before *C. ovis* in one experiment, and after *C. ovis* in another. The mean lesion diameters obtained in both experiments are given in Table II.

Since it was inconvenient to superinject at an interval shorter than 30 min. and since longer periods of up to 2 hr. between injection and superinjection gave no greater reduction, we adopted a 30 min. time interval in all prophylactic and therapeutic tests.

The results of a series of experiments, in which benzylpenicillin was given at three dose levels in prophylactic, simultaneous and therapeutic tests are expressed in the left-hand portion of Fig. 4, in which lesion diameters are plotted as ordinates against log dose. The horizontal dotted line represents the mean diameter over this series of tests with benzylpenicillin of the lesions caused by *C. ovis* alone, and the horizontal broken line the mean diameter of the lesions due to saline. In Fig. 5, results obtained with hedaquinium are expressed in the same way.

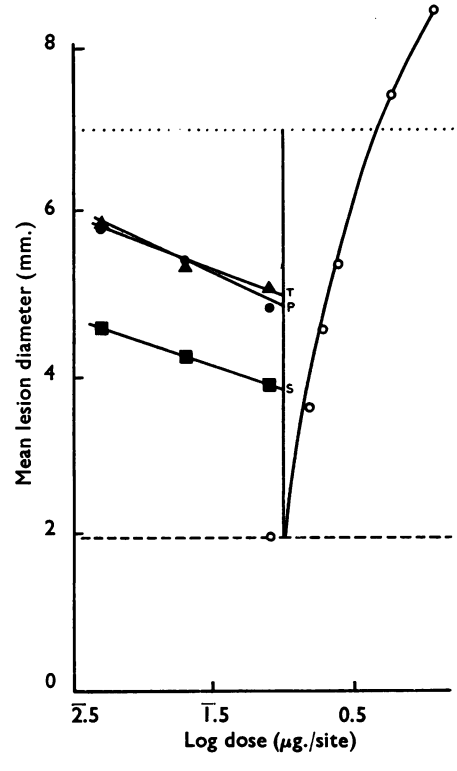

FIG. 5.—Relation of intradermal dose of hedaquinium in guinea-pigs to lesion diameter in the presence and absence of *C. ovis*. Method of test and symbols as in Fig. 4.

A similar graph was constructed for each drug, and the difference between the dotted and broken horizontal lines was taken as the maximal possible reduction. Response to a drug was expressed as % of this value (% maximal reduction, PMR).

Table III gives the mean PMR values obtained with dequalinium, hedaquinium, and benzylpenicillin in prophylactic, simultaneous and therapeutic tests. Results with oxytetracycline, cetrimide, and procaine penicillin in simultaneous tests are also given. It will be seen from Table III that in each type of test with each drug mean PMR increased with dose.

TABLE III

REDUCTION OF LESIONS DUE TO *C. OVIS* IN GUINEA-PIG SKIN BY ANTIBACTERIAL AGENTS ADMINISTERED INTRADERMALLY 30 MIN. BEFORE, SIMULTANEOUSLY WITH, OR 30 MIN. AFTER INJECTIONS OF *C. OVIS* AT THE SAME SITE

Mean PMR refers to mean % maximal reduction, namely % of the difference between mean lesion diameters after *C. ovis* alone and after saline alone.

Drug	Dose/ Site (μg.)	Mean PMR		
		Drug before *C. ovis*	Drug with *C. ovis*	Drug after *C. ovis*
Dequalinium chloride	0·05	22·9	43·8	24·2
	0·20	35·5	53·2	30·8
	0·80	65·4	55·4	40·9
Hedaquinium chloride	0·05	23·0	48·0	24·4
	0·20	30·1	55·2	31·6
	0·80	41·4	61·8	38·9
Benzylpenicillin	100	35·0	26·6	21·0
	400	38·9	37·9	26·9
	1,600	45·8	42·2	32·5
Oxytetracycline hydrochloride	56·25	—	8·0	—
	75·00	—	14·1	—
	100·00	—	15·7	—
Cetrimide	1·25	—	13·6	—
	2·50	—	39·8	—
	5·00	—	53·4	—
Procaine penicillin	62·5	—	6·2	—
	250·0	—	19·1	—
	500·0	—	20·3	—

Cortisone.—Two guinea-pigs received 0.05, 0.2, and 0.8 mg. cortisone and 2 pigs received 0.05, 0.2, and 0.8 μg. dequalinium in each of 4 sites. In addition all animals were given 4 control injections of 0.05 ml. saline. Thirty min. later *C. ovis* was superinjected at all sites. Dequalinium produced the expected reduction in lesion diameter, but no effect was observed after cortisone.

Drugs without C. ovis

Antibacterials.—All drugs at high doses exhibited toxicity, except procaine penicillin, which was too insoluble to allow sufficient intradermal doses to be given in 0.1 ml. volumes. Toxic lesions resembled those of *C. ovis*, but were flatter, and their diameters increased with increasing dose, as will be seen for benzylpenicillin and hedaquinium in the right-hand portions of Figs. 4 and 5. For reasons discussed below, we have taken as a practical measure of intradermal toxicity the maximal tolerated intradermal dose (MTID), which is the greatest dose producing a lesion no larger than that caused by the saline control injection.

Diphtheria Toxin.—When either dequalinium at 0.8 μg. or benzylpenicillin at 0.8 mg. were injected simultaneously with Schick toxin, the resulting lesions were not significantly smaller than those produced by toxin alone. The lesion produced by Schick toxin control was not significantly larger than that of saline control.

Old Tuberculin.—Six guinea-pigs, sensitized with killed *M. tuberculosis*, and treated subcutaneously with cortisone, and 6 other sensitized guinea-pigs, untreated with cortisone, were each given 6 intradermal injections of old tuberculin in doses of 160, 40, or 10 units. Analysis of variance showed that cortisone gave a highly significant reduction ($P<0.001$) in weal diameter, in confirmation of the finding of Long and Miles (1950).

In another type of experiment 9 guinea-pigs sensitized to *M. tuberculosis* were used. All animals received saline intradermally at two sites, of which three received 0.2 and 0.8 mg. cortisone, three 0.2 and 0.8 μg. dequalinium and three 0.2 and 0.8 μg. hedaquinium intradermally at each of two sites. All sites were superinjected with 100 units of old tuberculin. None of the intradermal treatments significantly reduced lesion diameter compared with the control mean ($P>0.05$).

DISCUSSION

Validity of Method

The question arose as to whether the antibacterial substances studied reduced the lesion of *C. ovis* by directly neutralizing bacterial toxin, by anti-inflammatory action or by damaging the bacteria.

The failure of benzylpenicillin and dequalinium to reduce the specific reaction to diphtheria toxin shows that they almost certainly did not act as antitoxic agents. We regard this result as typical of all antibacterial agents used.

Systemic cortisone reduced the response to old tuberculin; but intradermal dequalinium and hedaquinium did not. Cortisone, given intradermally, failed to reduce either the old tuberculin or *C. ovis* lesions. These facts made it highly improbable that the antibacterial compounds,

which were always given intradermally, acted as anti-inflammatory agents.

Since antitoxic and anti-inflammatory actions have been excluded, there remains the hypothesis that the antibacterials reduced the *C. ovis* lesions by direct damage to the bacteria. Two pieces of evidence are consistent with this hypothesis. First, all active substances were found to inhibit *C. ovis* strongly *in vitro*. Second, killed bacteria did not produce the typical lesion and bacteria damaged by penicillin before inoculation only produced a lesion at a time after injection which corresponded to the reappearance of growth *in vitro*.

Measurement of Toxicity

We have considered toxicity only as an intradermal response, since this tends to oppose any intradermal therapeutic effect. The curve relating log dose to diameter of toxic lesion might be expected on general grounds to be sigmoid; but the lower portion of this curve cannot be followed below the point at which saline itself produces a lesion, and we did not increase drug dosage to the point where the upper portion of the curve presumably becomes asymptotic (Figs. 4 and 5). These conditions made it impossible to measure toxicity at the point half-way between the upper and lower asymptotes which corresponds to the LD50 in dose/lethality curves. However, the truncation of the lower portion of the intradermal dose/response curve made it possible to express toxicity with reasonable precision as the maximal tolerated intradermal dose (MTID), which corresponds to the point at which the dose/response curve for toxicity meets or extrapolates to the horizontal of the saline lesion.

Measurement of Antibacterial Efficiency in vivo

To measure the efficiency of a drug *in vivo*, it is usual to relate therapeutic potency to toxicity. In experimental infections of the whole animal, this is done by means of the therapeutic ratio. Since, in the present work, such a measure did not apply, we used such graphs as those in Figs. 4 and 5 to relate intradermal antibacterial action to intradermal toxicity.

For two reasons, lesion diameter reached a minimum in each type of test at an optimal dose which was the MTID. First, none of the drugs was 100% effective at doses lower than the MTID, and at higher doses toxic effects opposed antibacterial effects. Second, the slope of the toxicity curve for each antibacterial drug studied was considerably greater than that for the antibacterial activity.

In Figs. 4 and 5, the lesion diameter curves have been extrapolated to cut, at P, S, and T, an ordinate erected at the MTID. The minimal lesions are indicated by these points, from which maximal PMR values have been derived. These and the corresponding MTID values are summarized in Table IV for all antibacterials used, except procaine penicillin.

TABLE IV

THE INTRADERMAL TOXICITIES OF SOME ANTIBACTERIAL AGENTS AND THEIR OPTIMAL EFFICIENCIES AGAINST EXPERIMENTAL *C. OVIS* LESIONS IN GUINEA-PIG SKIN

MTID refers to the maximal tolerated intradermal dose; for explanation of PMR, see the legend to Table III.

Drug	MTID (μg./Site)	PMR at MTID		
		Drug 30 min. before *C. ovis*	Drug Simultaneously with *C. ovis*	Drug 30 min. after *C. ovis*
Dequalinium chloride	1·6	67·8	60·2	44·1
Hedaquinium chloride	1·0	43·1	62·9	39·6
Benzylpenicillin ..	2,500	48·0	45·0	34·0
Oxytetracycline hydrochloride ..	79·4	—	15·8	—
Cetrimide	4·2	—	54·2	—

If drugs capable of reducing lesion diameter of *C. ovis* to that of the saline controls are to be compared, their relative merits might be measured as the ratio of the MTID to the smallest dose which produces 100% reduction of lesion diameter (minimal effective dose). This measure would correspond to a conventional therapeutic ratio.

Relation to Clinical Findings

It will be seen from Table III that almost all values obtained with dequalinium and hedaquinium were comparable. From Table IV it appears that benzylpenicillin possessed about three-quarters of the efficiency of dequalinium. Cetrimide was intermediate between these two drugs whereas oxytetracycline was only slightly active.

Several factors make it difficult to relate these results to those of clinical practice. First, many species of bacteria other than *C. ovis* are involved; second, natural lesions are usually well established before they are treated topically; and third, treatment usually consists in applying creams or paints. Nevertheless, our results with *C. ovis* probably agree with clinical impressions, in that all antibacterials used were somewhat, but not completely, effective.

We feel that the method described for evaluating topical antibacterials could be further developed, using other organisms more frequently met in

clinical practice. It seems reasonable, however, to suppose that if a drug was effective in this test against *C. ovis* and was active *in vitro* against another species, it would also act topically against the latter species.

We wish to thank Professor A. A. Miles for his advice and for the strain of *C. ovis*, Dr. A. Patterson for the preparation of *M. tuberculosis* and Mr. H. J. Fraser and Mr. G. K. A. Smith for photography. This work was carried out with the technical assistance of Miss Margery Boothroyd. Some preliminary experiments on other species were carried out by Mrs. M. Babbs.

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