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Experimental Bovine *Trichophyton verrucosum* Infection

Preliminary Clinical, Immunological and Histological Observations in Primarily Infected and Reinoculated Cattle

A. W. D. LEPPER*†
A.R.C., Institute for Research on Animal Diseases, Compton, Newbury, Berkshire

SUMMARY. The cutaneous application of different doses of viable Trichophyton verrucosum *to the unabraded skin of cattle of various ages resulted in clinically recognizable ringworm infection of varying extent and duration. Confluent lesions covering the whole inoculated area were produced by* 10^7 *viable units of the fungus, whereas the minimal infective dose of* 10^3 *viable units produced limited areas of infection only. The level of nutrition within the limits imposed had no effect on the extent or severity of lesions. The fungus was found to invade the keratinized portions of skin and hair of cattle of all ages at the same rate. However, both the cutaneous inflammatory response and the resolution of lesions were most rapid in older animals. The ability to eliminate infection more rapidly was associated with a marked delayed hypersensitivity response commencing 14 days after infection. Such hypersensitivity was not detectable by this means after the resolution of lesions.*

T. verrucosum *could not be isolated in culture from skin lesions until 21 days after inoculation and could only be isolated for half the period that lesions were present. Cattle were resistant to cutaneous reinfection with viable* T. verrucosum *on previously infected or fresh skin sites at 2 months and at more than one year after the resolution of primary lesions. A mild delayed hypersensitivity response developed in every site within 48 hr. of reinoculation. The intravenous inoculation of previously-infected cattle with* 10^4 *viable units of* T. verrucosum *resulted in an immediate-type cutaneous reaction at the original site of infection.*

Trichophyton verrucosum IS THE MOST common cause of ringworm infection in cattle (Ainsworth & Austwick, 1959). Skin lesions, resulting from the proliferation of the fungus in the stratum corneum, keratinized portions of the hair, and hair follicles, are accompanied by a marked local inflammatory reaction and usually resolve naturally after varying periods of infection. Clinical features of either naturally-occurring or experimental infections have been described by Hoerlein (1945); Ford (1956); Gentles & O'Sullivan (1957) and Kielstein & Balabanoff (1966) and histological features of the disease have been studied by La Touche (1952); Sellers *et al.* (1956) and by Kielstein (1967a). There is also limited experimental evidence to suggest that cattle that have recovered from experimental or naturally-occurring *T. verrucosum* infection are resistant to reinfection with this species of fungus for up to one year or more after the resolution of primary lesions (Hoerlein, 1945; Sellers *et al.*, 1956; Kielstein, 1967a).

The purpose of this work was to correlate certain clinical, histological and immunological aspects of experimental ringworm infection in young and adult cattle following cutaneous inoculation with different doses of viable *T. verrucosum*, and to determine the influence of age and the plane of nutrition on the duration and extent of lesions. In addition aspects of the response to reinoculation with *T. verrucosum* are described.

MATERIALS AND METHODS

Strain of T. verrucosum

Strain V. 6234 of *T. verrucosum* var. *discoides*, isolated from naturally occurring lesions of a calf was used in these studies. This was kindly supplied by Mr P. K. C. Austwick of the Central Veterinary Laboratory, Ministry of Agriculture, Weybridge.

* Present address: C.S.I.R.O. Animal Health Laboratory, Parkville, 3052, Victoria, Australia.

† In partial fulfilment of the Doctor of Philosophy Degree, London University.

Cultural Methods

For the purposes of primary isolation from experimental lesions, and for the maintenance of stock cultures *T. verrucosum* was cultured at 37°C. for up to 10 days on Sabouraud dextrose agar enriched with 3% yeast extract* plus 20 units of penicillin, 40 units of streptomycin and 0·5 mg. of cycloheximide per ml.

For the preparation of animal inocula submerged cultures of *T. verrucosum* were grown in 100 ml. amounts of glucose-peptone broth plus 20 units of penicillin, 40 units of streptomycin and 0·5% yeast extract. This medium was supplemented with the same quantities of inorganic salts used in Bacto Dubos broth for the culture of tubercle bacilli (Dubos *et al.*, 1950). Each batch of medium was inoculated with 2 ml. of a suspension of freshly ground 3 to 7-day-old colonies of *T. verrucosum* removed from the surface of solid medium. Liquid cultures were incubated at 37°C. for 6 days.

Preparation of Viable Inocula

Each batch of inoculum required for cutaneous application was prepared solely from submerged mycelium grown in 2 l. of culture medium; this was removed and washed in 200 ml. of distilled water. Twenty ml. amounts of this material suspended in distilled water were ground in Griffith's tubes. Forty ml. aliquots of the pooled, ground mycelium were added to 60 ml. of liquid 1% sterile agar* at 44°C. Thorough mixing was achieved by pouring the inocula into 50 ml. hypodermic syringes and expressing the cooled solidified mixture into sterile containers. Each batch of inoculum was stored for not more than 48 hr. before use. Inoculum for intravenous administration was made by grinding the mycelium in pyrogen-free distilled water and filtering the suspension through sterile gauze to remove the larger fragments.

The number of viable units of *T. verrucosum* in each batch of inoculum was determined by plating in triplicate a fixed volume of 10-fold dilutions of the aqueous suspension of freshly-ground mycelium in Sabouraud dextrose agar at 44°C. Counts were made of the number of colonies developing in the solidified medium after incubation at 37°C. for 7 to 10 days.

Preparation of T. verrucosum *Cell Sap*

Cell sap was prepared by ultra-sonic treatment of chopped mycelium from a 28 day liquid culture of *T. verrucosum*. Ultrasonication was carried out in 0·004M cysteine buffer, pH 7·2, at 4°C. using a Dawe Soniprobe, type 1130A, operated at maximum output. The sonicate was centrifuged at 1,000 rev./min. for 10 min. and the supernatant liquid containing the fungal cell sap was removed, Seitz-filtered, and stored at — 20°C.

* Difco, East Molesey, Surrey.

Cattle

A total of 28 female animals of the Friesian or Ayrshire × Red Poll breeds were used. They were between 3 and 22 months of age. These animals had been bred at Compton, reared in covered yards or loose-boxes, and were known to have been free from clinically-observable dermatophyte infection since birth. Animals on a medium plane of nutrition, whose diet had consisted of hay and concentrates and water, weighed approximately 330 kg. by 12 months of age. Animals on a lower plane of nutrition that had received unlimited quantities of hay only since weaning weighed 280 kg. at the same age.

Primary Inoculations

Cattle were divided into groups numbered I to VI, each group containing animals of approximately similar age (Table I), except Group III in which the ages were either 3 months or approximately 10 months.

Nine animals in Groups I and V, on the medium plane of nutrition, were each cutaneously inoculated with 10^7 and 10^4 viable units of *T. verrucosum* respectively.

Six animals comprising Group II, and on the lower plane of nutrition were each inoculated with 10^7 viable units of the fungus. All lesions that developed in each group were allowed to resolve naturally.

Reinoculation

A total of 11 animals from Groups I and II were cutaneously reinoculated with 10^6 viable units of *T. verrucosum* 2 months after the resolution of the primary infection. Inoculations (as shown in Table III) were made either on the same or on different sites to those previously infected. Ten animals, comprising Groups III and IV, that had not been previously infected were challenged at the same time and with the same dose of inoculum. One month later 5 of the cutaneously reinoculated animals from Group II were also challenged intravenously with 10^4 viable units of the fungus.

Finally, Groups I and III were cutaneously reinoculated with 10^3 viable units of *T. verrucosum* more than one year after the resolution of primary lesions (Table IV). The 3 animals in Group VI (Tables I and IV) were similarly challenged and served as susceptible 'controls' for this experiment.

TABLE I

PRIMARY EXPERIMENTAL *T. verrucosum* INFECTIONS: THE AGE AND PLANE OF NUTRITION OF ANIMALS IN RELATION TO DOSE OF INOCULUM, DURATION AND EXTENT OF LESIONS

Group No.	Animal No.	Age (months)	Plane of nutrition	Region inoculated	Units viable inoculum	Type of lesion	Duration of lesions (days)
I	1	3	Medium	Cervical	10^7	Confluent	126
	2	3					147
	3	5					147
	4	4					189
	5	5					147
	6	4					147
II	19	10	Low	Cervical	10^7	Confluent	77
	20	10				Confluent	70
	21	9				Multiple	105
	22	9				Confluent	70
	23	9		Costal		Confluent	56
	24	10		Cervical		Confluent	77
III	7	11	Medium	Cervical	10^6	Multiple	84
	8	10					84
	9	10					84
	10	10					84
	11	3				Solitary	71
	12	3					71
IV	27	22	Medium	Cervical	10^6	Multiple	Destroyed at 43 days
	28	21				Multiple	88
	29	17				Confluent	Destroyed at 36 days
	30	17				Multiple	88
V	16	8	Medium	Cervical	10^4	Solitary	42
	17	8		Cervical		Nil	—
	18	8		Gluteal		Solitary	56
VI	13	7	Medium	Cervical	10^2	Nil	—
	14	7				Solitary	63
	15	7				Nil	—

Cutaneous Inoculation

Skin sites were prepared by clipping hair to approximately 2 mm. in length within 10 × 10 cm. areas over the cervical, costal or gluteal regions, and these were inoculated by rubbing on a fixed volume of *T. verrucosum* suspension in 1% agar.

Intravenous Inoculation (Previously Infected Animals)

Fifteen ml. of a suspension of viable *T. verrucosum* in pyrogen-free distilled water were inoculated via the jugular vein using an aseptic technique.

Intradermal Skin Tests Using T. verrucosum *Cell Sap*

Skin tests were performed on non-infected and on infected cattle during primary experimental infections. Hair was clipped from skin sites selected over the costal region on 6 infected and on 5 non-infected animals and the thickness of each skin fold measured, using callipers. Paired sites on either side of each animal were intradermally inoculated with either 0·2 ml. of *T. verrucosum* cell sap or sterile cysteine buffer. Skin-fold thicknesses were again measured at these sites at 24, 48 and 72 hr. The character, size and duration of each response was recorded.

TABLE II

PRIMARY EXPERIMENTAL *T. verrucosum* INFECTIONS: OBSERVATIONS ON THE INCUBATION, MATURATION, SPREAD AND REGRESSION OF LESIONS IN RELATION TO AGE OF ANIMALS AND DOSE OF INOCULUM

Group No.	Animal No.	Age (months)	Units viable inoculum	Days after inoculation: First signs	Crust duration	Positive culture	Satellite lesions	Fungus in tissue	New hair growth	Complete healing
I	1	3	10^7	17	25–114	36*	36	7–91	63	126
	2	3		17	25–140	36*	36	7–91	63	147
	3	5		17	21–140	36*	29	21–63	91	147
	4	4		18	63–182	36*	—	21–106	63	189
	5	5		17	25–140	36*	36	14–98	49	147
	6	4		17	25–119	36*	42	7–98	56	147
II	19	10	10^7	14	21–63	21–49	None	7–35	63	77
	20	10		7	21–63	21–35		7–28	63	70
	21	9		14	28–91	21–77		7–70	63	105
	22	9		14	21–63	21–35		7–28	49	70
	23	9		14	21–28	21–28		7–35	49	56
	24	10		14	28–70	28–42		7–49	63	77
III	7	11	10^6	7	28–78	42*	42	17–57	71	84
	8	10		7	28–78	42*	42	14–50	78	84
	9	10		14	28–78	42*	42	28–57	78	84
	10	10		14	28–78	42*	42	42–63	71	84
	11	3		35	42–63	42*	—	29	56	71
	12	3		21	42–63	42*	35	No serial biopsies 47	56	71

* Single culture

Clinical Observations

All animals were examined daily after cutaneous inoculation and until the resolution of all clinically observable lesions. Intravenously inoculated animals were observed hourly for 6 hr. after inoculation and daily for 10 days thereafter.

Skin Biopsy

Skin biopsies were removed under lignocaine analgesia either by a rotary dermatome similar to that described by Evans *et al.* (1957) or by simple excision. During primary experimental infection biopsies were removed from either normal skin or from the inoculated area on 8 pairs of animals. Samples were removed 24 hr. after inoculation and at pre-determined intervals until the natural resolution of lesions. After the cutaneous reinoculation of 12 cattle, samples were removed at 24, 48 and 72 hr., and at varying intervals between 4 and 36 days after inoculation. Following the intravenous inoculation of such animals samples were removed from normal skin or from the previously infected skin site after 4 and 24 hr.

Portions of skin were fixed in 10% buffered formalin for 7 days, embedded in paraffin wax and 6 µm. sections cut and stained by the periodic acid Schiff method plus haematoxylin and eosin (P.A.S. + H. and E.), by a modified P.A.S. method (Gridley, 1953), by metachromatic sulphation (Kelly *et al.*, 1962) and by Giemsa.

Culture of T. verrucosum *from Skin Lesions*

Skin scrapings and individual hairs were removed from skin sites on 6 animals at weekly intervals after inoculation until the resolution of primary lesions and at 2, 7 and 14 days after inoculation in cutaneously reinoculated cattle. Scrapings with adherent hair fragments possessing 'spore sheaths' were identified using a plate microscope and inoculated on to the solid selective medium described above.

RESULTS

Clinical Observations during Primary Infections

The time taken for the development of the first visible signs of infection varied from 7 to

TABLE III

RESULTS OF RECHALLENGING 11 HEIFERS WITH 10^6 VIABLE UNITS OF *T. verrucosum* 2 MONTHS AFTER RESOLUTION OF PRIMARY LESIONS COMPARED WITH THE EFFECT OF THE SAME DOSE OF INOCULUM ON 10 ANIMALS PREVIOUSLY FREE FROM INFECTION

				Cutaneous challenge			
Group	Animal No.	Age (months)	Primary inoculation	Reinoculation same site	Reinoculation fresh site	Result	Duration (days)
I	1	9–11	6 Months previously	+	−	Nil	—
	3			+	−		
	6			+	−		
	4			−	+	Transient reaction	28
	5			−	+		
	2			−	+		
II	19	14–15	5 Months previously	+	+	Transient reaction	21
	20			+	+		
	21			+	−		
	23			−	+		
	24			+	−		
III	7	10–11	+			Clinical ringworm	84
	8		+				
	9		+				
	10		+				
	11	3	+				71
	12		+				
IV	27	17–22	+			Infection	43†
	28		+				88
	29		+				36†
	30		+				88

† Destroyed during primary infection.

35 days after inoculation. The longest incubation periods were observed in animals under 5 months of age that were inoculated with 10^7 or 10^6 viable units of *T. verrucosum* (Table II). The first macroscopic changes observed within the inoculated area were circumscribed erythematous swellings resulting in visible hair disturbance. These areas became enlarged by 18 to 20 days and were covered with a yellow exudate which matted together the bases of groups of hairs. Within the next 7 days these coalesced to form the characteristic raised, whitened, desquamating crusts from which most of the hair had disappeared (Fig. 2). Complete healing or resolution of lesions was said to have occurred when the crust had disappeared and the hair had regrown.

The extent of lesions depended on the dose of inoculum. The majority of animals of all age groups inoculated with 10^7 viable units of *T. verrucosum* developed confluent areas of infection covering the entire inoculated area. In many animals smaller circumscribed satellite lesions developed on the shoulder and other parts of the neck from 29 to 42 days after experimental inoculation (Table II). A dose of 10^6 viable units (Table I) generally produced multiple areas of infection which occasionally coalesced (Fig. 3) and also sometimes gave rise to satellite lesions. Doses of 10^4 or 10^3 viable units produced one or two solitary lesions of between 2 and 3 cm. in diameter in only a proportion of the inoculated animals. It was not possible to produce lesions with 100 viable

TABLE IV

RESULTS OF RECHALLENGING OF 12 HEIFERS WITH 10^3 VIABLE UNITS OF *T. verrucosum* OVER 1 YEAR AFTER RESLUTION OF PRIMARY LESIONS COMPARED WITH THE EFFECT OF THE SAME DOSE OF INOCULUM ON 3 ANIMALS PREVIOUSLY FREE FROM INFECTION

			Cutaneous challenge				
Group	Animal No.	Age (Months)	Primary inoculation	Reinoculation same site	Reinoculation fresh site	Result	Duration (days)
I	1	27–29	22 Months previously	+		Transient	15
	2				+	Nil	
	3				+	Transient	15
	4			+		Transient	
	5				+	Transient	
	6			+	−		
III	7	27–29	14 Months previously	+	−	Nil	
	8			+	−		
	9			+	−		
	10			+	−		
	11			+	−		
	12			+	−		
VI	13	7	+			Nil	
	14		+			Clinical ringworm	63
	15		+			Nil	

units of the fungus, and it was therefore concluded that the minimal infective dose for cattle with this particular strain of *T. verrucosum* was 10^3 viable units (Table I).

Confluent crust-like lesions generally persisted longer in young cattle, aged 3 to 5 months, inoculated with 10^7 viable units. In one animal the complete healing of lesions was not noted until 189 days after inoculation. In all older animals lesions of varying extent developed and the disease ran a more acute course. This was typified by a slightly shorter incubation period and a more rapid desquamation of the crust leading to the eventual spontaneous resolution of lesions. In the 2 animals (Nos. 4 and 21) that remained infected for the longest periods in Groups I and II (Table II) typical crust development was retarded. Complete hair loss occurred over the entire inoculated area leaving smooth dry skin for a period of 14 days or more before the appearance of thick crust.

The lower level of nutrition, at least within the limits imposed by these experimental conditions, did not appear to influence the duration or extent of lesions in cattle aged 9 to 10 months (Group II, Table I). Moreover the shortest period of infection of all was observed in one of these animals (No. 23, Group II, Table II) that was able to lick away the encrusted surface of the lesion.

The satellite lesions that developed 36 to 42 days after inoculation never persisted any longer than lesions in the primary inoculation site. *T. verrucosum* could never be isolated in cultures of skin scrapings until 21 days after inoculation; thereafter persistence of infection was variable, but every primary lesion yielded *T. verrucosum* between 36 and 42 days after inoculation (Table II). In the majority of animals positive cultural findings could be associated with the first appearance of the clinically observable crust. However, in the group sampled at weekly intervals such isolations could only be made for just over half the period that the crust persisted. For the remainder of this time it was no longer possible to obtain cultures of *T. verrucosum* from this material.

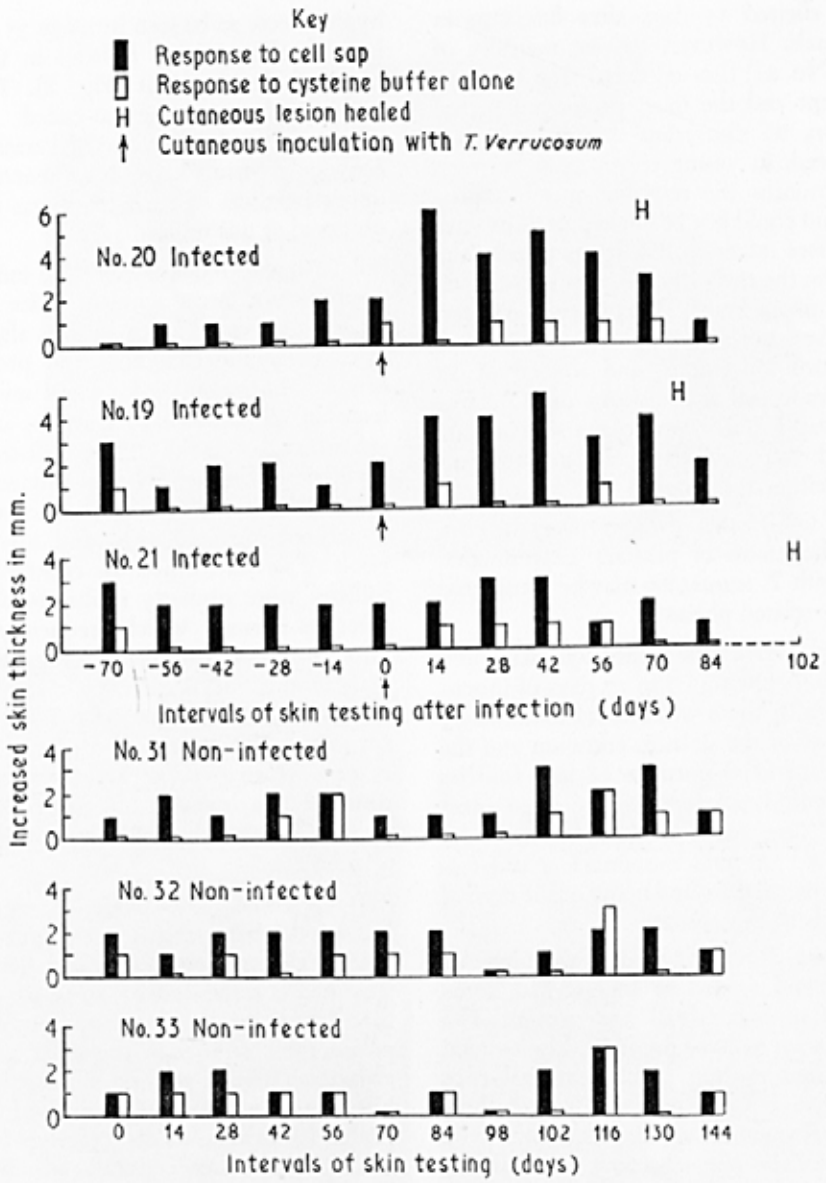

FIG. 1. Skin testing with *T. verrucosum* cell sap in infected and non-infected calves.

Intradermal Skin Tests in Primarily Infected Cattle

Intradermal inoculation of *T. verrucosum* cell sap produced a delayed-type hypersensitivity response in 4 of 6 animals tested at regular 2-weekly intervals after infection with the fungus (Fig. 1). An increase in skin thickness of 4 mm. or more was considered to be a positive response. Reactions reached maximum intensity 72 hr. after inoculation and were found to be most marked in cattle between 9 and 10 months of age that developed the more acute form of infection. Hypersensitivity responses

could be elicited 15 days after infection in these animals. However, in one member of Group II (No. 21) that exhibited retarded crust development and the most prolonged period of infection, no significant skin test response was observed. In young calves aged between 3 and 5 months the response was less pronounced and could not be elicited until 91 days or more after infection. This was usually the period when the clinically observable crust was of maximum thickness. Delayed-type skin test responses were not evident in any animals after the resolution of lesions, and a total of 12 similar intradermal inoculations of *T. verrucosum* cell sap failed to provoke a similar state of delayed hypersensitivity in non-infected animals of either age group.

Histological Observations during Primary Infection

The pathogenesis of primary experimental infection with *T. verrucosum* may be considered in 4 closely related phases:

(i) *Incubation*. During the incubation phase, and most commonly within 7 to 17 days of inoculation (Table II) there was a rapid invasion by *T. verrucosum* of the stratum corneum and the proximal superficial portions of hair follicles (Fig. 4). Long vegetative hyphae were most commonly observed in which septae were widely spaced. A mild mononuclear cell response was observed around many of the dermal blood vessels at this time.

(ii) *Maturation*. By 14 to 17 days the phase of maturation and spread of lesions had commenced in animals of all age groups. The fungus was seen to have progressively invaded the keratinized portion of the external root sheaths of hair follicles and primary ectothrix arthrospore formation was frequently observed at the level of the pilo-sebaceous ducts. By 21 days prolific arthrospore formation was evident at the ostia of hair follicles and in the softer external root sheath keratin of mature 'bed' hairs. By 28 days *T. verrucosum* had lifted the cuticle and invaded the cortex of actively growing hairs (Fig. 5) in which endothrix arthrospore formation was apparent (Fig. 6). Hyphae had also entered portions of the pilo-sebaceous ducts (Fig. 7). Similar vegetative hyphae were to be seen by 28 to 35 days in the keratohyalin zone in follicles in the catagen stage of development (Fig. 8). Their distal portions formed the so-called Adamson's fringe pattern in the zone of keratinization in actively growing hairs. Fragmentation of the upper portions of such hairs was commonly observed at this time.

(iii) *Climax of Inflammation*. The inflammatory response was most acute in older animals in which it occurred 28 to 49 days after inoculation. In younger animals the process often reached maximum proportions several weeks later. In all cattle massive serous and cellular exudation occurred from dilated dermal capillaries. Masses of polymorphonuclear leucocytes (PMNs) infiltrated the acanthotic and hyperkeratotic epidermis, and together with pools of serum formed the typical crust. Hair follicles were similarly infiltrated and micro-abscesses formed which frequently ruptured into the surrounding dermis (Fig. 9). Capillary beds in the mid-dermis were surrounded by masses of mononuclear cells. These varied in appearance from primitive reticular types to mature plasma cells. Hair fragments surrounded by masses of arthrospores were present in hyperkeratotic regions of the crust (Fig. 10).

(iv) *Regression*. The phase of regression of lesions was characterized by the growth of new hair in the majority of healed hair follicles. This had commenced by 49 to 63 days post-inoculation in all age groups but was completed most rapidly in the older animals. *T. verrucosum* could still be observed in some histological sections in this phase of the disease (Table II) although skin scrapings from these animals were often culturally negative. Compressed, degenerate hyphal elements were observed in dense masses of inflammatory exudate. Desquamation of this crust material was widespread leaving a slightly acanthotic epidermis beneath. Perifollicular areas of micro-abscesses were surrounded by granulation and fibrous tissue. Dermal perivascular areas were infiltrated with mononuclear cells and eosinophils.

FIG. 2. Confluent areas of infection 36 days after the inoculation of 10^7 viable units of *T. verrucosum*.

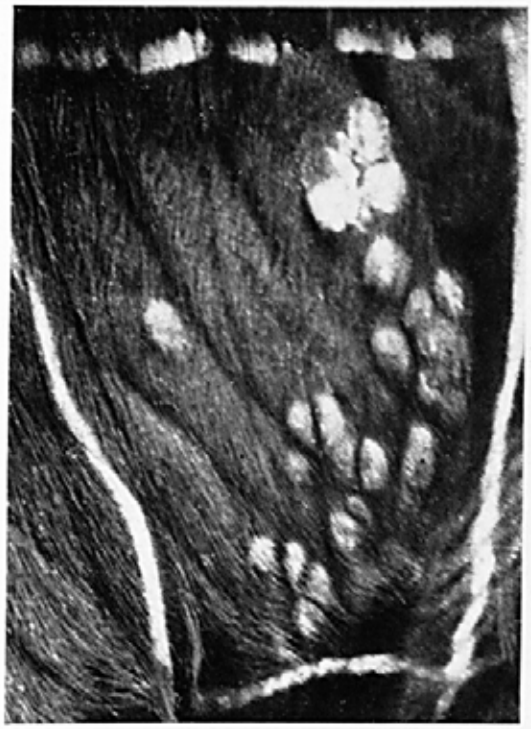

FIG. 3. Multiple coalescing lesions 22 days after the inoculation of 10^6 viable units of *T. verrucosum*.

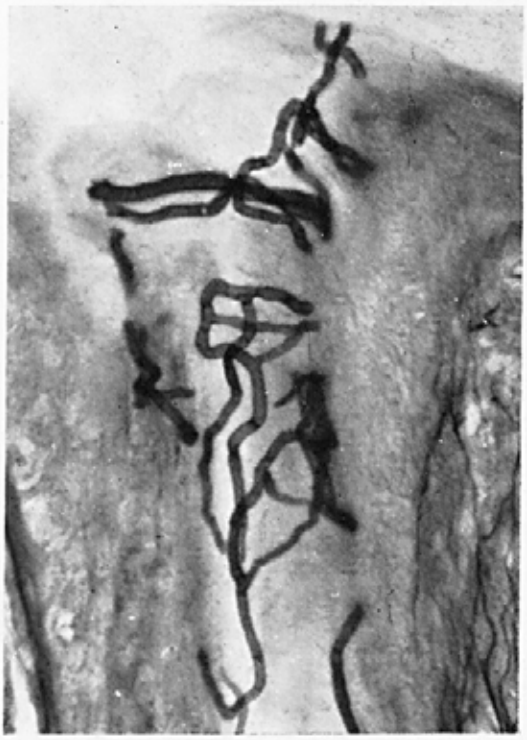

FIG. 4. Invasion of keratin in the upper portion of a hair follicle by *T. verrucosum* 7 days post-inoculation. Gridley × 800.

FIG. 5. *T. verrucosum* growing within and on the surface of the hair shaft. Gridley × 800.

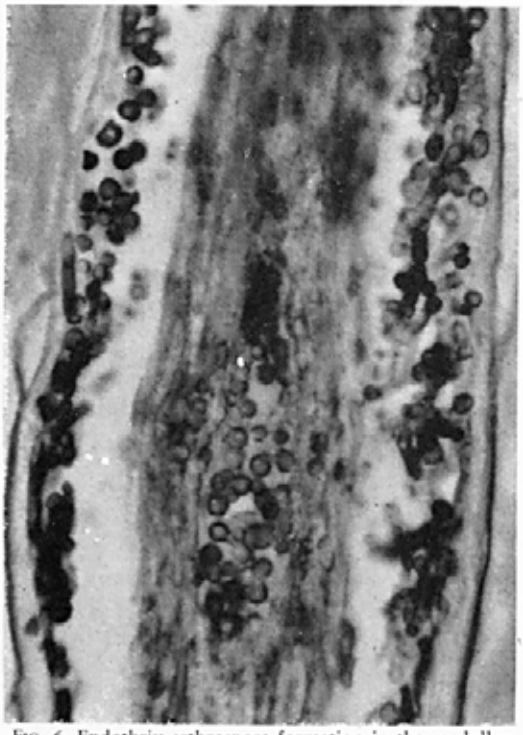

FIG. 6. Endothrix arthrospore formation in the medullary region of the hair shaft 28 days post-inoculation. Metachromatic sulphation × 1200.

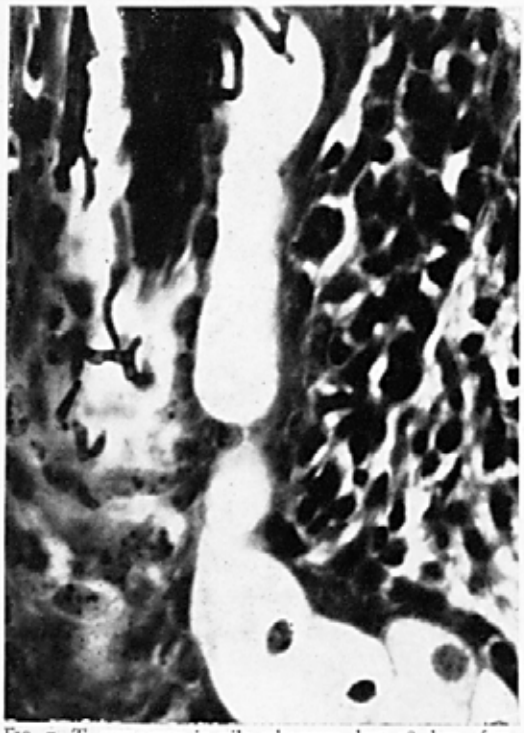

FIG. 7. *T. verrucosum* in pilo-sebaceous duct 28 days after inoculation. P.A.S. + H & E. × 800.

FIG. 8. Invasion of the keratohyalin zone of a hair follicle by *T. verrucosum* 49 days post-infection. P.A.S. + H & E. × 800.

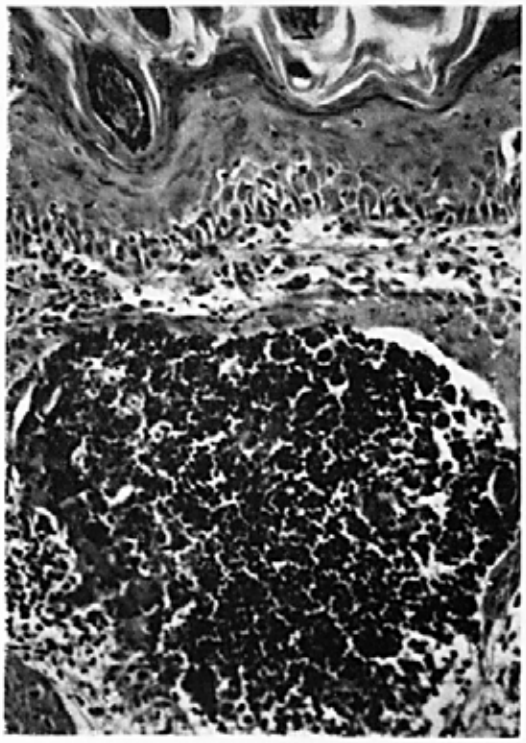

FIG. 9. Ruptured follicular micro-abscess in the dermis 42 days post-inoculation. P.A.S. + H & E. × 200.

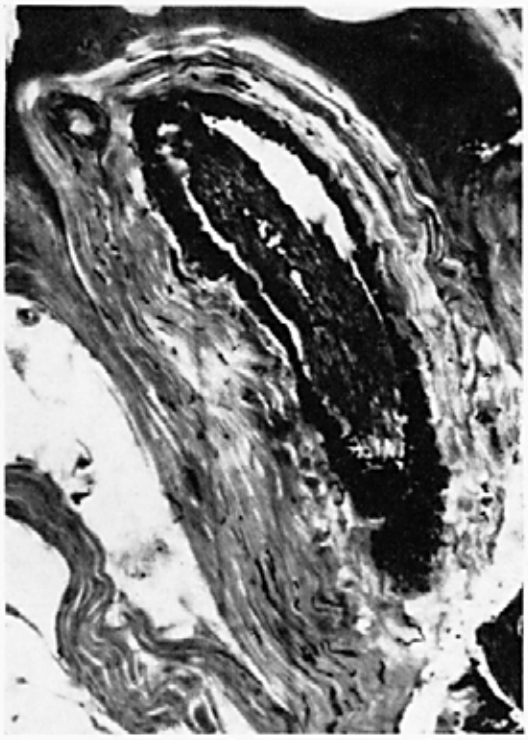

FIG. 10. Dense masses of arthrospores surrounding a fragment of infected hair in the hyperkeratotic crust 49 days post-inoculation. P.A.S. + H & E. × 200.

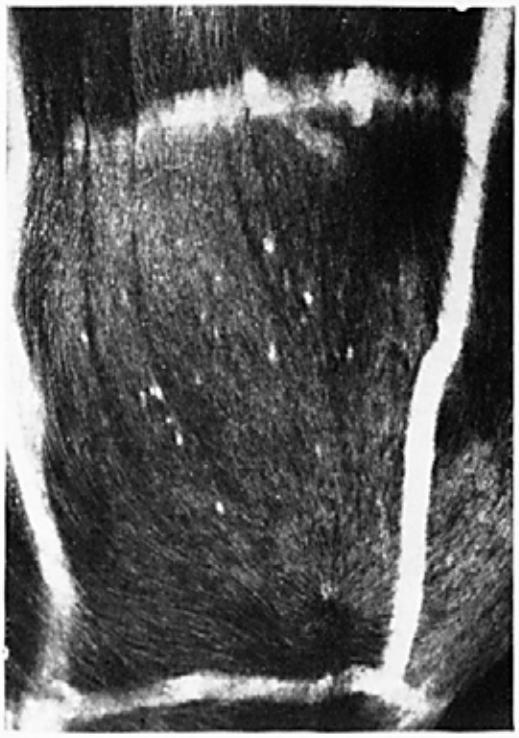

FIG. 11. Transient focal reactions persisting on the neck of a heifer 22 days after reinoculation with 10^8 viable units of *T. verrucosum*. Compare with primary infection Fig. 3).

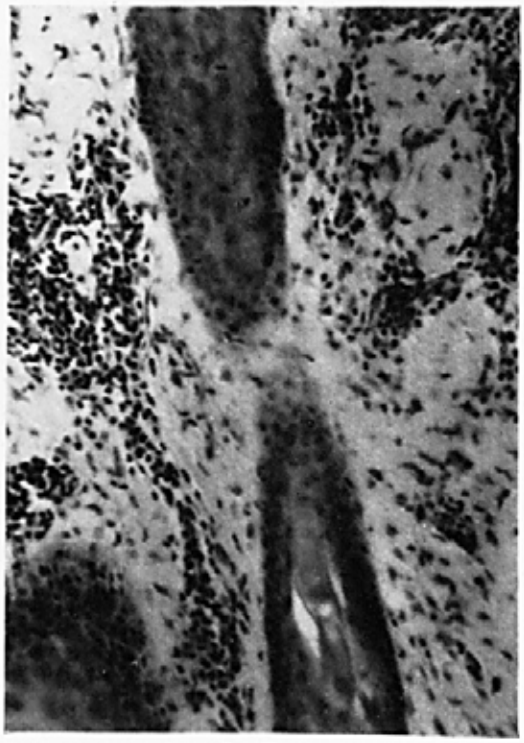

FIG. 12. Mononuclear cell infiltrates around dermal blood vessels 7 days after reinoculation with 10^3 viable units of *T. verrucosum*. P.A.S. + H & E. × 200.

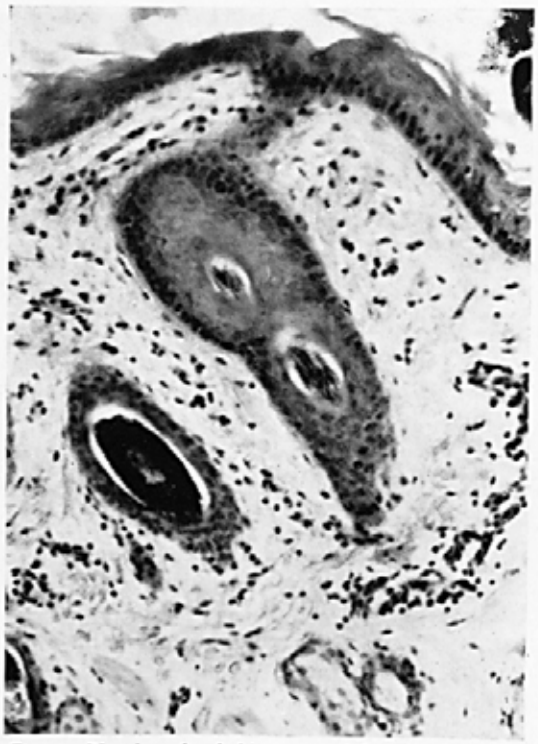

FIG. 13. Non-inoculated skin removed from the opposite side of the neck to that reinoculated with *T. verrucosum* 7 days previously. (Compare with Fig. 12). P.A.S. + H & E. × 200.

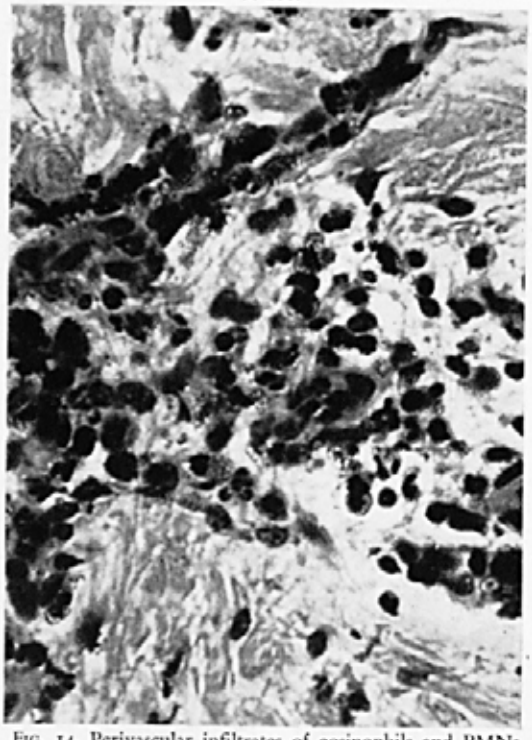

FIG. 14. Perivascular infiltrates of eosinophils and PMNs in the immediate-type response at the originally infected site 4 hr. after reinoculation of *T. verrucosum* via the intravenous route. Giemsa × 800.

Clinical Observations after Cutaneous Reinoculation of Previously Infected Cattle

The total of 11 heifer calves (Groups I and II, Table III) inoculated 2 months after spontaneous resolution of primary *T. verrucosum* lesions all proved to be resistant to reinfection. Three of 7 animals that were inoculated on the site previously occupied by the primary lesion showed no clinical reaction. The 4 animals inoculated at other sites developed small, patchy, erythematous areas which subsided in 48 hr. Scattered foci of serous exudate and scaling skin at the base of groups of hairs persisted without enlargement for up to 28 days (Fig. 11). Similar transient reactions were noted in 6 animals inoculated on fresh sites on the opposite side of the neck. *T. verrucosum* could not be isolated from skin scrapings taken 4, 10 and 28 days after inoculation from these areas. Ten animals (Groups III and IV, Table III) which were inoculated on the same day with the same dose of inoculum, and which had been free from clinically observable *T. verrucosum* infection since birth all developed typical multiple primary lesions (Fig. 2) within 17 to 21 days.

Twelve animals (Groups I and III, Table IV) infected in previous experiments also proved to be resistant to the same method of inoculation with 10^3 viable units of *T. verrucosum* over one year after the resolution of primary lesions. Transient exudative lesions were observed in 4 of them. The same batch of inoculum produced typical ringworm lesions in one of 3 calves (Group VI, Table IV) previously free from infection.

When inoculated intravenously with 10^4 viable units of the fungus in pyrogen-free distilled water, 5 animals which had been resistant to cutaneous reinoculation displayed immediate cutaneous inflammatory responses at the sites of the old lesions. These took the form of erythematous raised areas conforming almost exactly with the square sites covered by the previous infections. Disturbance of the coat pattern, erythema and swelling were evident within half an hour of inoculation. Slight restlesssness and elevated rectal temperatures of up to 104·5°F. were recorded in 4 of the heifers. All macroscopic signs of inflammation had disappeared 24 hr. after inoculation when rectal temperatures were also normal. No further systemic or cutaneous abnormalities were observed in these animals.

Histological Observations after Reinoculation

Histological changes resulting from cutaneous reinoculation with large and small doses of *T. verrucosum* were essentially the same. They took the form of a localized delayed hypersensitivity response. The perivascular cellular reaction at 24 hr. contained PMNs and small and large mononuclear cells. Swelling of the endothelial cells of dermal blood vessels together with perivascular oedema were also observed. Mild PMN and lymphocytic infiltration of portions of the epidermis occurred together with vacuolation of the cytoplasm and pyknosis of the nuclei in some basal cells. The ostia of some hair follicles contained small infiltrations of PMNs. By 48 hr. the perivascular infiltration consisted mainly of mononuclear cells and eosinophils and areas of moderate acanthosis were apparent in the epidermis (Fig. 12). The latter epidermal and vascular changes persisted for 21 days after inoculation although no proliferation of *T. verrucosum* was evident.

Skin biopsies removed from the acutely inflamed sites in resistant cattle 4 hr. after intravenous inoculation of viable units of *T. verrucosum* revealed marked vascular and infiltrative changes in the dermis and in the basal and middle layers of the epidermis. These were morphologically similar to a passive cutaneous anaphylactic response (Parish, 1965). Perivascular oedema was marked and so were swelling and detachment of the endothelium in venules. The perivascular exudate was composed almost entirely of eosinophils and some PMNs (Fig. 14). The lumina of sweat glands contained large numbers of PMNs, but sebaceous glands were virtually unaffected. Considerable intercellular oedema was apparent in the epidermis and PMN exudates occupied the larger intercellular spaces in the basal region and in the stratum malpighii.

DISCUSSION

The character and extent of the lesions produced in the non-scarified skin of cattle with agar suspensions of *T. verrucosum* in almost all cases depended upon the dose of inoculum. The minimal infective dose was the same as that determined for this organism in the rabbit (Cox & Moore, 1968). Further evidence that most areas of the skin surface are susceptible to infection (Pepin & Austwick, 1968) has been provided by the relative ease with which experimental infections were established on the neck, costal and gluteal regions. The moderate reduction in the plane of nutrition brought about by the exclusion of concentrated food from the diet of calves after weaning was not followed by more widespread chronic infection. It would thus appear that a marked deficiency of one or more essential vitamins (as suggested by Blakemore *et al.*, cited by Sellers *et al.*, 1956), or other dietary factors must occur before such lesions develop. The experiments indicated that the age of cattle had no influence on the susceptibility to infection although the incubation period and the duration of lesions were shorter in older animals. Kligman (1952) also observed in man that dermatophyte infections were often of shorter duration in adults than in children.

Histological studies regarding the mode of invasion and sporulation of the fungus in keratinized structures support the observations of La Touche (1952) and Sellers *et al.* (1956), including the finding of hyphae within the pilo-sebaceous ducts. Our cultural findings indicated that a period of up to 3 weeks usually elapsed before lesions within the inoculated area produced large numbers of infective arthrospores. *T. verrucosum* was not isolated from skin scrapings removed during the period of regression of lesions even though the agent was visible in sections of skin biopsies. This would indicate that certain host factors were responsible for the inhibition or death of the organism at that time.

The results of the intradermal skin tests with *T. verrucosum* cell sap showed that most cattle react with a marked delayed-type response only during clinical infection. Such findings support the conclusions of Jaksch (1963) and Kielstein (1967b). The results indicate that the delayed response is not clearly manifest in the young calf infected at 3 to 5 months of age until the infection has been established for nearly 3 months. The appearance of a marked skin test response in these animals heralds the commencement of resolution of lesions. Conversely, a more definite and prompt manifestation of the delayed-type response which occurs in animals infected later in life is associated with a shorter period of infection. A similar relationship between age and the ability to mount an allergic response was demonstrated by De Lameter (1942) in dermatophyte-infected guinea-pigs.

The demonstration of generalized resistance to reinfection in a total of 17 animals cutaneously reinoculated with large or small doses of *T. verrucosum* 2 months or a year or more after the resolution of primary lesions confirms that cattle develop a lasting acquired immunity to reinfection with this species of dermatophyte. The local response of cattle to cutaneous reinoculation with the fungus is relatively anergic compared with that of laboratory animals challenged with other species of dermatophyte. Contrary to some observations in laboratory animals (De Lameter, 1941; Wenk, 1962) no differences could be detected between the macroscopic or the histological aspects of the response to challenge at previously infected or freshly challenged skin sites. Histological changes at the reinoculated site were similar to those observed in chemical contact sensitivity (Waksman, 1960; Flax & Caulfield, 1963). It is still not clear whether these relatively mild inflammatory changes and the accompanying hypertrophy of keratinized structures were sufficient to alter the conditions favourable for the proliferation of *T. verrucosum* in the skin of reinoculated cattle. The observation of an immediate type response at the previously infected site after rechallenge with *T. verrucosum* via the intravenous route would indicate that tissue-sensitizing humoral antibody was present in this area. This type of response may bear a close similarity to the

cutaneous 'flare-up' observed after the intravenous challenges of chemically sensitized guinea-pigs (Polak & Turk, 1968).

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