Clin. exp. Immunol. (1970) **7**, 301–341.

IMMUNITY IN CUTANEOUS LEISHMANIASIS OF THE GUINEA-PIG

A. D. M. BRYCESON,* R. S. BRAY,† R. A. WOLSTENCROFT AND D. C. DUMONDE

Division of Immunology, Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London, W.6

(*Received* 25 *March* 1970)

SUMMARY

This paper describes the course of infection and development of immunity in guinea-pigs after intradermal inoculation of *Leishmania enriettii*, and the use of *in vivo* and *in vitro* techniques to characterize the immunological response to infection and artificial immunization.

Inoculation of 10^6 amastigotes into the ear produced a nodule which ulcerated in 2–3 weeks and healed in 8–16 weeks. 8% of animals developed cutaneous metastases which healed with the original lesions. Histology of the primary lesions showed epidermal necrosis overlying a mass of parasitized macrophages which, after 4–6 weeks, became surrounded and infiltrated by lymphocytes. Histological changes in the draining lymph node began after 3 days and proceeded for 6 weeks; both germinal centres and paracortical areas were hyperplastic and the medulla contained many plasma cells. Superinfection produced an 'isophasic' lesion, but reinfection after healing elicited only a delayed hypersensitivity response. Artificial immunization with soluble and insoluble antigenic extracts of *L. enriettii* in Freund's complete adjuvant partially protected against infection; extracts of other leishmanial species failed to protect. Immunological paralysis, attempted with intravenous injections of soluble antigen, increased the severity of subsequent infection.

Both infection and immunization were accompanied by delayed hypersensitivity which could be transferred passively by lymphoid cells. Cell-mediated immunity was studied *in vitro* by the ability of soluble leishmanial antigens to transform lymphocytes, to inhibit macrophage migration, and to induce the production of lymphokine factors from lymphocytes of sensitized animals. A target cell system was devised in which sensitized lymphocytes destroyed monolayers of parasitized macrophages. Cross reactivity of leishmanial with mycobacterial antigens was shown in skin tests and in target cell destruction, but not in cell transfer or in the other cell culture systems. The phagocytic activity of peritoneal macrophages from

* Present address: Department of Medicine, Ahnadu Bellow University, Zaria, Nigeria.

† Present address: Wellcome Parasitology Unit No. 2, Haile Sellassie I University, P.O. Box 1176, Addis Ababa, Ethiopia.

Correspondence: Dr. Dudley Dumonde, Division of Immunology, Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London, W.6.

recovered animals was increased for homologous but not for heterologous species of *Leishmania*; the growth of ingested organisms was not however reduced.

Circulating antibodies were not demonstrated by passive cutaneous anaphylaxis, or by agglutination of antigen coated sheep erythrocytes, in the sera of infected or convalescent animals, although some convalescent animals showed active cutaneous anaphylaxis. However, antibodies were demonstrated by both these techniques in immunized animals, which also showed anaphylactic and Arthus hypersensitivity when skin tested with the soluble antigens.

The results are taken to indicate that cellular mechanisms are prominent in the development of immunity of the guinea-pig against *L. enriettii*, and ways in which the host may eliminate the parasite are discussed. It is concluded that this model provides an experimental counterpart of human cutaneous leishmaniasis and that it is suitable for the analysis of the role of cell-mediated specific immunity in resistance to intracellular infection.

INTRODUCTION

Cutaneous leishmaniasis of the Old World, oriental sore, is characterized by a single, self-healing lesion which is followed by permanent immunity to reinfection and is accompanied by delayed hypersensitivity on skin testing with leishmanin and usually an absence of circulating antibodies (Berberian, 1944; Ansari & Mofidi, 1950; Dostrovsky & Sagher, 1945; Bray & Lainson, 1965, 1966, 1967). In the New World the primary lesion may be followed by destructive metastatic lesions of the nose or mouth, a condition known as espundia, in which both delayed hypersensitivity and circulating antibodies are demonstrable (Pessoa & Barretto, 1944; Bray & Lainson, 1965, 1966, 1967). In Venezuela and in Ethiopia patients are occasionally seen suffering from diffuse cutaneous leishmaniasis in which leishmanial nodules, which do not ulcerate or heal, cover large areas of the skin; in this syndrome the leishmanin test is negative (Convit, 1958; Bryceson, 1969, 1970). Visceral leishmaniasis, kala-azar, is characterized by massive reticulo-endothelial invasion and death; in this disease delayed hypersensitivity is absent before cure but there are antibodies in high titre (Manson-Bahr, Heisch & Garnham, 1959; Sen Gupta & Mukherjee, 1962; Heyneman, 1967). In order to understand these phenomena, which are summarized in Table 1, it is necessary to understand the processes by which healing occurs and immunity is maintained. It has been suggested that these processes are cell mediated and that the role of antibody is irrelevant or even detrimental (Garnham & Humphrey, 1969).

There are several difficulties in the selection of a suitable combination of host and parasite for the experimental study of leishmaniasis. Leishmaniasis is a zoonosis. A given species of parasite (e.g. *Leishmania tropica*) may have, in addition to its natural host (the gerbil) and Man, a liaison host (the dog) and a laboratory host (the mouse); and the behaviour of the parasite may be different in each. Depending on the choice of combination a strain of parasite may kill, produce a chronic indolent sore, a self-healing lesion or fail to establish itself, and any given host species may differ in its susceptibility to different strains of parasite. It is likely that the features of the immunological response to leishmanial infection will differ in different host–parasite combinations. Apart from Stauber and his colleagues (see Stauber, 1966), who have investigated infection in the hamster with *L. donovani*, few investigators have used inocula of defined size or infectivity, or have been able to quantitate

TABLE 1. Immunological features of leishmanial infections in man

Parasite	Disease	Self healing	Delayed hypersensitivity: During infection	Delayed hypersensitivity: After cure	Antibodies	Subsequent immunity	References
L. donovani	kala-azar	−	−	(±)	+ (CFT, HA, FAT, GD)	present†	e.g. Heyneman (1967);
L. donovani	PKADL*	−	(±)	+	decreasing	present	Sen Gupta (1962)
L. donovani (*L. infantum*)	infantile kala-azar	−	−	+	+ (CFT, HA, GD)	present	Bray (unpublished)
L. tropica	oriental sore	+	+	+	rare + (HA, PK)	present‡	Bray & Lainson (1966); Dostrovsky & Sagher (1945)
L. tropica	recidiva	−	+	+	−		Dostrovsky *et al.* (1952); Bray & Lainson (1967); Bryceson (1969, 1970)
L. tropica	diffusa	−	−	(±)	rare (low titre HA)		
L. brasiliensis	simple ulcer	+	+	+	− (HA)	present§	Bray & Lainson (1965, 1966, 1967); Convit & Kerdel Vegas (1965)
L. brasiliensis	espundia	−	+	+	+ (HA, FAT, GD)		
L. brasiliensis ('*L. pifanoi*')	diffusa	−	−	(±)	rare + (FAT, HA)		
L. mexicana	simple ulcer	+	+	+	− (HA, FAT, GD, PCA)	present	Lainson & Strangeways-Dixon (1963); Bray & Lainson (1965, 1966)
L. peruana	simple ulcer	+	+	+	− (FAT, GD, HA)	present	Battistini & Herrer (1946); Bray & Lainson (1966)

* Post kala-azar dermal leishmaniasis; CFT: complement fixation test; HA: indirect haemagglutination; FAT: fluorescent antibody test; GD: gel diffusion; PCA: passive cutaneous anaphylaxis; PK: Praustnitz-Kustner.

† Some develop PKADL. ‡ Some continue as recidiva. § Some develop espundia.

TABLE 2. Immunological features of leishmanial infections in experimental animals

Animal host	Parasite species	Route of inoculation	Site of infection	Outcome	Immunological features: DH	Antibodies	Immunity	Cross-immunity	References
Monkey	*L. mexicana*	ID	skin	heals	variable	±(PCA), −(HA)	present	*L. braziliensis*	Lainson & Bray (1966)
	L. braziliensis	ID	skin	heals	variable	±(PCA), −(HA)	present	*L. mexicana*±	Lainson & Bray (1966)
	L. tropica	ID	skin	heals			present	*L. infantum*	Parrot *et al.* (1927)
	L. donovani	ID/IV	sk/viscera	heals		+(HA)			Shortt & Swaminath (1925)
	L. enriettii	ID	none						Muniz & Medina (1948)
Dog	*L. infantum*	ID/IP	viscera	variable			partial	*L. tropica*	Nicolle & Comte (1909)
	L. tropica		sk/viscera	variable	+	+(FAT)	partial		Nicolle & Manceaux (1910)
	L. donovani	IP	sk/viscera	kills		+(FAT)	partial		Patton (1913)
Hamster	*L. donovani*	ID/IP	sk/viscera	kills		+	present		Chung & Wang (1939)
	L. tropica	ID/IP	sk/viscera	kills		+	present		Chung & Wang (1939)
	L. braziliensis	ID/IP	skin (viscn. ±)	chronic	−	−			Zeledon & Blanco (1965)
	'*L. pifanoi*'	ID	skin	chronic					Medina & Romero (1959)

	'*L. pifanoi*'	IP/IV	skin	chronic	−			Hyattee (pers. comm. 1969)
	L. mexicana	ID	skin (late viscn.)	chronic	−	+(Ha: late viscn.)	none	Mata *et al.* (1968)
	L. enriettii	ID	skin	transient	−			Muniz & Medina (1948)
		IP/IV	none					Alder & Halff (1955)
Mice	*L. tropica min.*	IP	viscera	kills		+	none	Laveran (1917)
	L. tropica maj.	ID	skin	chronic		−		Neal (1964)
	'*L. pifanoi*'	ID/IP	skin	chronic	−			Hyattee (pers. comm. 1969)
	L. mexicana	ID	skin (late viscn.)	chronic	−	−(+HA, GD: on viscn.)	none	Mata *et al.* (1968)
	L. enriettii	ID	skin ±	heals				Adler & Halff (1955)
	L. donovani	IP	viscera	heals		+	+	Shortt & Krishnan (1932)
Guinea-pigs	*L. enriettii*	ID/IV	skin	heals	+	−	present	Muniz & Medina (1948) Paraense (1953)
	all others	any	none					

Abbreviations: DH: delayed hypersensitivity; ID: intradermal; IP: intraperitoneal; IV: intravenous; viscn.: visceralization. Other abbreviations as in Table 1.

the results of infection, which has made the comparison of their results difficult. The literature concerning this field is summarized in Table 2. The conditions necessary for the successful protection of Man and animals by artificial immunization have not yet been clarified (Pessoa, 1941; Curban, 1942; Coutinho, 1954; Manson-Bahr & Southgate, 1964).

Cutaneous leishmaniasis of the guinea-pig is caused by *Leishmania enriettii*. This host–parasite combination is a natural one, which was discovered accidentally in a South American laboratory (Muniz & Medina, 1948; Adler & Halff, 1955). The disease is characterized by a single lesion which ulcerates and heals in 2–3 months (Paraense, 1953). It is accompanied by delayed hypersensitivity (Glazunova, 1965) and followed by long-lasting immunity to reinfection (von Kretschmar, 1965). Cutaneous metastases appear in a proportion of animals (Paraense, 1953; Adler & Halff, 1955). As this pattern so closely follows that of the disease in Man it seemed a suitable model in which to study the pathology of the infection and to determine the immunological response to *Leishmania*.

In this paper we describe the course of infection and the development of immunity in guinea-pigs infected with *L. enriettii*. We have examined by *in vivo* and *in vitro* techniques the features of the immunological response following infection and artificial immunization. We describe circumstances in which immunological paralysis results in increased susceptibility to infection.

MATERIALS AND METHODS

Organisms and antigenic extracts

The strain of *Leishmania enriettii* was kindly supplied by Dr R. A. Neal, Wellcome Research Laboratories, Beckenham, Kent. It was maintained by passage in guinea-pigs and on conventional N.N.N. blood-agar slopes. Leishmanial antigens were prepared as follows: promastigotes (leptomonads) of *L. enriettii* were grown in bulk in Hanks' balanced salt solution overlying N.N.N. medium (Bray & Munford, 1967) in Thompson flasks. The organisms were harvested and washed three times by centrifuging at 1000 *g* for 15 min in phosphate buffered saline, pH 7·2 (PBS). For initial experiments organisms were then resuspended in distilled water, lyophilized, triturated with distilled water and centrifuged at 105,000 *g* for 1 hr at 4°C (Bray & Lainson, 1966); the sediment was kept as 'crude' insoluble antigen (CIA) and the supernatant as 'crude' soluble antigen (CSA). In further experiments the washed organisms were sonicated in phosphate-buffered NaCl pH 7·2 (three periods of 1 min at 25,000 kc/s, on ice); urea was then added to a concentration of 8 M and the resulting suspension was incubated for 2 hr at 37°C, then dialysed five times against twenty volumes of 0·05 M ammonium bicarbonate:acetic acid buffer, pH 7·4 and then centrifuged at 105,000 *g* for 1 hr at 4°C. The deposit was kept as purified insoluble antigen (PIA) and the supernatant as purified soluble antigen (PSA). The soluble antigens (CSA, PSA) were freeze dried and assayed for protein content using bovine serum albumin as a standard (Lowry *et al.*, 1951). CSA usually contained about 10% protein and PSA 60–100% protein. Antigenicity of soluble antigens was checked by comparing the delayed (24 hr) hypersensitivity response in guinea-pigs convalescent from *L. enriettii* infection and skin tested with 100 μg, 10 μg and 1 μg antigen protein. Only batches of comparable antigenicity were used. The dose of soluble antigens used in all experiments was expressed as the protein weight, except where stated dilutions of crude soluble antigen were used (e.g. CSA 1/100). Antigens were similarly made from *L. donovani* (Strain L82), *L. braziliensis*

(Strain L23), *L. mexicana* (Strain L11) and *L. tropica* (Strain L6, see Bray, 1969). All antigens were stored at −20°C.

Infection, immunization and paralysis

Outbred Harley guinea-pigs weighing about 400 g were used. Amastigotes (Leishman-Donovan bodies) of *L. enriettii* were used for infecting guinea-pigs and were obtained from nasal lesions raised by injecting, 3–4 weeks previously, 10^6 amastigotes subcutaneously into the tip of the nose. Such a lesion, which contains the maximum number of organisms in relation to infiltrating cells, was dissected out as a nodule, washed, teased in Hanks' solution and lightly ground in a glass tissue grinder. The amastigotes were counted in a Neubauer chamber by phase microscopy and adjusted to 10^7 organisms/ml. Guinea-pigs were infected by intradermal injection of 10^6 organisms in 0·1 ml Hanks' solution on the dorsal surface of one ear. On occasion animals were multiply infected with a total of 2×10^6 organisms into the nose, both ears and the feet. Animals were most frequently immunized with 2 mg insoluble antigen (CIA or PIA) and 2 mg soluble antigen (CSA or PSA) of *L. enriettii*, suspended in 0·2 ml PBS and emulsified with 0·2 ml Freund's incomplete adjuvant (FIA), and given in divided doses intradermally into the four foot-pads. Other immunizing regimens used are presented in Tables 5 and 10 (see also 'Results').

Attempts were made to induce immunological paralysis in three ways: (i) by one intravenous injection of 2 mg PSA, (ii) by three intravenous injections of 10 mg PSA at weekly intervals and (iii) by one injection of 5 mg CSA into the mesenteric vein. One week later, animals were then either infected or immunized.

Passive transfer of delayed hypersensitivity was sought using peritoneal exudate cells, lymph nodes and spleen cells taken from animals at various stages of infection, immunization or paralysis. Peritoneal exudates were harvested 4–5 days after the intraperitoneal injection of 20 ml sterile paraffin oil; the cells were washed free of oil in Hanks' balanced salt solution to yield suspensions consisting of approximately 85% macrophages and 15% lymphocytes. Cell suspensions were prepared from lymph nodes draining sites of immunization or infection, and from spleens, which were dissected, teased in Hanks' solution and forced through a fine mesh sieve. Suspensions consisted of 80–95% lymphocytes. As judged by exclusion of 0·4% Eosin Y, cell viabilities were: peritoneal exudate cells, 99%; lymph node cells, 43–54%; and spleen cells, 54–63%. Each cell population was injected intravenously into two separate pairs of normal guinea-pigs in doses of 5×10^8 and 1×10^8 viable cells respectively. Recipients were tested immediately after cell transfer with 200 μg and 20 μg PSA and 50 μg tuberculin PPD (purified protein derivative). Skin tests were read 24 hr later.

Skin testing, antibody assay and resistance to infection

Skin testing was done with PSA given by intradermal injection into the shaved, depilated flank of the guinea-pig in doses of 100 μg, 10 μg and 0·1 μg of PSA in PBS; 10 μg PSA was found to be of similar potency to the standard Leishmanin test of 5×10^6 promastigotes in 0·1 ml of 0·5% phenol saline. Skin tests were examined at 15 min, 1, 4, 7½, 24 and 48 hr by measuring diameter of erythema and thickness (Schnelltaster callipers). Skin reactions were expressed as volumes of oblate ellipsoids $V = \frac{4}{3}\pi r^2 t$ (r = radius of erythema, t = thickness) which were found to be more closely related to dose of antigen than measurements of diameter or thickness alone.

In early experiments designed to study the timing of skin test positivity the area and intensity of skin reactions were delineated by an intravenous injection of 0·7 ml of 1% Evans' blue 15 min before reading the tests. Animals were then killed, skinned and the intensity of dye leakage on the underside of the skin was compared with that of control animals and scored as +++, ++, + or −.

Circulating antibodies were detected by passive cutaneous anaphylaxis (PCA), indirect haemagglutination (HA), and macrophage-cytophilia. Passive cutaneous anaphylaxis was elicited by injecting 0·1 ml of the serum to be tested intradermally into the shaved depilated flanks of fifteen normal guinea-pigs. After intervals each of 6, 12, 24, 48 and 72 hr three animals were given Evans' blue as above followed 15 min later by 20 μg of CSA by intravenous injection. The results were assessed by extent of dye leakage (see above). Indirect haemagglutination was carried out as described by Bray & Lainson (1967) using tanned sheep erythrocytes coated with CSA. Antibodies cytophilic for macrophages were sought by a modification of Boyden's (1964) technique using, as indicator particles, either CSA bisdiazotized to sheep erythrocytes (Jonas *et al.*, 1965) or suspensions of amastigotes and promastigotes [see (v) below]. Macrophage monolayers from unimmunized guinea-pigs [also see (v) below] were washed once with serum-free medium and were then treated with 1:20 dilutions in 199 medium of sera from experimental animals for 1 hr at 4°C or 22°C. Monolayers were then gently washed five times and were exposed to organisms (at 5×10^6/ml) or antigen-coated erythrocytes (0·5%) at the corresponding temperatures in serum-free medium 199 for a further 1-hr period. At the end of this time, gently-washed monolayers were examined for rosette formation; they were also fixed in Bouin's fluid and stained with Giemsa.

Resistance to infection of guinea-pigs in various experimental groups was determined by challenging individual animals with 10^6 amastigotes by intradermal injection into a previously uninfected ear. The state of this lesion and the presence of metastases were examined at weekly intervals for 9 weeks. In some animals the antibody and delayed hypersensitivity responses to the challenge infection were also examined.

Cellular reactions in vitro

(i) *Inhibition of macrophage migration by soluble antigen (PSA).* Oil-induced peritoneal exudate cells from experimental guinea-pigs were allowed to migrate from the cut ends of capillary tubes for a period of 20 hr into 15% guinea-pig serum–Eagle's medium containing PSA in concentrations up to 250 μg/ml. Inhibition of macrophage migration was expressed as a migration ratio (David *et al.*, 1964a).

(ii) *Antigen-induced lymphocyte transformation.* Lymph node cell suspensions, predominantly lymphocytes, were prepared as above. Cells were cultured at a concentration of 2×10^6/ml in Eagle's medium supplemented with 20% decomplemented guinea-pig serum, in the absence or presence of PSA or PPD, as 3 ml aliquots under air in sealed stationary bijou bottles at 37°C. Cultures set up in triplicate were harvested after 66 hr incubation; after 20 hr of incubation 1 ml of fresh tissue culture medium was added to all cultures. Eighteen hours before harvesting, 2 μCi of ^{3}H-thymidine (specific activity 5 Ci/mM—Amersham) were added to each culture. Lymphocyte transformation was measured by liquid scintillation counting of cellular incorporation of ^{3}H-thymidine (Oppenheim, Wolstencroft & Gell, 1967) and was expressed as disintegrations/min (dpm)/3 ml culture/2 μCi ^{3}H-thymidine.

(iii) '*Lymphokine*' *production.* This name has been given to soluble factors, other than

antibody, which are released by sensitized lymphocytes when incubated in the presence of specific antigen (Dumonde *et al.*, 1969). These factors, whose activity is non-specifically expressed, are considered to mediate delayed hypersensitivity. Their production is detected by their biological activities of which four are readily assayed: 'macrophage migration inhibition factor' (MIF), 'mitogenic factor' (MF), 'cytotoxic factor' and 'inflammatory factor'. We have looked for the presence of the first two activities (MIF and MF) in the supernatant fluids of lymphoid cells cultured in the presence of *L. enriettii* antigen. Lymphocyte culture supernatants for the assay of MIF and MF were prepared by incubating lymph node cells from individual animals at a concentration of 10^7 viable cells/ml in 20% guinea-pig serum–Eagle's medium with and without PSA at a concentration of 100 μg/ml for 20 hr at 37°C. Supernatants (5 or 10 ml volumes) were clarified by centrifugation. Supernatant which had not been incubated with antigen were 'reconstituted' (R) with antigen to the same concentration (100 μg/ml) as that of the 'preincubated' (P) supernatants. Supernatants were then sterilized by membrane filtration and stored at -20°C until tested for MIF and MF as described elsewhere (Wolstencroft & Dumonde, 1970). IFM activity was expressed as a migration ratio (P/R) utilizing peritoneal exudate cells from unimmunized guinea-pigs; (Dumonde *et al.*, 1969): MF activity was expressed as incremental dpm (P–R) utilizing lymph node cells from guinea-pigs injected with FIA (Wolstencroft & Dumonde, 1970).

(iv) *The presence of antibody on the surface of macrophages* from infected and immunized animals was sought by a rosette technique, using as indicator particles, either CSA bis-diazotized to sheep erythrocyte, or suspensions of amastigotes or promastigotes. Macrophage monolayers were overlaid with the indicator particles suspended in serum-free medium (see below) for 1 hr at 4°C and 22°C; after this they were washed gently, fixed, stained and examined for rosette formation.

(v) *Phagocytic and parasiticidal activities of macrophages* from animals in different experimental groups, were determined by studying the uptake and subsequent intracellular survival of *L. enriettii* amastigotes when added to macrophage monolayers. Starch-induced peritoneal exudate cells from experimental or from control normal guinea-pigs were suspended in serum-free medium 199 at 5×10^5 cells/ml and were allowed to settle in modified Carrell flasks (Zuckerman, 1945) for 1 hr at 36°C. After gently washing the monolayers with medium 199, these were exposed to *L. enriettii* by the addition of fresh amastigotes, suspended at 5×10^6 organisms/ml in medium 199 containing 10% normal guinea-pig serum, for 2 hr at 36°C, which resulted in parasitization of a high proportion of the macrophages. At the end of this time, cultures were washed with serum-free medium and were re-incubated in 30% serum-199 medium. After a further 4, 12, 24 and 48 hr, sample cultures were fixed in Bouin's fluid and stained with Giemsa. Comparison of parasite survival following the initial phagocytosis therefore provided an index of cellular resistance to intracellular infection. The results were expressed as the number of parasites per 100 macrophages and compared with those obtained with macrophages from normal animals.

(vi) *Action of sensitized lymphocytes on parasitized macrophages.* Monolayers of macrophages from normal unimmunized guinea-pigs were infected by exposure to *L. enriettii* amastigotes for 2 hr at 36°C (see above). After washing to remove free organisms, the monolayers were overlaid with lymph node cells, taken from animals in various categories of immunization or infection, and suspended in 199 medium containing 30% normal guinea-pig serum. Macrophage monolayers were formed at 5×10^5 cells/ml and lymph node cells (90% lymphocytes; 50% viability) were added at a total cell density of $2{\cdot}5 \times 10^7$/ml (50

lymph node cells per macrophage) or 5×10^7/ml (100 lymph node cells per macrophage). After 2, 12, 24, 48 or 72 hr of lymphocyte–macrophage interaction, cell monolayers were washed, fixed in Bouin's fluid, stained with Giemsa, and examined for cell density and survival of organisms (see also Bray & Bryceson, 1968). The number of organisms per 100 surviving macrophages was counted and the result expressed as a percentage of organisms in cultures receiving no lymphocytes. In control experiments, either the macrophages were unparasitized, or the lymphocytes were obtained from FIA-injected guinea-pigs. As a further index of parasite survival some supernatant fluids from mixed cell cultures were inoculated onto NNN slopes. Supernatant fluids were also tested on fresh parasitized macrophages to determine whether a cytotoxic factor (Kolb & Granger, 1968) was being generated in mixed culture.

RESULTS

Course of infection in previously normal guinea-pigs and their resistance to reinfection

Forty-five guinea-pigs were each infected in one ear. The state of this lesion and the presence of metastases were examined at weekly intervals for 9 weeks. Single animals were bled and killed daily for 7 days and then weekly for 9 weeks. The lesion and the draining (pre-auricular) lymph node were fixed in Carnoy's fluid, sectioned and stained with haematoxylin and eosin, Giemsa's stain (Bray & Garnham, 1962) and methyl-green–pyronin. Single animals were rechallenged with 10^6 organisms in the opposite ear at weekly intervals after the initial inoculation. The state of this lesion was examined weekly.

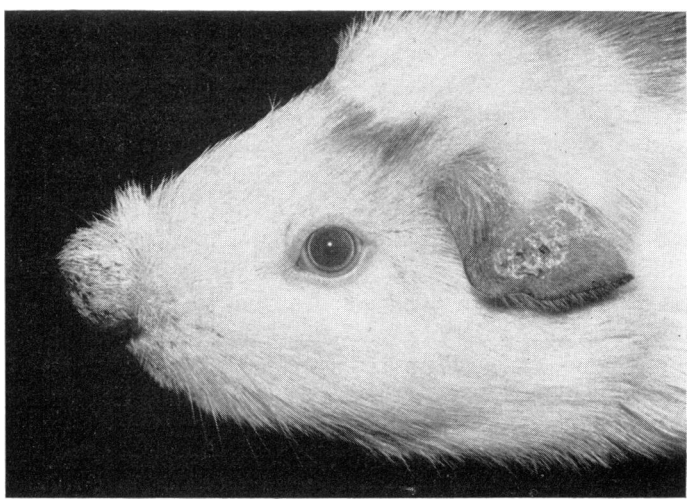

FIG. 1. 3-week infection of guinea-pig inoculated in the nose and ear with 10^6 amastigotes of *L. enriettii*.

Clinical course of infection

Within a week of inoculation a nodule became palpable on the ear; this grew rapidly and the overlying skin became hyperkeratotic. Within 2–3 weeks the lesion ulcerated and became

covered with a crust (Fig. 1). Between 4 and 6 weeks it reached its maximum size, about 2–3 cm in diameter by 0·5 cm in thickness, at which stage the cartilage of the pinna sometimes became eroded. Healing began by about 6–8 weeks and was complete by 9–16 weeks.

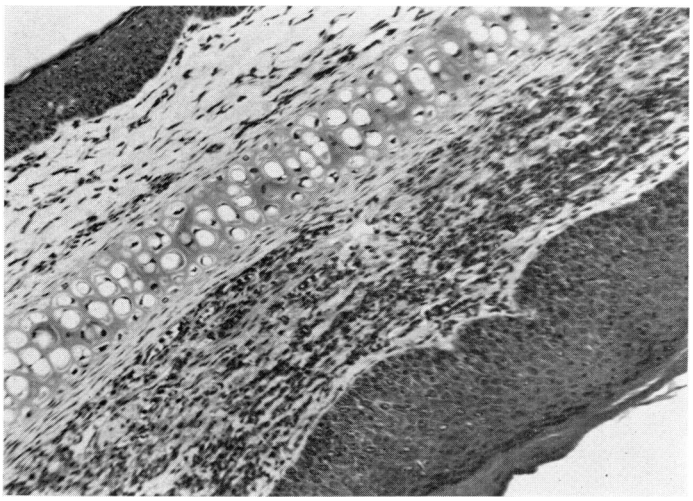

FIG. 2. 8-day infection, guinea-pig ear, showing dermal infiltration and epidermal changes. H and E ×260.

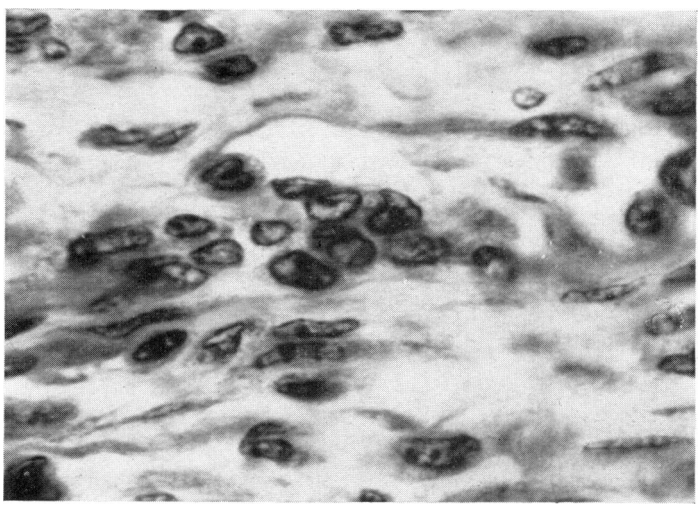

FIG. 3. 8-day infection; ear, showing mononuclear cell emigration from subcutaneous blood vessel. H and E ×2600.

In about 8% of infected animals metastases appeared on the other ear, nose, lip, feet or external genitalia. These metastases, which appeared at about 8 weeks and which did not always ulcerate, healed with or soon after the primary lesion. Lesions on the nose took and

grew well, with the same time course. After about 4 weeks, the lesion, on dissection, was clearly demarcated by a haemorrhagic zone.

Infection with 10^6 organisms could be established only on exposed surfaces of the skin

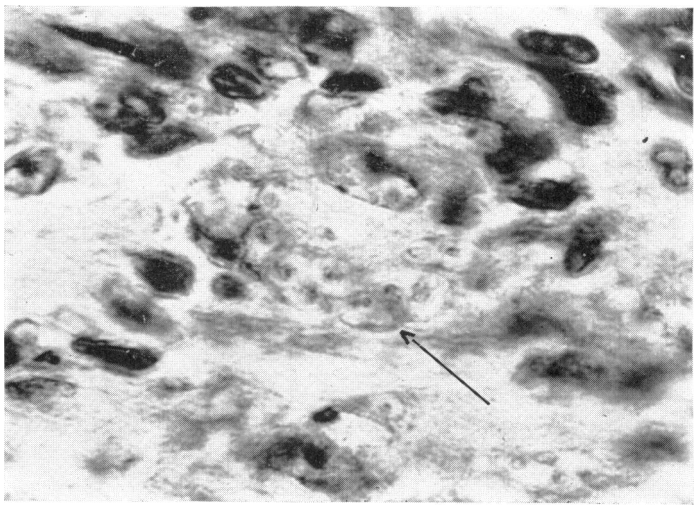

FIG. 4. 8-day infection; ear, showing collection of parasites within a macrophage and numerous uninfected mononuclear cells. H and E ×2600.

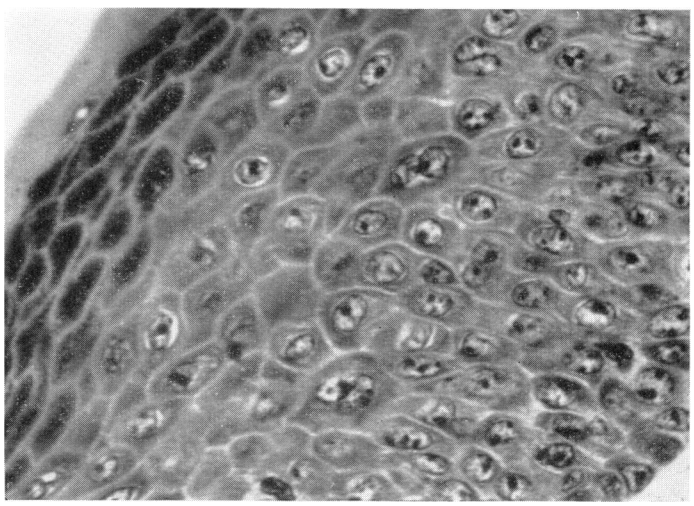

FIG. 5. 8-day infection; epidermis of ear, showing increase in thickness of all layers, with vacuolation of cytoplasm and condensation of nuclei of prickle cells. H and E ×1300.

and never on an area covered with hair. If amastigotes were inoculated onto the shaven back and the back was kept shaven for 1 week a lesion became established, which depilated that area and continued to grow. If the hair was allowed to grow back in the first few days after inoculation, no lesion appeared.

Histology of ear lesions

By the end of the first week, the epidermis was still intact, but the dermis was already heavily infiltrated with macrophages, some of which contained a few amastigotes (Figs. 2,

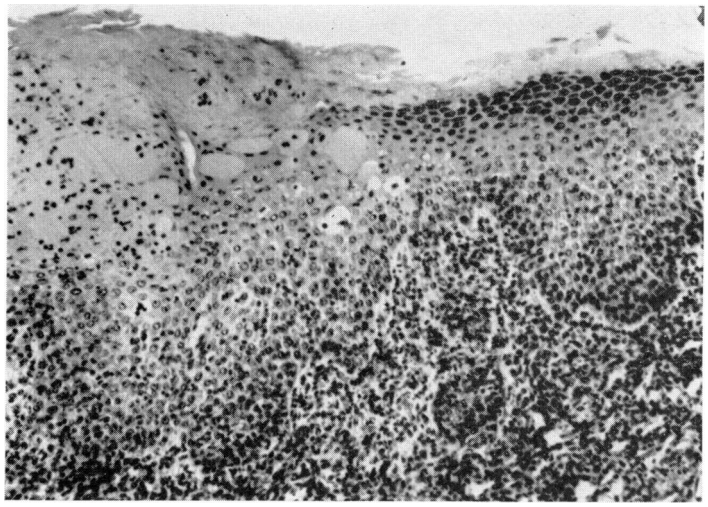

FIG. 6. 3-week infection; ear, showing origin of necrosis within the epidermis. H and E × 320.

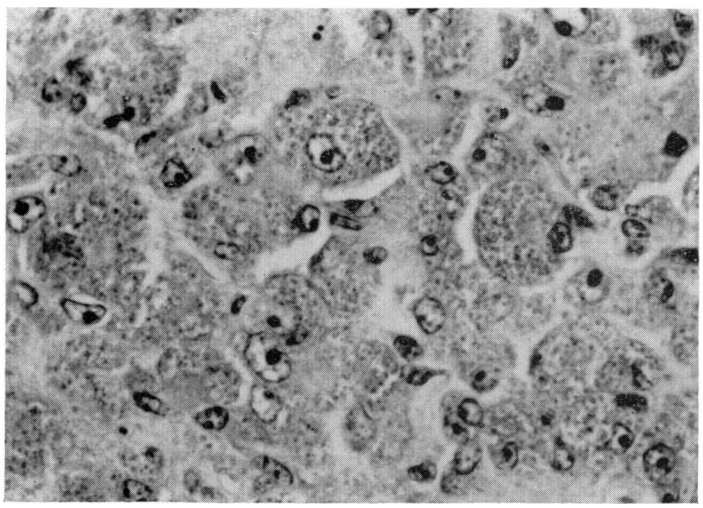

FIG. 7. 4-week infection; ear, showing heavily parasitized giant cells, some of them multinucleate. H and E × 1300.

3, 4). During the second week the basal layer of the epidermis became acanthotic. There was parakeratosis; prickle cell cytoplasm became vacuolated and the nuclei became condensed. The granular cell layer was thickened and there was an increase of keratin (Fig. 5). Necrosis

first appeared within the prickle-cell layer: ulceration followed and the ulcer was covered with a slough of keratin, nuclear debris and degenerating parasites (Fig. 6). The macrophage infiltration was even more intense and most of these cells contained many amastigotes

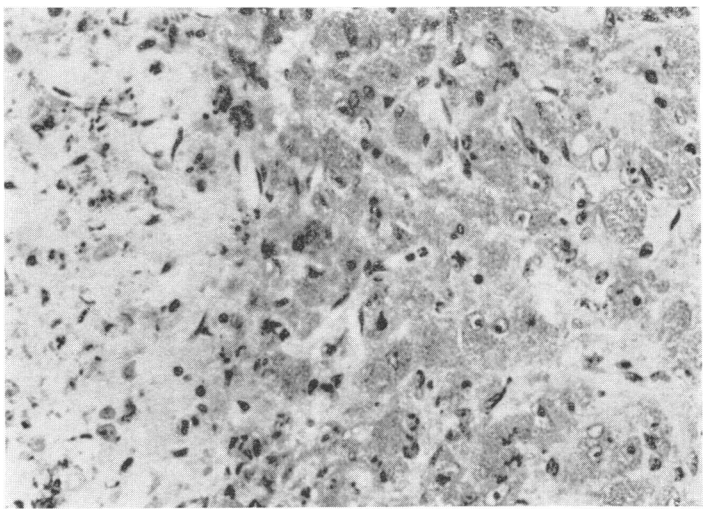

FIG. 8. 4-week infection; ear, showing infected macrophages immediately underlying the necrotic slough. H and E × 800.

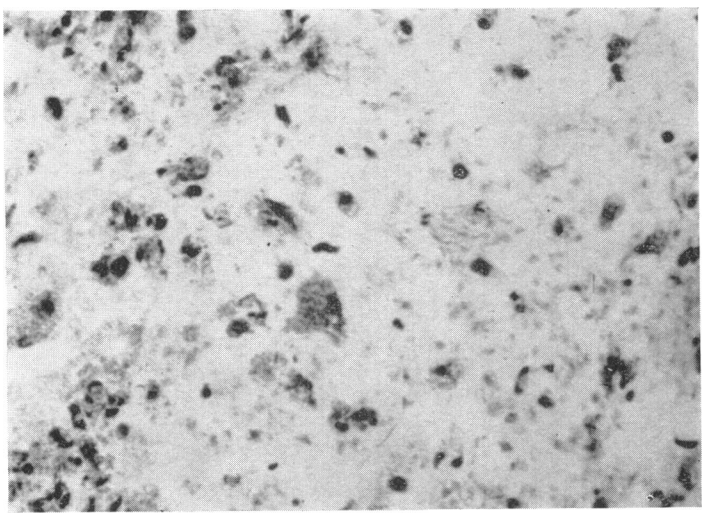

FIG. 9. Higher power view of Fig. 8 showing parasites and nuclear debris in slough. H and E × 1300.

(Fig. 7). Multinuclear, heavily parasitized giant cells were also seen. Occasional plasma cells were present. At the periphery of the lesion a few cells with rather less open nuclei and less bulky cytoplasm made their appearance. In the succeeding weeks, the ulcerated epidermis remained much the same. Underlying it directly was the parasitized mass of macrophages

(Figs. 8, 9) and around this lymphocytes more recognizably and more definitely built up (Fig. 10). Many of these lymphocytes were pyroninophilic. From about 4 weeks the lesion became demarked along the line of surrounding lymphocytes by a layer of loose tissue

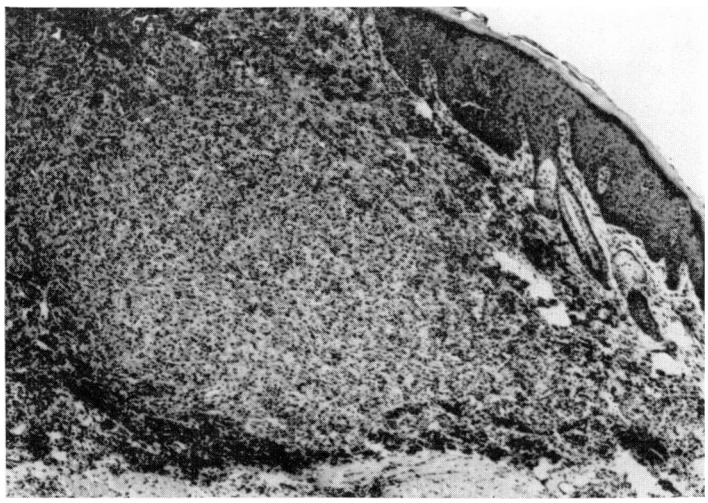

FIG. 10. 5-week infection; ear, showing zone of lymphocytic infiltration around the mass of infected macrophages. H and E ×130.

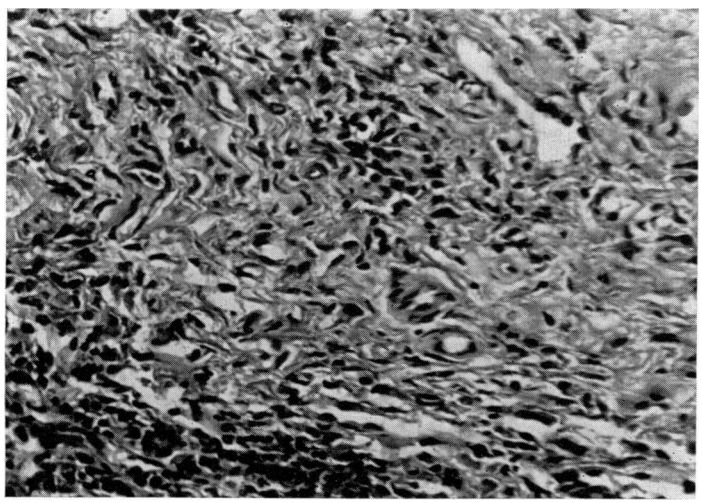

FIG. 11. 9-week infection; ear, showing healing lesion containing fibroblasts and lymphocytes without parasites. H and E ×640.

containing many small blood vessels. By 8 weeks fibroblasts could be seen at the periphery of the lesion. Slowly the number of infected macrophages decreased, the epidermis was restored and by 10 weeks only infiltration of the dermis with lymphocytes, fibroblasts and large mononuclear cells, together with some cell debris remained (Fig. 11).

Histology of draining lymph node

Changes in the draining lymph node started on the 3rd or 4th day of the infection. Germinal centres developed rapidly in the cortex and contained large pyroninophilic cells

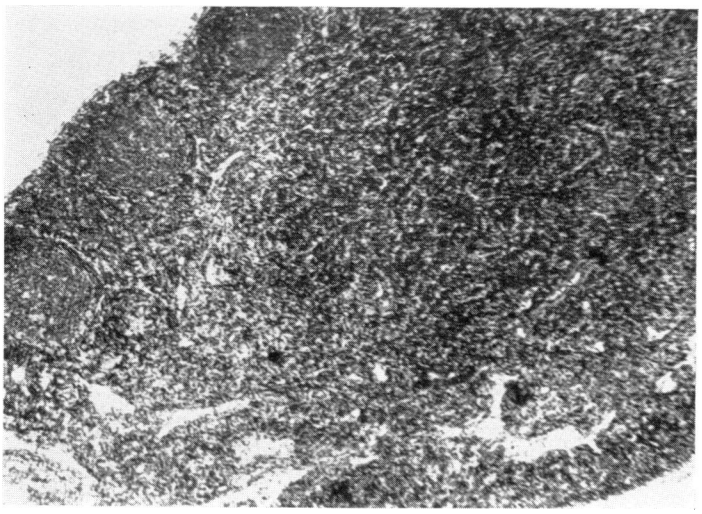

FIG. 12. 7-day infection; draining lymph node, showing development of paracortical area and of germinal centres in the cortex. H and E ×130.

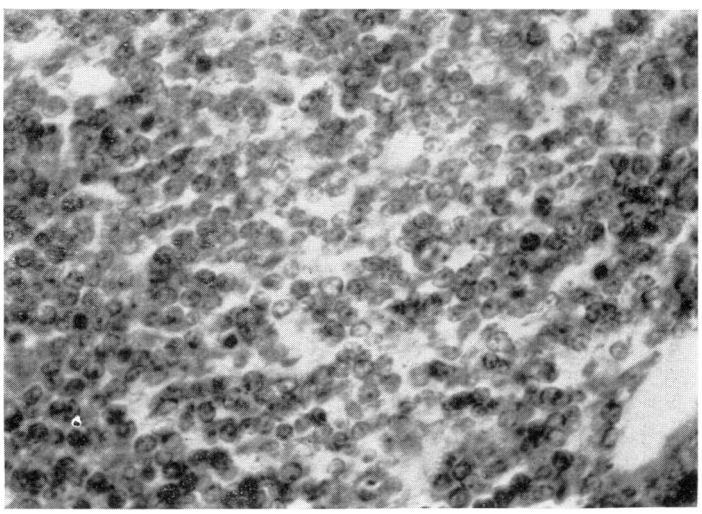

FIG. 13. High power view of Fig. 12; showing pyroninophilic cells in paracortical area. Methy green–pyronin ×1300.

undergoing mitosis. At the same time, the paracortical areas hypertrophied, with the appearance of many large pale pyroninophilic cells (Figs. 12, 13). These activities, which did not always proceed together at the same rate in individual animals, reached a peak

between the 4th and 6th week. The paracortical activity merged with that of the medulla where, by the 2nd week, many pyroninophilic cells were seen (Fig. 14); by the 4th week many

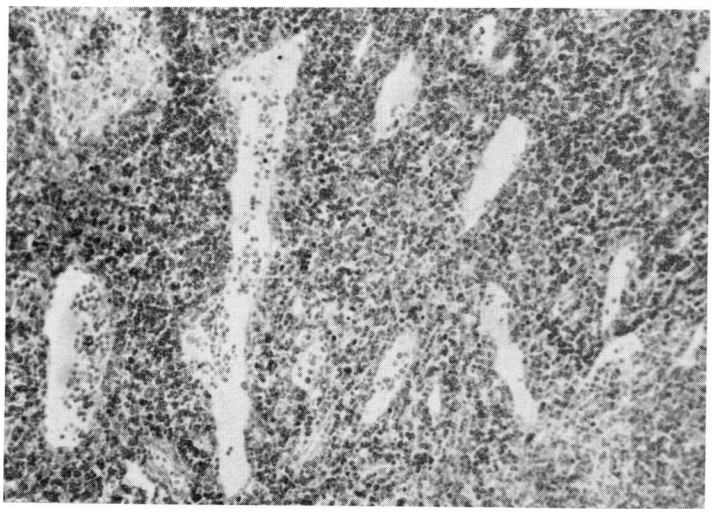

FIG. 14. 2-week infection; draining lymph node, showing pyroninophilic cells in medullary cords and scanty lymphocytes in efferent sinuses. Methyl green–pyronin × 320.

FIG. 15. 4-week infection. Draining lymph node, showing intense activity of germinal centres; paracortical areas divided by cords of pyroninophilic cells; and efferent lymphatics full of small round, non-pyroninophilic lymphocytes. Methyl green–pyronin × 130.

of these cells had assumed the appearance of plasma cells which could be shown by immunofluorescence to contain γ-globulin (Bryceson *et al.*, 1970). By 4–6 weeks the medulla was extremely active with masses of plasma cells and groups of non-pyroninophilic lymphocytes in the cords and large dilated efferent vessels full of small, non-pyroninophilic lymphocytes

(Fig. 15). Efferent lymphatics started to become dilated by the 2nd week and contained small lymphocytes. Macrophages containing amastigotes could be found in the subcapsular sinus from the 2nd day onwards (Fig. 16); and were most prominent about the 3rd–4th week when these channels were dilated. At this time, foci of pale-staining macrophages, several of them containing infected cells, were found between the germinal centres (Fig. 17). From the 4th–6th week onwards first the germinal centres and then the paracortical areas became less active and by 8 weeks the macrophage foci were no longer evident.

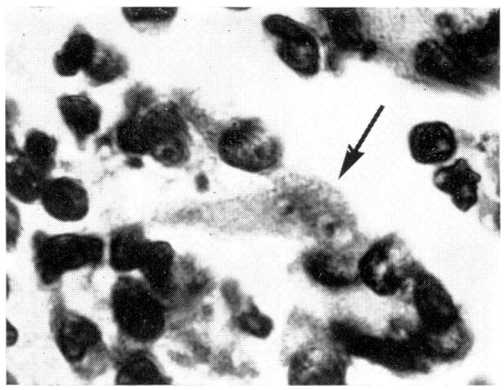

FIG. 16. 2-week infection; draining lymph node, showing two parasites in a macrophage lining the subcapsular sinus. H and E ×2600.

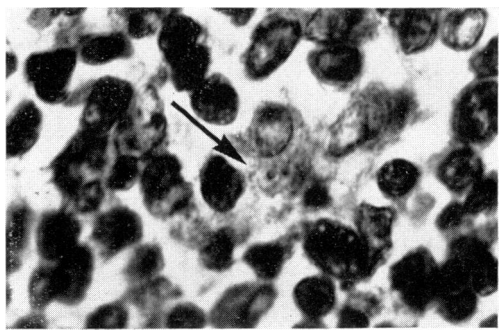

FIG. 17. 2-week infection; draining lymph node: focus (arrowed) of pale-staining macrophages containing parasites in paracortical area. H and E ×2600.

Histology of metastases

Metastatic lesions were often more deeply situated in the dermis than the primary lesion, which may be why they did not all ulcerate. Epidermal changes resembled that of the primary lesion but were less marked over the deeper lesions. The cellular infiltrate of the dermis usually paralleled that of the primary lesion. In the deeper lesions the infected macrophage mass was surrounded by lymphocytes and in the metastases that appeared latest the infiltrate consisted almost entirely of lymphocytes, plasma cells, fibroblasts and histiocytes in which parasites were scanty or invisible.

Resistance to reinfection

By the end of the first week there appeared to be some increased resistance as the challenge lesion healed within 4 weeks, i.e. before the primary. However, challenge lesions in 2, 3 and 4-week infected animals took 5 weeks to heal, indicating a fall off in acquired immunity until an 'isophasic' response appeared (Dostrovsky, Sagher & Zuckerman, 1953). Challenge lesions went through the same stages of nodule and ulcer. From the 5th week the challenge lesion never ulcerated and took 3, 2 and 1 weeks to heal in, respectively, 5, 6 and 7-week infected animals. Challenge in 8 and 9-week infected animals elicited a delayed hypersensitivity response only: infection was not established, even if the primary or metastatic lesions were still present and contained organisms.

Course of infection in artificially immunized animals

Eight pairs of guinea-pigs were immunized by each of the regimens shown on Table 3, which also shows that resistance to infection was not absolute in any group. Those receiving antigenic fractions with either adjuvant showed smaller, less destructive lesions and did not

TABLE 3. Artificial immunization of guinea-pigs against *L. enriettii* infection

Immunization procedure	Course of subsequent ear infections: Lesion at 6 weeks	Lesion at 8 weeks	Metastases
Saline alone	Large ulcer; cartilage eroded	Large ulcer	4 of 8 animals (solitary sites)
FCA alone	Large ulcer; cartilage eroded	Large ulcer	
2 mg CSA in saline	Large ulcer; cartilage eroded	Large ulcer	
2 mg CSA/2 mg CIA in saline	Large ulcer; cartilage eroded	Large ulcer	
2 mg CSA in FIA	Small ulcer	Healing	0 of 8 animals
2 mg CSA in FCA	Small ulcer	Healing	
2 mg CSA/2 mg CIA in FCA	Small ulcer	Healing	
2 mg CSA/2 mg CIA in FCA	Healed (4 weeks)	Healed	
'Paralysed' (3 × 10 mg PSA IV)	Large ulcer; cartilage eroded	Large ulcer 1 of 4 dead	4 of 6 animals (multiple sites)

Abbreviations: FCA: Freund's complete adjuvant; FIA: Freund's incomplete adjuvant; CSA: Crude soluble antigen; CIA: crude insoluble antigen; PSA: Purified soluble antigen; PIA: Purified insoluble antigen; IV: Intravenous.

develop metastases. Resistance to infection was most marked if both soluble and insoluble antigen fractions were given with FCA, in which case the infection healed within 4 weeks. Guinea-pigs immunized with antigens of *L. donovani*, *L. mexicana* and *L. braziliensis* in FCA were not protected against infection with *L. enriettii* although they had antibody titres to *L. enriettii* antigen comparable with those in animals immunized against *L. enriettii*. They also showed comparable delayed hypersensitivity reponses to all four antigens (Table 4).

Course of infection following immunological paralysis with soluble antigen

Animals 'paralysed' with 3 × 10 mg antigen were more susceptible to infection than normal. The lesion covered the ear within 2–3 weeks and there was severe ulceration and erosion of cartilage. By 4 weeks three of six had metastases. Four of these six animals were killed at 4 weeks for *in vitro* studies. Of the remaining two, one died at 6 weeks; it had metastases on the opposite ear, nose, scrotum and four feet. The surviving animal which also had multiple metastases, began to recover about the 8th week and was healed by 11 weeks. In two control animals simultaneously infected with the same batch and dose of amastigotes, the lesions

TABLE 4. Effects of immunization with heterologous leishmanial antigens on the resistance of guinea-pigs to infection with *L. enriettii*

Immunization procedure	HA titre*	Delayed hypersensitivity†	Course of subsequent ear infection: Lesion at 6 weeks	Lesion at 8 weeks
L. enriettii: 2 mg CSA/2 mg CIA in FIA	320	+	healed	healed; no metastases
L. donovani: 2 mg CSA/2 mg CIA in FIA	160	+	large ulcer; cartilage eroded	small ulcer; metastases
L. mexicana: 2 mg CSA/2 mg CIA in FIA	640	+	large ulcer; cartilage eroded	small ulcer; metastases
L. braziliensis: 2 mg CSA/2 mg CIA in FIA	640	+	large ulcer; cartilage eroded	small ulcer; metastases

* Haemagglutinating antibody (reciprocal titre) to *L. enriettii* antigen.
† To antigens of all four species (1:100 dilution of CSA).

were not as severe, although one developed metastases on the nose, scrotum and right hind foot. Both these animals healed simultaneously with the surviving 'paralysed' animal. The results, however, must be interpreted cautiously, as these animals were infected with 10^7 organisms (ten times the usual dose).

Histology of the lesions

Lesions were examined from four animals at 4 weeks. In two animals there were striking differences from the picture seen in control infected animals. In the dermis of the first animal there were huge giant cells characterized by a ring of histiocyte nuclei and cytoplasm crowded with parasites (Fig. 18). There was very little lymphocytic infiltration. There was also parasitized cells in the epidermis but other epidermal changes were slight; in particular, there was no parakeratosis, and ulceration was not preceded by necrosis of the prickle-cell layer (Fig. 19). In the other animal there were tubercles (Fig. 20), some with classical Langerhans giant cells and relatively scanty parasites (Fig. 21). This was the only animal in which we found a true tuberculoid picture.

Histology of regional lymph node

In three of the animals the draining lymph node, examined at 4 weeks, differed from that in control infected animals in several ways. In the cortex, germinal centres were large and

numerous and appeared to be full of holes; each of these holes contained one or two histiocytes which often contained parasites (Fig. 22). The paracortical areas were relatively small, with scanty lymphocytes but an increased degree of histiocytic infiltration. The

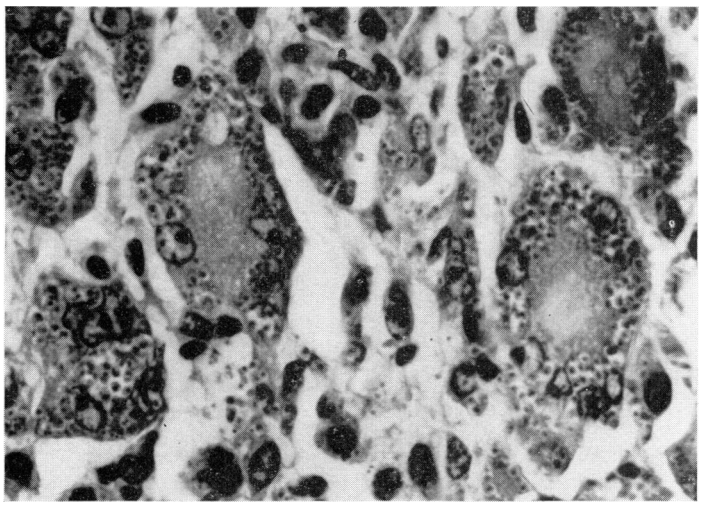

FIG. 18. 'Paralysed-Infected' animal 1; 4-week infection; ear, showing heavily parasitized multinucleate giant cells. H and E ×1300.

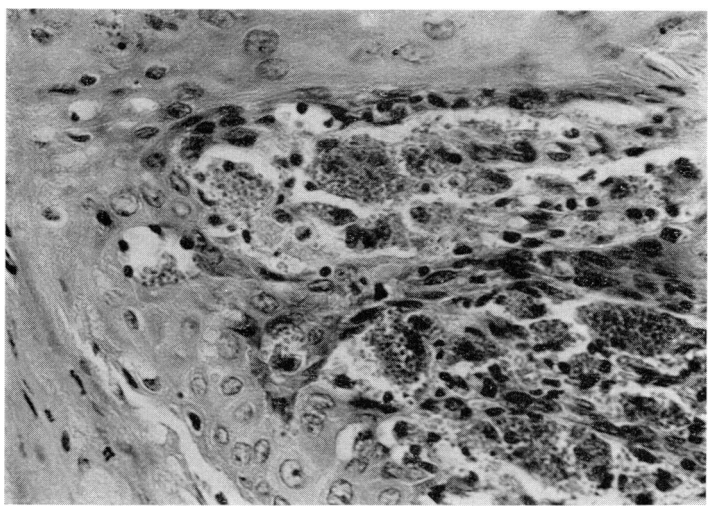

FIG. 19. Lower power view of Fig. 18; showing heavy parasitization extending into the epidermis. H and E ×640.

medulla contained many plasma cells in the cords, but few lymphocytes in the efferent vessels. Parasites were numerous in histiocytes throughout the node: in the subcapsular sinus, germinal centres, paracortical areas and efferent lymphatics.

In the fourth animal whose lesion showed a tuberculoid picture, the histology of the node at 4 weeks did not differ appreciably from that of the controls.

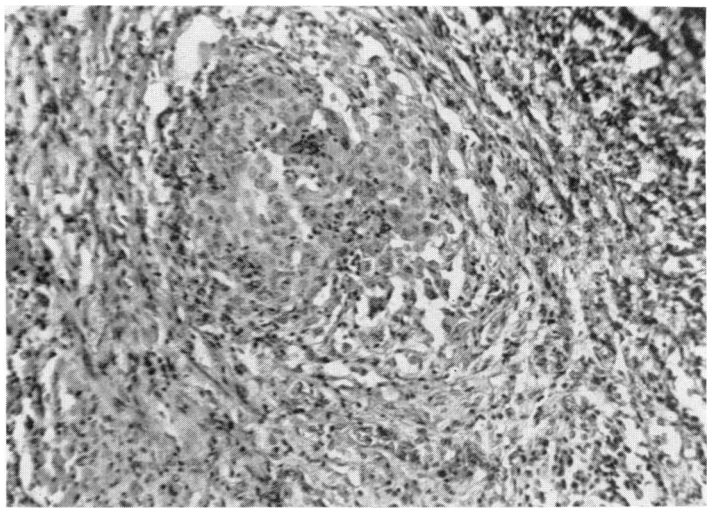

FIG. 20. 'Paralysed-infected' animal 4; 4-week infection; ear, showing epithelioid cells at centre of tubercle. H and E ×260.

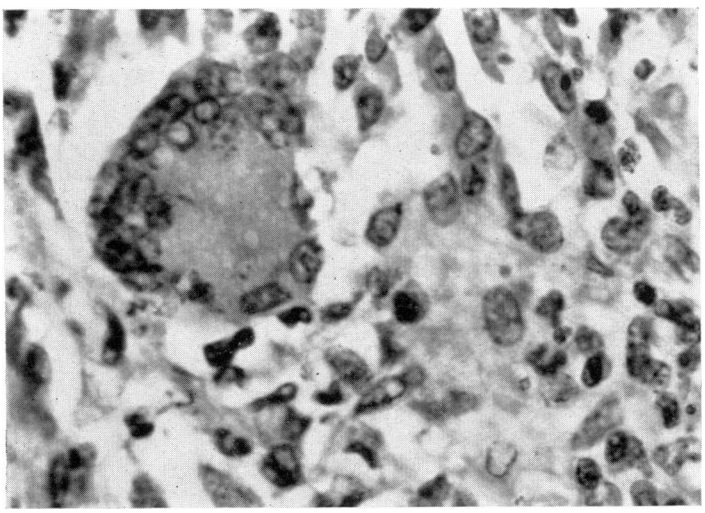

FIG. 21. Another field of Fig. 20; showing Langerhans giant cell containing granules and vacuoles but no parasites. H and E ×1300.

Antibody and hypersensitivity responses to immunization and infection

Design of experiments

Eighty-one guinea-pigs were each infected in one ear. At weekly intervals the state of the lesion was noted and nine animals were each skin tested with 100 μg, 10 μg and 1 μg of PSA.

After 15 min, 7½ hr and 48 hr the volume of the skin test was measured and two animals were, at each time, injected with Evans' blue and examined. The third animal, at each time, did not receive Evans' blue: it was bled out, and the serum stored at −20°C for antibody assay. Eight pairs of guinea-pigs were immunized by each of the regimens given in Table 3. Three weeks after immunization serum as assayed by HA and PCA, and intradermal skin tests were performed with CSA at concentrations of 1/100, 1/1000 and 1/10,000. Forty-two guinea-pigs were immunized with soluble and insoluble antigens of *L. enriettii* in FIA. One week and 6 weeks later, eighteen and twenty-four animals respectively were similarly skin tested with CSA. At 0–15 min, 3, 7½, 12, 24 and 48 hr later three animals each received

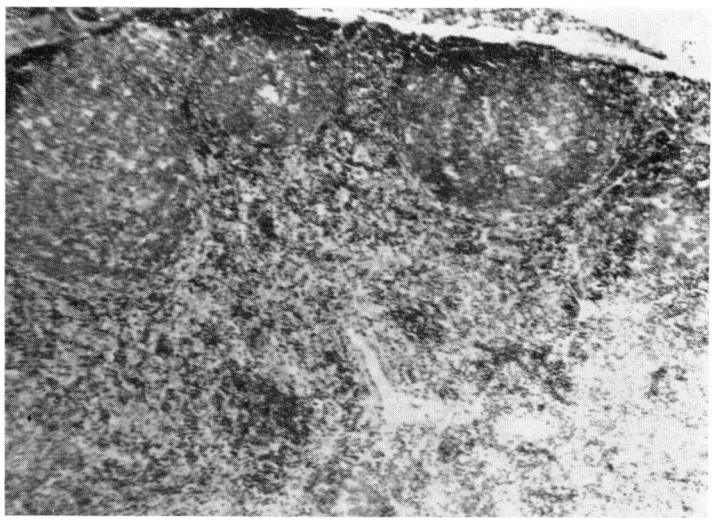

FIG. 22. 'Paralysed-infected' animal 3; 4-week infection; draining lymph node, showing infiltration of germinal centres and paracortical area with pale-staining histiocytes. H and E ×130.

Evans' blue intravenously and were examined. The 6-week animals were in addition tested with CSA at 1/10 dilution; and their skin tests were also examined at 5 hr.

Development of circulating antibody

Circulating antibodies could not be detected, either by passive cutaneous anaphylaxis or by indirect haemagglutination, following simple auricular infection with 10^6 organisms, at any time during the process of lesion formation, ulceration or healing. This was in contrast to the response to various immunization procedures, which resulted in the production of skin-sensitizing and haemagglutinating antibodies, provided that immunizing antigens were injected as emulsions with complete or incomplete Freund's adjuvant (Table 5). 2 mg PSA given intravenously 1 week before immunization did not prevent the development of antibodies (see also Table 5).

In additional animals immunized with a mixture of soluble and insoluble antigens emulsified in Freund's incomplete adjuvant, affinity of circulating antibody for homologous skin increased during the first 5 weeks after immunization; agglutinating antibodies also reached

peak titres at about 5 weeks (Table 6). Antibodies cytophilic for homologous macrophages could not be demonstrated at any time following infection, or following immunization with CSA and CIA in FIA.

TABLE 5. Antibody response (passive cutaneous anaphylaxis and indirect haemagglutination) of guinea-pigs 3 weeks after immunization or infection with *L. enriettii*

Infection or immunization	Haemagglutination titre (reciprocal)	Passive cutaneous anaphylaxis (6 hr skin fixation)
Saline	< 5	negative
Infection (1–9 weeks)	< 5	negative
FCA alone	< 5	negative
2 mg CSA in saline	< 5	negative
2 mg CSA/2 mg CIA in saline	< 5	negative
2 mg CSA in FIA	5	positive
2 mg CSA in FCA	5	positive
2 mg CSA/2 mg CIA in FIA	320	positive
2 mg CSA/2 mg CIA in FCA	320	positive
Paralysed-immunized*	1280	positive

* IV injection of 2 mg PSA; 2 weeks later 2 mg CSA and 2 mg CIA in FCA injected ID. For abbreviations see Table 3.

TABLE 6. Antibody response (passive cutaneous anaphylaxis and indirect haemagglutination) of guinea-pigs at intervals after immunization with *L. enriettii*

Weeks after immunization*	PCA: skin sensitization time†					Haemagglutinating antibody (reciprocal titre)
	6 hr	12 hr	24 hr	48 hr	72 hr	
Control	−	−	−	−	−	< 5
1 week	−	−	−	−	−	20
2 weeks	++	+	−	−	−	320
3 weeks	++	+	−	−	−	1280
4 weeks	+++	++	+	+	+	5120
5 weeks	+++	+++	+++	++	+	5120

* Animals immunized with 2 mg CSA/2 mg CIA in FIA (see Table 3).
† Interval between ID injection of serum and IV injection of antigen with dye.

Development of immediate and delayed hypersensitivity

In animals infected with 10^6 organisms, anaphylactic hypersensitivity (15 min skin responses) eventually developed at the stage of healing. Arthus sensitivity (3–4 hr skin responses) was never visible in infected or convalescent animals, although delayed hypersensitivity (24–48 hr skin responses) could be detected from the end of the first infection

week onwards. The extent of delayed hypersensitivity increased during the first 4 weeks of infection and persisted throughout healing, convalescence or re-infection. The onset of the stronger 24 hr skin reactions was detectable by an increased skin test volume at 7½ hr or even 4 hr after skin testing (Fig. 23). However, there was no increased vascular permeability to Evans' blue at any time during the development of delayed hypersensitivity reactions in infected animals.

Without the aid of Freund's adjuvants, soluble and insoluble *L. enriettii* antigens were only weakly immunogenic; for immediate hypersensitivity could not be induced, and delayed

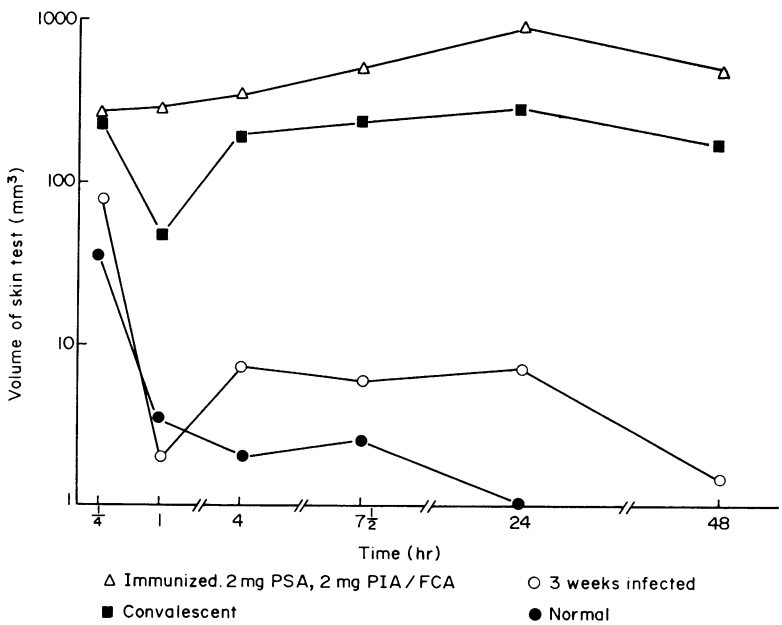

FIG. 23. Time course of mean responses to intradermal injection of 100 μg PSA in pairs of normal, infected, convalescent and immunized guinea-pigs. (See Table 3 for abbreviations.)

hypersensitivity was seen only in occasional animals (Group 3, Table 10). A mixture of soluble and insoluble antigens emulsified in Freund's incomplete adjuvant was capable of inducing both anaphylactic (15 min) and delayed (24 hr) hypersensitivity responses (Group 4, Table 10). Arthus-type sensitivity was noticeably poor: dye leakage could not be demonstrated at 3 hr and only with difficulty at 5 hr (Table 7). Antigens emulsified in Freund's complete adjuvant were strongly immunogenic, and induced anaphylactic, Arthus and delayed hypersensitivities. Arthus sensitivity did not appear until after the first week following immunization. Delayed hypersensitivity reactions in animals immunized with antigens in either adjuvant were always accompanied by increased vascular permeability to Evans' blue in skin reactions of 7½ hr or more duration (Table 7). The degree of increased vascular permeability in skin reactions of more than 1 hr duration was closely dependent on the skin test dose of soluble antigen (PSA or CSA).

A close relationship between reaction volume and skin test dose of antigen was particularly evident in delayed hypersensitivity (Fig. 24). Animals immunized with antigens in FCA

TABLE 7. Time course of vascular permeability of skin reactions* to *L. enriettii* antigen in guinea-pigs immunized with soluble and insoluble antigens in FIA

Dose of antigen used for skin testing	Guinea-pigs immunized 1 week previously						Guinea-pigs immunized 6 weeks previously						
	Time after intradermal injection of antigen at which skin test was examined:												
	0–15 min†	3 hr	7½ hr	12 hr	24 hr	48 hr	0–15 min†	3 hr	5 hr	7½ hr	12 hr	24 hr	48 hr
None (saline)	–	–	–	–	–	–	–	–	–	–	–	–	–
CSA 1/10	nd	nd	nd	nd	nd	nd	+++	–	++	++	+++	+++	+++
CSA 1/100	++	–	++	(±)	(±)	(±)	+++	–	–	++	++	++	++
CSA 1/1000	++	–	++	(±)	–	–	+++	–	–	–	++	++	++
CSA 1/10,000	+	–	+	–	–	–	+++	–	–	–	(±)	+	+

* Skin test site examined after intravenous injection of Evans' blue (see Methods).

† At this time unimmunized animals also gave a small immediate vascular leakage following ID injection of 1/10 or 1/100 CSA.

(±) Some animals positive, some negative. (CSA: crude soluble antigen; nd: not done).

TABLE 8. *In vitro* effect of sensitized lymphocytes on survival of *L. enriettii* in normal guinea-pig macrophages

Experimental series No.	Pretreatment of lymphocyte donor	Lymphocyte concentration	No. amastigotes (mean*) per 100 macrophages; hours after addition of lymphocytes: 2 hr	24 hr	48 hr	72 hr	Survival of amastigotes at 24 hr (%)
I	—	none	145	251	267	180	
II	—	none		818			100
	FIA only	5×10^7/ml	240	220	196		27
	Convalescent	5×10^7/ml	128	5	0		1
	FIA only	$2{\cdot}5 \times 10^7$/ml	238	400			45
	Convalescent	$2{\cdot}5 \times 10^7$/ml	153	20			2
III	—	none		574	543		100
	FIA only	5×10^7/ml		95	211		17
	FCA only	5×10^7/ml		5	0		1
	Convalescent	5×10^7/ml		10	0		2
IV	FIA only	$2{\cdot}5 \times 10^7$/ml		73			
	FCA only	$2{\cdot}5 \times 10^7$/ml		1			
	2 mg PSA/2 mg PIA in saline	$2{\cdot}5 \times 10^7$/ml		0			
	2 mg PSA/2 mg PIA in FIA	$2{\cdot}5 \times 10^7$/ml		0			
	2 mg PSA/2 mg PIA in FCA	$2{\cdot}5 \times 10^7$/ml		0			
	4-week infected	$2{\cdot}5 \times 10^7$/ml		0			

See Table 3 for abbreviations. * Means of 2–4 cultures.

TABLE 9. Cytotoxic effects of lymphocytes from convalescent guinea-pigs on macrophages coated with *L. enriettii* antigen

Macrophage monolayer	Pretreatment of lymphocyte donor	Lymphocyte concentration	No. macrophages (mean*) per 10 high power fields; hours after addition of lymphocytes: 12 hr	24 hr	Survival of macrophages at 24 hr (%)
Untreated	—	none		460	100
	FIA only	10^8/ml	172	216	47
	Convalescent	10^8/ml	192	168	37
Soaked in PSA (100 μg/ml) for 2 hr	FIA	10^8/ml	172	243	35
	Convalescent	10^8/ml	92	27	6

See Table 3 for abbreviations. * Means of duplicate cultures.

TABLE 10. Summary of immunological features following infection and immunization of the guinea-pig with *L. enriettii*

Animal group* and No.	Cutaneous hypersensitivity; time of reading skin tests:			Antibodies		Cell transfer of DH	Cellular reactions *in vitro*					Resistance to (re) infection
	15 min	3–4 hr	24 hr	PCA	HA		Phagocytic activity of macrophages	Macrophage migration inhibition	Lymphocyte transformation	Cell-free (lymphokine) factors	Target cell destr.	
(1) Infected (4 week)	−	−	+	−	< 5	+	normal	+	+	+	+	isophasic
(2) FCA alone	−	−	(±)	−	< 5	−	normal	−	−	−	+	absent
(3) Sol. + insol Ag	−	−	(±)	−	< 5	nd	normal	nd	nd	nd	+	absent
(4) Sol. + insol Ag/FIA	+	−	+	+	5	+	normal	+	nd	nd	+	slight
(5) Sol. Ag/FCA	+	+	+	+	5	nd	normal	nd	nd	nd	nd	slight
(6) Sol. + insol Ag/FCA	+	+	+	+	320	+	normal	+	+	+	+	marked
(7) Convalescent (8–10 weeks)	+	−	+	−	< 5	+	increased	+	+	+	+	complete
(8) 'Paralysed' immunized	+	+	+	nd	1280	+	nd	nd	(±)	nd	nd	nd
(9) 'Paralysed' infected	−	−	(±)	nd	40	nd	normal	reduced	−	nd	nd	reduced

Abbreviations: PCA: passive cutaneous anaphylaxis (6 hr skin fixation); Ha: reciprocal haemagglutination titre; DH: delayed hypersensitivity; Ag: antigen.

* Groups 1–6, 8 and 9 examined 3–4 weeks after infection and immunization.

Scoring: −, negative; +, positive; (±), marginal or variable; nd, not done.

generally gave larger 24-hr skin reactions than 4-week infected animals. Challenge with live organisms of convalescent or immunized animals increased their 24-hr skin reactions.

Passive transfer of delayed hypersensitivity to *L. enriettii* antigen was accomplished by lymphoid cells from animals infected and immunized 4 weeks previously (Fig. 25) but not 6 days previously. Transfer with 5×10^8 lymphoid cells was usually more successful than with 1×10^8 cells; and peritoneal exudates from sensitized animals were more effective than similar numbers of viable cells from spleen or lymph node. Fig. 25 also shows that peritoneal exudate cells transferred tuberculin sensitivity when they were derived from animals immunized with the aid of complete Freund's adjuvant. However, the small degree of delayed

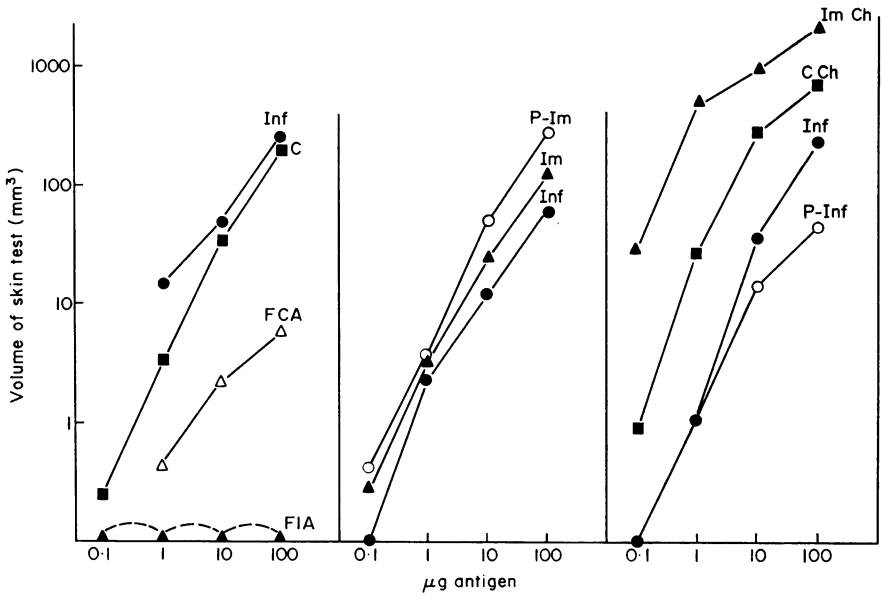

FIG. 24. Mean 24 hr skin responses to increasing doses of PSA in various groups of guinea-pigs. Each panel contains groups tested at the same time with the same batch of antigen. (Inf: 4-week infected; C: convalescent; FCA: injected with FCA alone; P-Im: paralysed with 2 mg intravenous PSA 2 weeks before intradermal immunization with 2 mg PSA/2 mg PIA in FCA; Im: immunized with 2 mg PSA/2 mg PIA in FCA; Im Ch: immunized and challenged with live parasites; C.Ch: convalescent and challenged with live parasites; P-Inf: 'Paralysed' with 3×10 mg intravenous PSA before infection. (See Table 3 for other abbreviations.)

hypersensitivity to PSA, exhibited by animals immunized with FCA alone (Fig. 24) was not transferable by 5×10^8 viable peritoneal exudate cells (Fig. 25).

Evidence that immune paralysis could suppress skin reactions of hypersensitivity was obtained with the group of six animals infected with 10^7 organisms 1 week after three weekly intravenous injections of soluble leishmanial antigen. Four weeks after infection, these animals had smaller delayed hypersensitivity reactions than did control infected animals (Fig. 24). In two animals tested at 6 weeks, delayed hypersensitivity could not be elicited. In the animal that recovered the delayed response became as strong as in the controls. A single intravenous injection of 2 mg PSA 3 weeks before immunization with antigen in

Freund's complete adjuvant did not reduce the delayed hypersensitivity response (Fig. 24) or the ability of peritoneal exudate cells to transfer delayed hypersensitivity (Fig. 25).

In vitro reactions of cellular hypersensitivity and cellular immunity

Inhibition of macrophage migration by soluble antigen

Peritoneal exudate macrophages from pairs of uninfected control guinea-pigs, injected with FCA alone, migrated normally in the presence of PSA in concentrations of 10–250 μg/ml (Fig. 26). The migration of macrophages from pairs of infected and immunized animals

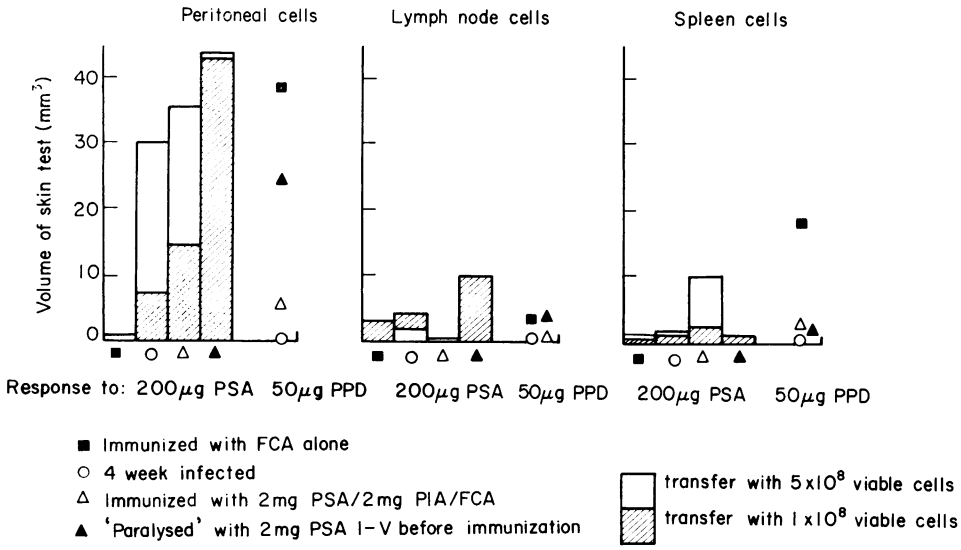

FIG. 25. Passive transfer of delayed hypersensitivity to normal guinea-pigs with lymphoid cells taken from groups of infected, immunized and 'paralysed–immunized' guinea-pigs. Recipients tested with 200 μg PSA immediately and read after 24 hr. (See Table 3 for abbreviations.)

was markedly inhibited by antigen (Fig. 26: groups 'Inf' and 'Im'). Two groups of 'paralysed-infected' animals were tested. In one of these (P-Inf 2), which had an extra 5 mg PSA intravenously 1 week after infection with 10^6 organisms, macrophage migration was not inhibited by concentrations of antigen which were active on a control infected group (Fig. 26) indicating that suppression of this test of cellular hypersensitivity could be achieved by a prolonged paralysing regime. In the other group (P-Inf: 3 × 10 mg PSA intravenously before infection with 10^7 organisms), migration-inhibition was not affected; this is the group whose altered clinical course, histology and skin reactions have been described above.

The sensitivity of the migration-inhibition test was determined by mixing various proportions of exudate cells from experimental animals with allogeneic exudate cells from unsensitized animals (David, Lawrence & Thomas, 1964b), in the presence of 250 μg/ml of PSA. Only 10% and 6% of cells, from infected and immunized animals respectively, were required to produce inhibition of normal guinea-pig macrophage migration under these conditions (Fig. 27). This method of cell dilution generally provides a more sensitive index

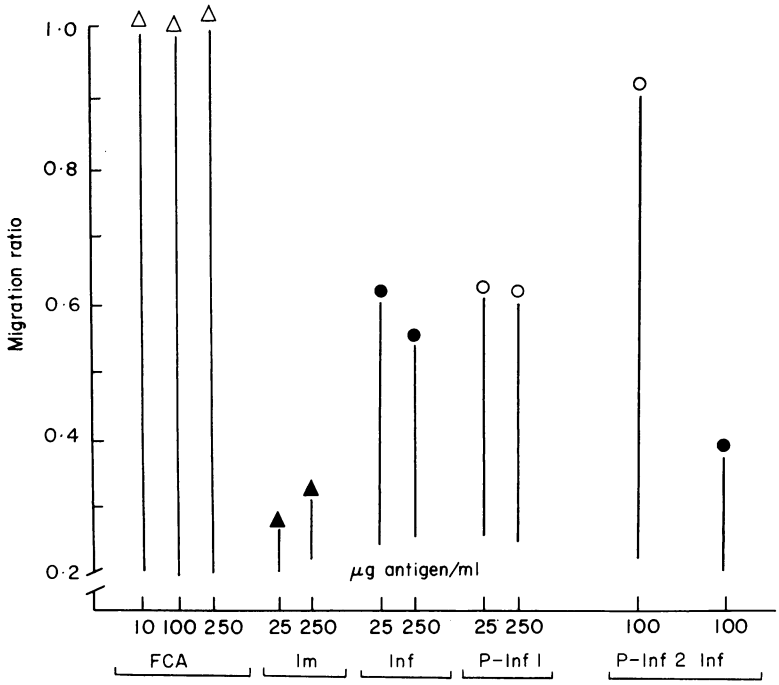

FIG. 26. Inhibition of migration of peritoneal macrophages from various groups of guinea-pigs in the presence of leishmanial antigen (PSA). Result expressed as a ratio of cell migration in the absence of antigen. (See Fig. 24 for abbreviations: 'P-Inf 2' received an extra dose of 5 mg PSA intravenously 1 week after infection.)

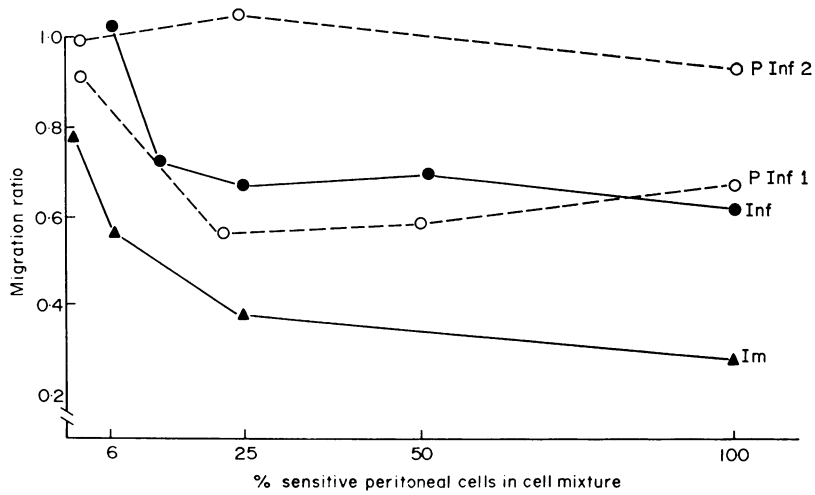

FIG. 27. Inhibition of migration of peritoneal macrophages in the presence of leishmanial antigen (100 μg/ml PSA). Cells of pretreated guinea-pigs were mixed in dilutions with cells of normal animals. Result expressed as a ratio of cell migration in the absence of antigen. (See Figs. 24 and 26 for abbreviations.)

of cellular hypersensitivity than is derived from antigen dilution experiments. Application of this technique to the paralysed-infected animals revealed that those in group 'P-Inf 1' displayed a similar degree of cellular hypersensitivity as did the control infected animals in contrast to the animals in group 'P-Inf 2' (Fig. 27).

Lymphocyte transformation

Lymphocytes from pairs or trios of infected (Fig. 28) and immunized (Fig. 29) animals were vigorously transformed by soluble antigen (PSA). Transformation was detected at

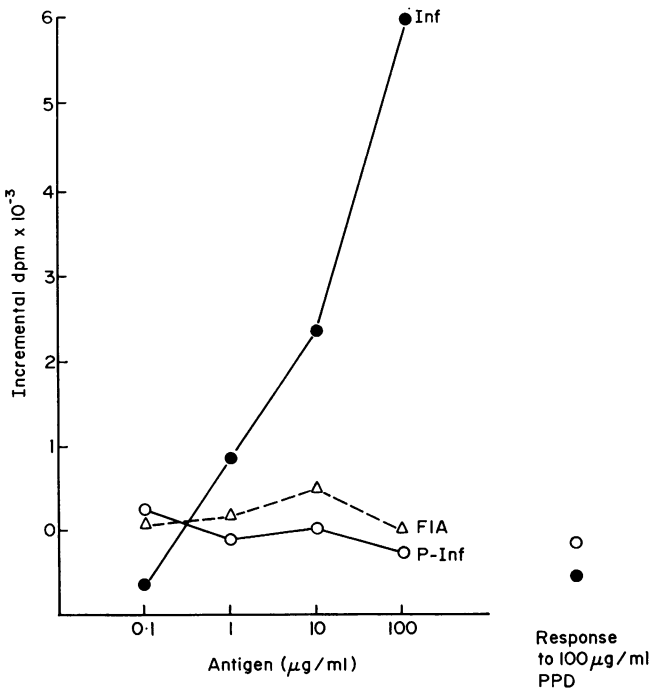

FIG. 28. Uptake of ^{3}H-thymidine in the presence of leishmanial antigen (PSA) and tuberculin (PPD) by lymph node cells from infected, paralysed-infected, and FIA injected guinea-pigs. (See Fig. 24 for abbreviations.)

1 μg/ml PSA and increased up to 100 μg/ml of antigen (the maximum dose used). Lymphocytes from infected animals were not transformed by PPD (Fig. 28). Lymphocytes from paralysed infected animals (P-Inf 1) failed to transform at any of the concentrations of PSA used (Fig. 28). Lymphocytes from the paralysed immunized group were unresponsive to low concentrations of PSA (to 10 μg/ml) and responded only to 100 μg/ml (Fig. 29). Lymphocytes from animals injected with FCA responded to PPD (Fig. 29).

Lymphokine production

Lymph node cells from three pairs of animals were tested for their ability to generate migration inhibition factor (MIF) and mitogenic factor (MF) when cultured with 100 μg/ml PSA. Both factors were generated by cells from one each of the pairs of infected and immun-

ized animals respectively (MIF activity: 30% and 35% inhibition of cell migration; MF activity: +1500 and +2200 dpm/culture). Cells from animals immunized with FCA alone failed to generate either factor. The results showed that cell-free mediators of cellular hypersensitivity could be generated by stimulation of sensitized lymphocytes with *L. enriettii* antigen.

Presence of macrophage-bound antibody

The results of adherence tests done at temperatures below 36°C showed no difference between the rosette-forming ability of macrophages from infected or convalescent animals or from animals immunized with antigens in FIA, when compared with macrophages from

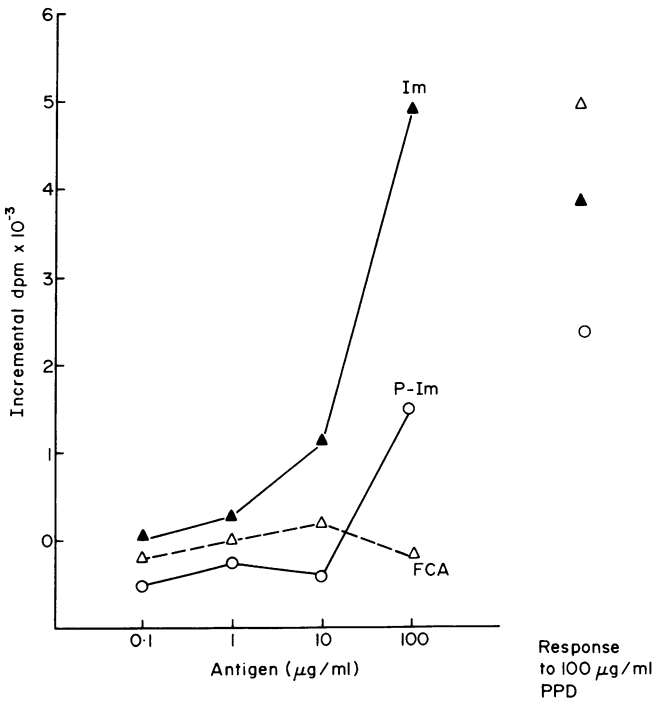

FIG. 29. Uptake of ^{3}H-thymidine in the presence of leishmanial antigen (PSA) and tuberculin (PPD) by lymph node cells from immunized, paralysed-immunized and FCA injected guinea-pigs. (See Fig. 24 for abbreviations.)

normal animals or from those injected with FIA alone. When exposed to antigen-coated erythrocytes at 4°C or 22°C, all monolayers showed only occasional adherence of erythrocytes to isolated macrophages; furthermore, no cellular adherence of *L. enriettii* amastigotes or promastigotes was observed under these conditions. It was concluded that cell-bound antibody was absent from the surface of peritoneal macrophages from these experimental animals.

Phagocytic and parasiticidal activities of macrophages

During the 2 hr allowed for phagocytosis of organisms at 36°C, macrophages from convalescent animals took up 1·8–5·7 (mean: 2·7) times as many amastigotes of *L. enriettii*

as did macrophages from normal or FIA-treated guinea-pigs. Both the number of infected macrophages and the number of organisms per macrophage were increased in 'convalescent' monolayers; however, the phagocytosis of amastigotes of *L. braziliensis* and *L. donovani*, and of formolized sheep erythrocytes, was not increased. The phagocytic activity of macrophages from 4-week infected animals or from artificially immunized animals was not increased above normal. Parasites in macrophage monolayers grew well over 72 hr, reaching maximum numbers between 24 and 48 hr; amastigotes taken up by convalescent macrophages appeared normal, and grew as well, over 48 hr, as did those taken up by macrophages from normal animals. It was concluded that whereas 'convalescent' macrophages had greater phagocytic activity for the homologous infecting organism, there was no evidence that this was accompanied by an increased ability to suppress the subsequent growth of such ingested organisms. This is contrary to the experience of Miller & Twohy (1969) who found that macrophages from infected mice showed some ability to inhibit the growth of *L. donovani* amastigotes.

Action of sensitized lymphocytes on infected macrophages

Lymphocytes from animals immunized with FIA alone had an equal effect on infected and non-infected macrophages, reducing their numbers to about 60% after 24 hr incubation; and reducing the number of parasites to 17–45% in different experiments (Table 8). Lymphocytes from convalescent animals reduced the number of macrophages to about 40% but the parasitized macrophages were selectively destroyed and only 1–2% of parasites survived in the monolayer at 24 hr. Parasite destruction was, however, complete by 48 hr (Series II and III, Table 8). Destruction of infected macrophages by convalescent lymphocytes began after only 2 hr. These lymphocytoxic effects were observed when $2{\cdot}5 \times 10^7$ or 10^8 lymphocytes/ml were used, but the effect with 10^7 lymphocytes was only slight. The supernatant fluids from six such 24-hr cultures that had been treated with $2{\cdot}5 \times 10^7$ or 5×10^7 cells were sterile upon inoculation onto NNN slopes; two taken from cultures treated with 10^7 cells were infective. However, fresh monolayers of infected macrophages were not destroyed by supernatant fluids from these cultures, or from supernatants prepared as for the production of lymphokine factors (see above, Methods).

Lymphocytes from guinea-pigs immunized with FCA alone (Series III and IV, Table 8) were, however, just as effective at destroying the parasitized macrophages as were those from convalescent animals. Likewise, lymph node cells from animals immunized with mixed *L. enriettii* antigens (emulsified with FIA, FCA, or suspended in saline without adjuvant) also showed cytopathic activity on infected macrophages (Series IV, Table 8). When uninfected macrophages pre-treated with soluble antigen were used as target cells they were destroyed by convalescent lymphocyte although not quite as completely as were parasitized macrophages (Table 9). Although the immunological specificity of this phenomenon was not demonstrated, comparison with the effects of unsensitized (FIA) lymphocytes revealed a six-fold diminution of the survival of macrophages after 24 hr.

DISCUSSION

Cutaneous leishmaniasis of the guinea-pig presents as a nodule at the site of inoculation, which ulcerates and then heals in 2–3 months. During the course of infection delayed

hypersensitivity is detectable, but at no time are haemagglutinating antibodies found. In animals immunized artificially antibody production accompanies delayed hypersensitivity. Resistance to reinfection develops early and is present before the primary lesion has healed and at a time when metastases from the primary infection are liable to appear. The purpose of this study has been to examine the features of the immunological response in order to assess the relative contribution of humoral and cellular mechanisms in the development of resistance to the infection (see Table 10).

Immunological response to infection and immunization

Dominance of the cell-mediated immunological response to intracutaneous infection was clearly shown by the early development and persistence of delayed hypersensitivity, the lack of haemagglutinating antibody, and the general absence of immediate hypersensitivity during the active phase of the infection. The response to artificial immunization (Table 10) revealed that whereas soluble extracts of killed organisms were capable of eliciting immediate and delayed hypersensitivities, they were only immunogenic for the guinea-pig when incorporated into Freund's adjuvants. The interesting association of anaphylactic and delayed cutaneous hypersensitivities, which followed immunization with a mixture of crude soluble and insoluble extracts of *L. enriettii* emulsified in FIA, was to some extent paralleled by the development of active cutaneous anaphylaxis at the stage of healing. Absence of Arthus reactivity was a noticeable feature of both the response to infection and to immunization with crude antigens in FIA. Despite these various similarities in the immunological response, animals immunized with crude antigens in FIA were far less resistant to challenge infection than were animals recovering from a primary infection.

Further examination of the delayed hypersensitivity responses in these two groups of animals revealed that strong 24-hr skin reactions were usually accompanied by indurated skin reactions visible at $7\frac{1}{2}$ hr. In the FIA-immunized group these skin reactions showed increased vascular permeability to Evans' blue; and in the early response to immunization the presence of such $7\frac{1}{2}$ hr skin reactions antedated the ability of animals to mount 24-hr skin responses (Table 7). On the other hand, in the early response to infection, 24-hr skin reactions could be elicited at a time (1 week) when $7\frac{1}{2}$ hr reactions were undetectable. Furthermore, increased vascular permeability to Evans' blue was never a feature of delayed hypersensitivity accompanying infection. Such early (6–9 hr) responses in immunized animals are thought to involve only part of the cutaneous vascular bed (Willms-Kretchmer, Flax & Cotran, 1967), which can be shown to develop increased permeability to intravascular protein as well as to haematogenous cells at this time (Morley *et al.*, 1970). Differences between the microvascular reactions of infected and adjuvant-immunized animals may be due to differences in the composition of inflammatory cell populations leaving the intravascular compartment in response to intracutaneous antigen. This alone may be an important factor in determining the fate of an intracutaneous challenge infection, and studies are in progress to determine whether there is a histological basis for this possibility.

The occurrence of haemagglutinating antibody did not run parallel with resistance to infection (Table 10), although there was a marked germinal centre response of lymph nodes regional to primary infections (see also Vasina, Demina & Glazunova, 1965). There was no evidence that antibody interfered with the development of resistance, for animals immunized with crude antigens in FCA developed considerable resistance to infection

(Table 10). We have since found that if 4 mg of urea-extracted antigen protein is given in FCA the recipient guinea-pig is completely resistant to challenge 4 weeks later; such an animal has a haemagglutinating antibody titre of 1/4056, and displays cutaneous anaphylaxis Arthus and delayed hypersensitivities when skin tested with the immunizing antigen. The rise in skin affinity of PCA antibody developing during FIA-immunization (Table 7) ran parallel with development of partial resistance in these animals; but the possible role of such antibody in combating established infection is far from clear. In this context, the development of active cutaneous anaphylaxis at the stage of healing might be expected to alter the microvascular response to challenge infection; and indeed, all the evidence suggests that immunity accompanying convalescence is sterile. If anaphylactic hypersensitivity is important in contributing to the rejection of *L. enriettii*, it might be anticipated that antibodies of the highest affinity for homologous skin would be the most effective. Anaphylactic antibodies are involved in the response of rodents to some helminthic infections (Ogilvie & Jones, 1969) even though cellular mechanisms are important in others (Dineen & Wagland, 1966). Serum from convalescent or immunized guinea-pigs provided no protection against *L. enriettii* infection when injected into normal recipients (unpublished observations) although immediate hypersensitivity could be transferred by this means. It is not known whether humoral and cellular immune responses of the guinea-pig are directed towards the same leishmanial antigens.

Development of the delayed hypersensitivity response to infection followed the appearance of paracortical activity in the regional lymph node and accompanied epidermal changes (necrosis of the prickle-cell layer and hyperkeratosis) which are reminiscent of the inflammatory response to skin homografts (Medawar, 1944). The ability of lymphoid cells to transfer delayed hypersensitivity also ran parallel with these events. Lymphocytic palisading and infiltration of the lesion became striking at about 4 weeks, when also the efferent lymphatics in the node contained the greatest number of lymphocytes. From then onwards the number of infected macrophages in the peripheral lesions declined, and the histological studies suggested that infected macrophages were being rejected through the ulcerated epidermis.

Some suppression of the resistance of normal animals to *L. enriettii* infection was achieved by the repeated intravenous administration of larger doses of soluble antigen. This suppression of resistance was shown by the rapid growth of florid lesions and by the extent of metastasis following subsequent infection. Two animals became moribund at the height of infection, and one died; but death was not due to visceralization, because organisms could not be recovered from the liver and spleen, which were of normal size. Recovery of the other animal was not delayed, and so its resistance was only temporarily suppressed. Study of the immunological response revealed that the paralysis was best indicated by suppression of lymphocyte transformation, less well by inhibition of macrophage migration and only poorly by skin testing. This was especially noticeable in the animals that were immunized after 'paralysis', and which showed unusually strong skin tests but significantly suppressed lymphocyte transformation (Figs. 23 and 28). In the paralysed-infected group (Figs. 23 and 27), the possible influence of the larger infecting dose (10^7 organisms) in augmenting paralysis is not known from these studies, which were necessarily limited by the difficulty of producing large quantities of soluble paralysing antigens. Taken together with the histological studies (Fig. 18) the results of these experiments added weight to the association of cell-mediated immune mechanisms with resistance to infection.

Role of cellular immune mechanisms in resistance to infection

Studies of lymphoid cell populations from infected and immunized animals extended histological evidence for a close involvement of lymphocytes and macrophages in the expression of resistance to *L. enriettii* infection. Sensitization of lymphocytes was inferred from the successful passive transfer of delayed hypersensitivity, the demonstration of lymphocyte transformation and inhibition of macrophage migration by protein-rich antigen, the phenomenon of lymphocyte-cytotoxicity, and the ability of lymphocytes from immune animals to generate lymphokine mediators when cultured with leishmanial antigen. However, there was little evidence implicating an active role for the macrophage in the expression of immunity to *L. enriettii.* Thus we could not demonstrate that peritoneal macrophages isolated from animals at the height of the immune response had any greater parasiticidal activity than macrophages from non-immune animals; there was no evidence for a macrophage-bound antibody, and no basis for a contributory role of cytophilic, opsonic or complement-fixing antibody. In fact, the increased ability of 'convalescent' macrophages to take up the organism would seem only to have promoted the state of parasitization.

In the light of these observations, we offer a simplified view of the role of the lymphocyte and the macrophage in resistance of guinea-pigs to this protozoal organism. Cutaneous infection with a limited number of amastigotes (10^6–10^7 organisms) would result in increasing parasitization of local macrophages and in the gradual accumulation of leishmanial antigen in the peripheral lesion and in the regional lymph node. An early response of lymphocyte sensitization might be favoured by the association of antigen with host macrophages but the full development of cellular resistance would await the accumulation of sufficient immunogen and the circulation of sufficient sensitized lymphocytes. When the lymphocyte population reaches a critical size (5–6 weeks) it would successfully interact with parasitized macrophages bearing antigen on their surface, or leaking antigen from intracellular compartments. This interaction would result in a cytotoxic effect on parasitized macrophages in the primary lesion, with concomitant death of the liberated parasites; and these interactions between parasitized macrophages and sensitized lymphocytes would be assisted by the local production of lymphokine factors. These proposals view the lymphocyte as the prime mover in resistance to infection, and the parasitized macrophage as an 'innocent bystander' (Dumonde, 1967), which having served to promote the immunogenicity of antigen, becomes immobilized, destroyed or rejected by the very cells whose sensitization it may have facilitated.

This descriptive theory does not explain all the features of the experimental system. Firstly, it is not known whether macrophages at different stages of parasitization are equally susceptible to the cytotoxic effects of sensitized lymphocytes; the presence of metastases in some animals resistant to reinfection suggests that intracellular sequestration of antigen may be a feature of metastatic lesions. Secondly, acquired cellular resistance of macrophages may only develop in hitherto unparasitized cells, and may be dependent on the simultaneous presence of accessible antigen and sensitized lymphocytes in proportions inappropriate for the expression of lymphocytoxic potential. We have not explored this possibility; but the demonstration of an isophasic response to reinfection (Dostrovsky *et al.*, 1953) is difficult to explain without the operation of some such cellular interaction. Thirdly, little is known of the antigenic or immunogenic characteristics of *L. enriettii.* It has often been suggested that

successful host–parasite relationships are favoured by antigenic sharing, antigenic masking, or weak immunogenicity of a parasite in its host; and it may be relevant that *L. enriettii* is far more immunogenic for the rabbit (which it will not infect) than for the guinea-pig (P. M. Preston and D.C.D., unpublished results), which is its 'natural' host.

Protection against infection with *L. enriettii* was conferred by artificial immunization only with homologous antigens in FCA, and not by antigens of *L. donovani*, *L. mexicana* and *L. braziliensis*. It is interesting that increased phagocytic activity of macrophages from convalescent animals was also confined to the homologous organism. Protection was not afforded by immunization with dead mycobacteria in adjuvant. Cross protection between *L. donovani* and *M. tuberculosis* in infected animals is, however, well documented (Goble *et al.*, 1963). It is not known whether this is a manifestation of nonspecific cellular resistance or is specific, due to the possession of common antigens. The existence of such antigens is implied and made use of in the complement fixation test for the diagnosis of human visceral leishmaniasis, in which the standard antigen is a mycobacterium, Kedrovsky's bacillus.

The present studies showed cross reactivity in two situations. Firstly, guinea-pigs immunized with FCA alone exhibited slight, but definite, delayed hypersensitivity to soluble protein antigens of *L. enriettii*. Secondly, monolayers of parasitized macrophages were equally susceptible to destruction by lymphocytes either from convalescent guinea-pigs or from guinea-pigs immunized with FCA with or without specific leishmanial antigens. Participation of cross-reacting antibody or of non-specific lymphokine activity in these situations cannot be excluded. On the other hand lymphocyte transformation and macrophage migration inhibition studies showed no cross reactivity between the two antigen systems and may reflect the *in vivo* specificity of cellular immunity. The observation that lymphocytes from animals immunized with antigens without adjuvant destroyed parasitized monolayers is of interest in that such animals exhibited only weak delayed hypersensitivity and had no antibody detectable by the methods used. The elucidation of specific and non-specific mechanisms of cellular resistance to infection (Youmans & Youmans, 1969) may require more detailed knowledge of the response to defined leishmanial antigens than we have obtained in this present study.

Cutaneous leishmaniasis as an experimental model

The predictable course of *L. enriettii* infection in the guinea-pig suggests that this model may be useful in three ways: firstly, in the analysis of cellular mechanisms of immunity to infection; secondly, in the study of host–parasite relationships; and thirdly, as a laboratory model of aspects of human leishmaniasis.

Analysis of the relationships between the different *in vitro* and *in vivo* phenomena which characterize this infection may be expected to supplement other experimental models in defining the role of lymphocyte, macrophages and antibodies in resistance to intracellular organisms. Manipulation of host response and parasite virulence may reveal their relative importance in the maintainance and healing of this chronic infection, and in the interpretation of epidemiological observations relating to the significance of delayed hypersensitivity in population protection. As a laboratory model of human disease the unmodified guinea-pig infection runs a course similar to that of oriental sore; and its metastases in normal and paralysed animals are reminiscent of some aspects of espundia and of diffusa forms of cutaneous leishmaniasis respectively. Our preliminary attempts to induce immunological

paralysis suggest that it may be possible to create and define situations even more closely resembling the non-healing forms of human cutaneous leishmaniasis, which in turn may improve our understanding of the immunological paralysis that characterizes fatal kala-azar. Our continued work is aimed at a more detailed analysis of the phenomena described in this present study.

ACKNOWLEDGMENTS

We thank Mrs Frances Guy, Miss Maureen Thomas, B.Sc., and Mr David Page, B.Sc., for their skilled technical assistance; Dr G. S. Panayi for help with macrophage migration; and the Arthritis and Rheumatism Council for general support. The work was initiated in the Department of Parasitology at the London School of Hygiene, W.C.1, and in the Department of Immunology at the Wright-Fleming Institute, London, W.2. We gratefully acknowledge grants from the World Health Organization (R.S.B.), the Wellcome Trust (R.S.B. and D.C.D.) and the Medical Research Council (G.965/307/B: D.C.D.). A.D.M.B. held a Wellcome Research Fellowship.

REFERENCES

ADLER, S. & HALFF, L. (1955) Observations on *Leishmania enriettii* (Muniz and Medina, 1948). *Ann. trop. Med. Parasit.* **49,** 37.

ANSARI, N. & MOFIDI, CH. (1950) Contribution a l'étude des 'formes humides' de leishmaniose cutanee. *Bull. Soc. Path. exot.* **43,** 601.

BATTISTINI, M.G. & HERRER, A. (1946) Intradermo-reación en la leishmaniosis tegumentaria en el Peru. *Rev. Med. exp. (Lima),* **4,** 109.

BERBERIAN, D.A. (1944) Cutaneous Leishmaniasis (Oriental Sore). *Arch. Derm. Syph. (Berl.),* **49,** 433.

BOYDEN, S.V. (1964) Cytophilic antibody in guinea-pigs with delayed type hypersensitivity. *Immunology,* **7,** 474.

BRAY, R.S. (1969) Bank of strains of *Leishmania.* WHO/LEISH/69. 8.

BRAY, R.S. & BRYCESON, A.D.M. (1968) Cutaneous leishmaniasis of the guinea pig. Action of sensitised lymphocytes on infected macrophages. *Lancet,* **ii,** 898.

BRAY, R.S. & GARNHAM, P.C.C. (1962) The Giemsa-colophonium method for staining protozoa in tissue sections. *Indian J. Malar.* **16,** 153.

BRAY, R.S. & LAINSON, R. (1965) The immunology and serology of leishmaniasis, I. The fluorescent antibody staining technique. *Trans. roy. Soc. trop. Med. Hyg.* **59,** 535.

BRAY, R.S. & LAINSON, R. (1966) The immunology and serology of leishmaniasis, IV. Results of Ouchterlony double diffusion tests. *Trans. roy. Soc. trop. Med. Hyg.* **60,** 605.

BRAY, R.S. & LAINSON, R. (1967) Studies on the immunology and serology of leishmaniasis, V. The use of particles as vehicles in passive agglutination tests. *Trans. roy. Soc. trop. Med. Hyg.* **61,** 490.

BRAY, R.S. & MUNFORD, F.M. (1967) On the maintainance of strains of *Leishmania* from the Guianas. *J. trop. Med. Hyg.* **70,** 23.

BRYCESON, A.D.M. (1969) Diffuse cutaneous leishmaniasis in Ethiopia, I. The clinical and histological features of the disease. *Trans. roy. Soc. trop. Med. Hyg.* **63,** 708.

BRYCESON, A.D.M. (1970) Diffuse cutaneous leishmaniasis in Ethiopia, III. Immunological studies. *Trans. roy. Soc. trop. Med. Hyg.* **64,** 380.

BRYCESON, A.D.M., RADWANSKI, Z., PRESTON, P.M., CHAFFEY, N. & DUMONDE, D.C. (1970) Fluorescent antibody studies of experimental cutaneous leishmaniasis in the guinea pig. (MS in preparation.)

CHUNG, H.L. & WANG, C.W.I. (1939) The immunity to infection with *Leishmania canis* of hamsters recently cured of *Leishmania donovani* infection. *China med. J.* **56,** 519.

CONVIT, J. (1958) Leishmaniasis tegumentaria diffusa: Neuva entidad clinicopatologica y parasitaria. *Rev. Sanid. Asist. soc.* **23,** 1.

CONVIT, J. & KERDEL VEGAS, F. (1965) Disseminated cutaneous leishmaniasis. Inoculation to laboratory animals, electron microscopy and fluorescent antibodies studies. *Arch. Derm.* **91,** 439.

COUTINHO, J.O. (1954) Observacões sôbre a vacinação preventiva com leptomas mortas na leishmaniose espontânea da corbaia *Leishmania enriettii. Folia clin. biol. (S. Paulo)*, **21,** 321.

CURBAN, G.V. (1942) Contribuição clinica ao estudo da vacinação preventiva na leishmaniose. *Rev. paul. Med.* **20,** 334.

DAVID, J.R., AL-ASKARI, S., LAWRENCE, H.S. & THOMAS, L. (1964a) Delayed hypersensitivity *in vitro*, I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93,** 264.

DAVID, J.R., LAWRENCE, H.S. & THOMAS, L. (1964b) Delayed hypersensitivity *in vitro*, II. Effect of sensitive cells on normal cells in presence of antigen. *J. Immunol.* **93,** 274.

DINEEN, J.K. & WAGLAND, B.M. (1966) The cellular transfer of immunity to *Trichostrongylus colubriformis* in an isogenic strain of guinea-pigs. II. The relative susceptibility of larval and adult stages of the parasite to immunological attack. *Immunology*, **11,** 47.

DOSTROVSKY, A. & SAGHER, F. (1945) The intracutaneous test in cutaneous leishmaniasis. *Ann. trop. Med. Parasit.* **40,** 265.

DOSTROVSKY, A., SAGHER, F. & ZUCKERMAN, A. (1953) Isophasic reaction following experimental superinfection of *Leishmania tropica. Arch. Derm.* **66,** 665.

DOSTROVSKY, A., ZUCKERMAN, A. & SAGHER, F. (1952) Successful experimental superinfection of *Leishmania tropica* in patients with relapsing leishmaniasis. *Harefuah*, **43,** 29.

DUMONDE, D.C. (1967) The role of the macrophage in delayed hypersensitivity. *Brit. med. Bull.* **23,** 9.

DUMONDE, D.C., WOLSTENCROFT, R.A., PANAYI, G.S., MATTHEW, M., MORLEY, J. & HOWSON, W.T. (1969) 'Lymphokines': Non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature (Lond.)*, **224,** 38.

GARNHAM, P.C.C. & HUMPHREY, J.H. (1969) Problems in leishmaniasis related to immunology. *Curr. Top. Microbiol. Immunol.* **48,** 29.

GLAZUNOVA, Z.I. (1965) Allergic reactions in guinea-pigs upon repeated inoculation with *Leishmania enriettii.* (In Russian). *Med. Parazit. (Mosk.)*, **34,** 582.

GOBLE, F.C., KONOPKA, E.A., BOYD, J.L. & LEWIS, L. (1963) Resistance to experimental leishmaniasis and tuberculosis induced by heterologous inocula. *Proc. 7th int. Congr. trop. Med. Malar.* **2,** 238.

HEYNEMAN, D. (1967) The effects of changes in environmental conditions of the distribution of leishmaniasis. W.H.O. Inter-regional travelling seminar on leishmaniasis, U.S.S.R. 1967. LEISH/WP/67.7.

JONAS, W.E., GURNER, B.W., NELSON, D.S. & COOMBS, R.R.A. (1965) Passive sensitisation of tissue cells, I. Passive sensitisation of macrophages by guinea pig cytophilic antibody. *Int. Arch. Allergy*, **28,** 86.

KOLB, W.P. & GRAINGER, G.A. (1968) Lymphocyte *in vitro* cytotoxicity: characterisation of human lymphotoxin. *Proc. nat. Acad. Sci. (Wash.)*, **61,** 1250.

LAINSON, R. & BRAY, R.S. (1966) Studies on the immunology and serology of leishmaniasis, II. Cross-immunity experiments among different forms of American cutaneous leishmaniasis in monkeys. *Trans. roy. Soc. trop. Med. Hyg.* **60,** 526.

LAINSON, R. & STRANGEWAYS-DIXON, J. (1963) *Leishmania mexicana:* the epidemiology of dermal leishmaniasis in British Honduras, I. The human disease. *Trans. roy. Soc. trop. Med. Hyg.* **57,** 242.

LAVERAN, A. (1917) *Les Leishmanioses.* Masson et Cie, Paris.

LOWRY, O.H., ROSEBOROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

MANSON-BAHR, P.E.C., HEISCH, R.B. & GARNHAM, P.C.C. (1959) Studies in leishmaniasis in East Africa, IV. The Montenegro test in kala-azar in Kenya. *Trans. roy. Soc. trop. Med. Hyg.* **53,** 380.

MANSON-BAHR, P.E.C. & SOUTHGATE, B.A. (1964) Recent research on kala-azar in East Africa. *J. trop. Med. Hyg.* **67,** 79.

MATA, A.D., RUIZ, C.B., GASCA, E.F. & MORALES, M.C. (1968) Infection experimental con cepas mexicanas del agente causal de la leishmaniasis cutánea. *Salud. púb. Méx.* **10,** 159.

MEDAWAR, P.B. (1944) The behaviour and fate of skin autografts and skin homografts in rabbits. *J. Anat. (Lond.)*, **78,** 176.

MEDINA, H. & ROMERO, J. (1959) Estudio clinico y parasitologico de una nueva cepa de Leishmania. *Arch. venez. Med. trop.* **3,** 298.

MILLER, H.C. & TWOHY, D.W. (1969) Cellular immunity to *Leishmania donovani* in macrophages in culture. *J. Parasit.* **55,** 200.

MORLEY, J., WILLIAMS, T.J., SLATER, A.J., CUBITT, D. & DUMONDE, D.C. (1970) Allergic inflammation, II. Simultaneous measurements of the accumulation of isotope-labelled erythrocytes and protein in reactions of delayed hypersensitivity. *Immunology* (To be submitted for publication.)

MUNIZ, J. & MEDINA, H. (1948) Leishmaniose tegumentar do corbaio (*Leishmania enriettii* n.sp.). *Hospital (Rio de J.)*, **33**, 7.

NEAL, R.A. (1964) Chemotherapy of cutaneous leishmaniasis: *Leishmania tropica* infections in mice. *Ann. trop. Med. Parasit.* **58**, 420.

NICOLLE, C. & COMTE, C. (1909) Kala-azar experimental du chien. *Arch. Inst. Pasteur. Tunis.* **4**, 199.

NICOLLE, C. & MANCEAUX, L. (1910) Reproduction experimentale du bouton d'Orient chez le chien. Originie canine possible de cette infection. *C.R. Acad. Sci. (Paris)*, **150**, 889.

OGILVIE, B.M. & JONES, V.E. (1969) Reaginic antibodies and Helminth infections. *Proc. 3rd Int. Symp. Canad. Soc. Immunol., Toronto*, 1968, p. 13.

OPPENHEIM, J.J., WOLSTENCROFT, R.A. & GELL, P.G.H. (1967) Delayed hypersensitivity in the guinea-pig to a protein-hapten conjugate and its relationship to in vitro transformation of lymph node, spleen, thymus and peripheral blood lymphocytes. *Immunology*, **12**, 89.

PARAENSE, L.W. (1953) The spread of *Leishmania enriettii* through the body of the guinea pig. *Trans. roy. Soc. trop. Med. Hyg.* **47**, 556.

PARROT, L., DONATIEN, A. & LESTOQUARD, F. (1927) Notes expérimentales sur le bouton d'Orient et sur la leishmaniose canine viscérale. *Arch. Inst. Pasteur. Algér.* **5**, 120.

PATTON, W.S. (1913) Is Kala-azar of Madras of animal origin? *Indian J. med. Res.* **1**, 185.

PESSOA, S.B. (1941) Segunda nota sôbre a vacinação preventiva na leishmaniose tegumentar americana con leptomonas mortas. *Rev. paul. Med.* **19**, 106.

PESSOA, S.B. & BARRETTO, M.P. (1944) Contribuição para o estudo da immunidade de leishmaniose tegumentar americana. *1 Reun. Ann. dos. Derm. Sif. Bras. (Rio de Janeiro)*, p. 137.

SEN GUPTA, P.C. (1962) Post-kala-azar dermal leishmaniasis. *Sci. Rep. Instituto Super. Sanitá.* **2**, 124.

SEN GUPTA, P.C. & MUKHERJEE, A.M. (1962) Intradermal test with *Leishmania donovani* antigen in post-kala-azar dermal leishmaniasis. *Ann. Biochem.* **22**, 63.

SHORTT, H.E. & KRISHNAN, K.V. (1932) A comparative study of susceptibility to infection with *L. donovani* in splenectomised and non-splenectomised mice. *Indian J. med. Res. Mem.* (Suppl. to *Indian J. med. Res.*), **19**, 109.

SHORTT, H.E. & SWAMINATH, C.S. (1925) Systemic infection of a monkey (*Macacus rhesus*) by intradermal inoculation of spleen puncture material from a case of Indian kala-azar. *Indian J. med. Res.* **13**, 149.

STAUBER, L.A. (1966) Characterisation of strains of *Leishmania donovani*. *Exp. Parasit.* **18**, 1.

VASINA, S.G., DEMINA, N.A. & GLAZUNOVA, Z.I. (1965) Morphologic and cytochemical investigations of lymph nodes and spleen in leishmaniasis in guinea pigs (Leishmania enriettii). (In Russian). *Me. Parazit. (Mosk.)*, **34**, 708.

VON KRETSCHMAR, W. (1965) Immunität bei der *Leishmania enriettii* Infektion des Meerschweinchens. *Z. Tropenmed. Parasit.* **16**, 277.

WILLMS-KRETSCHMER, K., FLAX, M.H. & COTRAN, R.S. (1967) The fine structure of the vascular response in hapten-specific delayed hypersensitivity and contact dermatitis. *Lab. Invest.* **17**, 334.

WOLSTENCROFT, R.A. & DUMONDE, D.C. (1970) *In vitro* studies of cell mediated immunity, I. Induction of lymphocyte transformation by a soluble 'mitogenic' factor derived from interaction of sensitised guinea-pig lymphoid cells with specific antigen. *Immunology*, **18**, 597.

YOUMANS, G.P. & YOUMANS, A.S. (1969) Recent studies on acquired immunity in tuberculosis. *Curr. Top. Microbiol. Immunol.* **48**, 129.

ZELEDON, R. & BLANCO, E. (1965) Experimental infections in animals with Costa Rican strains of *Leishmania braziliensis*. *J. Parasit.* **51**, (2 suppl.), 20.

ZUCKERMAN, A. (1945). *In vitro* opsonic tests with *Plasmodium gallinaceum* and *Plasmodium lophurae*. *J. infect. Dis.* **77**, 28.