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Establishment of Cutaneous *Leishmania enriettii* Infection in Hamsters

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A model of a self-healing type of cutaneous leishmaniasis was established in hamsters using the guinea pig parasite *Leishmania enriettii*. *L. enriettii* was passaged several times in hamsters without losing its infectivity for guinea pigs or for hamsters. The course of the infection in hamsters resembled that of guinea pigs, with the exception that the lesion at the site of parasite inoculation did not ulcerate and no metastatic lesions developed spontaneously. Moreover, unlike guinea pigs, infected or recovered hamsters were skin test unresponsive to various preparations of *L. enriettii* antigens. However, histological examination of draining lymph nodes showed features of a cell-mediated immune response, and in vitro inhibition of macrophage migration was demonstrable using peritoneal exudate cells from recovered animals and specific leishmanial antigen. Antibody was demonstrable by indirect immunofluorescence starting 1 week after infection. Recovered animals were immune to reinfection; however, the passive transfer of peritoneal exudate cells or serum from recovered animals did not confer protection against *L. enriettii* infection in normal animals.

The protozoon *Leishmania enriettii* was first identified and described as the causative agent of guinea pig leishmaniasis by Medina (12). Muniz and Medina (13) were able to reproduce and establish the disease experimentally in guinea pigs using this parasite. Since then this infection has been a useful model in the study of human cutaneous leishmaniasis. The same authors failed to establish the disease in human volunteers, rhesus monkeys, white rats, hamsters, mice, rabbits, dogs, préa (*Cavia aperea*), hare, and agouti (*Dasyprocata agoutii*) by subcutaneous or intraperitoneal injection of the culture form (promastigotes) or tissue form (amastigotes) of the parasite. Adler and Haff (1) reported that a transient infection was produced by subcutaneous inoculation of promastigotes or amastigotes into suckling mice. Heisch et al. (8) mentioned that *L. enriettii* would grow in the testicles of hamsters, but they did not elaborate on this observation.

In this paper we report the establishment of a self-healing type of cutaneous leishmaniasis in hamsters using *L. enriettii* as an infective agent. Observations on the course of the infection and the immunopathological response of the hamster to *L. enriettii* are also described.

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MATERIALS AND METHODS

***Leishmania* organisms.** A *L. enriettii*-infected guinea pig was obtained from A. D. M. Bryceson, and the infection was maintained by continuous passage of 10^7 amastigotes (subcutaneously nose to nose) in guinea pigs. Conventional NNN medium blood agar slopes overlaid with Hanks balanced salt solution containing 200 Units of penicillin and 200 μg of streptomycin per ml were used to determine viability of amastigotes and to obtain promastigotes from amastigotes.

Animals. Male Syrian hamsters, hairless (naked) hamsters (7), inbred C lac-strain hamsters, and outbred albino Hartley guinea pigs were used in this study. All animals were fed appropriate commercial pellet diets (E. Dixon & Sons, Ware, Hertfordshire) and given water ad libitum. Hay and cabbage were provided for guinea pigs twice a week.

Organisms. Amastigotes were prepared from infected guinea pig nose tissue as described previously (A. Belehu, Ph.D. thesis, Univ. of London, London, England, 1975). Promastigotes were obtained by culture as described above.

Course of leishmanial infection. The course of infection was assessed weekly by measuring the lateral thickness of the infected nose using a Schnelltäster (system AO2T, Kröplin Schluchtern, Hessen). At each time groups of six animals were killed, and the number of parasites in homogenized nose tissue (total hairless area excised) was counted. The weight of the auricular lymph nodes of these animals was also recorded.

Histology. The auricular lymph nodes and some tissues from the site of inoculation were prepared for paraffin sectioning, and slides were stained with hematoxylin and eosin or methyl green pyronin.

Preparation of parasite antigen. Purified soluble antigen (PSA) was prepared from promastigotes of *L. enriettii* according to the method of Bryceson et al. (4).

Skin testing. Skin testing was attempted using various doses of PSA. Homogenates or sonicates of amastigotes or promastigotes, suspended in 0.85% NaCl solution, were also used. Skin tests were performed on the ear, footpad, and shaved flanks. The dermal reaction was read at 24 and 48 h after skin testing by measuring skin thickness at the site of skin testing with a skin micrometer (3).

Indirect immunofluorescence. Six animals were bled every week after infection. Serum was separated and stored at −70 C for antibody assay. A suspension of amastigotes of *L. enriettii* free from guinea pig tissue was centrifuged at 276 × *g*, and the cell button was washed three times with 0.067 M phosphate-buffered saline (PBS) pH 7.2. After the final wash the concentration was adjusted to 10^5 organisms/ml. Organisms were dispensed to 10 clear wells on microscope slides coated with hydrophobic plastic sprays (Fluoroglide, Chemplast, Wayne, N.J.) as described by Voller and O'Neill (17). The organisms were allowed to dry on the slide at room temperature, and the slides were wrapped in absorbent paper and stored in a tin box at −20 C. When required, these were removed from the −20 C refrigerator and allowed to air dry. Twofold dilutions (from 1:4 to 1:4,096) of each test serum were made in PBS in 50-μl amounts. The prepared slides were then overlaid with dilutions of serum and incubated at room temperature (20 to 22 C) in a humid chamber for 30 min. This was followed by three 5-min washes with PBS, after which they were allowed to stand until almost dry. Organisms were stained for 30 min with fluorescein-conjugated swine anti-hamster globulin (Nordic Pharmaceuticals & Diagnostics, The Netherlands), diluted 1:40 in PBS, in a humid chamber at room temperature. After a further three washes in PBS the glass slides were allowed to dry and were mounted in 10% glycerol-PBS under a cover slip, which was sealed with transparent nail polish.

Stained smears of amastigotes of *L. enriettii* were examined with a Zeiss fluorescence microscope using a BG12 primary filter and a barrier filter Zeiss no. 47. The light source was an HBO 200-W mercury lamp. Immunofluorescence was graded visually by comparison with control smears exposed to dilutions of serum from uninfected animals. Immunofluorescence titers were expressed as the reciprocal of the highest serum dilution giving positive fluorescence.

Macrophage migration test. Migration inhibition of peritoneal exudate cells by leishmanial soluble antigen (PSA) was performed using the method of David et al. (6). Peritoneal exudates, induced by injecting 5 ml of sterile liquid paraffin intraperitoneally 6 days previously, were harvested from hamsters, recovered from *L. enriettii* infection. These were allowed to migrate from the cut end of capillary tubes (20 μl) for a period of 24 h at 37 C in a chamber containing 15% heat-inactivated (56 C) fetal calf serum in Eagle minimal essential medium with and without 10 or 100 μg of PSA per ml. As a control, peritoneal exudate cells from normal animals were also tested. After incubation for 24 h the area of migrated cells was projected onto paper and measured by planimetry. Quadruplicate capillaries were examined from each animal in each experimental group. The results were expressed as: migration inhibition = migration (without antigen − with antigen)/migration without antigen × 100. Inhibition of more than 10% was considered as positive evidence of migration inhibition of macrophages.

RESULTS

Course of infection with different doses of *L. enriettii*. The intradermal injection of 2 × 10^6 amastigotes or promastigotes of *L. enriettii* into the nose of Syrian hamsters resulted in the development of a chronic nonulcerating local lesion (Fig. 1), in all of over 300 animals observed. Infection was also achieved by injecting amastigotes subcutaneously into the footpad, ear, or testicle. These results were achieved with all three types of hamster used. Subsequent experiments were performed only on Syrian hamsters.

The time course and severity of the *L. enriettii* infection in the hamster varied depending on the dose of the inoculum. Maximal increase in nose thickness occurred 3 weeks after infection (Fig. 2), except when 2 × 10^3 parasites were used. With this smaller inoculum the development of the lesion was delayed and peaked at week 5. Healing was always complete by week 10, and after recovery animals were found to be immune to reinfection. Spontaneous development of metastatic lesions was not observed in hamsters infected with *L. enriettii*.

An infective dose of 2 × 10^6 was selected for further experiments, as this was the minimal dose of parasites that resulted in a measurable lesion, which developed relatively rapidly.

Serial transmission of parasites in hamsters. It was possible to passage amastigotes of *L. enriettii* in hamsters. So far 35 serial passages of parasites have been performed. After 25 transfers in hamsters, amastigotes of *L. enriettii* were still infective in guinea pigs.

Correlation of increase in lateral nose thickness and parasite counts. Table 1 shows the proportion of animals with viable parasites in the nose up to 14 weeks after infection. The number of animals with viable parasites decreased after week 6. Lateral nose thickness increased up to week 5 and then declined. This correlated with the parasite count in homogenized nose tissue, which also reached a peak after 5 weeks. There was less consistency after week 5, however, and nose thickening receded faster than loss of parasites.

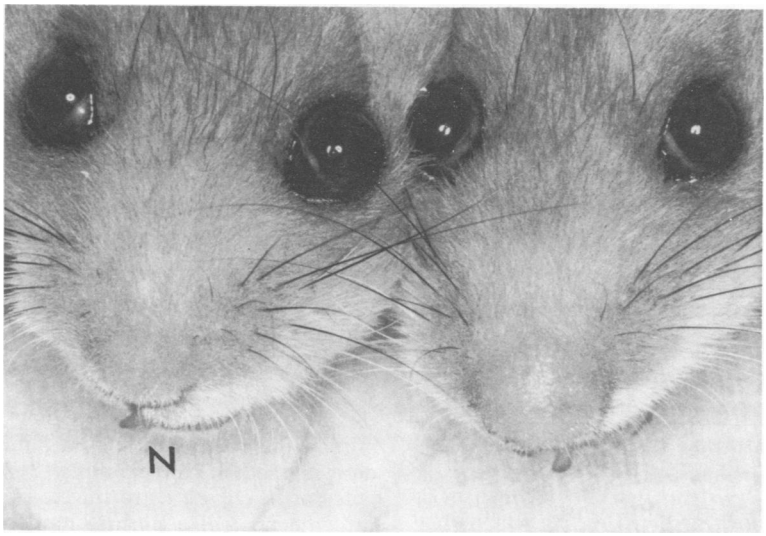

FIG. 1. *Nonulcerating nose lesion of a Syrian hamster 4 weeks after intradermal inoculation of 2 × 10⁶ amastigotes of L. enriettii into the nose, as compared to age-matched control (N).*

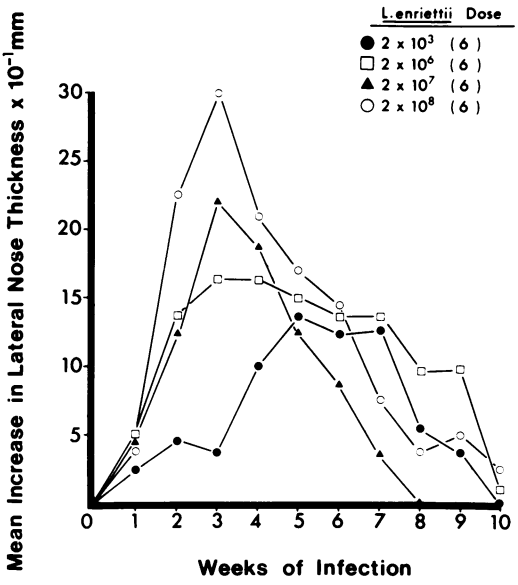

FIG. 2. *Course of infection in hamsters inoculated intradermally into the nose with various doses of amastigotes of L. enriettii. Numbers in parentheses represent number of animals used.*

TABLE 1. *Correlation between lateral nose thickness and parasite counts in hamsters after intradermal inoculation of 2 × 10⁶ amastigotes of L. enriettii into the nose*

Week of infection	Proportion of animals with viable parasites	Mean increase (%) in lateral nose thickness	Mean total parasite count of infected nose tissue[a] (× 10⁶)
1	6/6	21.0 ± 2.0[b]	6.1 ± 0.5
2	6/6	45.0 ± 5.3	8.4 ± 1.2
3	6/6	47.0 ± 15.1	8.7 ± 0.4
4	6/6	85.0 ± 10.8	13.8 ± 1.9
5	6/6	112.0 ± 22.9	25.3 ± 5.5
6	6/6	79.0 ± 31.4	6.1 ± 0.5
8	3/6	5.0 ± 1.0	2.9 ± 0.9
10	1/6	0.0 ± 0.0	2.4 ± 0.1
12	0/6	0.0 ± 0.0	
14	0/6	0.0 ± 0.0	

[a] All hairless area of the nose was removed and homogenized in a glass grinder, and parasites were separated by filtering the homogenate through a fine nylon mesh and the total number was determined using a hemocytometer.

[b] Mean ± standard deviation.

Response to infection in draining lymph nodes. The weight of the auricular lymph nodes reached a peak between 3 and 6 weeks after infection and declined as healing progressed (Table 2). Histological examination showed a gradual increase in the number of large pyroninophilic cells in the paracortical area, reaching a maximum at week 6. There was a gradual increase in germinal center formation and the appearance of plasma cells in the corticomedullary junction and medullary cords. These changes in the B-cell areas were maximal between 3 and 6 weeks after infection and then declined. An example of auricular lymph node histology is shown in Fig. 3.

Histology of the site of parasite inoculation. Histological examintion of the site of par-

TABLE 2. *Changes in auricular lymph nodes in response to L. enriettii infection of the hamster*[a]

Week of infection	Lymph node wt index[b]	No. of large pyroninophilic cells in the paracortex[c]
	6.7 ± 0.5[d]	11 ± 3[d]
1	7.5 ± 0.8	18 ± 5
2	8.2 ± 1.0	22 ± 8
3	8.4 ± 0.7	38 ± 7
4	12.8 ± 1.2	47 ± 7
5	12.9 ± 2.9	65 ± 14
6	9.8 ± 0.7	70 ± 16
8	10.8 ± 1.0	26 ± 11
10	10.6 ± 0.6	18 ± 13
12	7.7 ± 2.1	ND[e]
14	7.3 ± 0.9	ND

[a] Hamsters used: body weight, 55 to 65 g; lymph node weight, 3 to 5 mg.

[b] Lymph node weight index = (lymph node weight/body weight) × 10^5. The index of normal hamsters above 55-g body weight ranges from 6.5 to 7.5.

[c] In a microscopic field of 280 μm in diameter.

[d] Mean ± standard deviation of five values from five animals.

[e] ND, Not done.

asite inoculation 1 week after infection showed an infiltration with 40 to 50% polymorphonuclear leukocytes and 50 to 60% mononuclear cells, which were mainly lymphocytes. By week 2 to 3 the infiltrate was composed predominantly of monocytic cells occasionally parasitized with *Leishmania;* however, small lymphocytes, plasma cells, polymorphonuclear leukocytes, and eosinophils could also be seen. During the recovery phase an increased number of small lymphocytes, plasma cells, and some eosinophils was observed, with fragmented parasites lying in an extracellular position. The appearance of the mononuclear cell infiltrate in the nose is shown in Fig. 4.

Delayed-type hypersensitivity. Hamsters at any stage of infection or after recovery were totally unresponsive to skin testing with PSA, live amastigotes, or promastigotes or their sonicates.

Inhibition of macrophage migration. Table 3 shows that inhibition of macrophage migration in the presence of leishmanial antigen (10 μg of PSA/ml per culture) was demonstrable in vitro with peritoneal exudate cells from hamsters that had been inoculated with *L. enriettii* into the nose 12 weeks earlier and had recovered. It was not possible to demonstrate specific migration inhibition using 100 μg of PSA per ml in culture, as this dose caused similar inhibition of migration of macrophages from normal animals.

Antibody response. Immunofluorescence titers of sera taken from hamsters at different times after leishmanial infection were determined and recorded as the geometric mean of serum titers ($\log_2$) (Table 4). Antibody was detectable after 1 week. By week 4 of infection antibody levels reached their peak and remained so until week 6. After week 6, the level dropped slightly and then remained the same up to week 12 after infection.

DISCUSSION

In this study, experimental cutaneous leishmaniasis has been established in hamsters using *L. enriettii*. The course of *L. enriettii* infection in the hamster nose follows that in the guinea pig providing that a similar infective dose is used. If, for example, 2×10^7 amastigotes of *L. enriettii* are injected intradermally into the nose of a normal hamster there is a progressive increase in nose thickness, which reaches a peak 3 to 5 weeks after inoculation. After this time there is a progressive healing of the lesion, which eventually resolves after 10 to 14 weeks. Unlike the guinea pig, the primary lesion on the nose of the hamster does not ulcerate. No metastatic lesions were observed in hamsters even using a dose of 2×10^8 amastigotes, which is sufficient to cause these lesions in guinea pigs (2). The number of organisms inoculated into the nose made no significant difference to the course of the disease in hamsters, although higher numbers of the organisms resulted in a greater increase in nose thickness. Even with an infective dose of 2×10^8 amastigotes, the nose or ear lesions still resolved by week 10 to 12. This is different from the situation in guinea pigs. Bryceson et al. (2) have reported that a dose as high as 10^8 amastigotes injected intradermally into the dorsal surface of the ear caused metastatic infection in 20 out of 20 guinea pigs. These results might indicate that the susceptibility of hamsters to this organism lies somewhere between CBA mice, Wistar rats, and gerbils, which are completely resistant to *L. enriettii* (Belehu, Ph.D. thesis), and guinea pigs, which are highly susceptible to *L. enriettii* (12).

This sort of spectrum of susceptibility or resistance of hosts can also be observed with other species of *Leishmania* (3, 9). Guinea pigs are resistant to *L. tropica major* inoculated by any route, monkeys are susceptible but overcome the infection, mice develop a chronic infection, and hamsters die from the infection.

It has been found that peritoneal macrophages of hamsters take up amastigotes of *L. enriettii* injected intraperitoneally as detected by examination of Giemsa-stained preparations of peritoneal cells (Belehu, Ph.D. thesis). In

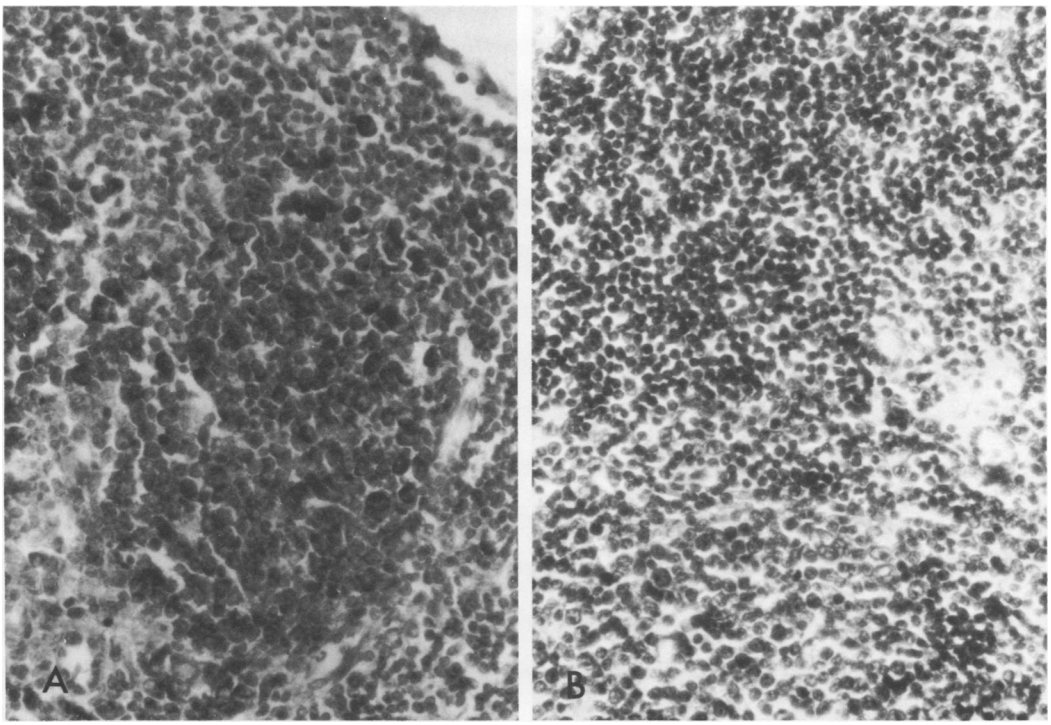

FIG. 3. *Histology of the auricular lymph node taken from a hamster 3 weeks after infection with 2 × 10⁶ amastigotes of L. enriettii into the nose. (A) Germinal center formation; (B) expansion of the paracortical area of the node. Hematoxylin and eosin, ×400.*

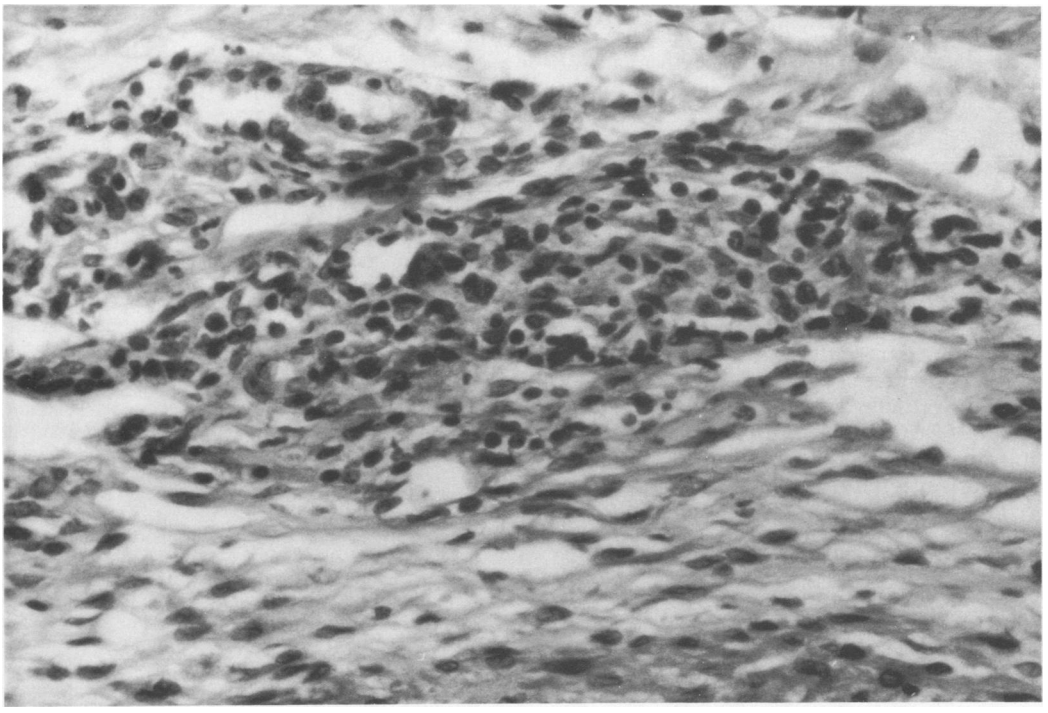

FIG. 4. *Mononuclear cells infiltrating the skin of the nose. Tissue taken 3 weeks after infection with 2 × 10⁶ amastigotes of L. enriettii. Hematoxylin and eosin, ×400.*

TABLE 3. *L. enriettii PSA-induced migration inhibition of hamster peritoneal exudate cells*

Group of animals	Concn of antigen (PSA) in culture (μg/ml)	Migration inhibition[a] (%)
Normal	10	0
	100	72 ± 15
Leishmania infected and recovered	10	62 ± 8
	100	46 ± 12

[a] Results of three experiments, six animals per group per experiment and four capillaries per treatment.

TABLE 4. *Antibody responses of hamsters infected with L. enriettii*

Week of infection	Immunofluorescence titer (log_2)
1	4.3 ± 0.5[a]
2	5.8 ± 0.6
4	7.2 ± 0.6
6	7.1 ± 0.6
8	6.5 ± 0.5
10	6.3 ± 0.2
12 (recovered)	5.4 ± 0.4

[a] Standard deviation of the arithmetic mean of immunofluorescence titer (log_2) of sera from five animals.

addition, unstimulated macrophages from the peritoneum of hamsters phagocytose amastigotes in vitro and appear to destroy them to some extent when examined 24 h after incubation. As this in vitro microbicidal capacity appears to be lacking in guinea pig macrophages (11), its presence in hamster cells may play some part in reducing the severity of the disease.

It was not possible to detect delayed skin test response to various preparations of *L. enriettii* antigens in hamsters at various times during the course of the infection or after recovery. However, contact sensitivity to 2,4-dinitrofluorobenzene or delayed hypersensitivity to a suspension of BCG whole bacilli is demonstrable in hamsters previously immunized with the corresponding antigens (Belehu, Ph.D. thesis). Krenkel (10) also demonstrated specific delayed-type hypersensitivity in hamsters infected with *Besnoitia* and *Mycobacterium tuberculosis*. It was possible, however, to demonstrate in vitro migration inhibition of peritoneal exudate cells from *L. enriettii*-recovered hamsters using soluble leishmanial antigens. In addition, histological examination of auricular lymph nodes of *L. enriettii*-infected or -recovering hamsters showed the presence of large pyroninophilic cells in the paracortical areas, the appearance of which is also characteristic of a cellular immune response (14, 15). It is unclear why no delayed hypersensitivity response can be detected using PSA, and further experiments are necessary to examine this.

Plasma cells were also seen in medullary cords and there was germinal center activity, which would indicate a humoral response (14) to the leishmanial antigens. Examination of the serum from infected or recovered hamsters by the fluorescent antibody method showed the presence of antibody directed against *L. enriettii*. The titer of this antibody increased during the course of the infection. The foregoing account showed that there is evidence that a mixed cellular and humoral response is mounted by hamsters against *L. enriettii*.

Attempts to reinfect hamsters that had recovered from a primary infection were completely unsuccessful, showing that immunity developed to the parasite even though delayed skin hypersensitivity to *L. enriettii* antigens could not be demonstrated. More detailed examination of the mechanism of this immunity is required before any conclusions can be drawn from these results.

It is felt, however, that *L. enriettii* infection in hamsters is a useful additional experimental model of human cutaneous leishmaniasis in addition to the infection of guinea pigs with *L. enriettii* (13) and of mice with *L. tropica* (16).

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Adler, S., and L. Haff.** 1955. Observations on *Leishmania enriettii* (Muniz and Medina, 1948). Ann. Trop. Med. Parasitol. 49:37-41.
2. **Bryceson, A. D. M., R. S. Bray, and D. C. Dumonde.** 1974. Experimental cutaneous leishmaniasis. IV. Selective suppression of cell-mediated immunity during the response of guinea pigs to infection with *Leishmania enriettii*. Clin. Exp. Immunol. 16:189-201.
3. **Bryceson, A. D. M., R. S. Bray, R. A. Wolstencroft, and D. C. Dumonde.** 1970. Immunity in cutaneous leishmaniasis of the guinea pig. Clin. Exp. Immunol. 7:301-341.
4. **Bryceson, A. D. M., P. M. Preston, R. S. Bray, and D. C. Dumonde.** 1972. Experimental cutaneous leishmaniasis. II. Effects of immunosuppression and antigenic competition on the course of infection with *Leishmania enriettii* in the guinea pig. Clin. Exp. Immunol. 10:305-335.
5. **Bryceson, A. D. M., and J. L. Turk.** 1971. The effect of prolonged treatment with antilymphocyte serum on the course of infections with BCG and *Leishmania enriettii* in the guinea pig. J. Pathol. 104:153-165.
6. **David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas.** 1964. Delayed hypersensitivity *in vitro*. 1.

The specificity of inhibition of cell migration by antigens. J. Immunol. 93:264-273.
7. **Festing, M. F. W., and M. K. Wright.** 1972. New semidominant mutation in the Syrian hamsters. Nature (London) 236:81.
8. **Heisch, R. B., R. Killick-Kendrick, M. W. Guy, and J. Dorrell.** 1970. The development of trypanosomes, leishmaniae and ascitic tumour cells in the testicles of laboratory animals. Trans. R. Soc. Trop. Med. Hyg. 64:679-682.
9. **Heyneman, D.** 1971. Immunology of leishmaniasis. *In* World Health Organization (ed.), *Leishmaniasis*. Bull. W. H. O. 44:499-514.
10. **Krenkel, J. K.** 1972. Infection and immunity in hamsters. Prog. Exp. Tumor Res. 16:326-367.
11. **Mauel, J., R. Behin, Biroum-Noerjasin, and D. S. Rowe.** 1975. Mechanisms of protective immunity in experimental cutaneous leishmaniasis of the guinea pig. I. Lack of effects of immune lymphocytes and of activated macrophages. Clin. Exp. Immunol. 20:339-350.
12. **Medina, H.** 1946. Estudo sôbre leishmaniose. I. Primerios casos de leishmaniose expontânea. Observados em cobais. Arq. Biol. Technol. 1:39-74.
13. **Muniz, J., and H. Medina.** 1948. Leishmaniose tegumentar do corbaio *(Leishmania enriettii* n.sp). Hospital (Rio de Janeiro) 33:35-59.
14. **Nossal, G. J. V., and G. Ada.** 1971. Antigens, lymphoid cells and the immune response, p. 61-85. Academic Press Inc., New York.
15. **Oort, J., and J. L. Turk.** 1965. A histological and autoradiographic study of lymph nodes during the development of contact sensitivity in the guinea pig. Br. J. Exp. Pathol. 46:147-154.
16. **Preston, P. M., R. L. Carter, E. Leuchars, A. J. S. Davies, and D. C. Dumonde.** 1972. Experimental cutaneous leishmaniasis. III. Effects of thymectomy on the course of infection of CBA mice with *Leishmania tropica*. Clin. Exp. Immunol. 10:337-357.
17. **Voller, A., and P. O'Neil.** 1971. Immunofluorescence method suitable for large application to malaria. Bull. W.H.O. 45:524-529.