Studies on the immunology and serology of leishmaniasis

II. Cross-immunity experiments among different forms of American cutaneous leishmaniasis in monkeys

Lainson, R.** Bray, R. S.***

Cross immunity between different species or strains of *Leishmania* in man and other animals is both a neglected and somewhat confused subject. Thus, some workers believe that recovery from a natural infection with *Leishmania tropica* will protect a person against *Leishmania donovani*, and SENEKJI (1943) in fact failed to infect two patients recovered from oriental sore with *L. donovani*, and another with *L. braziliensis*. On the other hand, PATTON (1922), NAPIER (1946) and MANSON-BAHR (1959, 1961) each felt that there is no cross immunity between kala-azar and oriental sore in man, and MANSON-BAHR (1961) found that patients cured of kala-azar were readily infected with *L. tropica*. It is known from the work of SOKOLOVA (1940), KOZEVNIKOV (1945, 1963), RODYAKIN (1957) and other Russian workers, that in central Asia recovery from an infection with the rural form of cutaneous leishmaniasis immunizes against the urban disease but, as a rule, not *vice versa*.
A few experiments with monkeys and dogs have been made in the past, but have far from cleared the situation. NICOLLE and MANCEAUX (1910) showed that *L. infantum* infection in monkeys conferred immunity to *L. tropica* and *vice versa*. LAVERAN (1914), too, was unable to infect with *L. donovani* a monkey recovered from *L. tropica*, but PARROT et al. (1927) claimed that they were able to do so. The subject has recently been reviewed by RANQUE et al. (1960).

ADLER and GUNDERS (1964) showed that long-past infections with *L. tropica* from Israel rendered two volunteers completely resistant to challenge with leptomonads and Leishman-Donovan (LD) bodies of *L. mexicana* from British Honduras. Except for this last experiment, leishmaniasis of the New World has been little investigated with regard to cross immunity, yet it is here that the most chronic, destructive and frequently incurable forms of the disease occur. Notable, of course, are espundia due to *L. braziliensis* and leishmaniasis tegumentaria diffusa caused by a supposedly different species, *L. pifanoi*.

For some years now the late professor Saul Adler urged us to investigate the possibility that some relatively mild form of cutaneous leishmaniasis might immunize man against these frightfully disfiguring diseases (see also ADLER, 1962). LAINSON and STRANGWAYS-DIXON (1963) showed that when leptomonads of *L. mexicana* were inoculated into human skin (other than that of the ear) a small lesion was readily produced, generally painless, neither greatly inconveniencing nor disfiguring, and self-curing after a few months. Persons with either natural or experimental infections were quickly rendered immune to further massive challenge with *L. mexicana*, even when their initial lesion was but one month old and still progressive. This immunity certainly persists for many years, and probably for life. We felt, therefore, that this parasite might prove a suitable tool in the sort of experiments professor Adler had in mind.

Clearly, in experiments with virulent strains of *L. braziliensis* or *L. pifanoi* we would be unable to use human volunteers, as clinical studies have shown that metastatic spread of *L. braziliensis* to the mucosae can occur many years after the primary lesion has disappeared.
Numerous workers, however (SANT’ANNA, 1913; WENYON, 1913; AMARAL, 1941; PESSŌA and BARRETTO, 1945) have indicated that the Rhesus monkey, *Macaca mulatta*, can be infected with *L. braziliensis*, as can baboons (*Papio*) and guenons (*Cercopithecus*), while our own experiments have shown that *L. mexicana* invariably produces lesions in Rhesus monkeys very similar to those in man. We, therefore, attempted to immunize Rhesus monkeys against two different strains of *L. braziliensis (sensu lato)* by prior infection with *L. mexicana*.

**MATERIALS AND METHODS**

Strains of *Leishmania* used

L1 *Leishmania braziliensis* from Ceará State, Brazil. Recovered from a patient with espundia in November, 1960.

L15 *Leishmania braziliensis* obtained from Pará State, Brazil, in August, 1963. Isolated from a man with an infection exactly resembling that described by the venezuelan workers as leishmaniasis tegumentaria diffusa, i.e. large nodular lesions spreading over much of the body surface and containing vast numbers of LD bodies, and a negative skin reaction to leishmanin. In Venezuela, at least, the causative agent of such a clinical condition is regarded as a distinct species, *Leishmania pifanoi* (MEDINA and ROMERO, 1959, 1962)

Up to now we had also referred to our strain L15 by this name. Recently, however, professor Adler informed us that this parasite is antigenically indistinguishable from undoubted strains of *L. braziliensis* from typical cases of espundia. In the present work, therefore, L15 will be regarded provisionally as *L. braziliensis (sensu lato)*.

All three strains were maintained in hamsters or mice, in which they produce large leishmaniomas after the inoculation of leptomonads or LD bodies intradermally into the skin of the body or nose.

**INOCULATION AND EXAMINATION OF MONKEYS**

Inoculation was by the intradermal route, the inocula being hamster or mouse lesions triturated in Hanks or Locke solutions and containing very large numbers of LD bodies. Infectivity of the parasites was checked by the inoculation of further hamsters and mice.

Monkeys were inspected regularly for evidence of developing lesions. Whether or not obvious lesions did develop, inoculation sites were examined for LD bodies in stained smears of exudate from the incised areas and by culture of this material in NNN medium.

**EXPERIMENTAL**

**Experiment 1**

Immunity to *L. mexicana* infection in the Rhesus monkey

Seven monkeys were inoculated with LD bodies of *L. mexicana* into the upper part of one ear and, by the 20th day, all developed a conspicuous swelling at the site of inoculation. Abundant LD bodies were found in the lesions, and inoculated NNN cultures rapidly produced leptomonads. At some time between the 20th and 60th days all the animals presented a typical chiclero’s ulcer, which slowly healed during the next month.
Both during and after infection, the monkeys were found to be completely resistant to re-infection with the same parasite. Thus, in Rhesus monkeys (as in man), *L. mexicana* evokes a remarkably rapid and solid immune response.

**Experiment 2**

**Immunity to *L. braziliensis* infection in the Rhesus monkey**

Four monkeys were each inoculated into the nose with LD bodies of strain L1 *L. braziliensis*. One month later all had developed a swelling at the site and scanty parasites were demonstrated in smears and by NNN culture. In three animals the lesions did not ulcerate, and slowly disappeared during the 3rd month; at this time parasites could no longer be detected. The swelling on the nose of the remaining monkey persisted, however, and ulcerated during the 3rd month. NNN cultures from this lesion were positive at six months.

Two of the recovered monkeys were challenged by inoculation of further LD bodies of strain L1 into the eyebrow. Both animals proved completely resistant to re-infection.

The 3rd recovered monkey was similarly resistant to challenge with strain L15 (*L. braziliensis* s.l.) into the skin of the ear, tail, knuckles and chest. A control, non-immune monkey received similar inoculations from the same suspension and in this case developed large nodular lesions up to 1 cm in diameter at all sites other than the chest. Lesions on the tail and nose became deeply ulcerated during the 2nd month and complete recovery was not achieved until the end of five months.

The remaining monkey of the series was still showing an active lesion of the nose due to strain L1 when it was challenged with strain L15, 4½ months after the original infection. Massive inoculations into the skin of the ear, tail, knuckles and chest resulted in small swellings up to 15 mm in diameter with parasites detected only with difficulty in the first three sites. The lesions developed little further and, after mild ulceration of those on the ear and tail, healed by the end of 2½ months.
at the same time as the nose lesion healed. It seems possible that _L. braziliensis_ was behaving here as does _L. tropica_ on re-inoculation (see BERBERIAN, 1944; DOSTROVSKY, 1952) – the lesion resulting from re-inoculation takes on the same state of development or regression as the original lesion, and both heal at the same time. In general it would appear that, like _L. mexicana, L. braziliensis_ infection in the Rhesus monkey induces a strong resistance to re-infection with the same parasite.

**Experiment 3**

Cross-immunity between _L. mexicana_ and _L. braziliensis_ in the Rhesus monkey

Six Rhesus monkeys, recovered from ear lesions due to _L. mexicana_, were challenged by the inoculation of LD bodies of strain L1 (_L. braziliensis_) into the nose. No lesions developed and repeated NNN culture over the subsequent four months failed to isolate parasites from the site of inoculation. Two control, non-immune monkeys inoculated with strain L1 parasites from the same suspension developed typical lesions containing LD bodies.

Conversely, two monkeys recovered from nose lesions due to strain L1 (_L. braziliensis_) were challenged by inoculation of _L. mexicana_ into the ear. Both animals developed transitory swellings at the site of infection and parasites were isolated only with difficulty up to the 4th week, when the nodules regressed and disappeared without ulcerating.

Two monkeys recovered from ear lesions due to _L. mexicana_ were then challenged with strain L15 (_L. braziliensis_ s.l.). One animal was inoculated in the skin of the chest, nose, ear, knuckles and tail, and the other in the tail alone. Three weeks later a small swelling appeared on the tail of each monkey, all other inoculation sites remaining normal. The tail lesions remained very small (about 10mm in diameter) and only mildly ulcerative before they regressed entirely at four to six weeks. Parasites could not be isolated in cultures made at the 4th week of infection.
DISCUSSION AND CONCLUSIONS

PESSÔA and BARRETTO (1945) attempted to immunize monkeys against American cutaneous leishmaniasis, using killed leptomonads. They were able to infect 100% of Rhesus monkeys with \textit{L. braziliensis}: of six monkeys inoculated with a vaccine of killed leptomonads, four were subsequently found immune to challenge with the living flagellates. With human subjects only 2.7% of 144 vaccinated persons later became infected, while of 683 non-vaccinated persons 15.6% eventually acquired infections when working in the same endemic focus, during the same period.

NICOLLE and MANCEAUX (1910) and PARROT et al. (1927) showed that \textit{L. tropica} infection in monkeys confers immunity to re-infection with this parasite only after the lesion has completely healed, and not always then. BERBERIAN (1944) and DOSTROVSKY et al. (1952) among others found that they could readily superinfect man with \textit{L. tropica} so long as parasites persisted in the primary lesion, but not after the lesion had healed.

In this respect, \textit{L. mexicana} differs sharply from \textit{L. tropica} in that our monkeys resisted re-infection while lesions were still developing (in one animal only 29 days after infection). This accords with previous observations on \textit{L. mexicana} infection in man, when it was shown that men with active lesions of only a month's duration were completely resistant to superinfection with further strains of this parasite (LAINSON and STRANGWAYS-DIXON, 1963)

Within the limits of our experiments we can say that present or past infections with \textit{L. mexicana} have completely protected Rhesus monkeys against massive challenge with a Brazilian strain of \textit{L. braziliensis} (L1) originating from a patient with espundia, to which they are normally susceptible.

\textit{L. mexicana} infection also partially immunized other monkeys against another strain (L15) of \textit{L. braziliensis} (\textit{sensu lato}) isolated from a case of Brazilian leishmaniasis tegumentaria diffusa. It
may be that this is merely a reflection of different degrees of virulence shown by two strains of the same parasite – supported by professor Adler’s insistence that strains L1 and L15 are antigenically identical. In this respect it is notable that strain L15 has exhibited a remarkable virulence in the human host, but with an absence of muco-cutaneous involvement; in the Rhesus monkey its behaviour has been essentially similar to that of L1 L. braziliensis, producing more severe lesions which are, however, self-limiting and undergo no metastatic spread. Clearly, strain L15 warrants further detailed study and it is outside the scope of this paper to consider its exact nature further, but it might be added that in the Rhesus monkey the L15 strain gives rise to a positive skin reaction to leishmanin though the patient from whom the strain was isolated has shown no skin reaction to leishmanin at any time.

Curiously enough, L. braziliensis infection in Rhesus monkeys only partially protected these animals against challenge with L. mexicana – partial in that the resulting lesions were very small, contained scanty parasites, failed to ulcerate and disappeared quickly.

Our results raise the question of possible active immunization of man against muco-cutaneous leishmaniasis by vaccination with living leptomonads of L. mexicana. Among forest workers in areas where espundia is a common hazard there is the possibility of either developing mucosal lesions (up to 70% of cases) or acquiring the verrucosa or metastasizing form of the disease. It might be considered, therefore, that a prior self-limiting, painless and single lesion of the chiclero’s ulcer type is a preferable alternative.

One of the factors needing consideration is the possibility of undue persistence or growth of the L. mexicana lesion. In this respect it may be noted that immunity to this parasite is conferred very early in the infection (unlike L. tropica infections) and specific chemotherapy could be given early on, if thought necessary, without limiting the effective immune response. Medical supervision and follow-up would be necessary also, as a safeguard against secondary bacterial or fungal infections of the L. mexicana lesion.
Whether such a live vaccine, giving rise to an active lesion, is preferable to treatment of any *L. braziliensis* infection as it arises, could be decided only by local conditions and medical opinion. Good results in the treatment of *L. braziliensis* (s.l.) infection have recently been claimed, with pyrimethamine (SOLANO and VARGAS, 1960) and glucantime and amphotericin B (SAMPAIO et al., 1960). *L. mexicana* infection responds well to the considerably less toxic range of pentavalent antimonials.

A number of incidental observations have arisen in this work and are worthy of mention. Firstly, it is clear that Rhesus monkeys are readily infected with *L. mexicana* and *L. braziliensis* and we have also produced a very extensive ear lesion, due to the former parasite, in *Cercopithecus aethiops*. LAINSON and STRANGWAYS-DIXON (1963), however, record failure to infect young spider monkeys (*Ateles geoffroyi*) with leptomonads of *L. mexicana*, and MIGONE (1913) failed to infect *Cebus* with *L. braziliensis*. It may be, then, that New World *Cebidae* are less susceptible to *Leishmania* of the New World than are the Old World *Cercopithecidae*.

Secondly, while Rhesus monkeys can easily be infected with *L. braziliensis* (at least, with the strains we were using), the resulting infection appears to be far less damaging than that frequently seen in man (e.g. espundia). Thus, in our Rhesus monkeys the nose lesions were small, mostly non-ulcerative and relatively short-lived. No metastatic spread or recrudescence was noted up to 18 months after the healing of primary lesions. After such periods some monkeys were killed and large portions of the nose triturated and cultured in NNN medium; our consistent failure to isolate parasites from such material suggests that infection terminated with the earlier healing of the initial lesion.

Thirdly, three of the seven monkeys with single ear lesions due to *L. mexicana* quite suddenly developed small secondary sores on the ears, face, arms, legs and scrotum. This occurred at a period when the primary lesion was regressing, between the 2nd and 3rd months of infection. In only one monkey were abundant LD bodies detected in
secondary lesions which healed at the end of a further two weeks. The spread of infection was so rapid and dramatic and the resulting lesions so dispersed that the parasites could only have been disseminated by the blood stream – presumably from the primary lesion, as no parasites could be isolated from the animal’s liver or spleen. While we are sure that these dispersed skin lesions were due to *Leishmania* in one monkey (as we recovered *Leishmania* from several sites) the origin of the lesions in the other two monkeys is not certain. In the 1st monkey the serum before spread contained no detectable precipitins or agglutinins. Immediately after the spread serum showed a powerful haemagglutination reaction and six precipitins. This, however, was not the case with the other two monkeys and in them the secondary lesions may have an aetiology unconnected with leishmaniasis.

In our experience, such a metastasis of *L. mexicana* has never been found to occur in man, but a similar dramatic spread of infection has been described in experimental *L. tropica* infection in man (ZUCKERMÁN and SAGHER, 1963). Precisely what causes such a phenomenon is not clear, but it is interesting to compare the resulting multiple lesions with those sometimes seen in patients suffering from cutaneous leishmaniasis in Costa Rica, Venezuela and other Latin American countries. The origin of excessive numbers of lesions, scattered at random over the body, has long been debated. From the comparative rarity of infected sandflies in nature it would seem unlikely that such infections are the result of separate transmissions, all at the same time; possibly a haematogenous spread, as described above, might also account for some of these cases of disseminated cutaneous leishmaniasis in man. The sudden appearance of immunoglobulins in the serum adds some colour to the theory of haematogenous spread.

**SUMMARY**

The Rhesus monkey (*Macaca mulatta*) was found to be readily infected with either *L. mexicana* or *L. braziliensis*, and cross immunity between the two parasites has been studied in these animals.
Monkeys recovered from *L. mexicana* lesions were completely resistant to re-infection with the same parasite.

Monkeys recovered from *L. braziliensis* infection were immune to further infection with the same strain of parasite.

Monkeys recovered from *L. mexicana* infection were completely resistant to challenge with a strain of *L. braziliensis* isolated from a patient with espundia, to which they were normally susceptible. On the other hand, monkeys immune to *L. braziliensis* could be infected with *L. mexicana* but the resultant infection was milder and short-lived.

The question of possible immunization of forest workers against muco-cutaneous leishmaniasis by vaccination with living leptomonads of *L. mexicana* is discussed. In areas where espundia is a common hazard it might be considered that a prior, self-limiting and single lesion of the chiclero’s ulcer type is a preferable alternative.

ADDENDUM

Since this paper was written the following Rhesus monkeys were inoculated intra-venously with Leishman-Donovan bodies of a strain of *L. donovani* from India.

- M 276 Recovered from *L. braziliensis* (L1) infection.
- M 287 Recovered from *L. braziliensis* s.l. (L15) infection.
- M 342 Recovered from *L. mexicana* infection.
- M 346 Recovered from *L. braziliensis* s.l. (Panama) infection.
- M 309 Recovered from *L. mexicana* infection. Partially immune to *L. braziliensis* s.l. (L15) infection.
- M 349 Uninfected control.
- M 373 Uninfected control.

Spleen biopsies were performed every 45 days or so after infection and splenic juice was examined microscopically and inoculated into cultures. Monkeys M 276, 287, 346 and 373 became infected, but with only very scanty parasites which were never demonstrated microscopically. Six months after infection only one monkey (M 276)
was found lightly infected at autopsy. During the six months the haemagglutination titre of serum from each monkey varied with time as follows:

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 276</td>
<td>1/10, 1/160, 1/2560, 1/800</td>
</tr>
<tr>
<td>M 287</td>
<td>1/10, 1/80, 1/160</td>
</tr>
<tr>
<td>M 342</td>
<td>1/20, 1/320, 1/2560, 1/400</td>
</tr>
<tr>
<td>M 346</td>
<td>1/5, 1/80, 1/160, 1/80</td>
</tr>
<tr>
<td>M 309</td>
<td>1/10, 1/40, 1/20, 1/80</td>
</tr>
<tr>
<td>M 349</td>
<td>0, 1/160, 1/80</td>
</tr>
<tr>
<td>M 373</td>
<td>0, 1/160, 1/80</td>
</tr>
</tbody>
</table>

The haemagglutination titre would seem to show that all monkeys became infected at a fairly low level, and some recovered spontaneously. It is obvious that recovery from infection by any of the *Leishmania* spp. causing american cutaneous leishmaniasis does not confer immunity in Rhesus monkeys to visceral infection with *L. donovani* from India.

REFERENCES


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