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MECHANISMS OF PROTECTIVE IMMUNITY IN EXPERIMENTAL CUTANEOUS LEISHMANIASIS OF THE GUINEA-PIG

II. SELECTIVE DESTRUCTION OF DIFFERENT *LEISHMANIA* SPECIES IN ACTIVATED GUINEA-PIG AND MOUSE MACROPHAGES

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SUMMARY

Macrophage activation as the effector mechanism in destroying *L. enriettii* in the guinea-pig, and *L. tropica* in the mouse, was tested *in vitro*. Activated guinea-pig macrophages, with enhanced anti-*Listeria* capacity had no effect on the survival of intracellular *L. enriettii*, irrespective of the antigen used. Activated mouse macrophages, on the other hand, destroyed ingested *L. enriettii* within 24–48 hr but had no effect on *L. tropica* during the same time period. It is suggested that the pathogenicity of a *Leishmania* parasite in a given host depends on the ability of the parasite to survive in the host's activated macrophages. The possible mechanisms by which *L. enriettii* evades destruction in activated guinea-pig macrophages are discussed.

INTRODUCTION

In the preceding paper on cell-mediated immunity (CMI) in guinea-pig leishmaniasis (Mauel *et al.*, 1975), it has been shown that sensitized lymphocytes from convalescent animals had no specific *in vitro* cytotoxicity for parasitized macrophages; that amastigotes of *Leishmania enriettii* survived equally well in explanted macrophages of either normal or *Leishmania* immune animals; and finally, that explanted peritoneal exudate cells (PEC) of animals immunized against HGG in Freund's complete adjuvant (FCA) became activated in the presence of the antigen and exhibited a marked bacteriostatic effect against *Listeria* monocytogenes, but had no effect on the survival of ingested *L. enriettii*.

Since macrophage activation is considered to be an integral part of CMI to a variety of infectious agents, other immunological methods of inducing macrophage activation were therefore studied. We here report the survival patterns of *Listeria* and two *Leishmania* species in guinea-pig and mouse macrophages following activation induced by *Leishmania* itself, or by more persisting antigens such as BCG and *Toxoplasma*.

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MATERIALS AND METHODS

In general, the materials and methods were as previously described (Mauel *et al.*, 1975).

Animals and parasites

Adult inbred C57Bl and DBA-2 mice were obtained from the breeding colony of the Swiss Institute for Experimental Cancer Research. Both strains proved susceptible to cutaneous infection with *L. tropica major*.

Leishmania tropica major

A strain of *L. tropica major* was obtained from Dr D. C. Dumonde. The parasite was maintained in DBA-2 mice in the form of a skin ulcer and in conventional NNN medium. Promastigotes of recently isolated parasites were used to avoid changes which may have occurred in the parasite after repeated *in vitro* cultivation.

Toxoplasma gondii

The low virulence strain C-56 of *Toxoplasma gondii* was kindly provided by Dr J. S. Remington. C57Bl mice were infected with ten to fifteen brain cysts or $20–30 \times 10^3$ trophozoites of strain C-56, and guinea-pigs with a five times higher dose. Chronic *Toxoplasma* infection was induced by subcurative treatment of animals with 40 mg% sulphadiazine and 1–2% sodium bicarbonate in their drinking water. The treatment was given 2 days after infection and was continued for 2 weeks. Animals were used from 2 months after initiation of infection.

Antigens

A partially purified antigen from *L. enriettii* was obtained from Dr R. S. Bray (Bryceson *et al.*, 1970). The antigen was used for activation of *L. enriettii*-immune guinea-pig macrophages at a concentration of 50 μg dry weight per millilitre. PPD was obtained from the Staten Serum Institute, Copenhagen, and was used at a final concentration of 100 u/ml. For preparation of *Toxoplasma* antigen, peritoneal cavities of moribund mice, inoculated 5–6 days earlier with strain C-56 of *T. gondii*, were washed with 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10 u of heparin per millilitre. Free trophozoites were counted in a haemocytometer and foetal calf serum was added to a final concentration of 10%. After 1 week at 4°C, the suspension was frozen and thawed three to four times, freed from cellular debris by centrifugation at 1500 *g* for 15 min and stored at −20°C. The final concentration of the antigen was equivalent to 5×10^5 trophozoites per millilitre.

Immunization of guinea-pigs with BCG

The Japanese strain of BCG was obtained from the Staten Serum Institute, Copenhagen, Denmark. A volume of 0·1 ml containing 2×10^6 units of BCG was inoculated into each foot pad. Animals were used for experimentation from 1 month after vaccination.

PPD-induced macrophage activation

Activation of PEC from BCG-immune animals was achieved in a similar manner to that described for activation with HGG (Mauel *et al.*, 1975). In brief, starch-induced PEC from normal and BCG-immune animals were incubated overnight in the presence of PPD

(100 u/ml). The cultures were then infected with either *L. monocytogenes* or *L. enriettii* and the survival of micro-organisms was determined.

Toxoplasma-induced macrophage activation

Krahenbuhl & Remington (1971) successfully activated normal guinea-pig macrophages by incubating them with *Toxoplasma*-sensitized lymphocytes and *Toxoplasma* antigen. In the present study, we used a similar technique to activate guinea-pig and mouse macrophages and test their microbicidal capacity against *Listeria* and *Leishmania.*

(a) *Macrophage preparation and parasitization.* Preparation of macrophage monolayers from guinea-pigs was previously described (Mauel *et al.*, 1975); for mouse cells, an essentially similar method was used. In brief, 3 days after inoculation of 2–3 ml of sterile starch solution into the abdominal cavities of normal C57Bl mice, PEC were collected, washed and adjusted to the desired concentration in DMEM containing 10% inactivated foetal calf serum. 2×10^6 and $0{\cdot}2 \times 10^6$ nucleated cells were placed in small Petri dishes containing four round coverslips, and in small flat-bottomed tubes respectively. Several hours were allowed for cell attachment. A suspension of *L. enriettii* amastigotes or *L. tropica* promastigotes was then added. A parasite:macrophage ratio of 5:1 was generally used. After overnight incubation, the macrophage cultures were washed to remove non-phagocytosed parasites, then normal or sensitized lymphocytes and antigen were added to appropriate dishes and tubes.

(b) *Lymphocyte preparation.* Spleens from C57Bl mice or guinea-pigs were removed aseptically and homogenized in ice-cold medium using a Ten-Broek glass tissue grinder. The cell suspensions were centrifuged at 300 *g* for 5 min, the supernatant fluids removed and the pellets resuspended in 5–10 ml of 0·8% cold ammonium chloride to lyse the red cells. After 5 min, the cells were centrifuged, resuspended in cold medium and allowed to stand for 10–15 min in a refrigerator to let the larger particles settle. Viable lymphocytes in the supernatant fluid were enumerated by trypan blue exclusion and suspended at the desired concentration in medium containing 10% serum. Addition of *Toxoplasma*-sensitized lymphocytes and *Toxoplasma* antigen to the macrophage monolayers invariably caused macrophage activation. A lymphocyte:macrophage ratio of 10:1 was generally used to activate the cells, but much lower ratios also proved effective. In experiments where *Listeria* was used as the target organism, the addition of lymphocytes and the antigen preceded the infection of macrophages with the bacterium.

Assay method

Survival of micro-organisms in monolayers was determined by direct microscopic observation and by quantitative assay methods as previously described (Mauel, Behin & Biroum-Noerjasin, 1973).

The tables represent experiments which have been performed at least three to four times.

RESULTS

Activation of macrophages from Leishmania-immune guinea-pigs

The ability of PEC from guinea-pigs which have recovered from *Leishmania* infection, to be activated by live parasites or by parasite antigen was tested. Amastigotes of *L. enriettii* or *Leishmania* antigen were added to PEC cultures and the survival of *Listeria* and *Leish-*

TABLE 1. *Leishmania*-induced activation of guinea-pig macrophages; effect on *Listeria monocytogenes* and *Leishmania enriettii*

Cultures*	*Listeria* (CFU† × 10^{-3}/ml) recovered at:						*L. enriettii* (× 10^{-6}) recovered at:			
	1 hr		5 hr		6 hr		48 hr		96 hr	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
N. PEC	44	28–60	—	—	196	168–224	—	—	—	—
N. PEC+*L.*	92	80–104	—	—	468	332–604	—	—	—	—
Im. PEC	30	20–40	—	—	242	200–284	—	—	—	—
Im. PEC+*L.*	8	8–8	—	—	14	12–16	—	—	—	—
N. PEC	112	100–124	400	344–456	—	—	1·8	1·7–1·9	1·8	1·5–2·2
N. PEC+*L.* Ag	120	112–128	720	736–704	—	—	1·6	1·3–2·1	1·6	1·4–1·9
Im. PEC	98	88–108	388	440–336	—	—	1·5	1·3–1·7	1·5	1·5–1·6
Im. PEC+*L.* Ag	66	52–80	48	32–64	—	—	1·4	1·2–1·7	1·7	1·6–1·9

Means are obtained from two or more replicate culture tubes.

* N = normal. Im = immune. *L* = *Leishmania*. Ag = antigen. PEC = peritoneal exudate cells.

† CFU = colony-forming units.

mania assessed (Table 1). The growth inhibition of *Listeria* in immune cells incubated with amastigotes or *Leishmania* antigen indicated a considerable degree of macrophage activation. However, despite this activation, the survival of *L. enriettii* was not adversely affected.

Effect of PPD-induced activation of guinea-pig macrophages

Addition of PPD to PEC of guinea-pigs infected with BCG induced macrophage activation. This was evident from inhibition of intracellular growth of *Listeria*. The growth of the bacterium was not inhibited in controls using BCG-immune PEC without antigen, or in PEC or normal animals irrespective of the presence or absence of the antigen. PPD-induced activation of guinea-pig macrophages had no effect on the survival of intracellular *L. enriettii* (Table 2).

Effect of Toxoplasma-induced activation of macrophages

(a) *In guinea-pigs.* Monolayers of normal guinea-pig macrophages invariably became activated in the presence of *Toxoplasma* antigen and spleen lymphocytes from guinea-pigs chronically infected with *Toxoplasma gondii* (Table 3). Overnight incubation of cell–antigen mixtures rendered normal macrophages highly resistant to *Listeria* challenge. Omission of the antigen from sensitized lymphocytes or addition of spleen cells from normal animals with or without *Toxoplasma* antigen did not induce activation. However, *Toxoplasma*-induced activation of guinea-pig macrophages was without effect on intracellular survival of *L. enriettii*.

(b) *In mice.* Normal mouse PEC were infected with either *L. enriettii* amastigotes or *L. tropica* promastigotes. After overnight incubation and subsequent washing, the parasitized macrophages were incubated with spleen lymphocytes from normal or *Toxoplasma*-infected

TABLE 2. PPD-induced activation of guinea-pig macrophages; effect on *L. monocytogenes* and *L. enriettii*

Cultures*	*Listeria* (CFU† $\times 10^{-3}$/ml) recovered at:				*L. enriettii* ($\times 10^{-6}$/ml) recovered at:			
	1 hr		6 hr		48 hr		72 hr	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
N. PEC	97	94–101	822	788–856	10·3	9–11·5	8·9	7·8–10
N. PEC+PPD	132	123–142	820	796–844	10·8	9·1–12·4	9·5	7·8–11·1
Im. PEC	134	116–152	1059	892–1226	11·2	10·8–11·5	11·5	10·5–12·8
Im. PEC+PPD	24	18–30	22	18–27	10·1	6–10	10·1	9·3–10·8

Means are obtained from two or more replicate culture tubes.

* N = normal. PEC = peritoneal exudate cells. Im = immune.

† CFU = colony-forming units.

mice with or without addition of antigen. Survival of the parasites was then assessed. Table 4 shows that *L. enriettii* and *L. tropica* survived differently in activated mouse macrophages. The activated mouse cells readily destroyed amastigotes of *L. enriettii* within 24–48 hr. In contrast, in parallel cultures, and within the same time limit, *L. tropica* was not adversely affected.

DISCUSSION

It has already been demonstrated that lymphocytes from *Leishmania*-immune animals have no specific cytotoxic effect on parasitized macrophages (Mauel *et al.*, 1975). The results presented in this study also indicate that survival of *L. enriettii* is not compromised in peritoneal macrophages of *Leishmania*-immune guinea-pigs. It was also found that addition of live

TABLE 3. *Toxoplasma*-induced activation of guinea-pig macrophages; effect on *L. monocytogenes* and *L. enriettii*

N. PEC incubated with:*	*Listeria* (CFU† $\times 10^{-3}$/ml) recovered at:				*L. enriettii* ($\times 10^{-6}$/ml) recovered at:			
	1 hr		5 hr		48 hr		72 hr	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
N. Ly	207	199–216	823	804–842	3·9	3·6–4·4	3·2	3·2–3·3
N. Ly+Ag	214	210–227	968	958–978	3·5	3·1–4·2	3·4	3·2–4
Im. Ly	217	219–215	850	820–880	4·1	3·8–4·3	4·1	3·8–4·6
Im. Ly+Ag	44	54–34	157	151–163	3·1	2·8–3·5	3·1	2·8–3·6

Means are obtained from two or more replicate culture tubes.

* N. Ly = normal lymphocytes. Im Ly = immune lymphocytes. PEC = peritoneal exudate cells. Ag = antigen.

† CFU = colony-forming units.

TABLE 4. *Toxoplasma*-induced activation of mouse macrophages; effect on *L. enriettii* and *L. tropica*

N. PEC incubated with:*	*L. enriettii* ($\times 10^{-4}$/ml) recovered at:				*L. tropica* ($\times 10^{-4}$/ml) recovered at:			
	24 hr		48 hr		24 hr		48 hr	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
N. Ly	162	129–194	314	281–346	116	108–122	272	230–309
N. Ly+Ag	262	222–276	400	351–435	207	191–219	338	311–360
Im. Ly	147	122–185	312	268–355	105	86–132	249	209–289
Im. Ly+Ag	1	0–2	0	0	179	146–217	197	193–212

Means are obtained from triplicate culture tubes.

* N. Ly = normal lymphocytes. Im. Ly = immune lymphocytes. Ag = antigen. PEC = peritoneal exudate cells.

parasites or its antigen to PEC of *Leishmania*-immune animals readily caused macrophage activation, as judged by their ability to destroy *L. monocytogenes*. However, *Leishmania*-activated macrophages were unable to destroy the ingested parasites. This observation suggests that as an infected guinea-pig becomes sufficiently sensitized to *Leishmania* antigen, macrophages in the lesion may undergo activation. Macrophages are considered to be important effectors of cell-mediated immunity to a variety of facultative bacteria, fungi and viruses (Nelson, 1972). There is less information available about the role of macrophages in resistance to protozoan parasites. However, it has been shown that amastigotes of *L. donovani* are destroyed inside macrophages from mice superinfected with the same parasite (Miller & Twohy, 1969; Bradley, personal communication). Remington *et al.* (1972) have shown that activated macrophages from mice chronically infected with *Toxoplasma gondii* or *Besnoitia jellisoni* or immunized with FCA have an enhanced capacity to kill intracellular *Toxoplasma*. Recently, Hoff & Frenkel (1974) have shown that *Besnoitia*-immune hamsters reduced the intraperitoneally inoculated homologous organisms 100–10,000-fold over a 5-day period, but that another parasite, *T. gondii*, increased 100–1000-fold over the same period. It was also claimed that specifically committed lymphocytes could instruct macrophages to reduce the homologous organism to a greater extent than the heterologous parasite. However, in the case of *L. enriettii*, we were completely unable to induce positive killing by guinea-pig macrophages, irrespective of the means of activation, including activation by specifically sensitized lymphocytes and *Leishmania* antigen.

How do *L. enriettii* evade destruction in activated macrophages? Either they may be resistant to lysosomal enzymes or they may prevent access of lysosomal enzymes to phagocytic vesicles. Jones & Hirsch (1972) have demonstrated that lysosomes do not fuse with phagosomes containing living *Toxoplasma*, whereas fusion does occur if the *Toxoplasma* are dead. Perhaps living *Toxoplasma* and *Leishmania* release factors which inhibit lysosomal fusion.

Infection of C57Bl mice with *Leishmania* showed interesting differences from the guinea-pig. *L. enriettii* did not produce lesions, although this parasite could multiply in mouse macrophages *in vitro*. However, activated mouse macrophages destroyed *L. enriettii*. On the

other hand, *L. tropica* produced lesions in mice, and grew equally well in non-activated and activated macrophages. These findings suggest that pathogenicity of *Leishmania* species in mice is related to ability to grow in activated macrophages, and the same may be true for the guinea-pig.

Species specificity of *Leishmania* killing in activated mouse macrophages appears to offer a useful model for the study of the killing processes of these cells. It would also be very interesting to investigate the behaviour of *Leishmania* species other than *L. enriettii*, such as *L. tropica*, in activated guinea-pig cells. Unfortunately, due to technical difficulties such as poor phagocytosis, the fate of *L. tropica* in activated guinea-pig macrophages could not be assessed.

What is the mechanism of acquired immunity in the guinea-pig? From circumstantial evidence of prominent cell-mediated response and little antibody response, Turk & Bryceson (1971) considered that the mechanism was cell-mediated. As reported in the previous paper, we have not been able to demonstrate that lymphocytes from immune animals are able to destroy parasitized macrophages. Nor have we been able to obtain evidence that macrophage activation plays a role in acquired immunity or healing, although as mentioned above, susceptibility to activated macrophages may render *Leishmania* non-pathogenic. Failure to demonstrate an *in vitro* system does not of course mean that cellular mediated responses are not effective *in vivo*. But the recent demonstration of serum antibody in animals with healing lesions or who are immune, and of membrane changes induced in living organisms by antibody (Doyle *et al.*, 1974), emphasize that it is unwise to consider protective immunity solely in terms of a cell-mediated response.

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