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Modification of cutaneous leishmaniasis in the guinea-pig by cyclophosphamide

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SUMMARY

Pretreatment of guinea-pigs with cyclophosphamide (Cy) (300 mg/kg) 3 days before cutaneous infection with *Leishmania enriettii* caused an increased intensity of the lesion at the site of infection and an increase in the incidence of widespread metastases. Decreased levels of circulating antibody were found from the first to fourth week after infection. Decreased delayed type hypersensitivity could only be detected beyond 4 weeks. Peritoneal macrophages obtained from guinea-pigs 3 days after Cy pretreatment showed increased rather than decreased ability to phagocytose *L. enriettii.* Phagocytosis of *L. enriettii* by peritoneal macrophages obtained from guinea-pigs pretreated with Cy 24 days previously was normal. It is suggested that more attention should be taken of antibody levels during the early phase of infection and that control of infection could be due to a synergism between antibody and cell-mediated immunity. Upset in the balance by suppression of either function might lead to the development of widespread metastatic lesions.

INTRODUCTION

Two possible mechanisms have been suggested for immunity to infection with *L. enriettii* in the guinea-pig. Initially it was considered that infection with this obligatory intracellular protozoan parasite was controlled by cell-mediated immune mechanisms (Bryceson *et al.*, 1970). This concept was supported by the observation that increased incidence of a disseminated form of the disease might be induced by treatment with anti-lymphocyte serum (Bryceson & Turk, 1971; Bryceson *et al.*, 1972). The demonstration of specific antibody like activity in the sera of guinea-pigs that had recovered from infection with this parasite has, however, raised the question as to whether humoral as well as cell-mediated immune mechanisms might play a role in the healing process of cutaneous leishmaniasis. Evidence for such a mechanism comes from two sources. In the first place the serum of guinea-pigs that had recovered from *L. enriettii* infection contained a factor that inhibited the growth of promastigotes of *L. enriettii* in culture (Rezai, Gettner & Behforouz, 1972). In addition specific antibodies have also been demonstrated by immunoflorescence during infection with this organism (Radwanski *et al.*, 1974).

The demonstration of the selective effect of cyclophosphamide (Cy) on lymphoid tissue (Turk & Poulter, 1972) suggested that pretreatment of guinea-pigs with this agent before infection might cast some light on the differential roles of cell-mediated immunity and humoral antibody in this infection. It was considered that this might be a particularly

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useful model as in some of the systems studied, increased delayed type hypersensitivity was demonstrated following a decrease in humoral antibody production (Turk, Parker & Poulter, 1972; Turk & Parker 1973).

MATERIALS AND METHODS

Animals. Albino male guinea-pigs of outbred Hartley strain, weighing 450–550 g and bred in the department were used. They were fed RGP pelleted diet (E. Dixon & Sons, Ware, Herts.) and water *ad libitum*. Hay and cabbage were provided twice a week.

Parasites. A *Leishmania enriettii*-infected guinea-pig was obtained from Dr A. D. M. Bryceson and the infection was maintained by continuous passage of 1×10^7 amastigotes in guinea-pigs from nose to nose.

Preparation of parasites for infecting animals and for culture. Parasites were obtained from nasal lesions raised by injecting 1×10^7 amastigotes s.c. into the tip of the nose. A portion of lesion was excised, washed, teased and lightly ground in a glass homogenizer containing Earle's balanced salt solution (Earle's BSS, Wellcome Laboratories) with 100 u/ml of penicillin and 100 μg/ml streptomycin (Crystamycin, Glazo Laboratories Ltd, Greenford). The suspension was filtered through a fine mesh of sterile gauze or nylon to remove any guinea-pig tissues. Amastigotes were counted by a standard method using a haemocytometer chamber (Bauer, Toro & Achermann, 1962) and the concentration was adjusted, as required.

Injection of cyclophosphamide. Cyclophosphamide 'Endoxana' kindly given by Ward Blenkinsop, Wembley, Middlesex, was used. Guinea-pigs were injected i.p. with 300 mg/kg body weight cyclophosphamide in a freshly made saline (0·15 M NaCl) solution (Turk & Poulter, 1972). The Cy was injected 3 days before infection.

Infecting animals. Experimental guinea-pigs were infected with 1×10^6 amastigotes of *L. enriettii* i.d. in the dorsal surface of the ear.

Course of infection. The course of infection was assessed by weekly examination of the state of ear lesions and the presence of metastatic lesions. Lesion size was measured and the volume calculated according to the method of Bryceson *et al.* (1972).

Preparation of tissues for histology. Lymph nodes were fixed in Carnoy's solution embedded in paraffin wax, sectioned at 5 μm and stained with pyronin methyl green or haematoxylin–eosin. Excised pieces of primary and metastatic cutaneous lesions were fixed in formol–acetic acid (40% formaldehyde: 200 ml, 90% ethanol: 1800 ml and glacial acetic acid: 80 ml) or 10% formol–saline. These were then embedded in wax, sectioned and stained with haematoxylin–eosin or methyl green–pyronin.

Preparation of parasite antigens. Antigens of *L. enriettii* used for skin testing and *in vitro* experiments were prepared according to the method of Bryceson *et al.* (1972). The purified soluble antigen of *L. enriettii* (PSA) was assayed for protein content using a simple ultraviolet spectrophotometric method as described by Waddell (1956). PSA contained about 40% protein according to this method. One batch of PSA only was used throughout this investigation.

Skin testing. Skin testing was performed by i.d. injection of 10 μg or 100 μg of PSA in 0·1 ml saline into the shaved flanks of guinea-pigs. As a control 0·1 ml of saline alone was injected. The dermal reaction was read at 24 hr after skin testing by measuring skin thickness at the injection site with a Schnelltäster (Bryceson *et al.*, 1970).

Antibody assay by immunofluorescence. Sera. Blood was taken from experimental animals every other week after infection by venepuncture of the hind limb. Serum was separated and stored at −70°C for antibody assay.

Antigens. Suspension of amastigotes of *L. enriettii* free from guinea-pig tissues were centrifuged at 276 *g* and the cell button was washed three times with phosphate-buffered saline pH 7·2 (PBS). After the final wash the concentration was adjusted to 1×10^5 organisms/ml. Organisms were dispersed to ten clear wells on microscope slides coated with hydrophobic plastic sprays (Fluoroglide: Chemplast, 100 Dey Road, Wayne, N.J.) as described by Voller & O'Neill (1971). The organisms were allowed to dry on the slide at room temperature, the slides were wrapped in absorbent paper and stored in a tin box at −20°C.

Indirect immunofluorescence staining. Microscope slides containing amastigotes were removed from the −20°C refrigerator and allowed to air dry. Two-fold dilutions (from 1:4 to 1:4096) of test serum was made in PBS in 50 μl amounts. The prepared slides were then overlaid with dilutions of serum and incubated at room temperature 20–22°C in a humid chamber for 30 min. This was followed by three 5-min thorough washes with PBS. The PBS was poured off and the slides allowed to stand until almost dry. Organisms were stained for 30 min with fluorescein-conjugated swine–anti-guinea-pig globulin (Nordic Pharmaceuticals and Diagnostics, 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 coverslip and sealed with a transparent nail polish.

Microscopic examination. 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 HBO

200 W mercury lamp. Immunofluorescence was graded visually by comparison with control smears exposed to dilutions of serum from uninfected animals. Immunofluorescence titres were expressed as the reciprocal of the highest serum dilution giving positive fluorescence.

In vitro phagocytosis of parasites by macrophages. Phagocytosis of *L. enriettii* amastigotes by macrophages was studied *in vitro* using a modified method of Bryceson *et al.* (1972). Caseinate or paraffin oil-induced peritoneal exudate cells from normal and infected guinea-pigs were collected in Earle's BSS containing antibiotics. After centrifugation at 276 *g* for 20 min the cell button was resuspended and washed three times. After the final wash the cells were resuspended in a small volume of Earle's BSS with antibiotics. Cell viability was assayed by the dye exclusion method (Pappenheimer, 1917) using a final concentration of 0·1% trypan blue stain. Total and differential cell counts were performed by the standard method (Bauer *et al.*, 1962). The cell concentration was adjusted to 1×10^6 viable cells/ml. Cell suspensions prepared from stimulated animals contained 60–80% macrophages. One millilitre of cell suspension was dispensed into each Leighton tube which contained a coverslip for the macrophages to adhere to. One hour after incubation at 37°C, non-adherent cells were removed. After gently washing the monolayers with Eagle's medium, each culture was exposed for 2 hr at 37°C to 1×10^7 freshly isolated amastigotes of *L. enriettii* in 1 ml of Eagle's minimum essential medium (MEM) (Wellcome Laboratories) containing 10% inactivated foetal calf serum and antibiotics. This resulted in the parasitization of macrophages. At the end of this time the cultures were washed carefully to remove non-adherent cells and extracellular parasites with Eagle's MEM, and reincubated in 20% inactivated foetal calf serum Eagle's MEM for a further 24 or 48 hr.

Cultures were terminated after 2, 24 and 48 hr of incubation. Sample monolayer cultures on coverslips were washed, fixed with methanol and stained with Giemsa. The results were expressed as infected macrophages (per cent) and number of parasites per infected macrophage.

RESULTS

Course of infection in Cy-treated animals

The initial lesion at the site of infection developed more slowly in Cy-pretreated animals. However, the lesion was much greater than in controls at 5 weeks and persisted for at least 10 weeks, although that in controls had resolved by 7 weeks (Fig. 1). These findings were consistent in two separate experiments with at least five animals in each group. Twenty per cent of controls developed metastatic lesions as might be expected following an infection with 1×10^6 amastigotes of *L. enriettii* into the dorsal surface of the ear (Bryceson *et al.*, 1972). However, in the Cy-pretreated group widespread metastases were found in the scrotum, feet, legs and nose in 89% of the animals (Table 1). Histologically the metastases

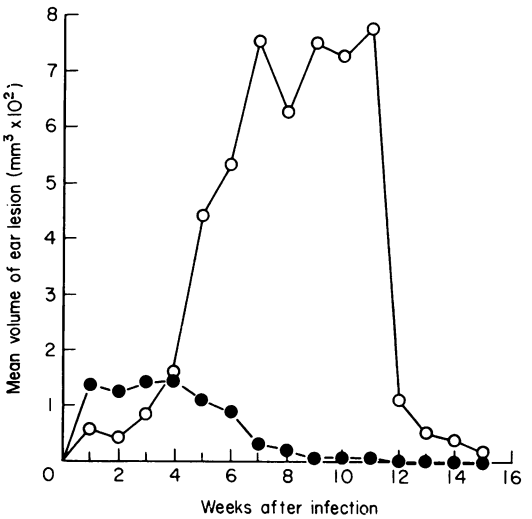

FIG. 1. Growth of cutaneous lesion at the site of inoculation of the amastigote form of *L. enriettii* in guinea-pigs pretreated with 300 mg/kg body wt of cyclophosphamide, i.p. 3 days prior to infection (○) and in non-pretreated guinea-pigs (●).

TABLE 1. The effect of Cy on the development of metastases in *L. enriettii* infection of guinea-pigs

Cy pretreatment	Proportion of animals developing metastatic lesions	Cumulative numbers of animals with metastatic lesions in relation to the time of infection						
		Week of infection						
		3	4	5	6	7	8	12
−	6/30 (20%)	0	0	1	2	5	6	6
+	23/28 (89%)	0	4	5	8	16	23	23*

* Eight out of twenty-three animals had died. Deaths started after week 6 of infection.

in both groups showed macrophages packed with parasites and a remarkable absence of lymphocyte infiltration. The main difference in histological appearance at the site of inoculation was a slight increase in the number of polymorphonuclear leucocytes in the Cy-pretreated group. Although there appeared to be a slight decrease in macrophage density in Cy-treated animals as compared with controls, there was no difference in parasite uptake by these cells.

Delayed hypersensitivity response

Guinea-pigs pretreated with Cy have a markedly depressed cutaneous delayed hypersensitivity to PSA at 4 weeks of *L. enriettii* infection (Table 2) with two different challenge doses of antigen. At week 13 the situation was rather more complicated. Using 100 μg PSA as a challenge dose, animals with metastatic lesions reacted less strongly than those which had recovered, whether or not they had been pretreated with Cy. With this dose there was no difference in reactivity between the Cy and the non-Cy-treated groups. When 10 μg of PSA was used for challenge the alternatives of recovery on the one hand or metastasis on the other, did not affect the intensity of the delayed hypersensitivity reaction, but at this dose Cy-pretreated animals which had recovered were less reactive than the corresponding animals which had not received Cy.

TABLE 2. Delayed skin hypersensitivity of guinea-pigs infected with *L. enriettii*

Week of infection	Cy pretreatment	Status of animals	Increase in skin thickness 24 hr after skin testing with:	
			10 μg PSA	100 μg PSA
4	−	Infected	1·6 ± 0·2*	2·7 ± 0·4
4	+	Infected	0·6 ± 0·3‡	0·9 ± 0·5‡
13	−	Recovered	3·0 ± 0·7	5·0 ± 0·5
13	−	With metastatic lesions	2·0 ± 0·5	2·9 ± 1·1†
13	+	Recovered	1·2 ± 0·4‡	5·2 ± 0·7
13	+	With metastatic lesions	1·7 ± 0·6‡	2·4 ± 0·8†

Significance: †$P < 0{\cdot}01$; ‡$P < 0{\cdot}001$ when compared to corresponding values of untreated animals with infection (week 4) or recovered from infection (week 13) (Student's *t*-test).

* Mean in mm (×10) ± standard deviation of values from at least five animals.

TABLE 3. Mean antibody titres in sera of guinea-pigs treated or untreated with Cy 3 days before *L. enriettii* infection

Cy pretreatment	Animals		Week of infection				
	State	Number	1	2	4	8	12
−	Self-healing	14	0·9±0·7*	4·4±0·6	8·4±1·3	8·7±1·0	7·3±0·9
−	Developing metastatic lesions	6	1·0±0·6	5·9±0·4	9·0±1·1	9·5±1·2	6·0±1·2
+	Self-healing	5	0·5±0·5	2·0±0·5‡	9·5±1·8	8·5±0·9	7·3±0·5
+	Developing metastatic lesions	15	0·5±0·5	2·4±0·7‡	5·4±0·1†	8·1±1·2	7·2±1·4

Significance: † $P<0·01$; ‡ $P<0·001$ when compared to corresponding values of untreated animals with self-healing infections (Student's *t*-test).

* Mean ± standard deviation of immunofluorescent titres (log_2).

Antibody response

Antibodies were detected by indirect immunofluorescence in sera of infected animals using amastigotes as the antigen. Table 3 shows the arithmetic means of log_2 of immunofluorescence titres of leishmanial antibody in the sera of infected guinea-pigs pretreated or not pretreated with Cy. Antibody titres were significantly depressed at weeks 2 and 4 in animals treated with Cy, and developing metastatic lesions. Cy-treated animals that did not develop metastases also had depressed antibody levels at 2 weeks but at 4 weeks the antibody titres were the same as in animals not given Cy. Animals not given Cy but which did develop metastases had similar antibody titres to animals without the diffuse lesions. After 8 and 12 weeks of leishmanial infection the antibody titres in sera from animals of all groups were similar.

Effect of Cy on macrophage phagocytic capacity in normal and infected guinea-pigs

Table 4 shows a comparison of the phagocytic ability of macrophages from animals infected 3 weeks previously with or without Cy pretreatment. As controls, macrophages

TABLE 4. *In vitro* phagocytosis of amastigotes of *L. enriettii* by macrophages from guinea-pigs* 3 weeks after infection with or without Cy pretreatment

Cy† pretreatment	Status of animals	Mean number of amastigotes per 100 macrophages			Infected macrophages (%)		
		Incubation period (hr)			Incubation period (hr)		
−	Uninfected	225±75‡	337±79	432±96	41±11	54±7	61±8
+	Uninfected	198±51	314±105	445±119	48±18	64±11	61±15
−	Infected	190±84	76±35	40±21	51±13	18±8	12±12
+	Infected	204±49	84±24	37±16	43±16	21±50	8±4

* Guinea-pigs were stimulated 4 days previously with sterile paraffin oil to obtain peritoneal exudate macrophages.

† Cy given i.p. 24 days before cells were harvested.

‡ Mean ± standard deviation from values of three cultures per animal. Three animals were used per treatment.

TABLE 5. *In vitro* phagocytosis of amastigotes of *L. enriettii* by macrophages from non-infected guinea-pigs*

Pretreatment of macrophages donors with Cy on day −3	Mean number of amastigotes per 100 macrophages			Infected macrophages (%)		
	Incubation period (hr)			Incubation period (hr)		
	2	24	48	2	24	48
−	331 ± 69†	441 ± 41	312 ± 94	58 ± 10	69 ± 17	66 ± 11
+	720 ± 201	739 ± 201	290 ± 85	75 ± 6	89 ± 5	70 ± 19

* Guinea-pigs were stimulated 2 days previously with 10% sodium caesinate to obtain peritoneal macrophages.

† Mean ± standard deviation from values of three cultures per animal per treatment; three animals were used per treatment.

from uninfected animals either untreated, or which had received Cy 24 days before cell harvest, were used. In all cases sterile liquid paraffin, injected 4 days before cell harvest, was used as an exudate stimulant. It can be seen that the Cy treatment had no effect on the phagocytic ability of normal or infected animals. However, after 24–48 hr culture the macrophages from infected animals contained a reduced number of phagocytosed parasites. This was true of Cy-treated or untreated infected guinea-pigs.

As the greatest effects of Cy treatment on lymphoid tissue seemed to occur after 3 days (Turk & Poulter, 1972) it seemed important to examine the phagocytic ability of macrophages taken from the peritoneum at this time. To avoid the complication of injecting Cy into a peritoneum already full of paraffin, sodium caseinate was used to stimulate the exudate as this only has to be injected 2 days prior to harvest.

The results of these experiments (Table 5) showed that macrophages from animals pretreated with Cy phagocytosed more than twice as many amastigotes as macrophages from normal animals. By 48 hr the number of amastigotes in macrophages from both groups had been reduced to a similar level. However, because of the much greater initial uptake of parasites by the macrophages from Cy-treated animals the reduction in this group was much greater.

DISCUSSION

Pretreatment of guinea-pigs with Cy before infection with *L. enriettii* has been found to provide a further model of the metastatic form of this disease similar to that produced previously by treatment with anti-lymphocyte serum. In these Cy-pretreated animals the primary lesion is larger than in untreated infected controls and the healing process is prolonged. This situation was associated with a decrease in antibody levels during the early phase of infection. It is of interest that there was no similar decrease in antibody levels in the small number of guinea-pigs that developed metastases spontaneously.

The effect of Cy pretreatment on the ability to develop delayed hypersensitivity reactions was more difficult to interpret. At 4 weeks after infection there was a decrease in reactivity in animals pretreated with Cy. Significant differences between the Cy group and controls were found with 10 μg PSA dose. However, with the 100 μg PSA dose differences depended not on whether it had developed metastases. Animals with metastases produced decreased reactions whether or not they had received Cy.

A further possible effect of Cy might have been on macrophage function. No defect in phagocytosis could be detected at the site of inoculation where there appeared to be a similar uptake of parasites within the macrophages of Cy-pretreated animals and controls.

Moreover, *in vitro* studies showed that Cy pretreatment of normal guinea-pigs 3 days before macrophage harvest, far from decreasing phagocytic potential showed some evidence of increasing it. However, the phagocytic ability of macrophages taken 21 days after infection was the same whether or not the guinea-pigs had been treated with Cy before infection.

Previous studies have shown that T-cell function is not greatly affected by Cy pretreatment. Although contact sensitivity and Jones–Mote reactions are increased in intensity in Cy-treated animals, the same treatment may cause some slight decrease in tuberculin reactivity following BCG vaccination or in tuberculin-like reactions to ovalbumin in animals sensitized with this antigen in Freund's complete adjuvant (Turk *et al.*, 1972; Turk & Parker, 1973). Moreover, Revell (1974) has found using a rosetting technique that there is a decrease in the total number of T as well as B lymphocytes in lymph nodes as well as in the peripheral blood, although B cells are affected to a far greater degree than T cells. Stockman *et al.* (1973) found that the effect of Cy on B cells was more severe and longer lasting than the effect on T cells.

In the present studies the effect of Cy pretreatment on delayed type hypersensitivity could not be detected until it was possible to demonstrate this reaction 4 weeks after infection. Thus it is not possible to say what effect there might have been on specific T-cell function during the first week after infection.

Antibody levels were detectable as early as 1 week after infection and were decreased by Cy during the first 4 weeks. Thus the earliest demonstrable effect of Cy in this system was on antibody production. The effect on delayed type hypersensitivity was not detectable until antibody production had begun to return to normal.

The present results therefore support the suggestion that more attention should be taken of the role of antibody in the early stages of *L. enriettii* infection of the guinea-pig (Rezai *et al.*, 1972; Behin *et al.*, 1975; Mauel *et al.*, 1975) especially during the first 2 weeks at a time when there are already significant levels of antibody and before significant delayed-type hypersensitivity can be detected by skin testing. It may be that control of this infection is due to a synergism between cell-mediated immunity and antibody production and that a decrease in either function can result in an increased incidence of widespread metastatic lesions.

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