Parasite Immunology 1983, 5, 95–108

The primary humoral immune response of European green lizards (*Lacerta viridis*) to *Leishmania agamae*

G.A.INGRAM & D.H.MOLYNEUX
Department of Biology, University of Salford, Salford M5 4WT

Accepted for publication 22 June 1982

Summary European green lizards, *Lacerta viridis*, produced relatively thermostable, dithiothreitol-sensitive, non-precipitating, agglutinins and complement-fixing antibodies (CFA) to *Leishmania agamae* administered subcutaneously (SC), intraperitoneally (IP) or orally (OR). Antibodies were also detected by the immobilization test (IMM) and by enzyme-linked immunosorbent assay (ELISA). The most sensitive method for the detection of stimulated immunoglobulins was ELISA. Antibodies were detected as early as 3 days post-infection with ELISA and between 5 and 7 for CFA, direct agglutination (DA) and indirect haemagglutination (IHA). In the case of IMM, the times of first detection varied from 14 to 28 days. Maximum CFA (2^{-8}), DA (2^{-8}), IHA (2^{-11}) and ELISA (2^{-16}) titres were reached from 42 to 49 days with significantly higher values occurring in the OR and IP groups. With IMM, maxima occurred after 5 or 6 weeks. Following exposure, two- to five-fold significant increases in serum lysozyme levels were demonstrated but the concentrations in sera following SC, IP or OR routes of antigen administration were not significantly different when the groups were compared with each other. The highest lysozyme values (approximately 12·3–12·5 μgml^{-1}) were found in the SC and OR groups when compared to the IP (7·40 μgml^{-1}).

Keywords: *Lacerta viridis, Leishmania agamae,* immune response, agglutinins, complement-fixing antibodies, ELISA, immobilization, lysozyme

Introduction

Besides mammals, the only other vertebrate group parasitized with *Leishmania* is the reptiles (Belova 1971a). In areas where mammalian leishmaniasis is endemic, lizards may play a role as *Leishmania* reservoirs in human infection since some reptiles are known to be hosts for many feeding sandfly species (Mutinga & Ngoka 1981).

Following the first report of flagellates in geckos morphologically similar to the promastigote stage of *L. tropica* (Sergent *et al.* 1914) other lizard species have been found infected with *Leishmania* (Nicoli 1963). To date, ten different species of saurian *Leishmania* have been described (Adler 1964; Wilson & Southgate 1979). They are either parasites of leucocytes, in particular monocytes (Dollahon & Janovy 1973; Edeson &

0141-9838/83/0100-0095$02.00 © 1983 Blackwell Scientific Publications

Himo 1973) and thrombocytes (Telford 1979), or occur in the intestine (Killick-Kendrick 1979). A review of the relevant literature has been presented by Wilson & Southgate (1979).

Reptilian *Leishmania* are usually non-infective to mammals (Safjanova & Aliev 1973) although in some cases they are pathogenic (Safjanova, Aliev & Koshelev 1972). In lizards naturally infected with reptilian *Leishmania* very low levels of infection are encountered (Belova 1971b). The first experimental infection of iguanids with *L. adleri*, a strain isolated from lizards, was described by Dollahon & Janovy (1973).

The only report of *Leishmania* in lacertidians was that of Heisch (1958) who isolated *L. adleri* from the heart blood of *Latastia longicaudata revoili*. Work upon the immune response in reptiles has been reviewed by Ambrosius (1976). Most of the studies concerned with immunity in lacertid lizards have involved the use of bacteria (Dimow 1968; Bukh 1940) or viral antigens (Sekeyova, Gresikova & Lesko 1970; Sixl, Sekeyova & Riedl 1971), with reports on antibody-dependent cytotoxic reactions (Jurd & Doritis 1977) and graft rejection (Worley & Jurd 1979).

This paper presents the results of a study on the humoral immune response of the European green lizard (*Lacerta viridis*) to *Leishmania agamae*.

Materials and methods

LIZARDS

European green lizards (*Lacerta viridis*) ranging in length from 165 to 279 mm (weight 10·8–42·7 g), were maintained at 27·5°C in an illuminated room in tanks, not more than four per cage, each containing sterilized gravel and refractory pipes. The animals were fed on live meal worms and small house crickets and were supplied with water.

PARASITES

Leishmania agamae (isolate *Agamae* 27; LV32), isolated from *Agama stellio*, were obtained from stock cultures at the Liverpool School of Tropical Medicine. The parasites were maintained in Novy, McNeal and Nicolle's medium—NNN (NaCl 6·67 gl^{-1} and 15·56 gl^{-1} agar in distilled water) to which was added 10% rabbit blood. The overlay consisted of a modified Locke's solution (NaCl 8 gl^{-1}; KCl 0·2 gl^{-1}; $CaCl_2$ 0·2 gl^{-1}; glucose 2·5 gl^{-1}; KH_2PO_4 0·3 gl^{-1} in distilled water). The culture media were inoculated with *L. agamae* and incubated at 25°C for 11–13 days after which time they were sampled and the cells counted.

EXPERIMENTAL PROTOCOL

Prior to infection the lizards were examined for the presence of naturally occurring trypanosomatids. They were anaesthetized by ether inhalation for 3 min and 0·5 ml blood was obtained from each by cardiac puncture. Blood smears were made in duplicate and bottles of sterile culture media (NNN/Locke's) were inoculated, incubated and examined for the presence of parasites. In addition, the gut contents of several crickets and meal

worms were examined both microscopically and by culture for the presence of trypanosomatid parasites.

Lizards were divided into five groups, each of 15 animals. The first group consisted of uninfected control animals and the second control group comprised lizards which had been given Locke's solution either subcutaneously (SC), orally (OR) or intraperitoneally (IP). The third group were injected IP with 0·5 ml $3{\cdot}78 \times 10^6$ *L. agamae* cells ml^{-1}. The fourth group received 0·05 ml of a similar concentration of parasites SC and each animal in the final group was individually fed OR three crickets, each of the latter containing 0·1 ml of an approximately $6{\cdot}4 \times 10^5$ suspension of *L. agamae*.

Crickets which had been injected with the *Leishmania* were sampled at regular intervals, between 1 min and 30 min before the OR treatment was performed. This examination was carried out in order to assess whether or not parasite inactivation or destruction had occurred due to the presence of 'natural' lysins or agglutinins within the insect haemolymph, or enzymes in the gut.

SERA

The lizards were anaesthetized, bled by cardiac puncture, killed and their lengths and weights recorded. The animals injected IP or orally fed were bled at 1-week intervals. Those injected SC were examined after 1, 3, 5, 15, 20, 25, 30 and 35 days. Control animals were bled at approximately weekly intervals. The blood was allowed to clot and kept overnight at 4°C. It was centrifuged at 3000 rpm for 5 min, the serum collected and stored at −20°C.

ANTIGEN EXTRACTS

Cultures of *L. agamae* were harvested, the promastigotes centrifuged at $5000 \times g$ for 15 min in chilled phosphate-buffered saline—PBS pH 7·2 (NaCl 8·5 gl^{-1}; Na_2HPO_4 1·07 gl^{-1} and NaH_2PO_4 0·39 gl^{-1} in de-ionized water), and the material stored at −20°C. After addition of cold PBS, the pellet was disrupted by sonication whilst the suspension was kept chilled. The sonicated suspension was then left overnight at 4°C and centrifuged for 30 min at $10\,000 \times g$. The supernatant protein concentration was measured by the method of Lowry *et al.* (1951) and stored at −20°C.

PRECIPITINS

Gel diffusion (GD): an immunodiffusion method as adapted for LKB glass plates was used as previously described by Ingram & Alexander (1980). Sera were challenged against concentrations of antigen extract ranging from 0·01 to 25 $mgml^{-1}$.

Counter-current electrophoresis (CCE): a modification of the method of Corkill (1977) was used and the details reported elsewhere (Ingram & Alexander 1980). The diameters of the wells were 3·5 mm, one of each pair of wells received 15 μl test serum and the other the same volume of the appropriate antigen dilution in PBS (0·1–30 $mgml^{-1}$). Electrophoresis was carried out at 6 volts cm^{-1} for up to 1 h. The incubation, processing of the plates and scoring system used have been described in previous papers (Ingram & Alexander 1976, 1977).

IMMOBILIZATION TEST

A modification of the method that Hines & Spira (1974) used for *Ichthyopthirius* was employed. Doubling dilutions of inactivated lizard sera were made in Locke's solution. To each dilution was added an equal volume of motile promastigotes (approximately 9×10^5 cells ml^{-1}). After incubation for 3 h at 25°C, the dilution series were examined for parasite motility and scored. Immobilization was taken as the dilution where 90% of the *L. agamae* failed to move progressively or display their characteristic motion. Tests were always run in duplicate together with the appropriate controls.

AGGLUTINATION

Direct agglutination (DA): doubling dilutions of inactivated lizard sera were made with PBS pH 7·4 containing 0·25 M NaCl. An equal volume of *Leishmania* ($1{\cdot}5 \times 10^6$ cells ml^{-1}) was added to each dilution and the mixtures incubated at 25°C for 2 h. The agglutination end point was taken as that dilution which just gave visible agglutination when compared with the controls.

Indirect (passive) haemagglutination (IHA): Weir's (1973) method was used. Sheep red blood cells were tanned with 0·0033% tannic acid coated with 0·85 $mgml^{-1}$ antigen extract in PBS. Doubling dilutions of inactivated lizard sera were prepared with PBS. To each dilution was added the same volume of 2·5% sheep red blood cells coated with antigen extract. Untanned, tanned and antigen-coated cells were included in the controls. The samples were incubated at 37°C for 1 h and overnight at 4°C. Agglutination was scored from 0 to 4+

COMPLEMENT-FIXING ANTIBODIES (CFA)

The complement fixation test was employed to determine the titres of CFA in lizard sera. The determination of any anti-complementary and haemolytic activities of the antigen extract, and the titrations of antigen, lysin and complement were as recorded by Ingram & Alexander (1977). The antigen extract was used at a concentration of 1·5 $mgml^{-1}$, complement at a dilution of 1 in 35, lysin at 1:500 and sheep red blood cells at 2%. Controls for lysin, complement, antigen and sheep erythrocytes were also set up. Fixation was allowed to proceed at 37°C for 1 h and the samples were scored. After further incubation overnight at 4°C, the trays were re-examined. The end point was taken as that dilution which gave 50% haemolysis.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The micro-ELISA technique used was adapted from Voller, Bartlett & Bidwell (1976). As a positive control, a rabbit anti-keyhole limpet haemocyanin (KLH) antiserum was challenged against KLH. Buffer and negative serum controls were also included. The concentration of the antigen extract used 40 μg ml^{-1}, rabbit anti-*Lacerta viridis* serum was used at a dilution of 1:100, goat anti-rabbit alkaline phosphatase at 1:2000 and the enzyme substrate at 1 $mgml^{-1}$. The end point antibody titre was taken as that dilution which was visibly different from the control wells.

Each end point in the antibody titrations was expressed as its $\log_{-2}$ value (i.e. 2^{-n}) and

the mean antibody titre as the $\log_{-2}$ geometric mean. The results were analysed statistically by the χ^2 test.

EFFECT OF DITHIOTHREITOL (DDT) AND HEAT

Sera were buffered at pH 8·6 with 0·01 M tris–HCl containing 0·15 M NaCl and DTT was added to each sample to give a final concentration of 0·05 M. The samples were incubated at 25°C for 1 h and examined for agglutinins and lysins, and by IMM and ELISA.

Samples of the same sera were heated at 56°C for 30 min and also examined for antibody activity.

LYSOZYME DETERMINATION

Serum lysozyme was measured by the agar (lyso) plate method of Osserman & Lawlor (1966). *Micrococcus lysodeikticus* substrate was used at a concentration of 50 mg 100 ml^{-1} and suspended in 1% purified agar in 0·07 M phosphate buffer pH 6·9 at 55°C. Twenty-two ml of the bacteria/agar mixture were poured onto glass plates, allowed to gel and incubated overnight at 4°C. Holes (3 mm diam.) were punched 15 mm apart in the agar and 10 μl serum dispensed into each well. A known control was included on each plate.

After incubation at 37°C for 3 h and overnight at 4°C, the plates were rinsed with PBS and placed for 2 min in 1·5% tannic acid. The diameters of the transparent hydrolysis zones were measured to the nearest 0·5 mm. The concentrations of lysozyme were read off a calibration curve constructed using dilutions of standard lysozyme ranging from 1 to 500 $\mu g ml^{-1}$.

Results

Eight LLS controls and 29 experimentally infected animals survived the duration of the experiments.

CONTROL ANIMALS

With reference to the values obtained for natural antibody activity in the ULS and LLS control groups, lizard sera possessing end point titres of over 2^{-2} for IMM and CFA, over 2^{-3} for both DA and IHA, and greater than 2^{-4} for ELISA were considered to be positive. The mean antibody titres of the control groups together with the standard errors of the means (SE) and ranges are given in Table 1.

IMMOBILIZATION

Immobilization (IMM) was first demonstrated in the OR and IP groups 14 and 28 days after infection respectively, with maximum titres being obtained after 5–6 weeks (Figure 1a). Lizards in the SC group were all considered to be negative.

When the two control groups were compared with each other, the results were not significantly different ($P > 0·05$). The OR and IP values were significantly different

Table 1. The group sizes, means ± standard errors of the means (ranges) of the immobilization (IMM), direct agglutination (DA), complement-fixing (CFA), indirect haemagglutination (IHA) and ELISA antibody titres ($\log_{-2}$) to *Leishmania agamae* in the sera of lizards from the different treatment groups

Group*	No. of animals	IMM	DA	CFA	IHA	ELISA
ULS	15	0·60 ± 0·19 (0–2)	0·8 ± 0·26 (0–3)	0·20 ± 0·14 (0–2)	1·27 ± 0·27 (0–3)	1·70 ± 0·32 (0–4)
LLS	8	0·38 ± 0·13 (0–2)	0·75 ± 0·27 (0–2)	0·38 ± 0·26 (0–2)	1·00 ± 0·35 (0–3)	1·63 ± 0·58 (0–4)
SC	8	0·88 ± 0·31 (0–2)	3·14 ± 1·11 (0–5)	2·00 ± 0·71 (0–4)	4·19 ± 1·48 (2–7)	6·00 ± 2·12 (4–9)
OR	10	2·80 ± 0·89 (0–5)	5·20 ± 1·64 (3·8)	5·40 ± 1·71 (3·8)	7·80 ± 2·47 (5–11)	11·00 ± 3·48 (8–16)
IP	11	1·82 ± 0·33 (0–4)	2·90 ± 0·87 (0–7)	3·45 ± 1·04 (0–7)	6·45 ± 1·95 (2–11)	10·18 ± 3·07 (4–15)

* ULS = normal lizard serum, LLS = Locke's solution control, SC = subcutaneous, OR = oral, IP = intraperitoneal.

($P < 0{\cdot}025$) from those of the controls but the SC were not. There was no significant difference between the OR and IP groups.

AGGLUTININS AND LYSINS (FIGURE 1B, 1C AND 1D)

Agglutinins and lysins were detected 5–7 days after *Leishmania* administration by the SC and OR routes, and between 3 and 4 weeks in the case of IP. Maximum titres were obtained on day 42 and day 49.

When DA, CFA and IHA values for ULS were compared with those obtained from the LLS group no significant differences were found. All three experimental groups were significantly different from the control groups ($P < 0{\cdot}01$). When the OR group was compared with that of the IP, a significant difference was found for DA ($P < 0{\cdot}05$).

ELISA (FIGURE 1E)

Antibodies were detectable by ELISA as early as 3 days in the SC group and on the first day of sampling, day 7, in the cases of the OR and IP groups. Maximum titres were reached after 6–7 weeks.

When the control groups were compared with each other the differences were not significant. Significant comparisons were found when the control groups were compared to the infected ones ($P < 0{\cdot}005$). The results for IP versus OR were not significantly different.

Of the sera from 29 surviving lizards infected with *L. agamae*, irrespective of route, 11 were positive for IMM, 15 for DA, 19 for CFA, 24 for IHA and 27 by ELISA.

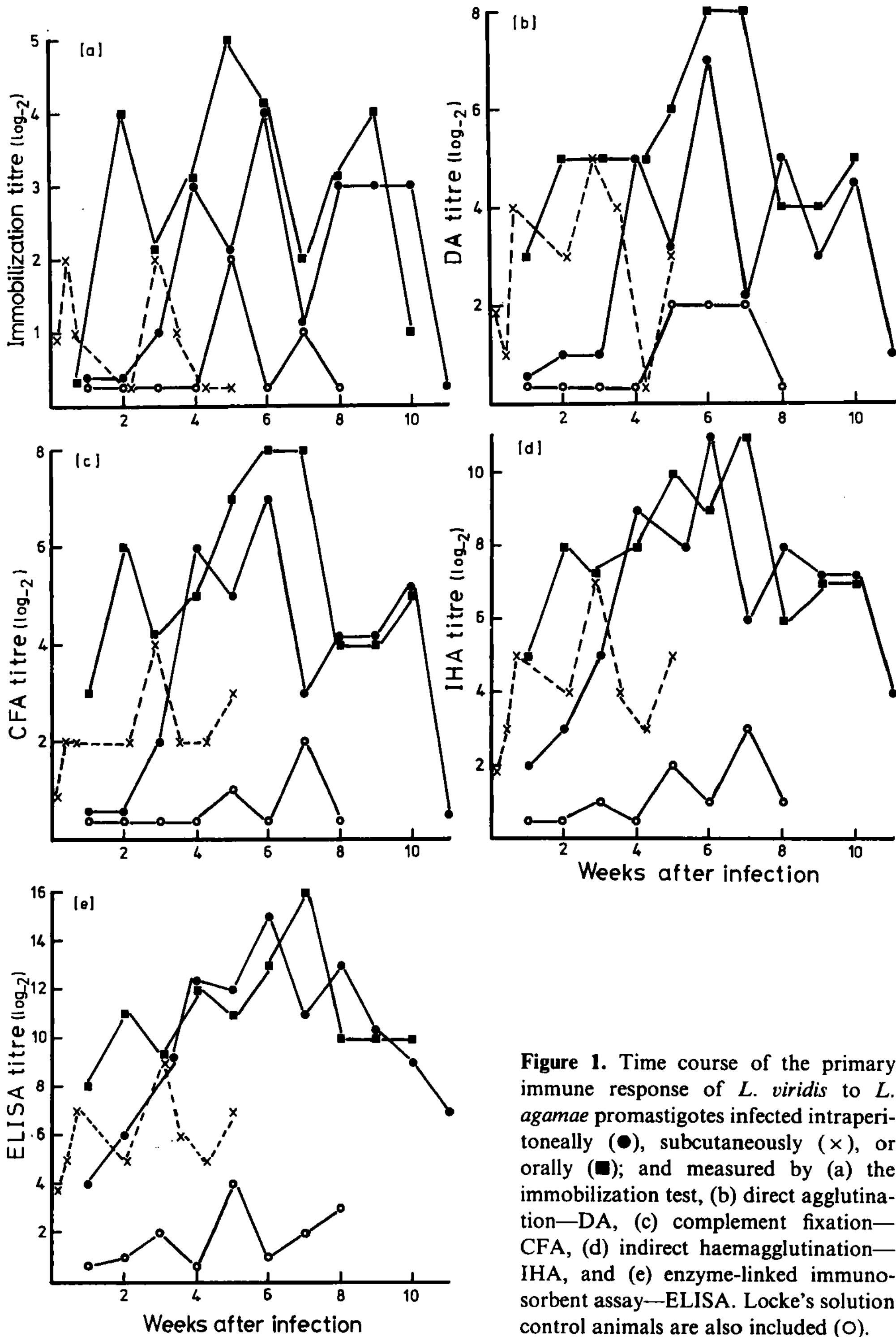

Figure 1. Time course of the primary immune response of *L. viridis* to *L. agamae* promastigotes infected intraperitoneally (●), subcutaneously (×), or orally (■); and measured by (a) the immobilization test, (b) direct agglutination—DA, (c) complement fixation—CFA, (d) indirect haemagglutination—IHA, and (e) enzyme-linked immunosorbent assay—ELISA. Locke's solution control animals are also included (○).

Table 2. The group sizes, means ± standard errors of the means (ranges) of the immobilization (IMM), direct agglutination (DA), complement-fixing (CFA), indirect haemagglutination (IHA) and ELISA antibody titres ($\log_{-2}$) in the sera of lizards after treatment with dithiothreitol (DTT)

Group*	No. of animals	IMM	DA	CFA	IHA	ELISA
ULS	15	0·20 ± 0·11 (0–1)	0	0	0	0·13 ± 0·09 (0–1)
LLS	8	0·13 ± 0·13 (0–1)	0	0	0	0·13 ± 0·13 (0–1)
SC	8	0·38 ± 0·15 (0–1)	0	0	0·25 ± 0·16 (0–1)	0·63 ± 0·26 (0–2)
OR	10	0·30 ± 0·15 (0–4)	0·60 ± 0·22 (0–2)	0·50 ± 0·22 (0–2)	1·00 ± 0·32 (0–3)	2·18 ± 0·53 (0–4)
IP	11	0·09 ± 0·09 (0–1)	0·44 ± 0·22 (0–2)	0·54 ± 0·25 (0–2)	1·00 ± 0·33 (0–3)	1·73 ± 0·43 (0–4)

* ULS = normal lizard serum, LLS = Locke's solution control, SC = subcutaneous, OR = oral, IP = intraperitoneal.

Table 3. The group sizes, means ± standard errors of the means (ranges) of the immobilization (IMM), direct agglutination (DA), complement-fixing (CFA), indirect haemagglutination (IHA) and ELISA antibody titres ($\log_{-2}$) in the sera of lizards after heating at 56°C for 30 min

Group*	No. of animals	IMM	DA	CFA	IHA	ELISA
ULS	15	0·33 ± 0·13 (0–1)	0·53 ± 0·18 (0–2)	0·07 ± 0·07 (0–1)	0·80 ± 0·20 (0–2)	0·97 ± 0·25 (0–2)
LLS	8	0·13 ± 0·13 (0–1)	0·50 ± 0·26 (0–2)	0·13 ± 0·13 (0–1)	0·38 ± 0·18 (0–1)	0·76 ± 0·25 (0–2)
SC	8	0·38 ± 0·26 (0–2)	1·37 ± 0·42 (0–3)	0·88 ± 0·35 (0–3)	2·63 ± 0·65 (0–6)	4·38 ± 0·53 (0–7)
OR	10	1·40 ± 0·34 (0–3)	3·70 ± 0·56 (1–7)	3·60 ± 0·60 (1–7)	6·00 ± 0·65 (3–9)	9·20 ± 0·68 6–13)
IP	11	0·90 ± 0·90 (0–2)	1·73 ± 0·51 (0–5)	1·91 ± 0·55 (0–5)	4·45 ± 0·77 (1–10)	7·73 ± 0·89 (2–11)

* ULS = normal lizard serum, LLS = Locke's solution control, SC = subcutaneous, OR = oral, IP = intraperitoneal.

The group mean IMM, DA, IHA, CFA and ELISA titres for the infected animals, SE and ranges are given in Table 1.

EFFECT OF HEAT AND DTT

Treatment of lizard sera with DTT resulted in the antibody titres in the controls being virtually negated whilst those in the experimentally infected groups were either drastically reduced to background levels or totally abrogated (Table 2). The titres in sera which had been heated at 56°C for 30 min were also reduced (Table 3).

PRECIPITINS

Neither controls nor infected animals showed precipitin activity to *Leishmania agamae* extracts by either GD or CCE.

LYSOZYME LEVELS (FIGURE 2)

The mean level of serum lysozyme of the ULS controls was 3·09 $\mu g ml^{-1}$ with an SE of 0·29 (range 0·95–4·9). For reptiles in the LLS control group the values were $2{\cdot}71 \pm 0{\cdot}55$ (0·95–5·9). In the cases of SC, OR and IP groups, the values were $12{\cdot}32 \pm 4{\cdot}75$ (2·3–34), $12{\cdot}45 \pm 4{\cdot}71$ (3·1–46) and $7{\cdot}40 \pm 2{\cdot}71$ (0·95–34) respectively. Sera from two lizards each had a high lysozyme concentration of 34 $\mu g ml^{-1}$ 25 and 30 days after SC injection although in the IP and OR treated animals, maximum values were found from day 49 to day 56.

Lysosyme levels were not significantly different between the ULS and LLS control animals. Significant increases occurred when the controls were tested against the three experimentally infected groups ($P < 0{\cdot}001$). The differences in lysozyme values were significant when comparisons were performed between the OR and the IP group ($P < 0{\cdot}001$).

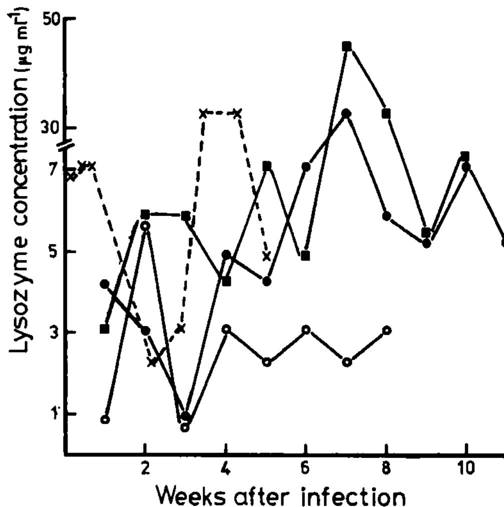

Figure 2. Lysozyme concentrations in the sera of *L. viridis* administered *L. agamae* promastigotes either intraperitoneally (●), subcutaneously (×), or orally (■). Control lizards given Locke's solution are also included (○).

Discussion

Low levels of 'naturally occurring' antibodies were detected in the sera of uninfected lizards. They may have been caused by the environmental presence of bacteria or other micro-organisms with related cell wall carbohydrate determinants. Such a relationship seems to occur between the human ABO blood group antigens and *Leishmania* (Greenblatt *et al.* 1981). On the other hand, these 'natural' antibodies may have been induced by the normal bacterial flora of the lizard intestine (Tan, Lim & Ishak 1978). Alternatively, this natural immunity may indicate previous infection with either *L. agamae* or other reptilian *Leishmania* spp. (Zuckerman & Lainson 1977) or certain mammalian leishmanias with common antigenic determinants (Aliev *et al.* 1972; Safjanova & Aliev 1973).

The times of first detection and maximum antibody titres against *L. agamae* are similar to those reported for the other reptiles following antigenic stimulation (Ambrosius & Frenzel 1972; Kanakambika & Muthukkaruppan 1972; Sekeyova *et al.* 1970). Significantly elevated antibody levels were found when both control groups were compared to each of the experimental groups. Overall, the orally infected lizards produced higher titres than those injected SC or IP. Possibly this is because the gut-associated lymphoid tissue (GALT) present in reptiles (Solas & Zapata 1980) has been stimulated by the *Leishmania* released from the house crickets by digestion in the gut. Some of the immunoglobulins from the stimulated lymphocytes naturally enter the general circulation via the hepatic portal system. Although the alimentary canal is claimed to be the most likely route of infection in sandflies (Killick-Kendrick 1979), this mode of entry is still controversial (Adler & Theodor 1957). Furthermore, it is doubtful whether the alimentary canal is the natural route of infection in lizards by reptilian *Leishmania* spp. (Killick-Kendrick 1979). However, irrespective of the route of laboratory infection leishmanial parasites were found in the GALT using immunoperoxidase-labelled immunoglobulins (Ingram & Molyneux, unpublished observations). In contrast, in the sea turtle, *Chelonia mydas*, no significant differences have been found with different injection routes (Benedict & Pollard 1972). In lizards other parameters such as nature and dose of the antigen are known to influence the immune response (Ambrosius 1976).

No precipitins were detected in *L. viridis* sera. In reptiles precipitation of antigen–antibody complexes appears to depend upon the nature of the antigen, with a requirement for high antigen concentration (Ambrosius & Frenzel 1972). Also, the immunoglobulins produced must have a relatively high affinity for the antigenic determinants (Frenzel 1971). Reptiles produce IgM which is an efficient agglutinin and CFA but a poor precipitin. In addition, they synthesize an IgG-like immunoglobulin (IgRAA—Atwell & Marchalonis 1976) which is a good precipitin but a weak agglutinin (Ambrosius 1976). In the present study a wide range of concentrations of *Leishmania* extract was used in both the GD and CCE tests. It is possible that the lack of precipitating activity was due to a low avidity between the leishmanial antigens and lizard high molecular weight (HMW) immunoglobulins and that no IgRAA was produced.

Inactivation of IgM by reduction with DTT or similar mercaptans such as 2-mercaptoethanol (2-ME) has been used to distinguish IgM from IgG (Beale & Kent 1977; Capel *et al.* 1980). In the present study, DTT either completely negated or reduced the antibody titres of immune *L. viridis* sera to background levels (Table 2). In reptiles,

high molecular weight IgM is produced early in the immune response and is 2-ME-sensitive whilst low molecular weight IgG-like molecules appear later and are 2-ME-resistant (Ambrosius 1970, 1976). In *L. viridis* there did not appear to be a shift from one type of antibody to the other. It is possible that a longer period of *L. agamae* administration may be required in order to stimulate IgRAA production. The reduction in antibody titres by heating the sera at 56°C for 30 min, further supports the idea that an immune macroglobulin, probably IgM, was the only immunoglobulin produced following primary antigenic stimulation in *L. viridis*. Similar results have been reported for other reptiles after injection with cellular antigens (Marchalonis, Ealey & Diener 1969; Wetherall & Turner 1972).

Natural lytic activity against *L. agamae* was very low even though lizards possess an active complement system (Gigli & Austen 1971) with the capacity to react with endotoxic cell wall antigens (Day *et al.* 1970). However, the immunoglobulins of *L. viridis* are capable of a high degree of complement fixation in the presence of antigen and the complement system of this species may be activated via the classical or the alternate pathway; a situation which is found in the host–parasite relationship of mammals (Santoro, Bernal & Capron 1979). Therefore, the induction of complement fixation may be an important mechanism by which lizards partly counteract infection with *Leishmania* promastigotes.

Levels of lysozyme in the control sera (1–5 $\mu g ml^{-1}$) were approximately half those reported for human sera (Zorn *et al.* 1980). Upon antigenic stimulation, a two- to five-fold increase in serum lysozyme concentration occurred. The higher similar mean titres for the SC and OR groups in contrast to the lower mean value for IP may reflect the mode of parasite transmission by skin bite or ingestion. The amounts of lysozyme in stimulated lizards are comparable to those found with certain human diseases (Cooper *et al.* 1980). No reports on the levels of lysozyme in the sera of lizards have been found in the literature.

In mammals, lysozyme has been shown to originate largely in monocytes and macrophages (Kokoshis & Di Luzio 1979). It has been shown that lizards experimentally infected with *Leishmania* phagocytose promastigotes, with blood monocytes and other leucocytes (Dollahon & Janovy 1973, 1974) as well as peritoneal exudate cells (Dollahon & Janovy 1973) being implicated.

In the present report no evidence of antigen was observed in blood smear leucocytes but antigen was demonstrated in several body organs (Ingram & Molyneux, unpublished observations) which is in accordance with the sites of detection in other poikilothermic vertebrates (Fänge, Lundblad & Lind 1976; Fletcher & White 1973). It is therefore possible that parasites may stimulate lysozyme release by lizard leucocytes although evidence for this is still lacking.

The increases in serum lysozyme levels paralleled the post-infection rise in antibodies. Similar findings have been reported in fish (Vladimirov 1968). Lysozyme may work either alone or in conjunction with antibody and complement to further enhance the reduction of infection. It is possible that lysozyme could play an important part in the intrinsic defence mechanisms, both natural and acquired, in lizards against protozoan parasites and assist in their destruction.

Apart from an immunological function, lysozyme participates in a digestive role. It can partially hydrolyse the chitinous exoskeleton of insects, including sandflies, taken in during feeding. This would partly account for the low levels of lysozyme in uninfected

lizards and the fractionally higher mean levels of the enzyme when orally given crickets containing *L. agamae*.

Acknowledgements

The authors wish to thank Drs S.L.Croft and J.B.Alexander for reading the manuscript and for their useful comments.

References

ADLER S. (1964) *Leishmania. Advances in Parasitology* **2,** 35

ADLER S. & THEODOR O. (1957) Transmission of disease agents by phlebotomine sandflies. *Annual Review of Entomology* **2,** 203

ALIEV E.I., SAFJANOVA V.M., NADZHAFOV H.Y. & GASANZADE G.B. (1972) Comparative serological study of *Leishmania tropica major* and *Leishmania tropica minor. Meditsinskaya Parazitologiya: Parazitarnye bolezni (Moskva)* **41,** 531

AMBROSIUS H. (1970) Some aspects of the phylogeny of immunity. *Annals of Immunology, Poland* **2,** 177

AMBROSIUS H (1976) Immunoglobulins and antibody production in reptiles. In *Comparative Immunology*, ed. J. J. Marchalonis, p. 298. Blackwell Scientific Publications, Oxford

AMBROSIUS H. & FRENZEL E.M. (1972) Anti-DNP antibodies in carps and tortoises. *Immunochemistry* **9,** 65

ATWELL J.L. & MARCHALONIS J.J. (1976) Immunoglobulin class of lower vertebrates distinct from IgM immunoglobulin. In *Comparative Immunology*, ed. J. J. Marchalonis, p. 276. Blackwell Scientific Publications, Oxford

BEALE D. & KENT C.M. (1977) The effect of dithiothreitol on agglutination and complement fixation by porcine 19 S IgM. *Biochimica et Biophysica Acta* **494,** 209

BELOVA E.M. (1971a) Reptiles and their importance in the epidemiology of leishmaniasis. *Bulletin of the World Health Organization* **44,** 553

BELOVA E.M. (1971b) Susceptibility of reptiles to infection with *Leishmaniae* and non-identified cultures of leptomonads. *Parasizologiya* **5,** 466

BENEDICT A.A. & POLLARD L.W. (1972) Three classes of immunoglobulins found in the sea turtle, *Chelonia mydas. Folia Microbiologica* **17,** 75

BUKH P.L. (1940) Humoral immunity from the viewpoint of comparative pathology. III. On the formation of agglutinins in cold-blooded animals. *Medichnii Zhurnal* **10,** 1153

CAPEL P.J.A., GERLAG P.G.G., HAGEMANN J.F.H.M. & KOENE R.A.P. (1980) The effect of 2-mercaptoethanol on IgM and IgG antibody activity. *Journal of Immunological Methods* **36,** 77

COOPER B.T., UKABAM S.O., BARRY R.E., ALLAN R.N. & READ A.E. (1980) Serum lysozyme activities in coeliac disease: an aid to diagnosis of malignant change? *Clinical Science* **59,** 25

CORKILL J.E. (1977) Counterimmunoelectrophoresis. *Current Topics in Medical Laboratory Sciences* **2,** 1

DAY N.K., GOOD R.A., FINSTAD J., JOHANNSEN R., PICKERING R.J. & GEWURZ H. (1970) Interactions between endotoxic lipopolysaccharides and the complement system in the sera of lower vertebrates. *Proceedings of the Society for Experimental Biology and Medicine* **133,** 1397

DIMOW I. (1968) Versuch zur künstlichen infektion von eidechsen (*Lacerta muralis*) mit Salmonella -und Arizonabakterien. *Zentralblatt für Veterinärmedizin, Reihe B* **13,** 587

DOLLAHON N.R. & JANOVY J. (1973) *Leishmania adleri*: *in vitro* phagocytosis by lizard leukocytes and peritoneal exudate cells. *Experimental Parasitology* **34**, 56

DOLLAHON N.R. & JANOVY J. (1974) Experimental infection of New World lizards with Old World lizard *Leishmania* species. *Experimental Parsitology* **36**, 253

EDESON J.F.B. & HIMO J. (1973) *Leishmania* sp. in the blood of a lizard (*Agama stellio*) from Lebanon. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **67**, 27

FÄNGE R., LUNDBLAD G. & LIND J. (1976) Lysozyme and chitinase in blood and lymphomyeloid tissues of marine fish. *Marine Biology* **36**, 277

FLETCHER T.C. & WHITE A. (1973) Lysozyme activity in the plaice (*Pleuronectes platessa* L.). *Experientia* **29**, 1283

FRENZEL E.M. (1971) Differences in the precipitin reaction between lower and higher vertebrates. In *Antigen–Antibody Reactions*, eds H. Ambrosius, J. Malberg & H. Schaffner, p. 40. Jena

GIGLI I. & AUSTEN K.F. (1971) Phylogeny and function of the complement system. *Annual Review of Microbiology* **25**, 309

GREENBLATT C.L., KARK J.D., SCHNUR L.F. & SLUTZKY G.M. (1981) Do *Leishmania* serotypes mimic human blood group antigens? *Lancet* **1**, 505

HEISCH R.B. (1958) On *Leishmania adleri* sp. nov. from lacertid lizards (*Latastia* sp.) in Kenya. *Annals of Tropical Medicine and Parasitology* **52**, 68

HINES R.S. & SPIRA D.T. (1974) Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). V. Acquired immunity. *Journal of Fish Biology* **6**, 373

INGRAM G.A. & ALEXANDER J.B. (1976) The immune response of brown trout (*Salmo trutta* L.) to injection with soluble antigens. *Acta Biologica et Medica Germanica* **35**, 1561

INGRAM G.A. & ALEXANDER J.B. (1977) The primary immune response of brown trout (*Salmo trutta*) to injection with cellular antigens. *Journal of Fish Biology* **10**, 63

INGRAM G.A. & ALEXANDER J.B. (1980) The immune response of the brown trout *Salmo trutta* to lipopolysaccharide. *Journal of Fish Biology* **16**, 181

JURD R.D. & DORITIS A. (1977) Antibody-dependent cytotoxicity in poikilotherms. *Developmental and Comparative Immunology* **1**, 341

KANAKAMBIKA P. & MUTHUKKARUPPAN V.R. (1972) The immune response to sheep erythrocytes in the lizard *Calotes versicolor*. *Journal of Immunology* **109**, 415

KILLICK-KENDRICK R. (1979) Biology of *Leishmania* in phlebotomine sandflies. In *Biology of Kinetoplastida*, eds W. H. R. Lumsden & D. A. Evans, vol. 2, p. 395. Academic Press, London

KOKOSHIS O. L. & DI LUZIO N. R. (1979) Serum lysozyme: an index of macrophage function. *Journal of the Reticuloendothelial Society* **25**, 85

LOWRY O.H., ROSEBROUGH N.J., FARR L. & RANDALL R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265

MARCHALONIS J.J., EALEY E.H.M. & DIENER E. (1969) Immune responses of the tuatara, *Sphenodon punctatum*. *Australian Journal of Experimental Biology and Medical Science* **47**, 367

MUTINGA M.J. & NGOKA J.M. (1981) Suspected vectors of lizard leishmaniasis in Kenya and their possible role in partial immunisation of the human population against *Leishmania donovani* in Kala-azar endemic areas. *Insect Science and Its Application* **1**, 207

NICOLI R.M. (1963) Le genre *Leishmania* R. Ross, 1903. *Bulletin de la Société de Pathologie Exotique et de ses Filiales* **56**, 408

OSSERMAN E. F. & LAWLOR D. P. (1966) Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *Journal of Experimental Medicine* **124**, 921

SAFJANOVA V.M. & ALIEV E.I. (1973) Comparative study of biological characteristics of the causal agents of zoonotic and anthroponotic cutaneous leishmaniasis in the USSR. *Bulletin of the World Health Organization* **49**, 499

SAFJANOVA V.M., ALIEV E.I. & KOSHELEV B.A. (1972) On biological characters of *Leishmania adleri* Heisch, parasite of lizards, pathogenic for mammals. *Parazitologiya* **6**, 206

SANTORO F., BERNAL J. & CAPRON A. (1979) Complement activation by parasites. *Acta Tropica* **36**, 5

SEKEYOVA M., GRESIKOVA M. & LESKO J. (1970) Formation of antibody to tick-bone encephalitis virus in *Lacerta viridis* and *L. agilis* lizards. *Acta Virologica* **14**, 87

SERGENT ED., SERGENT ET., LEMAIRE G. & SEVERNET G.L. (1914) Insecte transmetteur et réservoir de virus du clou de Biskra. Hypothèse et expériences préliminaires. *Bulletin de la Société de Pathologie Exotique et de ses Filiales* **7**, 577

SIXL W., SEKEYOVA M. & RIEDL H. (1971) Ein fund von antikörpern gegen Arboviren in *Lacerta agilis*. *Archiv für Hygiene und Bakteriologie* **154**, 609

SOLAS M.T. & ZAPATA A. (1980) Gut-associated lymphoid tissue (GALT) in reptiles: intraepithelial cells. *Development and Comparative Immunology* **4**, 87

TAN R.J.S., LIM E.W. & ISHAK B. (1978) Intestinal bacterial flora of the household lizard, *Gecko gecko*. *Research in Veterinary Science* **24**, 262

TELFORD S.R. (1979) Evolutionary implications of *Leishmania* amastigotes in circulating blood cells of lizards. *Parasitology* **79**, 317

VLADIMIROV V.L. (1968) Immunity in fish. *Bulletin Office International des Épizooties* **69**, 1365

VOLLER A., BARTLETT A. & BIDWELL D.E. (1976) Enzyme immunoassays for parasitic diseases. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **70**, 98

WEIR D.M. (1973) Ed. *Handbook of Experimental Immunology* Blackwell, Oxford

WETHERALL J.D. & TURNER K.J. (1972) Immune response of the lizard *Tiliqua rugosa*. *Australian Journal of Experimental Biology and Medical Science* **50**, 79

WILSON V.C.L.C. & SOUTHGATE B.A. (1979) Lizard *Leishmania*. In *Biology of the Kinetoplastida*, eds W. H. R. Lumsden & D. A. Evans, vol. 2, p. 241. Academic Press, London

WORLEY R.T.S. & JURD R.D. (1979) The effect of a laboratory environment on graft rejection in *Lactera viridis*, the European green lizard. *Developmental and Comparative Immunology* **3**, 653

ZORN S.K., STEVENS C.A., SCHACHTER E.N. & GEE J.B.L. (1980) The angiotensin converting enzyme in pulmonary sarcoidosis and the relative diagnostic value of serum lysozyme. *Lung* **157**, 87

ZUCKERMAN A. & LAINSON R. (1977) *Leishmania*. In *Parasitic Protozoa*, ed. J. P. Kreier, vol. 1, p. 58, Academic Press, London