Age, acquired immunity and the risk of visceral leishmaniasis: a prospective study in Iran

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SUMMARY

In order to investigate the phenomenon of age-related immunity to visceral leishmaniasis, a 1 year prospective survey was carried out on 5671 people in a *Leishmania infantum* focus in north-west Iran. The average incidence rate of infection since 1985 was 2·8%/year with all ages equally at risk. One in 13 infections in children led to visceral leishmaniasis (VL), and this ratio decreased significantly with age. Seroprevalence also dropped rapidly with age, suggesting that the same process may affect both clinical outcome and the humoral immune responses. Cell-mediated immunity was associated with a reduction in the seroconversion rate and an increase in the serorecovery rate. Even amongst people with no detectable cell-mediated immunity to *Leishmania*, the seroconversion rate decreased and the serorecovery rate increased with age. All current VL patients had a negative leishmanin skin test response. Hence, adults may develop protection against *L. infantum* through 2 processes, 1 dependent and 1 independent of acquired cell-mediated immunity.

Key words: *Leishmania infantum*, visceral leishmaniasis, acquired immunity.

INTRODUCTION

Most *Leishmania infantum* infections are thought to be subclinical and characterized by the development of a specific cell-mediated or humoral response but no symptoms (Pampiglioni *et al*. 1974, 1975; Badaro *et al*. 1986*b*). In endemic zones where *L. infantum* causes visceral leishmaniasis (VL) the great majority of cases tend to be children (Bettini, Maroli & Gradoni, 1981; Navin *et al*. 1985; Harrat *et al*. 1992; Edrissian, 1996). In contrast, where *L. infantum* infections cause cutaneous leishmaniasis all ages appear to be susceptible (Bettini *et al*. 1981; Gasanzade *et al*. 1990). The increasing frequency of HIV-*L. infantum* co-infections has also led to increases in the proportion of VL cases amongst adults in Europe (Alvar *et al*. 1997). However, the precise relationship between either age or acquired immunity and the relative rates of subclinical and clinical infections remains uncertain (Dye & Williams, 1993). Although cross-sectional immuno-epidemiological surveys can provide clues to the factors determining the course of *L. infantum* infection, the interpretation of cross-sectional patterns depends on precise information on the recovery rates of the immunological responses that are recorded. The interpretation may be confused further by the confounding effects of (1) time and age and (2) age and acquired immunity. Prospective surveys which record immune status and disease status at more than 1 time-point should, in principle, be able to distinguish the effects of time, age, and acquired immunity.

Here we present the result of a prospective study of *L. infantum* infection and disease in an endemic focus of VL in north-west Iran. The study population, comprising all ages, was diagnosed at 2 time-points both by the leishmanin skin test (LST), which detects the cell-mediated response, and the direct agglutination test (DAT), which detects the humoral response. The parameter estimates for infection and recovery (for both LST and DAT) derived from the prospective survey are incorporated into a mathematical model which makes explicit the biological processes responsible for the age prevalence curves for both the DAT and LST response. We then derive 3 estimates of the proportion of infections causing disease (on the basis of the historical, prevalent or incident cases identified from the survey), and show how this proportion changes with age in the absence of detectable acquired immunity.

MATERIALS AND METHODS

Study population and survey design

The study was carried out in the north-west of Iran in the neighbouring provinces of Ardabil (districts of Meshkin-Shahr and Ghermi) and East Azerbaijan

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(district of Kalaybar), which together comprise the principal endemic focus of visceral leishmaniasis in Iran (Edrissian *et al.* 1988; Soleimanzadeh *et al.* 1993; Edrissian, 1996). Nearly all the population in this region live between 500 and 2500 m above sea level. All parasites isolated from humans or dogs infected with visceral leishmaniasis in this region have been typed by isoenzymes as *L. infantum* (Edrissian, 1996; Mazloumi Gavgani, unpublished observations). The sandfly vectors have not been incriminated conclusively, but suspected vectors found in the focus include *Phlebotomus kandelakii*, *P. keshishiani*, *P. major* and *P. perfiliewi* (Nadim *et al.* 1992).

Thirty-eight villages were selected randomly for the serological survey, comprising 4·2% (17/403) of the villages in Meshkin-Shahr, 2·8% (10/352) in Ghermi, and 2·7% (11/405) in Kalaybar. About 30% of the households in each of the 38 villages were selected randomly for the survey (with informed consent). Largely due to absences, children were significantly better represented in the survey than adults: 30·5% of all children (up to 10 years old) in the 38 villages were surveyed, compared to 3·9% of the population above 10 years old. Demographic details were collected by questionnaire from the study population, and any history of visceral leishmaniasis was recorded and later confirmed by checking local MOH records. A total of 5390 individuals (1686 households) were included in the DAT survey carried out between August to December 1995, and 5172 individuals (96%) were serologically tested a second time between August and October 1996. The interval between the 2 surveys was 1 year for nearly all the study population, and for simplicity a 1 year period is assumed in all calculations of rates from the prospective survey data.

At the same time as the DAT survey, in 24 of the 38 study villages the LST was applied to 4068 individuals (1162 households), representing 14·1% of the population of these villages. All except 281 individuals of the LST study population was also tested by DAT in the first survey. A total of 3595 individuals (88%) received a second LST after a 1 year interval. The sampling bias for the LST survey was similar to that for the DAT survey, except that relatively few infants less than 1 year old were tested by LST (8·5%) compared to 23·6% tested by DAT. This was because the LST is difficult to administer to babies.

Direct agglutination tests

Finger-prick blood samples were spotted onto filter paper (Whatman no 4.), allowed to dry and stored at −20 °C prior to elution and diagnosis by DAT. Circles (6 mm diameter) were punched from the blood spots, equivalent to 10 μl of blood (Evergard, Linder & Lundbergh, 1988), and left overnight in 0·5 ml of citrate saline solution containing 1% foetal calf serum and 0·1 M 2-mercaptoethanol. The DAT antigen was prepared at the London School of Hygiene and Tropical Medicine by the standard method (WHO, 1996) from log phase promastigotes of a Sudanese strain of *L. donovani* (MHOM/SD/68/1S) grown in a modified αMEM, supplemented with foetal calf serum and antibiotics, and stored at 4 °C prior to use. The DAT was performed as described previously (Harith *et al.* 1986) with sequential dilutions of serum in a solution of 0·9% NaCl, 0·78% β-mercaptoethanol and 0·1% foetal calf serum. All samples were screened at a dilution of 1/100 in Iran (Tabriz Medical University), and positives were then re-tested and titrated out up to 1/102400 in London.

The cut-off point to designate infection with *L. infantum*, 1/1600, was chosen empirically by seeking the best correlations between the village incidence rates calculated by LST conversion and the village incidence rates calculated by seroconversion (using a series of different DAT titres to define seroconversion). The specificity of the DAT was confirmed by tests on healthy controls (from non-endemic sites) and on patients with other diseases (TB, toxoplasmosis and malaria).

Leishmanin skin tests

The leishmanin antigen used for skin testing was derived from *L. major* and prepared at the Pasteur Institute of Iran as previously described (Alimohammadian, Hakimi & Nikseresht, 1993). The volar surface of 1 forearm was injected intradermally with 0·1 ml of the antigen (at a concentration of 10^7 promastigotes/ml), and a control solution (0·1 ml of sterile pyrogen-free PBS with 0·01% thimerosal) was injected into the other arm. The LST response was measured 48 h later, and an induration width $\geqslant$ 5 mm was taken as a positive response. This cut-off point was tested empirically by confirming that there was no significant association between the distribution of induration sizes between 1 and 4 mm and the distribution of positive serological responses.

Statistical analysis

All regression analyses with age as an explanatory variable were carried out in EXCEL by least squares, dividing the population into 13 age groups with roughly equal numbers in each. For some analyses, regressions were only carried out on the first 10 age groups: i.e. on ages up to 10 years old. This cut-off was chosen on the basis of the evidence (described below) that this focus has only been endemic for about 10 years, and because a number of the parameter values tested appear to vary in a linear fashion up the age of about 10 years, after which they

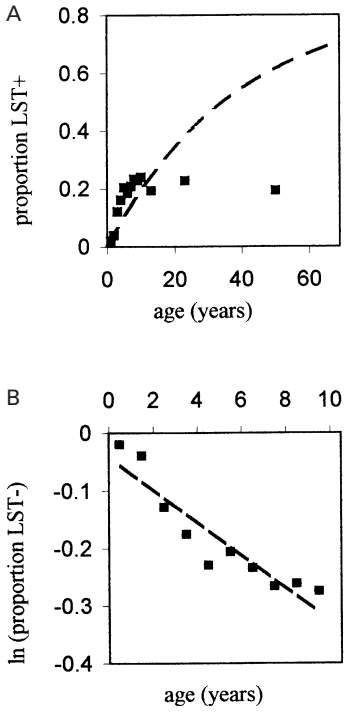

Fig. 1. (A) The relationship between age and the proportion of the population with a positive leishmanin skin test (LST) response. The observed data (points) are grouped into 13 age classes. The simulated data (––––) are generated from the equation: proportion LST+ at age $t = [\lambda/(\lambda+\rho)](1-e^{-(\lambda+\rho)t})$, where λ, the force of infection, and ρ, the recovery rate, were estimated from the prospective survey. (B) The relationship between age up to 10 years and the natural logarithms of the observed proportions of the population who are LST−. The broken line was fitted by least squares linear regression.

remain relatively constant. Chi-squared tests were carried out in Epi-Info and use the Yates corrected value. For all tests of association with age, the cut-off point of 10 years was selected in the contingency tables for the reasons described above and in order to minimize *post-hoc* decisions about age grouping.

RESULTS

The relationship between age and acquired cell-mediated immunity

The relationship between LST prevalence and age is characterized by a monotonically increasing curve which asymptotes at about 0·21 after 10 years (Fig. 1A). The apparent flattening in the curve was demonstrated statistically as a linear regression of ln[proportion LST−] against age was not significant ($F = 1{\cdot}50$, D.F. $= 1, 11$, $P = 0{\cdot}24$), whereas a quadratic regression was highly significant ($F = 7{\cdot}0$, D.F. $= 2, 10$, $P < 0{\cdot}01$, $r^2 = 0{\cdot}58$).

The 3 possible hypotheses to explain the asymptote in the age–prevalence curve were tested using the data collected in our surveys. The first hypothesis is that the LST response persists for a very short time (i.e. there is a high recovery rate). Simple infection-recovery models of leishmaniasis, as described previously (Davies *et al.* 1995), demonstrate that if the transmission rate is constant and age independent,

$$\text{the proportion infected at age } t = \lambda/(\lambda+\rho).(1-e^{-(\lambda+\rho)t}). \quad (1)$$

Hence, age prevalence should asymptote at $\lambda/(\lambda+\rho)$, where λ = the force of infection and ρ = the instantaneous recovery rate. It follows that the recovery rate would have to equal 3·8 times the force of infection in order to generate an asymptote at 0·21. We can discount this possibility as our prospective study detected a recovery rate of 0·004/year: 3 out of 684 who were LST+ at the first survey became LST− at the second survey 1 year later. This compares to an annual incidence rate of infection between the 2 surveys of 0·022/year (95% CI ±0·005), as 64 out of 2914 converted from LST− to LST+ (i.e. $\lambda = -\ln[1-0{\cdot}022] = 0{\cdot}022$/year). The divergence between the observed age prevalence data and the age prevalence curve predicted from our estimates of incidence and recovery rates (if constant with time and age) is clearly illustrated in Fig. 1A.

The second hypothesis to account for the age–prevalence asymptote is that teenagers and adults have a very small risk of infection, either because (1) the risk of being bitten by an infected sandfly decreases with age (e.g. due to an age-related behavioural change); or (2) the risk of being bitten by infected sandflies is highly variable amongst the study population, with about 21% at high risk (so all become infected by the age of 10 years) and the remainder with no significant risk (e.g. due to the location or nature of their homes). This hypothesis can be discounted again as the prospective study detected no significant association between age and LST incidence rate (Table 1). The mean LST incidence rate in children up to 10 years was 0·023/year (51/2214), which compares to 0·019/year (13/691) amongst the population greater than 10 years ($\chi^2 = 0{\cdot}31$, $P = 0{\cdot}31$).

The third hypothesis for the asymptote in the LST age–prevalence curve is that the transmission rate in this region has not been constant over time. An asymptote at 10 years of age would be expected if the region has been endemic for only 10 years. This hypothesis is supported by the Ministry of Health's records of VL cases reported annually in the 3 Districts surveyed: the mean annual number of cases in this region between 1980 and 1986 was 27 (range: 1–74), and between 1987 and 1994 was 238 (range: 127–493).

As LST prevalence apparently increases monotonically for the first 10 years of age, it is possible to estimate the mean incidence rate of infection during

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Table 1. The relationship between age and (1) the annual incidence rate during the prospective survey, according to the proportion developing either a positive leishmanin skin test (LST) or a positive direct agglutination test (DAT) response, and (2) the recovery rate of DAT responsiveness measured from the same survey

(*N*, number tested; *I*, annual incidence rate; *R*, annual recovery rate.)

	LST conversion			DAT conversion			DAT recovery		
Age (years)	*N*	No. converted	I (/year)	*N*	No. converted	I (/year)	*N*	No. recovered	R (/year)
0	33	1	0·030	208	6	0·029	13	2	0·15
1	165	5	0·030	430	16	0·037	31	8	0·26
2	293	6	0·021	476	17	0·036	39	14	0·36
3	221	6	0·027	354	14	0·040	30	12	0·40
4	277	7	0·025	425	14	0·033	34	16	0·47
5	236	6	0·025	351	10	0·028	27	13	0·48
6	266	4	0·015	436	12	0·028	30	17	0·57
7	243	6	0·025	388	8	0·021	29	18	0·62
8	290	6	0·021	498	8	0·016	21	15	0·71
9	190	4	0·021	286	5	0·017	13	11	0·85
10–14	257	6	0·023	435	7	0·016	15	13	0·87
15–29	283	4	0·014	379	6	0·016	18	16	0·89
30–70	160	3	0·019	197	3	0·015	9	8	0·89
Total	2914	64	0·022	4863	126	0·026	309	163	0·53

the previous 10 years by a linear regression of ln[proportion LST−] against age, t (in years) up to the age of 10 (Fig. 1B; F = 51·1, D.F. = 1, 8, $P < 0{\cdot}01$, $r^2 = 0{\cdot}86$):

$$\ln[\text{proportion LST}-] = -0{\cdot}042 - 0{\cdot}028[t]. \quad (2)$$

it follows that the mean annual incidence rate during the last 10 years = $1 - e^{-0{\cdot}028} = 0{\cdot}028$/year (95% CI ± 0·008), i.e. not significantly different from the incidence rate of infection detected during the prospective survey.

Effects of age and acquired cell-mediated immunity on the humoral response

The relationship between age and seroprevalence (Fig. 2A) is characterized by a peak of about 0·08 in the age group 1–6 years, followed by a rapid decline and a relatively constant low prevalence of about 0·04 in the population above 10 years. The relationship between age and the proportion with high DAT titres (> 1/3200) shows a similar pattern.

The factors that could explain this age–seroprevalence pattern were then investigated using the prospective survey data. First, the seroconversion rate could decrease with age. Inspection of the prospective data (Table 1) indicates that the seroconversion rate does decrease during the first 10 years of age, after which it stays relatively constant; and the seroconversion rate in children up to 10 years old, 0·029/year (110/3852), is significantly higher than the seroconversion rate in those above 10 years old, 0·016/year (16/1011): $\chi^2 = 4{\cdot}7$, $P = 0{\cdot}03$, RR = 1·8 [1·0–1·3]. Secondly, the serorecovery rate could increase with age. This is also apparent from inspection of Table 1; and the serorecovery rate in children up to 10 years old, 0·47/year (126/267), is significantly lower than that in people above 10 years old, 0·88/year (37/42): $\chi^2 = 5{\cdot}0$, $P < 0{\cdot}001$, RR = 0·47[0·45–0·63].

Next we investigate the evidence for the 2 processes which could explain these age-related changes in the humoral response to *L. infantum*: the development of acquired cell-mediated immunity (i.e. in response to prior *Leishmania* infections) or an age-related change in the immune system unrelated to acquired cell-mediated immunity (i.e. possibly in the absence of *Leishmania* exposure). As LST responsiveness is considered to be an accurate reflection of cell-mediated immunity acquired from prior *Leishmania* exposure, these 2 hypotheses can be distinguished by stratifying the prospective serological data according to initial LST status. Taking this approach, we find that the seroconversion rate is significantly lower amongst LST+, 0·0046/year (3/640), than amongst LST−, 0·038/year (103/2733): $\chi^2 = 17{\cdot}5$, $P < 0{\cdot}01$, RR = 8·0[2·6–25·3]. This effect remains highly significant even when the analysis is limited to children ⩽ 10 years ($\chi^2 = 15$, $P < 0{\cdot}01$; RR = 9·9[2·4–4·0]), and the same trend is apparent (but insignificant) amongst the population > 10 years (Fisher exact test, $P = 0{\cdot}08$). Similarly, the serorecovery rate is significantly higher amongst LST+, 0·95/year (35/37), than amongst LST−, 0·49/year (109/223): $\chi^2 = 23{\cdot}8$, $P < 0{\cdot}01$, RR = 2·0[1·8–2·5]. Again, this effect re-

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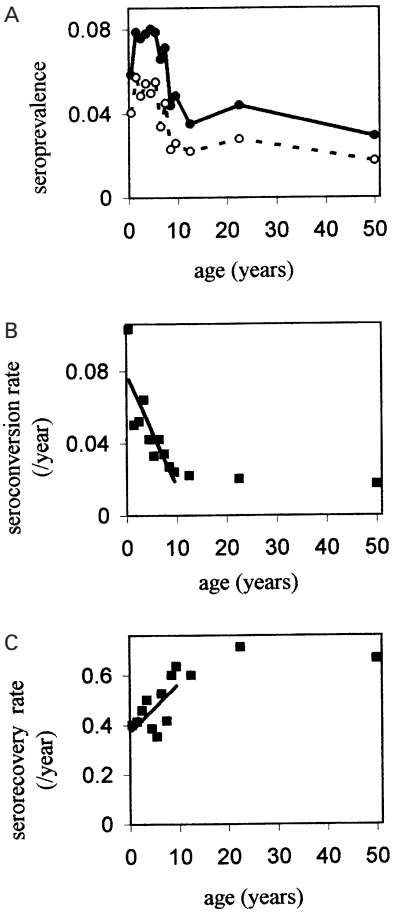

Fig. 2. (A) The relationship between age and the proportion of the population with a positive direct agglutination (DAT) response, using either 1/1600 (———) or 1/3200 (– – – – –) as the cut-off titre. (B) The relationship between age and the proportion who seroconverted during the prospective survey. The line was fitted by least squares linear regression on the data up to the age of 10 years. (C) The relationship between age and the proportion who serorecovered during the prospective survey. The line was fitted by least squares linear regression on the data up to the age of 10 years.

mains significant when the analysis is limited to children $\leqslant 10$ years ($\chi^2 = 25$, $P < 0{\cdot}01$; RR = 1·9[1·7–2·3]), and the same trend is apparent (but insignificant) amongst the population > 10 years (Fisher exact test, $P = 0{\cdot}24$).

Finally, we address the question: does the humoral response to *L. infantum* infection change with age in the absence of acquired immunity due to prior exposure (i.e. amongst LST−)? Inspection of Fig. 2B and C, respectively, suggests that amongst LST− the seroconversion decreases with age up to about 10 years after which it remains relatively constant, whilst the serorecovery rate increases with age up to 10 years and then stays relatively constant. Amongst LST− the seroconversion rate up to the age of 10 years, 0·043/year (91/2136), is significantly higher than amongst people above 10 years, 0·020/year (12/597): $\chi^2 = 5{\cdot}9$, $P = 0{\cdot}015$, RR = 2·1[1·1–3·8]; and amongst LST− the increase in the serorecovery rate from 0·46/year (89/193), for children up to the age of 10 years, to 0·67/year (20/30), amongst people over 10 years, has borderline significance: $\chi^2 = 3{\cdot}61$, $P = 0{\cdot}058$, RR = 1·45[1·08–1·96]. Both rates appear to change (amongst LST−) in a linear fashion with age up to 10 years, and the relationships with age t (in years) can be quantified by linear regressions within this age group:

$$\text{Seroconversion rate (/year)} = \lambda = 0{\cdot}0785 - 0{\cdot}0063t, \quad (3)$$

($F = 16{\cdot}9$, D.F. $= 1, 8$, $P = 0{\cdot}003$, $r^2 = 0{\cdot}64$).

$$\text{Serorecovery rate (/year)} = \rho = 0{\cdot}370 + 0{\cdot}0198t, \quad (4)$$

($F = 5{\cdot}4$, D.F. $= 1, 8$, $P = 0{\cdot}049$, $r^2 = 0{\cdot}33$).

Amongst LST+, no relationship was detected between either the seroconversion or serorecovery rate and age.

Drawing all these results together, the dynamics of change in both LST and DAT prevalence with age can be summarized by a 4-compartmental model (Fig. 3) with 8 rates defining the movement in both directions between pairs of compartments. For simplicity, the model does not allow an individual to change both his DAT and LST status simultaneously, i.e. within the same month. Estimates for the 8 monthly rates in the model were derived from the instantaneous rates which were themselves derived by solving pairs of simultaneous equations for the annual rates measured during the prospective

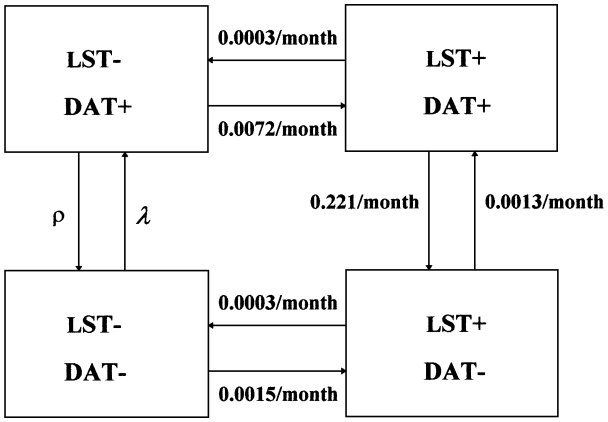

Fig. 3. Flow diagram depicting a model for the immunoepidemiology of *Leishmania infantum* in north-west Iran. Each person may have a positive or negative direct agglutination test response (D+ or D−), and a positive or negative leishmanin skin test response (L+ or L−). Hence, there are 4 possible states. The parameters describing the monthly rates of transition between states were derived empirically from the prospective survey. All are fixed, except for the rates describing the transition from L−D− to L−D+, λ, and the transition from L−D+ to L−D−, ρ, which vary with age as described in the text.

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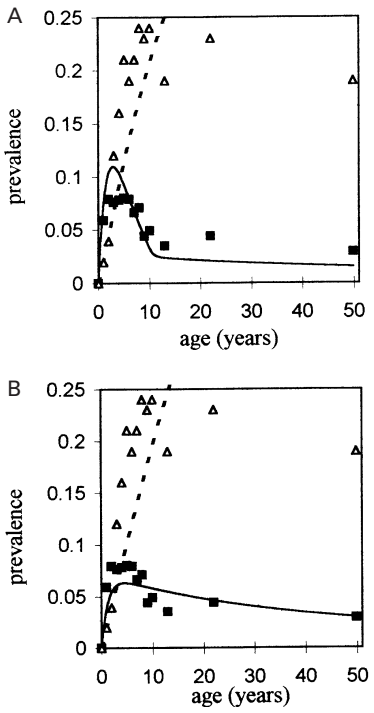

Fig. 4. The relationship between age and the proportions with a positive direct agglutination test (DAT) response (——— and ■) and leishmanin skin test (LST) response (– – – – and △). (A) The lines were simulated using the model depicted in Fig. 3, with age-dependent seroconversion and serorecovery rates amongst the LST− population (see text). (B) The lines were simulated using the same model but with all the rates fixed at their mean values (i.e. independent of age).

study as described above (Equation 1). Six of the 8 rates are fixed, whilst 2 rates (the seroconversion and serorecovery rates amongst LST−) vary with age up to 10 years after which they are fixed at the mean values above 10 years. By assuming that all 8 rates have been constant over time and that infection-induced mortalities have been insignificant, we can use this model to simulate the expected age–prevalence curves for both DAT and LST (Fig. 4) from a series of difference equations.

$$N^t = S^t + D^t + L^t + I^t, \quad (5)$$

$$S^{t+1} = S^t(1\text{–}0{\cdot}0015\text{–}\lambda) + D^t(\rho) + L^t(0{\cdot}0003), \quad (6)$$

$$D^{t+1} = D^t(1\text{–}0{\cdot}0072\text{–}\rho) + S^t(\lambda) + I^t(0{\cdot}0003), \quad (7)$$

$$L^{t+1} = L^t(1\text{–}0{\cdot}0003\text{–}0{\cdot}0013) + S^t(0{\cdot}0015) + I^t(0{\cdot}221), \quad (8)$$

$$I^{t+1} = I^t(1\text{–}0{\cdot}0003\text{–}0{\cdot}221) + D^t(0{\cdot}0072) + L^t(0{\cdot}0013). \quad (9)$$

N^t = the total population at age t (in months); S = the number LST− and DAT−, D = the number DAT+ and LST−, L = the number LST+ and DAT−, I = the number LST+ and DAT+: at the age (in years) designated by the superscript. λ and ρ are monthly rates which vary up to the age of 10 years, according to formulae derived from Equations 3 and 4, and are constant above 10 with values of 0·0028/month and 0·0896/month, respectively. The age–prevalence curves for both DAT and LST can then be simulated from the following equations:

$$\text{LST prevalence at age } t = (L^t + I^t)/N^t. \quad (10)$$

$$\text{DAT prevalence at age } t = (D^t + I^t)/N^t. \quad (11)$$

The adequacy of the model for explaining the age–prevalence curves is demonstrated by the reasonably good fit, at least qualitatively, of the simulated curves for LST prevalence and DAT prevalence to the observed data up to the age of 10 years (Fig. 4A). Above 10 years, the observed LST prevalence reaches an asymptote, as discussed above, whereas the simulated values continue to increase, as the model assumed (for simplicity) no change in transmission rate with time. The effect of this assumption on the DAT prevalence is much less significant because DAT prevalence (unlike LST prevalence) is not cumulative, as the serorecovery rate is so high. The simulated seroprevalence has the same characteristics as the observed data, increasing to a peak at 2 years 10 months old, after which it decreases rapidly to the age of 10, and then persists at low but relatively stable levels amongst the adult population. The relatively fast decline in seroprevalence amongst children is the direct result of the age dependency of the humoral response. This is demonstrated by inspection of a model simulation (Fig. 4B) which uses the same mean values for the serological parameters at every age. In this case, there is a very shallow peak seroprevalence at the age of 4 years and 7 months, and a very slight decline with age (a reduction from 0·063 at the peak to 0·058 at the age of 10) solely as a result of the increase in LST prevalence with age, i.e. due to acquired immunity.

Effects of age and acquired immunity on the ratio of infection : disease

The relationship between infection and disease was investigated using 3 different data sources: prevalent cases (i.e. with active infection at the time of the first survey, as demonstrated by a positive DAT response), incident cases (i.e. infection apparently occurred between the 2 surveys, as demonstrated by seroconversion), and historical cases (i.e. people with cured VL from before the first survey).

Amongst the seropositives at the time of the first survey 7·0% (23/330) developed VL. All the VL cases were LST−, and 20 had a DAT titre at the first survey of 1/3200 or more (which represents 9·6% of the 209 who had these high titres); the remaining 3 cases had a titre of 1/1600 (i.e. 2·5% of the 121 who had this titre) but they all developed a DAT titre of 1/3200 while they had the disease symptoms. In addition, 3·2% (4) of the 126 who seroconverted between the surveys developed VL. All 4 seroconverted to titres of 1/3200 or more (i.e. representing 12·1% of the 34 who seroconverted to

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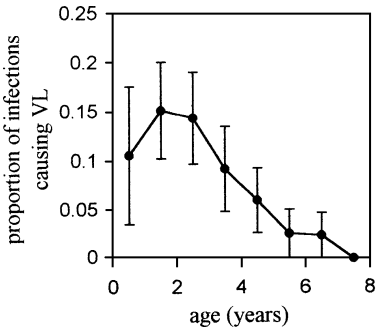

Fig. 5. The relationship between age and the proportion of infections causing visceral leishmaniasis (VL). Infection status was defined by a positive direct agglutination test response at the time of the first or second survey.

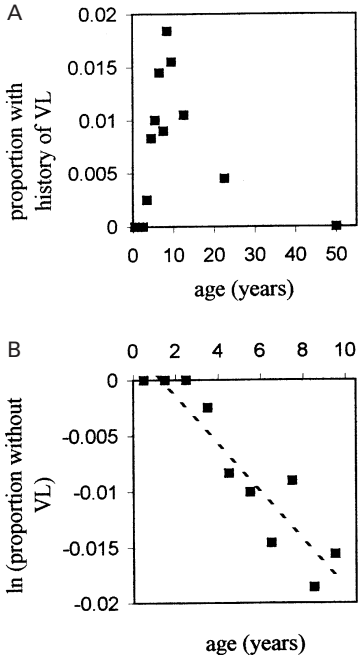

Fig. 6. (A) The relationship between age and the proportion of the population with a history of visceral leishmaniasis (VL), grouped into 13 age classes. (B) The relationship between age up to 10 years and the natural logarithms of the observed proportions of the population with no history of VL. The line was fitted by least squares linear regression.

these high titres), and all were LST− at both surveys.

The relationship between age and the ratio of infection: disease for the combined prevalent and incident cases is illustrated in Fig. 5. All the cases were less than 7 years old, and there was a significant decline in the proportion of infections causing VL up to the age of 7 years ($F = 32{\cdot}0$, D.F. $= 1, 6$, $P < 0{\cdot}01$, $r^2 = 0{\cdot}84$).

Amongst the total study population of 5671, 0·74% (42) had a history of VL prior to the first survey. All of these cases that were skin tested were LST+ (33/33), but only 17% (7/42) were DAT+. Five of the 7 DAT+ historic cases were recent, i.e. they had been cured less than 3 years prior to the serosurvey. All the historic cases had had their disease as children, with the peak frequency between 1 and 2 years old, declining steadily to a single case with VL at the age of 12.

The cumulative prevalence of VL (i.e. the proportion with a history of VL) increased with age up to about 10 years, after which a steep decline was observed in adults (Fig. 6). As for the cumulative LST prevalence, the cumulative prevalence of VL apparently increases monotonically for the first 10 years of age, so it is possible to estimate the mean incidence rate of VL during the last 10 years by a linear regression of ln[proportion without VL] against age t (in years) up to 10 years ($F = 50{\cdot}4$, D.F. $= 1, 8$, $P < 0{\cdot}01$, $r^2 = 0{\cdot}86$):

$$\ln[\text{proportion without VL}] = 0{\cdot}0028 - 0{\cdot}0022\,[t]. \quad (12)$$

It follows that the annual incidence rate of VL during the last 10 years $= 1 - e^{-0{\cdot}0022} = 0{\cdot}0022$/year (95% CI $\pm 0{\cdot}0003$). This represents 7·9% (95% CI $\pm 0{\cdot}8$) of the total incidence rate of infection during the same period (i.e. 0·0022/0·028), so that the ratio of infection: disease is 13:1.

DISCUSSION

Ratio of infection : disease

The mean percentage of *L. infantum* infections leading to VL in the north-west of Iran was estimated in 3 different ways. The lowest estimate, 3·2%, derived from the calculation of this percentage amongst those who seroconverted during the project. This may be an underestimate as some of the apparently healthy seroconverters could develop VL later. The same criticism applies to the estimate, 7·0%, that derives from the people who were seropositive at the first survey. This measure is also affected by a bias that would cause an overestimate: the serorecovery rates of people with subclinical infections tend to be greater than the serorecovery rates amongst people who develop VL (Badaro *et al.* 1986*a*), so that the latter are over-represented amongst seropositives. The most reliable estimate for the mean percentage of *L. infantum* infections leading to VL, 7·9%, derives from a comparison of the force of infection for LST conversions (0·028/year) and the force of infection for VL (0·0022/year), both calculated from age–prevalence curves up to the age of 10. As these figures relate to historical infections, the problem of delayed VL development does not apply. There is still some possibility of bias (leading to an underestimate) due to VL-related mortality. However, VL patients in this focus have fortunately been the subject of a major public health programme, so that mortality rates have been remarkably low (Edrissian, 1996).

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As expected, the mean percentage of *L. infantum* infections leading to VL decreases significantly with age, which explains why 93% of patients in this focus are children up the age of 12 years old (Edrissian, 1996). However, the observation that the cumulative risk of VL decreased rapidly with age in the population above 10 years old was surprising. If it is true that this focus has only been endemic for about 10 years, then the cumulative risk of *L. infantum* infection in the population above 10 years should be equal for all ages (as there is no evidence of age-related exposure). It follows that the reduction in cumulative VL in the older age groups cannot be due to acquired immunity, as the proportion who are LST+ is roughly constant above the age of 10 years. The most likely explanation is that the older age groups have some protection against VL which they have developed independently of prior exposure to *L. infantum*.

Comparisons with the few previously published estimates of the percentage of *L. infantum* infections causing VL, 5·4% (Badaro *et al*., 1986*b*) and 11·1% (Evans *et al*. 1992), are complicated by the differences in study design. In particular, the estimates will be heavily dependent on the age groups surveyed, the diagnostic tools and procedures used to identify subclinical infections, and the period of follow-up in order to check for the development of VL. Despite these caveats, the conventional wisdom appears correct that *L. infantum* infections are less likely than infections with its close relative *L. donovani* to cause disease: reported estimates of the percentage of *L. donovani* infections causing VL range from 16·7% in Kenya (Ho *et al*. 1982) and 17·9% in Ethiopia (Ali & Ashford, 1994*b*) to 66% in Sudan (Zijlstra *et al*. 1994). It is also true that the age distributions of VL cases are dependent on the parasite responsible. Whilst, VL caused by *L. infantum* is typically a disease of children (Bettini *et al*. 1981; Navin *et al*. 1985; Badaro *et al*. 1986*b*; Harrat *et al*. 1992; Edrissian, 1996), all age groups are prone to VL caused by *L. donovani* (Sharma *et al*. 1990; Chowdhury *et al*. 1993; Ali & Ashford, 1994*b*; Seaman *et al*. 1996). Genetic variation between *L. infantum* strains might also lead to geographic variability in pathogenicity.

Effects of age on humoral response

The relationship detected between age and the risk of VL is reflected in the relationship detected between age and the humoral response to *L. infantum* infection. Even amongst the LST− population, the rate of seroconversion decreased with age and the rate of serorecovery increased with age. This effect was most marked in children, so that the humoral response above the age of 10 was relatively constant. The explanation for these findings could be that the cell-mediated response to *L. infantum* infection is more effective at greater ages, eliminating the parasites more quickly, and therefore limiting the rate of antibody production in response to parasites in the blood. However, we cannot discount the hypothetical possibility that *Leishmania* infections which fail to cause LST conversion may still cause a reduction in the body's antibody response to subsequent infections. If this is true, as age and time exposed are confounders for populations living in endemic foci, the reduction in the humoral response with age could be a function of cumulative, but undetected, exposure to *L. infantum*.

The only previous prospective study of VL to report the relationship between age and the seroconversion rate identified an increase in adults (Ali & Ashford, 1994*a*). However, this Ethiopian study differed in 2 key respects from the one described here: the parasite was *L. donovani*, and the rate of LST conversion also increased with age (indicating that exposure to infected sandflies increased with age).

In our study, the mean life-time persistence of antibodies was 47 months in infants ($\leqslant$ 2 years when infected) but only 6 months in adults ($>$ 10 years). Previously reported studies of serorecovery rates following subclinical infections with *L. infection* also indicate that the life-time persistence of a humoral response varies between individuals, ranging from less than 1 month (Londner *et al*. 1988) to 1 year (Pampiglioni *et al*. 1974) or even 3 years (Badaro *et al*. 1986*a*), but this study is the first to demonstrate an association with age. In a Brazilian study (in which LST status was not tested), about half of the seropositive children less than 11 years old serorecovered in 1 year (Evans *et al*. 1992), equivalent to an average life-time persistence of 17 months, which is similar to the mean rate detected in our study. In contrast, the serorecovery rate reported following subclinical infections with *L. donovani* in Ethiopia was 80% in 6 months (Ali & Ashford, 1994*a*), equivalent to an average life-time persistence of 4 months. However, comparisons of different serological surveys should be interpreted cautiously due to the differences in the diagnostic tools and procedures used. For example, the antibodies detectable by DAT may be more persistent than the antibodies detectable by IFAT (Edrissian, 1996).

Effects of acquired immunity on humoral and clinical response

Experimental evidence suggests that the cell-mediated immunity acquired from subclinical infections with *L. infantum* or *L. donovani* reduces the risk of VL developing as a result of superinfection (Southgate & Manson-Bahr, 1967). This would explain why, as in this study, VL patients are always LST− when the disease is active. However, some studies have shown that LST+ people may occasion-

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ally lose their LST responsiveness (Ali & Ashford, 1993), possibly due to immunosuppression, and statistically significant protection has yet to be confirmed by follow-up studies of skin tested populations. Probably the most reliable estimate comes from a prospective survey of VL in Ethiopia which suggested that cell-mediated immunity provides 81% protection against clinical superinfection with *L. donovani* (Ali & Ashford, 1994*b*).

In the prospective study reported here, due to the small number of incident VL cases we were again unable to prove that cell-mediated immunity reduces the risk of disease, but we did demonstrate that cell-mediated immunity significantly reduces the risk of seroconversion and increases the rate of sero-recovery. As suggested above, this is indirect evidence that the cell-mediated response increases the speed with which parasites are killed following infection: high parasite loads lead to high antibody titres and to a relatively high risk of VL.

Whilst the characteristic reduction with age in the risk that *L. infantum* causes VL is not exclusively the result of acquired cell-mediated immunity, the latter does have some effect. For example, the development of acquired immunity might explain the following observations: (1) the relatively high susceptibility to VL demonstrated by previously unexposed adult visitors, e.g. soldiers or tourists, to endemic zones of *L. infantum* (Southgate & Oriedo, 1967); and (2) the relatively high seroprevalence (19·2%) that was reported amongst visitors to an endemic region in Italy in comparison with the seroprevalence in residents (3·7%) (Pampiglioni *et al.* 1974). The reduced risk for resident adults compared to visiting adults appears to apply even to people with no detectable cell-mediated immunity. The explanation can only be speculative. Possibly, the saliva injected by repeated uninfected sandfly bites to residents of endemic sites provides some protection against clinical infections with *L. infantum* (undetected by the LST), as there is now considerable evidence that sandfly saliva has an impact on the clinical response to *Leishmania* infections (Warburg *et al.* 1994; Hall & Titus, 1995).

If acquired immunity does play a significant role in determining the age distribution of VL cases, one would predict that the latter should be higher where the transmission rate is less. This could explain the reported increase in the average age of VL cases in India since the transmission rate was effectively cut by a control programme (Basu & Mallik, 1995). It might also explain the negative relationship in Iran between the average age of VL cases and the transmission rate in each province (Edrissian, 1996): the peak age in highly endemic provinces is between 1 and 2 years, compared to 3–4 years in provinces where VL is sporadic. However, the fact that young children are still the main risk group for VL in provinces where there is very little transmission adds further support to the hypothesis that acquired immunity alone is insufficient to explain the age distribution of VL cases.

Generality of the age–seroprevalence patterns

This study is the first to explain the age–seroprevalence curves in an endemic zone on the basis of empirically proven relationships between the humoral response and both age and cell-mediated immunity. In order to demonstrate the generality of these findings, one would need to measure these parameters in other endemic zones. Unfortunately, such data are unavailable. However, age seroprevalence curves in endemic zones of both *L. infantum* and *L. donovani* have been reported for a few large study populations, and it is instructive to compare the patterns previously observed. The pattern observed here, of a peak seroprevalence in young children followed by a rapid decline with age, has been observed previously in endemic zones of *L. infantum* in Iran (Edrissian *et al.* 1993; Edrissian, 1996), Iraq (Al-Alousi, Latif & Al-Shenawi, 1980), and Pakistan (Rab & Evans, 1994). In contrast, a number of other studies of *L. infantum* epidemiology have found that seroprevalence increases with age, up to at least 15 years in Brazil (Badaro *et al.* 1986*b*) and in Khazakistan (Shukina & Gorbunova, 1978), 20 years in Egypt (Faris *et al.* 1988) and in Khusistan province, Iran (Edrissian, 1996), or even 40 years in Azerbaijan (Titova *et al.* 1982). The explanation for these 2 patterns is unclear, but it might involve differences in the persistence of antibodies detected by the diagnostic tools used in the various studies. In contrast, seroprevalence in endemic zones of *L. donovani* (Perea *et al.* 1991; Seaman *et al.* 1992; Chowdhury *et al.* 1993; Ali & Ashford, 1994*a*; Zijlstra *et al.* 1994) apparently never peaks in young children. For *L. donovani*, unlike *L. infantum*, the age group with peak seroprevalence is principally dependent on the local patterns of exposure and not to age-related variation in susceptibility.

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