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Dichotomy of the human T cell response to *Leishmania* antigens. I. Th1-like response to *Leishmania major* promastigote antigens in individuals recovered from cutaneous leishmaniasis

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SUMMARY

The T cell response to antigens from *Leishmania major* promastigotes was investigated in peripheral blood mononuclear cells from Sudanese individuals with a history of cutaneous leishmaniasis (CL), Sudanese individuals with positive DTH reaction in the leishmanin skin test but with no history of skin lesions, and in Danes without known exposure to *Leishmania* parasites. Proliferation and production of interferon-gamma (IFN-γ) and IL-4 in antigen-stimulated cultures was measured. Lymphocytes from individuals with a history of CL proliferated vigorously and produced IFN-γ after stimulation with either a crude preparation of *L. major* antigens or the major surface protease gp63. These cultures produced no or only little IL-4. Also cells from leishmanin skin test-positive donors with no history of CL produced IFN-γ and no IL-4 in response to *L. major* antigens. Cells from the unexposed Danes were not activated by gp63. The cells from Danish donors produced either IFN-γ or IL-4, but not both cytokines after incubation with the crude preparation of *L. major* antigens. The data show that the T cell response to *Leishmania* antigens in humans who have had uncomplicated CL or subclinical *L. major* infection is an IFN-γ-producing Th1-like response.

Keywords cytokines T cells leishmaniasis immunoregulation gp63

INTRODUCTION

The leishmaniases are vector-borne diseases caused by obligate intracellular protozoans of the genus *Leishmania*. The diseases affect millions of people in tropical and subtropical parts of the world. The clinical manifestations of *Leishmania* infections in humans range from self-healing cutaneous ulcers to fatal systemic diseases. In addition, evidence is accumulating that in many individuals *Leishmania* infections run a subclinical course without symptoms, detectable only by development of specific immunological memory to leishmanial antigens [1–7].

The spectrum of clinical manifestations of human *Leishmania* infections can be mimicked in experimental infections of mice with *L. major*. In the murine model the outcome of the infection depends on activation of one of two subsets of T helper/inducer cells, Th1 and Th2. Murine $CD4^+$ T cells can be divided into Th1 and Th2 cells on the basis of the pattern of their cytokine production [8]. Th1 cells produce interferon-gamma (IFN-γ), IL-2 and lymphotoxin, whereas Th2 cells secrete IL-4, IL-5, IL-6 and IL-10. Functionally, Th1 cells have mainly been associated with DTH reactions, whereas Th2 cells have been related to B cell help, particularly to IgE-producing cells. The outcome of *L. major* infections in mice is closely related to preferential activation of Th1 or Th2 cells. Activation of Th1 cells is associated with recovery and resistance to reinfection, whereas activation of Th2 cells results in progressive disease [9,10]. A clear dichotomy in the T cell response to invading *Leishmania* parasites, as the one seen in mice, has not been demonstrated in humans. To investigate whether the different clinical presentations of *Leishmania* infections in humans are reflected by qualitative differences in the T cell response to *Leishmania* antigens, a series of experiments were carried out using peripheral blood mononuclear cells (PBMC) from different donor groups. Proliferation and production of IFN-γ was assessed after antigen stimulation *in vitro*. The T cell responses were further characterized by employing a recently developed method allowing measurement of antigen-induced IL-4 pro-

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duction [11]. In the present study the human T cell response to *L. major* was investigated. In humans *L. major* causes cutaneous leishmaniasis (CL). The typical presentation, the oriental sore, is one or a few skin ulcers, which heal spontaneously within a few months leaving small scars. In some cases the lesion spreads and may cause severe disfiguration.

Epidemics of CL can occur when *L. major* is introduced in naive populations in areas where the sandfly vector is present [12]. The village El Izergab, 15 km north of Khartoum, Sudan, was reached by such an epidemic late in 1985. In October 1990, when the epidemic was ceasing, less than 50% of the inhabitants of the village had had skin lesions [13]. However, more than 90% of the inhabitants had a positive DTH reaction in the leishmanin skin test, suggesting that a proportion of the population had been infected and developed T cell memory to the infecting agent without clinical disease [13]. This study describes the pattern of IFN-γ and IL-4 production by *L. major*-reactive T cells from leishmanin DTH-positive individuals living in El Izergab with and without a history of self-limiting CL, and by cells from Danes with no known exposure to *Leishmania* parasites. The cells were stimulated with either a crude preparation of *L. major* promastigotes or with a purified antigen, the abundant surface protease gp63.

MATERIALS AND METHODS

Donors and isolation of mononuclear cells

Leishmanin DTH-positive donors with and without a history of CL were from El Izergab village north of Khartoum, Sudan. The epidemiology of CL in this village in the years 1985–1990 has been described elsewhere [13]. The high proportion of leishmann skin test-positive individuals without a history of CL in the study population could reflect a high number of false positive reactions in the DTH test. However, no new cases were observed in the village for more than 1 year despite active transmission in neighbouring villages. This indicates that the population had become resistant to infection, probably after exposure to the parasite. Clinical examinations and leishmanin skin tests were performed on all donors as previously described [13]. Informed consent of individuals participating in the study was obtained. Heparinized blood was collected and PBMC were isolated by Lymphoprep (Nyegaard, Oslo, Norway) density centrifugation. The cells were frozen by a manually operated version of a recently described device for gradient freezing of cells under field conditions [14] and stored and transported in liquid nitrogen. Blood from Danish volunteers was collected and treated similarly, except that a stationary gradient cell freezer was used for the cryopreservation. Before use, the cells were rapidly thawed and washed. The viability of the cells was ascertained by trypan blue dye exclusion.

Antigens

A preparation of proteins from *L. major* (Baringo strain, Kenya, MHOM/KE/87/NLB 455) was made as previously described [15]. The crude *L. major* preparation was used in final concentrations of 700 μg/ml, 70 μg/ml, and 15 μg/ml. All results shown are from stimulation with 70 μg/ml, but results from the other two concentrations were comparable. gp63 was isolated after phospholipase C treatment by concanavalin A (Con A) affinity chromatography and fast protein liquid chromatography (FPLC) ion exchange chromatography (modified from [16]). Purity of the isolated protein was confirmed by silver stained SDS–PAGE, and identity was verifed by Western blotting using MoAbs (Hey *et al.*, unpublished data). The enzyme activity of the purified gp63 was inactivated by heating to 70°C for 15 min. gp63 was used at a concentration of 8 μg/ml in the cultures.

Purified protein derivative of tuberculin (PPD) and tetanus toxoid (TT) were purchased from Statens Seruminstitut (Copenhagen, Denmark). PPD was used at 12 μg/ml and TT was used at 3 μg/ml (final concentrations).

Lymphoproliferation and cytokine production

PBMC were cultured in RPMI 1640 with 10 mM HEPES, 20 U/ml penicillin and 20 μg/ml streptomycin (Gibco Ltd., Paisley, UK) with 15% heat-inactivated pooled normal human serum (NHS). The cells were incubated with antigens at $6{\cdot}6 \times 10^5$ cells/ml in volumes of 170 μl in round-bottomed microculture plates (Nunc, Roskilde, Denmark). The cultures were incubated for 7 days at 37°C, 5% CO_2 in a humidified atmosphere and pulsed with ^{3}H-thymidine (1·85 MBq/ml, 20 μl/culture) (New England Nuclear, Boston, MA) for the last 24 h of incubation. The culture supernatants were recovered and stored at −20°C for later determination of IFN-γ. The cells were harvested onto glassfibre filters and the incorporation of ^{3}H-thymidine into DNA was determined by measurement of β-radiation, using a Matrix β-counter (Packard, Greve, Denmark). All tests were done in triplicate. For each set of samples the median was recorded. The specific response was expressed as the difference between antigen-stimulated cultures and cultures incubated without antigen. A positive proliferative response was defined as stimulatory index (SI) $> 2{\cdot}5$ and specific response $> 0{\cdot}5$ kct/min. For the measurement of IL-4 release by antigen-stimulated cultures of PBMC, parallel cultures were cultured for 6 days and then pulsed with 1 μM of ionomycin and 50 ng/ml of phorbol myristate acetate (PMA) (both from Sigma, St Louis, MO) for 24 h before the culture supernatants from triplicate wells were harvested [11].

Cytokine measurements

IL-4 and IFN-γ in supernatants of cultures of PBMC were measured by ELISA as described elsewhere [11,17]. The detection ranges of the ELISAs were: 1–66 U/ml (specific activity of the reference protein $2{\cdot}5 \times 10^8$ U/mg) for IFN-γ and 30–2000 pg/ml for IL-4. The antigen-induced cytokine production was calculated as the difference between antigen-stimulated and unstimulated cultures.

Statistical analysis

Comparisons between groups were done by the Mann–Whitney rank sum test. $P < 0{\cdot}05$ was considered significant.

RESULTS

T cell responses to L. major

The lymphoproliferation and cytokine production in response to the crude *L. major* promastigote preparation in PBMC from leishmanin skin test-postitive Sudanese individuals with and without a history of cutaneous lesions, and in cells from presumably unexposed Danes, are shown in Fig. 1. The *Leishmania* antigens induced proliferation in 23 of 25 cultures of cells from previous CL patients. In leishmanin skin test-

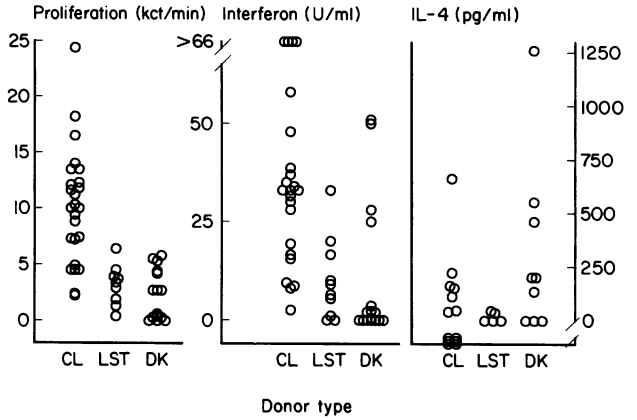

Fig. 1. Proliferation and production of IFN-γ and IL-4 in response to *Leishmania major* antigen by peripheral blood mononuclear cells (PBMC) from Sudanese individuals with a history of cutaneous leishmaniasis (CL), leishmanin skin test-positive Sudanese individuals with no history of CL (LST), and Danes with no known exposure to *Leishmania* parasites (DK). Proliferation is shown as ct/min $\times 10^3$ (ct/min from unstimulated cultures subtracted).

positive individuals without previous lesions, eight of nine cultures responded by proliferation. The number of proliferating cultures in samples from unexposed Danes was nine out of 15. The magnitude of the responses to *L. major* antigens by cells from history-negative, skin test-positive individuals was comparable to the responses by cells from the Danish donors. The responses by the cells from individuals with a history of CL were significantly higher than both the responses by cells from leishmanin-positive donors without a history of CL (median difference 7·3 kct/min, 95% confidence interval (CI) 3·9–10·1, $P = 0{\cdot}0004$) and the responses by cells from unexposed Danes (median difference 8·0 kct/min, 95% CI 5·7–10·8, $P < 0{\cdot}0001$). Cells from all three donor groups produced IFN-γ after stimulation with *L. major* antigens. The amount of IFN-γ produced in the group of cells from previous CL patients was significantly higher than in the two other groups (median differences 27·6 U/ml (95% CI 11·5–37·6, $P = 0{\cdot}0006$) and 32·5 U/ml (15.5–37·0, $P < 0{\cdot}0001$), compared with cells from leishmanin-positive donors with no history of CL and unexposed Danes, respectively). No statistically significant differences between the groups could be demonstrated in IL-4 production by the cells.

Relation between IFN-γ and IL-4 production in response to L. major *antigens*

When the concentrations of IL-4 in supernatants of *L. major*-stimulated PBMC from individual donors were related to the concentrations of IFN-γ (Fig. 2), a clear dichotomy was observed in the responses from unexposed Danes. These cells produced either IFN-γ or IL-4. The pattern of IFN-γ and IL-4 production by cells from Danish donors in response to *Leishmania* antigens is in general stable when tested on consecutive samples (Kurtzhals *et al.*, unpublished data). In contrast, cells from leishmanin skin test-positive individuals with and without a history of CL both showed a response clearly dominated by IFN-γ. Thus, in the individuals who had been exposed to *L. major*, the cells had a Th1-like response to a crude preparation of *L. major* antigens.

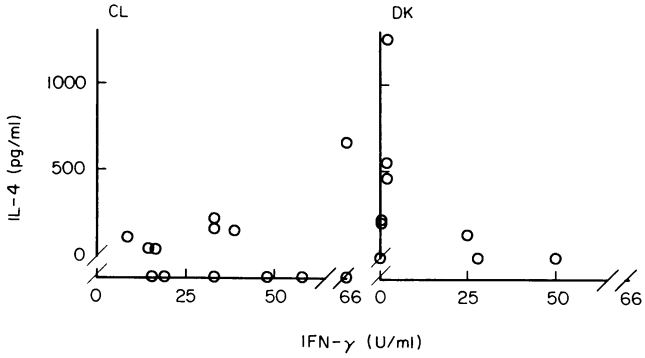

Fig. 2. IFN-γ and IL-4 production by peripheral blood mononuclear cells (PBMC) from Sudanese individuals with a history of cutaneous leishmaniasis (CL) and from unexposed Danes (DK) in response to a crude preparation of *Leishmania major* antigen. The cultures were pulsed with ionomycin and phorbol myristate acetate (PMA) before IL-4 was measured.

T cell response to gp63 purified from L. major

Cells from 18 of 21 Sudanese donors with a history of CL proliferated in response to gp63 purified from *L. major* (Fig. 3). In contrast, gp63 induced proliferation in only one of 10 samples from Danish donors. Likewise, IFN-γ levels above 5 U/ml were measured in 16 of 21 cultures of cells from Sudanese donors and in none of 10 cultures from Danish donors. gp63 induced IL-4 production in only two of 12 cell samples from Sudanese donors with a history of CL. Cells from these two donors did not produce IFN-γ after incubation with gp63.

T cell responses to PPD and TT

Varying proliferative responses to PPD and TT were seen in cultures of cells from all three donor groups (Fig. 4). Cells from all three donor groups produced IFN-γ but no or little IL-4 when incubated with PPD. This antigen can induce DTH reactions in humans, and has previously been found to produce a Th1-like response in human PBMC [11]. Some production of both IFN-γ and IL-4 was seen in TT-stimulated cultures.

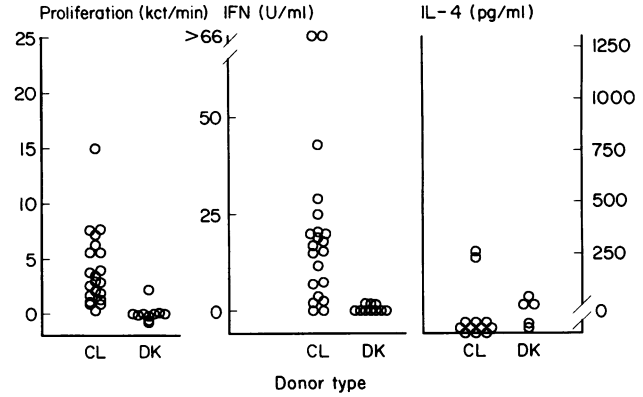

Fig. 3. Proliferation and production of IFN-γ and IL-4 in response to gp63 by peripheral blood mononuclear cells (PBMC) from Sudanese individuals with a history of cutaneous leishmaniasis (CL) and Danes with no known exposure to *Leishmania* parasites (DK). Proliferation is shown as ct/min $\times 10^3$ (ct/min from unstimulated cultures subtracted).

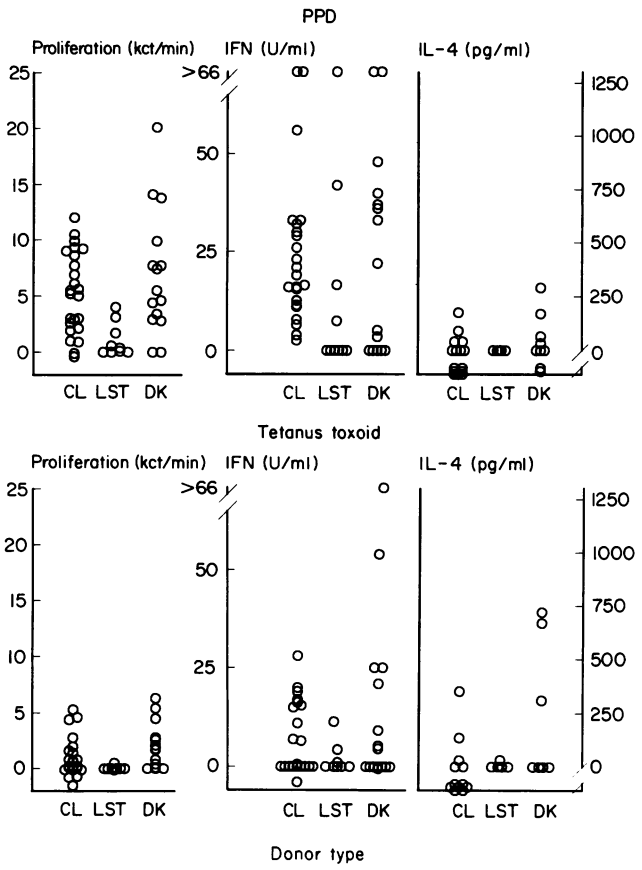

Fig. 4. Proliferation and production of IFN-γ and IL-4 by peripheral blood mononuclear cells (PBMC) in response to purified protein derivative (PPD) and tetanus toxoid. Proliferation is shown as ct/min × 10^3 (ct/min from unstimulated cultures subtracted).

DISCUSSION

The profound effect of differential activation of Th1 and Th2-like cells in murine leishmaniasis prompted us to explore patterns of cytokine production in human *Leishmania* infections. Although secretion of IFN-γ by *Leishmania*-reactive human T cells has been evaluated in some studies, the study of T cell subsets in human *Leishmania* infections has suffered from the lack of methods for the measurement of antigen-induced Th2 cytokine production. In this study we used an amplification step involving pulsing of the cultures with PMA and ionomycin for the measurement of IL-4 production [11]. The antigen-induced IL-4 production was calculated as the difference between values from antigen-stimulated cultures and cultures incubated without antigen. By use of this method it has previously been found that IL-4 production by PBMC in response to stimulation with TT correlates with the tetanus vaccination status of the donors [11]. The IL-4 secretion by PBMC measured by this method can be ascribed mainly to antigen-reactive $CD2^+$ $CD4^+$ T cells (Kurtzhals *et al.*, unpublished data). Experiments *in vitro* have shown that the two cytokines, IFN-γ and IL-4, can directly influence *Leishmania*-infected macrophages; IFN-γ induces parasite killing [18–22], an effect which is reported to be either antagonized [23,24] or synergized by IL-4 [22,25]. Local application of IFN-γ has been found to promote healing of human CL [26].

In this study the human T cell response to *L. major* promastigote antigens was investigated. PBMC from Sudanese donors with a history of self-healing CL proliferated vigorously when incubated with a crude *L. major* antigen preparation. Also cells from leishmanin skin test-positive Sudanese individuals with no history of clinical CL proliferated in response to the antigen preparation; however, the magnitude of the thymidine incorporation was comparable to that of cells from Danes with no known exposure to *Leishmania* parasites. The cells from the Danes were probably activated because of cross-recognition of leishmanial antigens [15,27,28].

The *Leishmania*-reactive cells from the unexposed Danes showed a dichotomy in the secretion of IFN-γ and IL-4, as the cell cultures produced only one of the two cytokines after incubation with *L. major* antigens. Cytokines secreted by *Leishmania*-reactive cells in unexposed individuals may contribute to the primary protection against parasite invasion of macrophages in human *Leishmania* infections [28].

In contrast to the dichotomy in the cytokine response to *L. major* in cells from unexposed Danes, PBMC from Sudanese donors with a history of self-healing *L. major* infections produced large amounts of IFN-γ and no or little IL-4 in response to the crude *L. major* antigen preparation. The preferential production of IFN-γ over IL-4 after *Leishmania* antigen stimulation suggests activation of cells resembling murine Th1 cells. The activation of Th1-like cells in individuals who have had spontaneously cured CL suggests that Th1-like cells have been expanded during infection with *L. major*. This is particularly interesting because the self-limiting nature of this disease resembles the course of *L. major* infection in mice with a Th1 response to the infecting parasite. In the murine system the Th1 cells are directly involved in resolution of the infection, as demonstrated by reconstitution experiments in immunodeficient animals [29]. The present finding of Th1-like response to *L. major* in humans suggests that cytokines from Th1-like cells (e.g. IFN-γ) may be important in the process of healing of cutaneous lesions in humans. The cells from leishmanin DTH-positive individuals without a history of skin lesions living in the same village as the donors with clinical CL also produced IFN-γ and no IL-4 in response to *L. major* antigen stimulation. It is possible that in these individuals the primary response had overcome the parasites before the lesions developed. If so, they may not have had prolonged exposure to the parasites, which may explain why the responses to *L. major* antigens were low in this group.

The abundant surface protein gp63 has been found to induce some protection in mice against *L. major* infections when used as a vaccine [30]. Previous studies on the human T cell response to gp63 have shown conflicting results. We have found that there was a lack of response in cells from Kenyan individuals cured from visceral leishmaniasis [17], and in a study from Israel cells from donors with previous CL also failed to respond to gp63 [31]. In contrast, in a study from Brazil gp63 induced proliferation and IFN-γ production in cells from patients with active or cured cutaneous, mucosal, or visceral leishmaniasis [32]. In the present study we found that cells from Sudanese individuals with a recent history of

uncomplicated CL were activated and proliferated after incubation with gp63 isolated from *L. major*. Furthermore, the cytokine pattern in all but two donors tested was Th1-like, suggesting that gp63-reactive T cells may take part in the protection against CL in humans. The gp63-reactive T cells have most probably been expanded during the infection, since no activation of T cells could be observed in the group of Danish donors without known exposure to *Leishmania* parasites.

The data presented in this study demonstrate the existence of a Th1-like response to *L. major* promastigote antigens in individuals with a history of *L. major* infection. Since the response in unexposed Danish individuals is either Th1- or Th2-like, it is likely that an up-regulation of *Leishmania*-specific Th1-like cells and a down-regulation of Th2-like cells occur during infection. Even though peripheral T cells may only to some extent reflect the situation in infected tissue, the present finding of Th1-like responses in individuals with a past history of self-healing CL is in striking contrast to the mixed Th1- and Th2-like response to *L. donovani* observed in individuals cured from visceral leishmaniasis [33,34]. Future investigations may clarify why human CL results in Th1-like responses, whereas human visceral leishmaniasis can induce a mixed response.

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