**In vivo** IL-12 and IL-8 production in hydatic patients and their possible diffusion in hydatid cysts

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**ABSTRACT.** Cystic echinococcosis (CE) is caused by infection with the larval stage of the cestode *Echinococcus granulosus*. It is one of the world’s major zoonotic infections. Variability and severity of clinical expression of this parasitosis are associated with duration and intensity of infection. They are also related to the variety of human immunological responses to the hydatic antigens. The aim of this work is to study the inflammatory response associated with human hydatidosis by evaluating the possible roles of the proinflammatory cytokines in hydatic patients. We investigated the patterns of IL-12 and IL-8 in serum from Algerian hydatic patients. Serum IL-12 and IL-8 levels are significantly higher in patients with hydatidosis than in control subjects. Furthermore, cytokines secretion correlates with disease statues (cystic localizations and clinical stage). These data indicate that infection with *E. granulosus* is associated with high levels of circulating IL-12 and IL-8. Moreover, our data, to our knowledge, constitute the first report of IL-12 and IL-8 diffusion into the hydatid cyst. Our results underline the permeability of the cyst wall to the soluble immune system of the host. The relationship between cyst fertility and cytokine infiltration indicates a strong host-parasite interaction. All these findings have important implications for the diagnosis of hydatidosis in humans.

**Keywords:** IL-12, IL-8, human hydatidosis

*Echinococcus granulosus* is one of the world’s major zoonotic infections. The larval stage is the causative agent of human hydatidosis. It constitutes a serious public health problem in various parts of the world, particularly in Algeria [1-3]. Human hydatidosis usually manifests as unilocular cyst(s) mainly located in the liver and/or lungs or other viscera of the intermediate host. The slow-growing cysts are able to survive for several years in chronic infections. In many cases, it appears to be refractory to the immunological responses of the host. Generally, the early phases of infection are asymptomatic and the diagnosis is based on the identification of cyst structures by radiology [4, 5]. Immunodiagnosis is considered to be an additional approach to confirm clinical findings [6, 7]. Treatment modalities for cystic echinococcosis include chemotherapy and surgery. However, surgery still remains the main therapeutic approach throughout the world [5, 8].

It seems that the variability and severity of the clinical expression of this parasitosis are associated with duration and intensity of infection. There are also related to the variety of human immunological responses to the several antigens. The study of cytokines with their multiple regulatory functional outcomes will be useful in designing strategies to develop early immunodiagnosis of hydatid disease. It could also open new perspectives in the understanding of the pathogenesis of this disease. In previous studies, we have shown the presence of IFN (a mixture of IFN-α, β and γ), TNF-α, and IL-6 activities in hydatid patients showing hepatic and pulmonary cystic localization. This production correlates with immunoreactivity versus parasitic antigen (Antigen 5) [9]. These cytokines would play a role in the initiation, intensity and duration of the immune response in human hydatidosis. Nevertheless, the exposure of these mechanisms requires the identification of other cytokines implicated in the immune process.

**Abbreviations:**

a = after
b = before
bLu = broken cyst in lungs
CE = cystic echinococcosis
cLi = partial calcified cyst in the liver
Ki = kidney
HCF = hydatid cyst fluid
HuIFN-γ = human IFN-γ
Li = liver
L-NMMA = N-monomethyl- L-arginine
Lu = lungs
O = ovary
To evaluate the role of inflammatory cytokines in the immune response against hydatidosis, we investigated in vivo IL-12 and IL-8 production in hydatid patients with different cyst localizations (before and after surgery). We have also studied the IL-12 and IL-8 levels in hydatid fluid and their correlation with cyst location and fertility.

PATIENTS AND METHODS

Patients

Fifty one blood samples were obtained from Algerian patients with Echinococcus granulosus infections (30 ± 13 years old, 68.63% men), tested before and after surgery (one week before and 24 h-72 h after surgical removal). Clinical diagnosis was surgically confirmed by the presence of cysts in each case (Department of Surgery and laboratory of parasitology, Mustapha Bacha Hospital, Algiers, Algeria). Subjects having secondary infection and other acute or chronic diseases were not included in this study. Ten healthy controls (mean age 25 ± 2 years, 70% women) (from the same region in Algeria) were included in this study. They did not present inflammatory disease or any sign of infection at the time of blood sample collection. In addition, none of the subjects had ever received blood transfusions or any medication. All participants gave their informed consent for the present study, which was carried out according to the guidelines of local Ethics Working Group.

Serum collection

Blood samples collected from healthy donors and patients were centrifuged at 2000rpm for 10 minutes to obtain the serum. Haemolysed serum was excluded from this study. All serum samples were stored at -70°C until cytokine determination. The hydatid fluids were collected from healthy donors and patients with multiple cysts localization: 100.2 ± 11.5 pg/mL of IL-8 in liver plus lung; 155.8 ± 5.75 pg/mL of IL-12 (p < 0.0001). Similarly, IL-12 levels in the hydatid patients (before surgery) are higher compared with levels in uninfected control subjects (e.g. 90 ± 10.23 pg/mL of IL-12 in liver plus lung versus 12.47 ± 1.58 pg/mL; p < 0.0001) (figure 1B). The same significant difference between patients and controls is also shown for all cyst locations.

Hydatid cyst fluid collection

Thirty seven hydatid cyst fluid (HCF) samples were obtained by aseptic puncture of hydatid cysts removed by surgery from hydatid patients. The hydatid cysts were mainly localized in the liver (Li, n = 15), lungs (Lu, n = 19) and less frequently in the ovary (O, n = 3). The HCF was filtered in order to eliminate the remaining hydatid membranes. It was centrifuged at 3000rpm (15min, +4°C) and the sediment checked for the presence of protoscolices. Finally, the supernatant of each cyst was stored at -70°C until cytokine determination. The hydatid fluids are classified into several groups. This classification is based on fertility (“+++”: high fertility- n = 13; “++”: medium fertility- n = 15; and “−”: no fertility- n = 9), and cyst location (Li: liver- n = 15; Lu: lungs- n = 19; and O: ovary- n = 3).

Cytokine detection

Levels of IL-8 and IL-12 in serum and cyst fluid samples were evaluated by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions (Immunotech). The plates were read on an ELISA reader (LABSYSTEM). These assays detected only human cytokines.

STATISTICAL ANALYSIS

All results were expressed as mean ± SD (standard deviation). Data analysis was performed with the SYSTAT 11. Student’s t test was used for comparisons between different groups (patients/controls, stimulated/unstimulated cells). Differences were considered to be statistically significant for p value < 0.05.

RESULTS

IL-8 and IL-12 levels in patients compared to healthy donors

Analysis of circulating cytokine production reveals that levels of IL-8 and IL-12 are elevated in all hydatid groups. Further, we observe a distinct pattern in patients and normal donors. As shown in figure 1A, patient serum samples (before surgery) have significantly higher IL-8 levels at all locations (e.g. 100.2 ± 11.5 pg/mL of IL-8 in liver plus lung) than serum samples from healthy donors (0 pg/mL) (p < 0.0001). Similarly, IL-12 levels in the hydatid patients (before surgery) are higher compared with levels in uninfected control subjects (e.g. 90 ± 10.23 pg/mL of IL-12 in liver plus lung versus 12.47 ± 1.58 pg/mL; p < 0.0001) (figure 1B). The same significant difference between patients and controls is also shown for all cyst locations.

Correlation between IL-8/IL-12 levels and disease stage

In order to determine a possible relationship between disease progression and cytokine production in vivo, serum IL-12 and IL-8 levels were assessed before and after surgery. The clinical stage seems to influence IL-8 and IL-12 production. Analysis of cytokine production before and after surgery shows that the IL-8 and IL-12 production observed following surgery is higher in all cases (e.g. in multiple cysts localization: 100.2 ± 11.5 pg/mL versus 155.8 ± 5.75 pg/mL of IL-8- p < 0.0001; and 90 ± 10.23 pg/mL versus 125 ± 14.94 pg/mL of IL-12- p < 0.0001) (figure 1). The same significant difference between clinical stages of disease (before and after surgery) is also shown for all cyst locations.

Correlation between IL-8/IL-12 levels and cyst location

Our results indicated in figure 1 suggest that cystic location is related to cytokine production. Interestingly, we observed that patients with multiple hydatidosis (liver and lung cysts) showed the most elevated serum IL-8 and IL-12 levels (100.2 ± 11.5 pg/mL and 90 ± 10.23 pg/mL respectively, before surgery). The hepatic cystic location is in
Figure 1
Serum levels of IL-8 (A) and IL-12 (B) in Algerian hydatic patients (b: before surgery; and a: after surgery- n = 51) and healthy controls (C- n = 10). Li+Lu: liver + lungs- n = 10; Li: liver- n = 09; Lu: lungs- n = 11; Ki: kidney- n = 4; cLi: partially calcified cyst in the liver- n = 06; bLu: broken cysts in lungs- n = 11. Cytokine levels were quantified as described in Materials and Methods and are presented as the mean ± SEM pg/mL. Significant differences between two groups (e.g. patients/controls) are indicated (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001). Differences were statistically significant for all groups.
second position (with 82.11 ± 4.7 pg/mL of IL-8 and 71.28 ± 9.9 pg/mL of IL-12, before surgery) followed by lung (with 68.5 ± 13.04 pg/mL of IL-8 and 37.73 ± 7.43 pg/mL of IL-12, before surgery).

We observed with interest that in patients with a rare cystic location (in kidney), serum IL-8 and IL-12 levels are very low, but remain higher than those measured in healthy controls (with 13.25 ± 2.5 pg/mL of IL-8 and 21.5 ± 2.04 pg/mL of IL-12, before surgery; p < 0.0001).

Furthermore, we have noted that the partial calcification of cysts reduces cytokine production measured in patients bearing intact hepatic cyst (46.42 ± 4.27 pg/mL versus 82.11 ± 4.7 pg/mL of IL-8 and 37.17 ± 8.59 pg/mL versus 71.28 ± 9.9 pg/mL of IL-12; p < 0.0001).

In contrast, those patients with a complication such as the breakdown of cysts show significantly higher levels of IL-8 (84.72 ± 9.86 pg/mL) and IL-12 (59.43 ± 12.41 pg/mL) in comparison with those bearing an intact pulmonary cyst (with 68.5 ± 13.04 pg/mL of IL-8; p = 0.003 and 73.73 ± 7.43 pg/mL of IL-12; p < 0.0001) (figure 1).

IL-8 and IL-12 levels in cystic fluid

Thirty-seven hydatid fluid samples were aseptically taken from fertile, hydatid cysts and acephalocysts of patients (see Patients and methods). Our observations show a correlation between cytokine levels and cyst fertility. The ELISA sandwich method revealed significant IL-8 and IL-12 levels in fluids from all fertile cysts located in the liver (with 1547.71 ± 89.68 pg/mL of IL-8 and 136 ± 9.86 pg/mL of IL-12) or lungs (with 793.17 ± 39.73 pg/mL of IL-8 and 103.17 ± 12.58 pg/mL of IL-12). In infertile hepatic cysts; levels of cytokines significantly decrease to 134 ± 10.15 pg/mL of IL-8 (p = 0.0001) and 55 ± 5 pg/mL of IL-12 (p < 0.0001) (figure 2).

Moreover, we have noted with interest that cyst location is related to the cytokine levels in cysts. In fertile cysts, an hepatic location shows the highest levels of cytokines (with 1547.71 ± 89.68 pg/mL of IL-8 and 1136 ± 9.86 pg/mL of IL-12) as compared to a pulmonary location (with 793.17 ± 39.73 pg/mL of IL-8; p < 0.0001 and 103.17 ± 12.58 pg/mL of IL-12; p < 0.001) (figure 2). The same observations were also noted in infertile cysts (figure 2).

DISCUSSION

Production of IL-8 and IL-12 in hydatid patients

To our knowledge, very little information is available concerning the role of IL-8, and IL-12 in human hydatidosis. Further, no previous studies have directly examined IL-12 and IL-8 production in Algerian hydatid patients, or their relationship to cyst location and disease stage. The present study is designed to assess the serum levels of IL-12 and IL-8 in Algerian patients with hydatidosis, and in healthy donors from the same community, as a control subjects. Our results provide evidence that levels of IL-12 and IL-8 are higher in hydatid patients.

This increase is in agreement with previous data on the role of cytokines in host anti-hydatic defence, and underscores the ability of the larval stage of *Echinococcus granulosus* to trigger cytokine production [9-12].

The role of cytokines in infection by various parasites is usually been investigated by measurement of cytokine levels in patients’ serum. This approach has a several advantages; one of them is the feasibility of assaying many samples quickly and easily. Refik *et al.* observed that IL-8 is increased in 11/28 (39.3%) of patients with cystic echinococcosis following surgery [13]. However, in this study, the patient population, and the disease stage when blood samples were obtained differed considerably from those in our study, which may explain the differences in the patterns of cytokine response. Rigano *et al.* detected IL-12 p40 mRNA almost exclusively in PBMC isolated from successfully treated patients at the end of chemotherapy [14]. We have previously measured antigen-driven cytokine production in patients with hydatidosis (before and after surgery). Our results showed high *in vitro* production of antigen-driven IL-12 and IL-8 by PBMC isolated from these patients [15]. The same observation was also reported by Drewec *et al.* in a related infection by *Echinococcus multilocularis*. These authors showed that substantial amounts of IL-8, and IL-12 were detected in alveolar echinococcosis patients [16]. Several intra-cellular parasitic diseases, such as infections by *Plasmodium*, showed similar serum IL-8 and IL-12 level increase. Expression of IL-8 mRNA has been demonstrated in placental malaria infection [17]. In addition, significantly elevated serum levels of IL-12, and IL-8 were found in severe cases of malaria versus healthy controls [18]. Courtoix *et al.* also reported that high levels of IL-8 were associated with parasite detection in human infection with *Trypanosoma brucei gambiense* [19].

IL-8 is a member of the C-X-C chemokine family. IL-8 is a proinflammatory, chemoattractant cytokine produced mainly by activated monocytes/macrophages [20-23]. IL-12 was originally termed natural killer cell stimulatory factor or cytotoxic lymphocyte maturation factor. This is produced mainly by activated macrophages/monocytes. It has a very important role in the initiation and regulation of the innate cellular immune response [24-26]. Taken together, these observations and our results suggest that IL-12 initiates a protective T helper type 1 immune response in hydatid disease. Emery *et al.* showed that IL-12 is of crucial importance in inhibiting larval growth after establishment of the *Echinococcus multilocularis* metacestode in the liver. These authors suggest that this cytokine could be of potential value in the treatment of human alveolar echinococcosis [27]. Other studies have also shown that IL-12 can be used as an adjuvant in conjunction with soluble antigens to enhance Th1 immune responses and to induce protection against re-infection by *shistosomases* [28].

IL-12 exerts its influence through upregulating IFN-γ [25, 26, 29]. IFN-γ has been shown to have *in vitro* anti-hydatic activity. Our *in vitro* experiments using L-NMMA (inhibitor for NOS) and anti-HuIFN-γ have shown that IFN-γ-induced nitric oxide production appears to be a critical mechanism for hydatid infection control [12]. IFN-γ also enhances the production of IL-12, creating a positive reinforcement loop which enhances Th1 type immunity [29]. Among the other possible mechanisms, IFN-γ may also
Hydatid cyst fluid levels of IL-8 (A) and IL-12 (B) in cyst samples surgically removed from Algerian hydatid patients \((n=37)\). The hydatid fluids are classified into several groups. This classification is based on fertility ("+++": high fertility- \(n=13\); "++": medium fertility- \(n=15\); and "+": no fertility- \(n=9\)) and cysts location (Li: liver- \(n=15\); Lu: lungs- \(n=19\); and O: ovary- \(n=3\)). Cytokine levels were quantified as described in Materials and Methods and are presented as the mean ± SEM pg/mL. Significant differences between two groups (e.g. liver cyst/lung cyst) are indicated (\(*p < 0.05\); \(**p < 0.01\); \(***p < 0.001\); and \(****p < 0.0001\)). Differences were statistically significant for all groups.

Figure 2
acts by induction of chemoattractants for immune cells. IL-8 release may provide a potential mechanism for attraction of effector cells into the invaded tissue by *Echinococcus granulosus*. A further study is needed to investigate the effect of IFN-γ on IL8 and IL-12 production elicited by hydatid antigens.

**Influence of clinical stage**

The higher serum IL-8 and IL-12 levels observed after surgery support the notion of a relationship between clinical stage and cytokine induction. Our results imply that the increase in cytokines levels correlates with the inflammatory state following surgical removal of cysts. These observations are in line with those reported by many other authors showing that major surgery induces a series of inflammatory responses such as elevation of body temperature and erythrocyte sedimentation rate, leukocytosis and increased acute phase reactants [30, 31]. Current evidence suggests that these responses are mediated by pro-inflammatory cytokines such as IL-8 and IL-12, which are produced locally and enter the blood stream very soon after surgery [32].

**Influence of cyst location**

In addition to the clinical stage, our results indicate that cyst location is related to the intensity of cytokine induction. Patients carrying multiple cysts (in liver and lungs) have higher serum cytokine levels. This increase might be associated with the presence of more than one cyst. Cytokine production is also higher in patients with liver cysts in comparison with those bearing cysts in the lungs. This difference may be attributed to several factors. Hepatic hydatid cysts are expected to provide the highest amounts of antigen, probably because of the good vascularity of this major filtering organ [33]. Moreover, Vidor et al. showed that hepatic cysts are more permeable than pulmonary cysts [34]. The data are in agreement with a previously published report indicating that cyst location determines the host immune response. For instance, a close relationship has been reported between cyst number, characteristics and, above all, location within the liver itself, and increases in blood level of IL-1, IL-2, IL-4 [35] and NO [10, 11].

**Detection of cytokines in cystic fluid**

To our knowledge, the present study is the first to show the presence of cytokines within hydatid cyst fluid. Ait-Aissa et al. had previously observed the presence of nitrite oxide in the cyst fluid [11]. Our results show that, as a consequence of antigenic burden and IFN-γ induction, IL-8, and IL-12 produced may diffuse and move into the cyst. However, our findings do not exclude the possibility of cytokine up-regulation in localized areas of the parasite sequestration (production by neighbouring cells). Further investigations are required to clarify this hypothesis. Furthermore, our results show that cytokine levels are associated with cystic fertility and the viability and vitality of the protoscoleces. In the same way, cytokine levels seem to be variable according to the location of the cyst. All these data suggest that the production and the diffusion of IL-8 and IL-12 inside hydatid cysts may be governed by anatomical and physiological parameters, including the location of the cyst and cystic fertility.

**CONCLUSION**

Collectively, this present study provides evidence that IL8 and IL-12 are actively produced during the course of human *Echinococcus granulosus* infection. Our results also show that cytokine secretion correlates with cystic localizations and clinical stage. Further, circulating cytokines or locally- produced cytokines could diffuse into the cyst. Our results suggest that these two pro-inflammatory cytokines are implicated, at least in part, in the host defence mechanism in hydatid disease. These findings underline the strong relationship between the immune system of host and parasite. Thus, the measurement of serum cytokines may allow for better monitoring and prediction of disease evolution in human hydatid patients. IL-12 is a useful clinical marker for disease activity and the protective Th1 response in hydatidosis. Combined with its role in down-regulating Th2 responses associated with pathology, IL-12 represents a powerful tool with which to manipulate the immune system. It is our hope, in future studies, to evaluate the clinical usefulness of IL-12 in the treatment of human echinococcosis.

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