Immune Suppression in Advanced Chronic Fascioliasis: An Experimental Study in a Rat Model

Núria Gironeś,1 M. Adela Valero,2 Maria A. García-Bodelón,2 Isabel Chico-Calero,1 Carmen Punzón,1 Manuel Fresno,1 and Santiago Mas-Coma2

1Centro de Biología Molecular “Severo Ochoa,” Departamento de Biología Molecular, Facultad de Ciencias, Universidad Autónoma de Madrid, Campus Cantoblanco, Madrid, and 2Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Burjassot-Valencia, Spain

Chronicity and Th2 immune responses are features of helminth infections in humans. The liver fluke promotes its own survival through several strategies to down-regulate the immune response of the host during the early phase of infection. However, there is no evidence that this modulation occurs much later. The immune response in advanced chronic fascioliasis was analyzed in an experimental rat model at 20 weeks after infection. Cytokine quantification in infected rat serum revealed basal levels. The predominant immunoglobulin (Ig) isotype was IgG1. Flow cytometry analysis of T cell (CD3+, CD4+, and CD8a+), B cell (CD45R+), and macrophage (CD11b+) populations in spleens showed no significant differences between infected and control rats. Mononuclear cell proliferation in the spleen in response to T and B mitogens was strongly inhibited in infected versus control rats. During early chronic infection, there is a predominance of a Th2 response, which decreases in advanced chronic infection characterized by a persistent immune suppression.

Today, fascioliasis is considered to be an important human disease caused by 2 liver fluke species, Fasciola hepatica and Fasciola gigantica (Fasciolidae), infecting the liver of a wide range of mammals [1] that show a marked variability in their immune response against infection [2]. There are several geographic regions that have been reported to be areas of hypoendemicity, mesoendemicity, and hyperendemicity for fascioliasis in humans, with prevalences and intensities ranging from low to very high [3–7]. In zones of hyperendemicity, the majority of adult human subjects are supposedly experiencing the chronic phase of disease, which lasts many years [4]. The liver fluke promotes its own survival through several strategies to down-regulate the immune response of the host during the early phase of infection. The liver fluke apparently secretes molecules, known as excretory/secretory (E/S) products, that modulate or suppress host immune responses [8]. However, to our knowledge, there is no evidence that modulation of the immune response occurs much later (i.e., in advanced chronic fascioliasis).

CD4+ T cells can be separated into 2 major subsets, Th1 and Th2, on the basis of their cytokine secretion patterns and function. Th1 cells produce many cytokines, including interferon (IFN)–γ and tumor necrosis factor (TNF)–α, and promote the activation of macrophages and the production of opsonizing antibodies. Th1 cells mediate a delayed-type hypersensitivity reaction and inflammatory responses. Th2 cells produce many other cytokines, including interleukin (IL)–4 and IL–10, and promote immediate-type hypersensitivity re-actions involving IgE, eosinophils, and mast cells. Usually, the cytokines of each T cell subtype are mutually
inhibitory for the differentiation and effector functions of the reciprocal subset, resulting in the polarization of the immune response to either type 1 or type 2 [9].

The Wistar rat model is a useful approach for pathological research into the advanced chronic period of fascioliasis, because results can be extrapolated to chronic infection in humans. In the rat, fascioliasis is considered to be an advanced chronic disease from 100 days after infection onward [10, 11]. Previous studies performed on T helper cytokines in rats basically concerned the early stages of fascioliasis, suggesting that *Fasciola* species, similar to other helminth parasites, induce a polarized Th2 response [12–14]. However, to our knowledge, no studies have investigated the immune response of *Fasciola* species in the advanced chronic stage in a rat model. Consequently, we investigated cytokine, antibody, and spleen mononuclear cell (Spm) proliferative responses in the advanced chronic period in a Wistar rat model, by use of a parasite isolate from an area where human fascioliasis is hyperendemic.

**MATERIAL AND METHODS**

**Animals and experimental design.** The study was approved by the institutional committee on animal care at the University of Valencia (Burjassot-Valencia, Spain). A liver fluke isolate was obtained from the area of the Nile Delta region (in Egypt) where human fascioliasis is hyperendemic (Behera Governorate). Ten male Wistar rats (Iffa Credo) (body weight, 80–100 g) were housed in microisolator boxes and were maintained in a pathogen-free room that was electrically heated and subject to a light:darkness cycle of 12 h of light and 12 h of darkness (i.e., conditions in compliance with the European Agreement of Strasbourg [15]). A balanced commercial rodent diet (Panlab Chow A04) and water were provided ad libitum. At the Department of Parasitology at the University of Valencia, metacercariae were obtained from experimentally infected *Galba truncatula* snails and were stored in freshwater at 4°C. Previous studies performed on T helper cytokines in rats basically concerned the early stages of fascioliasis, suggesting that *Fasciola* species, similar to other helminth parasites, induce a polarized Th2 response [12–14]. However, to our knowledge, no studies have investigated the immune response of *Fasciola* species in the advanced chronic stage in a rat model. Consequently, we investigated cytokine, antibody, and spleen mononuclear cell (Spm) proliferative responses in the advanced chronic period in a Wistar rat model, by use of a parasite isolate from an area where human fascioliasis is hyperendemic.

**Morphometric measurement techniques.** The body size of the liver fluke (expressed in millimeters squared) was measured using a computer image analysis system (Optimas 5; Optimas), with use of a color video camera (Sony DVC-930P) connected to a stereomicroscope [18]. The total parasitic area was calculated for each rat as the natural logarithm (with “ln” denoting the logarithm having base e) of the sum of the body area of all liver flukes present in the common bile duct (lnBA). For bivariate correlations, the lnBA was used.

**Blood sampling.** For serum preparation, whole blood was collected by means of venipuncture (in the great saphenous vein) at 7, 8, 9, 10, 11, and 12 weeks after infection. At 20 weeks after infection, blood samples were obtained through exsanguination. The blood was centrifuged at 760 ng at 4°C for 10 min. Serum samples were stored at –80°C until analysis.

**Blood eosinophils and leukocytes.** Blood eosinophil and leukocyte counts for each rat were calculated only after 20 weeks after infection, by use of an automatic blood analyzer (Cell-Dyn; Abbott Cientı́fica). Verification of white blood cell differentiation was performed through thin blood films stained with Giemsa, and at least 100 cells were counted on each film.

**Bile samples.** The bile specimens were cultured aerobically by inoculation onto a Mueller-Hinton plate and eosin-methylene blue agar, respectively. They were incubated at 37°C for 24 and 48 h, respectively.

**Proliferation assays.** Spleens from infected and control rats were extracted, and cells were obtained using a 40-μm mesh cell strainer (Becton Dickinson Labware). After red blood cells were lysed with water for 5 s, the cells were washed twice in Dulbecco’s modified Eagle medium (DMEM) and were resuspended in DMEM 10% fetal bovine serum (FBS). Cells were plated in 96-well plates (2 × 10^5 cells/200 μL) containing 5 μg/mL concanavalin A (ConA) and lipopolysaccharide (LPS), as indicated. Proliferation was measured by incorporating 1 μCi [^3H]thymidine (Amersham Pharmacia Biotech) per well during the last 24 h of a 3-day culture. Cells were then harvested on a glass-fiber filter, by use of a Cell Harvester (Skatron Instruments), and radioactivity was estimated.

**Flow cytometry assays.** A total of 10^7 Spm cells per sample, obtained as described above, were washed in PBS containing 1% bovine serum albumin and 1% FBS. Cells were incubated with antibodies to CD3+, CD4+, CD8α+, and CD11b+ coupled to fluorescein isothiocyanate and CD3+, CD45R+, and CD11b+
coupled to phycoerythrin (BD Pharmingen) at a concentration of 1 \( \mu \text{g}/10^6 \) cells, in PBS, at 4°C for 20 min in the dark. Cells were washed 3 times and were resuspended in 500 \( \mu \text{L} \) of PBS. Samples were analyzed in a FACSCalibur apparatus (BD Biosciences).

**Cytokine assays.** Serum cytokine assays were performed according to the directions of the manufacturers. Rat IL-4 Eli-pair, Rat IFN-\( \gamma \) Eli-pair, and Rat TNF-\( \alpha \) Eli-pair were from Diaclone Research. Rat IL-10 ELISA Set was from BD Biosciences. Rat IL-1\( \beta \) ELISA Set was from R&D Systems.

**IgG1, IgG2a, and IgE measurements in serum.** Serum IgG and IgE levels were determined using detergent-soluble *F. hepatica* extract in ELISA. Mature flukes were removed from the bile ducts of bovine livers. For the preparation of a detergent-soluble extract, adult flukes were killed by freezing at \(-70^\circ\text{C}\) and were washed in PBS and drained. One gram of wet weight of minced tissue was incubated in 10 mL of 50 \( \text{mmol/L Tris (pH 7.4)} \), 150 \( \text{mmol/L NaCl, 1% deoxycholate, 1 \( \mu \text{g/mL of protease inhibitors (leupeptin, pepstatin, and aprotinin)} \right. \), and 1 \( \text{mmol/L phenylmethylsulfonyl fluoride. The extract homog} \) 

Coating of the wells was performed in PBS at 4°C overnight. Blocking was done at room temperature for 1 h, with the use of PBS containing 0.2% Tween 20 and 3% low-fat dry milk. Wells were washed 3 times with PBS containing 0.05% Tween 20 and 1% low-fat dry milk. Serum samples were diluted 1:50 in PBS and were incubated at room temperature for 1 h. Wells were washed 3 times with PBS containing 0.05% Tween 20 and 1% low-fat dry milk. Antibodies against IgG1, IgG2a, and IgE coupled to horseradish peroxidase (Serotec) were used at 1:10,000 dilution and were incubated at room temperature for 1 h. Wells were washed 5 times with PBS containing 0.05% Tween 20 and 1% low-fat dry milk, and they were developed with phenylenediamine at room temperature for 30 min. Absorbance at 450 nm was measured in an EL 340 Biokinetics microplate reader (Biotek Instruments).

**Statistical analyses.** Cytokines, antibody responses, proliferation indexes, eosinophils, and leukocytes were compared using the nonparametric Mann-Whitney U test. All experiments were repeated 2 or more times, with similar results achieved in each case. Bivariate correlation (Spearman correlation) was used for the analysis of cytokine levels (TNF-\( \alpha \), IFN-\( \gamma \), IL-10, IL-4, and IL-1\( \beta \)), immunoglobulin subclasses (IgG1, IgG2a, and IgE), egg, number of parasites, and lnBA. \( P<.05 \) was considered to be statistically significant.

**RESULTS**

**Development of *F. hepatica* chronic infection in rats.** The results of the experimental infections are summarized in table 1. All infected rats were parasitized (mean no. of parasites, 3.3 worms/rat). The average number of eggs emitted daily increased progressively until 78 days after infection, and, afterward, a steady decrease in the shedding of eggs was observed until 95 days after infection, when the coprological study was completed. Significant differences in the average of the total values of leukocytes and percentages of neutrophils, lymphocytes, monocytes, and eosinophils were not detected between infected rats and control rats at 20 weeks after infection (table 2). Results of all microbiological analyses of the bile samples obtained from infected rats and control rats were negative.

**Immunoglobulin production during infection.** Specific IgG and IgE responses were induced in all infected rats (figure 1). A predominance of the IgG1 versus the IgG2a subclass was observed in serum samples from 7 to 12 weeks after infection.

---

**Table 1. Kinetics of infection and morphometric parameters observed up to 20 weeks after infection in 6 rats with chronic Fasciola infection.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP days</td>
<td>43–47 (43.8 ± 1.6)</td>
</tr>
<tr>
<td>epg, by week after infection</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>144–968 (377.3 ± 309.5)</td>
</tr>
<tr>
<td>10</td>
<td>1160–4264 (2457.3 ± 1120.7)</td>
</tr>
<tr>
<td>14</td>
<td>1200–4128 (2360.0 ± 1095.7)</td>
</tr>
<tr>
<td>Worms at 20 weeks after infection</td>
<td></td>
</tr>
<tr>
<td>Recovered per rat, no.</td>
<td>1–6 (3.3 ± 1.8)</td>
</tr>
<tr>
<td>Recovered,a %</td>
<td>5–30 (16.6 ± 9.3)</td>
</tr>
<tr>
<td>Body area,b mm²</td>
<td>270.5–719.7 (443.7 ± 217.0)</td>
</tr>
</tbody>
</table>

**Table 2. Leukocytes observed in the blood of 6 chronically infected rats at 20 weeks after infection and in 4 control rats.**

<table>
<thead>
<tr>
<th>Rats</th>
<th>Leukocytes, no. ( \times 10^3 )</th>
<th>Neutrophils, %</th>
<th>Lymphocytes, %</th>
<th>Monocytes, %</th>
<th>Eosinophils, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>1.1–4.7 (2.7 ± 1.4)</td>
<td>12–25 (18.1 ± 4.5)</td>
<td>62–74 (65.0 ± 11.8)</td>
<td>4–23 (11.3 ± 6.6)</td>
<td>3–6 (3.8 ± 1.3)</td>
</tr>
<tr>
<td>Control</td>
<td>1.3–5.3 (2.9 ± 1.6)</td>
<td>14–29 (20.4 ± 6.3)</td>
<td>56–70 (65.4 ± 5.6)</td>
<td>8–14 (9.8 ± 2.5)</td>
<td>1–4 (2.8 ± 1.3)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are range (mean ± SD).
A significant increase in IgG1 and IgG2a levels in the serum of infected rats, with respect to control rats, was detected. However, to a lesser extent, the IgE levels of infected rats had also significantly increased from 7 to 12 weeks after infection, in comparison with levels in serum samples from control rats. The bivariant correlation of IgG1, IgG2a, and IgE levels in each infected rat at 7, 8, 9, 10, 11, and 12 weeks after infection and the weekly average of egg production (i.e., the epg) at 7, 8, 9, 10, 11, and 12 weeks after infection was calculated (figure 2). A significant negative correlation was obtained between IgG1 levels and the epg ($r = -0.442; P < 0.05$) (figure 2). Significant correlations were detected between the percentage of eosinophils and IgG2a ($r = 0.724; P < 0.05$) and between lnBA and IgG1 ($r = 0.886; P < 0.05$).

**Cytokine production during infection.** At 7, 10, and 20 weeks after infection, cytokine quantification in serum samples from infected rats showed a predominance of Th2 cytokines versus Th1 cytokines, compared with quantification in serum samples from control rats (figure 3). No significant differences in the secretion of type 1 cytokines IFN-γ and TNF-α were detected between infected and control rats at any time points tested. Nevertheless, an increase in TNF-α production was observed among the infected rats (figure 3). IL-1β, IL-10, and IL-4 levels at 7 weeks after infection were significantly higher in infected rats than in control rats. IL-10 and IL-4 levels decreased at 10 and 20 weeks after infection. No significant differences were detected in the levels of secretion of IL-1β, IL-10, and IL-4 at 20 weeks between infected and control rats. Nor were any significant correlations found between the number of parasites and the TNF-α, IFN-γ, IL-10, IL-4, and IL-1β levels in each infected rat at 7, 10, and 20 weeks after infection. However, analysis of the association between the TNF-α, IFN-γ, IL-10, IL-4, and IL-1β levels in each infected rat at 20 weeks after infection and the parasitic area total of all parasites in the common bile duct (lnBA) revealed a significant negative correlation between lnBA and the TNF-α level ($r = -0.845; P < 0.05$). A significant negative correlation was observed between the IL-4 level and the average egg production (the epg) at 7 and 10 weeks after infection ($r = -0.765; P < 0.05$).

**Spleen cell proliferative responses.** Flow cytometry analysis of T cell (CD3+, CD4+, and CD8α+), B cell (CD45R+), and macrophage (CD11b+) populations in spleens showed no significant differences between infected and control rats at 20 weeks after infection (figure 4). In experiments performed to evaluate the proliferative response to mitogens (which are considered to be nonspecific) during the advanced chronic period of the disease, *Fasciola*-infected Spm cell ConA activation from parasitized rats showed a significant decrease in proliferation, compared with Spm cells of healthy rats ($P < 0.05$). When the cells were stimulated with LPS, a significant decrease in the proliferation by Spm cells of parasitized rats, compared with Spm cells of healthy rats, was also found ($P < 0.05$) (figure 5). In conclusion, a pronounced suppression of mitogen-induced proliferative response was observed. This immune suppression was detected in all parasitized rats, independent of the worm burden (1–6 liver flukes/rat)—that is, suppression can be generated by a single adult parasite.

**DISCUSSION**

The present study describes the immune response of fascioliasis in a rat model, showing that, in early chronic infection, there...
is an increase in type 2 cytokines that changes to a decrease toward the advanced chronic phase. This finding correlates with a predominance of IgG1-type immunoglobulins specific for *Fasciola* species antigens at different times after infection, which is characteristic of a type 2 response.

At 20 weeks after infection, Spm cells showed no statistical differences in lymphoid and myeloid spleen cell populations, nor in total leukocytes and the percentage of eosinophils. These cells were unresponsive to mitogens, compared with the Spm cells of control rats. This finding is indicative of a persistent immune suppression. All the above findings are in agreement with the findings of other studies of different host species that have included infected rats but that have been performed at earlier stages of infection [19–26]. The reason for this unresponsiveness is not yet known. The E/S *Fasciola* products and/or the presence of regulatory T (Treg) cells are possibly responsible for this effect; therefore, further research needs to be done. This is the first time that immune suppression has been described in advanced chronic fascioliasis in a rat model.

**Blood eosinophils and leukocytes.** Blood eosinophilia and leukocytosis have been described in different host species in fascioliasis [2, 27]. Eosinophils are involved in the antibody-dependent cell-mediated cytotoxicity and participate in the antibody-mediated destruction of juvenile forms of *F. hepatica* in a variety of host species [28]. In experimental fascioliasis in rats, eosinophilia has been described, reaching a maximum value in the acute phase (4 weeks after infection) and exhibiting a gradual decrease to 10 weeks after infection [29]. The data
obtained in the present study of advanced chronic fascioliasis show that, although in some rats, the rate of eosinophils is higher than that in the control rats, the average is only slightly higher, and significant differences were not detected. As in other helminth infections, eosinophilia is one of the hallmarks of human fascioliasis [30] and is probably related to the early diagnosis of the disease in developed countries (in the acute or early chronic phase), or it is likely that most of the parasites eventually become trapped in the liver parenchyma [4]. Reports of infections in areas where human fascioliasis is endemic show that eosinophilia is not always present and, in some cases, is a useless indicator of infection (e.g., in Peru, only 47% of patients with chronic cases show eosinophilia [31], which is likely to result from the delayed diagnosis in areas of hyperendemicity, where the parasite usually reaches the bile duct [4]).

**Immunoglobulin subclasses.** A predominant Th2 cytokine response, along with antifluke IgG1 in serum, was detected in the present study, which agrees with the findings of other investigators [32]. Antibody responses in rats in the acute and chronic phase (1–21 weeks) of disease show a marked predominance of IgG1 over IgG2a isotypes. During the first weeks after infection, IgG1 quickly increases, whereas IgG2a slowly increases and reaches the highest values at 5–7 weeks after infection [32]. Humans infected with *F. hepatica* develop specific antibodies of the IgM, IgA, IgE, and IgG class. The immunoglobulin responses of liver fluke–infected humans to E/S antigens and to a fluke cysteine proteinase, cathepsin L1, showed that the predominant isotypes elicited by infection were IgG1 and IgG4 [33]. An association between egg counts and specific IgG antibody levels exists in humans [34].

**Cytokines.** Previous studies of Th cytokines in rats concerned only the early stages of fascioliasis. During the first 2 weeks of infection, *F. hepatica* induced a transient Th0 cytokine profile, followed by down-regulation of the cellular response and the induction of a Th2 cytokine profile [12, 13]. In infected rats, the presence of higher levels of IL-10 and IL-4 at 7 weeks...
after infection and of IL-10 at 10 weeks after infection was observed, findings that agree with previous observations regarding early infection. These findings suggest that the induction of IL-10 and IL-4 may suppress IFN-γ production by Th1 cells and inhibit activation of macrophages. Nevertheless, cytokine production is absent in the advanced chronic phase. IL-4 limits the fecundity and survival of other helminths [35]. Likewise, we demonstrated that there is a negative correlation between the liver fluke epg and IL-4, at least until 10 weeks after infection. In some of the infected rats, TNF-α also increased at 10 weeks after infection, which does not correspond to a typical Th2 response. TNF-α is implicated in the regulation of Th2 responses in other helminth infections, apparently regulating worm expulsion [36]. Unfortunately, the role of TNF-α during liver fluke infection still remains unknown. IFN-γ and IL-4 play an important role in the establishment of chronic helminth infection, parasite development, and worm expulsion [37]. Moreover, IL-4 and IL-10 can act synergistically, inhibiting the production of reactive nitrogen oxides, which up-regulate IL-12 production and inflammatory responses [38].

**Lymphocyte proliferative responses.** To date, all mechanisms described merely explain the advantage of the parasite in the phases of rapid parasite growth within the liver parenchyma and establishment within the bile ducts—that is, as a mechanism to avoid an immune response during the first stages of liver penetration [22, 39, 40]. A parasite-induced immune suppression in advanced chronic fascioliasis has been demonstrated for the first time, which might correlate with the immunomodulatory effects of E/S products released by the adult fluke observed in vitro, in the absence of cytokine secretion. High doses of *F. hepatica* and *F. gigantica* E/S products released by the adult inhibit lymphoproliferation induced by ConA in lymphocytes from various animal species [18, 23, 25, 26]. The liver fluke molecules that may induce immune suppression have yet to be characterized, but they most likely are actively secreted by the parasite. Candidate immunosuppressive molecules include glycoconjugates sloughed from the parasite surface glycocalyx, phosphorylcholine-rich antigens [41], Kunitz-type serine proteinase inhibitors [42], cysteine proteinases [43], and cathepsin L proteases [44]. On the other hand, the role of T<sub>R</sub> cells in chronic helminth infections has been described elsewhere [45]. T<sub>R</sub> cells are characterized by the predominant production of IL-10 and/or transforming growth factor β (TGF-β). In our study, IL-10 was not induced in the advanced chronic phase, but other regulatory cytokines were not tested. To our knowledge, there are no studies of TGF-β during advanced chronic fascioliasis. Therefore, the role of T<sub>R</sub> cells cannot be excluded in the maintenance of immune suppression. Future experiments will address this hypothesis. The present study proves that immune response modulation occurs in advanced chronic fascioliasis. The proliferative response of Spm cells to ConA and LPS significantly decreased at 20 weeks after infection, which is in agreement with immune suppression probably caused by an unknown fluke E/S product. This phenomenon has also been detected in schistosomiasis. Many studies clearly demonstrate that the chronic phase of schistosomiasis is characterized by a state of immune hyporesponsiveness exhibited as a reduced ability of host cells to proliferate [46].

Chronicity, immune suppression, and Th2-type immune responses are characteristic features of human infection with multicellular parasites [47]. Immune suppression and Th2 responses have been attributed to chronic helminth infection. In both laboratory animals and ruminants, fascioliasis, like other helminth infections, is a potent inducer of Th2 responses that impair the ability to mount any effective Th1 responses against bacteria and other pathogens [48, 49]. A high risk of bacterobilia in advanced experimental chronic fascioliasis has been described in a rat model and supports this fact [50]. Extrapolation of the results obtained in the rat model to human infection gives a new dimension to chronic fascioliasis. The effect of advanced chronic fascioliasis on the immune system, particularly parasite-induced immune suppression, and the effect of cytokines controlling polarization of the Th1 or Th2 arms pose a great risk for individuals to acquire concomitant infections (with viruses, bacteria, protozoa, and other helminths). This observation of coinfections in humans is often found in areas where fascioliasis is endemic (e.g., the Nile Delta region in Egypt, which is where the fluke isolate used in the present study originated [51]).

**Acknowledgments**

We thank M. D. Bargues and M. Kouchbane (Valencia, Spain) for collecting and rearing lymnaeid snails; for liver fluke infection experiments of snails; and for obtaining, keeping, and providing the metacercariae used. We also thank M. V. Periago (Valencia, Spain) for her collaboration in experimental rat studies. We also acknowledge the facilities provided by M. Mostafa, Z. Youssef, and Y. Abd El-Wahab (Egyptian Ministry of Health and Population, Cairo, Egypt), the Behera Survey Team (Damanhour, Egypt), and F. Curtale and P. Bardusgni (Italian Cooperation, Italian Embassy, Cairo, Egypt). We thank the Servicio de Análisis Clínicos del Hospital Militar de Valencia (Spain) for the hematological determinations.

**References**

5. Mas-Coma S, Esteban JG, Bargues MD. Epidemiology of human fas-


