Activation-Induced Apoptosis in Peripheral Blood Mononuclear Cells during Hepatosplenic Schistosoma mansoni Infections

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It is well established that programmed cell death (apoptosis) is an important regulator of host responses during infection with a variety of intra- and extra-cellular pathogens. The present work aimed at assessment of in vitro spontaneous and phytohemagglutinin (PHA)-induced apoptosis in mononuclear cells isolated from patients with hepatosplenic form of S. mansoni infections. Cell death data were correlated to the degree of lymphoproliferative responses to PHA as well as to the serum anti-schistosomal antibody titers. A markedly significant increase in PHA-induced apoptosis in lymphocytes isolated from S. mansoni-infected patients was seen when compared to the corresponding healthy controls. However, a slight difference was recorded between the two studied groups regarding the spontaneous apoptosis. This was accompanied with a significant impairment of in vitro PHA-induced lymphoproliferation of T cells from S. mansoni patients. Data of the present study supports the hypothesis that activation-induced cell death (AICD) is a potentially contributing factor in T helper (Th) cell regulation during chronic stages of schistosomiasis, which represents a critically determinant factor in the host-parasite interaction and might influence the destiny of parasitic infections either towards establishment of chronic infection or towards host death.

Schistosomiasis is a widespread chronic helminthic infection that contributes to death of over half a million people yearly (Newport & Colley 1993). The major form of the disease pathogenesis results from chronic granulomatous response to parasite ova trapped in host tissues. Most infected individuals, however, tolerate chronic infection without debilitating illness. This is thought to occur due to down-modulation of the host's granulomatous response. Failure to modulate can ultimately lead to hepatic periportal fibrosis, portal hypertension and, finally death. The mechanisms associated with modulation of the granulomatous response have important implications for control of schistosome-induced symptoms (Moha et al., 1999). Following the peak of the granuloma formation, a spontaneous down modulation of the inflammatory response occurs with diminished Th2 cytokine production, decreased granuloma size and cumulative fibrosis (Boros, 1994). However, the factors involved in the regulation of the CD4⁺ Th cell response at the acute and chronic stages of infection are still being investigated.

This immunomodulation received considerable attention and may be mediated by a number of different mechanisms including CD4⁺ T cell unresponsiveness by way of anergy (Andrade & Warren, 1964), active suppression (Stadecker & Flores-Villanueva, 1994), anti-inflammatory cytokines, idiotypic networks (Pedras-Vasconcelos & Pearce, 1996) and apoptosis (Rathmell & Thompson, 2002). The precise role that apoptosis has in the regulating mechanisms associated with chronic schistosome infection is not fully understood. Most of our knowledge about the mechanisms of apoptosis and modulation associated with these processes are derived from the murine model of the disease. Whether the mechanisms of apoptosis and disease in humans parallel those observed in murine
activation-induced apoptosis in PBMCs during hepatosplenic S. mansoni infections is not yet clear. In addition, most of the previous studies concerning apoptosis in schistosomiasis have been focused on the acute stage of the infection (Estaquier et al., 1997), while low attention has been paid to apoptotic mechanisms at chronic stages of the disease.

Subsequently, the present study was carried out aiming at assessment of in vitro spontaneous and PHA-induced apoptosis in lymphocytes isolated from patients with chronic Schistosoma mansoni infection, in order to gain an insight into the role played by apoptotic mechanisms in the immunomodulation observed during the chronic stage of infection. Cell death data was further correlated to the degree of lymphoproliferation in response to PHA as well as to the serum anti-schistosomal antibody titres.

Subjects and Methods

Subjects

The present study was carried out on twenty patients with pure S. mansoni infection free from other helminthic diseases admitted to the outpatient clinic of the department of Parasitology at Medical Research Institute, Alexandria University. In addition, ten normal schistosomiasis-free individuals, matched in age and sex, were taken as a negative control group. Careful clinical examination was performed to exclude smokers, diabetics and hypertensive patients from the study which might affect different immunological parameters. All study individuals were subjected to thorough history taking, stool and urine examination as well as serological investigation for concomitant hepatitis B and/or C and estimation of anti-schistosomal antibodies. S. mansoni-infected patients were chosen according to the presence of specific eggs in stools by Kato technique (Abdelfattah et al., 1999) and anti-schistosomal antibodies in serum by indirect haemagglutination test (van Gool et al., 2002).

Blood Samples

Seven ml of peripheral blood were obtained from each subject under-study by veinpuncture; the sample was immediately divided into 2 portions: 5 ml on heparin for lymphocyte separation and assessment of lymphoproliferation and apoptosis. The other 2 ml volumes were left without anti-coagulant and were used for serum separation.

Separation of Peripheral Blood Mononuclear Cells (PBMCs)

The mononuclear cell fraction was separated from all heparinized blood samples under aseptic conditions by density gradient centrifugation over Ficoll-Histopaque (Deas et al., 1997). Viability of cells was tested by trypan blue dye exclusion test.

Assessment of Lymphocyte Proliferation

This was carried out by the ³H-thymidine uptake method (Vargas et al., 1996). Briefly, 100 µl of PBMCs cell suspensions were transferred into 6 wells of a sterile 96-wells flat-bottomed microtitre plate containing 100 µl RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin at a final concentration of 2x10⁵ cells/well. Phytohemagglutinin (PHA) (Difco) was then added at a final concentration of 1µg/well to triplicate wells leaving the other three wells unstimulated. The plate was then incubated at 37º C in a humidified CO₂ incubator in an atmosphere of 5% CO₂ and 95% air for 3 days. Eighteen hours prior to the end of the culture, all wells were pulsed with 1 µCi, ³H-thymidine. At the end of the culture, the cellular contents of the plate were harvested using plate harvester (Dynatech) and the radioactivity incorporation was monitored in a β-counter. The extent of lymphoproliferative response was expressed as stimulation Index (S.I), which was calculated according to the following formula:

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\text{S.I} = \frac{\text{Mean cpm for the PHA stimulated wells}}{\text{Mean cpm for the unstimulated wells}}
\]

Where cpm = counts per minute.

Assessment of Apoptosis

Spontaneous and activation-induced cell death (apoptosis) was monitored by the assessment of DNA and histone mono- and oligonucleosomes that reflect the internucleosomal DNA fragmentation. This characteristic feature of dead cells was assessed using the cell death detection ELISA plus kit (Adler et al., 1995) (LaRoche). Briefly, volumes of 20 µl from each culture supernatant were transferred into corresponding wells. Volumes of 80 µl of anti-histone and anti-DNA-POD were added to each well and mixed. The plate was covered with adhesive foil and incubated for 2 hours at room temperature on a plate shaker at 300 rpm. After incubation, well contents were thoroughly
removed by tapping and were rinsed three times with 300 µl washing solution. A volume of 100 µl substrate solution was added into each well and the plate was incubated in the dark at room temperature and then 10 µl stopping solution were added. The optical density was read immediately at 405 nm using an ELISA reader (BioRad). Background values were subtracted from absorbance measurements of samples. The specific enrichment of mono- and oligonucleosomes released into the culture supernatant was calculated and expressed as mU/5x10^3 cells, where mU= absorbancex10^3.

Statistical Analysis
The obtained results were analyzed statistically using the commercial program SPSS version 10. Results were reported as mean ± SE. Differences between groups were considered significant at P< 0.05.

Results
This study was conducted on a total of 20 patients with schistosomiasis mansoni as the only helminthic infection. They were 11 males (55%) and 9 females (45%). Their age ranged from 10-49 years with a mean of 23.85±2.38. In addition, 10 healthy individuals with no current or history of schistosomiasis were enrolled in the study as negative controls. They were 7 males (70%) and 3 females (30%); age range from 25–36 years with a mean of 28.8±1.10.

Anti–schistosomal antibody titre
Estimation of serum anti–schistosomal antibody titre was performed in all subjects under study by indirect haemagglutination (IHA) kit (Fumouze)®. Results were expressed as the reciprocal of highest serum dilution still capable of agglutinating RBC’s. The obtained results revealed that all S. mansoni–infected patients diagnosed primarily by the presence of eggs in their stools were further confirmed serologically by IHA to have anti–schistosomal antibodies with various titres; 1/160 (1 patient), 1/320 (3 patients), 1/640 (7 patients), 1/1280 (6 patients) and 1/2560 (3 patients). On the other hand, all controls were seronegative.

T Cell Proliferation
Results of T cell proliferation were summarized in figure 1 and represented as stimulation index (S.I.). The mean S.I. in schistosomal patients was 14.1±2.11 compared to 36.3±1.7 in negative control individuals which revealed that the lymphoproliferative response to polyclonal stimulation with PHA was significantly impaired in S. mansoni infected patients as compared to that of the healthy control subjects (t= 6.829, P= 0.0001; Fig 1).

Figure 1. Lymphoproliferation of PBMCs of normal controls and Schistosoma mansoni infected patients expressed as stimulation index (S.I). Proliferation was measured by [³H] thymidine incorporation.
Programmed Cell Death (Apoptosis)

Mean spontaneous cell death in controls was 0.123±0.015 mU while it was 0.158±0.011 mU after PHA stimulation. On the other hand, mean apoptosis in *S. mansoni* infected patients was 0.33±0.058 mU compared to 1.11±0.17 mU following PHA activation. The statistical analysis of the obtained data is illustrated in figure 2. The data revealed that only peripheral blood lymphocytes from *S. mansoni* infected patients manifested increased tendency to die in a programmed manner by apoptosis following stimulation with PHA (*P*=0.0001). On the other hand, lymphocytes from healthy controls did not show such a significant tendency for apoptosis following stimulation with PHA (*P*=0.077). In addition, the obtained results revealed a slightly significant difference between *S. mansoni* patients and controls concerning the spontaneous DNA fragmentation (*P*=0.019); but a markedly significant increase in PHA–induced apoptosis in lymphocytes from *S. mansoni* infected patients was recorded when compared to their partners isolated from healthy controls (*P*=0.0001).

**Figure 2.** Statistical difference between results of spontaneous and PHA-induced apoptosis in *S. mansoni* infected patients and healthy control subjects.

Correlation Analysis

The statistical analysis of the results revealed that a significant positive correlation was detected between the titre of anti–schistosomal antibodies and the lymphoproliferative response to PHA. In addition, a positive and strongly significant correlation was also found between the spontaneous and PHA activation–induced apoptosis (figures 3, a and b). Meanwhile, the results showed a negative correlation between antibody titre and the spontaneous and PHA activation–induced apoptosis (figure 3, c and d), but no correlation could be detected between magnitude of mitogen-driven blastogenesis and apoptosis.
Discussion

Schistosomiasis is one of the major communicable diseases of public health and socio-economic importance in the developing world (Kenawy & Rizk 2004). It remains a public health problem in Egypt, despite the continuous control efforts (Abo-Madian et al., 2004). This disease is caused by Schistosoma and represents a principal cause of chronic and debilitating illness in endemic areas (Cook et al., 2004). It has been established that schistosomes display a dual impact on the immune system where the granulomas formed around ova eventually trigger periportal fibrosis and portal hypertension (Singh et al., 2004), and larval stage known as schistosomulum appears to subvert the host defense mechanisms (Trottein et al., 2004). Apoptosis appears to play several fundamental roles within the host-parasite relationship that is ultimately reflected in an effect on the parasite population either mediated through an alteration in parasite fecundity or reduction in parasite numbers (Bosque et al., 2005).

Results of the present study revealed a significant impairment of the in vitro PHA-induced lymphoproliferative capacity of cells from Schistosoma mansoni infected patients as compared to those from healthy controls. Such data are in agreement with those of Zwingenberger et al., (1989) who found that peripheral blood lymphocytes from asymptomatic S. mansoni-infected patients proliferate poorly compared with acutely infected subjects after curative chemotherapy. One of the mechanisms that contribute to the diminished T cell proliferation, results from active suppression by IL-10 (Freeman et al., 2005). In addition, this down-regulation of lymphocyte proliferation is thought by Lenardo et al., (1999) to be an important mechanism in limiting tissue damage and

Figure 3. Correlation analysis of anti-schistosomal antibodies and the lymphoproliferative response to PHA
other side effects caused by sustained inflammatory responses.

The impaired in vitro lymphoproliferation may also be attributed to the in vivo exhaustion of naïve T cell repertoire or due to the release of atypical lymphocytes where the reduced T cell reactivity to PHA may be a consequence of fewer functionally reactive lymphocytes rather than to a decreased rate of proliferation (Kim et al., 2001).

Koetz et al., (2000) showed that reduced telomere length of lymphocytes indicate that a major portion of T cells have gone through an increased replicative history in vivo with subsequent exhaustion upon in vitro stimulation with PHA.

In addition, development of CD4+ T cell populations lacking CD28 and CD7 expression may be associated with lower replicative capacity and inability to proliferate (Martens et al., 1997) An interesting explanation for the reduced T cell responsiveness to mitogen comes from the study of Zurier et al., (1999) who found that, possible minor changes in fatty acid content of T cells may have profound effects on early T cell signaling and T cell suppression. Also, depletion of CD14+ cells that have a survival signal resulted in a much higher increase of activation-induced cell death (AICD) and decrease of T cell proliferation (Tang et al., 2004).

Programmed cell death (apoptosis) was monitored in the present study employing the DNA fragmentation method, either spontaneously or following in vitro activation by PHA mitogen. According to our data, lymphocytes isolated from chronic S. mansoni-infected patients, exhibited slightly increased spontaneous apoptosis as compared to their negative control partners. However the cell death difference between patients and controls was significantly amplified following in vitro activation by PHA mitogen.

The finding that spontaneous cell apoptosis was elevated at chronic stage of infection suggested that antigenic stimulation by parasitic antigens (schistosome egg and worm antigens) could be involved in the induction of apoptosis. Lundy et al., (2001) proved in their study on experimental animals, that AICD of CD4+T cells is involved in the immunoinflammatory response and consequently involved in the down modulation of granulomatous reaction during the chronic stage of schistosomiasis. Krammer (2000), demonstrated FasL, CD95L on the surface of CD4+, CD8+ T cells as well as on CD19+ B cells during the early Th1 stage of schistosome infection and, hence, concluded that soluble egg antigen (SEA) stimulated T cell apoptosis at this stage of infection. Also, Fallon et al., (1998) demonstrated that SEA had a little effect on cytokine production of splenic CD4+ T cells derived from uninfected mice. This in turn may lead to the hypothesis that AICD is a potential contributing factor in Th cell regulation, where this regulation of host cell apoptosis is a critical determinant factor in host-parasite interaction.

The results of the current study suggested that as the infection proceeds to chronicity, the proapoptotic machinery expands to a point at which the balance is shifted towards cell death. In the face of continued egg deposition and antigenic stimuli, AICD of activated lymphocytes that mediate the inflammatory response seems to be an important regulatory mechanism which diminished lymphocyte proliferation recorded in our results and consequently, the inflammatory cytokine production and granulomatous inflammation (Boros, 1989). This explanation was adopted by Ricco et al., (2003) during human malaria infection. They suggested that occurrence and intensity of lymphocyte apoptosis in blood samples were significantly increased when compared to healthy individuals. It appeared that CD4+, compared to CD8+ T cells, were more susceptible to apoptosis either in infected or non-infected individuals and that
an increased expression of Fas antigen was recorded after stimulation with parasite antigen.

Our data also agreed with another study by Pinheiro et al., (2004) on *Leishmania amazonensis* concerning spontaneous and mitogenetic proliferative responses of T cells. They observed that T cell anergy was not due to a decreased production of growth factors, cell necrosis, or defects in antigen presentation but was accompanied by excessive DNA fragments in lymph node cells, indicating accelerated apoptosis.

In accordance to our results, Bosque et al., (2005) suggested that selective apoptosis of sensitized CD4+, but not CD8+, T lymphocytes within granulomas contributes to the state of immunoregulation recorded at chronic stages of schistosomiasis.

Similar to our findings, a study by Chen et al., (2002) showed that lymphocytes isolated from the skin or skin-draining lymph nodes of naïve mice or mice immunized previously with radiation-attenuated cercariae of *S. mansoni*, failed to respond to antigens in the excretory/secretory products of skin-stage schistosomula that can release molecules in their secretions capable of inducing apoptosis of T cells in the skin. Based on this finding, it could be suggested that the parasite may use T cell apoptosis as a potential mechanism to reduce initial cellular responses in the skin. Rumbley et al., (1998) proved that splenocytes from infected mice were sensitive to mitogen-induced apoptosis, which was detected in histological spleen and granuloma sections. Meanwhile, splenic Th1 cells were more susceptible to apoptosis than Th2 counterparts and a high level of lymphocyte apoptosis in granuloma, but not in splenic cells in infected mice, was recorded. Stadecker et al., (2004) revealed that severe schistosomiasis is associated with persistently elevated pro-inflammatory Th1 type cytokines, whereas milder pathology is present when Th2 cytokines dominate. This scenario is supported by the pronounced pathology resulting from the obliteration of pathways that facilitates Th2 differentiation and by the development of more intense lesions in mouse strains that fail to down-regulate the Th1 response. The authors revealed that low-pathology mice exhibit enhanced CD4+ T cell apoptosis which may contribute to limited pathology.

These data are comparable to those indicated by Carneiro-Santos et al., (2000) concerning greater T cell apoptosis in patients with mild intestinal schistosomiasis than in those with severe hepatosplenic form of the disease. These studies lend relevance to T cell apoptosis as a possible means to regulating disease severity in vivo. Together, these studies further emphasize the important regulatory role played by apoptosis in schistosomiasis and demonstrate that this process may be an important mechanism in controlling morbidity in *S. mansoni* infected individuals.

Correlation analysis of the present data revealed a positive correlation between spontaneous and activation-induced apoptosis in *S. mansoni* infected individuals. This is an expected finding since PHA-stimulation is thought to reflect and amplify the apoptosis rates recorded in vivo in the same subject.

In addition, the anti-schistosomal antibody titer was negatively correlated with the level of apoptosis. This is supported by the finding of Lundy et al., (2001) who recorded increased expression of FasL on CD19+ B lymphocytes in *S. mansoni* infected mice. In addition, Jankovic et al., (1998) found that the effect of antibody in modulation of granuloma size is directed through Fc receptor. Meanwhile, Rezendi et al., (1997) proved that the down-regulation of in vivo granuloma size takes place by immune complexes.

It is likely that effective manipulation of host cell apoptosis will become feasible in the future, either through the use of soluble recombinant constructs and monoclonal
antibodies that interfere with the Fas/FasL death pathway, or through pharmacological agents that interfere with the cellular apoptotic machinery. Such reagents would be helpful in the management of the host immune response to parasitic infection. Hence, future studies of lymphocyte apoptosis during acute and chronic stages of \textit{S. mansoni} infections are recommended to clarify the apoptotic mechanisms displayed at both stages. Assessment of the underlying factors (parasite or host) that may orient the activation-induced cell death of lymphocytes during and after egg deposition is thought to be pivotal in determining the destination of schistosomiasis either towards chronic infection or towards hepatosplenic complications that may lead to death.

References


imported schistosomiasis by a combination of a commercial indirect hemagglutination test with *Schistosoma mansoni* adult worm antigens and an enzyme-linked immunosorbent assay with *S. mansoni* egg antigens. J Clin Microbiol; 40: 3432-7.

