

IMMUNOPATHOLOGY OF HUMAN SCHISTOSOMIASIS MANSONI. II. NK ACTIVITY AND STIMULATION BY SPECIFIC ANTIGEN.

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SUMMARY

Sixteen *S. mansoni* infected and untreated patients (5 with recent infection and 11 with chronic disease) were evaluated for their *in vitro* natural killer (NK) activity against the NK sensitive target K562 cell line. NK levels in 9 out of 11 patients (82%) with chronic disease were significantly lower (mean = 15 ± 6%), compared with patients recently infected (mean = 41 ± 9%, $p < 0.001$) and with the control group (mean = 38 ± 13%, $p < 0.001$). However, both patients and controls NK activity was stimulated by soluble adult worm antigens (SAWA), indicating that NK function even in the chronic stage of the infection is able to respond to the parasite antigens. These results suggest the possibility of NK cell participation as effector mechanism against *S. mansoni*.

KEY WORDS: Natural Killer cells (NK); *S. mansoni*.

INTRODUCTION

Natural Killer (NK) cells are a subpopulation of lymphoid cells defined in several species, including man, by their spontaneous ability to lyse certain tumor target cells *in vitro*¹¹. NK activity is found predominantly in a population of cells known as large granular lymphocytes (LGL)¹².

Increasing evidences indicate that in certain types of tumors and infection, NK cells behave as effector cells *in vivo*¹⁸. The levels of NK activity have been found elevated in children with malaria and in mice infected with *Trypanosoma cruzi* and *Toxoplasma gondii*, suggesting a possible effector role of these cells in parasitic diseases^{8, 9}. Studies in *Schistosoma mansoni* infected mice have shown contradictory results: some have demonstrated activation of the NK system

during the acute phase of the infection, whereas other investigations have failed to show activation during the first four weeks of the infection^{1, 2}. Currently, there is no evidence to support the participation of NK cells in any given aspect of Schistosomiasis or other helminthic infections. This prompted us to determine whether *S. mansoni* infected patients with recent or chronic infection express altered levels of NK activity and the possible susceptibility of NK cells to be stimulated by *S. mansoni* adult worm antigens.

MATERIALS AND METHODS

Patients

Sixteen *S. mansoni* infected patients with a mean age of 36 years (range 18-50) were studied.

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Patients population was drawn from individuals admitted to the Bilharzia Experimental Laboratory at the Tropical Medicine Institute of the Central University of Venezuela.

The diagnosis was established following a standardized protocol (Table 1). Five patients were studied 6-12 months after primary exposure to the parasite and were classified as recent infections, and 11 individuals with documented infection longer than two years, were classified as patients with chronic disease. Two patients from the later group presented extra hepatic granulomas diagnosed by biopsy, one in the cervix of the uterus and the second one in the rectum with acute proctitis. Blood samples were drawn

and processed immediately after collection. Stool samples were studied by standard Kato and formol ether techniques; the number of eggs was also determined by the Kato Katz technique¹⁴. In addition, all patients were tested for the presence of specific antibodies against eggs or adult worms by intradermal¹⁵, ELISA²¹, and circumoval precipitin test¹⁷. All but one of the selected patients were parasitologically positive by at least 2 out of 3 immunodiagnostic procedures; furthermore, no other intestinal parasites but *S. mansoni* was detected in these patients. Sixteen non infected healthy individuals, evaluated by parasitologic and immunological methods, matched by age and sex with the patients, were included as controls.

TABLE 1
Clinical and Immunoparasitological Features of Schistosomiasis Patients

| Type of infection | N of patients | Sex (f/m) | Age Range (years) | INTENSITY OF INFECTION | | | |
|-------------------------|---------------|-----------|-------------------|--------------------------------|--------------------|---------------------|------------------|
| | | | | Mean (range) eggs gr. of feces | COPT ¹⁷ | ELISA ²¹ | Intradermal test |
| RECENT (6-12 months) | 5 | 2/3 | 23-45 | 65 (25-100) | 100 | 80 | 100 |
| CHRONIC (> 2 years) | 11 | 6/5 | 16-50 | 20 (10-50) | 100 | 91 | 80 |

(*) -- COPT = % positive patients by circumoval precipitin test

(†) -- ELISA = % positive patients by immunoenzymatic assay

(***) -- Intradermal Test = % positive patients by Bilharzia

Cell Preparation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood (10IU/ml sodium heparin) by centrifugation on Ficoll-Hypaque gradient as previously described⁴. PBMC was resuspended in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY), 4 mM L-glutamine and 100IU/ml Penicillin-Streptomycin mixture.

Non-adherent peripheral blood lymphocytes (PBL) were obtained after depletion of adherent cells (mostly monocytes) and B cells by sequential incubation on plastic surfaces (30 min at 37°C) and nylon wool columns, following the Julius's Technique¹².

Source of adult worm antigen

Soluble adult worm antigen (SAWA) was obtained according to COLLEY et al.⁶. Lyophilized adult worms were suspended in RPMI 1640 and homogenized in a teflon glass homogenizer. The suspension was maintained at 4°C for 24 hours, frozen and thawed. Thereafter, the suspension was centrifuged at 12,000 g for 2 hours, the supernatant removed and sterilized by filtration through a 0.45µm pore diameter millipore filter. Protein concentration was determined according to LOWRY et al.¹⁶. The antigen was aliquoted and kept at -20°C until use.

Stimulation of NK function with antigens

Two million cells (PBL) from patients and controls were stimulated with different concen-

trations of worm antigen or streptokinase/streptodornase (SK/SD), a common recall antigen; after 18 hours of incubation at 37°C, the cells were washed 3 times with culture medium and used as effectors in the NK cytotoxicity assay. Optimal stimulating doses for the adult worm antigen (2.5-5 µg/ml) was determined by mean of doses-response curves with a fixed number of cells.

NK microcytotoxicity assay

A ⁵¹Cr-release assay was performed as previously described⁵. Briefly, the NK sensitive target cells, K562, were labeled with 200µCi of NA⁵¹CrO₄ (New England Nuclear Boston, Ma.) for 1 hour at 37°C; the cells were washed three times and resuspended at the desired concentration in RPMI 1640 containing 10% FCS.

A fixed number of target cells (4 x 10³ in 0.1 ml) was mixed in triplicate with effector cells at different ratios in a 96 well round bottomed microtiter plate. Control wells were filled with 0.1 ml of target cells plus 0.1 ml of culture medium.

After incubation for 4 hours at 37°C, the plates were centrifuged at 400 g for 10 min, and 0.1 ml of supernatant were taken for determination of isotope release. Percent of specific release was calculated by the fórmula:

$$\% \text{ Specific release} = \frac{\text{Experimental release (cpm)} - \text{Spontaneous release (cpm)}}{\text{Total release (cpm)} - \text{Spontaneous release (cpm)}} \times 100$$

Statistical Analysis

The Student's t-test for paired and non-paired data was used.

RESULTS

NK activity was determined in the PBL of two groups of selected *S. mansoni* infected patients, 11 with chronic disease (> 2 years infection) and 5 recently infected (< 1 year infection). As shown in Fig. 1, the NK levels in 9 out of 11 patients (82%) with chronic disease was signi-

ficantly lower (mean = 15 ± 6%), than that of recently infected (mean = 41 ± 9%; p < 0.001) and that of the control group (mean = 38 ± 13%; p < 0.001).

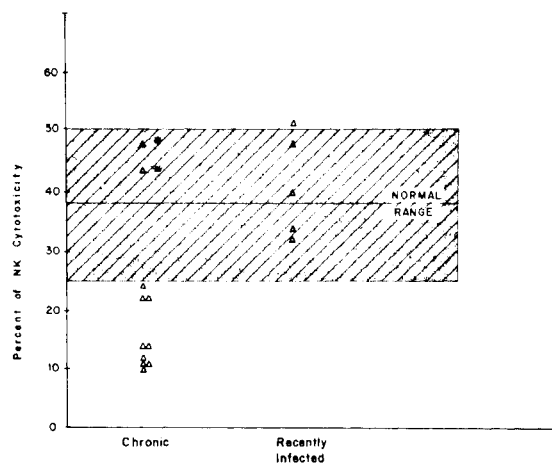


Fig. 1 — Basal NK activity in patients with *Schistosomiasis mansoni*, evaluated against K562 cell line in a 4 hours ⁵¹Cr release assay at 50:1 effector/target cell ratio.

* Patients with extra-hepatic granulomas.

It was interesting to note that the two remaining individuals with chronic disease and symptomatic extra-hepatic granulomas, showed a NK activity within normal range (also depicted in Fig. 1); differences observed among the recently infected patients and controls were not significant.

Activation of NK function by adult worm antigen

In order to assess whether NK function was susceptible to *in vitro* activation by parasite antigens, non-adherent cells from patients and controls, were stimulated for 18 hours with SAWA and subsequently used as effector cells in the cytotoxicity assay.

As shown in Table 2, a significant activation of NK function was achieved in both patients and controls; this activation was consistently observed when cells from chronic patients were stimulated with 2.5 µg/ml of SAWA as compared with controls or recently-infected individuals. In the later group, the activation was detected only at the lower effector target cells ration (E/T) and the higher doses of the antigen (5.0 µg/ml).

TABLE 2
Effect of adult worm antigen on NK activity in schistosomiasis mansoni

| Group | Stimulus µg Ag/ml | Percentage of Cytotoxicity | | |
|-------------------|----------------------|----------------------------|------------------------|------------------------|
| | | E/T Ratio | | |
| | | 100:1 | 50:1 | 25:1 |
| Chronic | 0.0 | 29 ± 10 | 15 ± 6 | 9 ± 4 ⁽¹⁾ |
| | 2.5 | 47 ± 10 ⁽¹⁾ | 30 ± 14 ⁽²⁾ | 23 ± 15 ⁽²⁾ |
| | 5.0 | 42 ± 8 ⁽¹⁾ | 28 ± 10 ⁽²⁾ | 21 ± 12 ⁽¹⁾ |
| Recently Infected | 0.0 | 58 ± 8 | 41 ± 9 | 30 ± 7 |
| | 2.5 | 60 ± 13 | 45 ± 11 ⁽³⁾ | 32 ± 7 ⁽³⁾ |
| | 5.0 | 62 ± 7 | 50 ± 4 ⁽²⁾ | 36 ± 2 ⁽¹⁾ |
| Controls | 0.0 | 53 ± 12 | 38 ± 13 | 26 ± 11 |
| | 2.5 | 60 ± 12 ⁽³⁾ | 45 ± 12 ⁽³⁾ | 34 ± 13 ⁽³⁾ |
| | 5.0 | 62 ± 11 ⁽²⁾ | 47 ± 13 ⁽²⁾ | 34 ± 12 ⁽¹⁾ |

NK activity was evaluated in basal condition and after estimation of 2×10^5 PBL (18h at 37°C) with 2.5 and 5.0 µg/ml of SAWA in a four hour ⁵¹Cr release assay.

(1), (2), (3) represent $p < 0.001$, $p < 0.01$, $p < 0.025$ respectively obtained when compared the basal NK activity vs SAWA stimulated as calculated by the Student's t test for paired data.

Activation of NK Function by SK/SD

Susceptibility of NK cells to be activated by a variety of antigen, including those of bacterial nature, allowed us to utilize the SK/SD recall antigen in order to evaluate the capability of the NK system in *S. mansoni* patients to respond to other stimulatory proteins²⁰. As shown in Table 3, both patients with recent infection and controls responded with a significant increase of NK function when stimulated with a dosis of 100 µg/ml SK/SD antigen.

TABLE 3
"In vitro" stimulation of NK activity by the SK/SD recall antigen in patients with Schistosomiasis Mansoni

| Groups | Percent of Cytotoxicity | | |
|---------------------|-------------------------|---------|-------|
| | Basal | SK/SD | P* |
| Patients (n = 4) | 58 ± 11 | 77 ± 16 | 0.025 |
| Controls (n = 4) | 61 ± 11 | 68 ± 9 | 0.05 |

2×10^5 PBL were estimated (18 hour at 37°C) with 100 µg/ml of SK/SD antigen and tested for NK activity in 4 hours ⁵¹Cr release assay.

* Student's test for paired data.

DISCUSSION

NK cells are considered a non-specific effector mechanism, susceptible of activation by a variety of viral, tumoral and some bacterial antigens^{15, 20}. The reports on the participation of this effector mechanism against parasites are rather scant. In several models such as *Plasmodium chabaudi*¹, *Babesia microti*¹, *Trypanosoma cruzi*⁸, and *Toxoplasma gondii*⁹ increase in NK activity have been found. More recently, a NK-mediated killing of *T. gondii* was demonstrated in vitro¹⁰. Experimental studies in recent *S. mansoni* infected animals have shown activation of the NK function which decreases as the infection became chronic; in humans, no previous report has compared NK function in patients with recent and chronic *S. mansoni* infection. In addition, as far as we know, there is not previous evidence of NK activation by *S. mansoni* antigens.

The results presented here show that *S. mansoni* chronically infected patients have a significant depressed NK activity as compared with that of recently infected individuals and the control group. By contrast with other reports on NK function in Schistosomiasis, our patients were only infected with *S. mansoni* and the parasite burden was lower⁷. Furthermore, our results are in contrast with those of BARSOU et al³ who did not find alterations of the NK activity in chronic *S. mansoni* infected patients.

Although the presence of inactive or defective NK cells was not examined in this study, it was of interest to find that soluble antigen from adult forms of the parasite (SAWA) and SK/SD stimulated significantly the NK function in both groups of patients. These observations indicate that non-intrinsic defect exists at the NK cell level and also suggests the possibility of NK cells participation as effector mechanism against *S. mansoni* particularly during the early stages of the infection.

The susceptibility of the different stages of the parasite to human NK cells should be investigated in order to gain further knowledge on the possible protective role of this effector mechanism against *S. mansoni*.

RESUMO

Imunopatologia da esquistossomose mansônica humana. II. Atividade NK e estimulação por antígeno específico.

Dezesseis doentes infectados e não tratados com *S. mansoni* (5 com infecção recente e 11 com doença crônica), foram submetidos a avaliação de atividade de células exterminadoras naturais (NK) "in vitro" frente a células alvo de linhagem K562. Os níveis de atividade das células NK em 9 de 11 doentes (82%) com a infecção crônica foram significativamente menores (média = $15 \pm 6\%$) quando comparados aos pacientes com infecção recente (média = $41 \pm 9\%$, $p < 0,001$) e aos indivíduos do grupo controle (média = $38 \pm 13\%$, $p < 0,001$). Porém, tanto nos doentes como nos controles, a atividade de células NK foi estimulada pelo antígeno solúvel do parasito adulto (SAWA), indicando que as células NK, mesmo na fase crônica da infecção, têm capacidade de responder ao antígeno dos parasitos. Estes resultados sugerem a possível participação das células NK no mecanismo efetor de defesa contra o *S. mansoni*.

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