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Enhancement of human natural cytotoxicity by *Plasmodium falciparum* antigen activated lymphocytes

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Summary

Mononuclear cells (MNC) isolated from malaria immune donors and from donors never exposed to malaria were stimulated in vitro with soluble purified *Plasmodium falciparum* antigens (SPag) or PPD. After 7 days of culture the proliferative response and the cytotoxic activity against the natural killer cell (NK cell) sensitive cell line, K562, were measured. It was found that SPag stimulation enhanced cytotoxic activity of MNC from donors whose lymphocytes exhibited a strong proliferative response to the antigen. MNC with low proliferative responsiveness showed increased cytotoxic activity if the MNC were preincubated with interleukin 2 (IL-2) for one hour before the start of the cytotoxic assay. SPag activation did not enhance the cytotoxic activity of MNC which did not respond to the antigen in the proliferation assay, and preincubation of these cells with IL-2 did not increase the activity. PPD stimulation enhanced the cytotoxic activity and induced strong proliferative responses in all MNC preparations. The role of NK cells in the protection against malaria is unknown, but they play a role in the protection against virus infection and in the immune surveillance against cancer. Our findings indicate that malaria antigens either directly or through the activation of immunoregulatory cells enhance the NK cell activity.

Key words: *Plasmodium falciparum;* NK cells; antigen activation.

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Introduction

It is generally accepted that acquired immunity to malaria is based on both humoral and cell-mediated mechanisms. The cell-mediated elements have not been well defined (Deans and Cohen, 1983), but it has been proposed that the stimulation of specific T-cells by parasite antigens triggers the activation of nonspecific effector cells that eliminate the parasite in immune individuals (Allison, 1984; Jensen et al., 1983; Ockenhouse and Shear, 1983). Neutrophils and products of activated macrophages have been shown to damage the parasite (Kharazmi and Jepsen, 1984; Ockenhouse et al., 1984). It is known that NK cells mediate the killing of virus infected cells and tumor cells (Ortaldo and Herberman, 1984; Pedersen, 1985). Their involvement in the killing of malaria parasites is not clear (Herberman, 1981). However, it has been suggested that these cells may play a role in the protection against *P. falciparum* infection (Allison, 1984). The present investigation was undertaken to establish if *P. falciparum* antigens could increase the NK cell activity in vitro, thus we examined the effect of soluble purified antigens isolated from *P. falciparum* culture supernatants on the NK cell activity of lymphocytes isolated from malaria immune Africans and from non immune controls. Furthermore, the effects of purified protein derivative of tuberculin (PPD), IL-2, and Interferon (IF) in this interaction were studied.

Materials and Methods

Preparation and culture of mononuclear cells. Heparinized blood was collected from Danish and from West-African donors. Isolation procedures were performed in Copenhagen and were always completed within less than 24 h after collection of the blood samples. Mononuclear cells (MNC) were isolated from heparinized blood by Lymphoprep (Nyegaard, Norway) density gradient centrifugation and washed three times in medium, RPMI 1640 supplemented with 15% pooled normal serum, L-glutamine (58.4 μg/ml), penicillin (20 IU/ml), and streptomycin (20 μg/ml). The cells were then frozen (controlled gradient freezing machine, Cryoson, GB) and stored in liquid nitrogen until testing. On the day of testing the MNC were thawed and washed twice in medium. Cell cultures were performed in microtiter trays (Nunc, Denmark) with 5×10^4 MNC in a final volume of 170 μl of culture medium per well. 20 μl of antigen were added to each well. After 7 days incubation the MNC were tested in proliferation assay and for cytotoxicity against K562 tumor cell line.

Proliferation assay

Cultures tested for proliferative response were pulsed with 3-H-thymidine (New England Nuclear, USA) 1 μCi per well after 6 days of culture and harvested on glass fibre filters with a harvesting machine (Skatron, Norway) 24 h later. 3-H-thymidine incorporation was measured in a liquid scintillation counter (TRI-CARB Packard, USA). The assay was performed in triplicate, and the median cpm was recorded.

Cytotoxicity assay

Cultures tested for cytotoxicity were collected from the trays on the 7th day, washed 3 times, and counted. Cell viability was determined by trypan blue exclusion and the MNC were then used as effector cells in the cytotoxicity assay. Some of the MNC were incubated with alpha-interferon (IF) (kindly provided by Dr. Robert Jordal, Copenhagen County Hospital, Gentofte, Denmark) in a final

concentration of 10 IU/ml, or purified interleukin 2 (IL-2) (Boehringer, Germany) in a final concentration of 30 IU/ml, for 1 h at 37°C, before they were incubated with the target cells.

Human leukemia cell line K 562 was used as target cells. 0.1 μCi $Na_2\ ^{51}CrO_4$ (New England Nuclear) were added to 2–8×10^6 target cells. After 1 h at 37°C, the labelled cells were washed through a 4 ml cushion of heat-inactivated fetal calf serum (FCS). The cell pellet was resuspended in RPMI with 10% FCS. 100 μl of target cells in a concentration of 5×10^4/ml and 100 μl of effector cells in a concentration of 4×10^6/ml were added to each well of round bottomed microtitre trays (Nunc), in triplicate. After incubation for 4 h at 37°C the cell mixture was centrifuged, 100 μl of each supernatant was transferred into tubes, and radioactivity was counted in a Packard scintillation gamma counter. Maximum experimental release was determined by measuring the radioactivity in supernatants from trays in which target cells were incubated with a 5% Triton solution. The spontaneous release was measured by incubating the target cells with medium, IL-2, or IF.

The *NK cell activity* was calculated as:

$$\frac{\text{cpm in sample} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100\%$$

Antigens. Two antigens were used to stimulate the MNC. 1. Purified protein derivative of tuberculin (PPD) (Statens Seruminstitut, Denmark) was used in a final concentration of 12 μg/ml. 2. Soluble purified malaria antigen (SPag). The isolation and purification of soluble antigens (exoantigens) from supernatants of *P. falciparum* cultures have been described in detail previously (Jepsen and Andersen, 1981). In brief, pooled supernatants from *P. falciparum* cultures (isolate F32/Tanzania) were added to a CNBr-Sepharose 4B column containing as ligand a pool of IgG from five clinically immune Liberian adults. Bound antigens were as eluted, pooled, concentrated, dialyzed, and tested for content of antigen in crossed immunoelectrophoresis (Jepsen and Axelsen, 1980). The end product, SPag, was diluted in medium and added to the cell cultures in concentrations optimal for MNC proliferation (Bygbjerg et al., 1985).

Blood donors. Blood was collected from 11 healthy West-Africans, who had no recent clinical history of malaria and no parasitaemia on examination of Giemsa stained thick blood films. As non immune controls, blood was collected from seven healthy Danes who had never been visiting areas with malaria transmission. None of the 18 donors took antimalarials or other medication. Lymphocytes from all donors were tested for their reactivity to SPag in proliferation assay before they were chosen for this study. Donors with a proliferative response to SPag <1000 cpm were defined as nonresponders. Donors with responses between 1000 und 10,000 cpm were defined as low-responders, and donors with responses >10,000 were defined as high-responders.

Statistics: The Wilcoxon matched pairs signed rank test and the Wilcoxon rank sum test were employed. $P \leq 0.05$ was considered significant.

Results

Fig. 1 shows the proliferative responses in unstimulated culture, and in cultures stimulated by malaria antigen (SPag), or PPD. All MNC preparations proliferated in response to PPD, and there were no statistically significant differences in the PPD responses of donors non immune or immune to malaria. The proliferative response to SPag was higher in immunes than in non-immunes. Five Danish donors and one African donor did not respond to the antigen, 1 Danish donor and 7 African donors showed low responses, while 1 Danish donor and 3 African donors showed high responses. The SPag responders showed no correlation between the responses to PPD and to SPag, median PPD response in SPag low and high responders being 42.1 and 38.1 Kcpm, respectively.

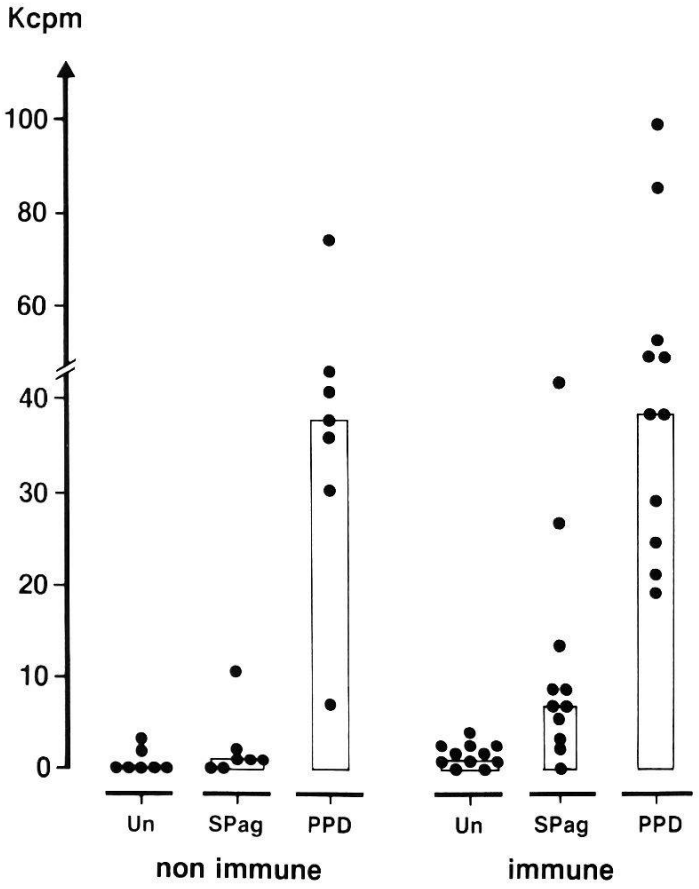

Fig. 1. 3-H-thymidine incorporation by unstimulated (Un), and soluble purified malaria antigen (SPag), and PPD stimulated mononuclear cells. The cells were isolated from non immune and immune donors as indicated. Columns represent the median incorporation of each group.

Table 1. NK cell activity (mean ± SEM of 18 experiments) of unstimulated and PPD stimulated MNC and effect of preincubation with interleukin 2 (IL-2) or alpha-Interferon (IF) for one hour before the cytotoxic assay

	Unstimulated %	PPD %
Before preincubation	5±2	32±4*
After preincubation with IL-2	7±2	35±4
After preincubation with IF	5±2	38±4**

* NK cell activity higher than in unstimulated MNC ($p \leq 0.01$).

** NK cell activity significant higher than activity of PPD stimulated MNC before preincubation ($p \leq 0.01$).

Table 1 shows the NK cell activity in unstimulated and PPD-stimulated MNC cultured for 7 days, and the effect of preincubating the MNC with IL-2 and IF for one hour before the cytotoxic assay. PPD stimulation enhanced the NK activity of all MNC preparations. There was no correlation between the proliferative response and the NK activity in the PPD-stimulated cultures (data not shown). Preincubation with IF increased the cytotoxicity of PPD-stimu-

Table 2. NK cell activity of unstimulated (un) and malaria antigen (SPag) stimulated MNC, isolated from 11 immune and 7 non immune (#) individuals. Effector cells marked +IL2 or +IF were preincuabted with interleukin 2 or alpha-interferon. The donors are grouped into SPag nonresponders, SPag low-responders, SPag high-responders according to their proliferative response to SPag.

	Effector cells					
	un %	un+IL2 %	un+IF %	SPag %	SPag+IL2 %	SPag+IF %
Nonresponders						
1	2	0	2	4	4	2
2#	4	2	1	6	6	5
3#	7	23	6	12	11	17
4#	0	0	0	3	0	5
5#	2	0	2	2	2	1
6#	1	1	3	2	1	1
Median	2	1	2	4	3	4
Nonresponders						
7#	3	4	5	3	11	4
8	0	7	0	1	8	7
9	17	24	15	9	40	45
10	22	24	18	26	41	37
11	0	2	0	5	13	3
12	0	1	0	2	35	15
13	0	0	0	2	9	1
14	0	0	0	0	10	4
Median	0	3	0	3	12*	6
High-responders						
15#	11	11	20	25	29	45
16	12	24	26	26	21	43
17	0	0	0	38	35	40
18	0	3	0	11	18	13
Median	6	7	10	26**	25	42

* NK cell activity higher than the activity of SPag+IL2 in MNC isolated from nonresponders ($p<0.01$).

** NK cell activity higher than the activity of SPag in MNC isolated from nonresponders ($p<0.01$).

lated cells significantly ($p \leq 0.01$), whereas IL-2 had no statistically significant effect.

The data in Table 2 show that there was no difference between the NK cell activity in unstimulated MNC from non-, low-, and high-responders. Furthermore, in unstimulated MNC preincubation with IL-2 and IF did not change the activity significantly. SPag stimulated MNC from high-responders showed

higher cytotoxic activity than low- or nonresponders (26% versus 3% and 4%, respectively, $p \leq 0.01$). After preincubation with IL-2 SPag stimulated MNC from low-responders show higher cytotoxic activity than cells isolated from nonresponders (12% versus 3%, $p \leq 0.01$). Preincubation with IL-2 did not increase the NK cell activity of SPag activated MNC from non- or high-responders. Preincubation with IF increased the NK cell activity in SPag stimulated MNC from the SPag responders (low- and high-responders, $p \leq 0.01$), but did not change the activity of MNC from nonresponders.

Discussion

We have earlier reported that immunoadsorbent purified soluble antigens isolated from *P. falciparum* culture supernatants (SPag) had little mitogenic effect on lymphocytes isolated from individuals never exposed to malaria, whereas lymphocytes isolated from former malaria patients responded to the antigen in a proliferation assay (Bygbjerg et al., 1985). Lymphocytes isolated from most malaria immune individuals responded to SPag, the responding cells were found in the population of OKT4 positive (T-helper cells), and high levels of IL-2 were produced only in cultures responding strongly to the antigen (Theander et al., 1986).

The present study demonstrates that SPag activation enhances the cytotoxic activity of MNC cultures with a strong proliferative response. NK cells are mainly functionally identified as cells mediating spontaneous cytotoxicity against NK-sensitive cells, e.g. the classical K562 target cell line. The bulk of the measured cytotoxic activity is probably mediated through NK cells (Chang et al., 1983), however, it cannot be excluded that cells of the monocyte/macrophage series might have contributed to the observed cytotoxicity (Hammerstrøm, 1979). The enhancement of the cytotoxicity in SPag stimulated cultures from high-responders could be due to a direct stimulation of the NK cells by SPag, or may reflect an indirect stimulation of the NK cells through mediators secreted from activated lymphocytes, such as IL-2 and IF. Preincubation of MNC with IL-2 increased the NK activity in cultures with low response to SPag, whereas IL-2 preincubation had no effect on the NK activity in unstimulated MNC or in SPag-activated MNC from non-responders. This finding may indicate that activation in low-responders resulted in the expression of IL-2 receptors on the cells mediating cytotoxicity. That addition of IL-2 did not increase the NK activity in PPD-stimulated cells may reflect previous exposure to IL-2 secreted from activated T-lymphocytes during the incubation period. In most cultures responding to SPag and PPD, addition of IF enhanced the NK cytotoxicity, whereas addition of IF had no enhancing effect on unstimulated cells or in cultures not responding to SPag. The finding that IF exerts its effect on NK cells from antigen-activated cultures might indicate that antigen activa-

tion of the cultures results in the expression of IF receptors on the cells mediating cytotoxicity.

Evidence is accumulating that NK cells play an important role in the immune surveillance against cancer and for the protection against virus infections (Herberman, 1981; Ortaldo and Herberman, 1984; Pedersen, 1985). The role of these cells in the protection against parasitic infections, including malaria, is unknown (Allison, 1984; Deans and Cohen, 1983; Herberman, 1981). It has been proposed that the cellular part of the protective immune response in malaria immune individuals is regulated by immune T-cells that recognize parasite antigens and initiate a process which results in the activation of non-specific effector cells eliminating the parasite (Allison, 1984; Jensen et al., 1983; Ockenhouse et al., 1984). This study shows that malaria antigens can stimulate NK cells in vitro, either directly, or more likely, through the activation of antigen-specific lymphocytes that stimulate the NK cells. Whether parasite antigen-induced activation of NK cells plays a major role in the protection against the parasite in vivo is an open question. Theoretically such an activation could increase the resistance to virus infection and reduce the risk for tumor development in malaria immune individuals.

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