Effects of Human Natural Killer (NK) Cells on *Plasmodium falciparum* Infected Red Cells*

Wanpen Chaicumpa, D.V.M., Ph.D.
Arunsri Chatkaeomorakot, B.Sc. Med. Tech.
Jintana Patarapotikul, M.Sc. Trop. Med.
Sornchai Looareesuwan, M.D.

SUMMARY **Null cells were separated from the peripheral blood of 22 healthy donors using Ficoll-hypaque gradient centrifugation, carbonyl iron phagocytosis, E-rosetting and 19S-EAC binding assays, respectively. These cells were used as effector cells against ^{51}Cr-labelled *Plasmodium falciparum* infected red cells (targets) at the effector: target ratios of 5:1 and 10:1 respectively. Our results demonstrated directly the role of natural killer cells against malaria.**

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The observation that natural killing is absent only in the most severe state of immuno-deficiency is somewhat indicative that it is among the most primitive form of host defense and surveillance mechanisms.[1] Spontaneous or natural killer cells constitute one of the several naturally occurring defense mechanisms in the immune response. Natural killer cells are operative in the protection against infections and tumours.[2] It is also possible that these cells may have a physiological role in regulating or interacting with normal primitive stem cells in bone marrow.[3-5]

The role played by the natural killing system in innate resistance to infections by viruses and other micro-organisms is well established. Infection with *Listeria monocytogenes* in mice resulted in an increase in NK activity.[6] The active role of NK cells in resistance to persistently virus-infected cells has also been demonstrated,[7] while markedly depressed NK reactivity could be observed in donors afflicted with severe viral infections.[8] Haller and Lindenmann[9] have observed that athymic (nude) mice were resistant to the detrimental effects of a number of myxoviruses, mainly influenza A. Yang *et al.*[10] have shown that the major cell population induced by vaccinia virus is composed of NK cells. The inhibition of growth of *Cryptococcus neoformans* in culture by NK cells was elaborated upon by Murphy and Mc Daniel.[11] Moreover, increased NK activity has been reported in cases of experimental trypanosomiasis,[12] in *Schistosoma mansoni* infections[13] and upon challenge with *Histoplasma capsulatum.*[14] Nude mice were also observed to be more resistant to *Candida albicans* infections than normal mice.[15] Varying degrees of innate resistance to malaria in unprimed inbred mouse strains can be attributed to the level of NK cells.[16] Similar observations were reported by Eugui and Allison.[17] Likewise, the role played by natural killer cells in the unsuccessful development of cerebral malaria in nude mice could not altogether be overlooked.[18] In humans, our recent investigation[19] was suggestive of a possible role played by natural killer cells in the course of malaria infection. It was found that on the one hand there were no significant differences between normal individuals and patients with acute malaria regarding NK cell activity (as measured by ^{51}Cr release assay using K562 cells as targets) and on the other hand that there was a marked decrease in activity in contrast with a pronounced numerical increase in the null cell population among convalescing malaria patients. The possibility that the observed results could be attributable to the recruitment of young, poorly equipped NK cells incapable of exerting their effects has been postulated, in order to compensate for the depleted null

*From the Department of Microbiology and Immunology and the Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.
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cell population consequent to the active role played by the mature NK cells during the acute phase of the disease.

In this report, ^{51}Cr-labelled malaria-infected red cells were used as a direct target for the demonstration of the role of NK cells with regard to malaria parasites.

MATERIALS AND METHODS

Effector cells (E): Mononuclear cells were isolated from 25 ml of peripheral blood of healthy individuals using Ficoll-Hypaque density-gradient centrifugation.[20] The contaminated phagocytes were removed by repeating carbonyl iron ingestions and magnet applications.[19] Subsequently, mature T cells were depleted by the method modified from "total E-rosetting" described by West *et al.*[21] In brief, equal volumes of mononuclear cells (at 5×10^6 cells/ml), foetal calf serum and 0.5% sheep red blood cells were mixed. The mixture was incubated at 37°C for five minutes then centrifuged at 250 x g for ten minutes before being stored at 4°C for one hour. After the incubation, the cells were recentrifuged at 450x g for another ten minutes then most of the supernatant was discarded. The cells were restored with a small volume of RPMI 1640 medium and overlayed onto FH. The free red blood cells and the E-rosette-forming cells were removed by centrifugation. Free lymphocytes (mature B cells and null cells) were collected from the white ring at the interface of the medium and the FH. These cells were washed once and restored with RPMI 1640 medium. Subsequently, mature B cells were removed from the cell preparation by 19S-EAC rosetting.[19] The maximum subagglutinating dilution of 19S fraction of rabbit anti-human O red cells, fresh mouse serum as a source of complement and 2.5% human O red cells were used to prepare 19S-EAC. Lymphocytes binding this complex and free 19S-EAC were removed by FH gradient centrifugation. The remaining free lymphocytes (null cells) were collected and adjusted to 5×10^6 cells/ml in RPMI 1640 medium. The cells were used as effector cells (E) in the experiments.

Target cells (T): *Plasmodium falciparum* infected human red blood cells labelled with ^{51}Cr were used as target cells in the experiments. They were prepared by culturing *P. falciparum* from the patients' blood in 60 mm petri dishes. Thus, 4 ml of 8% infected cell suspension were pipetted into RPMI 1640 medium supplemented with HEPES buffer (Sigma), 0.2% $NaHCO_3$, 10% heat inactivated human AB serum and 30 μg/ml gentamicin sulphate. The culture dish was kept in a humidified candle jar containing 5% CO_2 at 37°C. The culture medium was changed daily until high parasitaemia at the stage of trophozoites was obtained. The whole culture was then carefully overlayed onto Percoll and centrifuged to enrich the percentage of parasitaemia. The enriched parasitized red cells (ranging from 20% to 80%) were collected, washed once and restored with a small volume of culture medium. The cells were then labelled with ^{51}Cr by adding 0.1 ml (about 100 μCi) of $Na_2\ ^{51}CrO_4$ to the petri dish. The petri dish was kept in a candle jar for one hour. Thereafter, the cells were washed with fresh culture medium, restored to the required concentrations (10^6 infected red cells/ml and 5×10^5 infected red cells/ml) and used as target cells in the experiments.

The human AB serum employed in target cell cultures and in the tests was freed from antibody to *P. falciparum* as detected by indirect immunofluorescence. The serum also failed to agglutinate red blood cells used for culturing the parasites.

Cytotoxic assay: The methodology employed was essentially that of Chaicumpa *et al.*[19,21] Thus, equal volumes (100 μl) of effector cells and the target cells at the E:T ratios of 5:1 (5×10^5 null cells: 10^5 infected red cells, and in effect the target contained 2.5×10^4 to 4.0×10^5 uninfected red cells) and 10:1 (5×10^5 null cells: 5×10^4 infected red cells + 1.25×10^4 to 2×10^4 uninfected red cells) respectively, were pipetted into triplicate wells of a flat-bottomed 96-well microplate. Three sets of controls were included for each of the tested E:T ratio. The first set of controls was for determining spontaneous release. To each well of the first control set, 100 μl of the target at concentrations similar to those in the test system and 100 μl of the culture medium were added. The second set of controls was for determining the total radioactivity counts of the target used. Thus, the content of the wells was essentially the same as that of the first control set except that the content was mixed thoroughly before sampling. The third set of controls contained mixture of effector cells and ^{51}Cr-labelled normal red cells (the same batch as that used to culture the target cells) at concentrations similar to those of the test system. The plate was incubated at 37°C in a candle jar for 4 hours, then it was spun at 250x g for five minutes. One hundred microlitres of the samples (usually the supernatants except for the total count) were harvested from each well and tranferred into appropriately labelled 10X75 mm test tubes for radioactive counting. The contents of the total count wells were mixed by pipetting up and down for 5 times prior to harvest. The following formula was used to compute the percentage of cytotoxicity:

$$\%\ \text{cytotoxicity} = \frac{\text{test cpm}^* - \text{spontaneous release cpm}}{\text{total cpm} - \text{spontaneous release cpm}} \times 100$$

*counts per minute, average of triplicate wells.

RESULTS

The morphological appearance of typical human natural killer cells, which were used as effector cells, is shown in Figure 1. The cells are a distinct population of lymphocytes with abundant neutrophilic cytoplasm containing localized azurophilic granules. The ^{51}Cr-labelled infected red cells which were used as targets, are shown in Figure 2. The percentage of cytotoxicity from the experiments performed at E:T ratios of 5:1 and 10:1 are shown in Tables 1 and 2 respectively.

Among the 22 healthy individuals tested, five of them (22.7% of the total) (Nos. 6, 13, 14, 16 and 20) did not show any significant lysis of the target cells above the background spontaneous release when their effector cells were used at an E:T ratio of 5:1 Also, no activity was observed when the null cells of the same five individuals and those of volunteer No. 22 were used at an E:T ratio of 10:1. However, the null cells of the other individuals demonstrated a significant release of ^{51}Cr from the parasitized target cells. The overall results, therefore, revealed significant cytotoxicity of the null cells towards the red blood cells with *P. falciparum*.

It was observed from the third control set run in conjunction with the tests that the null cells of all the individuals tested did not have any cytotoxic effect upon the ^{51}Cr-labelled normal red blood cells since the cpm released in the presence of null cells were similar to the cpm resulting from spontaneous release.

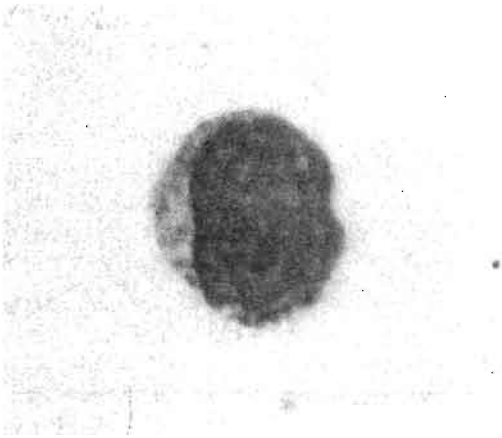

Fig. 1 Human natural killer cell. (Giemsa, x 1000)

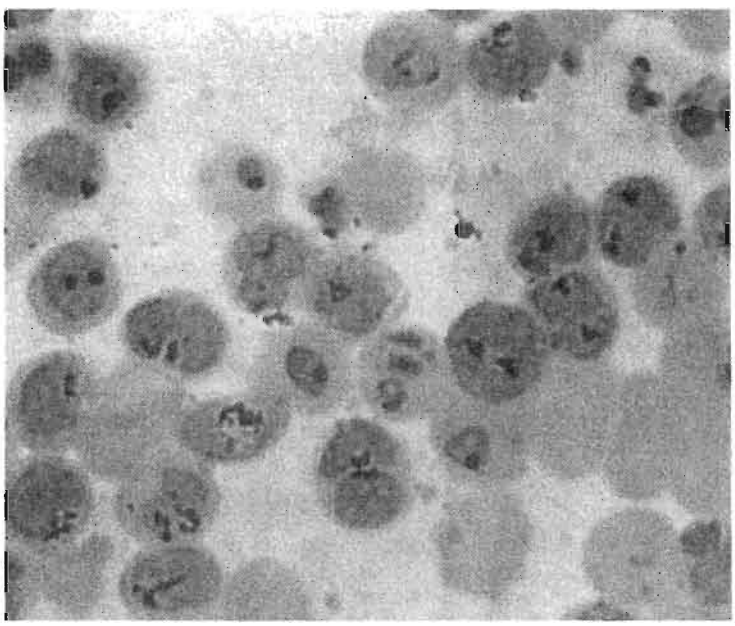

Fig. 2 Showing *P. falciparum* infected red cells which were used as targets. (Giemsa, x 1500)

DISCUSSION

The direct activity of human natural killer cells against *P. falciparum* infected red cells has never been reported. Although some evidence has suggested a role of NK cells against malaria,[19,22] the assay systems employed to back up the hypothesis were quite indirect. The NK cell levels were found to be raised in malaria-infected African children, with a positive correlation between the degree of parasitaemia and the lytic activity of NK cells towards the K562 cell line.[22] Our initial approach taken to study the role of NK cells in malaria also used the K562 cells as targets in cytotoxic assay.[19] Therefore, the data presented herein, for the first time, demonstrates a direct cytotoxic effect of NK cells towards malaria parasites. Along with the tests, the results from the controls, using radioactively labelled normal red cells as the targets for NK cells, provides substantial evidence to support the contention that human natural killer cells destroyed only the parasitized red cells. These findings, together with the observation that the human AB sera used in culturing the parasites were free of antibodies to malaria parasites and human red blood cells, exclude the possible role of an antibody-dependent mechanism acting through K cells in the null cell pools.

In the preparations of null cell-riched fractions, it was observed

Table 1 Percentage of cytotoxicity from the tests (effector: ^{51}Cr-labelled infected red cells) and the third control set (effector: ^{51}Cr-labelled uninfected red cells) at E: T = 5:1

Volenteer No.	Cytotoxicity (%) Test	Control
1	4.4	0
2	11.9	0
3	13.6	0
4	4.1	0
5	20.4	0
6	0	0
7	8.3	0
8	32.4	0
9	12.9	0
10	24.9	0
11	13.2	0
12	4.1	0
13	0	0
14	0	0
15	6.3	0
16	0	0
17	4.9	0
18	3.8	0
19	4.8	0
20	0	0
21	6.5	0
22	1.8	0

Table 2 Percentage of cytotoxicity from the tests (effector: ^{51}Cr-labelled infected red cells) and the third control set (effector: ^{51}Cr-labelled uninfected red cells) at E: T = 10:1

Volunteer No.	Cytotoxicity (%) Test	Control
1	9.6	0
2	12.6	0
3	7.0	0
4	19.6	0
5	16.4	0
6	0	0
7	24.3	0
8	53.7	0
9	36.1	0
10	17.0	0
11	23.0	0
12	3.6	0
13	0	0
14	0	0
15	1.9	0
16	0	0
17	9.8	0
18	1.6	0
19	15.2	0
20	0	0
21	4.2	0
22	0	0

that the contamination of T cells (as detected by allowing the null cell pools to react with sheep red blood cells under the conditions of E-rosette formation) does not exceed 5 per cent. Experiments using T cells recovered from E-rosettes, at the concentrations much higher than those found in the null cell pools, as effector cells against infected red cell targets were performed. No cytotoxicity was observed from these experiments. Thus the role of cytotoxic T cells in the system was excluded.

Reasons may be given to explain why the null cell pools of some individuals were refractory to the *P. falciparum* infected red cells. One explanation would be that the E:T ratios employed were unsuitable for the null cells of those individuals. It is now evident that the highly effective NK cells in human peripheral blood are HNK-1$^+$, T_3^-, M_1^+ cells while the NK cells with HNK-1$^+$, T_3^+, M_1^- are less effective.[23] Individuals' null cells which were not cytotoxic towards the *P. falciparum* infected red cells might have had higher ratio of HNK-1$^+$, T_3^+, M_1^- to HNK-1$^+$, T_3^-, M_1^+ than those from individuals who showed significant cytotoxicity. Support for this explanation would be based on the findings that the number of subpopulations of lymphocytes can be altered especially during infection.[24-26] The individuals which were selected as "healthy individuals" in this study, although apparently healthy, may have harboured certain subclinical infections which resulted in abnormal ratios of lymphocyte subpopulations. The end result would be that their null cells would be unable to create cytotoxicity to the malaria infected red cells at the E:T ratios used in our assay.

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