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POSITIVE CORRELATION BETWEEN DEGREE OF PARASITEMIA, INTERFERON TITERS, AND NATURAL KILLER CELL ACTIVITY IN *PLASMODIUM FALCIPARUM*-INFECTED CHILDREN[1]

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Natural killer (NK) cell activity and interferon levels have been measured in the peripheral blood of children acutely ill with *Plasmodium falciparum* infection. The NK cell levels were found to be raised in the malaria-infected children, with a positive correlation between the degree of parasitemia and lytic activity. Comparatively high titers of antiviral activity was discovered in sera from the majority of *P. falciparum*-infected children, again positively correlating with the degree of parasitemia and NK levels. The characteristics of the antiviral factor indicated α-type interferon to be the dominating agent involved. Addition of exogenous interferon *in vitro* potentiated the NK levels of PBL from normal children while having no significant impact on cells from malaria-infected children.

The mechanisms responsible for protective immunity in malaria are manifold. Both humoral (1–3) and cell-mediated (4, 5) reactions have been shown to play a role. In rodents, studies indicate the presence of both specific and nonspecific reactions acting against the malaria parasite (2, 3, 5–7). At the cellular level, a novel cell type mediating natural killer (NK)[3] activity against tumors (8) has been suggested as perhaps playing a role in the resistance of mice against the parasite (9, 10). No studies on NK levels in human malaria have previously been reported to our knowledge.

Evidence exists that interferon (IFN) is produced in rodent malaria (11), with a subsequent impact on the parasites (12), in contrast to a negative report on IFN production in human malaria (13).

IFN is known to have a highly significant impact on the immune system besides its antiviral and growth-inhibitory properties, and the major endogenous agent in the regulation of NK activity is IFN (14–16).

The present study assesses i) the level of NK activity in the peripheral blood of human beings with acute *Plasmodium falciparum* infection, ii) the level of IFN in the serum of such patients, iii) the possible correlation between IFN production and NK levels in the individual patients, and iv) the ability of peripheral blood mononuclear leukocytes (PBL) from malaria patients to respond to IFN contact *in vitro* as measured at the NK cell level.

Our present results show comparatively high IFN titers in sera of Nigerian children with acute *P. falciparum* infection. NK levels in such children are raised above normal levels and can not be further enhanced *in vitro* by the addition of exogenous IFN in contrast to PBL from normal control children. The results and their possible implications are discussed.

MATERIALS AND METHODS

PBL were obtained from venous blood by using heparin followed by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation to remove erythrocytes and granulocytes (17). Donors were malaria-infected children who had 1 to 3 wk history of illness with no known previous history of chemotherapy. Their healthy brothers and sisters, ranging in age from 6 mo to 5 yr of age, served as controls. Mononuclear cells were washed twice with phosphate-buffered saline (PBS), resuspended in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES[3] buffer, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol and were then ready for use. Target cells in the NK cell assay were K562 cells, a human erythroid leukemia cell line known for its high sensitivity toward NK lysis (18). The leukemia cells were kept growing in the above-indicated medium. When used as targets in cytolytic assays, K562 cells were adjusted to 3×10^6 cells/ml in RPMI 1640 with 10% FBS, and 300 μCi $Na_2{}^{51}CrO_4$ (Radiochemical Centre, Amershams, England) were added and kept with the cells for 90 min at 37°C. The cells were then washed thrice followed by resuspension to a concentration of 10^6/ml.

In cytotoxicity assays measuring NK cell activity, all experiments carried out were done at a single effector:target ratio, 25:1, using round-bottom microtiter plates (Falcon Products) in triplicate as previously described (8). In brief, PBL (5×10^5/100 μl) in medium alone or in the presence of 100 IU/ml of human IFN of α-type (kindly provided by Dr. Hans Strander, Karolinska Hospital, Stockholm, Sweden) for 1 hr at 37°C in a humidified 5% CO_2 incubator to allow maximal NK activity to become induced (14). At the end of the incubation period, 2×10^4 ^{51}Cr-labeled K562 cells were added to each well followed by a further incubation for 4 hr at 37°C. The plates were then centrifuged at 500 × G for 10 min, after which 100 μl of the well content were removed for counting in a gamma-isotope counter. The specific lysis was calculated from the ^{51}Cr-release using the formula

$$\% \text{ Specific release} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum release cpm} - \text{spontaneous cpm}}$$

Spontaneous and maximal release cpm were determined by incubating the target cells with medium only or 1% Nonidet P-40, respectively.

The degree of parasitemia was determined in a double-blind assay, using standard technology with thin and thick smears of fresh blood on glass slides, fixing with absolute methanol for 5 min, and staining for 30 min with 5% Giemsa dye in distilled water.

Tests for possible presence of humoral antibodies against K562 in the sera of the malarial patients and the normal controls were carried out using 2 different approaches. In the 1st, indirect immunoglobulin binding was evaluated using K562 cells in 1:5 dilutions of normal or malarial patient sera (10^6 cells/50 μl) and incubating in the cold for 60 min. The cells were then washed thrice in PBS without serum but with 1% ovalbumin and incubated with ^{125}I-protein A for another 60 min at 4°C. The cells were then washed thrice again and counted in a gamma-isotope counter (19). In the 2nd approach, we looked for possible antibodies in the various human sera for K562 using normal PBL as potential effector cells against K562 cells, which had been preincubated with normal or malaria sera at different dilutions for 1 hr at 4°C and washed before adding the cells. The K562 cells used as targets in this assay system looking for antibody-dependent cell-mediated

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[2] Reprint requests and all correspondence to current address: Dr. E. A. Ojo-Amaize, Division of Parasitology, Department of Microbiology, New York University Medical Center, 550 First Avenue, New York, NY 10016.
[3] Abbreviations used in this paper: NK, natural killer; PBL, peripheral blood mononuclear leukocytes; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ADCC, antibody-dependent cell-mediated cytolysis; IFN, interferon.

cytolysis (ADCC) were radiolabeled as in the cytolytic assay described above.

IFN assay. Human amnion cells (WISH; American Type Culture Collection, Bethesda, MD) were grown as monolayers in flat-bottomed Nunclon 96-well microtiter plates using MEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal calf serum (FCS; Flow Laboratories, Irvine, UK). The medium was replaced with 0.1-ml volumes of 2-fold dilution of serum, initial dilution 1/5. A series of dilutions of the human leukocyte reference interferon (G-023-901-527; National Institutes of Health, Bethesda, MD) was included on each microtiter plate. After 24 hr the medium was removed and replaced with fresh medium containing vesicular stomatitis medium (VSV, Indiana strain) at a multiplicity causing complete cytopathic effects in unprotected cells within 24 hr. At that time the cultures were rinsed, fixed, and stained with a crystal violet-formaldehyde-ethanol solution and observed under a microscope. The highest dilution still protecting at least 50% of the WISH cells provided the IFN titer. Observed antiviral activity of the serum samples was expressed in reference leukocyte IFN units per milliliter. One antiviral unit in our assay corresponded to approximately 0.3 reference IFN units.

Characterization of antiviral activity in human sera. The antiviral activity in malaria patient sera was analyzed using a pool from the 16 patients studied. This pool was tested for its ability to resist pH 2 treatment (assaying for human gamma-IFN activity (20)) and for sensitivity inactivation by sheep anti-human α-IFN serum (kindly provided by Dr. K. Cantell, Central Public Health Laboratory, Helsinki, Finland). The antiserum had a titer when undiluted for 4.5×10^5 α-IFN units and 3×10^3 β-IFN units. Serum samples from the malaria patients were incubated for 2 hr at 37°C with antiserum capable of inactivating approximately 4500 IFN units, that is, roughly a 20-fold antibody excess. Positive controls included α-FIN from Dr. C. Kantell as well as γ-IFN obtained from lentil lectin-stimulated human buffy coats with properties described elsewhere (L. Rönnblom, K. Funar, and G. Alm, manuscript in preparation).

RESULTS

Malaria patients have PBL that express an increased level of NK activity, which is postively correlated to the degree of parasitemia. Sixteen malaria patients with clinically manifest, acute *P. falciparum*-induced disease and the same number of age-matched normal children were analyzed for their NK activity as measured at the level of their PBL. The malaria patients were also ranked according to degree of parasitemia in blood smears. Only 1 fixed effector: target ratio was used against the NK-susceptible target K562 (25: 1) due to the small volume of blood drawn from each patient. The results are shown in Table I. Two things are obvious. There exists a highly positive correlation between the degree of parasitemia and the NK activity, with the highest mean (46.3% specific lysis) of NK cells being seen in the patients with very high degrees of parasitemia. The patients with malaria infection did have a significantly higher NK level activity compared with the control group. However, the patients with only very low degrees of parasitemia had a mean of NK lytic activity (10.0% specific lysis) that was not different from the mean of the control PBL (11.2% lysis).

In vitro incubation with IFN will induce an increase in NK activity in PBL from normal humans but not from malaria patients. IFN is known to be a dominating regulatory agent of NK activity *in vitro* and *in vivo* (14–16, 21–23). We reasoned that if the malaria patients were producing IFN, their PBL may be unresponsive to a further stimulation *in vitro* by added exogenous IFN, as is the case for PBL from tumor patients undergoing IFN treatment (23). As seen in Table I, although the majority of the samples tested (particularly from normal donors) had comparatively low NK values at the fixed ratio used, certain donors yielded cells with quite high specific lytic activity. This ensured that the target cells used, K562, were functioning as a sensitive target in the present assays and that the NK-sensitive fraction of the target population did constitute a majority of the cells. Accordingly, incubation experiments with IFN *in vitro* of the peripheral blood cells would allow "room" for measurable increases to take place with regard to NK activity. The results of this experimental approach, carried out on the same cell populations that were studied without IFN in Table I, are shown in Table II. Preincubation for 1 hr at 37°C with 100 IU α-IFN/ml caused a significant increase in the NK levels of PBL from the normal donors while failing to cause any enhancement of lytic activity in the PBL from the malaria patients.

Demonstration of circulating IFN in the plasma from human malaria patients: Positive correlation between IFN titers and NK levels. A likely explanation for the findings in Table II would be the actual presence of high titers of IFN in the malaria patients whose potential NK cells had already been stimulated to optimal function. Plasma from each individual normal or malaria-infected child obtained at the same time as the NK assay were thus assessed for possible presence of free IFN as described in *Materials and Methods.* The results are shown in Figure 1, where it can be seen that remarkably high titers of IFN could be detected in the majority of the plasma from *P. falciparum*-infected children. In Figure 1 are plotted the actual IFN titers in relation to the NK activity observed in the individual patient. A clear-cut positive correlation is noted between IFN titers and NK cell levels. We consider this proof that IFN is responsible for the high NK levels observed in malaria patients compared with normal donors, as shown in Table I. Only 2 out of 16 of the healthy children had detectable levels of IFN in their plasma, and then only with a titer of 16 and 31 IU/ml. These 2 normal children were also the very 2 with unusual high levels of NK levels in the absence of added IFN (49.6% and 38.8% specific lysis, respectively), further supporting the point of endogenous IFN levels being associated with NK levels in humans. Note, however, that the 2 normal children with detectable IFN titers had abnormally

TABLE I

Positive correlation between the degree of parasitemia in malaria-infected children and NK-activity

Patient No.	Age	Score of Level[a] of Parasitemia	% Specific Lysis
1	1 yr, 6 mo	+++	86.44
2	11 mo	+++	38.18
3	5 yr	+++	35.44
4	3 yr	+++	34.23
5	1 yr	+++	37.17
6	2 yr	++	29.00
7	5 yr	++	27.99
8	4 yr	++	23.28
9	10 mo	+	19.13
10	5 yr, 6 mo	+	17.46
11	8 mo	±	17.58
12	3 yr	±	12.37
13	2 yr	±	9.06
14	7 mo	±	8.00
15	2 yr	±	9.13
16	1 yr, 6 mo	±	3.90
CONTROL			
1	4 yr	N.D.	49.59
2	8 mo		38.83
3	7 mo		18.66
4	1 yr, 6 mo		7.69
6	1 yr		7.43
7	10 mo		7.36
8	6 mo		6.54
9	5 yr		6.37
10	1 yr		5.84
11	6 yr		5.77
12	11 mo		4.98
13	3 yr		4.18
14	2 yr, 6 mo		3.91
15	2 yr		2.66
16	1 yr, 6 mo	N.D.	1.80

[a] Parasitemia was not determined in the normal children. The symbols for the score of level of parasitemia in the malaria-infected children, denote the following level and range of parasitemia: ± 1 to 2%; + 3 to 5%; ++ 6 to 10%; and +++ 11 to 15%. The level of parasitemia was determined by thin blood smears and expressed as percent infected RBC. P values as calculated by the Student *t*-test: $p < 0.05$ for mean of malaria-induced NK compared with normal NK-levels.

TABLE II

Effect of exogenous IFN in vitro on normal and malaria-induced NK level[a]

	Mean % Specific Lysis ± SE	
	Without interferon	IFN added *in vitro*[b]
Normal children	11.20 ± 3	27.29 ± 6
Malaria-infected children	25.52 ± 5	30.21 ± 7

[a] Specific lysis of ^{51}Cr-K562 cells by NK-cells. Mean of total number of PBL recovered from Ficoll-Hypaque gradients: Normal children 11×10^6/ml and malaria-infected children 13.5×10^6/ml.

[b] P values as calculated by the Student *t*-test: $p < 0.05$ for NK-level of normal children as affected by IFN *in vitro*; $p < 0.2$ for effect of adding IFN *in vitro* on malaria-induced NK-level compared with normal NK-level with IFN added *in vitro*.

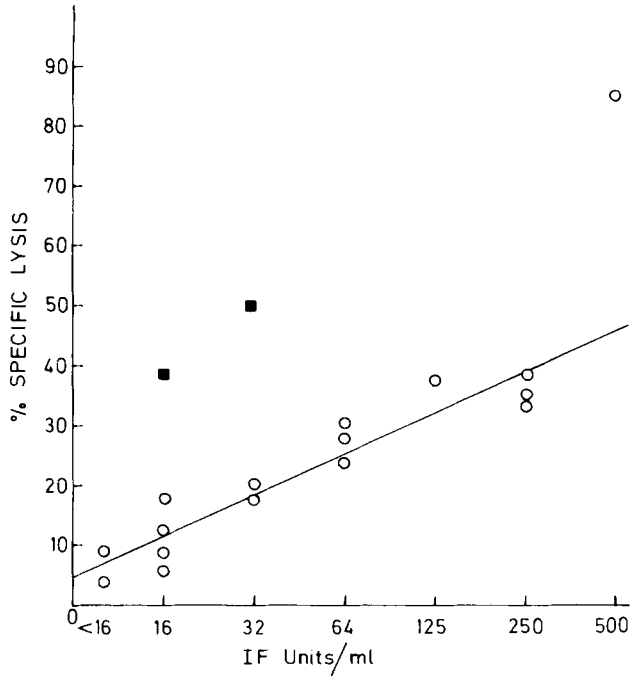

Figure 1. Correlation between NK activity and IFN titers. ■, NK-level of 2 normal children with high serum IFN levels; ○, NK-level of 16 malaria-infected children with their serum IFN levels. IFN units were plotted in log scale; NK activity as % specific lysis against K562.

high NK levels compared with the malaria patients when plotted in Figure 1. This would thus suggest that the rise in NK levels in malaria via endogenous IFN is not taking place with the same efficiency that it would under normal conditions. The actual reason why 2 of the normal humans had this detectable level of IFN could not be further analyzed for technical reasons.

Failure to find demonstrable antibodies against the target (K562) in normal or malaria-infected children. K562 represents a classical target for human NK cells (18). It is a human erythroid leukemia cell line with no, or very little, HLA antigens on its surface (24). The fact that it is erythroid in lineage could constitute a possible problem in the malaria system with the actual effector mechanism being analyzed. Auto-antibodies directed against erythrocytes are known to be frequent in malaria (25). We reasoned that it was thus conceivable that the lytic activity observed may not necessarily be due to an actual increase in NK cells in the malaria patients, but could be caused by an additional, antibody-dependent mechanism acting in parallel with the NK cells in mediating cytolysis. NK cells can also function as K cells in such ADCC systems (26, 27), and it has been shown in the human system that antigen-antibody complexes in antibody excess may physically arm potential killer cells *in vivo* (28). Two sets of approaches were used in studying this possibility. A direct search for IgG antibodies in the sera of normal or malaria-infected children failed to disclose such antibodies, as shown in Figure 2. Furthermore, attempts to induce an increase in the NK activity of normal human PBL by preincubating isotope-labeled K562 cells with plasma from normal or malaria-infected children all failed to demonstrate any ADCC-inducing antibodies in any of the samples tested, as exemplified in Figure 3. From this we conclude that there is no reason to believe that ADCC reactions played any detectable role in causing the results observed in this study.

Characteristics of the antiviral activity found in malaria sera. The antiviral activity of a pool from the 16 malaria patients studied was now analyzed with regard to pH 2 stability and reactivity with an antiserum directed predominantly against human α-IFN as described in *Materials and Methods.* The results of this analysis are shown in Table III. Approximately 75% of the antiviral activity was neutralized when using the anti-human α-IFN antiserum, whereas no reduction in activity was observed subsequent to pH 2 treatment. As control IFN preparations, we used a γ-IFN preparation containing as a contaminant approximately 5% α-type IFN (G. Alm, unpublished work) as well as Sendai virus-induced α-IFN. The control IFN preparations behaved according to expectation. Close to complete elimination of γ-IFN activity was thus achieved by the pH 2 treatment, with the residual activity most likely being due to the contaminating α-IFN. Likewise, the anti-α-IFN antiserum caused a complete elimination of α-IFN activity without detectably reducing the γ-IFN preparation. We would thus conclude that in children with acute *P. falciparum* infections in the stages observed in the present study, the dominating IFN activity in the circulation is of the α-IFN type. The remaining 25% of antiviral activity not neutralized by pH 2 or anti-α-IFN antiserum remains to be characterized.

DISCUSSION

In the present study we have analyzed the status of NK cell activity in the peripheral blood of children acutely ill with *P. falciparum* malaria. Our results were quite clear-cut in demonstrating an increased NK activity in peripheral blood of malaria-infected children. The levels of NK lysis were shown to be positively correlated with the degree of parasitemia as independently assessed. IFN is known to constitute a major regulatory agent for NK activity in both mouse and man (14, 15). Likewise, IFN has been reported in some studies to have a beneficial impact with regard to malaria in mice if given early during the infection (12). We could demonstrate sizable IFN titers in the majority of children with acute *P. falciparum* malaria. This increase in IFN titers could be shown to be positively correlated with the increase in NK activity as well as the degree of parasitemia. In contrast to the earlier negative report, the present patients had had no prior chemotherapy known to us. Furthermore, our pateints were children, in contrast to the previous report involving human adults, with the majority being infected with another plasmodium species, namely, *P. vivax* (13).

The fact that the IFN titers showed a striking positive correlation with the increase in NK activity in the individual patients strongly suggested that IFN was the actual cause for the observed increase in lytic ability. Further support for endogenous IFN regulating human NK activity came from the chance observation that 2 of the normal children had low but detectable IFN levels (see Table I). Those same 2 children also displayed unusually high NK activity. The "relative" level of NK cells was also higher than expected compared with the corresponding levels found in malaria patients

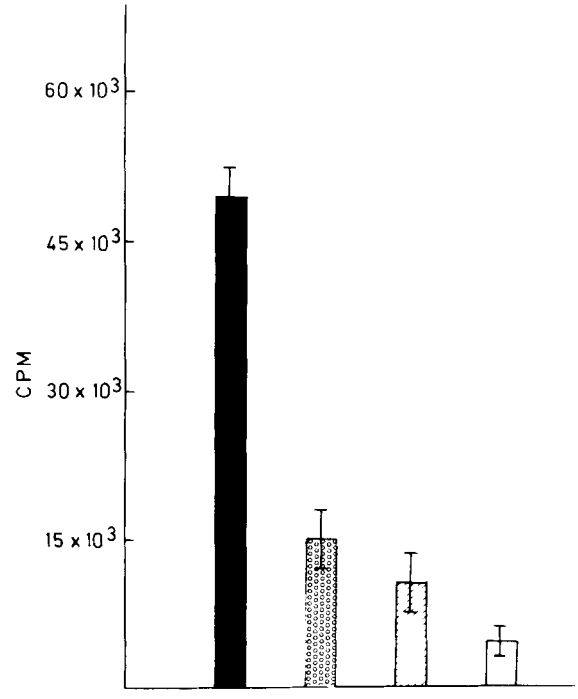

Figure 2. Failure to find IgG antibodies binding to K562 cells in sera from malaria patients. ■, Anti-human lymphoblastoid fluid; ▩, anti-human malaria serum (average cpm from 16 sera); ▨, normal human serum (average cpm from 16 sera); □, PBS. Assay carried out in 2 steps with ^{125}I-protein A as 2nd reagent.

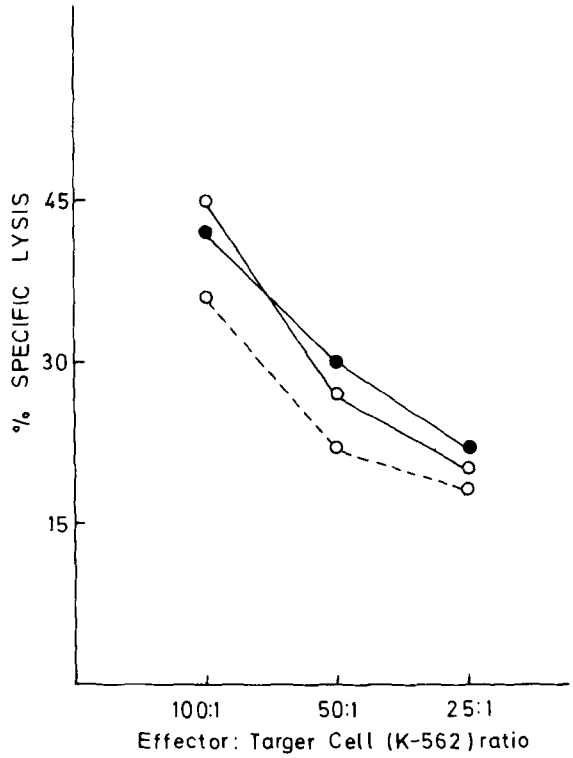

Figure 3. Failure to detect ADCC-inducing antibodies against K562 in sera from malaria patients. O—O, Effector + K562 (NK activity only). ●—●, Effector + normal serum-coated K562 (average % lysis by 2 sera that gave the highest and lowest cpm in Figure 2). O—O, Effector + malaria serum-coated K562. (Average % lysis by 2 malaria sera that gave the highest and lowest cpm in Fig. 2). Sera were used undiluted in the cytolytic assays.

TABLE III

Characterization of the malaria-induced IFN

Tested Material	Type of Treatment[a]					
	pH_2	pH_2 + anti-IFN-α	pH_7	pH_7 + anti-IFN-α	No dialysis	Dialysis + Anti-IFN-α
Malaria serum	100	25	100	25	100	25
IFN-α[b]	100	0	100	0	100	0
IFN-γ[c]	5	100	100	100	100	100

[a] The figures represent residual antiviral activity in per cent of nontreated material (= undialyzed).

[b] IFN-α is IFN induced by Sendai virus in the same type of cells.

[c] IFN-γ is immune IFN produced in human peripheral blood lymphocytes by lentil lectin. Anti-IFN-γ was not used in these experiments because it was unavailable.

(see Fig. 1). This in our minds would indicate that patients with malaria may not respond with optimal NK activity when encountering increases in IFN levels, suggesting a relative degree of unresponsiveness. Our malaria patients may have had still higher NK levels earlier in the disease. Evidence for such an early response with regard to both IFN production and NK response is available in studies of murine malaria (29). Our attempts to increase the lytic activity of NK cells in malaria patients by *in vitro* incubation with IFN failed to cause any significant enhancement of NK activity, in contrast to the increase observed when potential effector cells were derived from normal children. The peripheral blood cells of malaria patients would thus seem to be largely refractory to any further increase in lytic capacity by the addition of exogenous IFN.

Several reasons may exist why those PBL were refractory to further stimulation by exogenous IFN *in vitro*: i) the NK cells had already been stimulated to optimal levels by endogenous IFN *in vivo*, as suggested by our results in line with (9, 10); ii) partial attrition of NK cells from circulation may have occurred as a result of the infection. In support of the latter possibility are the findings that the relative distribution of subgroups of lymphocytes can be strikingly changed during acute falciparum infection in children (30–32). Since no unique marker is yet available for human NK cells, we could not test this last alternative directly in the present study.

Malaria patients are known to frequently produce auto-antibodies, particularly directed against erythrocytic components (25). Because the target used for the NK cell assay was an erythroid leukemia cell line, K562, although used at a preerythroid stage, we deemed it important to exclude the possible existence of antibody-dependent cytolytic activity in addition to NK cells in the malaria patients (26, 27). We failed to detect anti-K562 antibodies in the sera of malaria patients in 2 independent assays. NK cells are known from many studies to constitute the predominant lytic effector cell type against K562 in the peripheral blood from normal humans as well as from patients with a variety of clinical disorders (33). Altogether, we thus see no reason to consider any additional/other effector cell type to be responsible for the anti-K562 lysis observed in the present studies.

Finally, we explored the underlying molecular nature of the antiviral activity found in the sera from malaria children. Approximately three-fourths of the activity displayed features typical of human α-IFN. No evidence was observed for any significant levels of immune, γ-IFN molecules. Whether the remaining 25% of antiviral activity does correspond to β-IFN or has some other underlying basis remains to be solved. We would, however, like to add a note of caution that the relative proportions of IFN types as well as the actual titers produced would be expected to vary in relation to the clinical history of the patient.

The immune response against malaria infection is known to be extremely complicated. It would be largely premature to consider the present data as describing anything other than yet another immune phenomenon associated with this parasitic disease. There exist reports in murine malaria that both IFN treatment (12) and NK cell levels (9, 10, 29) are linked in a positive manner to the course of the infection. No evidence is yet available, however, that NK cells can interfere directly with the parasites in their intracellular or extracellular stages. Neither is it known whether IFN directly affects the malaria parasites or whether its impact is mediated in a secondary manner via its complex immunoregulatory capacities. Further experiments in animal model systems would thus be required to analyze the possible relevance of the present findings in a more stringent manner.

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NONSPECIFIC IMMUNOSUPPRESSIVE FACTORS IN MALIGNANT ASCITES: FURTHER CHARACTERIZATION AND POSSIBLE RELATIONSHIP TO ERYTHROCYTE RECEPTORS OF HUMAN PERIPHERAL T CELLS[1]

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Nonspecific suppressor factor has been purified from malignant human ascites through partial delipidation, ammonium sulfate precipitation, ACA-22 gel filtration followed by goat anti-human IgG immunoadsorbent chromatography. Molecular weight estimated by SDS-PAGE under mild reducing conditons is 50,000 and 25,000 under vigorous reducing conditions. It inhibits PHA, Con A, and PWM responses of peripheral lymphocytes by 50% at microgram concentrations per milliliter *in vitro* and inhibits plaque-forming cell response to SRBC at 100 μg/mouse *in vivo*. It also inhibits 2-way mixed lymphocyte reactions and natural killer cell activities. It inhibits SRBC rosette formation of peripheral T cells and appears to be an erythrocyte receptor of peripheral T cells.

A degree of immunosuppression commonly accompanies a variety of virus-induced tumors (1–6), chemically induced tumors (7), experimental amyloidosis (8), liver cirrhosis (9), infection (10), and pregnancy (11). The precise mechanism is not known, but numerous reports indicate that noncytotoxic immunosuppressive humoral factors in normal or malignant serum may play a role (12–15). Unlike the serum blocking factors that contain specific antibodies to tumor-associated antigens (16), the nonspecific suppressive factors inhibit responses to a spectrum of unrelated antigens. The cellular origin of these factors is not known. Studies from this institution (17) and others (18) have shown that malignant ascites fluids are abundant sources of these factors. Some investigators have reported that tumor extracts also contain nonspecific suppressive factors (19, 20). We have shown that the suppressive factors in malignant human ascites fluids (3 ovarian and 2 colonic cancer ascites fluids) exist as macromolecular complexes and that the active component can be extracted and purified as a a smaller m.w. (50,000), potent immunosuppressive moiety (21). This was

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