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Polymorph function and cellular immunity in Nigerians with pulmonary tuberculosis

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Summary

Polymorphonuclear leukocyte intracellular killing of *Staphylococcus aureus* and chemotactic indices as well as T and B lymphocyte subpopulations were determined in 35 healthy controls and 70 Nigerians with pulmonary tuberculosis. The patients were radiologically classified into exudative, fibro-cavitary, miliary, and mixed lesions groups. Migration inhibition factor (MIF) production and glucose-6-phosphate dehydrogenase (G-6-PD) levels were also determined in 40 patients and 20 controls. The polymorph function as shown by chemotaxic and killing index was significantly reduced ($P < 0.05$) in all patients when compared to the controls. Reduced G-6-PD levels though found in patients when compared to the controls was not significant. A reversal of the T helper/ T suppressor ratio was found in all tuberculosis patients when compared to the controls as a result of decreased T helper cell numbers. T helper cells as well as the MIF index tended to be lowest in the miliary lesions group.

Résumé

Le taux de leucocyte polynucleaires intracellulaire tueur de *Staphylococcus aureus*, les indices chimiotactiques ainsi que les sous population des lymphocytes T et B ont été determiné chez 35 personnes en sante considéré comme contrôles, et 70 patients souffrant de tuberculose pulmonaire. Les patients ont été classifié radiologiquement en groupes exudatif, fibro cavitaire, miliaire, et lesion mixte. La production du taux de facteur d'inibition de migration (MIF) et de glucose-6-phosphate deshydrogenase (G-6-PD) ont aussi été determiné chez 40 patients et 20 contrôles. La fonction polymorphe tel que demontré par chimiotaxie, et par index d'élimination a été reduite de manière significative ($P < 0.05$) chez tous les patients, l'orsquelles ont été comparés aux contrôles. Les taux peu élevés de G-6-PD quoique recncontré chez les patients n'a pas montré une difference significative, l'orsquelle ont été comparé à ceux des contrôles. Une reverse du ratio T-helper /T suppressor a été observé chez tous les patients souffrant de tuberculose, par rapport aux contrôles. Le resultat étant la consequence de la reduction du nombee des la cellules T helpers. L'index des cellules T Helper ainsi que celle des MIF tends à être tres faible dans le groupe de patients souffrant de lesions milliares.

Introduction

Pulmonary tuberculosis is a disease produced by infection with *Mycobacterium tuberculosis* and is one of the most important specific communicable diseases in the world [1]. The lipid containing components of the bacterial cell wall are active in evoking direct and immunologically induced effects on cells [2]. The virulence factors such as the sulfatides prevent the release of lysosomal enzymes from mononuclear phagosomes, while other factors like the c-mycosides and the toxic cord factor, form shields around the bacterium [3]. A large bacterial load in the patient leads to the induction of a large population of suppressor cells and anergy [2,4].

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Tuberculo-immunity is primarily cell-mediated and is known to involve T lymphocytes and macrophages [5,6], but recently, Appleberg and Silva [7] have also demonstrated the involvement of neutrophils. Functionally, neutrophils respond to non-specific inflammatory stimuli such as the complement activation products C5a, formylmethionyl peptide of bacterial origin, platelet-activating factor, and leukotriene B4. Neutrophils have also been suggested to be influenced by immunocompetent cells, particularly the T cells [8].

Changes in the levels of immune cells and their activities have been reported and studies on Caucasians have demonstrated changes in the polymorph function and T lymphocyte values amongst such patients [9,10,11]. Experiments have shown that gamma interferon, tumour necrosis factor, interleukin-2, calcitriol, retinoic acid, most which may be T cell derived, are capable of inducing a significant amount of intramonocyte/microphage killing of *M. tuberculosis* [12]; they are also known to enhance neutrophils effector functions [8]. Additionally, Interleukin-8 has been shown to facilitate migration of neutrophils to sites of bacterial infection [13].

This study was carried out to assess the chemotactic and intracellular killing indices of neutrophils and to relate these to T lymphocyte numbers and function as determined by the production of MIF [14]. G-6-PD levels were also assessed as a possible underlying factor contributing towards impairment of polymorph function [15,16]. Furthermore, pan T cells, T helper, T suppressor, and B cells were evaluated to determine the extent of their involvement in the pathogenesis of various radiological types of tuberculosis.

Patients and methods

A total of 70 Nigerian patients above the age of 15 years (age range of 17 to 62 years and mean of 31 years) were studied while attending the Chest Unit of Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. All patients were confirmed to be sputum positive for the acid fast bacilli (AFB) only. Peripheral blood samples were drawn prior to initiation of chemotherapy. Thirty-five healthy control subjects (age range of 16-67 years and mean of 31 years), were also included. The radiological classification of chest lesions was done according to the previous study of Okpapi and Onyemelukwe [17] and divided into exudative fibro-cavitary, mixed, and miliary lesions groups. Full blood count and differential counts were done routinely. Polymorph and lympocytes numbers and percentages were recorded.

Neutrophil bactericidal assay

The assay involved the procedure described by Phillips *et al* [18]. A stock culture of *Staphylococcus aureus* (NCTC 8530) was maintained on Columbia agar slopes. The intracellular killing ability was determined by mixing together viable neutrophils, *staphylococcus aureus* (antigen), pooled human serum, and Medium 199. A time period of 10 minutes was allowed before sonicating a portion of the neutrophils and diluting downwards in a microtitre tray containing nutrient broth. After allowing 3 hours for digestion of bacteria, another portion of neutrophils was sonicated and inoculated and diluted downwards in a microtitre tray containing broth. The growth pattern was determined using the Most Probable Number

Table of Halverson and Zeigler [19]. The killing index was calculated as a ratio of the number of viable bacteria after incubation (t = 3) and number of viable bacteria ingested (t = 0). The lower the killing index value calculated, the better the killing ability.

Chemotaxis assay

This was determined using the method of Nelson *et al* [20]. The directed migration of neutrophils under agarose towards an antigen (zymosan-activated serum) and the random migration towards medium 199 was measured. Quantitation of chemotaxis (as an index of A/B) was done by projecting the pattern and measuring the linear distance of cell migration from the margin of the well toward the chemoattractant (A) and the distance the cells have migrated from the margin of the well towards the control medium (B).

G-6-PD assay

The enzyme levels were assayed using the quantitative, ultraviolet kinetic determination of the enzyme by the spectrophotometric method of Lohr and Waller [21]. The conversion rate of $NADP^+$ to NADPH was measured as an indicator of G-6-PD activity in erythrocytes.

Lymphocyte typing

Lymphocytes were harvested from whole blood applying the method of Gupta and Good [22]. Lymphocyte subtyping was done with monoclonal antibodies using the immnunofluorescent technique as described in the Becton-Dickinson Source Book [23]. Fifty microlitres of the lymphocyte suspension were mixed with 20 mirolitres of the specific monoclonal antibody (anti-Pan T, CD4, CD8, B cells) and screened for fluorescent staining of cells under ultraviolet miroscopy.

The MIF production assay

This was done using the method of Rosenberg and David [24]. Capillary tubes containing packed white blood cells were incubated for 24 hours at 37°C in migration chambers filled with RPMI medium containing 100IU/ml PPD (test) or RPMI medium containing 15% fetal calf serum alone (control). The percentage migration inhibition was calculated using the formula:

$$\%\% \text{ inhibition} = 1.0 - \frac{\text{test area}}{\text{control area}} \times 100$$

Statistical analysis

Analsyis of variance and comparisons of means using the *t* test was applied on results obtained.

Results

The majority of the patients presented with bilateral lung involvement (45 patients) and were divided into four groups depending on the type of radiological lesions: (a) predominantly exudative (23 patients), (b) predominantly fibro-cavitary (20 patients), (c) mixed lesions (18 patients), and (d) miliary lesions (9 patients).

Polymorph function

A significantly impaired polymorph function was found ($P < 0.05$) when all patients were compared to controls with both the chemotactic and intracellular killing abilities of the neutrophils reduced. The least defective polymorph function was found in those with exudative lesions while the most defective were in the miliary group. Increasing killing ability was depicted by decreasing killing index values while the reverse was the case for the chemotactic index.

Table 1: Polymorph function, G-6-PD levels and MIF indices among TB patients and controls

		Patients	Controls	*P value*
Absolute neutrophil count (x10^9/L)	$\bar{X}$ ± 2 SD	n = 70 4.1±1.7	n = 35 2.4 ± 1.3	
	range	1.0-9.2	1.3-4.3	
hemotaxtis	$\bar{X}$ ± 2 SD	n = 70 1.9 ± 1.2	n = 35 0.5 ± 0.3	
	range	0.8-5.2	1.3-4.3	< 0.05
Kiling Index	$\bar{X}$ ± 2 SD	n = 70 1.9± 1.2	n = 35 0.5 ± 0.3	
	range	0.01-6.5	0.1-1.3	< 0.05
G-6-PD level (u/g Hb)	$\bar{X}$ ±2 SD	n = 40 6.8 ± 7.0	n = 20 8.0±2.6	
	range	0-13	6.2-10.2	< 0.05
MIF	$\bar{X}$ ± 2 SD	n = 40 60 ± 36	n = 20 52 ± 7	< 0.05

No statistical significant differences ($P > 0.05$) were found between G-6-PD levels of all patients and the controls. A negative correlation r = – 0.4, ($P < 0.05$) was found between G-6-PD levels and neutrophil intracellular killing indices among the tuberculosis patients. A similar negative correlation r = – 0.8, ($P < 0.05$) was also found between these two parameters in the controls. Migration inhibition to PPD showed a significant difference between all patients and the control group ($P < 0.05$).

Relationship to radiological types

Patients with fibro-cavitary lesions had the highest mean value (77 ± 20%) for MIF while patients in the miliary group had the lowest mean value (35 ± 20%). Statistical analysis was carried out on the exudative lesions group (10 patients; $\bar{X}$ ± 2SD 70 ± 28) and fibro-cavitary lesions group (13 patients $\bar{X}$ ± 2SD 60 ± 14) where the differences found were statistically significant ($P < 0.05$). Statistical analysis was not carried out on the miliary lesions group because of the few number of patients. (Table 2).

Table 2: Polymorph function in relation to radiological classification of TB patients

		Exudative	Fibro-cavitary	Mixed	Miliary
Neutrophil count (x 10^9 /L	no.	23	20	18	9
	± 2SD	4.6 ± 5	3.2 ± 1.9	3.8 ± 3.0	5.1± 6.3
	range	1.8-9.0	1.2-5.9	1.1-5.7	1.0-9.0
Chemotactic index	no.	23	20	18	9
	± 2SD	1.2 ± 2.2	1.5 ± 1.9	1.7± 2.7	4.2± 1.2
	range	0.01 -5.6	0.02-3.8	0.02-4.2	3.0-6.8
Killing index	no.	23	20	18	9
	± 2SD	1.2 ± 2.2	1.5 ± 1.9	1.7 ± 2.7	4.2 ± 1.2
	range	0.01 -5.6	0.02-3.8	0.02- 4.2	3.0-6.8

G-6-PD levels were highest in the miliary lesions group ($\bar{X}$ ± 2SD of 11.2 ± 6) and lowest in the fibro-cavitary group with a mean ± 2SD of 4.2 ± 7.5 ($P < 0.05$). The mean ± 2SD in the exudative lesions group was 5.1 ± 8.0 ($P < 0.05$) and 7.4 ± 4.4 in the mixed lesions group ($P < 0.05$).

The worst polymorph function results were found in the miliary lesions group (9 patients) with both the chemotactic and the killing indices being significantly different ($P < 0.05$). The differences in values of polymorph function indices among the exudative, fibro-cavitary, and mixed lesions group were not very high, although they were significantly different compared to the controls ($P < 0.05$).

Lymphocyte populations

Total T lymphocytes were lower in percentage and absolute numbers in all patients when compared to the controls ($P < 0.05$). T helper cells (CD4) were similarly lower in patients than in the controls ($P < 0.05$). T suppressor cell percentage but not the absolute value, were significantly higher in

patients. The B cells had higher percentages and absolute values in patients as compared to healthy controls ($P < 0.05$).

Table 3: Lymphoctes subpopulation in tuberculosis patients and controls

		Patients (40) ± 2SD	Controls (20) ± 2SD	*P* Value
Pan T cells	%	53 ± 6	66 ± 3	< 0.05
CD3	Absolute	1.1 ± 0.8	1.5 ± 0.3	< 0.05
T helper	%	24.5 ± 9	18.5 ± 3	< 0.05
CD4	Absolute	0.7 ± 0.7	1 ± 0.6	< 0.05
T suppressor	%	24 ± 9	19 ± 6	< 0.05
CD8	Absolute	0.7 ± 0.4	0.5 ± 0.3	< 0.05
B cells	%	33 ± 6	22 ± 8	< 0.05
	Absolute	0.8 ± 0.2	0.5 ± 0.4	< 0.05

Discussion

A significantly impaired polymorph function consisting of reduced directional movement (chemotaxis) and reduced killing ability were found in the patient group when compared to the controls. The greatest impairment in killing ability was in those with miliary lesions although the directional movement of their neutrophils was similar to other radiological groups. Caplin *et al* [25] have, however, suggested that radiological changes cannot strictly be related to the extent of impairment of the immune function as the x-ray changes could have occurred some time prior to the laboratory study.

The miliary group also had the highest neutrophil count and highest G-6-PD levels, thus implying that these do not necessarily represent a normal neutrophil function since the killing ability of neutrophils was lowest in this group. Kampschmidt [26] has suggested that due to chronic stimulation of the bone marrow by T cell products like interferon, tumour necrosis factor, and granulocyte-monocyte colony stimulating factor, an increase in immature circulatory neutrophils, which are unable to perform their function efficiently, occurs. T lymphocytes mediated increase in the bactericidal activity of the neutrophil has been observed to occur after the initial acute phase of infection [8].

This study implies that defective polymorph function could be as a result of T cell mediation. Evidence is presented in the miliary lesions group where the lowest T helper cell numbers are accompanied by the greatest impairment in mean killing index as well as the lowest MIF production value. The patients in the exudative lesions group presented with the best polymorph function indices as well as the highest mean T helper cell values.

G-6-DP levels were also studied to determine the underlying cause of detective intracellular killing. This enzyme is involved in the stimulation step of the hexose monophosphate shunt pathway which is an important component of the intracellular killing system of neutrophils. A negative correlation ($r = -0.4$) was found between G-6-PD levels and intracellular killing indices, meaning that as the enzyme level increases functionally, intracellular killing becomes enchanced. Thus, a background G-6-PD deficiency may be contributory to neutrophil dysfunction especially when 20-40% of the population of the tropical malaria endemic regions may also be G-6-PD deficient [4]. It is also possible that G-6-PD deficiency was acquired with the inflammatory process as it was more marked in the excudative and fibrocavitary group, and normal in the miliary patients with defective killing indices.

The chemotactic abnormality observed in this study could be due to an enrichment of the circulation with a population of neutrophilic cells with abnormal microtubule function [27]. Immature neutrophils can also be implicated since 60% of the patients presented with toxic granulations of their neutrophils. This chemotactic abnormality probably occurred as a result of factors dependent on the neutrophils since the assay technique used for chemotaxis in this study utilized a standard extrinsic medium (pooled control serum) for all cases.

Decreased percentage and absloute T lymphocyte values observed in this study could be due to their localization into tissues [28,29] or due to the presence of serum inhibitory substances that block T cell receptors [30]. As tuberculosis patients present with a wide spectrum of immnulogical and clinical characteristics, an analysis of the T cell subsets provides an insight into the understanding of the immune mechanism operating in the pathogenesis. Kaufmann [31] has suggested that protection is critically dependent on the balance between the T helper (CD4) and 1 suppressor cells CD8 observed in this study and findings were similar to those of Shiratsuchi and Tsuyuguchi [32] and Onwubalili *et al* [33]. The reversal in the T helper/suppressor ratio was especially prominent in the miliary group where grossly impaired polymorph function was also found. A defect in the immunomodulating effect of T cells on polymorph function as suggested by Campbell [8] may be a possible cause of the impairment.

An increase in the percentage B lymphocytes was also observed in tuberculosis patients with the highest values being in the miliary lesions group and is in keeping with the findings of Lenzini *et al* [34], which described the dominant immune reaction in miliary tuberculosis patients to be humoral and B cell mediated. An apparent increase in circulating B cell numbers could also be contributed to a reduction in circulating T cells, since T cells and microphages are the primary cells involved in the immune response to tuberculosis and may thus be trapped in the pulmonary lesions. This study has shown that neutrophil function and various other aforementioned parameters of cellular immunity are decreased in tuberculosis patients. These may be due to a decreased stimulatory effect of T lymphocytes and their products, although it has recently been shown that antigene-pulsed neutrophils can reduce proliferation of resting lymphocytes [35] suggesting that neutrophil-lymphocyte interaction is bilateral, mutual, and complex.

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