

Full Length Research paper

Cell-mediated immunity in Type 2 diabetes mellitus patients in diabetic ketoacidosis, patients with controlled Type 2 diabetes mellitus and healthy control subjects.

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Accepted 19 July, 2010

Immune modulation accompanying metabolic dysfunction can adversely affect outcome of a non infectious disease such as type 2 diabetes mellitus (T2DM). Peripheral T lymphocyte subsets were analyzed with monoclonal antibodies via manual counting by the indirect immunofluorescence method, in type 2 Diabetes mellitus patients in DKA, controlled T2DM patients as well as normal healthy controls (NHC). There was a significant decrease in CD4+ T cells alongside a non significant increase in CD8+ T cells in patients with diabetic ketoacidosis (DKA) compared to controlled T2DM patients and normal healthy controls. Ratio of CD4/CD8+ T cells was also lowest in DKA. These T cell changes in patients with DKA reflect an abnormal immunoregulatory mechanism in parallel with an impaired metabolic process and may lead to enhanced beta cell damage and increased susceptibility to DKA crisis in T2DM patients.

Keywords: Cell mediated immunity, diabetic ketoacidosis, Type 2 diabetes mellitus, metabolic dysfunction, T lymphocytes

INTRODUCTION

Diabetic ketoacidosis (DKA) is an extremely serious metabolic consequence of diabetes mellitus (DM) due to a state of relative or absolute insulin deficiency and a relative or absolute increase in counter regulatory hormones (Hamburger and Rush, 1993). A triad of acidosis, ketosis and hyperglycaemia are often characteristic of this condition, with DKA also being associated with poor control of DM, precipitating infections or diseases and first presentation with diabetes. Despite recent advances in therapy, mortality and morbidity in DKA remain alarmingly high especially in developing countries like Nigeria.

Previous studies had found impaired white cell function, defective mobilization of polymorphonuclear leucocytes and alteration of leucocyte sialic acid and sialidase activity in patients with DKA (Chari and Nath, 1984). More recent studies have demonstrated abnormal cellular

and humoral immunity in patients with DKA (Habib et al, 1998), while research over the last decade has suggested an association between lymphocyte membrane receptor *in vitro* glycosylation and altered lymphocyte function leading to lowered immunocompetence in patients with DKA (Ifere, 2000). These results suggest a discordant immune profile in these patients.

Although, DKA is generally considered a consequence of type 1 DM with insulin deficiency associated with beta cell damage, studies have shown that DKA is associated with type 2DM as well (Carroll and Schade, 2000). There is a high incidence of type 2 DM in Zaria, Nigeria and DKA is associated with type 2 DM in this environment. Reasons postulated for the prevalence of DKA in type 2 DM in Zaria include hyperglycaemia (indicating poor metabolic control) as well as the stimulus of endemic infections or underlying disease.

Since ambient glucose concentrations can profoundly affect lymphocyte response and consequently the

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immune response to infections and disease, this may be responsible for the development of conditions favouring the presence of DKA in type 2 DM patients. This study therefore examined numerical peripheral T- lymphocyte populations in patients with type 2 DM with DKA in Zaria at presentation and compared with that of matched type 2 DM patients in good metabolic control as well as matched normal healthy control subjects.

PATIENTS AND METHODS

Patients

The study involved patients from the medical wards (for patients in diabetic ketoacidosis) and the Endocrinology Clinic (for controlled diabetic patients) of the Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

A total of twenty-one (21) patients admitted to the Medical wards in diabetic ketoacidosis (DKA) with an 'unacceptable' glycaemic index reflected by a blood glucose level $>13\text{mmol/L}$, serum bicarbonate $<20\text{mmol/L}$ and positive ketonuria with or without an altered mental state (Khan and Khan, 2000) were enrolled.

Twenty (20) non symptomatic patients with controlled type 2 diabetes mellitus attending the Endocrinology clinic for routine follow-up at three monthly intervals who were age and sex matched with the patients in DKA served as controls.

Healthy volunteers from the hospital and laboratory staff, relations of patients, as well as members of the local community who agreed to participate in the study served as normal healthy controls. All the subject groups were sex (gender) matched while ages differed by no more than three years.

Exclusion criteria

Potential subjects from any group were excluded if they were positive for HIV, had any overt clinical condition or refused consent. Patients with Type 2 diabetes mellitus alone were excluded if they had poor drug compliance and an 'unacceptable' index of glycaemic control.

Ethical approval

The study was approved by the Ethical and Scientific Board of the A.B.U.T.H. and informed consent using consent forms approved for the protocol was obtained from all patients or their next of kin and control subjects.

Specimen Collection

Twenty (20) millilitres (ml) of blood was drawn from each subject. Three (3) ml of this was transferred to an EDTA bottle for determination of the basic haematological indices. Seven (7) ml was dispensed in a sterile plain bottle for separation of serum in two aliquots. One aliquot was used for the biochemical determination of urea and electrolyte profiles and blood glucose levels in each patient (Tietz, 2001), while the other aliquot was stored in tubes containing drops of Trasylol™ (aprotinin) to inhibit degradation of cytokines. Serum in the tubes was subsequently stored at -20°C for assay of cytokines. Ten (10) ml of blood was collected in preservative free heparin tubes (SIGMA, USA) for the extraction of peripheral blood lymphocytes. Blood samples were taken from all research subjects on receipt of consent, at confirmation of

diagnosis and before the administration of any drugs. Blood samples were taken from normal healthy controls after obtaining consent and treated similarly. Urine samples were also collected from diabetic patients for the detection of glucose, proteins and ketones and in support of clinical diagnosis, stool, urine and sputum samples were assessed for microbiological evidence of infection in patients where possible.

Complete blood count

All subjects and controls in the study had a complete blood count done using the standard procedures of Dacie and Lewis (1991) currently in practice in the Haematology laboratory of the teaching Hospital. These standard procedures included haemoglobin and haematocrit determinations, differential and total white blood cell counts, platelet and reticulocyte counts. Blood films for malaria parasites were also taken. Absolute lymphocyte counts were deduced from the total white blood cell counts and the percentage differential lymphocyte counts as follows:

$A / 100 \times B = C$ where

A = percent differential lymphocyte count

B = total white blood cell count $\times 10^9/\text{L}$

C = absolute number of cells $\times 10^9/\text{L}$

Assessment of Cellular Immunity

Separation of lymphocytes

The differential gradient centrifugation method of Gupta and Good (1989) as employed by Musa et al (2010) was used. Briefly, ten (10) ml of heparinised blood was diluted 1:2 with Eagle's minimum essential medium (MEM) adsorbed with fetal calf serum. This was layered in 7 ml aliquots on 3 ml of Ficoll-Histopaque (SIGMA, USA) density gradient medium in 10 ml tissue culture tubes (SIGMA, USA). The mixture was centrifuged (MSE,UK) at 1800 rpm for 30 minutes at room temperature and pure lymphocytes harvested by gently removing the lymphocyte layer at the Ficoll-MEM interface with a pasteur pipette and transferring to another 10 ml tissue culture tube. Lymphocyte viability was tested by trypan blue exclusion test. The cells were then washed thrice with MEM by suspending in the medium and centrifuging at 1800 rpm for 10 minutes at room temperature. After the final wash, 4×10^6 of the cells were suspended in 1 ml of MEM containing 100ug/ ml penicillin, 100ug/ ml streptomycin and 5% heat inactivated adsorbed fetal calf serum (Wellcome, UK) for lymphocyte enumeration by immunofluorescence microscopy.

Immunofluorescence microscopy technique

Lymphocyte subsets (CD3, CD4, CD8) were enumerated using the method described in the Becton- Dickinson (BD-Pharmingen,USA) source book and corresponding monoclonal antibodies (UCHT1, QS4120, QS4122; Ancell™ Corporation, USA) conjugated to FITC (Musa et al, 2010). Cells were incubated for 30 minutes in crushed ice in fifty (50) uL aliquots. This was taken from the lymphocyte suspension separated as described above and placed in small plastic tubes, with 20 uL of the appropriate monoclonal antibody added. The cells were then settled by centrifuging at 1000 rpm for 5 minutes at 4°C (Chilspin, MSE, UK) and washed twice. After the final wash, the cells were suspended in 50 uL of MEM and placed on a sterile glass slide to be kept in the dark at 4°C for 15 minutes. Direct fluorescence analysis was carried out using a fluorescent microscope (Leitz Orthoplan, Germany). Random fields

Table 1. Clinical characteristics of patients with DKA, controlled T2DM patients and normal healthy controls (NHC)

Characteristics of the Patients	Group 1 DKA patients N = 22	Group 2 T2DM patients n =22	Group3 Normal healthy controls n = 20	p – value
Age	46 ± 4	48±15	43 ± 6	NS
Gender	M:F 2.8: 1	M:F 2.8:1	M:F 2.9:1	NS
BMI (kg/m ²)	23 ± 4	25 ± 5	27 ± 6	NS
Glucose mmol/L	25 ± 2	5 ± 0.4	4 ± 1	<0.05* [#]
Fasting blood glucose	17 ± 4	5 ± 0.4	4 ± 0.6	<0.05* [#]
2 hr postprandial	26 ± 9	8 ± 0.7	6 ± 0.6	<0.05* [#]
Bicarbonate mmol/L	15 ± 3	25 ± 2	26 ± 2	< 0.05* [#]
Diastolic BP	133 ± 18	131 ± 38	113 ± 9	<0.05 [#] §
Systolic BP	85 ± 9	81 ± 24	74 ± 9	<0.05 [#] §

Numbers seen are mean ± SD. P values are the results of two tailed Students t-test. * Indicates a significant (p < 0.05) difference between DKA patients and controlled T2DM patients, # signifies a statistically significant (p < 0.05) difference between DKA patients and normal healthy controls while § indicates significant (p < 0.05) statistical difference between T2DM patients and normal healthy controls.

Table 2. Factors associated with DKA

Factors	DKA patients (n = 22)
Newly diagnosed diabetes (ignorance)	8 (36%)
Poor drug compliance	6 (27%)
Infection (fever, cough etc)	16 (73%)
Other medical illness (hypertension etc)	5 (23%)
Unknown factors	<1%

were screened in the dark by selecting a field, viewing under normal light to record the total number of cells in the area, then viewing the same field under ultra violet (uv) light filter and recording the number of cells which stain bright apple green peripherally as positive. Several fields were scanned until a total of 200 lymphocytes were counted. The numbers of positive cells were then expressed as a percentage of the total count and the absolute number of cells calculated.

Statistical analysis

Data was analyzed by the use of computer *SPSS version 11.5* and *GraphPad Prism 4.0* statistical programmes, and statistical significance was calculated using Student's unpaired two-tailed t-test. Comparisons were made between:

DKA crisis patients and controlled type 2 DM patients,

DKA crisis patients and normal healthy controls,

controlled type 2 DM patients and normal healthy controls.

A p value of less than 0.05 was considered to be significant.

RESULTS

Demographic and clinical features in diabetic ketoacidosis study

The clinical characteristics of the patients are shown in Table 1. Of the 22 patients with DKA, 17 (77%) were

males and 5 (23%) were females, while the controlled type 2 DM patients were made up of 14 males (64%) and 8 (36%) females. Seventeen (77%) of the DKA patients were above 40 years of age while 5 (23%) were below 40 and eighteen (82%) of the controlled DM patients were above 40 years with 4 (18%) below 40. Average age of patients with DKA was 46 ± 4 (range 15-75) years and 48 ± 9 (range 15-75) years for controlled DM patients. There was no significant difference in the age and gender distribution of the two groups (p>0.05). Hospital admission for DKA occurred in 9 (41%) patients newly diagnosed with diabetes and in 13 (59%) patients previously diagnosed as having diabetes. The mean duration of hospitalization was 8 ± 2 days for patients with DKA. The mean body mass index (BMI) for patients with DKA was 23 ± 4 kg/m² compared to 25 ± 5 kg/m² in controlled DM patients. The main documented clinical associations with DKA are as shown in Table 2 and include poor compliance with insulin therapy (27%), infection (55%), associated medical illness (23%) and lack of prior knowledge of diabetes (36%).

The commonest presenting complaints of patients with DKA were polyuria, polydipsia and weight loss despite increased appetite along with instances of fever, vomiting, bloody stools, respiratory distress, blurred vision, body weakness and inability to speak or walk. Two

Table 3. Haematological indices in patients in DKA, controlled T2DM and normal healthy controls (mean + SD)

Parameters	DKA patients n=22	T2DM patients n=22	Normal healthy controls n=20	p- value
PCV %	35 ± 1	37 ± 4	40 ± 5	p<0.05*
WBC x 10 ⁹	9 ± 0.7	6 ± 2	7 ± 2	p<0.05*#
Neutrophils(%)	70 ± 2	43 ± 7	51 ± 10	p<0.05*#§
Lymphocytes(%%))	28 ± 2	56 ± 7	46 ± 9	p<0.05*#§
Platelets	235 ± 9	248 ± 61	227 ± 56	p>0.05

Numbers are mean ± SD. P values are the results of two tailed Student's t-test. * Represents a significant (p <0.05) statistical difference between DKA patients and normal healthy controls, # indicates a significant (p<0.05) difference in values between DKA patients and controlled T2DM patients, while § shows a statistically significant difference between T2DM and normal healthy controls.

Table 4. Lymphocyte subsets in DKA, controlled T2DM patients and normal healthy controls (mean +SD)

Cell subset	DKA n=22	T2DM n=22	NHC n=20	p-value
CD3%	40 ± 6	49 ± 5	52 ± 7	< 0.05* ^{§NS}
ABS CD3	1.01 ± 0.5	1.67 ± 0.7	1.59 ± 0.6	< 0.05* ^{§NS}
CD4%	18 ± 4	28 ± 4	30 ± 4	< 0.05* ^{§NS}
ABS CD4	0.47 ± 0.3	0.94 ± 0.4	0.93 ± 0.4	< 0.05* ^{§NS}
CD8%	22 ± 4	20 ± 2	22 ± 4	> 0.05
ABS CD8	0.56 ± 0.3	0.67 ± 0.3	0.67 ± 0.2	> 0.05
CD4/CD8 ratio	0.84	1.40	1.36	<0.05* ^{§NS}

Numbers are mean ± SD. P values are the results of two tailed Student's t-test. * Indicates a significant (p < 0.05) statistical difference between patients with DKA versus normal healthy controls, § represents a significant (p<0.05) difference in patients with DKA versus controlled T2DM while NS shows a non significant (p>0.05) difference between T2 DM patients and normal healthy controls.

DKA patients had Kussmaul respiration and acetone breath. On admission 15 (68%) of DKA patients had an impaired mental status in terms of an altered sensorium i.e. lethargy or stupor and 7 (32%) had coma i.e. loss of consciousness. The occupational distribution of patients with DKA included five students, three farmers, two widows, one housewife, four civil servants and five casual labourers. There were eleven (50%) deaths in patients with DKA. All patients and controls in the study were seronegative for HIV

Haematological indices in diabetic ketoacidosis

The haematological parameters in patients in DKA, controlled DM patients and normal healthy controls are shown in Table 3. The complete white blood cell count and neutrophil percentages in patients with DKA (9 ± 0.7; 70 ± 2) was significantly higher compared to controlled DM patients (6 ± 2; 43 ± 7) and normal healthy controls (7 ± 2; 51 ± 10) (p<0.05) while there was no significant difference in WBC and neutrophil values between controlled DM patients and normal healthy controls. Differential lymphocyte counts were significantly higher in controlled DM patients and lower in patients with DKA

compared to normal healthy controls (p<0.05). The difference in differential lymphocyte percentages between patients with DKA and controlled patients was also significant. Haematocrit (PCV) levels between both groups (35%; 37%) were lower than normal healthy controls (40%). Similarly, platelet counts were higher in controlled DM patients compared to patients with DKA and counts in both groups were higher than in normal healthy controls although not significantly.

T cell subsets in diabetic ketoacidosis

The absolute and percent total T (CD3+) and CD4+ T-cell subset values (Table 4.) in patients with DKA were significantly lower than those for steady state controls as well as normal healthy controls on all counts (p<0.05). Values for CD4+ cells were most significantly depleted (p < 0.05). There was no significant difference in absolute or percent CD8+ T cell counts in patients with DKA, stable DM patients and healthy controls (p>0.05). However there was a significant difference in CD4 to CD8 ratios in patients with DKA compared to stable DM patients and normal healthy controls (p<0.05).

DISCUSSION

The study demonstrates abnormalities in cell mediated immunity in the peripheral T lymphocyte subset in patients with DKA when compared with healthy subjects and controlled type 2 diabetes mellitus (T2DM) patients. All the patients in DKA in this study were T2DM patients. A significant difference was also detected in numerical peripheral blood T lymphocytes between healthy subjects and those with controlled T2DM. The evidence of an imbalance in cell mediated immunity found in patients with DKA in this study suggests a possible relationship between glucose control, abnormal cellular immunity and the development of DKA. This implies that ambient glucose concentrations in a non infectious disease like diabetes mellitus can act as a stressor affecting immune modulation and outcome of disease.

The findings in the early seventies of Brodie and Merlie (1970), Ragab *et al.* (1972) and MacCuish *et al.* (1974) highlight this fact, indicting the heterogeneity of DM as a reason for differences in T cell populations and function. Brody and Merlie (1970), reported marked depression of the transformation response to phytohaemagglutinin (PHA) by lymphocytes from six elderly diabetic patients when compared to normal subjects. Ragab *et al.* (1972) on the other hand, found no difference in the PHA response of a larger number (23) of diabetics and controls. Interestingly, the disparity between their results was paralleled by the metabolic state of the diabetics at the time of the study. Patients studied by Brodie and Merlie (1970) were poorly controlled and hyperglycaemic while those of Ragab *et al.* (1972) were well controlled and normoglycaemic. Similarly MacCuish *et al.* (1974) comparing PHA responses in well and poorly controlled diabetics found depressed responses only in the latter group. Diabetics with DKA in this study were hyperglycaemic compared to stable diabetics and normal healthy controls suggesting that the significant differences in their lymphocyte subsets could be a reflection of metabolic disturbance. Hyperglycaemia is exaggerated in DKA by increased levels of circulating catecholamines, glucagon and cortisol. These have profound effects on lymphocytes which may reflect in the reduced T cell levels in patients with DKA in this study as opposed to T2DM and NHC and indicate a link between the immune and endocrine systems.

White blood cells including lymphocytes and their functions are also globally affected by ambient glucose concentrations (Alberti, 1977) and this is regarded as the likeliest explanation for the increased suspicion of infection in patients with DKA. Infections play a role as disease precipitating factors and many of them are known to be associated with a reduced CD4/CD8 ratio. It was not possible to identify via microscopy and culture infectious causes in DKA patients in the study. Infection was suspected based mainly on clinical signs in 73% of our patients with diabetic ketoacidosis as they were adm-

itted into the study on the premise of 'no overt infection'. These clinical signs included fever, diarrhoea and cough. Infections are known to decrease levels of corticosteroid binding globulin leading to increased levels of circulating free corticosteroids (Cooper and Stewart, 2003). Corticosteroids released during stress are immunosuppressive in vivo (Chambers and Schauenstein, 2000). In acutely ill patients this results in an increase in cortisol production that is roughly proportional to the severity of the illness (Cooper and Stewart, 2003). This stress induced endogenous release of glucocorticoids possibly due to an anti-infectious response is a potential mechanism of lymphocyte apoptosis (Hotchkiss and Karl, 2003) that may be responsible for the reduced numbers of CD3+ and CD4+ T cells in patients with DKA and steady state T2 DM patients in this study. Fever is closely associated with malaria in this environment and malaria is associated with reduced CD4+ and CD8+ T cell numbers.

The reversibility of DKA with the administration of insulin is also a very interesting phenomenon buttressing the case in point of metabolic dysfunction and lowered immunocompetence in T2DM. Although 50% of the patients with DKA eventually died due to the severity of their conditions, the other half survived on standard care involving insulin therapy. Kitabchi *et al.*, (2004) studying the in vivo activation of CD4 and CD8 lymphocytes in patients with DKA on admission and at resolution by measuring de novo growth factor receptors found increased levels of growth factor receptors in association with markers of oxidative stress in patients with DKA. Consequently, in the likelihood that DKA causes immunosuppression, Kitabchi *et al.*, (2004) proposed that DKA changes T lymphocytes to insulin sensitive tissues as a compensatory mechanism. Insulin prevents apoptotic cell death from various stimuli by activating the phosphatidylinositol 3- kinase-Akt pathway (Hotchkiss and Karl, 2003) and prevention of lymphocyte apoptosis. Insulin also reduces plasma glucose concentrations in DKA and lowers mortality rates (English and Williams, 2004). Insulin as a secreted hormone preventing apoptosis is similar to cytokines producing signals inhibitory to apoptosis. Such cytokines are said to be crucial for cell survival indicating vital parallels between endocrine and cytokine signalling necessary for maintaining the balance between life and death. Consequently this protective mechanism of insulin in DKA may indicate the termination of apoptotic mechanisms due to inflammation, hyperglycaemia as well as hypoinsulinaemia. Bakari and Onyemelukwe (2004) in their study of plasma insulin response to oral glucose challenge in patients with type 2 DM (T2DM) in Zaria, found low insulin outputs and proposed that the underlying defect in African type 2 diabetes could be beta cell malfunction with consequent low insulin outputs in the population. T cells rather than autoantibodies are said to be responsible for this defect which could alter the

insulin-glucagon ratio to the extent that DKA is precipitated in a type 2 diabetes mellitus patient (Yu *et al.* 2006).

The slightly elevated CD8+ T cells in patients in DKA in this study in conjunction with significantly low levels of CD4+ T cells would indicate acute critical illness with consequently an overload of the immune response. In such a situation, there is stimulation of suppressor CD8+ T cells and limitation of antigen induced lymphocyte proliferative responses by the production of suppressor factors. Additionally, the elevation of CD8+ T cells observed in this study may be as a result of sequestration of CD4+ T cells to the pancreas in T2DM involving anti-islet T cell mediated pathogenic mechanisms (Yu *et al.* 2006). The similar presence of higher numbers of suppressor (CD8+) T cells in normal human controls as compared to stable diabetic patients (T2DM) could imply the presence of non overt sub clinical infections such as preimmune malaria in these patients.

In conclusion, the study shows that impaired cell mediated immunity is prevalent in T2DM patients with DKA. Aberrant glucose concentrations, hypoinsulinaemia, infections or underlying disease can exert enough stress to affect the immune cell population and influence adversely the outcome of T2DM in Nigerians. This may be reflected in a lowered immunocompetence (a deranged immune cell milieu and function) and metabolic dysfunction as is seen in DKA.

As this was a case control study with the attendant limitations of the study group being defined by outcome, it becomes difficult to categorically establish a causal status of hyperglycaemia and deficient T lymphocytes. Further studies on these abnormalities of the cell mediated immune response in DKA and the role of insulin in correcting this anomaly are required.

ACKNOWLEDGEMENT

The authors wish to acknowledge the excellent technical assistance of Dr T.S.Kene in the statistical analysis of data.

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