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Immunological responses of Gambians in relation to clinical stage of HIV-2 disease

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SUMMARY

This study describes a broad spectrum of cellular and antibody-mediated immune responses found in 28 asymptomatic and 37 symptomatic Gambian patients with HIV-2 infection. It shows that these responses vary according to the stage of infection as described by three clinical staging systems. The first system was a local one based on the signs used for the WHO Bangui clinical definition of AIDS, the second, suggested by WHO, was based on a performance scale, and the third was that used by the Centre for Disease Control. Asymptomatic patients had significantly lower mean CD4 counts, lymphoproliferative and interferon-gamma (IFN-γ) responses and lower IgG and IgM antibody responses to keyhole limpet haemocyanin (KLH) than controls. These measurements and the size of the skin test reaction to purified protein derivative (PPD) or Candida antigen declined significantly according to the stage of infection. Mean values of the serological markers β_2-microglobulin and neopterin and antibody titres to Epstein–Barr virus capsid antigen (EBVCA) rose significantly according to severity of disease. The Gambian or WHO clinical staging systems, which are easy and cheap to apply, may serve as an alternative to sophisticated and expensive immunological measurements when trying to stage disease and predict prognosis.

Keywords HIV-2 clinical stage

INTRODUCTION

The literature pertaining to HIV-1 research is replete with accounts of its damaging effects on the immune system [1]. The virus infects and damages both CD4 [2] and antigen-processing cells [3]. The upshot of these effects is anergy to skin test antigens [4], low lymphocyte proliferative responses to both mitogens and antigens, together with impaired production of cytokines [5], and poor control of viral [6] and bacterial pathogens such as *Mycobacterium tuberculosis* [7] and *Streptococcus pneumoniae* [8]. B cell responses are also deranged, resulting in high serum immunoglobulin levels [9] and abnormally low responses to some immunogens, including vaccines [10].

In contrast, the full spectrum of immune damage caused by HIV-2 has yet to be charted. A community-based study in Bissau [11] and a cross-sectional study of prostitutes in The Gambia [12] have shown that CD4 cells are depleted in subjects with asymptomatic HIV-2 infection and that, in prostitutes, proliferative responses to mitogens and antigens are depressed and serum β_2 and neopterin levels raised. A hospital-based study in Senegal compared CD4 counts, immunoglobulin and β_2-microglobulin levels in asymptomatic and symptomatic subjects infected with either HIV-1 or HIV-2. The authors concluded that the correlation between immunological abnormalities and clinical status was similar for the two infections [13].

In this study we report the results of a wide range of immunological tests in Gambians at various stages of HIV-2 infection. We show that immune damage is correlated closely with the stage of disease as described by a local system based on the WHO Bangui definition of AIDS [14], by a clinical classification system suggested by the World Health Organization (WHO) [15], or by that developed at the Centre for Disease Control (CDC) [16].

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PATIENTS AND METHODS

Asymptomatic seropositive subjects, including those with generalized lymphadenopathy, were recruited from the Sexually Transmitted Diseases (STD) clinic at the Medical Research Council Hospital, The Gambia. Control subjects for this group were HIV$^-$ subjects matched by sex and age, who were recruited from this clinic. Their immunological tests were done at the same time as the seropositive patients. Controls and patients

were recruited only after their STD infection had been treated successfully. Patients with symptoms and/or signs suggesting chronic HIV infection who presented at the Royal Victoria Hospital in Banjul or at the MRC Hospital Fajara were screened for HIV antibodies. If positive, they were recruited into this study, which ran for 2·5 years.

Each HIV$^+$ patient received a detailed clinical examination, and on the basis of the history and the clinical findings was thereafter assigned a score on the Karnofsky performance scale. Bronchoscopy and/or gastrointestinal endoscopy was undertaken when appropriate. Within 2 weeks of presentation patients had received a haematological, immunological and microbiological investigation, including lymph node biopsies or aspirates if clinically warranted. Many of the symptomatic patients were near to death or were suffering from chronic or subacute associated infections like tuberculosis [17]. Thus it was not practical to perform the immunological tests after successful treatment of these infections, as was the case for the asymptomatic group.

HIV-2$^+$ patients were staged according to three clinical staging systems. The first was a local system based on the WHO Bangui definition of AIDS which requires a patient to have two major signs of disease (weight loss > 10% body weight, chronic diarrhoea > 1 month or prolonged fever > 1 month) coupled with at least one minor sign (persistent cough > 1 month, generalized pruritic dermatitis, recurrent herpes zoster, oropharyngeal candidiasis, chronic disseminated herpes simplex infection, generalized lymphadenopathy). The Gambian system which requires that patients should be seropositive for HIV is as follows: stage 1 (asymptomatic) includes asymptomatic subjects with or without lymphadenopathy; stage 2 (AIDS-related complex (ARC)) includes symptomatic patients who do not meet the definition of AIDS but had at least two AIDS-related signs or symptoms; stage 3 (AIDS) includes symptomatic patients with AIDS.

The second system was based on the WHO system [15] which was modified by using the Karnofsky performance scale instead of the WHO performance scale. The latter scale, which devolves around the amount of time the patient is bedridden during the day, could not be applied as we did not ask this question in the early part of the study. The WHO performance scale 1 was equated to a Karnofsky score of 100 (normal, no complaints), scale 2 to a score of 80–90 (symptomatic, but able to carry on with normal work), scale 3 to a score of 60–70 (unable to work, able to live at home and care for most personal needs), and scale 4 to a Karnofsky score of 50 or below (unable to care for self, disease progressing rapidly).

The third was the CDC classification system [16] which was condensed into four main categories: asymptomatic HIV infection (CDC2), generalized lymphadenopathy (CDC3), constitutional disease with one or more of the following: fever for more than 1 month, 10% weight loss, diarrhoea lasting 1 month (CDC4a), and severe disease (CDC 4b, c, d, e).

Sera were tested for HIV-1 or HIV-2 antibody with two, type-specific competitive ELISA Assays (Wellcome Diagnostics, Dartford, UK) and by a type-specific Western blot (New LAV Blot I and New LAV Blot II, Pasteur, Marnes la Coquette, France), and only included if the ELISA and blot tests were HIV-2$^+$. Three patients with dual infections were excluded.

Informed consent was obtained from all subjects and counselling offered on request of the HIV test result. The study was approved by the MRC/Gambia Government Ethical Committee and by the National AIDS Committee.

Each subject was skin tested with 10 units of purified protein derivative (PPD; Evans Medical, Horsham, UK) and *Candida albicans* antigen diluted 1:20 (Hollister-Steir, Spokane, WA) and the reactions read 48 h later. A positive response was defined as skin induration equal to or more than 5 mm in diameter. Each subject received 5 mg keyhole limpet haemocyanin (KLH; Calbiochem, La Jolla, CA) subcutaneously.

Whole-blood samples drawn into heparinized tubes were processed within 3–6 h of collection. Lymphocytes were separated on Lymphoprep TM (Nycomed Diagnostics, Oslo, Norway) and stained with the appropriate MoAbs (Becton Dickinson, Oxford, UK). The percentages of various subpopulations of lymphocytes were determined with a FACScan flow cytometer (Becton Dickinson). The total leucocyte count was obtained using a ZF Coulter Counter (Coultronics, Margency, France) and a 100-cell differential count performed manually on Leishman-stained blood films by two experienced observers. Lymphocyte proliferation assays were performed in triplicate in RPMI 1640 containing 7·5% AB serum or, in the case of pokeweed mitogen (PWM), 10% fetal calf serum (FCS) with either 2 μg/ml purified phytohaemagglutinin (PHA; Wellcome, Beckenham, UK), a 1:100 dilution of Candida antigen, 100 U/ml of PPD or a 1:200 dilution of PWM (Flow Labs, Irvine, UK). Control wells without mitogen or antigen were set up in triplicate. After incubation at 37°C for 3 days (with PHA) or 6 days (with antigens), 1 μCi of ^{3}H-thymidine was added to each well. After a further 18 h incubation, the cells were harvested and ^{3}H-thymidine incorporation assessed using a 1205 β-plate liquid scintillation counter. Results were expressed as $\log_e$ (ct/min stimulated cells − ct/min unstimulated cells) ($\log_e \Delta$). Interferon-gamma (IFN-γ) and immunoglobulin levels were measured in the cell culture supernatants using ELISA methods [18, 19]. IFN-γ responses were found to be obviously dichotomous, with a clear division at 40 U/ml. Thus a positive response was defined as one greater than 40 U/ml.

Serum antibodies to KLH were measured 4–6 weeks after immunization by ELISA [20], antibodies to Epstein–Barr viral capsid antigen (EBVCA) were measured by indirect immunofluorescence and serum β_2-microglobulin and neopterin levels were measured by radioimmunoassay using commercial kits (Pharmacia β_2-micro RIA, Pharmacia, Uppsala, Sweden; Immutest Neopterin, Henning-Berlin, Berlin, Germany).

Statistical analysis

A natural logarithm transformation was used to normalize the distribution of β_2-microglobin, neopterin, antibodies to EBVCA and the proliferative responses to PHA and PPD. Statistical tests were made against a χ^2 distribution for categorical variables, and using the *t*-test for continuous variables. The statistical package SAS was used for all analyses.

For all variables a statistical comparison was made between the seronegative control group and the asymptomatic HIV-infected group.

Within the HIV$^+$ patients the disease classifications or stages were considered as equal intervals along a continuum. Excluding the control group, each variable was tested for a linear trend across disease classifications. For all variables the deviations from the linear trend were not significant.

The Spearman rank-correlation coefficient was calculated to assess the correlation between variables.

RESULTS

The mean age of the 65 patients with HIV-2 infection was 35·4 years (range 20–60) and the male to female ratio was 0·85.

Immunological variables according to the Gambian staging system

Skin tests. Delayed hypersensitivity to PPD or Candida antigen, as measured by a positive skin test or by the mean size of the reaction, was increasingly likely to fail in the later stages of disease (Table 1). However, asymptomatic subjects showed a similar prevalence of positive reactions and degree of reactivity as controls. The size of the skin reaction to PPD was significantly correlated with the CD4 count ($r=0·25$, $P<0·05$), percentage CD4 cells ($r=0·33$, $P<0·01$) and the lymphocyte proliferative response to PHA and PPD ($r=0·33$ and $r=0·39$ respectively, $P<0·01$ in both cases).

Lymphocytes and lymphocyte subsets. Table 2 shows that all parameters measured except the percentage and number of CD8 cells tended to diminish with the severity of disease. In addition, asymptomatic patients had significantly lower values than controls. In contrast, the percentage CD8 cells rose significantly, but their numbers stayed relatively constant as the disease progressed. Two (7%) patients in stage 1, two (13%) in stage 2 and 12 (55%) in stage 3 of the infection had a CD4 count of $<200\times10^9/l$ (see Fig. 1).

Table 1. Skin reactivity to purified protein derivative (PPD) and *Candida albicans* extract according to the Gambian Clinical Staging System

		Percent positive		Mean reaction ± s.d. (mm)	
Category	*n*	PPD	Candida	PPD	Candida
Control	81	77	75	8·7±6·2	8·2±5·6
Stage 1	27	63	56	8·0±6·6	5·7±6·0
Stage 2	14	14	21	3·0±5·8	2·0±2·8
Stage 3	22	14	27	1·7±4·4	2·7±4·3
*P**		>0·10	0·05	>0·10	<0·10
P†		<0·001	<0·05	<0·001	<0·05

* Significance of difference between control and asymptomatic subjects (Stage 1).

† Significance of trend according to stage of infection. Control group excluded.

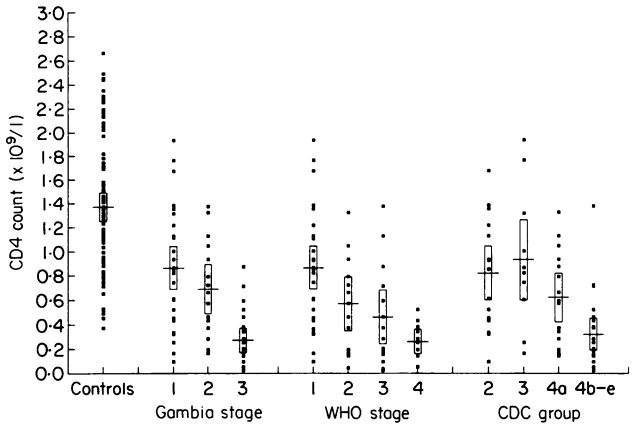

Fig. 1. CD4 counts according to different clinical staging systems. Bar signifies mean, box encompasses 95% confidence interval of mean.

Lymphoproliferative, IFN-γ and immunoglobulin responses to mitogens and antigens. Table 3 shows lymphoproliferative and cytokine responses to both mitogens and antigens were related to stage of disease, and that these trends were more marked in the case of the PHA-stimulated cultures. Control patients had significantly higher values than asymptomatic patients for all parameters tested except IFN-γ responses to PHA. PHA and PPD responses were significantly correlated with CD4 counts ($r=0·48$ and $r=0·39$ respectively, $P<0·01$ in both cases). Similar results were obtained when the above tests were performed in media containing 7·5% autologous plasma.

Resting levels of IgG and IgM secreted by lymphocytes were high, and IgG levels increased significantly according to the stage of disease. Only cells from control subjects produced a marked and significant increase in IgG and IgM when stimulated with PWM (data not shown).

Serological markers of cellular and viral activation infection. Table 4 shows that β_2-microglobulin and neopterin levels rose significantly according to stage of disease, and were inversely related to CD4 counts ($r=-0·43$ and $-0·42$ respectively, $P<0·01$ in both cases). However, the mean values of the asymptomatic and control groups were not significantly different.

All subjects were positive for antibodies to EBVCA. Mean titres of the various groups rose as disease progressed, but this trend was only just statistically significant (Table 4). On average,

Table 2. Lymphocyte subsets according to Gambian Clinical Staging System. Mean values ± s.d. are shown

Category	Lymphocytes ($\times10^9/l$)	CD4 cells ($\times10^9/l$)	Per cent CD4	CD8 cells ($\times10^9/l$)	Per cent CD8	CD4:CD8
Control ($n=82$)	3·31 (1·07)	1·37 (0·56)	41·1 (9·0)	1·15 (0·44)	35·3 (8·5)	1·27 (0·49)
Stage 1 ($n=28$)	2·90 (1·03)	0·86 (0·48)	28·5 (11·0)	1·26 (0·65)	43·0 (13·6)	0·79 (0·51)
Stage 2 ($n=15$)	2·59 (1·19)	0·69 (0·39)	26·8 (11·2)	1·26 (0·67)	48·5 (10·4)	0·58 (0·28)
Stage 3 ($n=22$)	2·34 (1·64)	0·27 (0·23)	12·7 (8·7)	1·24 (0·83)	54·5 (15·4)	0·28 (0·25)
*P**	<0·10	<0·001	<0·001	>0·10	<0·001	<0·001
P†	>0·10	<0·001	<0·001	>0·10	<0·001	<0·001

* Significance of difference between control and asymptomatic subjects (Stage 1).

† Significance of trend according to stage of infection. Control group excluded.

Table 3. Lymphoproliferative and IFN-γ responses to phytohaemagglutinin (PHA) and to purified protein derivative (PPD) according to Gambian Clinical Staging System. Means ± s.d. are shown

	PHA responses		PPD responses	
Category	Proliferative $\log_e$ Δ ct/min	IFN-γ % positive	Proliferative $\log_e$ Δ ct/min	IFN-γ % positive
Controls	10·0 ± 0·8 (n = 80)	87·7 (n = 65)	9·0 ± 1·3 (n = 68)	57·7 (n = 71)
Stage 1	9·4 ± 1·2 (n = 25)	87·5 (n = 24)	7·4 ± 2·1 (n = 17)	29·2 (n = 24)
Stage 2	8·8 ± 1·9 (n = 12)	60·0 (n = 15)	7·8 ± 1·3 (n = 10)	8·3 (n = 12)
Stage 3	7·6 ± 1·6 (n = 21)	23·5 (n = 17)	6·2 ± 2·1 (n = 16)	4·5 (n = 22)
P*	< 0·02	> 0·10	< 0·001	< 0·02
P†	< 0·001	< 0·001	< 0·10	< 0·02

* Significance of difference between control and asymptomatic subjects (Stage 1).
† Significance of trend according to stage of infection. Control group excluded.

Table 4. Serological measurements according to Gambian Clinical Staging System. Means ± s.d. are shown

Category	$\log_e$ β_2-microglobulin (nmol/l)	$\log_e$ neopterin (nmol/l)	$\log_e$ titre antibody to EBV viral capsid antigen
Control	0·71 ± 0·38 (n = 60)	1·60 ± 1·52 (n = 65)	3·80 ± 1·09 (n = 75)
Stage 1	0·89 ± 0·38 (n = 22)	2·19 ± 1·41 (n = 26)	4·47 ± 1·10 (n = 25)
Stage 2	1·12 ± 0·41 (n = 11)	2·80 ± 1·50 (n = 11)	4·67 ± 0·86 (n = 12)
Stage 3	1·81 ± 0·55 (n = 16)	4·11 ± 0·47 (n = 15)	5·15 ± 1·23 (n = 18)
P*	0·05	> 0·10	0·01
P†	< 0·001	< 0·001	0·05

* Significance of difference between control and asymptomatic subjects (Stage 1).
† Significance of trend according to stage of infection. Control group excluded.
EBV, Epstein–Barr virus.

asymptomatic subjects had significantly higher titres than control subjects.

Antibody responses to KLH infection. Table 5 shows that IgM antibody responses to KLH, a neoantigen, diminished markedly and significantly according to stage of disease, but this trend was not so obvious in the case of IgG antibody. Control subjects had significantly higher IgM and IgG responses than asymptomatic subjects.

Immunological variables according to the WHO Staging System

Mean CD4, CD8 counts, CD4/CD8 ratios, serum $\log_e$ β_2-microglobulin and $\log_e$ neopterin levels and mean size of PPD skin reactions were calculated for subjects at each stage of disease according to the WHO staging systems. Table 6 shows that all these measurements except the CD8 counts showed highly significant trends ($P < 0{\cdot}001$). Two (7%) patients in stage 1, four (31%) patients in stage 2, six (43%) patients in stage 3, and four (40%) patients in stage 4 of the infection had CD4 count $< 200 \times 10^9/l$ (see Fig. 1).

Immunological variables according to the CDC classification system

The above immunological variables were also grouped according to the CDC system. Once more all the measurements except the CD8 counts showed highly significant trends ($P < 0{\cdot}001$) according to the grouping of the patients. Figure 1 shows that one (6%) patient in group 2, one (9%) in group 3, three (20%) in

Table 5. Antibody responses to keyhole limpet haemocyanin (KLH) 4–6 weeks after immunization according to Gambian Clinical Staging System. Means ± s.d. are shown

Category	n	Titre IgG ($\log_{10}$)	IgM (optical density)
Control	53	2·00 ± 0·50	0·83 ± 0·44
Stage 1	17	1·69 ± 0·42	0·43 ± 0·27
Stage 2	10	1·64 ± 0·40	0·25 ± 0·18
Stage 3	13	1·42 ± 0·20	0·18 ± 0·21
P*		< 0·05	< 0·001
P†		< 0·10	0·01

* Significance of difference between control and symptomatic subjects (group 1).
† Significance of trend according to stage of infection. Control group excluded.

Table 6. Immunological variables according to WHO Clinical Staging System. Means ± s.d. are shown

Variable	Stage			
	(*n* = 22–28)*	(*n* = 8–13)	(*n* = 11–14)	(*n* = 7–10)
CD4 cells × $10^9/l$	0·86 (0·48)	0·57 (0·39)	0·46 (0·42)	0·26 (0·16)
CD8 cells × $10^9/l$	1·26 (0·65)	1·32 (0·86)	1·21 (0·73)	1·21 (0·74)
CD4/CD8 ratio	0·79 (0·51)	0·49 (0·29)	0·41 (0·36)	0·29 (0·17)
$\log_e$ β_2-microglobulin nmol/*l*	0·89 (0·38)	1·03 (0·36)	1·83 (0·58)	1·59 (0·50)
$\log_e$ neopterin nmol/*l*	2·19 (1·41)	2·59 (1·61)	4·05 (0·65)	3·86 (0·77)
Size PPD reaction (mm)	8·00 (6·65)	3·15 (6·05)	2·15 (5·34)	1·20 (2·70)

*Numbers for each test varied within this range.

group 4a and 11 (50%) in group 4(b–e) had a CD4 count below $200 \times 10^9/l$. The mean CD4 count of the asymptomatic patients (group 2) was similar to that of patients with generalized lymphadenopathy (group 3).

DISCUSSION

We wish to highlight a number of the findings of this study. Asymptomatic subjects with HIV-2 infection were found to be immunologically damaged, resulting in a depressed number of CD4 cells and low responses to immunization with KLH, a neoantigen. The poor antibody responses, which are also seen in the early stage of HIV-1 infection [21], may explain why asymptomatic subjects may be susceptible to bacterial septicaemia [8]. Thus, if prophylactic immunization is to be considered in this group, pneumococcal or other vaccines should be given as early as possible after the diagnosis of HIV infection. It is also of interest that delayed hypersensitivity reactions, as manifest by positive skin tests, were intact in most asymptomatic subjects. These patients may still be protected against *Myco. tuberculosis*, which is frequently reactivated in anergic subjects in the later stages of HIV infection [22].

Our simple staging system, which was based on the WHO Bangui definition of AIDS, correlated well with the degree of immunological damage, as did the CDC and WHO clinical staging systems. The latter is perhaps most surprising, as it is primarily based on a performance scale which in our modification, using the Karnofsky score, devolved around the ability of the patient to work and to care for himself. The practical importance of our findings is that these simple clinical systems may act as alternatives for sophisticated and expensive immunological measurements when trying to determine prognosis. This, of course, is not to deny the central role that damage to CD4 lymphocytes, triggered by HIV infection, plays in the decay of the immune system. Our data, like those of many others, showed that many immune tests such as lymphocyte proliferative responses, skin tests and β_2-microglobulin and neopterin levels, correlated strongly with CD4 counts [12]. This substantiates our previous finding that the CD4 count is a powerful predictor of survival in symptomatic Gambian patients [17] despite the fact that the measurement was made when other infections such as tuberculosis, which in theory could also influence the CD4 count, were still being treated. In practice we have found minor, and insignificant, differences between CD4 numbers in HIV^- Gambians with pulmonary tuberculosis and in control subjects [22].

The recent proposal from CDC that a CD4 count of less than $200 \times 10^9/l$ in an HIV^+ patient should be used to define AIDS might not be entirely appropriate for The Gambia [23]. We found that 20% of our symptomatic patients who did not meet the WHO or CDC definition of AIDS had counts below this level, as did 7% of asymptomatic patients. One difficulty in establishing global standards for laboratory parameters of AIDS, such as the CD4 count, is that normal values are very different in different groups of people. Thus our laboratory has found that prostitutes, uninfected with HIV, have on average 30% more CD4 cells than uninfected subjects attending an STD clinic [12], and twice as many CD4 cells as villagers of a different ethnic group living in a rural area.

There is a growing body of evidence which suggests HIV-2 is less virulent than HIV-1 [17, 24]. However, at the moment there is no consistent and convincing evidence that there are marked differences in the degree of immune damage caused by the two infections either in the asymptomatic [12,13] or symptomatic stage of disease [13,17]. Ultimately a larger immunological study, ideally involving incident cases of HIV-1 and HIV-2, will be necessary if the real differences in the effects of the two infections on the immune system are to be discerned.

We conclude that HIV-2 causes a similar spectrum of immunological changes as HIV-1, and that these are closely related to the clinical stage of disease.

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