JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2011. Vol. **5**(2), p. 775-780

Cytokine Profile of Patients among Aspergillosis and Human Immunodeficiency Virus Co-infection from Central India

Desh D. Singh[1] and Vinod Singh[2]*

[1]Department of Microbiology, Chhatrapati Shahuji Maharaj Medical University, Chowk, Lucknow - 226 003, India.
[2]Department of Microbiology, Barkatullah University, Bhopal 462 026, India.

(Received: 16 March 2011; accepted: 21 April 2011)

Aspergillosis (AS) infection arises in more than 50% of human immunodeficiency virus (HIV) transmits a disease to Indian patients. This study was carried out to analyze the immunophenotypic and intracellular cytokine profile of patients with HIV- AS co-infection s. Fifteen patients with HIV- AS co-infection and 15 each with AS alone and healthy individuals were studied. Immunophenotypic analysis and intracellular cytokines were measured using appropriate antibodies on a flowcytometer. Percentage of CD3+ did not differ significantly in the three groups. The ratio of CD4+: CD8+ was reversed among patients with CA and HIV-AS CD19+ and CD25+ were present on fewer cells of healthy individuals but this was not statistically significant. Significantly higher percentage of cells of patients with AS and HIV-AS were CD69 positive. Interferongamma (INF-γ) and tumour necrosis factor-alpha (TNF-α) levels are significantly reduced in the CD4+ cells of patients with HIV- AS when compared with those with AS and healthy individuals. In CD8+ cells of patients with HIV- AS, levels of TNF-α are higher when compared with the other two groups. Interleukin-2 (IL-2) producing cells were not significantly different in any of the above subsets. Monocytes in individuals with HIV- AS had significantly higher interleukin-6 (IL-6) and TNF-α. T-helper cells among patients with HIV- AS have significantly lower cytokine production. T-suppressor cells and monocytes produce more TNF-α. These findings may be significant in view of recent attempts to treat HIV- AS coinfected Patients with anti-TNF therapy.

Key words: Aspergillosis, HIV, Central India population.

Worldwide, Aspergillosis (AS) is the most frequent coinfection in individuals with human immunodeficiency virus (HIV) type 1 infection[1]. It is the most common risk factor for the development of active AS both reactivation of a latent *Aspergillus fumigates (AF)* infection and progressive primary AS are substantially more common in HIV-1 infected patients[2-8]. The resurgence of AS has been attributed, in part, to the HIV-1 epidemic in both developed countries and developing countries[9-11]. Sixty percent to seventy percent of AS cases occurred in HIV-1 infected individuals in Zambia and in India 1.2% of newly diagnosed AS patients are HIV sero-positive[12-15]. There is a mutual interaction between HIV-1 and *AF* infection[16]. On the one hand, HIV-1 infection predisposes to the development of active AS and on the other hand, the course of HIV-related

* To whom all correspondence should be addressed.
Mob.: +91-09425712910; Res.: 0755- 6458209
E-mail: vinodsingh63@yahoo.co.in

immunodeficiency is worsened by active *AF* infection [17].

The relationship of host immune responses to the development of AS at different times during progression of HIV-1 disease is not clear[18-21]. Cytokines play an important role in the process of host defense against *AF* [22-24]. The production of interferon-gamma (INF-γ) appears to be crucial to the control of *AF* infection[25]. Impaired production of IFN-g correlates with progression of immunodeficiency and is probably related to abnormalities in the interleukin- 12-INF-γ axis [26]. Tumour necrosis factor-alpha (TNF-γ) plays an essential role in preventing reactivation of persistent AS, modulates the pulmonary expression of specific immunologic factors and limits the pathological response of the host[27]. Because cytokines function in a micro-environmental, cell specific fashion; cell-specific cytokine profiles may provide important information about both the immune network at various stages of clinical HIV infection and also the immune response of HIV-infected persons to associated co-infections[28-32] .We examined the intracellular cytokine levels of T-helper type 1, Thelper type 2, pro-inflammatory and anti-inflammatory cytokines among patients with HIV-AS co-infection, AS alone and healthy controls. We carried out this study to determine the immunophenotypic and intracellular cytokine profile of our patients with HIV-AS co-infection.

MATERIAL AND METHODS

Patients were sequentially selected from OPD of King Georges Medical College and associated Hospitals. Fifteen patients with both HIV and active AS, 15 patients with AS alone and 15 healthy controls were selected. The HIV infection was diagnosed on the basis of positivity on a panel of three enzyme linked immunosorbent assays (Genedia, Korea; Lab Systems, Finland; and Xcyton Diagnostics Ltd, India). Clinical history was taken and examination performed on all patients.

To determine the immunophenotypic characteristics of the T-helper cell population, 10 mL of the Corresponding antibody conjugated with FITC (Sigma, USA) was added to 100 mL of whole blood and incubated at room temperature for 15 minutes. At the end of the incubation, lysing solution was added and incubated for 10 minutes at room temperature. The washed and fixed cells were then analysed on the flowcytometer (FACScan, Becton Dickinson, Mountain View, CA). Ten thousand cells were computed and analysed using Cell Quest Program (Becton Dickinson, Mountain View, CA). Dead cells were excluded by forward and side scatter gating. Statistical markers were set using irrelevant isotypematched controls as reference. Peripheral blood mononuclear cells (PBMCs) were separated from the whole blood by Ficoll-Hypaque gradient method. Cells were then cultured in RPMI- 1640 (1×106 cells/mL) in the presence of PHA (2mg/mL) for 48 hours in a CO_2 incubator at 37°C. Monensin was added to the culture six hours prior to harvest and cells were then separated from the culture by centrifugation. For intracellular cytokine detection, cells were fixed by using fixative at 4 °C for 30 minutes and then permeabilised using permeabilisation buffer for 30 minutes at 4°C. Permeabilised cells were then labeled with fluorochrome conjugated monoclonal antibodies against the cytokines to be detected. Labeled cells were then washed and analysed on the flowcytometer.

Statistical Analysis

All results are expressed as mean ±SD. Comparisons of means between the two groups was carried out using unpaired t-test. The three groups were compared using analysis of variance (ANOVA). A value less than 0.05 were regarded as significant.

RESULTS

Fifteen patients with HIV-AS co-infection were studied. Aspergillosis was newly diagnosed in all of them. They were all males (mean age 32.7±4.4, range 25-38 years). Five had pulmonary AS and remaining 10 had extra pulmonary AS (AS lymphadenopathy). Patients with AS alone (n=15) (mean age 31.6±6.4, range 22-40 years). All except one of them were males and had pulmonary AS. All healthy individuals were males (mean age 29.2±3.2, range 21-36 years).

The CD3+ percentage (Table 1) was not significantly different in the three groups. The CD4+ percentages were significantly higher in

healthy individuals as compared to the other two groups ($p<0.05$). The CD4+ and CD8+ ratio was reversed among patients with AS and HIV-AS. The CD19 and CD25+ positive cells were present in lesser percentage of cells of healthy individuals but this was not statistically significant. Significantly higher percentage of cells among patients with AS and HIV-AS were CD69 positive ($p<0.05$).

Percentage (mean±SD) of cells with intracellular cytokine levels in CD4+ T-cells are shown in the table 2. The IL-2 levels were significantly higher among patients with AS, with or without HIV infection, when compared with

Table 1. Immunophenotypic characteristics, shown as percentage, in patients with AS, HIV-AS and healthy control subjects

	CD3+	CD4+	CD8+	CD19+	CD25+	CD69+
AS (n=15)	65.3±5.3	28.0±12.9	33.5±11.8	12.4±6.0	5.1±1.0	15.8±1.6
HIV-AS (n=15)	60.3±9.8	25.0±12.9	34.6±10.2	10.2±5.0	4.0±1.0	10.1±1.1
Healthy control subjects	75.3±11.6	36.0±5.5	28.2±8.3	8.7±2.0	2.5±1.1	4.0±1.1

CD4+ percentages were significantly higher in healthy individuals as compared to the other two groups ($p<0.05$). Patients with AS, with and without HIV had significantly higher CD69+ numbers as compared with healthy control subjects ($p<0.05$)
AS=Aspergillosis; HIV-AS=Co-infection with human immunodeficiency virus and Aspergillosis

Table 2. Intracellular cytokine measurement, shown as percentage, in T-helper cells in patients with AS, HIV-AS and healthy control subjects

	CD4+IL-2+*	CD4+IFN-γ +†	CD4+TNF-α+‡
HIV-AS (n=15)	6.1±0.7	2.9±0.9	1.0±0.4
AS (n=15)	8.5±3.9	18.4±5.2	7.2±1.7
Healthy controlsubjects (n=15)	3.6±4.3	8.1±2.9	2.6±0.8

*=IL-2 levels were significantly higher among patients with AS, with or without HIV infection ($p<0.05$) when compared with healthy individuals
†=IFN-γ levels were considerably lower among patients with HIV-AS ($p<0.05$) when compared with the other two groups. Patients with AS had significantly higher levels of IFN-γ when compared with healthy individuals ($p<0.05$)
‡=TNF-α levels were significantly higher among patients with AS alone when compared with the other two groups ($p<0.05$)
IL=Interleukin; IFN-γ=Interferon-gamma; TNF-α=Tumour necrosis factor-alpha; HIV-AS=Co-infection with human immunodeficiency virus and Aspergillosis; AS=Aspergillosis

Table 3. Intracellular cytokine measurement, shown as percentage, in T-suppressor cells in patients with AS, HIVAS and healthy control subjects

	CD8+IL2+	CD8+IFN-γ+*	CD8+TNF-α+**
HIV-AS (n=15)	5.5±0.6	12.0±1.8	46.2±3.2
AS (n=15)	6.6±1.0	8.4±1.0	<1
Healthy controlsubjects (n=15)	4.4±1.0	23.0±3.7	<1

There was no significant difference between the three groups of patients as far as IL-2 levels are concerned.
*=Healthy control subjects had significantly higher levels of IFN-γ when compared with the other two groups ($p<0.05$) **=HIV-AS patients had significantly higher levels of TNF-α when compared with the other two groups ($p<0.01$) IL=Interleukin; IFN-γ =Interferon-gamma; TNF-α=Tumour necrosis factor-alpha; AS=Aspergillosis; HIV-AS=Co infection with human immunodeficiency virus infection and Aspergillosis

healthy individuals (P<0.05). The IFN-a and TNF-α levels were significantly reduced in CD4+ cells of patients with HIV-AS when compared to those with AS alone and healthy individuals (p<0.05).

When CD8+ T-cells were analysed (Table 3), patients with HIV-AS had IFN-γ levels lower than the other two groups of individuals (p<0.05). The IFN-γ was significantly less among patients with AS alone when compared with those who were healthy or dually infected (p<0.05). On the other hand, TNF-γ levels were significantly lower in healthy and AS infected individuals when compared with those with dual infection. The IL-2 levels did not differ significantly between the three groups.

Among CD14+ cells, IL-6 and TNF-α levels were significantly higher among HIV-AS co-nfected individuals when compared with those with AS alone and healthy individuals (p<0.05) [Table 4].

Table 4. IL-6 and TNF-α level, shown as percentage, in CD14 positive cells in patients with AS, HIV-AS and healthy control subjects

	CD14+IL-6+	CD14+TNF-α
HIV-AS (n=15)	25.0±2.4	46.3±3.3
AS (n=15)	15.7±2.4	8.6±1.0
Healthy control subjects (n=15)	10.3±3.9	5.8±1.0

Patients with HIV-AS had significantly higher levels of IL-6 and TNF-α when compared with the other two groups (p<0.01) IL=Interleukin; TNF-α=Tumour necrosis factor-alpha; AS=Aspergillosis; HIV-AS=Co-infection with human immunodeficiency virus infection and Aspergillosis.

DISCUSSION

In this study, immunophenotypic characteristics and intracellular cytokine levels of T-ymphocytes and monocytes associated cells in Indian patients with AS alone or co-infected with HIV infection were studied. The CD4+ percentage was lower in patients with AS when compared to healthy individuals. Thisis in keeping with the median CD4+ counts of Indian patients with pulmonary AS of 242/mL and that of extrapulmonary AS of 175/mL.10 Numbers of cytotoxic T-lymphocytes and B cells were greater among patients with AS when compared with healthy individuals, but this did not reach statistical significance. As expected, CD4+ percentage in patients with HIV-AS co-infection was lower than that in normal individuals. Cells with CD69+ (an activation marker) were present in a higher proportion of PBMC's in patients with AS, when compared with controls. There was no significant difference between those with and without HIV infection. The CD25+ levels were not significantly different in the three groups. One previous study has demonstrated impairment of IL-2 signalling by demonstrating the median concentration of soluble IL-2 receptor-a being much lower in the HIV-AS coinfected compared to those with AS alone soluble TNF-receptor.

All cytokines tested were present in a greater percentage of helper T-cells in patients with AS alone, when compared to those with dual infection. Advanced HIV infection is associated with impaired T-helper cell function. This is the likely reason for lower IL-2 and IFN-γ levels in patients with HIV-AS co-infection, when compared to patients with AS alone. There are data to suggest that the immunosuppression of AS is not only immediate but is also long lasting, presumably relating to a primary abnormality in T-cell function. This is partly due to immunosuppressive cytokines, early in the course of *AF* infection.14 Patients with advanced immunosuppression are unable to mount a significant inflammatory response that can reflect in high levels of TNF-α levels. The source of TNF-α is probably better reflected in monocytes, where the levels are much higher.

There was no significant difference in IL-2 levels in the cytotoxic T-cells (CTLs) in the three subgroups of individuals. The response with IFN-γ in CTLs was lower in AS patients with and without HIV, when compared to healthy individuals. There is data to show that circulating HIV-1 specific CTLs release IFN-γ in an epitope-specific and human leukocyte antigen (HLA) class I-restricted fashion, paralleling HIV-1 specific cytotoxic response of these cells. The picture is less clear when we determine what happens to co-infected individuals as most studies have not determined these levels in specific subsets of cells. The TNF-α levels are the only one that is elevated in CTLs of these patients. These would be in keeping with wasting and other constitutional symptoms associated with advanced HIV infection.

In the subset of cells represented by monocytes, IL- 6 and TNF-α level were elevated. This reflects the role of these cells in the inflammatory process associated with advanced HIV infection. In both the instances the levels of patients with AS alone were intermediate between those with dual infection and healthy individuals.

One problem with studies like these is that while there are comparisons available with plasma levels of cytokines in other settings, there is little to compare by way of intracellular cytokines in specific subset of cells in patients with HIV-AS co-infection. While plasma levels would reflect the composite effect of all the factors regulating cytokine levels in the system, deciphering the role of subset of cells becomes that much more difficult. Further, follow-up studies are required to determine the changes in cytokine levels over time to see how anti-Aspergillus and antiretroviral therapy changes the intracellular levels of the cytokines.

REFERENCES

1. Denning, D. W. "Invasive aspergillosis".*Clin Infect Dis* 1998; **26**: 781-805.
2. Mishra, B. B., Singh, Desh. D., Kishore, N., Tiwari,V. K., Tripathi, V. "Novel antifungal constituents isolated from the seeds of *Aegle marmelos* Corr. collected in India". *Phytochemistry.* 2010; **71**: 230-234.
3. Singh, Desh. D., Singh, Rambir, Tiwari, V. K. and. Mishra, B.B "Antimicrobial Activity Studies on 1-cyclopropyl-2-ethyl-5floro-6-(4-methylpiperazine-1-yl)-1H-bezimidazole" *J.chemtracks* 2007; **9**: 97-100.
4. Yadav, V., Gupta, J., Mandhan, R., Dabur, R., Chhilar, A.K., Singh, Desh. D. and Sharma G L. "Investigation on anti-Aspergillus properties of bacterial products. *Letters in Applied Microbiology*". 2005; **41**: 309-314.
5. Walsh, T. J. and Dixon, D. M. Pulmonary aspergillosis and the clinical spectrum: *Eur J Epidemo*. 1989; **5**(2): 131-142 .
6. Greenberger, P.A. In:Adkins, N.F., Yunginger, J.W., Busse, W,W., Bochner, B.S., Holgate, S.T., Simons, F. E. R., (eds). "Allergic bronchopulmonary aspergillosis. Middletons Allergy, Principles and Practice". 2nd edn. Philadelphia: *C V Mosby,*: 2003; 1353-/1371.
7. Groll, A. H., Shah P. M., Mentzel, C., Schneider, MJust-Nuebling, G., and Huebner, K., "Trends in the postmortem epidemiology of invasive fungal infections at a University Hospital". *J. Infect*. 1996; **33**: 23-32.
8. Indian Pharmacopeiea "Microbiological assays and tests". Appendix 9. 1996; P A-100-A-A108.
9. Kurup, V.,P., Madan, T., Sarma, U. P., "Allergic bronchopulmonary aspergillosis: recent concepts and considerations" . In: Domer JE, Kobayashi GS (eds). The Mycota XII Human Fungal Pathogens. Berlin-Heidelberg: Springer-Verlag : 2004; 225-/241
10. Randhawa, H.S. & Khan, Z.U. "Acute pulmonary mycoses in India: Current status and laboratory diagnosis aspects". *Ind J Tub* 1987; 34: 3-11.
11. Sharma GL and Sarma PU "Luminescent immunoassay for detection of specific antibodies in allergic bronchopulmonary aspergillosis. Serodiagn". *Immunotherp. Infect. Disease* 1993; **5**: 186.
12. Vogeser, M., Haas, A., Aust, D. and Ruckdeschel G. "Postmortem analysis of invasive aspergillosis in a tertiary care hospital". *Eur J Clin Microbiol Infect Dis* 1997; **16**: 1.
13. Kurup, V.P., Fungal allergens. *Curr allergy Asthma* Rep 2003; **3**: 416-/423.
14. Meyer, J.D. "Fungal infection in bone marrow transplants patients" *Semin. Oncol*. 17: 10 (1990).
15. Cazzadori, A., Di Perri, G., Todeschini, G. "Transbronchial biopsy in the diagnosis of pulmonary inûltrates in immunocompromised patients" *Chest*. 1995; **107**: 101-106.
16. Girish Kumar, C.P., Menon, T., Rajasekaran, S., Sekar, B., Prabu, D., "Carriage of Candida species in oral cavities of HIV infected patients in South India". Mycoses. 2008 Jun 3.
17. Menon, T., Herrera, M., Periasamy, S., Palanivelu, V., Sikhamani, R., Wickes, B. "Oral candidiasis caused by Kodamaea ohmeri in a HIV patient in Chennai, India". Mycoses. Jun 15 (2009). [Epub ahead of print] Sundaram, S., Kalyanasundaram, R and Rangaswamy, V., "Serological diagnosis of pulmonary aspergillosis". *Mycopathologia*, 1981; **75**: 93-99.
18. Rhodes, J.C., "Aspergillosis". In: Murphy JW, Friedman H, Bendinelli M (eds) fungal infections and immune responses, London: Plenum Press, 1993: 360-404.
19. Rollides E, Holmes A, Blake C, Pizzo PA, Wash TJ. Impairment of neutrophil antifungal activity against hyphae of *Aspergillus fumigatus* in children infected with Human Immunodeficiency Virus infection. *J Infect Dis*

1993; **167**: 905-11.

20. Hochhaus G, Brookman L, Fox H, et al. Pharmacodynamics of omalizumab: implications for optimised dosing strategies and clinical efficacy in the treatment of allergic asthma. *Curr Med Res Opin* 2003; **19**: 491-8.
21. Casaulta C, Fluckiger S, Crameri R, Blaser K, Schoeni MH, Time course of antibody response to recombinant Aspergillus fumigatus antigens in cystic fibrosis with and without ABPA. *Pediatr Allergy Immunol*. 2005; **16:** 217-25.
22. Grazziutti, M. L., Rex, J. H., Cowart, R. E., Anaissie, E. J., Ford, A. and Savary, C. A., "*Aspergillus fumigatus* conidia induce a Th1-type cytokine response". *J. Infect. Dis.* 1997; **176**: 1579-1583.
23. Kurup, V. P., Banerjee, B., Hemmann, S., Greenberger, P. A., Blaser, K and Crameri R "Selected recombinant *Aspergillus fumigatus* allergens bind specifically to IgE in ABPA". *Clin. Exp. Allergy* 2000; **30**: 988-993.
24. Aberg, J.A., Watson, J., Segal, M., Chang, L.W "Clinical utility of monitoring serum cryptococcal antigen titers in patients with AIDS-related cryptococcal disease". *HIV Clin Trials*. 2000; **1**: 1-6.
25. Pappas, P.A., Bustamante, B., Ticona, E., Hamill, R. J., Johnson, P.C., Reboli, A. "Recombinant Interferon-ã1b as adjunctive therapy for AIDS-related acute cryptococcal meningitis". *J Infect Dis.*2004; **189**: 2185-91.
26. Kedzierska, K., Ellery, P., Mak, J., Lewin, S. R., Crowe, S. M. &Jaworowski, A. "HIV-1 down-modulates c signaling chain of FccR in human macrophages: a possible mechanism for inhibition of phagocytosis". *J Immunol* 2002; **168**: 2895-2903.
27. George, J., Murakawa, Jeff, D., Harvell, Paul, Lubitz., Sanford, Schnoll., Sue, Lee., and Timothy, Berger. "Cutaneous Aspergillosis and Acquired Immunodeficiency Syndrome". *Arch Dermatol.*2000; **136**(3): 365-369.
28. Kedzierski, L., Montgomery, J., Bullen, D., Curtis, J., Gardiner, E., Jimenez-Ruiz, A. & Handman, E. "A leucine-rich repeat motif of Leishmania parasite surface antigen 2 binds to macrophages through the complement receptor 3". *J Immunol* 2004; **172**: 4902-4906.
29. Castro, N.M., Rodrigues, W. Jr., Freitas, D.M., Muniz, A., Oliveira, P., Carvalho, E.M., "Urinary symptoms associated with human T-cell lymphotropic virus type I infection: evidence of urinary manifestations in large group of HTLV-I carriers" *Urology*; 2007; **69**: 813-818.
30. Carvalho, E.M., Bacellar, O., Porto, A.F., Braga, S., Galvao-Castro, B., Neva, F. "Cytokine profile and immunomodulation in asymptomatic human T-lymphotropic virus type 1-infected blood donors" *J Acquir Immune Defic Syndr* 2001; **27**: 1-6.
31. Ponte, E.V., Lima, F., Araujo, M.I., Oliveira, R.R., Cardoso, L.S., Cruz, A. A. "Skin test reactivity and Der p-induced interleukin 10 production in patients with asthma or rhinitis infected with Ascaris". *Ann Allergy Asthma Immunol* 2006; **96**: 713-718.
32. Geramizadeh, B., Kheirandish, P., Fatheezadeh, P., Jannesar, R. "Fine needle aspiration of subcutaneous masses caused by Aspergillus: A report of 2 cases". *Acta Cytol*. 2005; **49**: 666-8.