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Study of some lymphocyte subset counts and cytokine levels in cryptococcosis associated with AIDS

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Summary

Approximately 100 new cases per year of cryptococcosis in HIV+ patients are observed in Muñiz Hospital, 35% of them suffer a fatal outcome within the first four weeks after diagnosis in spite of treatment. Apparently there is not a useful parameter that allows a clear prediction of this early fatal outcome of the disease. The aim of this study is to determine some cytokine levels and several lymphocyte subpopulations counts in order to correlate these results with the evolution of the disease.
Forty HIV+ patients suffering culture confirmed cryptococcosis were enrolled in this study, 8 HIV+ patients without cryptococcosis and 8 healthy individuals with negative serology for HIV were included as controls. The following determinations were done in all cases: CD3+, CD4+, CD8+, CD16+CD3-, CD19+ cell counts, IL-1; IL-12, TNFα in serum and TNFα in CSF. Ten cases with cryptococcosis and AIDS were controlled three months after treatment. The average of CD4+ and NK cell counts in patients before treatment were 22/µl and 90/µl respectively; IL-1 levels were higher in the patients than in the healthy control group, conversely IL-12 levels did not show significant differences in the three studied groups. Serum concentrations of TNFα were higher in patients than in the control group and were not modified after treatment, conversely antifungal medication diminished IL-1 concentration and remarkably increased NK cell counts. At the same time antigen levels in serum and CSF decreased.
The results obtained seem to show that the immunological alterations observed in these patients are those characteristically exhibited in severe HIV disease and that some parameters such as CD8+ cell counts lower than 200/µl, less than 50 CD4+/µl, more than 50 pg/ml of TNF-α and serum capsular antigen titer higher than 1:5000 seem to predict a rapidly fatal course of infection.

Key words Cryptococcosis, AIDS, Immunity, Cytokines

Estudio de subpoblaciones linfocitarias y nivel de algunas citoquinas en la criptococosis asociada al sida

Resumen

En el Hospital Muñiz se registran alrededor de 100 casos nuevos anuales de criptococosis asociada al sida y un 35% fallece antes de las 4 semanas aun a pesar de efectuar el diagnóstico e instituir tratamiento rápidamente. Hasta ahora no hay índices claros que permitan predecir qué enfermo tendrá una evolución fatal. Por este motivo decidimos investigar el estado de algunos mecanismos inmunes que el organismo pone en juego frente a *C. neofomans* y que variaciones se producían luego de tres meses de tratamiento.
Se estudiaron 40 pacientes VIH+ con criptococosis sistémica confirmada, independientemente de su gravedad y se utilizaron como grupos control ocho enfermos VIH+ sin criptococosis y ocho individuos sanos. Se determinaron los niveles de subpoblaciones linfocitarias CD3+, CD4+, CD8+, CD16+CD3- y CD19+, interleuquina-1 (IL-1), interleuquina-12 (IL-12), TNFα séricos y TNFα en LCR de los pacientes con criptococosis meníngea. En 10 pacientes que pudieron controlarse después de tres meses de tratamiento, se les repitieron los dosajes.
Se encontró que los recuentos de células CD4+ de los pacientes tenía valor promedio de 22 células/µl; y las células NK ~90/ µl. Los niveles de IL-1 eran muy inferiores a los de los individuos sanos en tanto que los niveles de IL-12 no mostraron diferencia entre los tres grupos. El valor de TNFα sérico fue mayor que en la población sana pero no se modificó después del tratamiento. Por el contrario disminuyó la concentración de IL-1 y aumentó considerablemente el número de células NK y disminuyeron los títulos de antigenemia y antigenorraquia. Los resultados obtenidos indicarían que las alteraciones inmunológicas que presentan estos pacientes, corresponden a las características que se evidencian en las formas muy avanzadas del sida. Por otra parte ciertos parámetros inmunológicos tales como los recuentos de linfocitos CD4+ y CD8+ inferiores a 50/µl y

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200/µl respectivamente, las concentraciones de TNFα mayores de 50 pg/ml y los títulos de antígeno capsular en suero de 1:5000 o más, se relacionaron con una rápida evolución fatal.

Palabras clave Criptococosis, Sida, Inmunidad, Citoquinas

Cryptococcosis is the most frequent systemic mycosis associated with AIDS. Its incidence varies from 4% of AIDS patients in the United Kingdom to near 33% in Central Africa [1]. In Argentina, cryptococcosis incidence in HIV positive patients was calculated at about 10% [2].

Approximately 100 new cases per year of cryptococcosis in AIDS patients are observed in the Muñiz Hospital in Buenos Aires. This systemic mycosis is one of the most frequent causes of death; 35% of cases die during the first four weeks after diagnosis in spite of the specific treatment [3]. This high mortality rate is also detected in other parts of the world [4].

Altered mental status, high opening pressure of CSF, lymphocyte counts lower than 20 cells/µl, CSF capsular antigen levels over 1/32 and the isolation of *Cryptococcus neoformans* from different clinical samples have been identified as important indicators of severe disease[5]. However, all these signs are commonly observed in our patients and we did not detect a clear correlation between them and the fatal outcome during the first month of evolution. Cytokines and different lymphocyte subsets are involved in the specific and nonspecific responses to this mycosis [6]. The aim of this study is to determine a possible correlation between some cytokines level, several lymphocyte subset counts and capsular antigen titers in serum, CSF and urine with the patients'evolution.

PATIENTS, MATERIALS AND METHODS

Patients. A total of 56 individuals divided in three groups were studied

- *Group I*: 40 AIDS patients with culture proven cryptococcosis; mean age 30 years (range: 21-43 years); 34 males and six females.
- *Group II*: eight HIV positive patients without cryptococcosis; mean age 27 years (range: 24-37 years), seven males and one female.

Patients belonging to the groups I and II presented an HIV infection serologically detected by ELISA and confirmed by Western blot

- *Group III*: eight healthy volunteers; mean age 32 years (range: 21 to 45 years), six males and two females.

C. neoformans was isolated from the following clinical specimens: blood cultures: 25; CSF:31; urine: seven; sputum: two; bone marrow aspiration: two and hepatic biopsy: one. In the majority of patients the fungus was cultured from more than one clinical specimen.

Immunological tests

1. Blood and serum samples: 12-15 ml of blood were obtained by venous puncture and then 2.5 ml were added to plastic tubes containing EDTA as anticoagulant and gently mixed by inversion. The remaining part of each sample was poored into plastic tubes without anticoagulant and allowed to clot, serum was separated by centrifugation at 2000 rpm and then stored at -20°C until the moment of use.

2. Lymphocyte subset counts. CD3+; CD4+; CD8+; CD16+CD3- and CD19+ subset counts were performed with a flow cytometer Cytoron Absolute (Ortho Diagnostic Systems, USA). Control: Nonbinding control monoclonal antibody for FITC/PE/C y P - conjugated monoclonal antibody reagents; nonreactive with human leukocyte cell surface antigens IgG1; IgG2a. Monoclonal antibodies: 10 ml of the following reagents were used in each determination: CD4 IgG2a OKT4A clone; CD8 IgG2a OKT8F clone; CD3 IgG2a OKT3 clone; CD16 IgG1, 3G8 clone; CD19 IgG1 OKB19A clone (Ortho Diagnostic Systems). The entire blood of each patient was gently mixed by inversion and 100 µl of each sample containing 1 x 10^4 to 1.5 x 10^6 lymphocytes were used. Absolute count were determined directly when using the Ortho Immunocount flow Cytometry System. For properly calibrate the system, Ortho-Count Calibration Kit (Product Code 310300) was employed.

3. Cytokine concentrations. IL-1, IL-12, TNFα in serum and TNFα in CSF were determined by ELISA technique with Quantikine kits (R&D systems, USA). The reaction was carried out according to the instructions from the manufacturer. All the tests were run in duplicate.

4. Capsular polysaccharide antigen of C. neoformans. Levels in serum, CSF and urine were determined by latex agglutination technique with the Crypto-latex antigen detection system kit (Immunomycologics, USA). The determinations were carried out following the manufacturer's recommendations.

All the tests were carried out at the time of diagnosis and three months later when possible.

Statistical analysis. Comparisons among the three groups were made by analysis of variance and student's t test was used to compare the results obtained in Group I before and after treatment or between the patients who died in the first four weeks and those who survived more than three months [7].

RESULTS

In table 1 the concentration of polysaccharide antigen in serum, CSF and urine in the 40 patients with cryptococcosis are presented. The average and the standard deviation of each determination were considered. Only 25% of the patients were controlled three months after starting specific treatment, this low proportion of cases was due to the short survival time of many cases and the high proportion of patients who abandoned clinical control.

Table 1. Capsular polysaccharide antigen levels in serum, CSF and urine in the 40 patients with cryptococcosis (mean values).

Group I	N	Antigen titer average (standard deviation)		
		Serum	CSF	Urine
Total	40	4294 (3451)	1069 (1840)	361 (768)
<4 weeks survival	8	6586 (2153)	5025 (1023)**	28 (16)*
>3 months survival	10	3255 (1374)	289 (124)	1.7 (1.2)

Significant between the groups with different survival time: *p<0.05, **p<0.01.

Table 2. Lymphocyte subset counts and cytokine concentrations in the three groups analyzed. Average (standard deviation).

Parameter	Group I	Group II	Group III
Lymph. subset	**cell/µl**	**cell/µl**	**cell/µl**
$CD3^+$	421 (234)**	756 (479)	1775 (724)
$CD4^+$	22 (19.7)	132 (176)	1060 (413)
$CD8^+$	354 (284)	536 (315)	627 (292)
$CD16^+CD3^+$	98 (81.2)	112 (96)	238 (74)
$CD19^+$	79 (47.6)	108 (67)	119 (48)
Lymph. total	614 (343)	840 (463)	2155 (936)
Cytokines	**pg/ml**	**pg/ml**	**pg/ml**
IL-1	19.8 (12.3)	54.2 (25.7)	4.3 (0.4)
IL-12	9.5 (1.6)	10.9 (1.7)	9.8 (1.3)
TNF-α	81.9 (47.2)	38.7 (39.6)	15.2 (0.9)
TNF in CSF	115.1 (157.9)		

Significant different with control group: *p<0.05, **p<0.01.

Table 3. Comparative results of lymphocyte subset counts and cytokines concentration between patiens who died before four weeks and those who survived more than three months average (standard deviation).

	Survival time	
	< 4 weeks >	**3 months**
No of patients	**8**	**10**
Lymphocite subset (cell/µl)		
$CD3^+$	253 (196)	596 (378)
$CD4^+$	16 (31)	132 (19)
$CD8^+$	194 (112)	511 (374)
$CD16^+CD3^+$	117 (74))	47 (53)
$CD19^+$	58 (42)	113 (64)
Lymphocyte total count	429 (159)	746 (417)
Cytokines (pg/ml)		
IL-1	26.7 (21.2)	42.3 (24.7)
IL-12	6.4 (1.1)	7.6 (1.8)
TNF-α	136.6 (184.2)	58.1 (63.3)

Table 4. Modifications of the analyzed parameters after three months of treatment (10 patients) average (standard deviation).

Parameter		Pre-treatment	Post-treatment
Lymphocyte subset (cell/µl)	$CD3^+$	596 (312)	560 (356)
	$CD4^+$	28 (31)	43 (29)
	$CD8^+$	511 (228)	424 (223)
	$CD16^+CD3^+$	47 (29)*	138 (49)
	$CD19^+$	113 (74)	60 (68)
	Lymphocyte count	746(358)	793(417)
Cytokines (pg/ml)	IL-1	42.3 (39.8)	5.7 (7.4)
	IL-12	7.2 (1.2)*	12.3 (3.4)
	TNF-α	58.1 (37.9)	58.9 (42.6)
C. neoformans capsular antigen (titer)	Serum	3255 (1478)	1424 (1079)
	CSF	289 (136)	141 (108)
	Urine	1.7 (12.8)	1.5 (9.6)

*Significant difference p<0.05.

In table 2 lymphocyte subset counts and cytokine concentrations in the three groups of patients are analyzed, considering the average values of each determination as well as their standard deviations. The results obtained in all the considered determinations in patients who survived less than four weeks and in those who survived more than three months are comparatively exhibited in Table 3; all the results are expressed as the average of the obtained results and their standard deviations.

In table 4 a comparison between the results obtained at the beginning of the treatment and 3 months later is presented, all the parameters are taken into account in this table

DISCUSSION AND CONCLUSIONS

The patients with cryptococcosis and AIDS studied in this research were in an advanced stage of HIV infection. They presented with very low lymphocyte counts of all the cell subsets, the CD4+ cells average count was 22 cells/µl and the CSF inflammatory response was extremely scarce showing less than 20 cell/µl in all the studied cases. Furthermore, those patients who died during the first month of evolution showed very low CD8+ cells counts (average 194 cells/µl).

The role of CD4+ cells in the control of cryptococcal infection is very well known [6,8]. In an experimental cryptococcal pulmonary infection in Balb/c mice, it was demonstrated that CD4+ and CD8+ cells combined to mediate a prominent pulmonary inflammatory response against *C. neoformans*. It was also observed that CD8+ cells are able to secret IL-2 and IFN-γ, two very important cytokines in the mediation of a defensive response in this infection [8]. According to these experimental observations CD8+ cells seem to play a secondary but important role in the cryptococcosis outcome.

The TNFα and IL-1 levels were highly superior to those of the healthy persons studied in the control group. Mannoprotein, glucuronoxylomannan and galactoxylomannan induce TNFα production in a dose-dependent manner. Mannoproteins are significantly more potent than any other of the cryptococcal components. Monocytes are the cell population mainly responsible for TNFα production and plasma complement is necessary for TNFα induction by the cryptococcal components [9].

TNFα has a beneficial role in the outcome of murine cryptococcosis by increasing phagocytosis of encapsulated yeasts [12]. However, in AIDS patients TNFα may lead to increased replication of HIV in latency infected cells and progression of the disease [10,11]. We observed that TNFα levels were four folds higher in those cases who suffered a fatal evolution during the first four weeks than in those patients who presented a longer and less severe outcome. According to this observation TNFα level seems to be an important parameter in the prediction of fatal evolution.

No clear correlation was detected between IL-1 concentrations and the severity of cryptococcal infection, although the IL-1 levels in HIV positive patients with cryptococcosis were higher than those observed in healthy volunteers but lower than in the AIDS patients without cryptococcosis.

The titers of polysaccharide capsular antigen of *C. neoformans* in serum and CSF were extremely high, specially in the eight patients who died in less than one month after diagnosis. The CSF antigen titers were 20 folds higher than those observed in the cases who exhibited a survival time longer than three months. In the latter group of patients antigen concentration in serum gradually lowered during treatment according to patients clinical improvement.

Only two significant variations were detected in those cases who were studied after three months of treatment: an increase of the NK cells count and a higher IL-12 concentration. Patients with AIDS often have impaired NK cell functions and low NK cells count usually indicates progression of the disease [5]. The role of NK cells has been studied in murine cryptococcosis, they apparently play a role during the early stage of pulmonary infections as nonspecific resistance mechanism [13]. IFN-γ which is an important mediator of resistance to *C. neoformans*, is also able to induce the augmentation of splenic NK cells activity during cryptococcal infection.

Little is known about the importance of NK cells during the advanced disseminated infection [12].

Among the parameters which have been studied in this research, those that contribute to a great extent in the prediction of a rapid fatal outcome seem to be a low CD8+ cells count (<200 cells/µl) together with CD4+ cells count lower than 50 cells/µl, high TNFα concentration (>50pg/ml) and a very high capsular antigen titer in serum (>1/5000). The isolation of *C. neoformans* from several clinical samples in a single patient is also an important sign of severity.

It is impossible to know if these alterations, observed in the different parameters, are due to the advanced stage of HIV disease or to the severity of the cryptococcal infection; nevertheless some of them seem to be of interest in predicting the fatal evolution.

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