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Circulating Antigen of Toxoplasma gondii in Patients with AIDS: Significance of Detection and Structural Properties*

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With 1 Figure

Abstract

232 sera and 40 cerebrospinal fluid samples of altogether 125 patients in stages III or IV of a HIV-infection were tested for circulating antigen of *Toxoplasma gondii* by means of a three-layer enzyme-linked immunosorbent assay. Circulating antigen was detected in 32 sera of 20 patients (= 16% of all persons investigated). These ELISA results were reexamined by an Immunoblot following a SDS-PAGE and confirmed in most cases. In addition, this test system led to a partial characterization of the circulating antigen; it consists of at least two proteins with atomic mass units of 27 and 57 kd respectively.

The antigenemia was correlated with IgG- and IgM-antibody titres, with clinical symptoms, and with pathological findings also. Our results indicate that the detection of circulating antigen in sera offers a rapid and efficient method for the diagnosis of an acute toxoplasmosis in AIDS-patients.

Zusammenfassung

Zirkulierendes Antigen von *Toxoplasma gondii* in AIDS-Patienten: Bedeutung des Nachweises und strukturelle Eigenschaften.

232 Seren und 40 Liquores von insgesamt 125 Patienten in Stadien III oder IV einer HIV-Infektion wurden mittels eines Dreischicht-Enzym-Immuntests auf zirkulierendes Antigen von *Toxoplasma gondii* untersucht. Zirkulierendes Antigen wurde in 32 Seren von insgesamt 20 Patienten (= 16% der untersuchten Patienten) nachgewiesen. Die im Enzym-Immuntest erhobenen Befunde wurden in einem Immunoblot nach Auftrennung mittels SDS-PAGE überprüft und in den meisten Fällen bestätigt. Mit diesem Testsystem gelang auch eine Charakterisierung des Antigens; es besteht aus mindestens zwei Proteinen mit Atommassen von 27 und 57 kd.

Die Antigenämie wurde mit den festgestellten IgG-, IgM-Antikörpertitern, ebenso mit klinischen Symptomen und pathologischen Befunden in Beziehung gesetzt. Die Ergebnisse zeigen, daß der Nachweis von zirkulierendem Antigen wesentlich zu einer raschen und sicheren Diagnose einer akuten Toxoplasmose bei AIDS-Patienten beitragen kann.

Introduction

The diagnosis of an acute toxoplasmosis by conventional serological tests basing upon titration of IgG or IgM antibodies is difficult in patients who are severely immunocompromised. This difficulty is caused particularly by the insignificantly low titers or even by the absence of specific antibodies in the sera of most AIDS-patients infected with *Toxoplasma gondii.* However, the appearance of a circulating antigen (cag) in sera during the acute phase of a *Toxoplasma* infection has repeatedly been demonstrated in men as well as in animals (1, 9, 14, 16, 21). Methods for the detection of cag are therefore promising to be useful diagnostic tools for the diagnosis of acute toxoplasmosis also in patients with AIDS.

Materials and Methods

Patients' sera and cerebrospinal fluids. 232 serum samples and 40 cerebrospinal fluids of 125 patients in different stages of a HIV-infection, classified according to the CDC-system (5), were examined for their contents of cag, of IgG, and of IgM antibodies against *Toxoplasma gondii.* The samples were taken from routineously supervised patients; in most cases there was no suspicion of an acute *Toxoplasma* infection. The serological results were compared with clinical findings indicating an active toxoplasmosis and/or with other diagnostic data.

Serological procedures. The detection of cag was performed by an enzyme-linked immunosorbent assay as described by *Hassl* et al. (9), except for the following modifications: for the production of the hyperimmune serum, rabbits were orally immunized with 106 trophozoites of *Toxoplasma gondii,* strain BK. The parasites had been cultivated and multiplied in mouse peritoneal cavities (mice: Him: OF1 (Swiss) SPF). Booster infections of 106 trophozoites were given at days 10 and 21 p.i. IgG antibodies from the antiserum pool (indirect hemagglutination assay titer: 1: 256 000) were isolated by affinity chromatography on protein A-sepharose according to the manufacturer's recommendation (Pharmacia Ges.m.b.H., Vienna, Austria). The $_{F(ab)2}$ fragments were separated from the F_c parts and from undigested IgG by a second chromatography step on protein A-sepharose. Furthermore, EIA serocluster plates (Costar, Cambridge, MA, USA) were used in the cag-ELISA. For the detection of specific IgG antibodies an indirect fluorescent antibody test (IFA) was used. It was performed in a standardized manner according to the recommendations of the Bundesgesundheitsamt of the FRG (4), using a goat anti-human IgG/FITC conjugate (Behringwerke AG, Marburg, FRG). IgM antibody titers were measured by an IgM-IFA, performed in the same way as the IgG-IFA, except for the periods of incubation which lasted 2 h. A goat anti-human IgM/FITC conjugate (Atlantic antibodies, Scarborough, ME, USA) was used. Rheumatoid factor activity was routineously determined by a RapiTex-RF assay (Behringwerke AG) and removed if detected.

Western blot. All sera with antigenemia, except for six, were also examined in an Immunoblot. 50 µl of cag-containing serum were precipitated with 28% ammonium sulfate (v/v) at 20 °C, centrifuged at 8750 g for 2 min, and resuspended in 10 µl sample buffer of the SDS-PAGE containing 5% mercaptoethanol. The SDS-PAGE was performed on a PAA 4/30 gradient gel (Pharmacia Ges.m.b.H.) according to the manufacturer's recommendations. The electrophoresis was run for 450 Vh. Thereafter, the proteins were transferred to nitrocellulose sheets (7 $Vxcm^{-1}$, 480 Vh) and identified by an enzyme-linked immunosorbent assay. In short: The sheets were soaked in ELISA buffer, then incubated with cag-ELISA conjugate (1 :50 in ELISA buffer, 2 h, 37°C) before adding the substrate (H_2O_2 plus 4-chloro-1-naphtol).

Table 1. Results of serological tests for *Toxoplasma gondii* antigen and for specific antibodies in sera and cerebrospinal fluids of 20 patients with an antigenemia

No.	Patient sex	Ma.	Days*	AIDS stage	Serological tests cag	IFA	IgM	Symptoms, clinical diagnosis, remarks
1	m	s	0	IV B	+	64	neg	fever, encephalitis,
		s	12	IV	—	64	neg	—> therapy
2	m	s	0	IV B	+	1000	16	hemiparesis, CT:
		1	0	IV B	—	16	nd	focus, —> therapy
		s	90	IV B	+	1000	16	"toxoplasmosis recrudescence"
3	f	s	0	III	+	16	nd	fever
4	m	s	0	IV D	+	neg	neg	
		s	37	IV D	+	neg	neg	
		s	54	IV D	+	neg	neg	
		s	85	IV D	+	neg	neg	
		s	98	IV D	+	16	neg	
		s	110	IV D	+	64	neg	"toxoplasmosis"
5	m	s	0	nd	—	1000	neg	CFA: 5, IHA: 256
		s	15	nd	—	256	neg	
		s	122	nd	—	64	neg	
		1	302	IV A	—	neg	neg	
		s	324	IV B	+	64	neg	--> therapy
		s	420	IV	—	64	neg	
6	m	s	0	nd	—	256	nd	
		s	168	IV	—	256	nd	
		s	289	IV C	+	16	neg	CNS-foci
7	f	s	0	IV A	+	64	16	
		s	117	IV A	+	64	16	
		s	263	IV A	+	64	16	
8	m	s	0	IV A	+	1000	neg	fever
		s	21	IV A	+	1000	neg	fever
		s	75	IV A	+	1000	neg	fever, -> therapy
		s	172	IV A	—	256	neg	
9	m	s	0	IV A	+	16	neg	night sweat
		s	49	IV A	—	neg	neg	
10	m	s	0	IV	+	16	neg	CNS: ring-enhancing
		1	0	IV	—	neg	neg	lesions
11	m	s	0	III	+	4000	64	lymphadenopathy, → therapy
		s	49	III	-	1000	neg	
12	m	s	0	IV D	+	64	neg	
		s	13	IV D	+	64	neg	autopsy: acute toxoplasmosis

Patient No.	sex	Ma.	Days*	AIDS stage	cag	Serological tests IFA	IgM	Symptoms, clinical diagnosis, remarks
13	m	s	0	IV A	—	16	neg	
		s	98	IV A	—	16	neg	
		s	111	IV A	+	16	neg	lung-toxoplasmosis,
		1	111	IV A	—	16	neg	---> therapy
		s	125	IV A	—	16	neg	
14	f	s	0	III	—	16	nd	
		s	219	IV A	+	16	neg	
15	m	s	0	III	—	neg	nd	
		s	308	III	—	neg	neg	
		s	344	IV	+	neg	neg	
		s	400	IV	+	neg	neg	
16	m	s	0	IV A	—	neg	nd	
		s	189	IV A	—	neg	neg	
		s	202	IV A	+	16	neg	lymphadenopathy
17	m	s	0	III	+	16	neg	
18	m	s	0	IV A	+	16	neg	lymphadenopathy
19	m	s	0	IV A	—	neg	neg	
		I	0	IV A	—	neg	neg	
		s	97	IV A	+	neg	neg	
		1	97	IV A	—	neg	neg	
20	m	s	0	III	+	256	neg	

Ma: Material: s = serum, 1 = liquor
* days after the first investigation
Serological tests: cag = enzyme-linked immunosorbent assay for *Toxoplasma gondii* antigen, IFA = indirect fluorescent antibody test, IgM = IgM-indirect fluorescent antibody test. Antibody test results are expressed as reciprocals of titers.
nd = no data available

Results

Serum samples

In 32 sera of 20 patients (16%) antigen was detected by the ELISA. From these, 16 patients were in stage IV, only 4 suffered from AIDS in stage III, although this stage group comprised 51% of all investigated persons. In none of the cerebrospinal fluids cag could be found. The results of the serological tests done with the samples of the patients with an antigenemia and the state of health of these patients are listed in Table 1. False positive results due to rheumatoid factors were not detected.

Immunoblot

The results of the immunochemical analysis are shown in Fig. 1. The first three sera of patient 4 and the sera of patients 12 and 19 could not be included into this investigation because of insufficient serum amounts available. In 17 of the 26 investigated sera

two or more immunoreactive bands could be demonstrated. All sera contained the two strongly reacting bands at 27 kd and 57 kd. Some minor bands with a.m.u. between 65 kd and 25 kd were detected in some sera (Fig. 1) No reactions ocurred with sera giving negative results in the ELISA or with controls at all.

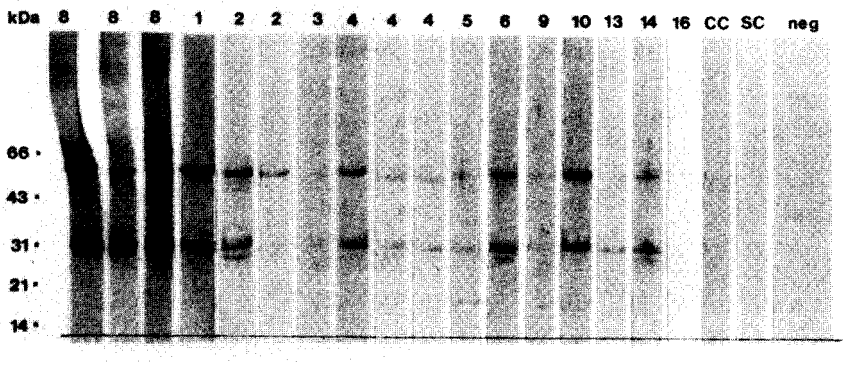

Fig. 1. Immunoblot following a SDS-PAGE for the detection of Toxoplasma antigen circulating in sera of HIV-infected patients. Numbers of sera correspond to the numbers of the patients listed in tab. 1. In the conjugate control (CC) PBS-buffer was used instead of serum. In the serum control (SC) positive serum (No. 4) was separated but buffer instead of conjugate was applied. As a negative control (neg) sera were separated which did not react in the cag-ELISA. The positions of the molecular weight standards are shown on the left.

Discussion

Toxoplasmosis is one of several opportunistic infectious disease that effect patients with an acquired immunodeficiency syndrome. Acute toxoplasmosis, especially with lesions in the central nervous system, has emerged as a major cause of death in AIDS-patients suffering from infections due to opportunistic agents (6, 10, 22, 23). Unfortunately, the diagnosis of an acute *Toxoplasma* infection may be very difficult or even impossible as few techniques, preferably imaging methods, have been reported to be useful for this purpose (19). Antibody titration, which is the usual diagnostic procedure for the detection of a *Toxoplasma* infection in an immunocompetent person, commonly fails to uncover an acute infection in AIDS patients (10, 11, 15, 20, 23). However, an alternative serological procedure, the detection of cag, is considered to be useful for serodiagnosis (17, 19), as antigenemia has repeatedly been described to be indicative of an acute infection phase (1, 9, 14, 16, 21). At present, open questions exist concerning the significance of the detection of an antigenemia in patients with AIDS, and no data are available concerning the structure of the antigen circulating in the sera of such patients.

We detected cag in 28 sera of 16 patients suffering from severe AIDS symptoms (group IV), that means that probably 26% of the patients in stage IV had acute toxoplasmosis. In patients of stage III antigenemia was found only in 6% (4 out of 64).

This striking difference of the prevalence rates among the two groups apparently reflects the frequency of reactivation and dissemination of a latent *Toxoplasma* infection in the course of AIDS. Our results support the suggestion of *Pohl* and *Eichenlaub* that an exacerbation of a latent *Toxoplasma* infection will occur in most moribund AIDS patients (19). Until now, we found two convincing cases of such a terminal exacerbation, patients No. 4 and 12, who both died of broad consumption shortly after the last tests revealing an antigenemia. Unfortunately these two patients had not undergone a chemotherapy. If such terminal exacerbation of latent *Toxoplasma* infections really occurs regularly, on an average every second Austrian AIDS patient would be affected. This high prevalence is due to the 50% infection rate of adults with *Toxoplasma gondii* in Central Europe (3). This epidemiological situation may be the reason why the prevalence of active toxoplasmosis in AIDS patients of stage IV, as detemined by us, correlates conspiciously well with the prevalence in the FRG (30%) (19). A comparison of data on the prevalence of acute toxoplasmosis among AIDS patients on an international scale may not be significant as the infection rates with *Toxoplasma gondii* differ considerably throughout the world.

Although detection of cag in cerebrospinal fluids has been expected as being helpful for the diagnosis of CNS-toxoplasmosis (17), we could, surprisingly, not find cag in any cerebrospinal fluid sample, not even in cerebrospinal fluids of patients with an antigenemia and lesions in the brain (patients No. 2, 10). A possible explanation would be the lack of immunolysis of *Toxoplasma* trophozoites in the cerebrospinal fluid.

As far as available, clinical and pathological data of the patients with an antigenemia support the assumption of an active toxoplasmosis (Table 1). The results of antibody titrations on the other hand are inconclusive in the majority of cases. Except for two cases (patients No. 2, 11) the detection of specific IgG and IgM antibodies would certainly not have led to the diagnosis of an active toxoplasmosis. The mere demonstration of specific antibodies must be considered simply as an indication that an infection of the patient with *Toxoplasma gondii* has taken place, but does not allow any conclusion concerning the infection phase. The lack of specific antibodies is, indeed, of no diagnostic value, especially in the late phase of AIDS. This can impressively be demonstrated by the antibody titers of patient 4.

As regards the separation of cag in the electrophoresis two points are noteworthy. On one hand, not all sera giving positive results in the ELISA, reacted in the immunoblot also. This might be due to the disintegration of the antigen during the electrophoresis either or to a too small quantity of antigen in some sera. On the other hand, all sera reacting in the immunoblot showed very similar band patterns. The molecular weight analysis revealed two main protein bands at 57 and 27 kd in all sera. Minor bands between 65 and 25 kd were found in some sera. These results coincide very well with results obtained by previous investigations on cag in sera of different intermediate hosts (9). This congruence is very likely an expression of a certain specificity of the antigen detecting antibodies used by us and, thus depends on the immunization mode applied to the antibody donors.

A 27 kd as well as a 57 kd protein is a prominent antigen of *Toxoplasma* membrane described by several authors (e.g. 7, 8, 13, 18). Thus, the destruction of *Toxoplasma* cells and the following release of some proteins may be the cause for the antigenemia, a suggestion which has already been expressed by *Hughes* and *van Knapen (12).* Furthermore, a large enzyme (nucleoside triphosphate hydrolase, 220 kd) has been described as a circulating antigen in mice (2). It is not impossible that the antigens detected by us are fragments of such a secretion product.

The results of this study indicate that a *Toxoplasma* antigenemia in AIDS patients should be considered as an expression of multiplication and subsequent destruction of parasites. Thus, the detection of an antigenemia may be helpful for the establishment of a conclusive diagnosis, especially in patients with clinical symptoms indicating an active toxoplasmosis or in patients with brain lesions. No doubts, tests for the detection of cag will prove to be essential tools in the serodiagnosis of a toxoplasmosis in patients with AIDS.

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