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Antigens of Toxoplasma gondii Recognized by Sera of AIDS Patients Before, During, and After Clinically Important Infections*

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Summary

A longitudinal study of different parameters of the immune responses to *Toxoplasma gondii* was performed with sera of AIDS patients taken during and after clinically important *Toxoplasma* infections. Follow-up of patients lasted for 9 months on an average. The titres of the specific IgG and IgM antibodies were measured by an indirect fluorescent antibody test (IFAT), and the appearance of circulating antigens of *Toxoplasma gondii* was determined in 88 sera of 18 patients with CNS (6 cases), pulmonary (1), lymph-node toxoplasmosis (1), or asymptomatic primary infections (2), respectively. The profiles of the IgG antibodies reacting with a lytic antigen originating from a pool of trophozoites of six different *Toxoplasma* strains were examined by means of an SDS-PAGE followed by an immunoblot.

Although numerous antigen bands were recognized by the sera of patients with clinically important infections, an antigen pattern characteristic of an acute infection could not be discovered. The majority of these sera, however, recognized bands at 27 and 57 kd; proteins of these molecular weights are components of the circulating antigens. In patients without any indication of a *Toxoplasma* infection, small amounts of antibodies reacting with 34-38 kd antigens were detected. The results of this study demonstrate that seropositivity to *Toxoplasma gondii* in AIDS patients determined by routine serological methods (e.g. IFAT) may be very heterogenous even if identical titres are found; it simply results from different combinations of various antibodies which can only be detected by the immunoblotting technique.

Cordially and gratefully dedicated to Professor Dr. Dr. h. c. *H. Flamm* on the occasion of his 60th birthday.

Zusammenfassung

In einer Langzeitstudie wurden insgesamt 18 AIDS-Patienten wiederholt — durchschnittlich neun Monate lang — auf Antikörper gegen *Toxoplasma gondii* und zirkulierendes Antigen untersucht. Bei 12 Patienten lag eine Toxoplasma-Infektion vor, im einzelnen handelte es sich um 6 ZNS-, 1 Lungen- und 1 Lymphknoten-Toxoplasmose, sowie um zwei Frischinfektionen. Bei sechs Patienten konnte kein Hinweis für eine Infektion mit *Toxoplasma gondii* gefunden wurden. Insgesamt wurden 88 Seren dieser 18 Patienten in einem Indirekten Immunfluoreszenztest (IIFT) quantitativ auf spezifische IgG- und IgM-Antikörper sowie mittels eines ELISA auf zirkulierende Antigene untersucht. Außerdem wurden die Bandenmuster der IgG-Antikörper in einem Immunoblot nach einer SDS-PAGE eines Lysats eines Pools von Trophozoiten von sechs verschiedenen *Toxoplasma-Stämmen* bestimmt und miteinander verglichen.

Seren von Patienten mit klinisch relevanten Infektionen erkannten zahlreiche verschiedene *Toxoplasma-Antigene,* allerdings konnte ein nur für akute Infektionen charakteristisches Antigenmuster nicht gefunden werden. Die meisten dieser Seren markierten jedoch — neben vielen anderen — Antigene mit Molekulargewichten von 27 und 57 Kd; Antigene dieser Molekulargewichte werden regelmäßig in zirkulierenden Antigenen gefunden. In den Seren von Patienten, bei denen weder klinisch noch serologisch ein Anhaltspunkt für eine Infektion mit *Toxoplasma gondii* festgestellt werden konnte, konnten dennoch Antikörper gegen Antigene des verwendeten Lysats mit Molekulargewichten zwischen 34 und 38 Kd gefunden werden. Die Ergebnisse dieser Studie zeigen, daß die durch herkömmliche Routinetests (z. B. IIFT) festgestellte Seropositivität gegen *Toxoplasma gondii* bei AIDS-Patienten selbst bei identischen Titern sehr heterogen ist und auf durchaus unterschiedliche Kombinationen verschiedener Antikörper zurückzuführen ist. Für die Aufdeckung dieser Unterschiede eignet sich der Immunoblot.

Introduction

Toxoplasmosis, particularly that of the central nervous system, but also in a generalized form affecting various other organs, is a well known, very serious and life-threatening complication in AIDS patients. The disease may result from a reactivation of a latent infection (in the USA e.g., almost all cases of CNS toxoplasmosis are of this type) or from a primary infection (this type is, besides reactivation, apparently of considerable importance in Central Europe). Unfortunately, serodiagnosis of toxoplasmosis is difficult in AIDS patients: due to the compromised immune system, specific IgM antibodies can commonly not be produced by the patient even during life threatening infections; IgG titres are usually low and, if evaluated solely, inconclusive (12). Although the demonstration of ciruculating antigens of *Toxoplasma gondii* is obviously a useful diagnostic tool for the uncovering of toxoplasmosis in patients suffering from AIDS (5, 8), a precise serodiagnosis presently demands a rather complicated and sophisticated examination procedure (6). Therefore, new simple serological procedures for diagnostic purposes would be of great benefit; they are being developed (17). Longitudinal data on the dynamics of antibody formation during the course of AIDS is therefore of great importance.

In this study, we have used the protein-blotting technique (Western blot) to detect antigens of *Toxoplasma gondii* that are recognized by IgG antibodies of AIDS patients before, during, and after acute infections. Acute infections may be exacerbations of old infections with clinical symptoms on the one hand or primary infections with or without clinical symptoms, on the other. In addition, we were also particularly

interested in the question whether so-called natural antibodies (13) appeared in sera of AIDS patients without *Toxoplasma* infections, whether there was a response of the immunsystem to the appearance of circulating antigens, and whether certain antigens of *Toxoplasma gondii* were recognized by "indicative" antibodies during the acute stage of the infection.

Materials and Methods

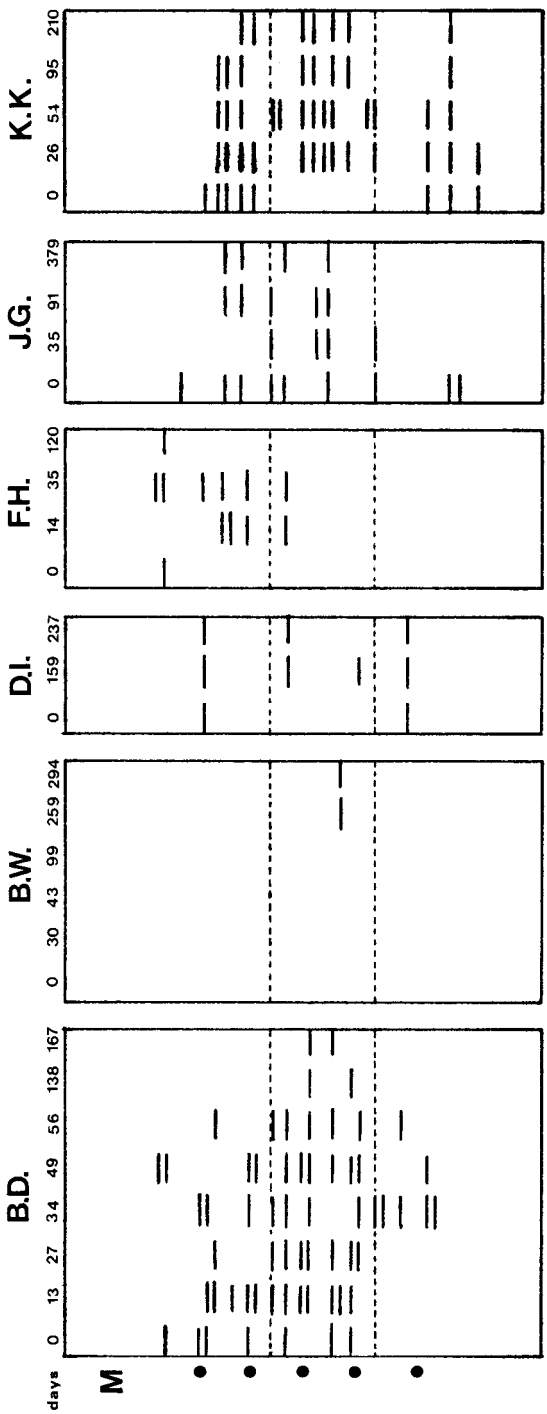
Patients' sera. 88 sera of 18 patients, all in stage IV of HIV infection as classified according to the CDC-system (2), were tested for circulating antigens, for IgG, and for IgM antibodies against *Toxoplasma gondii.* The samples were taken from routinely supervised patients; in 11 cases, toxoplasmosis (6 CNS, 1 pulmonary, 1 lymph-node toxoplasmosis, 1 reactivation without striking clinical signs, 2 primary infections without clinical symptoms) was diagnosed clinically and/or serologically and/or "ex iuvantibus", respectively. One patient had an asymptomatic old infection, and in 6 patients, no evidence of an infection with *Toxoplasma gondii* was found, neither by any serological test nor by the presence of any typical clinical symptom.

Serological procedures. The detection of circulating antigens was performed by a four-layer enzyme-linked immunosorbent assay as described previously (5).

For the detection of specific IgG antibodies, an indirect fluorescent antibody test (IFAT) was used, manufactured by the Institute of Hygiene, Vienna. It was performed in a standardized manner according to the recommendations of the Bundesgesundheitsamt of the FRG (1), using a goat anti-human IgG/FITC conjugate (Behringwerke AG, Marburg/Lahn, FRG).

In parallel, the IgM antibody titres were measured by an IgM-IFAT, performed in the same way as the IgG-IFAT, 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 routinely determined by a RapiTex-RF assay (Behringwerke AG) and removed if detected.

Western blot. Trophozoites of six different *Toxoplasma* strains (RH, BK, KB, Alt, 928, T) were harvested from the peritoneal cavities of laboratory mice (Him: OF1 SPF, source: Versuchstierzucht Himberg, Himberg/Austria) infected two or three days previously. About 10^9 trophozoites of each *Toxoplasma* strain were pooled, washed three times in physiological saline (5 min, 100 g), resuspended in distilled water, and sonicated (3 min, step 3 of a Sonifer B-30, Branson Sonic Power Co., Danbury, CT). The supernatent was lyophilized and resuspended in reducing sample buffer (1 M Tris pH 6.8, 10% SDS, 5% mercaptoethanol) to a protein concentration of 10 mg x ml ^{1} and boiled for 10 min. This antigen solution (10 µl per lane) and a commercially available molecular weight marker (Bio-Rad Laboratories, Richmond, CA) were separated in an SDS-PAGE (GE 2/4 LS system, PAA 4/ 30 gel, Pharmacia LKB Biotechnology, Vienna, Austria) according to the manufacturer's recommendations. After a separation of 480 Vh (125 V constant), the gels were washed in blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3 + 20% methanol) and thereafter, the proteins were transferred overnight to nitrocellulose sheets (7 V x cm-^{1}, 425 Vh) in a BioRad blotting chamber. The sheets were cut into strips; the strip containing the molecular weight marker was stained with amidoblack (staining: 10 min in 65 ml a.d. + 25 ml methanol + 10 ml acetic acid + 0.1 g amidoblack; destaining: 2 x 10 min in 65 ml a.d. + 25 ml methanol + 10 ml acetic acid); the other strips were soaked in ELISA-buffer for 2 h to block free binding sites. Thereafter, patients' sera, all diluted 1 : 11 in ELISA buffer, were applied to the strips for 2 h, then, conjugate (Peroxidase-conjugated IgG fraction goat antihuman IgG, Cappel, West Chester, PA), diluted 1: 1000 in ELISA buffer and, finally, substrate (0.01% H_20_2 plus 4-chloro-1-naphtol) was added. The reaction was stopped by removing the substrate after 30 min.


K.K.
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J.G.
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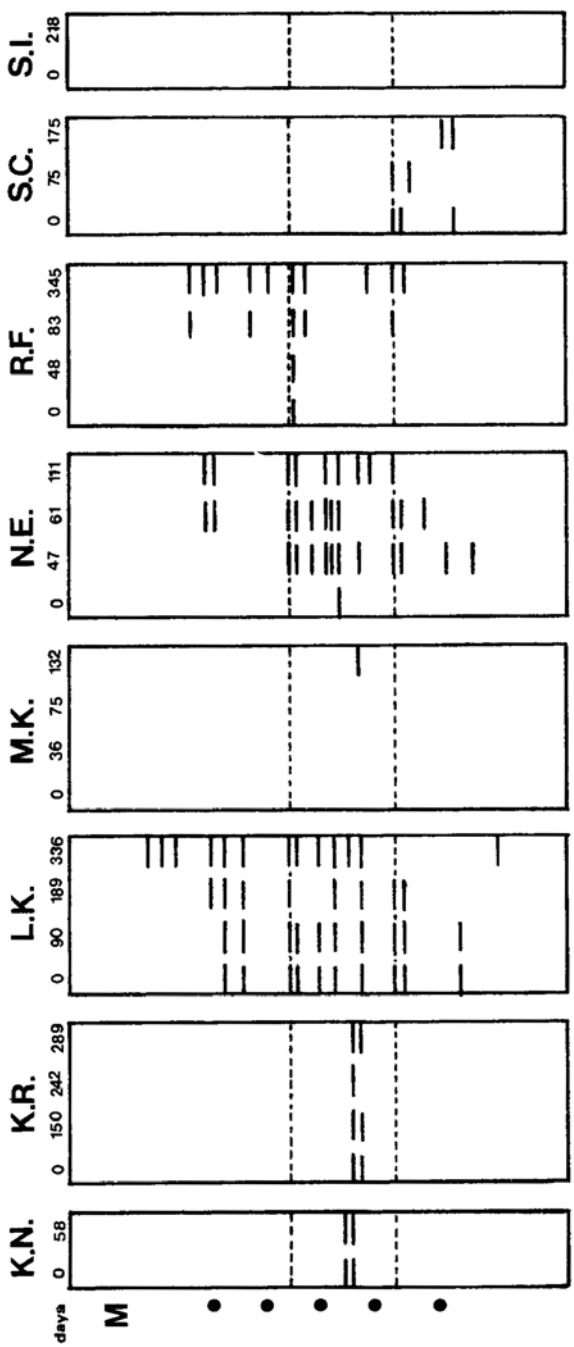
S.I.
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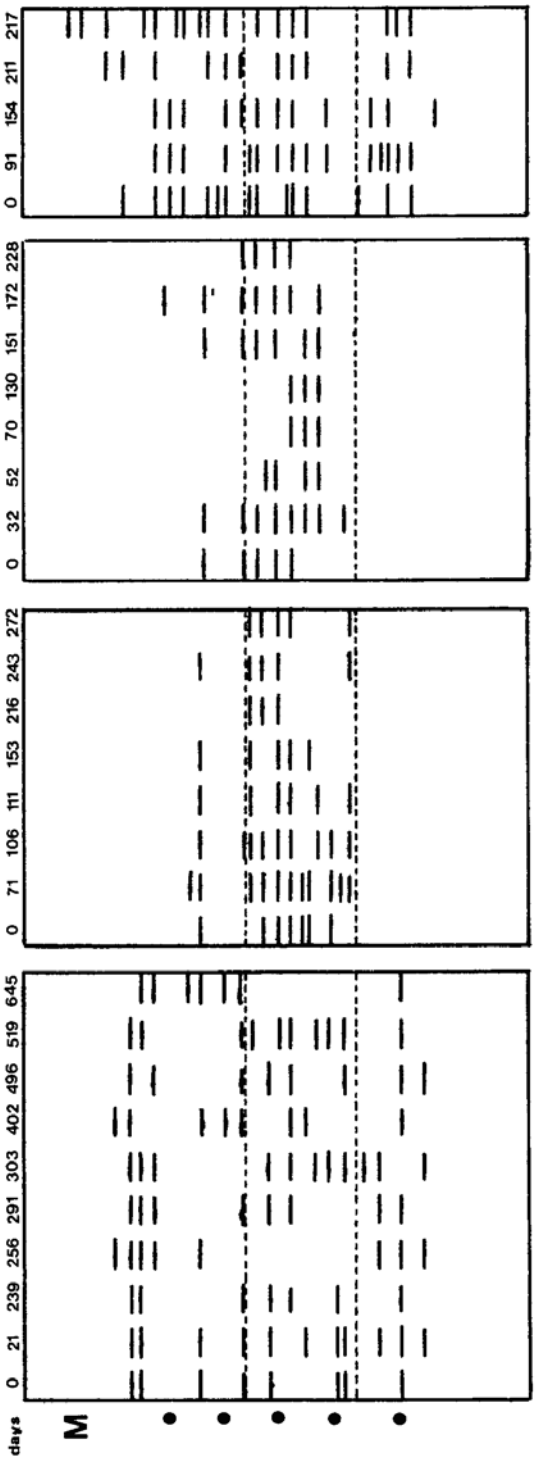

Fig. 1. Immunoblots following SDS-PAGE for the detection of anti-*Toxoplasma* IgG antibodies circulating in the sera of AIDS patients. Designation of sera corresponds to the designations shown in Table 1. Dotted lines: molecular weights of the main proteins of circulating *Toxoplasma* antigens. M: molecular weight marker; from top to bottom: 97.4, 66.2, 42.7, 31, 21.5 kd.

Results

The results of the serological tests done with the sera of the 12 AIDS patients suffering from *Toxoplasma* infections and nosological data are listed in Table 1. 21 serum samples from 6 patients seronegative for *Toxoplasma gondii* (patients B.W., K.N., K.R., M. K., S. C., S. I.) were examined and found to be negative in both the cag and the IFAT assays. The results of the immunochemical analysis are shown in Fig. 1. Due to the fact that many bands on the immunoblots were faint and did not reproduce well in photographs but were important for evaluation, the immunoblotting results have been presented as drawings. Fig. 2 presents a photograph of an immunoblot of sera of persons without HIV infection (controls).

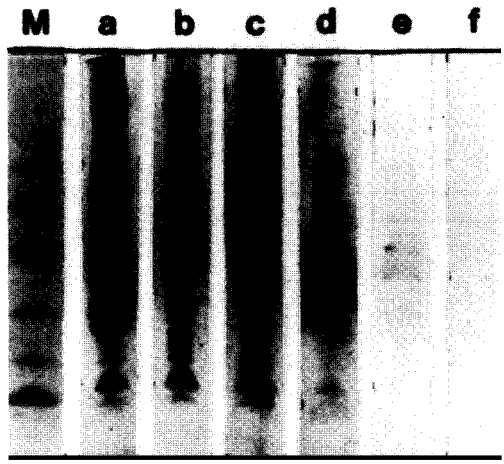

Fig. 2. Immunoblots of sera of non-HIV-infected persons. Technical procedure identical with that described under Material and Methods. Titres of *anti-Toxoplasma* antibodies as determined in an IFAT: Serum a: 1 : 1000; b and c: 1 : 64; d: 1 : 16; e and f: neg. M: molecular weight marker; from top to bottom: 94, 67, 43, 30, 20.1, 14.4 kd.

Numerous bands with molecular weights between 180 kd and 18 kd could be demonstrated in the sera of patients with clinically important infections. In the sera of patients with CNS toxoplasmosis and in the sera of patients with pulmonary or lymph-node toxoplasmosis, bands with a.m.u. of 32-33 and 36-37 kd, respectively, could be located. Only a few or no bands at all were found in sera without demonstrable IgG titres. Generally spoken, toxoplasmosis, presenting itself as exacerbation as well as primary infection, is characterized by the appearance of IgG antibodies directed against proteins with high molecular weights.

Discussion

Toxoplasma gondii is one of the most dangerous opportunistic pathogens in patients with an acquired immunodeficiency syndrome. Toxoplasmosis, especially that of the central nervous system, is in fact a major cause of death in AIDS patients (4, 9, 16, 22).

Table 1. Results of serological testing for *Toxoplasma gondii* antigen and for specific antibodies in sera of 12 AIDS patients with toxoplasmosis

Patient	Sex	Days	cag	IFAT	IgM-IFAT	Remarks
Patients with CNS toxoplasmosis:						
F.H.	m	0	pos	16	neg	
		14	pos	16	neg	Headache, therapy
		35	neg	16	neg	
		120	neg	16	neg	
J. G.	m	0	pos	64	neg	Fever, therapy
		35	neg	64	16	
		91	neg	64	16	
		379	neg	64	neg	
K.K.	m	0	neg	256	neg	
		26	neg	256	neg	
		54	pos	1000	neg	Therapy
		95	neg	64	neg	
		210	neg	256	neg	
L. K.	m	0	pos	1000	16	Foci in the CNS, hemiparesis, therapy
		90	pos	1000	16	Toxoplasmosis recrudescence
		189	neg	256	neg	Fansidar prophylaxis
		336	pos	64	neg	Remission of the CNS-toxoplasmosis, therapy renewed
S. M.	m	0	pos	1000	neg	Fever
		21	pos	1000	neg	Fever, therapy
		239	neg	256	neg	
		256	neg	256	neg	
		291	neg	64	neg	
		303	neg	64	neg	
		402	neg	256	neg	
		496	pos	256	neg	Remission of CNS toxoplasmosis, therapy
		519	neg	256	neg	
		645	neg	64	neg	
V.S.	m	0	neg	256	neg	
		91	neg	1000	neg	Headache, fever, therapy
		154	neg	256	neg	
		211	neg	256	neg	
		217	neg	256	16	
Patient with pulmonary toxoplasmosis						
B.D.	m	0	neg	16	neg	
		13	pos	16	neg	Therapy
		27	neg	16	neg	
		34	neg	16	neg	
		49	neg	16	neg	
		56	neg	16	neg	
		138	neg	16	neg	
		167	neg	neg	neg	

Table 1. Continued

Patient	Sex	Days	cag	IFAT	IgM-IFAT	Remarks
Patient with lymph-node toxoplasmosis						
U. W.	m	0	neg	64	16	
		71	neg	64	16	CMV infection
		106	pos	64	16	Therapy
		111	neg	64	neg	
		153	neg	64	neg	
		216	neg	16	neg	
		243	neg	64	16	Remision
		272	neg	64	16	
Patients with primary infections						
N.E.	m	0	neg	neg	neg	
		47	neg	16	neg	
		61	pos	64	neg	
		111	neg	1000	neg	
R. F.	m	0	pos	neg	neg	Therapy
		48	neg	neg	neg	
		83	neg	16	neg	
		345	neg	16	neg	
Patient with toxoplasmosis without severe clinical signs						
V.K.	m	0	neg	16	neg	
		32	pos	16	neg	
		52	neg	16	neg	
		70	neg	16	neg	
		130	neg	neg	neg	
		151	neg	neg	neg	
		172	neg	16	neg	
		228	neg	neg	neg	
Patient with asymptomatic old infection						
D.I.	f	0	neg	16	neg	
		159	neg	16	neg	
		237	neg	16	neg	

days: days after the first investigation
Serological tests: cag = enzyme-linked immunosorbent assay for the detection of *Toxoplasma gondii* antigen, IFAT = indirect fluorescent antibody test, IgM-IFAT = IgM-indirect fluorescent antibody test. Antibody test results are expressed as reciprocals of titres. Remarks: clinical signs, time of therapy, "ex iuvantibus" diagnosis.

Unfortunatly, diagnosis of toxoplasmosis is difficult in AIDS patients. Imaging methods may be helpful (12) but are not indicative. The laboratory diagnosis is based on attempts to demonstrate trophozoites in brain biopsy material (12) or rather complicated and time-consuming serotests (6, 17). Antibody titration, which is the common diagnostic procedure for the detection of a *Toxoplasma* infection in immunocompetent persons, has its shortcomings in patients suffering from AIDS: As a

rule, specific IgM antibodies cannot be produced by these patients, and the titres of specific IgG antibodies are frequently low, even in cases with a fatal course of the disease. Thus, the question arises whether the demonstration of a certain pattern of antibodies may be indicative.

A serious problem arising during serological studies is the appearance of *Toxoplasma* strain-specific antigens (20, 21). Such strain-specific antigens have been regarded as being responsible for the antibody diversity observed among sera of AIDS patients (21). In order to overcome this problem we decided to use a pool from six different *Toxoplasma* strains as an antigen for immunoblotting. Among our patients, a considerable heterogeneity in the composition of the different antibodies could be observed which is by no means less broad than that observed by *Weiss* et al. (21) (see Fig. 1). Thus, in our opinion, the antibody diversity in the sera of patients suffering from toxoplasmosis observed by different authors is more probably due to the individual ability of antigen recognition than to the appearance of strain-specific antigens. Therefore, we conclude that no indicative IgG antibody pattern characteristic of an acute infection seems to exist. This assumption is supported by the observations of several authors (3, 14, 15, 18, 19). In contrast, two bands (35 and 38 kd) have been described to appear regularly during CNS toxoplasmosis in AIDS patients (21). These antigens may correspond to the bands at 32-33 and 36-37 kd found by us in almost all sera of patients with toxoplasmosis. Undisputed, however, is the fact that a high serum antibody titre is always connected with a wide antibody diversity, in AIDS patients as well as in immunocompetent persons (14, 19, 21). Control tests with sera of HIV-negative but Toxoplasma-positive persons give some support to our assumption that *Toxoplasma* infections in immunocompetent persons show a greater — but compared to each other more similar — variability of antibodies than infections of HIV patients (Fig. 2). Two of our patients (N.E., R.F.) were patients with a primary, fresh Toxoplasma infection. Similarly to observations of other authors (14, 19), we followed the development of antibody patterns with an increasing complexity during the course of the primary infection. In the serum of patient N.E., a primary antibody band appeared at 35 kd. A band recognizing an antigen with this molecular weight has been described by other authors as the primary antibody answer (14, 19).

The phenomenon of antibodies to *Toxoplasma gondii* occurring in sera of non-infected persons has been described; these antibodies reacted with a number of different antigens with molecular weights between 15 and 205 kd (13). In the sera of three patients (B.W., K.N., K.R.) who did not exhibit any clinical or serological signs of a *Toxoplasma* infection, we found antibodies against 34-38 kd antigens. This is exactly the antigen range to which the primary IgG response is elicited (14, 19). It is, however, very difficult and, strictly spoken, impossible to exclude a very old infection in these cases with absolute certainty. Thus, the 35 kd antibodies could possibly also be interpreted as remnants of the immune response during an infection before the onset of AIDS. On the other hand, it is noteworthy that these antibodies could not be detected in a patient (D.I) with an old infection.

In a previous study, we have described the appearance and the structure of circulating *Toxoplasma* antigens in sera of AIDS patients with toxoplasmosis (5). Circulating antigens consist of at least two proteins with molecular weights of 27 and 57 kd. As some of the patients shown in the present study produced circulating antigens, too, (Table 1), the question arises whether AIDS patients are still able to produce an immune answer to the circulating antigens. As can be seen in Fig. 1, antibodies directed against a 27 or a 57 kd antigen appear in the sera of most patients with toxoplasmosis

(B.D., J.G., K.K., L.K., N.E., R.F., S.M., U.W., V.K., V.S.). In some patients, such antibodies are produced promptly after or during the appearance of circulating antigens (B.D., K.K., R.F.). Thus, circulating antigens seem to be highly immunogenic, even in patients with AIDS.

Finally, it should be mentioned that we have observed amounts of IgG antibodies of more than 40 mg x $m1^{-1}$ in some sera of AIDS patients (7). Such an overwhelming IgG production is regularly found in AIDS patients (10, 12). This formation may alter the patterns of specific antibodies against *Toxoplasma gondii*, although there is no toxoplasmosis. We could, however, not detect any interference of this unspecific IgG stimulation with the serological surveillance conducted by us, neither an influence on the titres of the specific antibodies measured in the IFAT (7), nor on the antibody patterns (Fig. 1).

We conclude that in the sera of AIDS patients as well as in those of immunocompetent persons, there is a vast antibody diversity following toxoplasmosis. However, the interpretation of antibody patterns in the sera of AIDS patients is heavily influenced by the decrease of the antibody formation capacity. Therefore, for routine diagnosis, antibody titrations with established serological methods (such as IFAT) can be regarded as sufficient, if the problems well known in the serodiagnosis in AIDS patients are observed (5, 12). It is, however, out of question that the use of immunoblotting techniques is essential for clarification of many theoretical basic questions which are still open in the field of immunobiology of toxoplasmosis in man.

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