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Anti-HIV antibodies in the CSF of AIDS patients: a serological and immunoblotting study

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SUMMARY CSF and serum samples from 16 AIDS patients were tested for the presence of anti-HIV antibodies either by classical serological methods or by an immunoblot technique based on agarose gel isoelectric focusing and transfer of the specific IgG antibodies onto HIV antigens-loaded nitrocellulose sheets. This method enabled the demonstration of an intrathecal synthesis of anti-HIV oligoclonal IgG antibodies, often superimposed on diffuse polyclonal production, in 14 patients. The two negative cases were devoid of neurological signs or symptoms. However, two patients classified in stage II of the disease (asymptomatic infection) displayed an intrathecal synthesis of anti-HIV antibodies.

The involvement of the nervous tissue by the human immunodeficiency virus (HIV) is now firmly established, as this virus has been detected in brain cells and isolated from the CSF of seropositive patients with neurological disorders related to the acquired immune deficiency syndrome (AIDS).[1-4] The most frequent clinical picture is a progressive dementia characterised by impaired memory and concentration, psychomotor slowing and behavioural disturbances, followed in the most advanced stage of the disease by a severe loss of cognitive functions, mutism and incontinence.[5-7] Early motor deficits may occur, such as ataxia, leg weakness, and tremor. Other clinical manifestations of the central nervous system (CNS) involvement by this retrovirus include vacuolar myelopathy,[8] aseptic meningitis,[5] meningoradiculitis[5 6 9 10] and possibly cerebrovascular complications.[5]

In addition to the isolation of the virus from the CNS and CSF, an intrathecal synthesis of HIV antibodies was detected by enzyme-linked immunosorbent assays (ELISA), which showed a greater percentage of HIV-specific IgG in CSF than in serum.[11-13] We describe here the results of an immunoblot technique[14 15] which allowed us to detect HIV specific oligoclonal IgG bands and to demonstrate an intrathecal synthesis of these antibodies in 14 out of 16 AIDS patients.

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Patients

Sixteen HIV seropositive patients (15 males, 1 female; age 19–66 years) were investigated at the St-Luc Hospital; eight black and four white heterosexuals had been living in Central Africa; the four other whites were European homosexuals (table 1). They were classified in AIDS groups I to IV on the basis of the criteria of the Centers for Disease Control (CDC).[16] Two patients (stage II) were asymptomatic, one (stage III) presented with a persistent generalised lymphadenopathy (PGL) and 13 developed the full-blown clinical picture of AIDS (stage IV). All of these 13 patients exhibited various neurological signs or symptoms, with the exception of patient number 2. In addition, two of them suffered from necropsy-proved *Toxoplasma gondii* encephalitis (patients numbers 3 and 6). All patients but one (patient number 16, stage II) had a low lymphocytes absolute count and a low CD4/CD8 ratio (below 0·5) (table 2). All but three had high serum IgG levels (above 14 g/l, results not shown).

Materials and methods

Samples

CSF and serum samples were stored at −20°C. IgG concentration was determined by immunonephelometry and an IgG index calculated (CSF IgG/serum IgG:CSF albumin/serum albumin; upper reference value: 0·70).

Enzyme linked immunosorbent assay

The serum and CSF HIV antibodies were screened by an enzyme-linked immunosorbent assay (ELISA) manufactured by Pasteur Production or by Wellcome. In the first

Table 1 *Clinical data*

Patient no	Age (y)	Sex	Race	Homosexual	General examination	Neurological examination	Opportunistic infection	Stage
1	35	M	B	–	weight loss, fever, myalgia, cervical adenopathy.	left facial palsy, irritability.	–	IV
2	40	M	B	–	fever, generalised lymph-adenopathy, hepatosplenomegaly, severe weight loss.	normal	*Pneumocystis carinii* pneumonia.	IV
3	53	M	B	–	fever, asthenia, renal failure.	left hemiparesis of acute onset, seizure, drowsiness.	labial herpetic vesicles, buccal candidiasis, lung tuberculosis.	IV
4	59	M	W	–	normal	normal but previous history of transient speech difficulties and confusion.	cerebral toxoplasmosis. –	II
5	19	M	W	+	generalised lymphadenopathy, weight loss.	left facial palsy.	CMV in urine.	IV
6	30	M	B	–	weight loss, fever, Kaposi's sarcoma, lymphadenopathy.	right hemiparesis, seizure and progressive dementia.	cerebral toxoplasmosis.	IV
7	52	M	W	–	generalised lymphadenopathy.	right hemiparesis, myoclonus at the left hand.	CMV in urine.	IV
8	58	M	B	–	lymphadenopathy, hepato-splenomegaly, asthenia.	cerebellar ataxia and dysmetria, nystagmus, left facial palsy.	–	IV
9	46	M	B	–	fever, weight loss, generalised lymphadenopathy.	apathy, confusion, seizure, myoclonus, progressive dementia.	buccal and pulmonary candidiasis, Pseudomonas pneumonia.	IV
10	57	M	W	–	weight loss, diarrhoea, gut lymphoma.	progressive dementia, myoclonus.	buccal candidiasis, Herpes simplex virus in saliva.	IV
11	66	M	B	–	inguinal lymphadenopathy.	bradypsychia followed by drowsiness and coma.	–	IV
12	40	M	W	+	axillary lymphadenopathy, diarrhoea, severe weight loss.	apathy, bradypsychia.	CMV in stool, urine, saliva and oesophageal candidiasis.	IV
13	38	M	W	+	fatigue, Kaposi's sarcoma, diarrhoea, generalised lymphadenopathy.	confusion, disorientation and progressive dementia.	buccal candidiasis, CMV in urine.	IV
14	56	M	W	+	fever, weight loss, generalised lymphadenopathy.	confusion, drowsiness.	*Pneumocystis carinii* pneumonia, Herpes simplex virus in saliva.	IV
15	44	F	B	–	asthenia, generalised lymphadenopathy.	normal	–	III
16	58	M	W	–	normal	normal but complaints of dizziness, headache.	–	II

Table 2 *Biological data*

	Serum		*Cerebrospinal fluid*					
Patient No	*anti-HIV antibody ELISA N1<300*	*CD4/CD8 ratio*	*anti-HIV antibody ELISA N1<300*	*Cells/mm³ N1<5*	*Protein content N1<0·5g/l*	*Oligoclonal bands restricted to CSF on agar electrophoresis*	*IgG index N1<0·70*	*Oligoclonal anti-HIV band restricted to or predominant in CSF*
1	2137	0·41	755	11	0·34	+	0·84	+ + +
2	2545	0·08	1589	18	0·45	–	1·13	0
3	1668	0·45	1247	6	0·87	–	1·46	+ +
4	1789	0·34	539	3	0·41	–	0·76	+
5	2473	0·26	1017	36	1·02	+	0·86	+ +
6	1177	0·027	672	0	0·28	+	1·04	+ +
7	1573	0·15	993	5	0·39	+	0·92	+ + +
8	2495	0·28	1472	6	0·48	+	0·75	+ +
9	492	0·09	988	14	0·26	+	1·27	+
10	1623	0·06	1316	6	0·44	+	1·3	+ + +
11	1989	0·08	744	2	0·94	–	0·71	+ +
12	1981	0·063	877	0	0·30	–	0·64	+
13	1242	0·06	403	0	0·38	–	0·78	+ +
14	1061	0·35	529	0	0·23	–	0·61	+
15	2677	0·26	2288	6	0·50	–	0·46	0
16	2294	0·7	1050	29	0·44	–	0·92	+ +

one, samples were incubated with both viral and mock prepared antigens, the numerical result being the difference in optical densities. The second test was based on the use of viral antigen captured by specific antibodies attached to the well. In each case the presence of specific antibodies was confirmed by an indirect fluorescent antibody assay, performed on HIV infected CEM cells[17 18] and by Western blot analysis for sera.

Immunoblotting technique

Agarose gel plates were prepared with 0·36 g Isogel agarose-EF for electrofocusing (LKB 1818, Sweden) and 4·3 g sor-

bitol (Merk, W-Germany) containing 2 ml ampholine (pH range 3·5–9·5) (LKB).

Antigens were prepared from the supernatant of culture medium of CEM cells infected by LAV-1 (reference virus supplied by F Barre and J C Cherman, Institut Pasteur, Paris) which was filtered through a 0·45 μm filter and mixed with an equal volume of 0·2% Triton × 100. Control antigens were prepared from the same culture medium of non-infected cells. The nitrocellulose sheet was loaded with HIV antigens by overnight incubation at room temperature and washed in Tris buffered saline (TBS, Tris 20 mM, NaCl 500 mM, pH 7·5) containing 0·05% Tween (Technicon Diagnostics) for at least 45 min with three changes.

Sera were diluted in distilled water to the same IgG concentration as the paired CSF samples. Ten μl were then applied side by side and isoelectrically focused for 90 min at 10°C in a LKB Multiphor Unit. Unprecipitated proteins were removed by application on the gel surface of a filter paper moistened in phosphate buffered saline (PBS). Precipitated proteins were blotted onto the control antigens, or HIV antigens-loaded nitrocellulose sheet under a uniform weight of 1 kg for 90 min at 10°C. The immunoblot was then washed in TBS-Tween 0·05% and incubated with biotinylated anti-human IgG antiserum (Dako Lot 016, Copenhagen, Denmark) diluted 350 times in TBS containing 0·3% bovine serum albumin (BSA, Calbiochem) for 90 min at room temperature. After three washings for 15 min each in TBS-Tween 0·05%, the immunoblot was incubated with the streptavidin-biotin-peroxidase complex (Amersham, England) diluted 400 times in TBS containing 0·3% BSA. Staining was performed in presence of 4 chloro-1-naphtol (Biorad, England).

In addition, the total IgG pattern of the focused samples was revealed by a similar immunoblotting procedure on nitrocellulose sheet loaded with an anti-human IgG antiserum (Dako).

Results

CSF data (table 2)

The protein content was most often normal and was moderately increased in only three cases up to 1·02 g/l. A slight pleocytosis (range: 6 to 29 cells/mm^3) was observed in nine out of 16 patients. Agar gel electrophoresis revealed oligoclonal bands restricted to or more marked in CSF than in serum in only seven samples. The IgG index was increased in 13 out of 16 patients.

Anti-HIV ELISA

All patients were seropositive, and all native CSF contained significant titres of anti-HIV antibodies (table 2). However, such positive results in CSF did not enable a distinction to be made between passive transudation of anti-HIV antibodies from blood and local synthesis. We therefore tested paired serum and CSF samples after prior dilution to the same IgG concentration in both fluids. Three sera (Patients number 6, 9 and 13) became negative while the corresponding CSF remained positive. Six pairs of samples (Patients number 1, 2, 5, 6, 7 and 10) were further diluted and higher titres were observed in CSF from four of them (Patients number 1, 6, 7 and 10), indicating a possible local synthesis (fig 1).

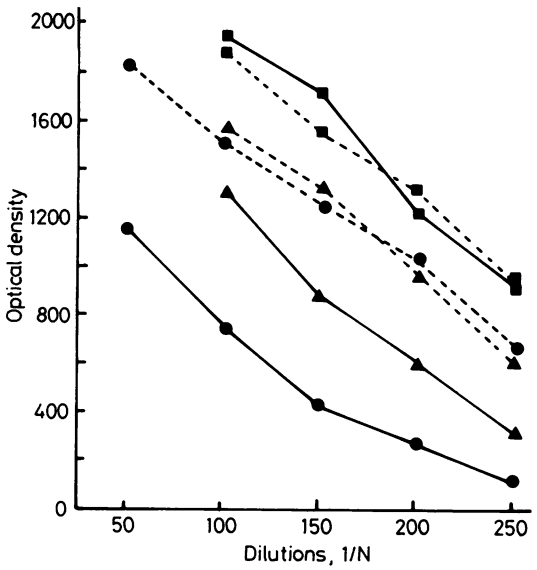

Fig 1 *Anti-HIV Elisa results from serial dilutions of three pairs of sera (continuous lines) and CSF (broken lines) at equal IgG concentrations. No differences were observed between CSF and serum from patient number 2 (■), indicating a similar antibody content. In contrast, CSF from patients number 6 (●) and number 7 (▲) contained higher amounts of antibodies than the corresponding sera, indicating a local production.*

Immunoblotting data

This technique enabled us to demonstrate a local synthesis of anti-HIV IgG in all but two patients (fig 2; table 2). No reaction was observed when the immunoblot sheet was coated with control antigens (results not shown). The locally produced antibodies in CSF had clear oligoclonal patterns, superimposed or not, on a diffuse polyclonal increase by comparison with the paired sera tested at the same IgG concentration. The occurrence of such a local synthesis was graded from 0 to + + + by visual inspection of the immunoblots (table 2). The two negative patients had similar oligoclonal bands in both serum and CSF after dilution at the same IgG concentrations ("mirror effect") and this ruled out an intrathecal synthesis of anti-HIV antibodies (for example, Patient 2, fig 2).

The two negative patients (Nos 2 and 15) had no neurological signs or symptoms. It should be noted that for Patient 16 who complained of only headache and dizziness and for Patient 4, who had transient speech difficulties and confusion, an intrathecal syn-

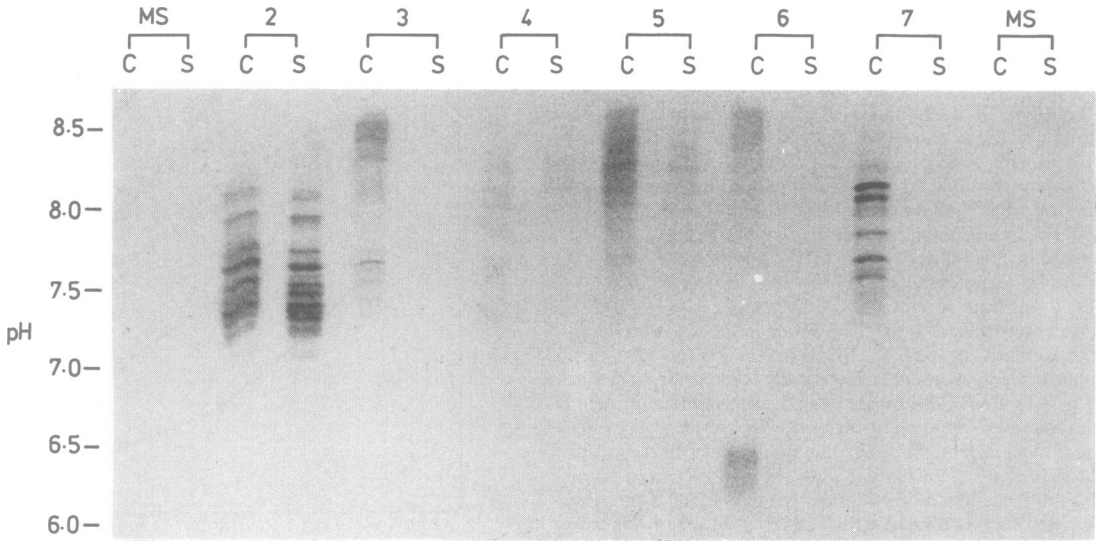

Fig 2 *Immunoblots of HIV-specific IgG from unconcentrated CSF (C) and the corresponding serum (S), diluted to the same CSF IgG concentration, after isoelectric focusing in agarose gel. Samples from two multiple sclerosis (MS) patients were used as controls. In lane 2 (Patient number 2), HIV-specific IgG oligoclonal bands are identical in CSF and serum ("mirror effect", i.e. no local production). In lanes 3 to 7 (Patients number 3 to 7), staining of HIV-specific oligoclonal and polyclonal IgG is restricted to the CSF or more clearly marked in CSF than in the corresponding serum.*

thesis of anti-HIV antibodies was demonstrated. However, the two patients were classified in stage II (asymptomatic infection), with a normal neurological examination.

When the pattern of all focused IgG was compared with the pattern of anti-HIV antibodies, it was obvious that numerous IgG bands were not anti-HIV antibodies. In contrast, faint IgG bands may have strong antibody activity (fig 3). Oligoclonal anti-HIV IgG bands were detected by immunoblotting in 7 CSF samples in spite of the absence of oligoclonal bands in agar gel electrophoresis (table 2).

Discussion

In normal conditions, CSF IgG originate from blood, as demonstrated by studies in humans with radiolabelled IgG.[19] The proportion of IgG antibodies against a given antigen should therefore be identical in both fluids in the absence of intrathecal synthesis. In contrast, when an immune reaction occurs within the CNS, the proportion of locally produced antibodies will be higher in CSF than in serum. Presence of anti-HIV antibodies in native CSF from all patients may be due to either passive transudation from blood across a normal or impaired blood-brain barrier, or a local synthesis within the CNS. In the first case, but not in the second one, proportions of specific anti-HIV antibodies will remain identical in CSF and serum. We therefore decided to test paired CSF and serum at the same IgG concentration, in order to compare directly the results of the ELISA tests and the immunoblots of both fluids.

From the immunoblots analysis, we were able to detect a local synthesis of anti-HIV antibodies in 14 out of the 16 patients under study. This high frequency is similar to results obtained by semi-quantitative methods.[12 13] It should be noted that neurological examination was normal for only four of our 16 patients (Nos 2, 4, 15 and 16). Patient 2 had high CSF anti-HIV titres, but in identical proportion in CSF and serum, as shown by serial dilutions of both fluids at equal IgG concentration on the one hand (fig 1) and by the "mirror effect" observed on immunoblots on the other (fig 2, lane 2). Similar findings were observed for Patient 15. A local production of specific antibodies was therefore ruled out in these two cases.

Patient 4 had a previous history of transient speech difficulties and confusion but was asymptomatic (stage II) when the CSF was collected. Perhaps these neurological symptoms were due to the initial infection, which may be coincident with an acute, self-remitting encephalopathy.[20 21] Patient 16 complained only of dizziness and headache but without objective signs and was also classified in stage II. However, both displayed an intrathecal synthesis of anti-HIV antibodies, as shown by the immunoblot technique.

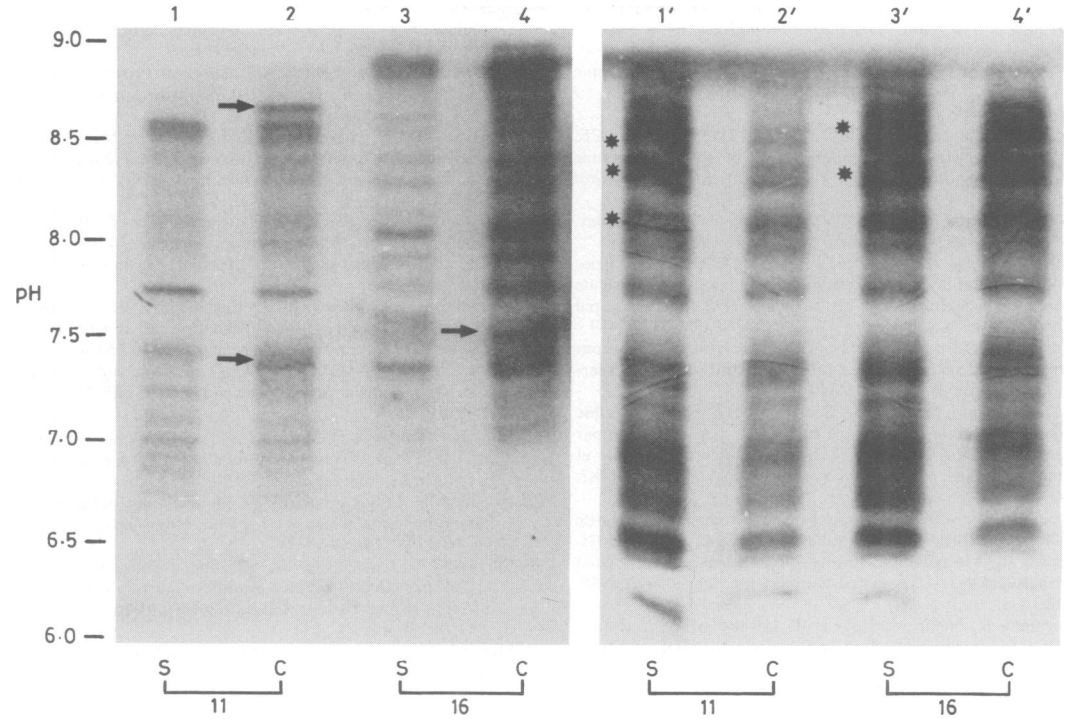

Fig 3 *Immunoblots of HIV-specific IgG CSF (C) and sera (S) of Patients 11 and 16 (left) and of all focused IgG of the same samples (right). Arrows indicate discreet anti-HIV oligoclonal bands restricted to the CSF. Asterisks indicate IgG bands with faint or no anti-HIV antibody specificity.*

The occurrence of such an intrathecal synthesis is likely to be due to the presence of the virus itself within the CNS. The recovery of the virus from the CSF would be very frequent, even in patients without neurological symptoms or disease.[22] From a clinical point of view, the two patients classified in stage II, but with local synthesis of antibodies, could be tentatively considered as being in stage IV of the disease and included in therapeutic trials.

The other CSF data were much less clinically relevant. The slight increase of the protein content and the pleocytosis observed in some patients, as already reported[23] was not correlated with the local synthesis of specific antibodies as demonstrated by our immunoblots study. The increase of IgG index was very frequent (13 patients out of 16) but it was impossible to establish whether this was due to synthesis of antibodies against opportunistic infectious agents or HIV itself. The occurrence of oligoclonal bands restricted to the CSF in agar gel electrophoresis was observed in only seven patients; this method is therefore definitely less sensitive than the immunoblot technique and did not enable the antibody specificity of such oligoclonal bands to be proved.

HIV infection is associated with an increase in B cells activation which results in a polyclonal production of immunoglobulins and hypergammaglobulinaemia.[24] This aberrant production may have also a mono- or oligoclonal pattern especially in patients with Kaposi's sarcoma when studied by high resolution technique as isoelectric focusing.[25] As shown in fig 3 all these oligoclonal immunoglobulins are not necessarily specific anti-HIV antibodies.

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