Characterization of Antibody Activity in Oligoclonal Immunoglobulin G Synthesized Within the Central Nervous System in a Patient with Tuberculous Meningitis

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Thin-layer polyacrylamide gel isoelectric focusing of cerebrospinal fluid (CSF) and serum obtained from one patient 48 and 65 days after the onset of tuberculous meningitis revealed five oligoclonal immunoglobulin zones in CSF without any counterpart in serum, indicating local immunoglobulin production. Subsequent immunofixation with specific antisera revealed that three of the zones consisted of immunoglobulin G lambda, and two consisted of immunoglobulin G kappa and immunoglobulin G lambda present simultaneously. Immunofixation with Mycobacterium tuberculosis and bacillus Calmette-Guérin (BCG) as antigens and autoradiography revealed zones of specific antibodies in the CSF which, regarding mobility, corresponded to oligoclonal and polyclonal CSF immunoglobulin G zones. No antibody activity was detectable in the corresponding serum, indicating that the antibodies present in CSF were synthesized within the central nervous system. In seven control patients (three with multiple sclerosis, four with chronic inflammatory central nervous system diseases of unknown cause) with oligoclonal CSF immunoglobulin, no evidence for local production of antibodies against M. tuberculosis or BCG was detectable. Immunofixation with M. tuberculosis or BCG as antigens and autoradiography may prove to be a useful diagnostic complement to conventional techniques in patients with suspected tuberculous meningitis.

Although cell-mediated immunocompetence is of the utmost importance for the course and outcome of human tuberculous infections, the humoral immune response might also play an important role. In tuberculous meningitis (TM), an intensive humoral immune response within the central nervous system (CNS) and characterized by the intrathecal synthesis of oligoclonal immunoglobulins may occur (2; J. Kinnman, A. Frydén, S. Eriksson, E. Möller, and H. Link, Scand. J. Immunol., in press). The phenomenon of local immunoglobulin synthesis is characteristic of a great variety of inflammatory disorders affecting the CNS (for a review, see reference 5). The antibody character of locally produced oligoclonal immunoglobulin G (IgG) has been defined in some of these disorders, e.g., in subacute sclerosing panencephalitis (8, 13), whereas in others, such as multiple sclerosis, the specificity of oligoclonal IgG synthesized within the CNS has been only partly clarified (R. Roström, H. Link, M. A. Laurenzi, S. Kam-Hansen, E. Norrby, and B. Wahren, Ann. Neurol., in press).

Characterization of locally produced immunoglobulin in etiologically well defined inflammatory CNS diseases, such as TM, is of importance for two reasons: (i) identification of the antibody detectable in locally produced immunoglobulin should be evaluated as a diagnostic tool and, therefore, its specificity and its appearance as well as disappearance during the course of disease should be established and (ii) careful analysis of, inter alia, the temporal profile of the immunoglobulin synthesized within the CNS regarding amount, class, and antibody specificity may increase our understanding of the local immune response in disorders of hitherto unknown pathogenesis, such as multiple sclerosis.

In this paper, oligoclonal immunoglobulin produced within the CNS in one patient with TM is characterized regarding class, light chain type, and antibody specificity. Using antigen immunofixation and autoradiography, it is shown that some of the oligoclonal IgG zones detectable in the patient’s cerebrospinal fluid (CSF) by thin-layer polyacrylamide gel (PAG) isoelectric focusing (IEF) contain antibodies directed against Mycobacterium tuberculosis and against bacillus Calmette-Guérin (BCG).

MATERIALS AND METHODS

Subjects. (i) Patient with TM. A previously healthy 33-year-old woman contracted signs and symptoms suspected to be due to TM on 21 March
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1976. The diagnosis was confirmed by identification of *M. tuberculosis* by culture of three consecutive CSF specimens taken on 4, 9, and 20 April. Direct microscopy did not reveal *M. tuberculosis* in the CSF. The patient's condition improved after treatment with tuberculostats instituted on 11 April. Steroids were not given. She was discharged completely recovered 3 months later. Lumbar punctures were carried out on several occasions after admission, and a serum specimen was always taken simultaneously (see Table 1).

(ii) Control patients. CSF and serum for antigen immunofixation were also obtained from three patients with clinically definite multiple sclerosis and four patients with chronic meningoencephalitis of unknown etiology. Repetitive CSF cultures for *M. tuberculosis* in the four latter patients were negative. All seven patients were ambulatory. They displayed oligoclonal immunoglobulin on routine agarose gel electrophoresis of CSF.

Routine CSF and serum studies. After cell counting by phase-contrast microscopy, the CSF was centrifuged. Albumin, IgG, and IgA were determined simultaneously in the CSF and serum, as were the CSF/serum albumin ratio as a variable for the blood-brain barrier function and the CSF IgG index [i.e., the ratio (CSF IgG/serum IgG):(CSF albumin/serum albumin)] and the corresponding CSF IgA index as variables for the demonstration of intrathecal synthesis of IgG and IgA, respectively. The age-dependent normal values previously described for the CSF/serum albumin ratio were used (11). The upper normal values for the CSF IgG index and the CSF IgA index in our laboratory are 0.7 and 0.6, respectively. Elevated values indicate synthesis within the CNS of the immunoglobulin in question (9). The IgG synthesis rate within the CNS was also calculated (12). Our upper normal value is 10 mg/24 h.

Thin-layer PAG IEF. IEF was carried out on Ampholine-prepared thin-layer PAGs (PAG plates; LKB, Stockholm, Sweden), pH range 3.5 to 9.5, as previously described (3). In short, CSF concentrated by ultrafiltration at 4°C in colloidium bags (Membranfilter; Sartorius, Göttingen, Federal Republic of Germany) to an IgG level of about 3 g/liter and serum diluted with 0.05 M NaCl to the same IgG level were applied to the gel at a pH between 6.2 and 6.4 at amounts of 10 μl, corresponding to about 30 μg of IgG. On every plate pooled blood donor serum diluted with 0.05 M NaCl to an IgG level of about 3 g/liter was run as a reference, and carboxy-hemoglobin was run as a marker. The protein patterns obtained on PAG IEF of CSF were compared with those of the corresponding serum and of normal serum. To define the results, the pH range was divided into three regions: I, pH 3.5 to 6.4; II, pH 6.5 to 8.6; III, pH 8.7 to 9.5. Zones found in CSF in pH regions II and III in addition to those seen on PAG IEF of the corresponding serum run in parallel were considered to represent oligoclonal immunoglobulin (3, 4a).

The investigations carried out on CSF and serum after PAG IEF are summarized in Fig. 1.

Immunofixation with specific antisera. For the characterization of immunoglobulin bands (regarding class and light chain type) found on PAG IEF, im-

Antiserum immunofixation for definition of class and light chain type of immunoglobulin zones

PAG IEF carried out on CSF and serum obtained simultaneously

Incubation with cellulose acetate strips dipped in monospecific antiserum

Incubation with antigen-containing agarose gel plates

Specific antigen-antibody complexes are formed in the PAG plate

Autoradiography for detection of antibodies complexed in the agarose gel plate

Comparison of zone patterns with those from reference run of CSF and serum

Fig. 1. Scheme of procedures used for classification of oligoclonal immunoglobulin zones demonstrable by thin-layer PAG IEF of CSF and serum from a patient with TM.

Immunofixation was done after the run, using monospecific antisera (Dakopatts, Copenhagen, Denmark) against Y chains, a chains, and kappa and lambda Bence-Jones proteins (4, 4a).

Antigen immunofixation and autoradiography. (i) *M. tuberculosis* and BCG. *M. tuberculosis* strain 37 (kindly supplied by B. Wahren, National Bacteriological Laboratory, Stockholm, Sweden), inactivated by heating to 100°C and freeze-dried, was diluted in 0.05 M NaCl, BCG (National Bacteriological Laboratory), freeze-dried, was similarly diluted. Before use in the antigen immunofixation experiments, the preparations of *M. tuberculosis* and BCG were sonicated in the cold with a 150-W Ultrasonic Disintegrator (mark 2; MSE, Crawley, England) with a 3-mm exponential probe at an amplitude of 12 to 14 μm for 10-s bursts and centrifuged at 3,000 × g for 10 min at room temperature. The supernatants thus obtained were used as antigens in antigen immunofixation. Three different concentrations of the antigens were used, namely, 400, 50, and 5 μl, respectively, of *M. tuberculosis* per 5 ml of agarose gel, and 500, 50, and 5 μl, respectively, of BCG per 5 ml of agarose gel.

(ii) Viral antigens. Ten percent (vol/vol) suspensions of the Lec strain of measles virus cultivated in Vero cells, complement fixation titer of 160 (kindly supplied by E. Norrby, Karolinska Institute, Stockholm, Sweden); herpes simplex virus (HSV) type 1 cultivated in baby hamster kidney cells, complement fixation titer of 320 (E. Norrby); concentrated extracellular mumps virus propagated in chick oviduct membranes, complement fixation titer of 160 (D-449; Orion, Helsinki, Finland); and Mycoplasma pneu-
moniae, complement titer of 32 (D-433, Orion), were kept at \(-20^\circ\)C until used as reference antigens in the antigen immunofixation experiments. Before use, the viral antigen preparations were sonicated as described above for three 10-s bursts and centrifuged for 10 min at 1,500 \(\times g\) at room temperature. The supernatants were used as antigens in antigen immunofixation. The final concentrations per 5 ml of agaroase gel were 150 \(\mu l\) for measles virus preparation, 10 \(\mu l\) for HSV, 50 \(\mu l\) for mumps virus, and 200 \(\mu l\) for P. moniae. The concentration of each type of antigen per 5 ml of gel was determined in calibration experiments.

(ii) Detection of antibodies by antigen immunofixation and autoradiography. CSF and the corresponding serum were run in duplicate on PAG IEF, one specimen being used for detection of antibodies in immunoglobulin zones, the other as reference. The presence of specific antibodies was detected by antigen immunofixation followed by incubation with \(^{125}\)I-labeled (code IMS 30; Radiochemical Centre, Amersham, England) rabbit anti-human immunoglobulin antibodies (code A-107, Dakopatts) and autoradiography as described elsewhere in detail (10; Ros trom et al., Ann Neurol., in press). In short, the PAG IEF plate to be used for antibody detection was quickly washed with phosphate-buffered saline, pH 7.2 to 7.4, immediately after IEF. The antigen-containing agarose gel plates were placed with the gel in direct contact with the PAG IEF plate, with a thin layer of phosphate-buffered saline in between. Only the pH region 6.5 to 9.5, i.e., pH regions II and III, was covered. PAG IEF and antigen-containing agarose gel plates were kept together for 5 to 8 min, during which time antibodies moved to the agarose gel plates to form antigen-antibody complexes. Up to five different antigen plates were successively placed on the same PAG IEF plate. After washing the plates in phosphate-buffered saline overnight and repeatedly pressing them under filter paper (10 min) and washing them in PBS (15 min), the plates were incubated with \(^{125}\)I-labeled rabbit anti-human immunoglobulin antibody for 12 h. The plates were then washed in phosphate-buffered saline, pressed under filter paper three times, and finally washed in distilled water, pressed, and air dried. The antibodies precipitated on the antigen-containing agarose gel plates were indicated by autoradiography carried out for 6 to 36 h with Kodak X-Omat L. To avoid artefacts on autoradiography, the first plate to be used for antigen immunofixation contained only agarose (agarose control plate). Some autoradiograms obtained from such plates revealed faint zones mostly in the vicinity of the application slit, which corresponded to protein zones seen on PAG IEF. The occurrence of such artefacts was efficiently prevented by a quick phosphate-buffered saline wash of the PAG IEF plates immediately after the run and before subsequent antigen immunofixation.

The zone patterns obtained at autoradiography were compared with those from PAG IEF of the corresponding CSF and serum run in parallel as references and also with those obtained after Coomassie blue staining of the PAG IEF plate after it had been used for antigen immunofixation. Intrathecal antibody synthesis was concluded to occur when (i) one or more of the zones on autoradiography were observed in CSF only but not in the patient’s serum and (ii) one or more of the zones on autoradiography were distinctly more pronounced in CSF than in the patient’s serum.

**RESULTS**

The patient with TM displayed throughout the observation period—even at the time when she had recovered clinically—evidence of intrathecal synthesis of IgG as reflected by elevated CSF IgG index values and increased IgG synthesis rate per 24 h (Table 1). The CSF IgA index was elevated between days 48 and 97, indicating IgA synthesis within the CNS. On routine agarose gel electrophoresis, two oligoclonal immunoglobulin bands were found in all four CSF specimens, but not in the corresponding sera.

**Immunoglobulin class and light chain type of oligoclonal zones found in CSF in the patient with TM.** PAG IEF of CSF and serum from the patient 48 and 65 days after the onset of symptoms due to TM revealed five zones in CSF in pH region III, i.e., pH 8.7 to 9.5, without any counterpart in the corresponding serum, reflecting the presence of oligoclonal immunoglobulin. Immunofixation with monospecific antisera revealed that all five zones consisted of IgG, three of them with light chains of the lambda type and the remaining two with light chains of types kappa and lambda present simultaneously (Fig. 2).

**Demonstration of antibody synthesis within the CNS.** Antigen immunofixation of CSF and serum obtained on days 48 and 65 after the onset of TM (Table 1), using *M. tuberculosis* as the antigen, revealed on the autoradiogram five zones in the CSF specimen from day 48 and three zones in the CSF specimen from day 65, but no zones in the corresponding serum (Fig. 3). These zones thus represented antibodies against *M. tuberculosis* synthesized within the CNS. Similar zone patterns present only in CSF were also obtained when BCG was used as the antigen.

Two of the zones of antibodies against *M. tuberculosis* and BCG found on the autoradiograms of CSF corresponded regarding mobility to two of the five zones identified as oligoclonal IgG. The remaining *M. tuberculosis*-specific antibody zones from the autoradiograms corresponded to polyclonal IgG zones.

With HSV, measles virus, mumps virus, and *P. moniae* as reference antigens, the only abnormality found was one zone of HSV antibodies in CSF and serum obtained on day 48 and two zones of HSV antibodies in CSF and serum obtained on day 65. These zones were
### Table 1. CSF findings in a patient with TM

<table>
<thead>
<tr>
<th>Days after onset of disease</th>
<th>$10^6$ Leukocytes/liter (polymorphonuclears/mononuclears)</th>
<th>CSF/serum albumin ratio</th>
<th>CSF IgG index</th>
<th>IgG synthesis rate/24 h (mg)</th>
<th>CSF IgA index</th>
<th>CSF/serum glucose ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>102 (14/88)</td>
<td>26.0</td>
<td>0.8</td>
<td>51</td>
<td>ND</td>
<td>0.2</td>
</tr>
<tr>
<td>48</td>
<td>58 (1/57)</td>
<td>14.7</td>
<td>1.2</td>
<td>54</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>65</td>
<td>38 (0/38)</td>
<td>16.0</td>
<td>1.1</td>
<td>43</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>97</td>
<td>13 (0/13)</td>
<td>9.5</td>
<td>1.0</td>
<td>19</td>
<td>0.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Normal value, ≤6.8.
* Normal value, ≤0.7.
* Normal value, <10 mg.
* Normal value, ≤0.6.
* Normal value, >0.5.
* ND, Not done.

All runs were repeated on another occasion, using the same antigens, with the same results.

For reference, CSF and the corresponding serum from each of seven patients with other neurological disorders and oligoclonal immunoglobulin in CSF were also investigated by immunofixation with *M. tuberculosis* and BCG as antigens and subsequent autoradiography. No zones of intrathecally synthesized antibodies against either *M. tuberculosis* or BCG were demonstrable on the autoradiograms.

### DISCUSSION

TM is a rare disease in Scandinavia, and therefore material from only one patient was available for this investigation. From previous studies it is known that oligoclonal immunoglobulin bands may appear in CSF in TM already during the first days of the disease (E. C. Laterre, personal communication). However, oligoclonal immunoglobulin is probably not found in CSF in every patient with TM. We have found this in 1 out of 2 patients (Kinnman et al., in press), and Laterre has found this in 5 out of 13 patients (2).

Immunofixation with monospecific antisera revealed a predominance in number of oligoclonal IgG lambda bands in the CSF from our patient with TM. A predominance of IgG lambda has previously been demonstrated in acute aseptic meningitis with oligoclonal bands in CSF (1, 7), whereas a predominance of IgG kappa has been registered in multiple sclerosis (7). It is therefore possible that the oligoclonal immune response within the CNS, with respect to intrathecal production of predominantly IgG kappa or IgG lambda, differs in various inflammatory CNS diseases.

Two of the five oligoclonal IgG zones demonstrable on PAG IEF of CSF obtained on two different occasions from our patient with TM contained antibodies directed against *M. tuberculosis* as well as BCG. These antibody zones

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**Fig. 2. Patterns from thin-layer PAG IEF and subsequent immunofixation of CSF (A through C) and serum (D) from a patient with TM meningitis, using antisera against $\gamma$ chain (A and D) and Bence-Jones proteins type kappa (B) and type lambda (C). Arrows denote oligoclonal IgG zones.**

much stronger in CSF than in serum, indicating intrathecal synthesis. The mobilities on the autoradiograms of the HSV-specific zones differed from those of the *M. tuberculosis* and BCG-specific zones.

The zones of HSV antibodies had their counterparts in polyclonal IgG zones in CSF and serum.
Fig. 3. Patterns from thin-layer PAG IEF of CSF and serum from a patient with TM obtained 48 (A and B) and 65 (G through H) days after onset and the corresponding autoradiograms obtained after immunofixation with M. tuberculosis (C, D, I, and J) and BCG (E, F, K, and L). Arrows on A and G denote zones of oligoclonal IgG present in CSF only, whereas the remaining arrows denote zones of intrathecally synthesized antibodies which corresponded to some of the patient’s oligoclonal CSF IgG zones found at pH values above 8.6 (pH region III) and to some of the polyclonal CSF IgG zones. These zones were easily distinguished on the gel plates, but were not reproduced with desirable sharpness in the photos.

were not found in the corresponding serum specimens, and they were therefore considered to represent specific oligoclonal IgG antibodies synthesized within the CNS. However, not all oligoclonal IgG zones detectable by PAG IEF of CSF from our patient with TM were identified as containing specific antibodies against M. tuberculosis, and their antibody character remained unknown. Furthermore, intrathecally synthesized antibodies against M. tuberculosis were also found in polyclonal IgG zones. The occurrence of intrathecally synthesized specific antibodies migrating in polyclonal IgG zones has also been observed in, e.g., subacute sclerosing panencephalitis (J. Shorr, B. Rostrom, and H. Link, J. Neurol. Sci., in press).

It is not known when the oligoclonal IgG started to appear in the CSF of our patient with TM, but the earliest available specimen, from day 14 after onset, revealed oligoclonal IgG bands which were also found in the specimen from day 97, when the patient had recovered clinically. Nor is it known how long the oligoclonal response in CSF may persist after onset of TM. CSF and serum obtained from a completely recovered patient 1 year after the onset of TM and studied by immunofixation with M. tuberculosis and BCG as antigens and by autoradiography did not reveal any intrathecal antibody synthesis. This patient had normal findings on routine CSF studies on this occasion, but it is not known whether he had ever had an oligoclonal immunoglobulin response within the CNS (unpublished data).

The significance of the finding of HSV antibodies synthesized within the CNS and migrating in polyclonal IgG zones in our patient with TM is not known. Intrathecally produced HSV antibodies migrating in polyclonal IgG zones may be found in patients with multiple sclerosis and also in a few normal controls (Rostrom et al., Ann. Neurol., in press). It may reflect the effect of polyclonal B-cell activation.

The diagnosis of TM is difficult and can still only be confirmed by culture of CSF, giving results after 4 to 6 weeks. Thus, therapy must be instituted on the basis of clinical findings and unspecific laboratory data. Antigen immunofixation and autoradiography introduced by Nordal et al. (10) have the advantage of being rapid. PAG IEF as the initial step for protein separation and identification of oligoclonal immuno-
globulin zones (Roström et al., Ann. Neurol., in press) is superior to the agarose gel electrophoresis initially used (10), because PAG IEF has higher sensitivity and reproducibility (Roström et al., Ann. Neurol., in press). The possibility of immunofixation with M. tuberculosis or BCG, and subsequent autoradiography, as a diagnostic tool should be tested on larger numbers of patients with TM in order to define, inter alia, the frequency of positive results and the earliest appearance of them. Furthermore, although the seven patients included as controls in the present study were negative regarding presence of intrathecal production of antibodies against M. tuberculosis and BCG, additional patients should be studied in order to establish the specificity of the test.

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LITERATURE CITED