Detection of Xanthochromia in Cerebrospinal Fluid, *Ainsley H. Chalmers* and Michelle Kiley* (Departments of Haematology and Neurology, Flinders University Medical Centre, Bedford Park, South Australia 5042, Australia; * author for correspondence: Department of Haematology, Flinders University Medical Centre, Bedford Park, South Australia 5042, Australia; e-mail Chalmers.AH@Flinders.edu.au)

Lumbar puncture (LP) still has an important role to play in the diagnosis of subarachnoid hemorrhage. Although computed tomography (CT) scanning has replaced LP as the investigation of first choice, within 24 h of ictus 5% of cases will show no evidence of hemorrhage on CT scanning; this percentage is as high as 50% by 1 week, 30% after 2 weeks, and 0% after 3 weeks *(1)*.

By definition, xanthochromia is the yellow discoloration indicating the presence of bilirubin in the cerebrospinal fluid (CSF) and is used by some to differentiate in vivo hemorrhage from a traumatic LP. In contrast to CT, CSF xanthochromia is present in all patients up to 2 weeks postictus and is still present in 70% of patients at 3 weeks *(1, 2)*. A minimum period for CSF bilirubin detection is 12 h postictus *(2)*. Thus, the detection of bilirubin in CSF appears to be the test of choice at late time points. Spectrophotometry of CSF in the visible region is, in general, considered more sensitive than visual examination, with peaks at 415 and ~440–460 nm indicating the presence of hemoglobin (Hb) and bilirubin, respectively *(1, 2)*. The problem with this test is that it is not known which CSF bilirubin absorbance indicates a clinically significant bleed. We have developed a simple quantitative method that attempts to address this question.

CSF was collected aseptically by LP for routine biochemical and microbiological investigations. The supernatants from CSF specimens microcentrifuged at 13 000*g* for 1 min were scanned between 360 and 800 nm in a spectrophotometer (Model 7500, Beckman Instruments). In most CSF samples, volumes collected were <500 μL and were scanned in 100-μL microcuvettes with a 1-cm light path. Volumes ≥500 μL were scanned in 1.5-mL cuvettes. After a scan was completed, the spectrum was autoscaled, and tangents were drawn from ~530 to 360 nm (Fig. 1). Perpendiculars to this tangent were measured in mm at 415 nm for Hb (a) and 440 nm for bilirubin (b). For pure bilirubin, this ratio of a/b was 1, whereas for Hb it was 8. In our scans, full-scale absorbance was represented by an 82-mm scale; hence the absorbance of Hb was a/82 multiplied by the full-scale absorbance, and the absorbance of bilirubin was b/82 multiplied by the full-scale absorbance. Because Hb increases the absorbance of bilirubin at 440 nm, the following correction to bilirubin absorption was made:

$$\textit{net bilirubin absorbance} = \frac{b/82 \times \textit{full-scale absorbance} \times (8 - a/b)}{7}$$

where 7 is 8 (a/b ratio for pure Hb) minus 1 (a/b ratio for pure bilirubin). Thus $(8 - a/b)/7$ allows for the contribution of the Hb absorbance at 415 nm to the bilirubin absorbance at 440 nm. The net bilirubin absorbance was used to quantify the xanthochromia.

In this study we looked at three groups: (*a*) controls representing 16 patients who underwent elective CT myelography, (*b*) patients considered positive xanthochromia with net bilirubin absorbances >0.015, and (*c*) patients with equivocal values with net bilirubin absorbances from 0.005 to 0.014.

In our control samples, net bilirubin absorbances ranged from 0 to 0.007, with a mean of 0.0023. The five patients with absorbances >0.015 all had subarachnoid hemorrhage: four because of aneurysmal rupture (proven angiographically) and one because of severe head injury. In our experience, at least three of these patients had a negative result for xanthochromia by direct inspection. Hb absorbances of these specimens, with net bilirubin absorbances in parenthesis, were 0.022 (0.022), 0.047 (0.026), 1.27 (0.93), 0.22 (0.18), and 0.2 (0.048). Thus, the Hb absorbances were variable and in four of the five samples were no different to those found in control specimens (range, 0–0.35; mean, 0.077 ± 0.1 SD).

Seven patients had net bilirubin absorbances between 0.005 and 0.014. Of these, four showed no abnormalities present by CT head scans and cerebral angiography. The other three did not undergo angiography, but were considered by a consultant neurologist to have migraine. At this time, we report values between 0.01 and 0.015 as borderline positive and values >0.015 as positive.

The scans of six patients shown in Fig. 1 were typical of the scans obtained. In patients A and C, considerable Hb was present, but only patient C was reported and confirmed clinically as positive for xanthochromia. With patient B, the 415/440 ratio was close to unity, indicating almost pure bilirubin, but the absorbances were low enough to consider this patient's result as being negative. In patients D, E, and F, although the absorbances of Hb were similar (0.022), the net bilirubin absorbances and consequent clinical reports were different: 0.006 (negative), 0.003 (negative), and 0.022 (positive), respectively.

The report that one can use a CSF Hb absorbance at 415 nm of >0.023 as a possible index of intracranial hemorrhage *(3)* is not tenable. For example, in one of our positive samples, the absorbance at 415 nm was 0.022. In addition, the finding that CSF proteins >1 g/L can produce absorbances >0.023 at 415 nm would suggest that absorbance measurements solely at 415 nm would yield low sensitivity in relation to determining xanthochromia *(4)*. It makes good sense, therefore, to do a full spectral scan because it is the spectral hump between 440 and 460 nm that is clinically significant for xanthochromia.

One CSF specimen kept at room temperature (23 °C) for 48 h gave absorbances for Hb of 0.03, 0.09, and 0.31 at 0, 24, and 48 h, respectively. The absorbance due to

bilirubin was 0 over these 3 days, suggesting that CSF does not need to be analyzed immediately, because there was no detectable alteration of Hb to bilirubin within the CSF after collection. This is consistent with the finding that Hb requires the enzyme hemoglobin oxidase present in macrophages within the arachnoid and choroid plexus to be converted to bilirubin *(5)*.

Addition of bilirubin (Sigma Chemical Co.) in increasing amounts to CSF (net bilirubin absorbances, 0.024–0.3) yielded a recovery of bilirubin calculated by this method of 98.2% (± 6.0%, n = 9). A quality-control sample was prepared by diluting serum 100-fold in water and adding a known amount of bilirubin. The interday CV of this quality-control sample analyzed by three separate ana-

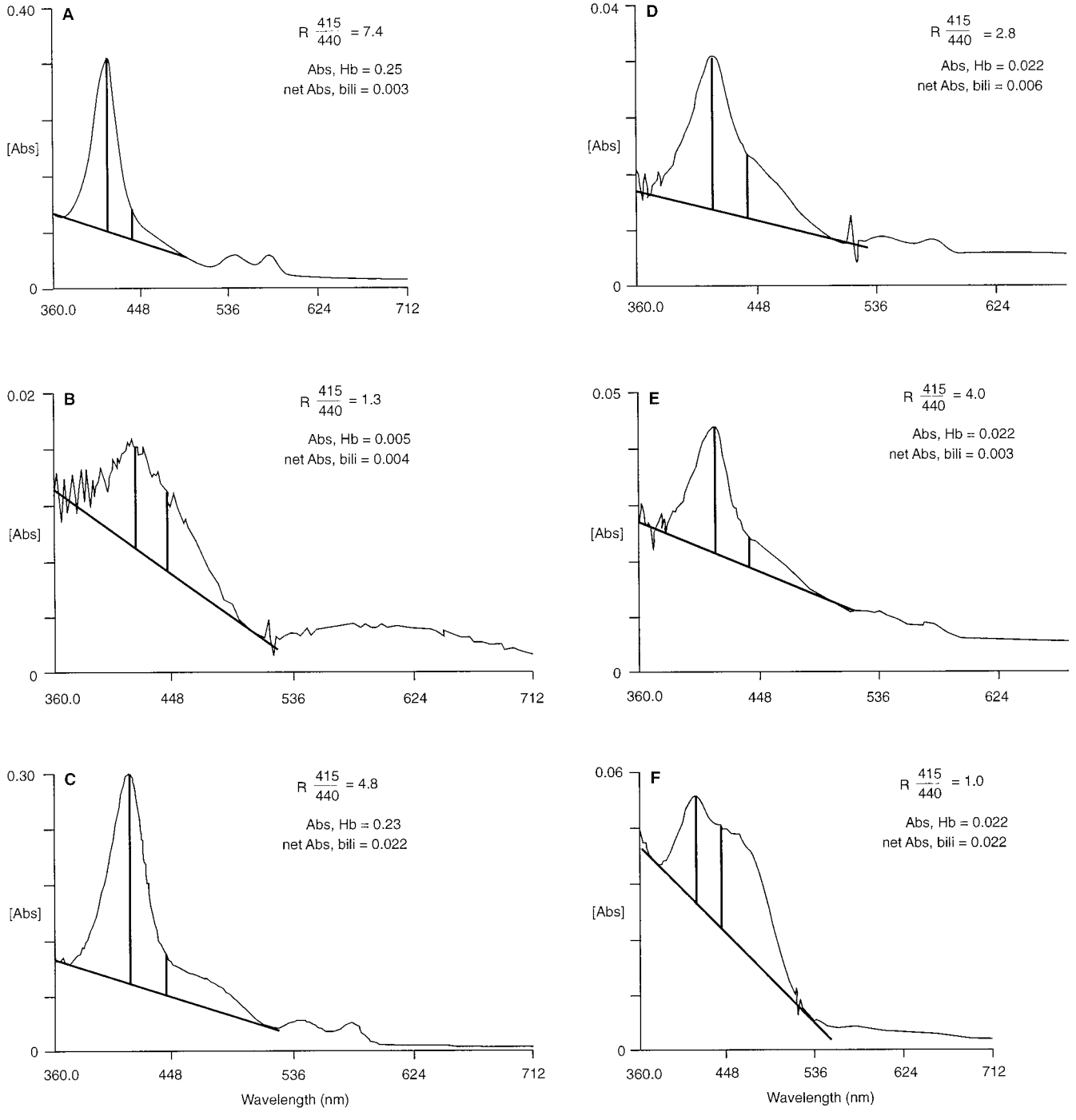

Fig. 1. Spectrophotometric scans on CSF specimens from six patients with suspected intracranial hemorrhages.
Drawn tangents (*vertical lines*) are the absorbances at 415 and 440 nm.

lysts 13 times over a 1-month period was 6.0% (mean net bilirubin absorbance, 0.0346 ± 0.0021). The intraday CV was 3.3% on this sample (mean absorbance, 0.0377 ± 0.00123, n = 12).

The correlation between serum bilirubin and CSF bilirubin was −0.058 (n = 31), indicating that the CSF bilirubin absorbance was not because of diffusion of bilirubin into the CSF or a consistent contamination of the CSF with vascular blood during the LP. In all our positive results to date, the clinical follow up has confirmed positive xanthochromia.

In conclusion, this test is simple, inexpensive, can easily be interpreted, and thereby overcomes the confusion in diagnosing subarachnoid hemorrhage *(6–8)*. We have used it for the last 20 months in over 50 patients, and it has proven clinically useful.

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Interferences with Two-site Immunoassays by Human Anti-mouse Antibodies Formed by Patients Treated with Monoclonal Antibodies: Comparison of Different Blocking Reagents, *Jochen Reinsberg* (Zentrum für Frauenheilkunde und Geburtshilfe, Universität Bonn, Sigmund-Freud-Strasse 25, D-53127 Bonn, Germany; fax 49-228-2874651)

The most widely used approach for reducing interferences with two-site immunoassays by human anti-mouse antibodies (HAMAs) developed by many patients after exposure to murine immunoglobulins is to include high amounts of nonspecific mouse IgG within the assay buffer *(1, 2)*. Recently, Mössner et al. *(3)* have developed a polymerized form of murine IgG (MAK33), which they found to be superior to normal mouse IgG for blocking HAMA interferences. In contrast, we observed false-positive values for the cancer antigen 125 (CA 125), because of HAMA interferences in samples from ovarian cancer patients treated with the anti-CA-125 antibody OC125, which could be corrected by preincubation with mouse IgG but not with MAK33 *(4)*. The aim of the present study was to examine whether treatment with other monoclonal antibodies gives rise to additional HAMAs that are insensitive to MAK33 and to further characterize this specific HAMA response.

Sixty-four serum samples were obtained from 51 ovarian cancer patients who, in the course of several clinical studies *(5–8)*, had received multiple infusions of one of four murine monoclonal antibodies: OC125 (16 patients), B43.13 (8 patients), ACA125 (18 patients), and B72.3 (9 patients). The procedures followed in this study were in accordance with the standards of the ethical committee of our faculty. The interfering HAMA activity of the samples was quantified with a bridging HAMA assay (HAMA-ELISA medac, Medac) involving polyclonal murine IgG in both capture and detection steps before and after preincubation (30 min at room temperature) with the following: (*a*) polyMAK-33 (MAK33), a polymerized murine $IgG_{1,\kappa}$ preparation (gift of Boehringer Mannheim, Mannheim, Germany); (*b*) Immunoglobulin Inhibiting Reagent (IIR) a formulation of immunoglobulins targeted against HAMAs (gift of Bioreclamation, East Meadow, NY); (*c*) polyclonal mouse IgG (reagent grade, Sigma Chemical Co.); and (*d*) mouse IgG of different subclasses, purified from myeloma proteins (Sigma) of the plasmacytoma cell lines UPC-10, HOPC-1, MOPC-141, and FLOPC-21.

Comparison of blocking reagents. In 35 samples, the interfering HAMA activity (ranging from 1162 to 55 397 μg/L) could be blocked completely by each of the three blocking agents (HAMA activity <100 μg/L after preincubation with 0.7 g/L MAK33, 2 g/L IIR, or 0.7 g/L polyclonal mouse IgG). In contrast, in 29 samples, the interfering HAMA activity (ranging from 1160 to 1 648 000 μg/L) could be eliminated only by IIR and polyclonal mouse IgG, respectively, whereas after preincubation with MAK33, increased HAMA values were still measured (Fig. 1). Residual HAMA activity insensitive to MAK33 was observed in most samples obtained from patients treated with OC125 fragments (16 of 18) compared with a considerably lower incidence in the other treatment groups (5 of 16 treated with B43.13, 4 of 19 treated with ACA125, and 4 of 11 treated with B72.3).

Specificity of the HAMA response insensitive to MAK33. To characterize the specificity of the HAMAs insensitive to MAK33 and to clarify how far they are directed against isotypic determinants not expressed on the $gamma_1$ heavy or the kappa light chain presented by MAK33, six samples (three OC125 patients, two ACA125 patients, and one B72.3 patient) were preincubated with supplementary 0.3 g/L murine IgG of different subclasses in the presence of 0.7 g/L MAK33. Nearly complete inhibition (73–99%) was achieved in all six samples by the $IgG_{2a,\kappa}$ antibody UPC-10, whereas the $IgG_{2a,\lambda}$ antibody HOPC-1 and the $IgG_{3,\kappa}$ antibody FLOPC-21 were ineffective in two samples, and in two samples only weak inhibition (17–35%) was observed. This indicates that the HAMAs of at least four samples bind to epitopes on the UPC-10 antibody