A pseudo-cryptococcal artefact derived from leucocytes in wet India ink mounts of centrifuged cerebrospinal fluid

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Abstract

Wet India ink mounts of cerebrospinal fluid (CSF) are useful in the laboratory diagnosis of cryptococcal meningitis. Pseudo-cryptococcal artefacts in such mounts have been attributed to leucocytes in CSF but their mode of formation has not been explained. This report describes the reproduction of such an artefact in cryptococcus free CSF-leucocyte mixtures that had been subjected to high speed centrifugation. The viscosity of DNA that could provide a morphological pseudo-capsule, and the yellow-green fluorescence of the pseudo-capsular material on staining with acridine-orange, suggest that lymphocytic nuclear DNA, which possibly leaked out after damage to the lymphocyte membrane by centrifugation, was responsible for this artefact.

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Keywords: leucocyte artefacts; India ink; cryptococcosis

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Cryptococcosis is increasing in importance as an opportunistic infection because of the increasing use of therapeutic immunosuppression, increasing incidence of HIV infection, and increase in numbers of susceptible aging people. Conventional laboratory methods for the diagnosis of cryptococcal meningitis with cerebrospinal fluid (CSF) include microscopy (wet India ink negative stain), capsular antigen detection (capsular antigen sensitised latex agglutination test), and culture of *Cryptococcus neoformans*.

Wet India ink mounts of CSF have sometimes shown elements that resemble capsulated cells of cryptococcus (fig 1), although culture on conventional media for fungi and tests for capsular antigen gave negative results. These elements were considered artefacts that probably originated from leucocytes in the CSF. As far as we are aware only one comment, "Lysed lymphocytes in an India ink preparation of a CSF sample have been mistaken for *Cryptococcus neoformans*," exists in the literature[1] on this artefact; no information, however, was given on the mode of its formation.

This report describes the reproduction of this artefact in cryptococcus free CSF mixtures with normal human leucocytes that had been centrifuged.

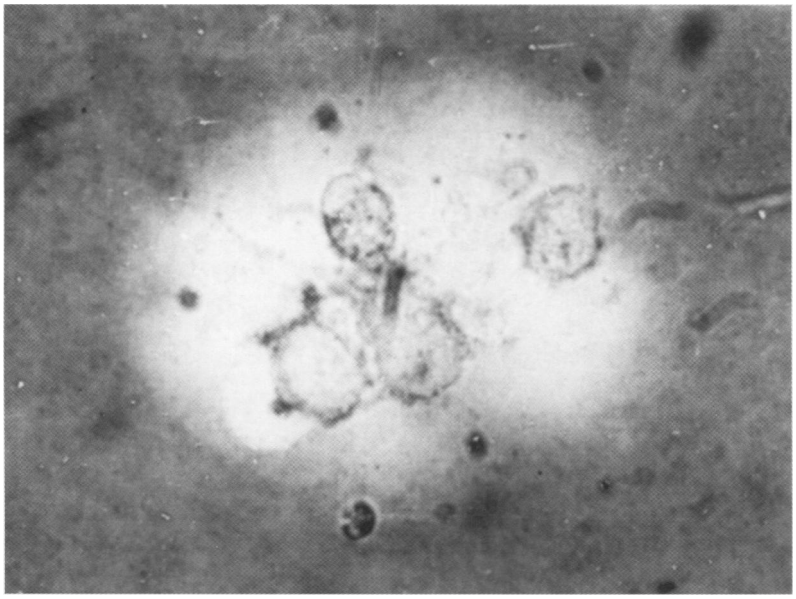

Figure 1 Pseudo-cryptococcal artefact in a clinical specimen of centrifuged CSF from a patient suspected of having meningitis (wet India ink mount).

Materials and methods

CEREBROSPINAL FLUID

Samples of surplus CSF after routine laboratory tests, which were negative (by microscopy and culture on blood agar at 37°C) for aerobic bacteria and for cryptococcus (wet India ink microscopy, tests for capsular antigen by the latex agglutination test (Latex-Crypto antigen detection system; Immuno-Mycologics, Inc, Norman, Oklahoma, USA), and culture on Sabouraud dextrose agar at 30°C), were used in all the tests. Different batches of such CSF were used in replicate tests.

LEUCOCYTES

Buffy coat cells in freshly donated blood (or within seven days of collection) were treated with Tris-ammonium chloride buffer (pH 7.2) to lyse contaminating red cells, washed, and resuspended in physiological saline for addition to CSF.

CSF containing leucocytes (approximately 100 leucocytes/ml) were subjected to a variety of treatments; a fresh mixture was used for each set of tests:

(1) incubation at 25–29°C and at 37°C in air, at 37°C in 5% CO_2, and at 4°C in air, for seven days, with daily examination
(2) evaporation and concentration by leaving the container with its cap loosened, on the bench at room temperature (25–29°C) for seven days, with daily examination
(3) sonication (Labsonic 2000 (Braun, Germany), 8 mm probe) in an ice bath for variable periods from 30 seconds to four minutes

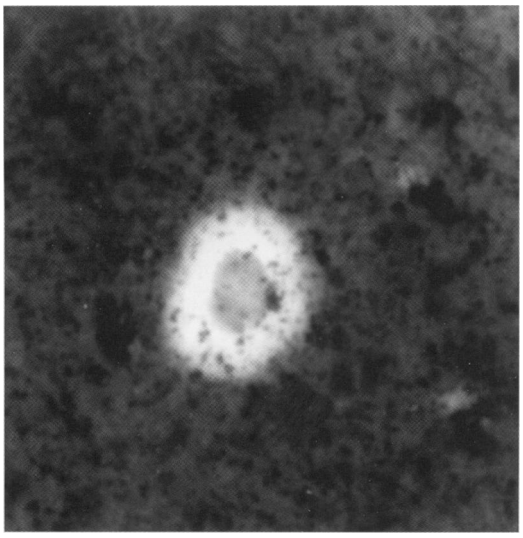

Figure 2 Pseudo-cryptococcal artefact in CSF-leucocyte mixture after centrifugation at 28 000 ×g for 15 minutes at 4°C (wet India-ink mount).

(4) centrifugation at 26°C at 650, 1458, 2590, and 4000 ×*g* for 15 minutes at each speed, and at 4°C at 6000, 13 500, 24 000, and 54 000 ×*g* for 15 minutes, at each speed. The deposit was resuspended in a quarter of the original volume of the suspension for the preparation of wet India ink mounts, and smears for staining with acridine orange.

Buffy coat leucocytes, diluted 10-fold in physiological saline, for comparison with CSF-leucocyte mixtures, were centrifuged at 6000 ×*g* at 4°C for 15 minutes and the deposit was resuspended in a quarter volume of saline for similar tests.

Wet India ink mounts were made on the samples, treated as described above, with an equal volume of commercial India ink (Pelikan, Hannover, Germany) scanned under ×25 and ×40 objectives.

Smears of CSF-leucocyte mixtures and of buffy coat leucocytes in saline that contained the artefacts in India ink mounts were fixed by mild warmth as for bacterial smears, stained with 0.01% aqueous acridine orange, and examined under ultraviolet light with a blue filter, with ×25 and ×40 objectives.

Results

The only treatment of CSF-leucocyte mixtures that resulted in the production of the pseudo-cryptococcal artefacts (fig 2) was centrifugation at 6000 ×*g* at 4°C. Greater intensities of centrifugation caused disruption of the leucocytes.

In CSF-leucocyte mixtures that produced this artefact some leucocytes appeared distorted or crenated, their nuclei were not clearly demarcated, and it was not possible to distinguish neutrophils from lymphocytes. These artefacts were not of lysed leucocytes, as has been described,[1] because in most preparations the leucocyte cell was visible within the pseudocapsule (fig 2). These abnormal shapes and pseudocapsulation were also seen with some leucocytes in saline, without CSF, that had also been centrifuged at 28 000 ×*g*. Such pseudocapsulated leucocytes were absent in preparations of buffy coat leucocytes in saline that had been centrifuged at 365 ×*g* comparable with the absence of this artefact in CSF-leucocyte mixtures that had also been centrifuged at this lower speed.

On acridine-orange stained smears, these artefacts in CSF-leucocyte mixtures showed a central, yellow-green fluorescence, indicating the presence of the leucocyte's nucleus, with peripheral stippling outside the cell, also yellow-green in colour, suggesting the presence of DNA (whether in pure form or with other nuclear or cytoplasmic constituents) around the cell. Such peripheral stippled fluorescence was not seen on similarly stained smears around buffy coat leucocytes, without CSF, which had been centrifuged at 365 ×*g*, and which had no pseudocapsular artefacts, as mentioned earlier.

Discussion

These artefacts could prompt a diagnosis of cryptococcosis despite a negative result with the latex agglutination test for cryptococcal capsular antigen and negative culture, when either the numbers of putative cryptococcal cells are small or when the yeast is non-viable because of prior antifungal treatment.

Although it was claimed that lysed lymphocytes could produce such artefacts,[1] the leucocytes in the present study were visible within the pseudocapsule. This suggests that the viscous DNA from the nucleus of the leucocyte had leaked out from the cell following the centrifugation to produce the pseudocapsule. The production of the leucocyte derived artefact is probably attributable to damage of the leucocyte's cell membrane by centrifugation, leading to egress of intracellular DNA, which was viscous enough to simulate a capsule. If this did occur, it has a parallel in the use of centrifugation to enhance infection of cells in the shell vial culture technique with chlamydia and cytomegalovirus. The effect of centrifugation at relatively low speeds on infection of cells by these microorganisms is not caused by mere sedimentation of these microorganisms with the cells, but probably results from alterations in the permeability of the cell membrane allowing entry of these microorganisms into the cells. In the case of our leucocytes, the centrifugation and consequent damage to their cell membranes could have caused the egress of intra-leucocytic DNA. The intensity of centrifugation (6000 ×*g*) that produced this artefact in our experimental CSF-leucocyte mixtures is, however, not usually used on clinical specimens of CSF in the examination for bacteria and yeasts. The appearance of these artefacts in conventionally processed clinical specimens (with relatively low speeds of centrifugation of 1458 ×*g* or less), as in fig 1, and not in the experimental CSF-leucocyte mixtures centrifuged at similar speeds, is difficult to explain. It could, however, be due to the effect of unidentified factors in the CSF, such as chemical (drugs) or biological constituents, similar to the effect of polyethylene glycol in

the hybridoma technique, combined with the low intensities of centrifugation.

Giemsa stained smears of a patient's CSF that had this artefact showed only lymphocytes without neutrophils, indicating that it was the lymphocyte from which this artefact arose.

Our results suggest that if sufficient numbers of cryptococci are present on a Gram stained smear, centrifugation should be avoided before microscopy with the India ink technique. If centrifugation is to be used, caution is warranted in interpreting such artefacts as cryptococcus, especially when the capsular antigen detection test and culture give negative results.

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Measurement of haemoglobin using single drops of skin puncture blood: is precision acceptable?

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Abstract

The study aimed to investigate local concerns about clinically important discrepancies between repeat HemoCue haemoglobin measurements from single drops of blood. Two biomedical scientists and two health visitors each obtained a series of paired haemoglobin values by fingerprick sampling from healthy volunteers. Seven of 20 paired values obtained by health visitors and three of 20 obtained by scientists from the first drop of blood forming at the puncture site differed by ≥ 10 g/l; 11 of 20 paired values obtained by health visitors and one of 20 by the scientists from the fourth drop of blood differed by ≥ 10 g/l. After collecting and mixing a number of drops in EDTA tubes before analysis, seven of 40 paired values differed by > 5 g/l, and none by > 10 g/l. Pooling drops of blood before analysis improves precision of HemoCue haemoglobin measurement and allows users to achieve results comparable to those obtained by experienced laboratory staff. Measurement of haemoglobin from single drops of skin puncture blood should be discontinued.

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Keywords: haemoglobin measurement; skin puncture blood sampling

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The HemoCue portable haemoglobinometer (HemoCue Ltd, Sheffield, UK) has been available commercially for some years and has been found to be reliable and to give precise and accurate results.[1 2] Nevertheless, concerns exist among local general practitioners about clinically important differences sometimes found between repeat HemoCue measurements made on single drops of skin puncture (capillary) blood. This study was performed to investigate the problem.

Subjects and methods

Two biomedical scientists and two health visitors took blood from four groups of 10 healthy adult volunteers. The health visitors had experience of using HemoCue in their daily work, and the biomedical scientists had considerable experience of skin puncture blood collection. All had received training by staff of HemoCue Ltd in the proper use of the instrument. Three samples were collected from one standard lancet puncture to the middle finger of each hand, producing three paired haemoglobin measurements from each volunteer. Blood from the first and fourth drops forming at the site was collected into HemoCue cuvettes, that from the second and third drops were wiped away with a sterile swab. About 20 drops were then taken into a miniaturised EDTA tube (Teklab Ltd, Co Durham, UK). After mixing, the haemoglobin of this sample was measured using both HemoCue and a Bayer H1 automated blood counter (Bayer plc, Newbury, Berks, UK). Venous blood from both arms of a further 10 volunteers was collected by one biomedical scientist and haemoglobin measured using both instruments. Coefficients of variation obtained by the health visitors and scientists on each sample type were determined, as was the mean and the 95% confidence intervals of the differences between duplicate measurements. Differences between paired values were assessed using the Wilcoxon signed rank test.

Results

Figure 1 shows the differences between paired values obtained with HemoCue; the results are summarised in table 1. Seven of 20 paired values obtained by health visitors and three of 20 samples obtained by the biomedical scientists from the first drop of blood differed by ≥ 10 g/l. Eleven of 20 paired values obtained by health visitors and one of 20 obtained by the biomedical scientists from the fourth drop