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Influence of storage temperature on estimates of tumour necrosis factor in plasma samples from patients with cerebral malaria

Following the discovery of the possible role of tumour necrosis factor-α (TNFα) in the pathophysiology of diseases such as *Plasmodium falciparum* malaria, determination of the concentration of this cytokine in plasma has become increasingly important. Experimental studies indicate that TNFα is associated with bad outcome, and several possible mechanisms behind this association have been proposed (Clark *et al.*, 1989; Grau *et al.*, 1989; Kwiatkowski *et al.*, 1993). Significant discrepancies in TNFα concentrations measured in the same samples but by different assays or different experimental procedures have been reported (Kossodo *et al.*, 1995). If the role of TNFα in malaria is to be elucidated, the methods used for assaying the cytokine ought therefore to be standardized. The aims of the present study were to see if a commercial immuno-assay gave reproducible results and whether the temperature at which plasma samples were stored (-20 *v.* -190°C) had any influence on the results obtained.

The subjects were children with malaria who were admitted to the health centre in the village of Guadalupe, Lobata District, São Tomé, between June and November of 1993. (Malaria is endemic on the island of São Tomé, which lies in the Gulf of Guinea.) Children were enrolled if they had cerebral malaria, according to the criteria of the World Health Organization (Warrell *et al.*, 1990; no directional response to painful stimulus and ≥ 6 h since the last convulsion, if one had occurred), had detectable parasitaemias with the asexual forms of *P. falciparum*, had not taken antimalarial drugs before admission (confirmed by Lelijveld—Kortmann urine test for 4-amino-quinolines; Bruce-Chwatt, 1986) and if their parents or guardians gave informed consent. Children who were found to have severe infections other than malaria, to be positive for sickle-cell [by the test of Lévy-Lambert (1973)], HIV-1 (by HIV I-ELISA; Wellcome), HIV-2 (by HIV II-ELISA; Quilaban) or hepatitis B surface antigen (by HbsAg-ELISA; Abbot), or to have >10 leucocytes/μl cerebrospinal fluid (collected by lumbar puncture) were excluded. All patients were admitted for 7 days of chemotherapy: a loading dose of quinine dihydrochloride (20 mg salt/kg in a 4-h perfusion containing 5% dextrose), followed by perfusions of 10 mg salt/kg every 8 h until oral tablets could be given.

Before treatment, a venous blood sample was collected from each of the 12 subjects [seven girls and five boys, aged 4–9 years, with mean (S.E.) age of 5.3 (1.4) years], into sterile tubes with EDTA (Monovette®, Sarstedt). Each sample was immediately centrifuged ($625 \times g$ for 15 min at room temperature) and the plasma divided into two equal subsamples in plastic tubes. One of the tubes from each subject was then stored and shipped to the Institute of Tropical Medicine (ITM) in Antwerpen, Belgium, in liquid nitrogen at -190°C. The other samples were stored at -20°C and shipped to the ITM in a portable refrigerator; all arrived still frozen. Specimens were stored for a maximum of 3 months.

At the ITM, the concentration of TNFα in each plasma sample was determined, after thawing at room temperature, in microtitre plates, using a commercial, solid-phase, enzyme-amplified-sensitivity immuno-assay (EASIA; Medgenix, Fleurus, Belgium). This assay, a double-sandwich ELISA, makes use of monospecific, polyclonal, rabbit antibodies to purified, recombinant cytokines, has a sensitivity threshold of 3 pg/ml and no cross-reaction with TNFβ. Samples stored at

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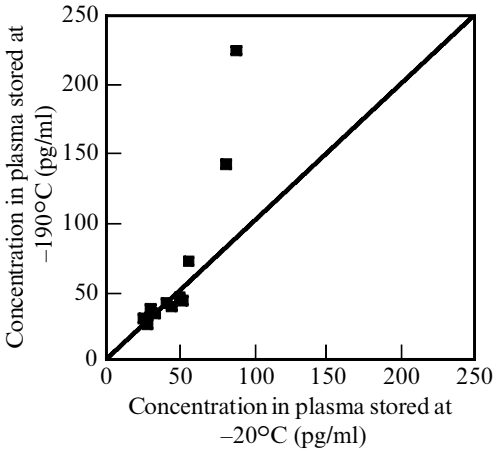

Fig. Estimates of tumour necrosis factor-α concentration (■) in plasma samples from 12 patients with cerebral malaria, after the samples were stored for ≤ 3 months at -20 or -190°C.

-190°C were assayed in duplicate whereas those stored at -20°C were assayed only once. A standard curve, obtained colorimetrically, was used to determine TNFα concentrations from optical densities. Because of the small sample size, graphical displays and standard, non-parametric statistical techniques were used. Intra-class correlation coefficients were calculated following the method described by Bernard and Lapointe (1987).

The TNFα concentrations determined in plasma stored at -190°C ranged from 31.2–217.7 pg/ml (median = 43.8 pg/ml) and were very reproducible (kappa = 0.99). However, TNFα concentrations in the two subsamples from each subject were only very similar (kappa = 0.86) if they fell below 75 pg/ml. When the cytokine was present at higher concentrations, the estimates of concentration based on the subsamples stored at -20°C were always significantly lower than in the corresponding subsamples stored at -190°C (kappa = 0.16; Fig.). It seems likely that the cytokine degrades more (and quite rapidly) at the higher storage temperature, probably as the result of proteolytic attack, to which it is very susceptible (Aggarwal *et al.*, 1985). It is unclear why this effect should only be apparent with relatively high concentrations of the cytokine; degradation may be an exponential function of time, resulting in higher discrepancies between samples with the higher, initial concentrations.

Although too few samples were investigated to draw final conclusions, even though all variables except storage temperature were kept constant, the present results indicate that different storage temperature may significantly affect subsequent estimates of TNFα concentration in plasma samples. There is, at present, no recommended storage temperature and inaccuracies caused by storage should be taken into account when interpreting data from different studies. Although storage at -20°C obviously has many practical advantages, it is apparently less reliable than storage at -190°C. Further studies are needed to determine the full effects of storage temperature on plasma TNFα.

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