THE VALUE OF CEREBROSPINAL FLUID LEUKOCYTE AGGREGATION IN DISTINGUISHING THE CAUSES OF MENINGITIS IN CHILDREN

Ian C. Michelow

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree

of

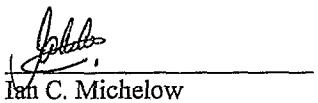
Master of Medicine in the branch of Paediatrics

Johannesburg, 1998

Degree awarded with distinction on 29 June 1999

DECLARATION

I, Ian Charles Michelow declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Paediatrics at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.



Ian C. Michelow

20th day of October, 1998

ABSTRACT

We evaluated a previously proposed method to distinguish bacterial from viral or aseptic meningitis. Cerebrospinal fluid (CSF) samples from 109 children with meningitis (67 bacterial, 23 viral and 19 aseptic) were compared on the basis of a predefined leukocyte aggregation score (LAS). The median LAS was 32.1% (range 0-84.1%) in the bacterial group, 0% (range 0-16.6%) in the viral group and 0% (range 0-20.7%) in the aseptic group. The LAS performed better than peripheral white cell count (WCC), serum C-reactive protein and CSF WCC in diagnosing bacterial meningitis. It was equally as sensitive as CSF protein and serum TNF-α, IL-1β, IL-6 and IL-8 but inferior to a combination of blood culture, CSF Gram stain and CSF culture. Significant correlations were found between the LAS and a number of inflammatory markers. Prior antibiotics, duration of symptoms prior to admission and traumatic lumbar punctures did not effect the LAS. HIV-1 status lowered the score in viral/aseptic cases. The test may be useful as an early screening tool but its sensitivity does not surpass the currently employed methods.

ACKNOWLEDGEMENTS

I would like to acknowledge the dedicated effort of my co-researcher Mark Nicol who spent many hours helping me supervise the project and count endless numbers of cells. I found the insight and guidance of my supervisor, Professor Pettifor invaluable. The house staff, registrars and nurses were always eager to cooperate despite demanding workloads, and I am grateful to them for making the study possible in such a short period of time. I would also like to express my appreciation of the financial and technical support from the staff at the National Institute for Virology particularly Professor Schoub, Dr Blackburn, Caroline Tiemessen, Claudia Chezzi, Ezekiel Maselesele and Lee Alagiozoglou. The advice regarding statistical analysis from Robin Bailey and Pam Sonnenberg was extremely helpful. Dennis Mendelsohn was patient as usual and his comments were very useful. I am grateful to the University of the Witwatersrand for allowing me to have access to the patients at Johannesburg, Chris Hani Baragwanath and Coronation Hospitals and especially the parents and children who participated in the study. Michaela and my parents, Berenice and Cecil were full of encouragement and help whenever I called on them.

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1.0 INTRODUCTION

Bacterial meningitis continues to pose a life-threatening risk to affected children (1). The prognosis of this severe disease is influenced by early diagnosis and prompt appropriate treatment (2). In comparison, aseptic meningitis carries a low risk of serious sequelae (3). The challenge which faces clinicians and researchers is to find a reliable means to distinguish between the causes of meningitis as rapidly as possible. It is this problem which the present research report seeks to address.

In the first section of this report, a review of the relevant literature is presented, the objectives of the present study are set out and a definition of the terminology is provided. An analysis of the results is then presented and these are interpreted in the context of previous reports, with a discussion regarding the limitations of this study. Finally the conclusion and recommendations are submitted.

1.1 LITERATURE REVIEW

The literature is filled with research attempts to discover the ideal investigative tool for meningitis. The following account summarises such reports, highlights the limitations which preclude their routine application, and contextualises the present study.

1.1.1 Standard investigations for meningitis

The immediate aetiological diagnosis of meningitis may be complex for a number of reasons. Equivocal clinical features on presentation may render physical assessment unreliable (4). In order to identify the causative pathogen accurately, a comprehensive investigation of the cerebrospinal fluid (CSF) usually needs to be conducted (5). The tests which are commonly utilised, however, have limited value. For example, the CSF cellular response and biochemical analysis (protein and glucose) often overlap in cases of meningitis due to different causes (5,6). Gram stains of CSF are reported to have sub-optimal sensitivity (7,8). Latex agglutination of CSF detects 36% - 100% of the common causes of meningitis (9-11) and therefore its routine use for initial assessment is controversial (12). For these reasons, cultures of the CSF and blood remain the gold standard for the diagnosis of bacterial meningitis (13) despite the delay of at least 24 hours before results are available (5,14).

1.1.2 Additional diagnostic tests

In the past a number of other tests have been proposed to facilitate a rapid aetiological diagnosis. Peripheral white blood cell (WBC) count and serum C-reactive protein (CRP) fail to distinguish the causes adequately (15,16). Cerebrospinal fluid CRP has been reported in some studies to have no diagnostic value (17,18) whereas others conclude that the test is useful (19-21). Cerebrospinal fluid lactate is a product of neuronal anaerobic glycolysis. Its level reflects the severity of cerebral hypoxia (22) but it has a poor specificity (23) and predictive value (24) in distinguishing the causes.

Many studies have investigated the pathophysiological role and diagnostic value of various cytokines in meningitis (1,25-28). In the context of bacterial meningitis, tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were found to have sensitivities of 80% and 75%, and specificities of 100% and 99% respectively (29). Studies investigating interleukin-6 (IL-6) (30,31) and interleukin-8 (IL-8) (32,33) reflect disagreement about their utility. Granulocyte colony-stimulating factor has been shown to be elevated in meningitis in a non-specific pattern and has a significant correlation with the number of neutrophils in the CSF (34). Although some of the listed cytokines may be useful in differentiating bacterial from viral meningitis, their determination requires sophisticated equipment and trained laboratory staff.

The measurement of serum procalcitonin levels has attracted much attention recently. Some data suggest that this peptide is a sensitive indicator of bacterial sepsis and meningitis (35,36). Larger confirmatory studies are awaited.

The increased CSF content of numerous other compounds is non-specific for bacterial meningitis. These include amino-acids (37), fibrin and fibrinogen degradation products (38), biopterin and homovanillic acid (39), free sialic acid (40), lactoferrin and alpha-1-antitrypsin (17), and vasopressin (41). A decrease in cyclic adenosine monophosphate has been found in bacterial meningitis but the technique has methodological weaknesses (42). In addition, the limulus lysate test was shown to be useful only for Gram-negative meningitis (43).

1.1.3 Diagnostic confounders

In addition to the limitations of the tests described above, two other factors may impede the diagnosis of meningitis. Firstly, antibiotics administered prior to lumbar puncture, may inhibit the bacterial growth in CSF samples (5,44-47). In such situations the resulting so-called partially treated meningitis may be difficult to distinguish from true viral or presumed aseptic meningitis.

Second, in populations where tuberculous meningitis is prevalent, as is the case in this study population, the diagnosis is particularly difficult because accepted techniques as described above are often inadequate in making a reliable diagnosis of this disease (48,49).

1.1.4 The need for a new test

In practical terms, antibiotics are often initiated empirically and the duration of therapy is guided by the culture result (if informative) and the clinical progress (5). For this reason an inexpensive screening test with optimal sensitivity and specificity enabling a rapid, simple and accurate diagnosis of the cause of meningitis, would make a significant contribution to patient management and impact beneficially on their outcome.

The aim of the present study was to establish the reliability of an early aetiological diagnostic test which could avoid the indiscriminate use of antibiotics and facilitate appropriate therapeutic decisions. The test is based on the phenomenon of leukocyte aggregation.

1.1.5 The phenomenon of leukocyte aggregation

A review of the literature indicates that an increased tendency of leukocytes to aggregate spontaneously in the blood occurs in a number of disease states, for example bone sepsis (50), post-immunization (51) and myocardial infarction (52). This phenomenon can be simply detected by the leukergy test which was first described by Fleck (51). Despite extensive investigation, the biological mechanism of this phenomenon has not been fully elucidated. It is thought that leukocyte aggregation is not only a manifestation of the adherence between leukocyte cell membranes but also reflects the potential interaction between leukocytes and endothelial cells. Aggregate formation therefore plays a role in the immune response by recruiting more cells to the sites of inflammation (53,54).

Recently Garty et al (55) described a novel technique of applying the above phenomenon to CSF. They postulated that the degree of leukocyte aggregation could be used to differentiate between the various causes of meningitis. Subjects included 100 paediatric patients ranging in age from 2 weeks to 14 years with proven or presumed meningitis. They defined a CSF "leukocyte aggregation score" (LAS) as the number of aggregated leukocytes as a percentage of 100-300 counted white cells. The number of proven bacterial and viral cases were 15 and 10 respectively. Cases of aseptic meningitis predominated (62%) and were presumed to be of viral origin. The specificity of the LAS for detection of bacterial meningitis was calculated to be 100% when an aggregation cut-off point of 15% was used. The sensitivity was 94% if the partially treated patients were excluded but 88% if included. No correlation was demonstrated between the LAS and other indices of meningeal inflammation (eg. pleocytosis).

1.1.6 A modified new diagnostic test

The preceding review reveals the deficiencies of a wide spectrum of common and experimental investigations for the rapid and accurate diagnosis of meningitis. This highlights the critical need to advance existing and develop new approaches to the investigation of this severe disease.

We modified the method of Garty et al (55) and applied it within a hospital setting to a population in which HIV-1 and tuberculosis are prevalent (56). The potential value of this investigation resides in the fact that infants and children with acute non-bacterial meningitis can appear toxic and it may be difficult to distinguish these cases from bacterial meningitis purely on clinical grounds and routine laboratory tests (5). The availability of a cheap, bed-side test may help clinicians target cases for appropriate treatment, thus reducing the expense and potential adverse effects of unnecessary antibiotic therapy and prolonged hospitalization.

1.2 PURPOSE AND OBJECTIVES

The purpose of the study was to investigate the validity and reliability of an early test for differentiating bacterial meningitis from other causes of meningitis in children.

The specific objectives included:

1) the standardisation of the method of calculating the leukocyte aggregation score (LAS) in CSF.

2) the calculation of the LAS cut-off to achieve the optimal sensitivity, specificity and likelihood ratios for bacterial versus non-bacterial meningitis in children.
3) the investigation of the relationship between the LAS and commonly used CSF investigations, Human Immunodeficiency Syndrome (HIV-1) status and CSF cytokines.
4) the determination of the effect on the LAS of prior antibiotics, duration of symptoms prior to admission and traumatic lumbar punctures.

1.3 DEFINITION OF TERMINOLOGY

The **leukocyte aggregation score (LAS)** is the proportion of aggregated leukocytes in the CSF expressed as a percentage of 300 to 500 counted white cells on a slide (using the higher figure whenever possible). In accordance with the technique of Garty et al (55), cells are considered aggregated when three or more nuclei are placed less than one cell diameter apart.

2.0 MATERIALS AND METHODS

2.1 INCLUSION CRITERIA

Patients were enrolled in the study if the following criteria were met: 1) a diagnosis of meningitis was suspected and 2) sufficient CSF was available and 3) the CSF leukocyte count exceded 30×10^6/L if the age was one month or less (57) or 10×10^6/L if older than one month (2) and 4) informed consent was obtained.

2.2 STUDY POPULATION

Over a five month period (July to November 1997), 116 patients were enrolled at Johannesburg, Chris Hani Baragwanath, and Coronation Hospitals. Three patients were excluded because of improperly prepared slides. The median age of the 113 study patients was 9 months (range 11 days - 14 years). The sex distribution was 59 males and 54 females.

2.3 OUTCOME MEASURES

The type of meningitis was classified retrospectively according to the following predefined criteria . A diagnosis of **bacterial meningitis** was made if the CSF culture or Gram stain or latex agglutination was positive or the blood culture yielded a clinically significant growth.

A diagnosis of **viral meningitis** was confirmed by a positive CSF enteroviral culture or a polymerase chain reaction (PCR) test for CSF enteroviral RNA or positive mumps serology for immunoglobulin M. This approach can detect up to 90% of cases in the local population (58).

Tuberculous meningitis was considered to be **confirmed** if the CSF examination revealed acid-fast bacilli or a growth of *Mycobacterium tuberculosis* (TB) or positive culture of gastric aspirates or sputum. **Probable** disease was presumed if there was a suggestive CSF cellular response and biochemical profile and any of the following criteria (48): characteristic clinical signs (59), positive tuberculin skin test in children under 5 years of age (≥15mm or ≥5mm if symptomatic HIV disease) (60), suggestive chest radiological features (48) or typical brain CT scan features (49).

Cases which had sterile cultures and did not meet the above criteria were classified as **aseptic meningitis.**

2.4 CEREBROSPINAL FLUID INVESTIGATIONS

Cerebrospinal fluid was collected for slide preparation as part of a routine diagnostic lumbar puncture for all patients undergoing investigation for suspected meningitis, provided that a sufficient amount could be obtained.

The medical staff who performed the lumbar punctures were trained in the method of slide preparation according to a standard protocol as follows: two drops of CSF were placed directly from the lumbar puncture needle onto one end of a clean slide which was held along the edges at 45°. The CSF was permitted to track down by gravity leaving a fine film. The slide was then laid flat and air-dried. Thereafter the primary investigators fixed the slide with methanol within 48 hours and stained it using haematoxylin.

Patients were admitted to the study if the inclusion criteria had been met and consent had been obtained. The following investigations were performed on the CSF of study patients: leukocyte aggregation scoring (Section 2.5), cellular and biochemical analysis, Gram stain and culture according to standard laboratory techniques. Latex agglutination was used at the discretion of the medical and microbiology staff if a bacterial cause was suspected but not proven.

Viral culture and a standardised in-house semi-nested polymerase chain reaction (PCR) test for enteroviral RNA were performed on CSF stored at 4°C if a bacterial cause was not confirmed within 72 hours of admission. The PCR system used the HighPure Viral RNA Extraction Kit for RNA extraction and the Titan RT-PCR system (both Boehringer Mannheim, GmbH, Germany) for reverse transcription and first round PCR amplification with primers described by Rotbart (61) . In order to increase the sensitivity of enteroviral RNA detection, a seminested PCR was then performed according to Rotbart (61) and Leparc et al (62). First and second round amplification products were detected by gel electrophoresis.

Excess CSF of enrolled patients (n=59) as well as controls (n=22), who had lumbar punctures performed during the same study period for investigation of pyrexia and whose CSF was normal, was stored at –70°C and cytokine assays (Biotrak ELISA system for TNF-α, IL-1β, IL-6, IL-8, Amersham International plc, England) were performed on these specimens at the end of the study period.

2.5 LEUKOCYTE AGGREGATION SCORE CALCULATION

The slide counts were performed by one of the two primary investigators. In each case the investigator was blinded to the aetiology. Three locations for leukocyte counting (proximal, central and distal) were selected along the length of the slide instead of one as described by Gaity et al (55). We chose this approach in the light of a prior pilot study of 10 patients which revealed differential aggregation scores at each site and in order to assess further the variability of the counts which may be intrinsic to the test. A total of 300 to 500 cells were counted at each site (the higher figure whenever possible) or a combined count of all three sites if there were insufficient cells. The proportion of aggregated cells was expressed as a percentage of total cells counted (the leukocyte aggregation score). A pilot study of 15 CSF specimens revealed that intra-observer variability was less than 5% and inter-observer variability was less than 5-10% (the higher percentage in cases with an LAS of more than 50%). This variability was checked and shown to be consistent throughout the study.

2.6 BLOOD TESTS

The patients had the following blood tests: full blood count, C-reactive protein, glucose, blood culture and mumps immunoglobulin M (IgM) if a viral cause was suspected at 72 hours after admission.

2.7 HIV-1 TESTING

Patients were selectively tested for HIV-1 at the discretion of the attending medical staff for clinically indicated reasons and their parents/guardians were counselled according to routine hospital practice after informed consent had been obtained. In those cases not tested initially, excess serum and plasma was stored at -70°C and unlinked HIV-1 testing was performed concurrently at the end of the study period.

Modified Center for Disease Control and Prevention (CDC) criteria (63) for diagnosing HIV-1 infection were applied. The requirements were as follows: 1) in children aged >18 months, two positive serum HIV-1 enzyme-linked immunosorbent assay (ELISA) tests (Vironostika HIV Uni-Form II plus O, Netherlands) and 2) in children aged ≤ 18 months, the group was divided into asymptomatic or symptomatic depending on the presence of symptoms and signs. If the child was asymptomatic, a positive serum ELISA test and a positive HIV-1 PCR for viral load determination was required. If symptomatic, a single confirmed positive ELISA test fulfilled the criteria.

Amplification of HIV-1 RNA from plasma samples was performed using the Amplicor HIV-1 Monitor Test (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA) according to the manufacturer's instructions. This test uses the PCR method for product amplification with the range of detection being 400-750 000 HIV-1 RNA copies/ml plasma.

2.8 ETHICAL CONSIDERATIONS

The study was approved by the University of the Witwatersrand Committee for Research on Human Subjects (clearance certificate: M970616). In each case a parent or guardian gave informed and signed consent for the patient to participate in the study (Appendix A). Standard hospital policies were adhered to for HIV-1 testing and the investigation and management of meningitis. In all cases optimal care and confidentiality was ensured.

2.9 STATISTICAL METHODS

The data were collected and encoded for confidentiality by the primary investigators. A database was compiled and checked using Epi Info version 6.04b (CDC, USA). Analysis was performed using Epi Info, Statistica (StatSoft Inc, USA 1998) and Analyse-it (Analyse-It Software, UK 1998) with a probability < 0.05 considered significant.

Comparisons of groups of continuous variables were made using the Mann-Whitney U, Kruskal-Wallis ANOVA and Wilcoxon matched pairs tests for non-parametric data where appropriate, and categorical data were compared using Chi-squared and 2-tailed Fisher's Exact

tests (for small expected frequencies). Correlations between variables were assessed using Spearman's rank correlations for non-parametric data.

The optimal sensitivity and specificity for the various diagnostic tests were derived from the receiver operator characteristic (ROC) curve and compared by means of area under the curve . Likelihood ratios for a positive test result (LR+) and negative test result (LR–) were calculated as the best measure of post-test probability of bacterial meningitis, using the formulae (64) :

LR(+)=sensitivity/(1–specificity) and

LR(–)=(1–sensitivity)/specificity.

The higher the LR(+), the better the test is at ruling in the disease and the lower the LR(–), the better the test is at ruling out the disease. The result can be easily converted to a post-test probability using a nomogram. This method has the advantage of being generalisable to other study populations with different prevalence of disease because it is derived from the sensitivity and specificity, and not from the positive and negative predictive values which are wholly dependent on prevalence of disease.

3.0 RESULTS

The 113 patients were classified into 5 categories of meningitis as follows: 67 bacterial (59.3%), 23 viral (20.3%), 19 aseptic (16.8%), 3 TB meningitis (2.7%; 2 confirmed, 1 probable) and 1 neurocysticercosis (0.9%). In view of the small sample sizes, the last 2 categories were excluded from statistical analysis. The aetiologies are listed in Table 3.1.

TABLE 3.1 Aetiology of meningitis in study patients

Category	**Aetiology**	**Number (%)**
Bacterial (n=67)	*N. meningitidis*	21 (31.3)
	S. pneumoniae	19 (28.4)
	H. influenzae b	17 (25.4)
	S. agalactiae	6 (9)
	E. coli	2 (3)
	S. pyogenes	1 (1.5)
	Erwinia species	1 (1.5)
Viral (n=23)	Enterovirus	18 (78.3)
	Mumps	5 (21.7)
Aseptic (n=19)	Unknown	19 (100)

Table 3.2 shows the sensitivities of the standard diagnostic tests for the bacterial meningitis patients. Two cases with negative cultures were classified as bacterial on the basis of positive Gram stains, and one case of recurrent *Streptococcus agalactiae* (positive CSF culture at initial presentation) was diagnosed on the basis of latex agglutination alone.

TABLE 3.2 Sensitivity of standard investigations in bacterial meningitis patients

Positive Investigation	Number of Patients	Sensitivity (%)
B/C [1]	36	57.1
G stain	54	80.6
CSF culture	57	85.1
CSF culture or G stain	59	88.1
CSF culture or B/C [1]	64	95.5
G stain or B/C [1]	64	95.5
CSF culture or B/C [1] or G stain	66	98.5

B/C Blood culture; G stain Gram stain

[1] not done in 4 bacterial, 2 viral and 3 aseptic meningitis cases

The diagnosis of enteroviral meningitis in 18 patients was based on PCR for viral RNA. The organism was isolated by viral culture in one patient. The remaining 5 cases of viral meningitis had positive IgM serology for mumps.

The results of the laboratory findings of the three categories of patients are shown in Table 3.3. Analysis of variance indicated that there was a significant difference in the variables between the 3 categories, except for the peripheral white cell count. Further analysis showed that the discrepancy was not due to differences between the viral and aseptic groups, except for the serum CRP which appeared to contribute to the difference noted. In view of this, the viral and aseptic meningitis groups were presumed to have similar aetiologies and were combined for various comparative analyses.

TABLE 3.3 Laboratory results

	Bacterial	**Viral**	**Aseptic**	**p value** [1]
n	67	23	19	-
Median peripheral WBC ($\times 10^9$/L) [2]	15.5	15.8	12.8	0.67 (0.49)
Median serum CRP (mg/L) [3]	200.0	33.5	12.5	**<0.001 (0.03)**
Total CSF WCC ($\times 10^6$/L)	848	48	45	**<0.001** (0.72)
CSF PMN ($\times 10^6$/L)	582	18	24	**<0.001** (0.49)
CSF Lymphocytes ($\times 10^6$/L)	75	17	16	**0.005** (0.85)
Median CSF Protein (g/L) [4]	2.55	0.50	0.53	**<0.001** (0.29)
CSF:Serum Glucose ratio	<50%: 49 ≥50%: 3	3 13	3 10	**<0.001** [5] (0.99) [6]
LAS (%) median (range)	32.1 (0-84.1)	0 (0-16.6)	0 (0-20.7)	**<0.001** (0.69)

WCC white cell count; CRP C-reactive protein; PMN polymorphonuclear

[1] Kruskal-Wallis ANOVA for non-parametric data comparing 3 groups of variables. Values in parentheses refer to comparisons of viral and aseptic groups using Mann-Whitney U tests

[2] no data for 1 patient in each category

[3] no data for 4 bacterial, 1 viral and 1 aseptic

[4] no data for 2 bacterial and 1 aseptic

[5] 2-tailed Fisher's exact test comparing bacterial to viral and aseptic

[6] 2-tailed Fisher's exact test comparing viral to aseptic

Comparative analysis revealed that there was no significant difference in age and sex distribution between the bacterial and viral/aseptic meningitis groups (Table 3.4). The duration of symptoms and antibiotic utilisation prior to admission did not differ significantly between

the two groups. The antibiotics used were enteral amoxicillin (n=16), enteral cotrimoxazole (n=5) and parenteral cefotaxime (n=1). When analysed separately, antibiotics were shown to have been used to an equivalent extent in the bacterial and aseptic groups. Therefore, in the absence of any evidence of bacterial infection in the aseptic group, it is unlikely that antibiotics contributed substantially to any cases of "partially treated" meningitis that may have been classified as aseptic (Table 3.4).

TABLE 3.4 Comparative data of patients

	Bacterial	**Viral / Aseptic**	**p value**
Median age (months)	9.0	8.6	0.25 [1]
Male : female ratio	0.91	1.33	0.34 [2]
Onset of symptoms prior to admission [3] (< 48 hours : ≥48 hours)	0.91	0.86	0.90 [2]
Antibiotic usage [4] (usage : no usage)	0.26	0.27 0.19 (Aseptic alone)	0.92 [2] 0.75 [5]

[1] Mann-Whitney U test
[2] Chi-squared test
[3] data unavailable for 1 viral/aseptic and 4 bacterial cases
[4] data unavailable for 4 bacterial cases
[5] 2-tailed Fisher's exact test (bacterial vs aseptic)

Of the 42 patients diagnosed as having viral or aseptic meningitis, 31 had CSF pleocytosis less than or equal to 100×10^6 cells/L , 5 had between 100 and 300×10^6 cells/L inclusive, 3 patients between 300 and 500×10^6 cells/L inclusive, and 6 more than 500×10^6 cells/L . The distribution of data was bimodal (Figure 3.1).

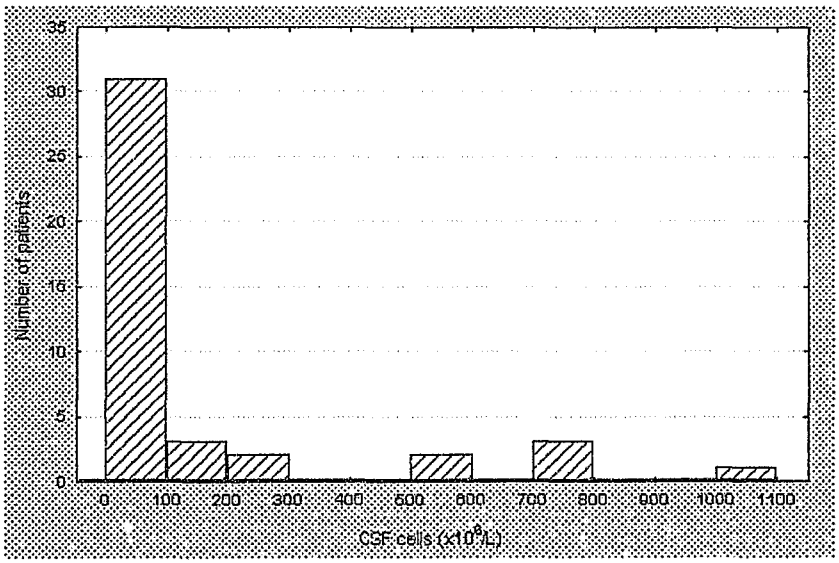

Figure 3.1 Distribution of CSF cells in viral/aseptic meningitis patients

The HIV status was tested in 109 patients. Twenty six (23.9%) were positive and the remainder (76.1%) were negative.

The mean LAS was significantly greater in the bacterial group than the viral and aseptic groups (Table 3.3; Figure 3.2). However scores varied within each category according to the position on the slide at which cells were counted. In the bacterial cases (Figure 3.3), there were significant differences in scores between the top and middle sites as well as the middle and bottom sites, increasing towards the bottom (Wilcoxon matched pairs test, n=64, p=0.01 and p=0.001 respectively). In the viral/aseptic cases no significant difference in scores between the sites was demonstrated (Wilcoxon matched pairs test, n=16, p=0.18

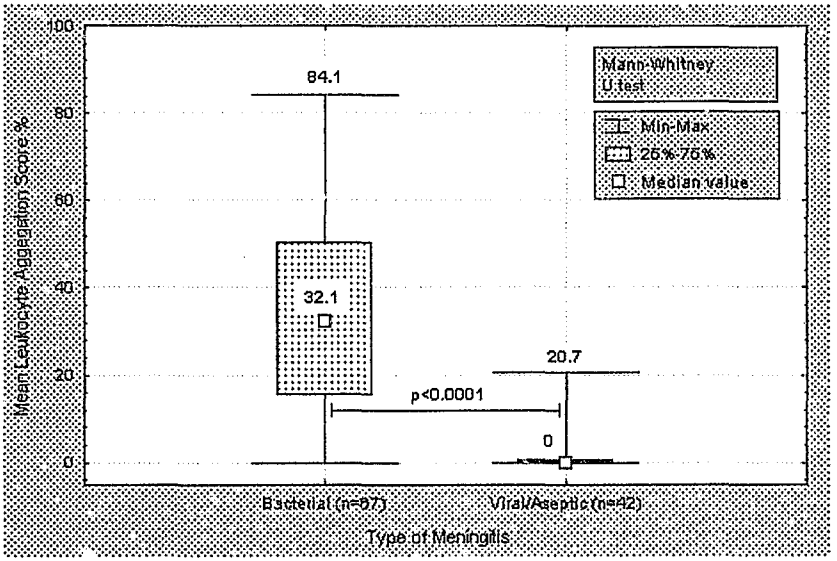

Figure 3.2 CSF leukocyte aggregation in meningitis

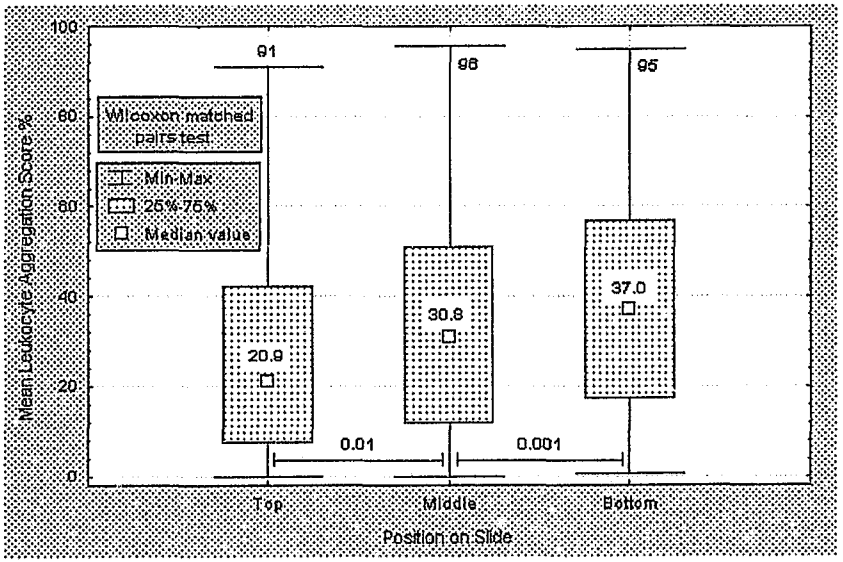

Figure 3.3 Leukocyte aggregation in bacterial meningitis according to position on slide

p=0.07 respectively). Cases were excluded from this analysis if insufficient cells necessitated single global counts.

Analyses were performed to investigate associations between the LAS and several variables. There was no relationship between a positive history of antibiotics prior to admission and the LAS in bacterial meningitis (Mann-Whitney U test, p=0.6, n=63). Similarly, no association was found between the duration of symptoms prior to admission and the LAS in either bacterial or viral/aseptic meningitis groups (Mann-Whitney U tests, p=0.76, n=63 and p=0.98, n=41 respectively).

Spearman's rank correlation tests showed no association between peripheral white cell count and the LAS in bacterial meningitis (p=0.73) but a weak correlation in viral/aseptic meningitis (r_s=0.32, p=0.04). No correlation was found between serum CRP and the LAS in the viral/aseptic meningitis group (r_s=0.14, p=0.4), however a statistically significant (although weak) relationship between these two variables was found in patients with bacterial meningitis. The upper limit of detection for CRP was 200 mg/L (Figure 3.4).

Figures 3.5–3.8 show the correlations between the LAS and CSF protein and total CSF white cell counts in both categories of disease. The associations were weak with a broad scatter of plots. Comparisons of cytokines (TNF-α, IL-1β, IL-6, IL-8) between the 2 diagnostic categories and a control group revealed significant differences (Figures 3.9–3.12). Correlations between the LAS and the listed cytokines are shown in Table 3.5

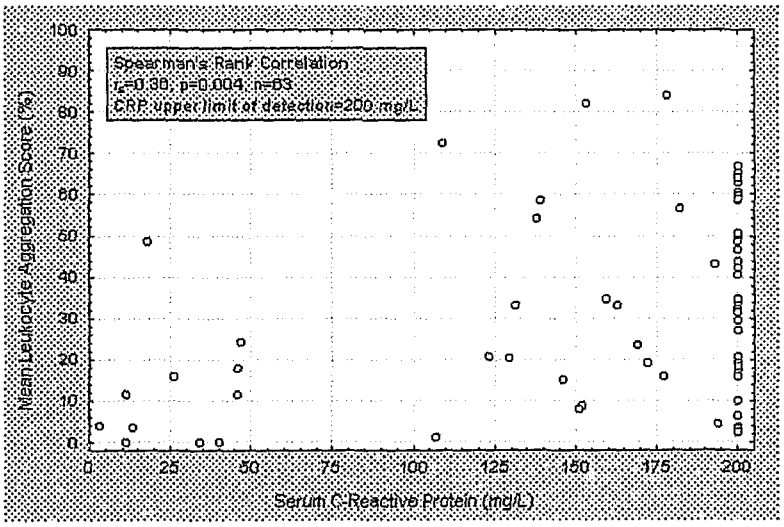

Figure 3.4 Relationship between serum CRP and mean Leukocyte Aggregation Score in bacterial meningitis patients

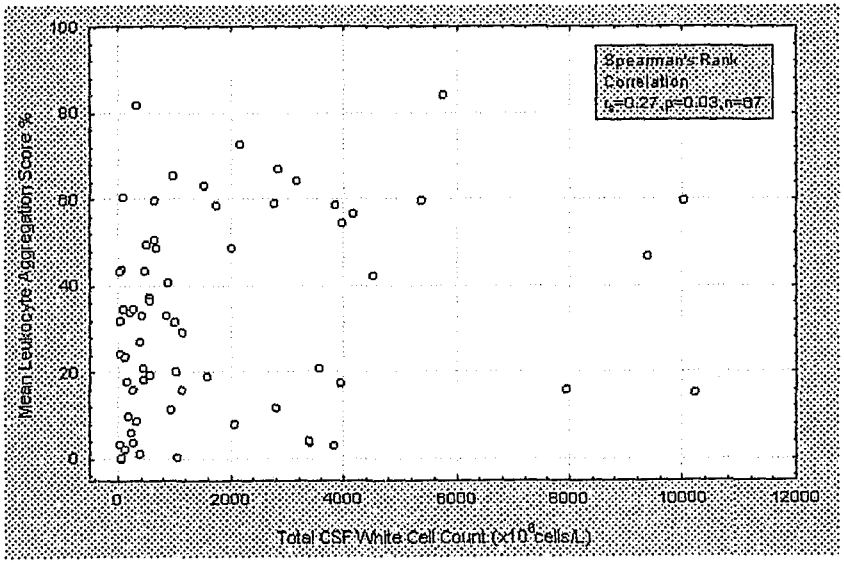

Figure 3.5 Relationship between CSF WCC and LAS in bacterial meningitis

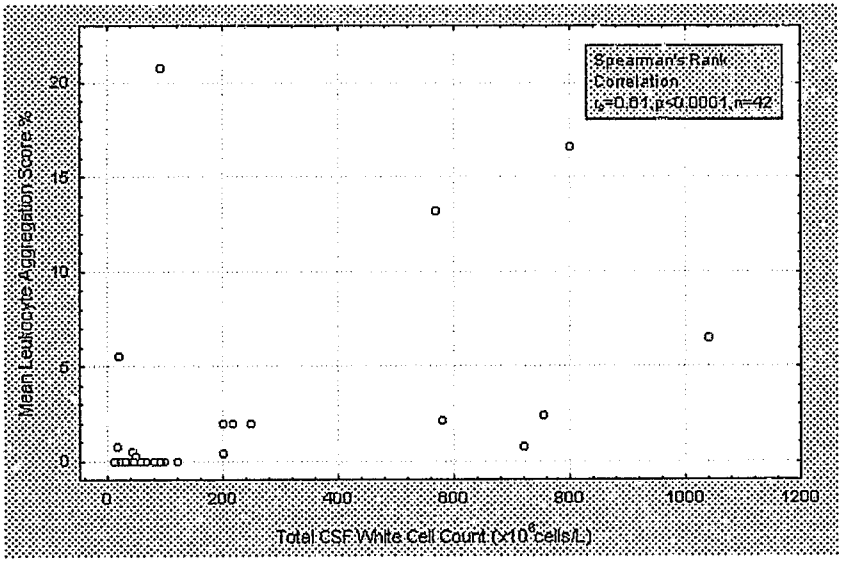

Figure 3.6 Relationship between total CSF WCC and LAS in viral/aseptic meningitis

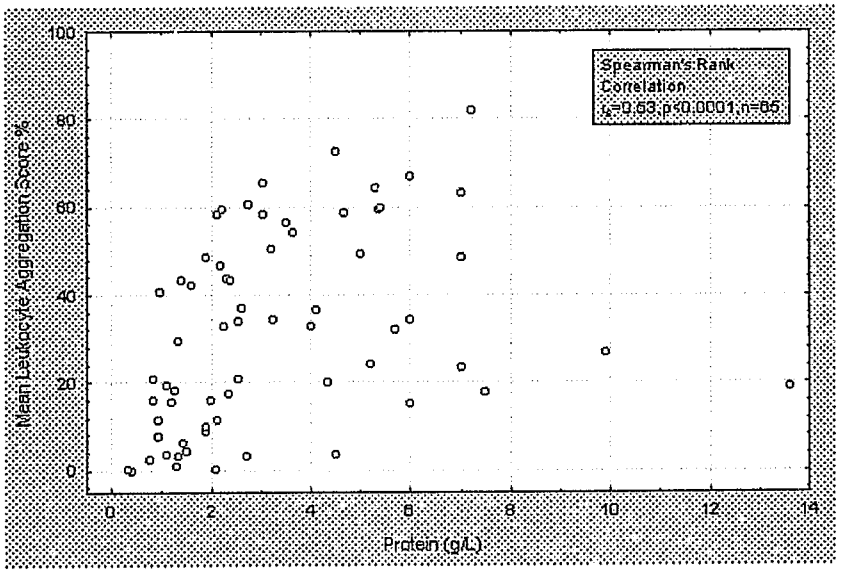

Figure 3.7 Relationship between CSF protein and LAS in bacterial meningitis

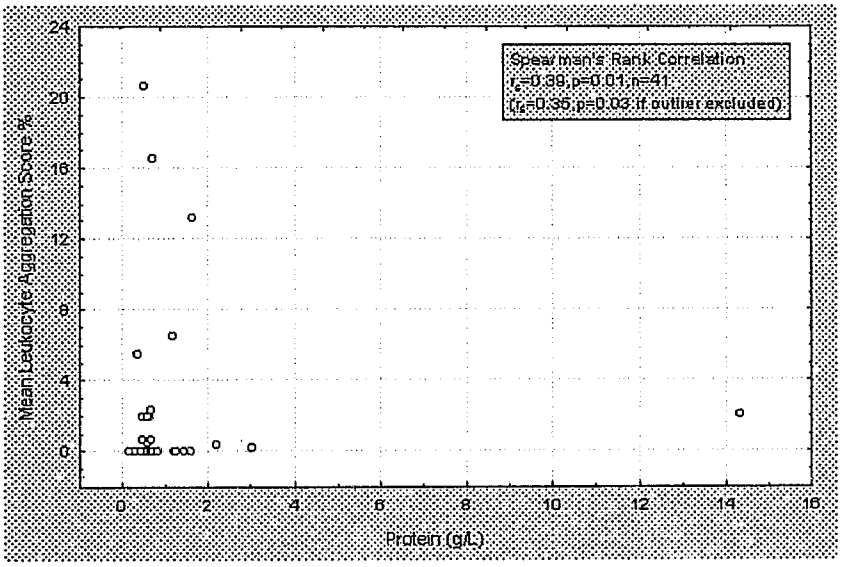

Figure 3.8 Relationship between CSF protein and LAS in viral/aseptic meningitis

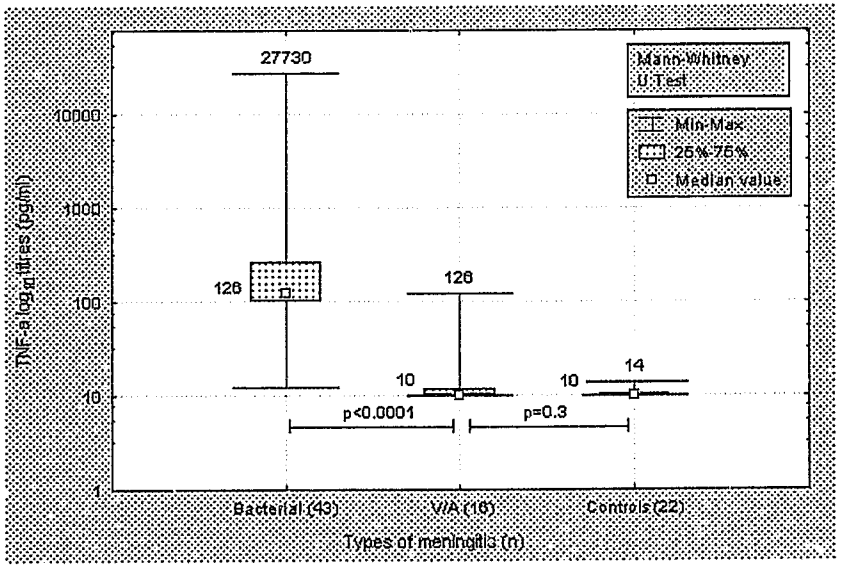

Figure 3.9 Comparison of TNF-α in bacterial and viral/aseptic meningitis patients and controls

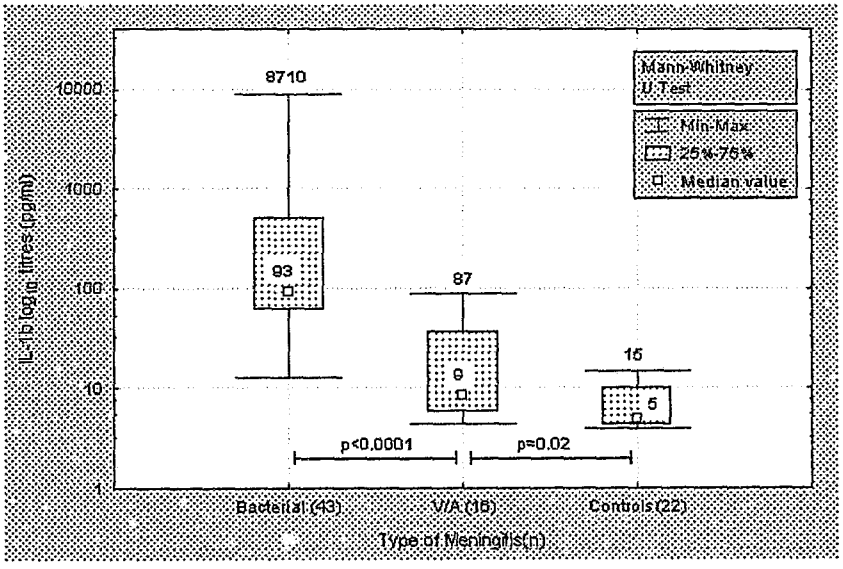

Figure 3.10 Comparison of IL-1β in bacterial and viral/aseptic meningitis patients and controls

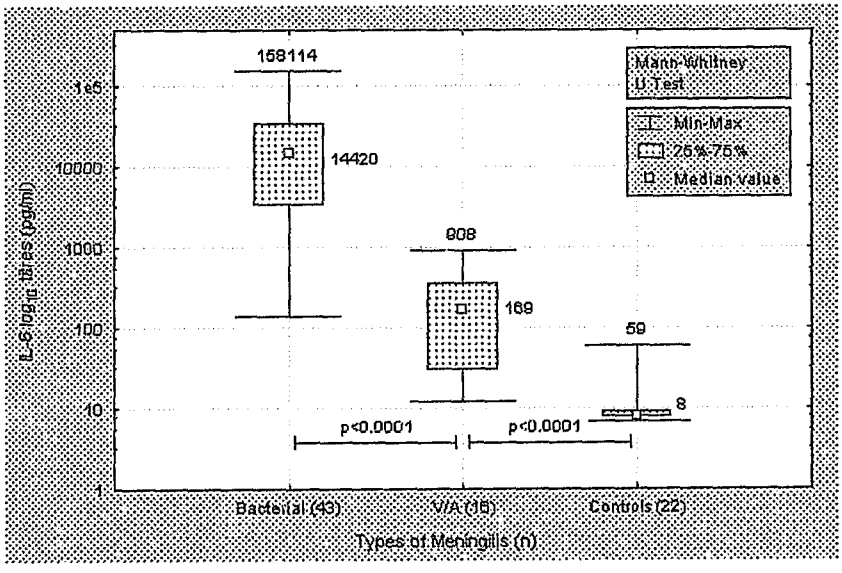

Figure 3.11 Comparison of IL-6 in bacterial and viral/aseptic meningitis patients and controls

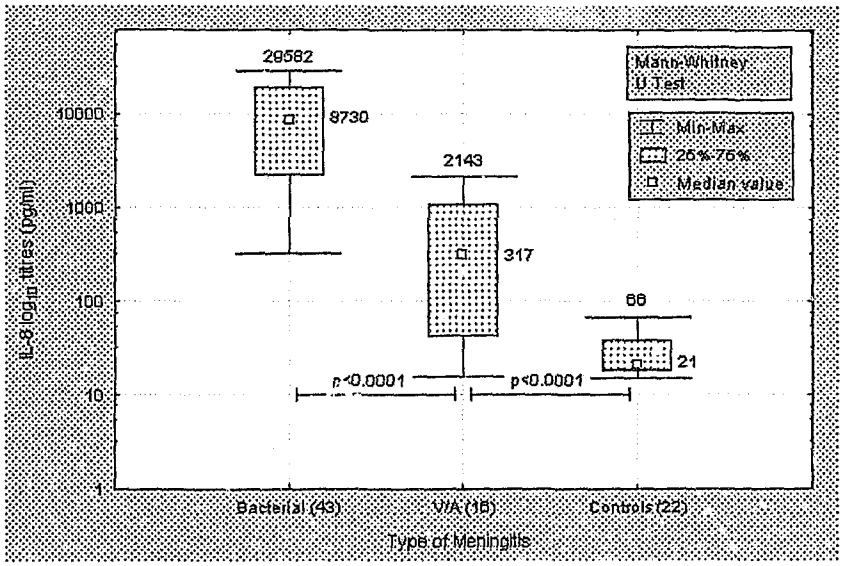

Figure 3.12 Comparison of IL-8 in bacterial and viral/aseptic meningitis patients and controls

TABLE 3.5 Spearman's Rank Correlations between mean LAS and selected cytokines

Cytokine	**Bacterial** r_s (p) (n=43)	**Viral/Aseptic** r_s (p) (n=16)
TNF-α	0.25 (0.10)	0.27 (0.32)
IL-1β	0.48 (**0.005**)	0.32 (0.23)
IL-6	0.45 (**0.005**)	0.38 (0.15)
IL-8	0.35 (**0.05**)	.22 (0.40)

HIV status had no demonstrable association with the LAS in the bacterial category (Mann-Whitney U test, p=0.4, n=67) but was significantly associated with a lower LAS in the viral/aseptic group (Figure 3.13).

There was no significant difference in the number of CSF red blood cells (RBC) between the

bacterial and viral/aseptic meningitis groups (Mann-Whitney U test, p=0.75). In addition, no correlation existed between CSF RBC and the LAS for either bacterial or viral/aseptic meningitis (r_s =0.003, p=0.98).

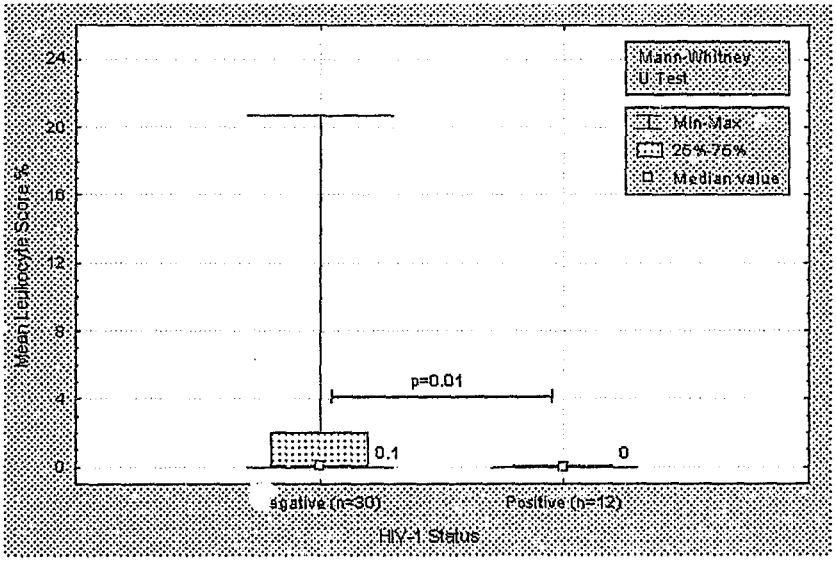

Figure 3.13 Relationship between HIV-1 status and LAS in viral/aseptic meningitis patients

The areas under the receiver operator characteristic (ROC) curves were used to compare the LAS with various diagnostic tests. The LAS curve was significantly better than the curves for peripheral WBC count (p<0.001), serum CRP (p=0.03) and CSF total WCC (p=0.01) but not for CSF protein (p=0.2), TNF-α (p=0.3), IL-1β (p=0.2), IL-6 (p=0.4) and IL-8 (p=0.1) (Figures 3.14–3.17).

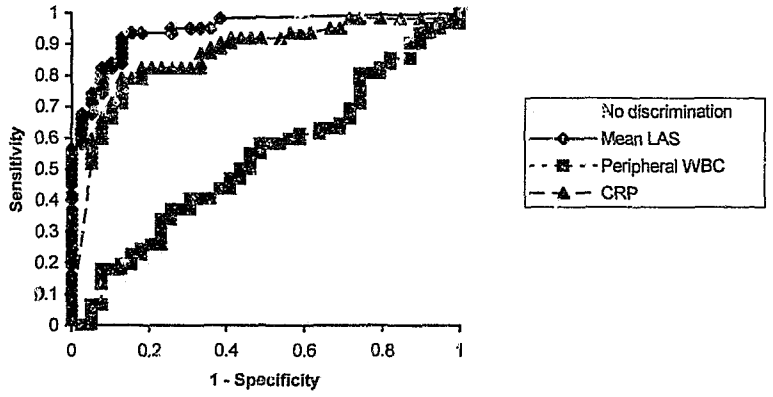

Figure 3.14 Receiver Operator Characteristic curves for mean LAS, peripheral white cell count and C-reactive protein

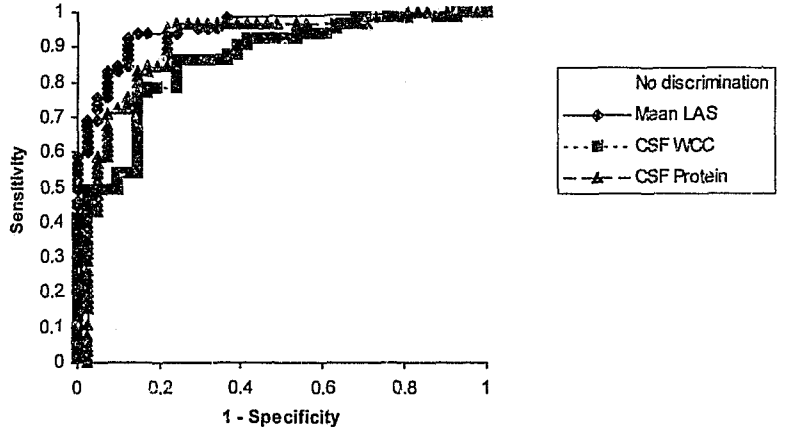

Figure 3.15 Receiver Operator Characteristic curves for mean LAS, CSF white cell count and CSF protein

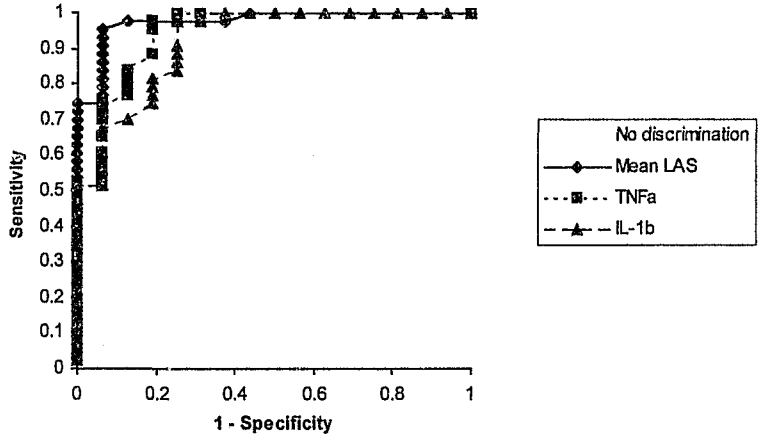

Figure 3.16 Receiver Operator Characteristic Curves for mean LAS, TNF-α and IL-1β

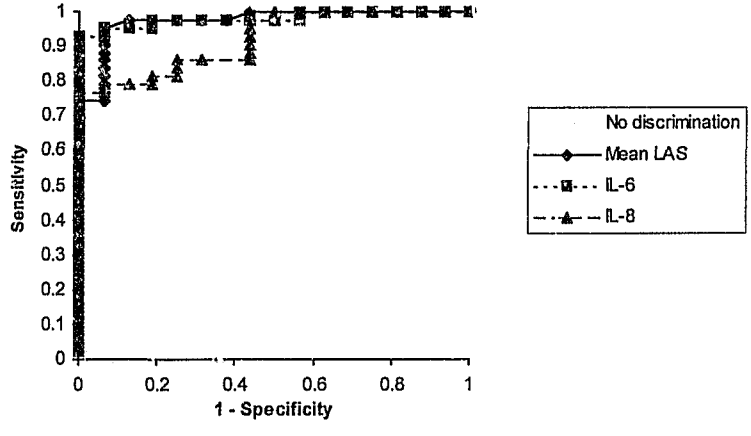

Figure 3.17 Receiver Operator Characteristic Curves for mean LAS, IL-6 and IL-8

The ROC curves were also used to derive LAS cutoff values corresponding to optimal sensitivities and specificities. In view of the fact that there was a significant correlation between the CSF total WCC and the LAS in viral /aseptic meningitis, additional analyses were undertaken to assess whether the test performed better if selectively applied. A CSF total WCC $\leq 300\times10^9$/L was chosen for this purpose because there was a bimodal distribution of cell counts among the viral/aseptic group centred around 300×10^9/L cells (Figure 3.1), and because at higher levels, antibiotics may be started empirically despite a negative test thus reducing the potential value of such an investigation (Table 3.6).

TABLE 3.6 Total CSF WCC, mean LAS cutoff values and corresponding sensitivity, specificity and likelihood ratios

Mean LAS cutoff (%)	Total CSF WCC in all patients (n=109)			Total CSF WCC $\leq 300\times10^6$/L (n=54)		
	Sensitivity (%)	Specificity (%)	LR (+/−)	Sensitivity (%)	Specificity (%)	LR (+/−)
0	98.5	64.3	2.8/ 0.02	94.4	75.0	3.8/ 0.07
1	95.5	76.2	4.0/ 0.06	88.9	86.1	6.4/ 0.13
2	94	83.3	5.6/ 0.07	88.9	94.4	15.9/ 0.12
3	92.5	88.1	7.8/ 0.09	83.3	94.4	14.9/ 0.18
4	86.6	88.1	7.3/ 0.15	72.2	94.4	12.9/ 0.29
5	85.1	88.1	7.2/ 0.17	72.2	94.4	12.9/ 0.29
7.5	83.6	92.9	11.8/ 0.18	66.7	97.2	23.9/ 0.34
10	79.1	92.9	11.1/ 0.22	61.1	97.2	22.6/ 0.40
15	76.1	95.2	15.9/ 0.25	61.1	97.2	22.6/ 0.40
20	62.7	97.6	26.1/ 0.38	50	97.2	17.9

LAS leukocyte aggregation score; CSF WCC cerebrospinal fluid white cell count; LR likelihood ratio

4.0 DISCUSSION

There are no early reliable diagnostic tests which can accurately and conveniently differentiate between bacterial and non-bacterial causes of meningitis in children. We modified a technique proposed by Israeli authors (55) and analysed its utility in children with bacterial, viral and aseptic meningitis. One hundred and nine patients who fulfilled predefined inclusion criteria were prospectively studied. A fresh sample of CSF was placed on a slide at the time of a diagnostic lumbar puncture. The 67 bacterial cases were compared to a combined category of 23 viral and 19 aseptic cases, which were presumed to have similar aetiologies on the basis of laboratory results. The degree of leukocyte aggregation was calculated and comparative analyses were conducted to evaluate the validity, the intra- and inter-observer variability, and susceptibility of the proposed test to confounding factors.

We showed that there were no significant differences in age, sex, exposure to antibiotics or duration of symptoms prior to admission in the 2 categories of disease that we studied: bacterial and viral/aseptic. In contrast to this, we demonstrated significant differences between these groups for the following standard tests used in investigating meningitis: serum CRP, total CSF WCC, CSF neutrophils and lymphocytes, CSF protein and glucose ratios. The limiting factors for these tests (except for CSF protein), however were the poor sensitivity and specificity as derived by the ROC curves. The peripheral white cell count was the only parameter studied that was not able to discriminate at all between the disease groups. In addition, assays of TNF-α, IL-1β, IL-6 and IL-8 differed significantly between the disease

groups and a control group. The highest levels of cytokines were found in the bacterial category.

In order to establish the reliability of the leukocyte aggregation test, we evaluated the technique of slide preparation and aggregate enumeration. We confirmed that intra- and inter-observer variability did not exceed 5% although in patients with the LAS greater than 50%, the inter-observer variability increased to a maximum of 10%. Considering that patients who had high levels of aggregation invariably had bacterial meningitis, we concluded that such variability did not effect the outcome (the highest LAS for the viral/aseptic category was 20.7%). Furthermore, our results clearly demonstrated that the degree of leukocyte aggregation increased towards the bottom of the slide in the bacterial cases and, although not reaching significance, there was also a trend towards greater aggregation nearer the bottom of the slide in viral/aseptic cases. This finding concurs with a previous study on blood involving a similar technique of leukocyte aggregation. The authors concluded that a random selection of a part of the slide may cause misinterpretation and recommended three counts at the proximal, central and distal portions of the slide in order to improve the reproducibility of the test (65). In the light of this finding, we used three separate scores and a mean score for each slide if there were sufficient cells, otherwise a single global score was used as a mean LAS.

The mean LAS was significantly greater in the bacterial group as was reported previously (55) although we found some overlap in the LAS between the disease groups. We compared the mean LAS per disease category with the conventional investigations and cytokine assays in order to evaluate the test's clinical suitability. It performed better than blood cultures, Gram stains or CSF cultures with regard to sensitivity when compared to each test independently,

however the combination of CSF culture, Gram stain or blood culture was the most sensitive method (Tables 3.2 and 3.6). The utility of the test was further analysed using ROC curves, which clearly showed that it performed significantly better as a diagnostic test than peripheral WBC count, serum CRP and CSF total WCC. There were no statistically significant differences between the LAS curve and the curves of CSF protein, TNF-α, IL-1β,IL-6 and IL-8 (Figures 3.15–3.18). This finding demonstrates the diagnostic usefulness of CSF protein as well as the value of the LAS test which is an easily applied and cheap method when compared with the sophisticated and expensive techniques listed above.

In order to recommend a new test, the sensitivity, specificity and likelihood ratios of a positive and negative test need to offer definite advantages. We assessed these parameters of the leukocyte aggregation test by deriving the sensitivities and specificities which corresponded to a range of LAS cutoff values from the ROC curve. Considering that the intention of a rapid bedside test is to screen accurately for bacterial meningitis the selected LAS cutoff would need to correspond to a high sensitivity and a low likelihood ratio (–), which would rule in disease. Table 3.6 lists the sensitivities, specificities and likelihood ratios for a range of LAS cutoff values. Optimal values may be considered 0–3%, however the higher the cutoff the lower the sensitivity. These cutoff values are substantially lower than the 15% previously reported as optimal (55).

The Israeli study did not find any correlations between the LAS and other markers of inflammation (55). Similarly other studies have reported no relationship between elevated CSF cytokines and other inflammatory markers in cases of bacterial meningitis (27,66,67).

However, we found statistically significant correlations between the LAS and serum CRP, CSF protein, CSF total WCC, IL-1β, IL-6 and IL-8 (but not TNF-α) in the bacterial meningitis patients. Similarly, in the viral/aseptic meningitis cases there were correlations with peripheral WBC count, CSF protein and CSF total WCC. The fact that there is a wide scatter of plots suggests that the clinical relevance of these findings is weak. The relationship between CSF cell count and degree of aggregation may partly be explained by greater inter-cellular interaction because of closer proximity of activated cells.

In view of the tendency to find greater leukocyte aggregation in viral/aseptic cases within the high range of CSF total WCC (which may detract from the ability to distinguish aetiologies), we analysed the efficiency of the test in patients with a CSF total WCC $\leq 300\times10^9$/L. This value was selected as it was the midpoint of a bimodal distribution (Figure 3.1). This approach did not offer any advantage with regards to sensitivities and likelihood ratios (–) (Table 3.6).

HIV-1 status was considered to be relevant to this study because recent reports have demonstrated that the disease stimulates the expression of cell surface molecules involved in leukocyte adherence, for example integrins of the CD11 and CD18 type and intercellular adhesion molecule-1 (ICAM-1) (68,69). It also induces the secretion of circulating forms of certain adhesion molecules such as soluble vascular adhesion molecule-1 (70). Increased levels of cytokines such as IL-1β and IL-6 have been detected in the CSF of HIV-1 infected patients (71). HIV-1 infected monocytes have also been shown to have a significantly increased tendency to aggregate (69). These characteristics may affect the CSF leukocyte aggregation pattern and are therefore an important consideration. Our findings did not

corroborate the theoretical implications of the previous reports. A positive HIV-1 status was associated with a lower mean LAS in the viral/aseptic cases (although the sample size was small) but no differences were noted in the bacterial cases.

Traumatic lumbar punctures introduce blood and possibly other circulating adhesion molecules into the CSF which may theoretically modify inter-cellular interactions and therefore, may affect the leukocyte aggregation. However, we did not find any evidence of such an effect.

The precise mechanism of leukocyte aggregation in blood or CSF is not known. Studies on blood have generated various hypotheses regarding the pathophysiology; whether these are similar for CSF requires further investigation. The theories can be summarised as follows: 1) membrane-mediated mechanisms and 2) plasma factors. Cellular adhesion molecules (CAMs) are protein receptors found on leukocyte and endothelial cell membranes. Three categories, the integrins, selectins and immunoglobulins function to maintain vascular and tissue integrity. However in disease states, they act in unison with cytokines to contribute to inflammatory tissue injury and cellular aggregation (72).

Furthermore, activated neutrophils express CD11b/CD18 glycoproteins, which are part of the integrin family of surface receptors and mediate cellular aggregation (54,73). Fibronectin, which is included in the integrin group of adhesion molecules, has also been shown to play a role in aggregation (54,74). L-selectin is an adhesion molecule on the surface of neutrophils and when activated, initiates the steps that lead to aggregation (75). Plasma mediators include

platelet-activating factor (76), the third and fifth components of complement (53) among others (54).

A limitation of our study was the insufficient number of TB meningitis cases and therefore the unknown effect of TB on the LAS. For this reason our results cannot be generalised to communities with a high prevalence of TB. The technique itself, although simple, is vulnerable to technical difficulties, necessitates extensive cell counts, requires a functional microscope, and does not increase the sensitivity and specificity over other currently employed tests.

5.0 CONCLUSION

No single clinical or laboratory test can make an early, accurate and convenient diagnosis of the aetiology of meningitis in children. Usually a combination of tests is required and often antibiotic therapy is initiated empirically. Our observations suggest that the finding of leukocyte aggregation in the CSF may be a helpful adjunct for the early diagnosis of bacterial meningitis. The proposed technique which we modified to enhance test reliability in diagnosing bacterial meningitis, performed better than peripheral WBC count, serum CRP, CSF total WCC, blood culture, CSF Gram stain or CSF culture (when compared independently), and equally as well as CSF protein and sophisticated tests measuring various cytokines. The most sensitive and specific method was a combination of blood culture, CSF culture or Gram stain, although this approach may be limited by a delay in diagnosis of at least 24 hours.

The proposed leukocyte aggregation test is limited however by possible technical difficulties, and the need for extensive cell counts and a functional microscope. The effect of TB meningitis needs to be assessed before the test can be recommended in TB endemic areas.

The optimal aggregation cutoff values as assessed by likelihood ratios (–) ranged from 0% to 3% with a sensitivity which declined from a maximum of 98.5% to 92.5%. No advantage was found by applying the test selectively to those patients with less than 300×10^9 cells/L of CSF. We recommend that a specific LAS cutoff value should be selected according to the

investigator's preferred emphasis on better sensitivity or specificity and that the listed likelihood ratios are used to calculate post-test probability.

The phenomenon of aggregation has not been fully elucidated, but it appears that diverse inflammatory mechanisms are involved as evidenced in this study by statistically significant correlations between the degree of aggregation and inflammatory markers like serum CRP, CSF WCC, CSF protein and cytokines. Prior antibiotics, duration of symptoms prior to admission and traumatic lumbar punctures did not effect the aggregation score. HIV-1 status had no effect on aggregation in bacterial cases but the association with lower aggregation scores in viral/ aseptic cases requires further investigation.

APPENDIX A

INFORMATION AND CONSENT FORM

Dear parent / guardian and child,

Your child is thought to have a serious disease called **meningitis**, which is due to an infection of the membranes around the brain. There are different types of germs (organisms) that can cause this disease and it is important to know which germ is causing the infection because the correct treatment depends on this. However, the tests done in the laboratory at present are not always able to tell us the precise cause.

Your doctor will be taking some spinal fluid to check for infection of the brain using the standard tests. We are doing a study to check whether clumping of certain cells in the spinal fluid can help us diagnose the cause of meningitis. We would like to :

1) do an extra test on the **spinal fluid** which your doctor will be collecting in any event, and
2) do an extra **blood test**, if necessary, when your doctor next takes blood to help make the diagnosis.

Taking part in this study is voluntary. The treatment will not change in any way if you refuse. You can change your mind and withdraw from the study at any time. The results of all tests will be confidential.

If you are willing to allow your child to take part in this study, please sign below.

	Parent/guardian	Child (if able to understand: ±12yrs)
Name:	_____	_____
Signature:	_____	_____
Witness:	_____	Date: _____

If you have any questions, please contact:

Dr Ian Michelow

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