Complement activation in neonatal infection

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Abstract

To investigate the usefulness of indices of complement activation in the diagnosis of infections in the neonatal period, activation products C4d, Ba, and C3d were measured in 42 babies with a putative diagnosis of infection based on clinical/laboratory criteria, and compared with conventional clinical and haematological criteria of infection and with C reactive protein. The diagnosis of sepsis was confirmed by culture and identification of organisms in 17. Fourteen babies in whom infection was not suspected formed the control group. In babies with proved infection, concentrations of the fragments C4d, Ba, and C3d were higher than in babies with suspected infection in whom microbiological tests were negative, and concentrations of Ba and C3d were higher than in controls. C reactive protein and the platelet count were not significantly different in babies with proved infection and those with negative microbiological tests, but in the latter, C reactive protein concentrations were

higher than in controls. Of the indices studied, high concentrations of Ba predicted microbiologically proved infection with the highest sensitivity (47.1%) and specificity (92.0%). Ba thus seems to be useful as an early indicator of infection in the neonatal period.

Bacterial infections in the neonatal intensive care unit are a major cause of morbidity and mortality.¹⁻³ The early and reliable diagnosis of sepsis in the neonate remains an important goal, but to date no single marker of infection, apart from culture and isolation of the relevant micro-organism, has been identified. Microbiological screening has the disadvantage of requiring 24 to 48 hours to provide results, while potentially life threatening neonatal infections must be treated immediately. Other, more indirect markers of infection, such as the white cell, platelet, and neutrophil counts and C reactive protein concentrations, have been shown to provide some diagnostic help,⁴⁻¹⁰ but do not vary solely in response to the presence of infection.^{6, 11-13}

The complement system is comprised of a series of over 30 proteins which are an essential component of host protection against a range of pathogenic organisms.¹⁴ The complement cascade is activated directly by bacteria and antigen-antibody complexes, and the degree of complement activation could, therefore, provide early and specific evidence of bacterial infection (fig 1). To examine the possibility that triggering of the complement cascade reflects the presence of infection in the neonatal period, we studied the concentrations of complement fragments released during complement activation using techniques recently developed in our department.¹⁵⁻¹⁷ The efficacy of complement activation in distinguishing infected from non-infected neonates is compared with other widely used indicators such as the platelet, neutrophil, and white cell counts and the concentration of C reactive protein.

Subjects and methods

STUDY DESIGN

Babies included in this study were allocated to two main diagnostic groups: the controls, comprising a group of babies without suspicion of infection from whom blood was being taken for management purposes, and babies with the diagnosis of infection. The clinical/

Figure 1 Complement activation pathway. Classical pathway activation in the neonate could be initiated by immune complexes composed of maternal antibody and bacterial antigens. The alternative pathway is activated by lipopolysaccharides (LPS) in bacterial cell walls. Both pathways converge at the level of C3 and the membrane attack pathway leads to formation of the membrane attack complex (MAC). Concentrations of the activation products C4d, Ba, and C3d provide a measurement of complement activation through the classical, alternative, and membrane attack pathways, respectively.

laboratory criteria for this diagnosis are given in table 1 and were assessed independently by two physicians (GL and HG), who were unaware of the outcome of microbiological tests, complement measurements, or C reactive protein concentrations. On the basis of these criteria, babies with suspected infection were further subdivided into those in whom infection was considered to be probable and those in whom it was only considered possible. All babies with suspected infection – possible or probable – were screened microbiologically by microscopy and culture of swabs (ear, nose, endotracheal tube, umbilicus) and fluid (blood, cerebrospinal fluid, urine, and gastric aspirate). Infection was considered proved in babies who had positive blood or cerebrospinal fluid cultures or +++ cultures with pure growth of a single organism from urine or more than one swab site. The performance of putative predictors of infection (clinical/laboratory criteria, C reactive protein, platelet count, and complement activation markers) could then be compared with microbiological screening, used in this study to define the true disease state. Measurement of complement activation and C reactive protein was carried out by investigators who were unaware of clinical details.

SUBJECTS

Fifty six babies (28 boys, 28 girls) with a median gestational age of 33 weeks (range 25–40), median age at time of study 1 day (range 1–73) and median birth weight 1700 g (range 434–4180) who were admitted to the neonatal intensive care unit of King's College Hospital were studied.

Forty two babies with clinically suspected infection were screened microbiologically. In 17 babies, sepsis was proved, the causative organisms being: *Staphylococcus epidermidis* (n=6), group B streptococcus (n=3), *Streptococcus faecalis* (n=2), *Pseudomonas aeruginosa* (n=2), *Escherichia coli* (n=1), *Proteus mirabilis* (n=1), *Streptococcus viridans* (n=1), and bacteroides species (n=1). Fifteen of these babies had clinical/laboratory criteria indicating probable infection and two were classified as having possible infection.

Fourteen babies had no suspicion of infection, and these formed the control group (seven boys, seven girls) with a median gestational age of 34 weeks (range 26–40), median age at time of study 1 day (range 1–73), and median birth weight 1580 g (range 840–4180). There were no significant differences in gestation, age, or birth weight between controls and babies with suspected infection, irrespective of whether the microbiological screen was positive or not.

Blood collected in a final concentration of edetic acid of 10 mmol/l and remaining after routine haematological tests had been performed (0.5–1 ml) was centrifuged at 1000 g at 4°C for 15 minutes and the plasma was stored at –70°C. When there was insufficient plasma for all parameters to be measured, analyses were performed in the following order of priority: C reactive protein, Ba, C3d, and C4d.

C4d, Ba, AND C3d MEASUREMENT

C4d concentrations were determined by a nephelometric technique.¹⁶ Briefly, plasma was brought to 11% final concentration of polyethylene glycol 6000 (PEG) (Sigma Chemicals) and centrifuged at 1500g for 30 minutes at 4°C. This manoeuvre precipitates C4 and its larger fragments, leaving C4d in the supernatant. The concentration of C4d was then measured by laser nephelometry, using anti-C4 intact antiserum (Behring Diagnostics). Ba and C3d plasma concentrations were measured using similar techniques and antifactor B and anti-C3d antiserum (Dakopatts) after treatment of the plasma samples with high PEG concentrations.^{15 18} C4d and C3d results were expressed as percentages of 100% C3d and C4d standards, obtained by exhaustively activating complement through the classical pathway by incubating normal human serum with heat aggregated immunoglobulin. Ba results were expressed as percentages of 100% Ba standard, obtained by exhaustively activating the alternative complement pathway by incubation of normal human serum with inulin.

C REACTIVE PROTEIN MEASUREMENT

Plasma concentrations of C reactive protein were measured using a commercially available kit (Behring Diagnostics). Latex particles coated with anti-C reactive protein antiserum are aggregated by serum C reactive protein and the light scatter measured by nephelometry. Results were expressed in mg/l.

STATISTICAL ANALYSIS

Values of the complement indices, C reactive protein, white cell, neutrophil, and platelet counts, gestational age, and birth weight in each test group conformed to a normal distribution according to the Kolmogorov-Smirnov goodness of fit test and were compared using one way analysis of variance (ANOVA) and multiple Student's *t* tests. Statistical computations were performed using the Statistical Package for the Social Sciences (SPSS-X) of the University of London Computer Centre Amdahl 5980/300. Frequency distributions

Table 1 Clinical criteria used to diagnose infection in neonates

(1) Possible infection		
At least one of the following:		
Prolonged rupture of membranes		Maternal fever
Gram stain positive in gastric aspirate		Fever (37.5–38°C)
Meconium staining		Vomiting
Increased gastric aspirate		Mild abdominal distension
(2) Probable infection		
One each of the following clinical and laboratory criteria:		
(a) Clinical:		
Lethargy		Apnoea
Poor spontaneous movement		Temperature instability
Bradycardia		Hypotension
Cyanosis		Grunting
Diarrhoea		Haematochezia
Severe abdominal distension/tenderness		Sclerema neonatorum
Petechiae		
(b) Laboratory		
White cell count ($\times 10^9/l$)	First 48 hours	After 48 hours
Neutrophil count ($\times 10^9/l$)	<3	>24
	<2.5	2.5 < >7.5

Table 2 Means (SD) and their statistical comparison in subjects with suspected infection (possible or probable) and controls

	(1) Controls	Babies with suspected infection		ANOVA (F ratio)	Student's <i>t</i> tests (<i>t</i> value)			
		(2) Possible	(3) Probable		(1) v (2)	(2) v (3)	(1) v (3)	(1) v (2 and 3)
C4d (%)	22.0 (9.9)	16.5 (8.5)	32.0 (17.4)	4.36*	1.19†	-2.75*	-1.34†	-0.60†
(n)	(6)	(10)	(17)					
Ba (%)	63.5 (27.8)	48.5 (22.9)	120.3 (71.3)	12.03***	1.68†	-4.35***	-2.61*	-1.02†
(n)	(12)	(21)	(19)					
C3d (%)	23.6 (8.6)	20.8 (4.9)	31.9 (8.9)	10.85***	1.11†	-4.75***	-2.42*	-0.83†
(n)	(9)	(19)	(18)					
C reactive protein (mg/l)	16.8 (15.5)	38.8 (46.8)	47.7 (46.3)	2.37†	-1.69†	-0.61†	-2.39*	-2.07*
(n)	(14)	(22)	(20)					
Platelet count ($\times 10^9/l$)	320 (78)	267 (106)	223 (105)	2.63†	1.29†	1.19†	2.39*	1.91†
(n)	(14)	(22)	(20)					

†=Not significant.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

were analysed using the χ^2 test with Yates's correction for small numbers and similar levels of significance were obtained using Fisher's exact probability test.

Results

COMPLEMENT MEASUREMENTS

When all babies with suspected infection were considered as a whole, concentrations of C4d, Ba, and C3d were similar to those of controls (table 2). In babies in whom infection was only considered possible, concentrations of these complement activation products were also similar to those of controls. Babies with clinical/laboratory criteria indicating probable infection had C4d concentrations similar to those of controls, but significantly higher concentrations of Ba and C3d. Babies with probable infection had concentrations of C4d, Ba, and C3d that were significantly higher than those in babies with possible infection.

Babies with suspected infection but negative microbiological screens had concentrations of C4d, Ba, and C3d similar to those of controls (table 3, fig 2). In contrast, Ba and C3d were significantly higher in babies with proved infection when compared with controls, though C4d concentrations were similar in the two groups. Concentrations of C4d, Ba, and C3d were significantly higher in patients with proved infection than in those with suspected infection but with negative microbiological screens. There was no relationship between the type of organism, whether Gram positive or negative, and the concentrations of any of the complement fragments.

To assess the relationship between the frequency of high concentrations of complement

fragments and the outcome of microbiological screening in babies with suspected infection, mean concentrations in the sera from the controls + 2SD were used as the upper limit of normal. Concentrations of Ba greater than the mean for controls + 2SD were found in eight of 17 neonates with proved infection and two out of 25 in whom microbiological screen was negative ($\chi^2=6.81$; $p < 0.01$). High concentrations of C4d were found in four out of 17 neonates with proved infection but in none of the 25 with negative sepsis screens ($\chi^2=3.42$; $p > 0.05$). High concentrations of C3d were found in two out of 17 babies with proved infection and one neonate with a negative sepsis screen ($\chi^2=0.11$; $p > 0.05$).

C REACTIVE PROTEIN

When all babies with suspected infection were considered as a whole, concentrations of C reactive protein were significantly higher than controls (table 2). In those considered to have only possible infection, C reactive protein was higher than in controls but the difference did not reach statistical significance ($p=0.10$). Babies with clinical/laboratory criteria indicative of probable infection had C reactive protein concentrations significantly higher than controls. Concentrations of C reactive protein were similar in babies considered to have possible infection and those with probable infection.

Babies with suspected infection but whose microbiological screening tests were negative had significantly higher C reactive protein than controls (table 3, fig 2) and higher concentrations than babies with microbiologically proved infection, though this was not statistically significant ($p=0.44$). Babies with proved

Table 3 Means (SD) and their statistical comparison in subjects with suspected infection (proved microbiologically or not) and controls

	(1) Controls	Babies with suspected infection and microbiological screen		ANOVA (F ratio)	Student's <i>t</i> tests (<i>t</i> value)		
		(2) Negative	(3) Positive		(1) v (2)	(2) v (3)	(1) v (3)
C4d (%)	22.0 (9.9)	18.1 (9.4)	36.1 (17.8)	6.84**	-0.85†	-3.49**	-1.80†
(n)	(6)	(14)	(13)				
Ba (%)	68.5 (27.8)	48.2 (26.9)	134.1 (66.9)	19.69***	1.59†	-5.67***	-3.43**
(n)	(12)	(24)	(16)				
C3d (%)	23.6 (8.6)	21.9 (7.0)	31.9 (8.4)	7.93**	0.58†	-3.97***	-2.45*
(n)	(9)	(21)	(16)				
C reactive protein (mg/l)	16.8 (15.5)	47.6 (48.7)	36.2 (42.7)	2.53†	-2.29*	-0.78†	-1.61†
(n)	(14)	(25)	(17)				
Platelet count ($\times 10^9/l$)	320 (78)	248 (111)	245 (103)	1.78†	1.74†	0.06†	1.82†
(n)	(14)	(25)	(17)				

†=Not significant.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2 Concentrations of the complement fragments (A) C4d; (B) Ba, (C) C3d, and (D) C reactive protein in control babies and neonates with suspected infection divided according to the outcome of microbiological tests.

infection had higher concentrations of C reactive protein than controls, though not significantly so ($p=0.12$).

Concentrations of C reactive protein greater than the mean of the controls + 2SD were found in five of 17 neonates with proved infection and in nine out of 25 in whom the microbiological screening tests were negative ($\chi^2=0.01$; $p>0.05$).

HAEMATOLOGICAL MEASUREMENTS

In all babies with suspected infection considered as a whole and those who were considered only to have possible infection, white cell, neutrophil, and platelet counts were not statistically different from those of the controls. Babies with clinical/laboratory criteria indicative of probable infection had platelet counts significantly lower than those of the controls but the counts were similar to those in babies in whom infection was only considered possible.

The white cell, neutrophil, and platelet counts were not statistically different in babies with microbiologically proved infection compared with those in babies with suspected infection whose microbiological screening tests were negative and those of controls.

Using previously established criteria for the definition of abnormal white cell (<5.0 or $>20.0 \times 10^9/l$),⁵ neutrophil (<2.0 or $>7.5 \times 10^9/l$),¹¹ and platelet counts ($<150 \times 10^9/l$)⁷

there were no significant relationships between abnormal values of these and the outcome of microbiological screening tests in babies with suspected infection ($\chi^2=0.02$, 0.08 , and 0.04 , respectively).

PERFORMANCE CHARACTERISTICS OF LABORATORY INDICATORS OF NEONATAL INFECTION

The sensitivity and specificity of the laboratory indicators C4d, C3d, Ba, C reactive protein, and white cell, neutrophil, and platelet counts in predicting infection are shown in table 4, using microbiologically proved infection as the reference disease state. The values of each parameter that are considered abnormal are those described above. The three markers of complement activation had the highest specificity of any laboratory index, and Ba provided the most sensitive marker of infection. When a high value of any of the three complement fragments was used as the criterion for prediction of infection, no improvement in performance was obtained (sensitivity 52.9%, specificity 88.0%).

Discussion

The diagnosis of infection presents one of the most difficult problems in the care of preterm babies. Current clinical and haematological diagnostic criteria, while identifying babies

Table 4 Performance characteristics of laboratory indicators of neonatal infection

Indicator	% Sensitivity*	% Specificity†
C4d	30.7	100.0
Ba	47.1	92.0
C3d	12.5	95.2
C reactive protein	29.4	64.0
White cell count	17.6	76.2
Platelet count	5.9	84.2
Neutrophil count	29.4	63.2

*Measurement of how well the presence of infection was predicted.

†Measurement of how well the absence of infection was predicted.

who are eventually proved to be infected, inevitably include many who are not, yet they all undergo intensive chemotherapy. Many studies have been performed to sharpen the criteria, employing other individual or groups of tests, but with only limited success.⁴⁻⁷ The present study was initiated to investigate the usefulness of measuring indices of complement activation in diagnosing neonatal infection. As complement forms an important arm of the innate immune system, it provides protection against infection during the neonatal period, when the specific, acquired immune system is still relatively immature.¹⁸ The concentrations of fragments C4d, Ba and C3d, which are released after cleavage of their parent molecules C4, factor B and C3, provide both unequivocal evidence, and a measurement, of complement activation proceeding through the classical, alternative, and common pathways.¹⁹ It follows that their concentrations may reflect infectious episodes in the neonate and be of use in the early diagnosis of sepsis.

In the present study, neonates with a presumptive diagnosis of infection made on conventional clinical and haematological grounds had no significant increase in concentrations of complement fragments. However, when the diagnosis was made using an accepted reference test, namely the culture and identification of organisms from relevant sites, high concentrations of Ba and C3d were present in the infected group, indicating an increase in activation through the alternative and common pathways, but normal concentrations were found in the group with negative cultures. Moreover, concentrations of C4d, Ba, and C3d were higher in babies with positive than in those with negative cultures. High levels of complement activation, therefore, may be able to identify infected neonates in whom a diagnosis of possible sepsis is made.

By comparison, other widely accepted markers of infection, such as C reactive protein⁸⁻¹⁰ and the platelet count,⁷ failed to discriminate between infected and non-infected neonates. In our study, C reactive protein was the only parameter which was higher in neonates with any suspicion of infection, whether possible or probable. At first sight, this finding lends apparent support to the concept that C reactive protein may be useful in managing the neonate with suspected sepsis. Further examination shows, however, that concentrations of C reactive protein were not statistically different from controls in babies from whom organisms were cultured, while in babies with negative infective screens C reac-

tive protein was higher than in controls. These results question the use of C reactive protein as a marker of neonatal infection. They suggest that its popularity rests largely on its tendency to confirm the clinician's suspicion, rather than its ability accurately to identify infected neonates.

The explanation for complement activation in infected babies could be the presence of preformed, maternal antibodies directed against micro-organisms. The antibodies that cross the placenta belong to the IgG isotype, and are efficient activators of the complement system.¹⁴ Activation could also take place after direct triggering of the alternative pathway by the lipopolysaccharide cell walls of bacteria.²⁰ The fact that evidence of complement activation is found in infected babies is not surprising, therefore, and it provides a potential tool with which to identify this population.

This is the first study to demonstrate that high values of indices of complement activation are found in neonates with microbiologically proved infection. The relative performances of complement activation indices, C reactive protein and white cell, neutrophil, and platelet counts are reflected in the sensitivity and specificity with which they were capable of predicting culture proved infection. C reactive protein and haematological counts lack the performance characteristics required of a useful laboratory marker of neonatal infection. Fragment Ba, on the other hand, has a combination of high specificity and reasonable sensitivity in the prediction of culture positive infectious episodes in the neonatal period. As complement activation products can be measured within hours, as opposed to the 24-48 hours required for blood, fluid and swab cultures, fragment Ba could offer the clinician an early marker, providing a much needed adjunct to the clinical diagnosis of infection in neonatal intensive care.

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