

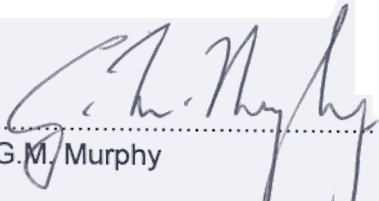
Ephemeral fever - a biochemical model for inflammatory disease in cattle and sheep.

Proceedings Fifth Symposium Arbovirus Research in Australia, 28 August - 1 September 1989. CSIRO/QIMR Brisbane, Australia. (Uren, M.F., Blok, J., and Manderson, L.H., Eds) pp.268-274 (1990).

by

Murphy, G.M., St.George, T.D. and Uren, M.F.

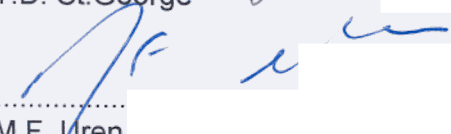
The increasing spectrum of test data available from cutaneous myiasis of sheep and ephemeral fever had shown that similar changes were occurring in the two disease entities, hence the title. Experience was transferred from the experiments with sheep in the planning stages, so that the results could be compared. The cattle were housed close to the biochemistry laboratory. Dr St.George and Dr Uren participated in the design of the experiments and shared the clinical observations. I wrote the paper.


G.M. Murphy
I agree with this statement.

Date 30/5/00


T.D. St. George

Date 30/5/2000


M.F. Uren

Date 18.7.00

EPHEMERAL FEVER — A BIOCHEMICAL MODEL FOR INFLAMMATORY DISEASE IN CATTLE AND SHEEP

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Our previous studies (St. George *et al* 1984; Uren and Murphy 1985; Murphy *et al* 1986) have provided a biochemical basis for the clinical signs shown by cattle affected with ephemeral fever. Most marked are the changes in circulating levels of calcium. We argued (Murphy *et al* 1986) that the rapid onset of the clinical signs — tachycardia, muscle tremor, ruminal stasis, inability to swallow and recumbency, were consistent with hypocalcaemia (plasma Ca ≤ 2.0 mM L⁻¹); and that these changes were direct consequences of a viraemia induced pathophysiology.

In this paper we comment on the time course of virus recovery in experimentally infected cattle and the onset of symptoms. We have also assessed the role of blood pH with respect to circulating levels of ionized calcium (Ca_i).

The changes that occur in plasma copper (Cu), iron (Fe) and zinc (Zn) during the course of an ephemeral fever viraemia are similar to those found during bacterial infection and inflammatory events. The inflammatory nature of ephemeral fever is well documented (St. George *et al* 1986). We are also studying the pathogenesis of other inflammatory diseases. Myiasis (flystrike) occurs as a result of the infestation of the fleece of sheep by larvae of the Australian sheep blowfly — *Lucilia cuprina*; and (myiasis) fits this disease category (Broadmeadow *et al* 1984). Subsequent studies (Guerrini *et al* 1988; Murphy *et al* 1987; Murphy, unpublished data) have shown that high levels of plasma ammonia (NH₃) occur during this disease and that these NH₃ levels are associated with pathological changes in the brain. We have taken this opportunity to monitor plasma NH₃ levels during the course of ephemeral fever infections.

Experimental Methods

Measurements were performed on 2 groups of experimentally infected cattle: Group 1 consisted of 8 yearling Herefords and Group 2 of 4 animals of comparable type. During both experiments cattle were confined, but not tethered, within an airconditioned isolation unit. Feed and water were available *ad lib*.

All animals were inoculated IV with a known strain (M6) of virulent BEF virus. Group 1 animals were observed hourly (temperature, respiration, physical signs etc) and bled 4 hourly. Group 2 animals were observed and bled each 4 h.

Appropriate venous jugular blood samples were taken from Group 1 for haematology, virus isolation, blood pH and macro (Ca, Mg) and micro (Cu, Zn, Fe) cation analyses. Virus isolation was performed essentially as described by Uren *et al* 1989. Blood pH was determined immediately using a blood gas analyser (Gilford 1312, Gilford Instruments). Plasma calcium and magnesium assays were performed by automatic clinical analyser techniques (Olympus Reply) and plasma micro elements (Cu, Zn, Fe) by atomic absorption spectroscopy.

Plasma ammonia, glucose and phosphate levels were determined in Group 2 animals. Plasma ammonia levels were analysed using a kit method (Ammonia Mono-test, Boehringer-Mannheim) and glucose and phosphate by appropriate autoanalyser techniques (Olympus Reply). Due to the costs involved, plasma NH₃ levels were measured only from the start of the temperature rise.

Results

All animals in both groups demonstrated clinical signs of ephemeral fever. Since the incubation period varied between animals in each of the experiments, all results have been adjusted to the onset of fever, ($t = 0$ h).

Affected animals showed fever ($>40^{\circ}\text{C}$), a neutrophilic leucocytosis, a marked drop in circulating Ca levels (>0.2 $\mu\text{M L}^{-1}$) and changes in micro cation levels. Figures 1 and 2 show the mean values of these changes in Group 1 animals relative to the onset of fever. Circulating virus particles were detected at least 32 h before any clinical expression of fever and peak virus levels occurred about 8 to 12 h prior to this event (Fig 1).

Blood pH increased by more than 0.1 pH units to >7.48 . These changes are closely related to the fever pattern but lag the increases in respiration rate by almost 12 h (Fig 3). There was a strong inverse relationship between blood pH and total plasma Ca (Fig 4).

Figure 5 shows the mean effects of ephemeral fever on plasma NH₃, glucose and phosphate levels in Group 2 cattle. Plasma NH₃ levels doubled to 60 M L⁻¹ within 24 h of the onset of fever, and levels remained elevated for more than 72 h. Plasma phosphorus levels fell at least 12 h prior to fever and reached a frankly hypophosphataemic state (≤ 1.0 mM L⁻¹) 24 h

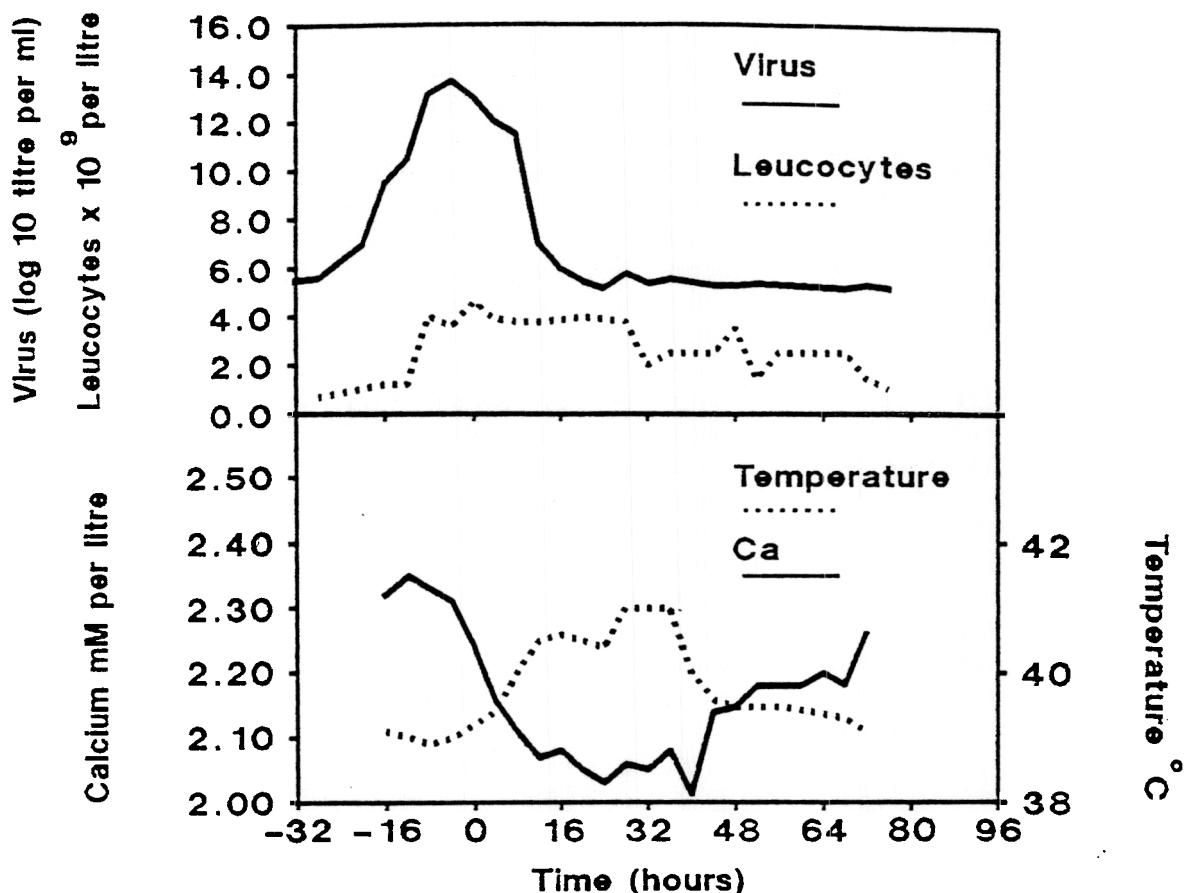


Figure 1. Cattle infected with BEF virus: isolation of virus and changes in circulating leucocyte levels, plasma calcium (Ca) and temperature, relative to the onset of fever (Time = 0 h).

after fever started. Glucose levels show a steady increase to reach a hyperglycaemic level (≥ 4.5 mM L⁻¹) at the onset of fever. Levels then fell sharply to approach frank hypoglycaemia (≤ 3.0 mM L⁻¹) 36 h after the onset of fever. The temperature response and physical signs and symptoms indicated Group 2 animals underwent only a mild reaction.

Table 1 compares the relative changes in a range of physiological parameters in ruminants affected by either ephemeral fever, flystrike or milk fever.

Discussion

The physiological responses to ephemeral fever infection reported in this paper mimic those recorded in our earlier studies (St. George *et al* 1984; Uren and Murphy 1985; Murphy *et al* 1986). What has not previously been available however, is the time base for virus circulation. This knowledge now allows more meaningful interpretation of the physiological disturbances.

In a previous study (Murphy *et al* 1986) we argued that the changes in plasma Cu, Fe and Zn during ephemeral fever were analogous to those occurring during bacterial infection and other inflammatory events. This relationship when coupled with the observation by St. George (St. George *et al* 1986) of high levels of plasma

interferon in ephemeral fever infected cattle but prior to the onset of fever, supported the contention that the physiological disturbances were orchestrated in response to and by, a lymphokine cascade. However, in the absence of an appropriate trigger, this remained purely speculative.

This speculation is now ended by the

TABLE 1

Response of plasma calcium (Ca), iron (Fe), zinc (Zn), copper (Cu), ammonia (NH₃), glucose and phosphate (Pi) levels and blood pH in cattle affected by ephemeral fever virus (BEF) milk fever (neoparturient hypocalcaemia) and sheep suffering fly strike (invasion of the skin and fleece by larvae of the Australian sheep blowfly *Lucilia cuprina*). Arrows indicate the direction and sequence of change relative to expected physiological values.

	System Response		
	B.E.F.	Flystrike	Milk fever
Plasma Ca	↓ ↓	↓	↓ ↓ ↓
Fe, Zn	↓	↓	↓
Cu	↑	↑	↑
NH ₃	↑	↑ ↑ ↑	?
Glucose	↑ ↓	↑ ↓	↑ ↓
Pi	↓	↓	↓
Blood pH	↑ ↑	↑ ↑	?

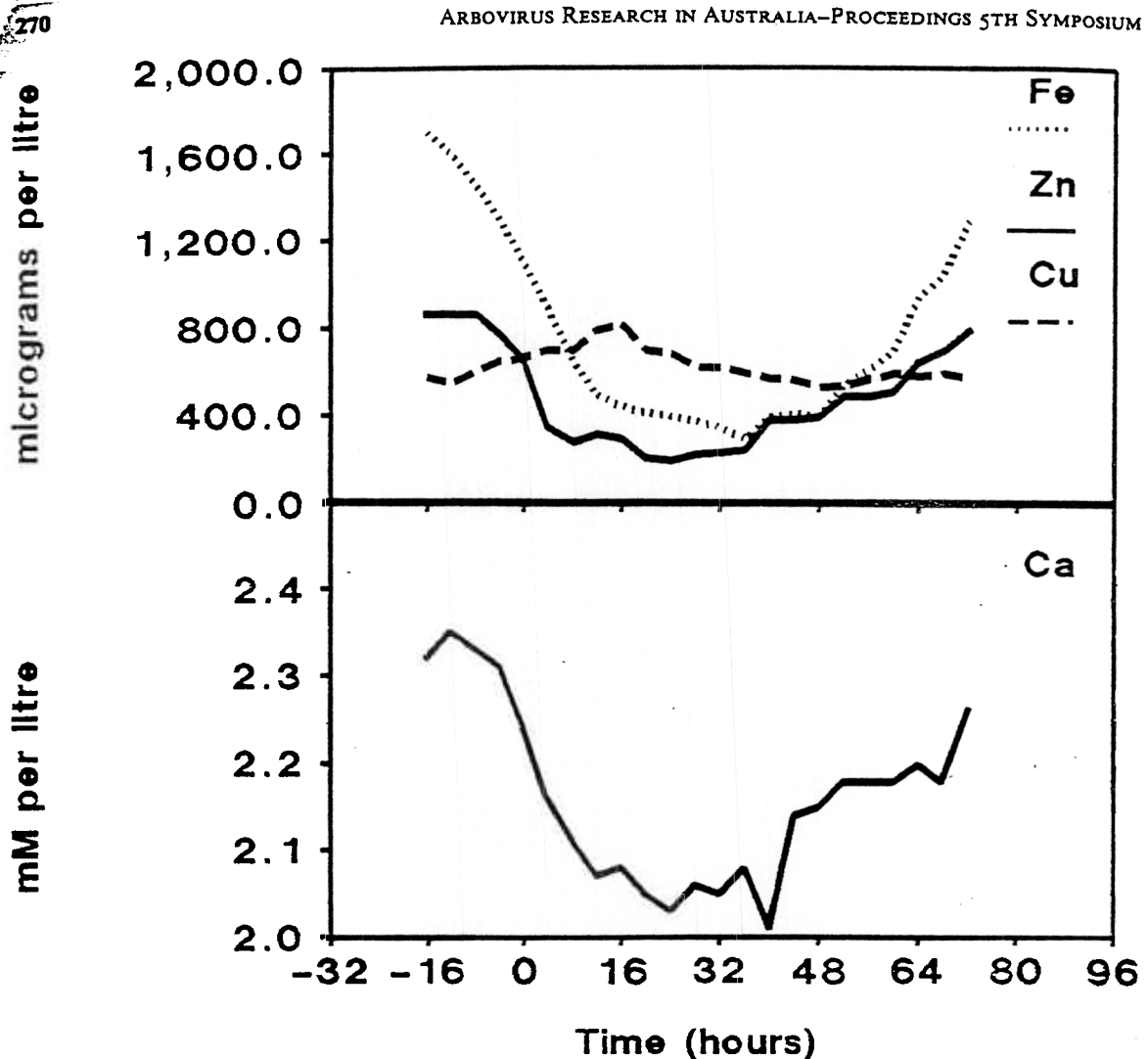


Figure 2. Cattle infected with BEF virus: changes in plasma calcium (Ca), iron (Fe), copper (Cu) and zinc (Zn) levels relative to the onset of fever (Time = 0 h).

unequivocal evidence of the presence of BEF virus. Peak viral circulation occurred 12 h prior to the onset of fever. This provides a suitable time base to explain the physiological disturbances as a consequence of a lymphokine cascade rather than attributable to pyrexia *per se*. In our previous study (Murphy *et al* 1986) we showed that calcium homeostasis was compromised well before animals refused to eat or ruminal stasis occurred. This study (Fig 1) shows that plasma calcium levels begin to fall at or about the peak of circulating virus. Whilst yet unproven, it seems reasonable to suggest that in addition to those factors identified in our earlier study as involved in this disturbed calcium homeostasis, (Murphy *et al* 1986) we should now include leucocytes. Recent evidence (Forehand *et al* 1989) indicates a significant increase (approximately 3 to 4 fold) in intracellular calcium levels in LPS primed human neutrophils. As indicated in this study and elsewhere (St. George *et al* 1986) BEF infection is characterised by a rapid increase in circulating neutrophils. This neutrophilic

leucocytosis parallels the recovery of virus (Fig 1) and the fall in total plasma calcium. This is consistent with the general conclusion arrived at by van Miert (1985) in his review of calcium homeostasis in non-specific inflammatory disease.

Previously we had speculated (Murphy *et al* 1986) that the dramatic fall in the ionizable fraction of circulating calcium may be influenced by blood pH. The data presented in Figure 4 confirm this concept which agrees with the known effect of pH on formation of the albumin calcium complex.

The behaviour of plasma ammonia has not previously been studied in ephemeral fever viraemia. However the twofold increase observed in mild reactors in this experiment (Fig 5) has potentially great clinical significance although it was not unexpected. Dinarello (1984) commented on the protein degradation resulting from the direct action of interleukin-1. He indicated that protein catabolism is greatly increased to meet the demands of the acute phase

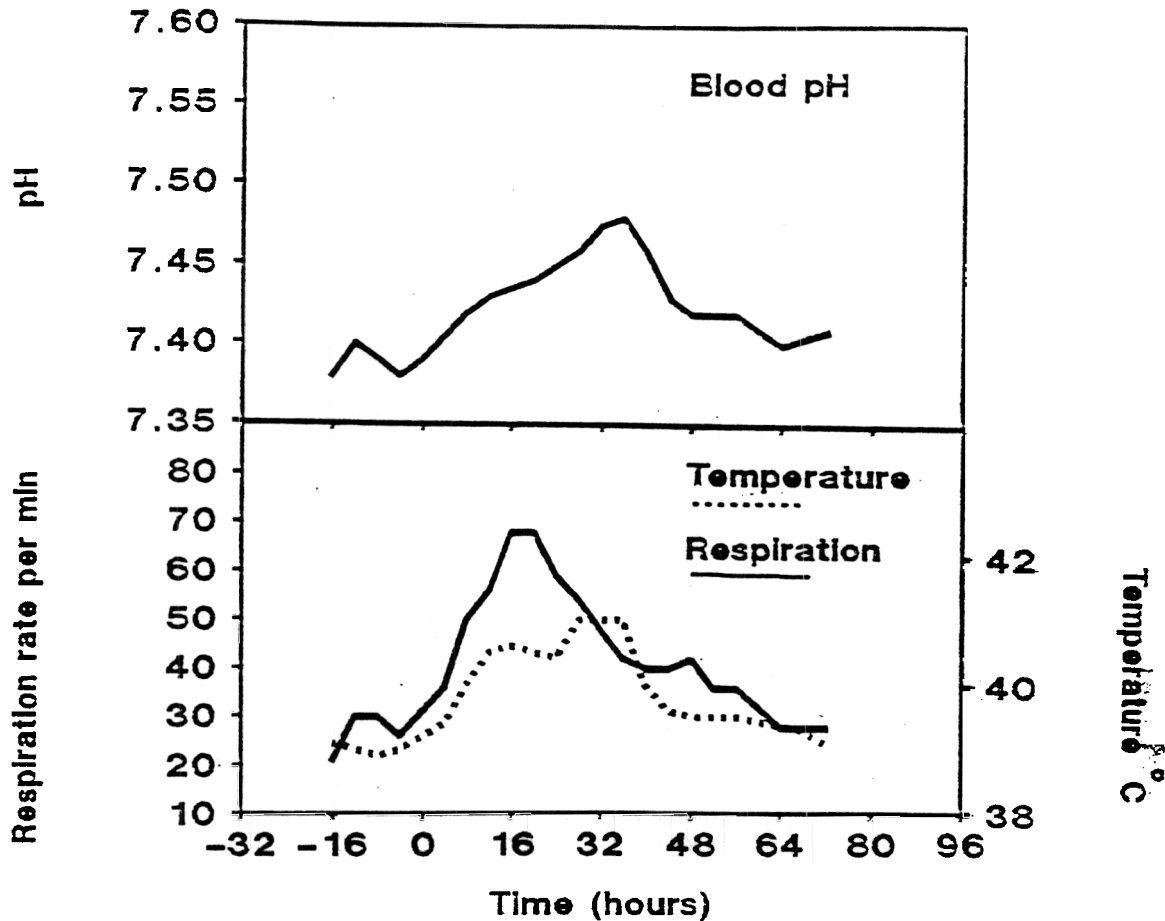


Figure 3. Cattle infected with BEF virus: changes in blood pH, Temperature and respiration rate relative to the onset of fever (Time = 0 h).

reaction for increased hepatic protein synthesis and the caloric demands of fever. Arguably, plasma NH_3 levels would rise under these conditions. The results shown in Figure 5 are consistent with this observation. The clinical significance of these ammonia levels is less obvious.

Infestation of the skin and fleece of sheep by larvae of the Australian sheep blowfly (*Lucilia cuprina*) results in pathophysiological changes which cause significant morbidity and in severe cases, death (Broadmeadow *et al* 1984). Typically, infested sheep show fever ($>40^\circ\text{C}$) respiratory distress, tachycardia, anorexia, and oligouria (Broadmeadow *et al* 1984). Haematological changes emphasise a neutrophilic leucocytosis with a high percentage of toxic band forms; and disruption of the vascular endothelium and the presence of intravascular fibrin clots in the lungs and kidneys are consistent with disseminated intravascular coagulation (Dimmock 1984). Collectively these signs point to an acute inflammatory and toxic response to unidentified factors. Recent studies (Guerrini 1988; Guerrini *et al* 1988) identify chronic hyperammonaemia (plasma $\text{NH}_3 \geq 200 \text{ ML}^{-1}$) combined with alkalosis (blood pH ≥ 7.50) as major factors in the physiological response to

myiasis. In particular these conclusions are supported by the necropsy evidence of pulmonary oedema and spongy degeneration and vacuolation of the white matter tracts of the brain (Guerrini *et al* 1988). These changes are physical evidence of ammonia toxicity in sheep (Singer and McCarthy 1971; Hooper 1972). The hyperammonaemia is thought to result from both absorption of larval secretions through the skin as well as from the acute phase response. Murphy (unpublished data) has shown that *L. cuprina* larval secretions contain in excess of $120 \text{ mM NH}_3 \text{ L}^{-1}$. Fever and alkalosis exacerbate all of the effects by increasing the fraction of total ammonia that is present in the highly toxic lipid soluble form (Visek 1984). More recently, less acute studies with the same disease (Murphy, unpublished data) have shown similar brain lesions occurred in sheep when plasma ammonia levels ranged between 120 to $150 \text{ } \mu\text{M L}^{-1}$ and body temperatures remained above 40°C for 72 h. Affected sheep were moribund and in some cases comatose prior to euthanasia.

Similar physical symptoms and temperatures of like duration, are not uncommon in severe ephemeral fever reactors (St. George *et al* 1986; Murphy *et al* 1986). It is tempting to speculate what role hyperammonaemia may play in the

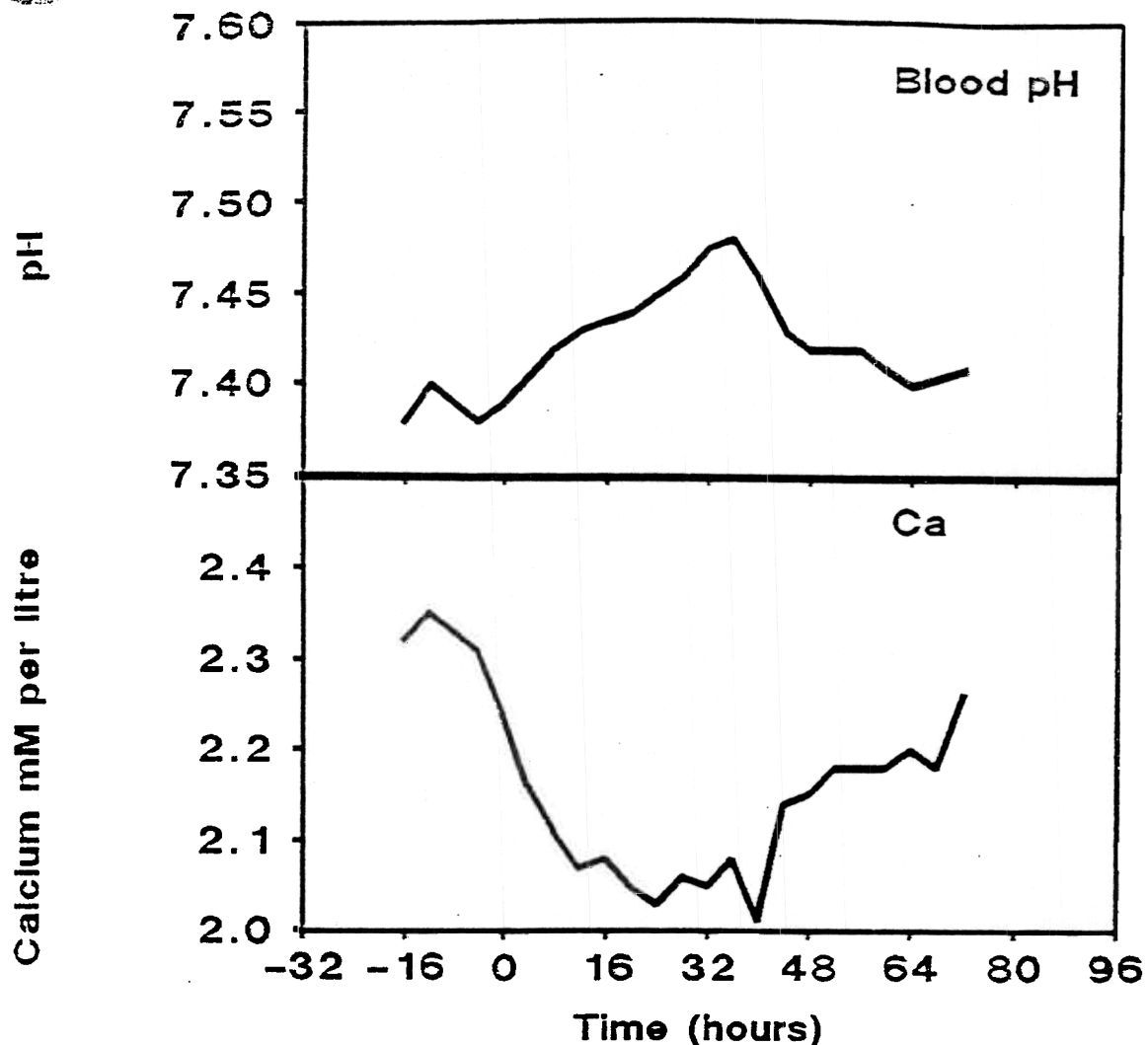


Figure 4. Cattle infected with BEF virus: changes in blood pH and plasma calcium (Ca) relative to the onset of fever (Time = 0 h).

continuing ataxia and/or paralysis seen in refractory field cases of ephemeral fever (Hill and Schultz 1977). Likewise the pulmonary oedema frequently encountered in severe ephemeral fever reactors is totally consistent with the known toxic effects of ammonia (Singer and McCarthy 1971).

The behaviour of plasma glucose and phosphorus during the course of ephemeral fever induced viraemia have not previously been reported. The initial hyperglycaemia reveals a similar physiological response to that induced by endotoxin (van Miert 1985). More importantly a similar picture is observed during myiasis in sheep (Murphy *et al* unpublished data) and in cattle suffering from milk-fever (neo-parturient hypocalcaemia) (Kronfeld 1971).

Milk fever continues as a source of serious economic loss in dairy cattle worldwide. Physiologically, milk fever or parturient paresis presents as an acute and sudden hypocalcaemia — often within hours of birth. Plasma calcium

levels fall precipitously to less than 1.5 mM L^{-1} and affected animals show dramatic responses to infusion (intravenously or intraperitoneally) of buffered solutions of calcium salts (Hibbs 1950). Clinically, affected animals show: depression, muscle tremor, recumbency (sternal progressing to lateral in severe cases), temporary paralysis of limbs, ruminal stasis and tachycardia. A low but variable percentage of affected animals do not recover within the usual 1-2 days of treatment and these animals suffer a progressive loss of reflexes, decline into a coma and die.

Given this clinical description of milk fever and the dramatic response, in most instances, to calcium therapy it is not surprising that this disease is widely held to occur because of an inability of the parathyroid gland to maintain calcium homeostasis in the face of the sudden upsurge in demand for calcium by the mammary gland (Kronfeld 1971). However careful consideration of the clinical expression of the disease and its pathophysiological similarities to ephem-

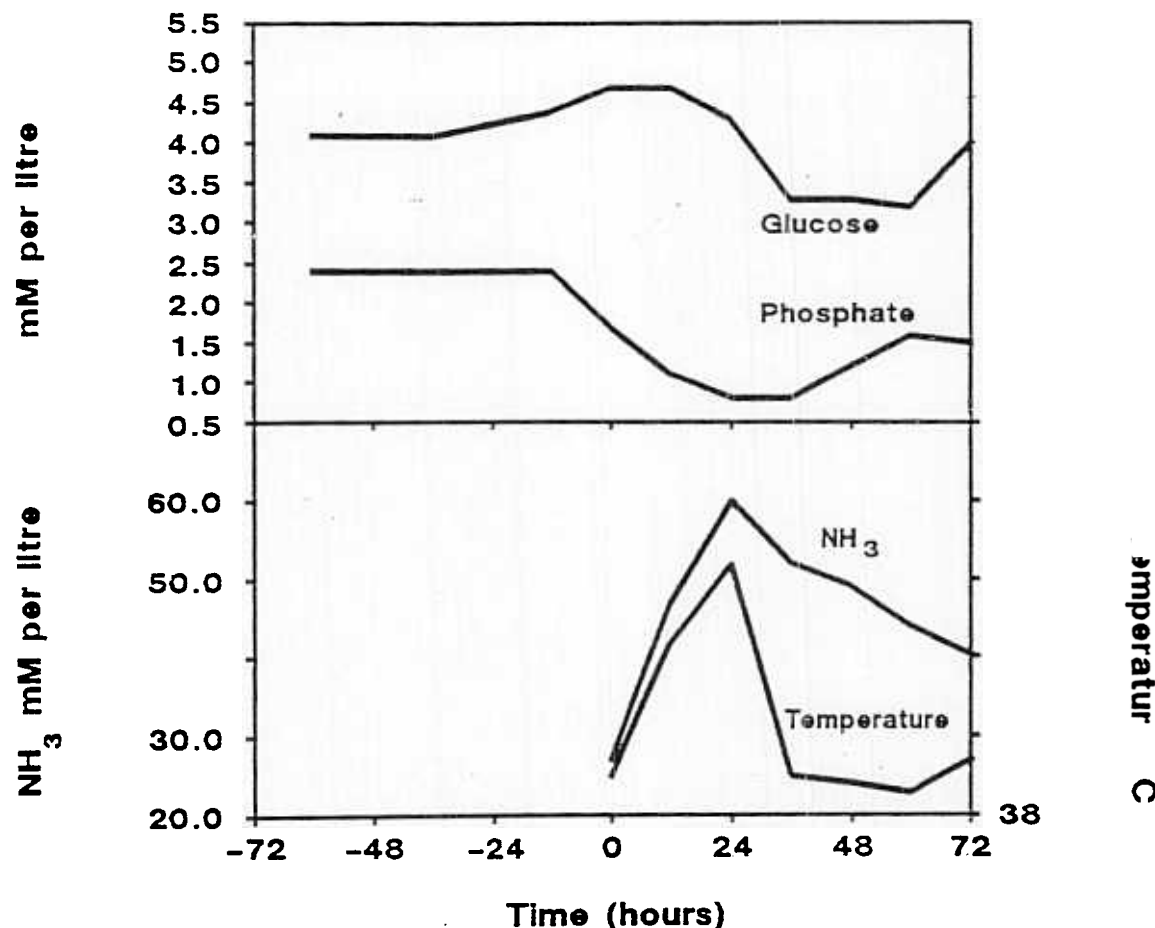


Figure 5. Cattle infected with BEF virus: changes in plasma glucose, phosphate and ammonia (NH₃) levels and body temperature relative to the onset of fever (Time = 0 h).

eral fever and fly strike, has lead us to re-examine the physiological processes leading to milk fever. Our contemporary findings (St. George, Murphy and Uren, unpublished data) involving both normal and milk fever affected animals are presented in Table 1. More importantly these results are compared with the physiological response to ephemeral fever and fly strike.

Careful consideration of the data in Table 1 indicate many common responses in ruminant animals suffering what are apparently 3 unrelated diseases — an insect borne rhabdoviral infection in cattle (ephemeral fever), an insect initiated toxemia/inflammatory reaction in sheep (fly strike), and an acute hypocalcaemic event following parturition in cattle (milk fever). We believe the unifying theme is that all represent the host response to an inflammatory process.

We would argue that our current studies (Table 1, St. George, Murphy and Uren, unpublished data) indicate that additional mechanisms are involved in the expression of milk fever. Furthermore we believe that careful assessment of the biochemical and physiological responses to inflammation as identified through our studies with ephemeral fever, will delineate the underlying

ing pathways in early milk fever (neo-parturient hypocalcaemia).

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MECHANISMS OF IMMUNITY TO BEF

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THE IMMUNE RESPONSE

The humoral response to BEF has been well documented (Cybinski 1987; Snowdon 1970). IgG - specific neutralising antibody can be detected 4 to 5 d after the onset of clinical signs and tends to peak 5 to 30 d later (Snowdon 1970). Although maternal antibody will protect calves against experimental challenge with BEF virus (St. George *et al* 1986), cattle possessing high titres of antibody can be susceptible to challenge (Tzipori and Spradrow 1978; Della-Porta and Snowdon 1979). To resolve this paradox, experiments were designed to determine the extent to which cell-mediated immunity influences the outcome of BEF infection. This approach would have obvious benefits in the design of an effective vaccine, particularly in determining the duration of immunity following vaccination.

Lymphokine Assays

Tumor necrosis factor (TNF) and interleukin 1 (IL-1) were assayed during experimental infection with BEF. Both of these lymphokines are known to have multiple overlapping biological activities (Dinarello *et al* 1988). These activities include both pathogenetic and immunological processes and it has been proposed that both these mediators play a major role in the clinical expression of BEF (Uren and Murphy 1985). Serum samples were assayed for the presence of TNF using the murine WEHI-13B myelomonocytic cell line. Cells were dispensed onto 96 well microtitre plates at 10^5 cells/well. TNF is toxic to the WEHI cell line and results are expressed as a reciprocal of the TNF concentration in the original sample. The extent of cell death was measured by the ability of live cells to

cleave the dye MTT (3-(4, 5-Dimethylthiazol-2-yl) - 2,5-diphenyl tetrazolium bromide) according to the method of Mosmann (1983). TNF levels rose markedly at the time of the onset of pyrexia and remained elevated for 24-48 h (Fig 1). A return to normal values was closely correlated with a return to normal levels of the rectal temperatures.

Serum samples were assayed for IL-1 activity according to the method of Dinarello *et al* (1977). Calf thymocytes were incubated for 72 h in the presence of concanavalin A and doubling dilutions of test serum samples. Proliferation was assessed by an overnight incubation with 0.5 μ Ci/well of [3 H] thymidine followed by liquid scintillation of incorporated radioactivity. Changes in serum levels of IL-1 closely mirrored changes in the rectal temperatures of all affected cattle (Fig 2). These results clearly demonstrate the co-ordinated effect that the release of TNF and IL-1 has on the temperature response. Recently it has been suggested that TNF has 2 separate effects: the induction of PGE₂ and the induction of IL-1 (Dinarello *et al* 1988). If both TNF and IL-1 are released at different intervals

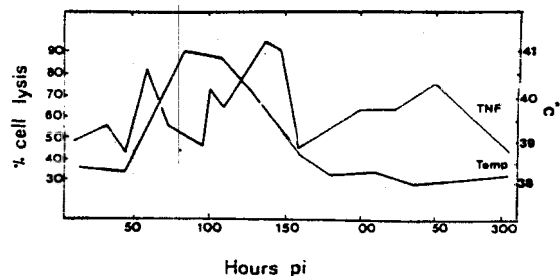


Figure 1. Tissue necrosis factor response of BEF infected cattle during the acute stage of disease.