THE LEUCOCYTOSIS OF TRAUMA

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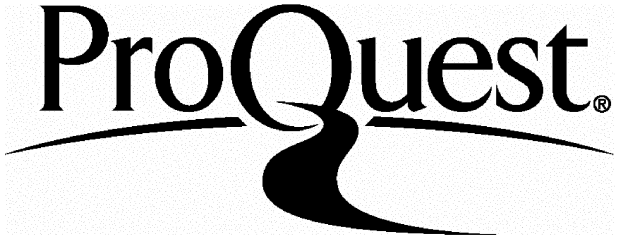

ABSTRACT

The studies in this thesis have attempted to establish the existence of the leucocytosis of trauma, to clarify its features, and to examine its possible implications for the immune response.

110 patients were studied within three hours of moderate or major injury, and a significant difference ($p<0.0001$) established between their leucocyte counts and those of 110 normal controls.

Differential leucocyte counts of 42 patients gave evidence of a very early neutrophilia and lymphocytosis. The neutrophilia was maximal at 3-4 hours post-injury and resolved slowly. The lymphocytosis resolved rapidly and was commonly replaced by lymphopenia. Serial phenotype studies of lymphocytes in 28 patients showed significant reductions ($p<0.01$) in total lymphocytes, CD4 and CD8 lymphocytes between blood samples taken less than 4 hours and 12-24 hours after injury. CD4 lymphocyte counts were low in 24 patients (85%), of which 6 (20%) were critically low ($<0.25x10^9$/L). 22 patients (78%) also had low CD8 counts.

Neutrophils of trauma patients exhibited reduced adhesion to nylon fibre when suspended in their own plasma, but not in tissue culture medium. Trauma plasma induced a reduction in adhesion of normal donor neutrophils, which was reversed by pre-incubation of the neutrophils with propranolol. The leucocytosis of trauma may therefore be due in part to reduced neutrophil adhesion induced by raised adrenaline levels.

Studies of neutrophil microbicidal pathways within 6 hours of injury in 23 patients showed normal responses in 21 (91%). In 8 patients studied 8 hours or more after injury, only one (12.5%) had normal responses.

Electron microscopic studies of neutrophils in 17 trauma patients showed no evidence of systemic degranulation within the first four hours after injury.

Taken together, these results indicate the existence of a leucocytosis of trauma, which is composed of normal cell lines. Derangements of cell function appear after 4-8 hours, and the early post-trauma phase may offer the opportunity for development of therapies to avoid later sepsis and multiple organ failure.

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PREFACE

In the United States of America, improvements in trauma care during the last two decades have led to a dramatic decrease in early mortality (Cales and Trunkey, 1985). The evidence for the effectiveness of such improvements during the "golden hour" is overwhelming, but emphasis has now shifted towards the remaining problem of late mortality from sepsis and multiple organ failure. It has been estimated that 78 percent of all non-neurological late trauma deaths are due to sepsis (Miller et al, 1982), despite optimal trauma care.

The literature on the immune consequences of trauma is unwieldy due to the lack of a precise definition of "trauma", and untested extrapolations are commonly made between the effects of surgical, thermal and traumatic injury. For the purposes of this research study, "trauma" is taken to mean any injury to the human body caused by the application of kinetic energy. This definition thus includes most accidents involving deceleration, but excludes thermal and surgical injury.

Defects in neutrophil and lymphocyte function have been reported after all types of injury in man, often lasting days to weeks after the initial insult. Neutrophils have also been implicated in the development of tissue damage following trauma and shock, as found in the Adult Respiratory Distress Syndrome, yet the kinetics of these cells immediately after traumatic injury remain largely unstudied.

This project was planned in an attempt to clarify the phenomenon of a leucocytosis observed in many recently injured patients. The existence of such a phenomenon is scarcely recognised in the literature of haematology, and even less well defined.

The aims of the study have been to establish the identity and source of the leucocytes involved, using both light and electron microscopy to assess the morphology of neutrophils and lymphocytes. Functional studies have also been undertaken to assess the adhesive properties of the neutrophils and the integrity of their bactericidal mechanisms, factors which may affect the quality of immune defences following trauma.

The coordinating role of CD4+ (T-helper) lymphocytes is now well recognised, and the final study examines changes in T-helper and T-suppressor cell subsets over time following injury.

THE HISTORICAL PERSPECTIVE

Trauma and Man

Man has never been a sedentary animal. The fossil record of Homo sapiens and his ancestor, Homo erectus, contains evidence of healed injuries and also of traumatic death. The bones of animals found with these relics and the remnants of camp fires suggest that early Man was a good hunter. In those early days the threats to Man included accidental falls, wild animals and human rivals.

The earliest records of attempts to repair injuries are found in the Ebers and Edwin Smith papyri, discovered in the 19th Century. These papyri, dating from the third millenium BC, contain a surgical treatise on the treatment of wounds and other injuries in ancient Egypt (Encyclopaedia Britannica: 1984).

Hindu physicians, from 800 BC onwards, developed diagnostic medicine using all five of the human senses. One of their discoveries was the crepitus produced by the rubbing together of broken ends of bones.

Almost independently, the great civilisation in Greece began to advance medical knowledge. Empedocles, a Greek philosopher who lived in Sicily during the fifth century BC, is credited with the saying "Blood is life". He advanced the theory that the soul, or "pneuma", was carried in the blood vessels of all animals and could be seen escaping as an ephemeral cloud when blood was shed. When Greece fell to Rome, Alexandria in Egypt became the refuge of classical scholarship. It was here that Claudius Galen studied after qualifying as a doctor at the Temple of Aesculapius in Greece in AD 140. Galen then returned home and served for four years as physician to the gladiators. Forbidden by the morals of the time to dissect human bodies, he advanced the study of anatomy by dissecting the beasts killed during the games.

He wrote more than three hundred books, many of which contained extrapolations to the human body of observations made on animals. He developed the pneumatist theory to include the idea that blood was formed in the intestines and acquired three spirits during its passage through the body.

With the fall of Rome, Europe was left in the grip of a restrictive Christian Church which inhibited original thought throughout the dark ages until the Renaissance. Galen's discoveries, both accurate and false, were adopted as undisputed truth for the next thousand years.

Most wounds encountered by military surgeons until the fourteenth century were either incised, due to the sword, or penetrating, caused by the arrow. Experiments by an English Franciscan monk, Roger Bacon, led to the discovery of the recipe for gunpowder in 1242. Bacon did not develop this discovery for military use, but by the beginning of the next century its use in guns had commenced. The pattern of injuries seen in war then changed - compound fractures and contaminated wounds became commonplace, and so too did death from gangrene. At that time, the concept of infection had not yet been realised, and the gangrene was thought to be due to the poisonous nature of gunpowder. The use of cautery became widespread and mortality rates were high. Although amputation of damaged limbs was later introduced, this was often performed too late to affect the outcome.

The Renaissance provided the environment for challenging orthodox thought, and many common men were to assist in the transformation of medical science. Amongst them was the father of modern Surgery, Ambroise Pare (1510-1590), who became an apprentice barber-surgeon at the Paris Hotel-Dieu in 1533 and later went on to gain fame as the surgeon to four French monarchs. Pare was in the service of the French army, whose surgeons used cautery with boiling oil to treat gunshot wounds, and hot irons to coagulate haemorrhaging vessels.

However, during a battle of 1536, supplies of oil ran out, and Pare tried dressing wounds with a simple salve of egg yolk, rose oil and turpentine. He soon discovered that the wounds treated in this way healed better, and in 1545 he reported his findings in "La methode de traicter les playes faites par les arquebusques et aultres bastons a feu" ("The method of treating wounds made by harquebuses and other guns"). Being written in French rather than scholarly Latin, Pare's work was ridiculed, as was his idea of re-introducing the ligation of bleeding vessels in preference to cautery. His later popularity ensured a more humane and effective management of battlefield injuries, even before the discovery of organisms.

The Discovery of Infection and Blood Cells

Knowledge of the microscopic structures became possible during the seventeenth centry with the discovery of lenses. Anton van Leeuwenhoek (1632-1723), a draper in Delft, Holland, developed a hobby of grinding lenses and using them to observe the natural world. He was the first to recognise the red corpuscles in blood, estimating their size with great accuracy. He also discovered bacteria, which he termed "animalcules", but did not appreciate their significance.

Jean Senac, a French physician in the mid-18th century, first described the white blood cells, which he had seen in blood and lymph. William Hewson, a pupil of John and William Hunter, went on to investigate leucocytes in more detail, and in 1843, Thomas Addison described how these cells migrate from the blood into inflamed tissue. At about the same time, in Vienna, Ignaz Semmelweiss (1818-1865) discovered that the epidemic of puerperal sepsis in his maternity hospital was being spread from cadavers to the mothers on the hands of attending doctors. He was prompted to his theories by the death of a colleague, Kolletschka, who developed symptoms like those of puerperal fever after cutting a finger whilst carrying out an autopsy on a dead mother.

By insisting that his staff and students cleansed their hands in chloride of lime before examining patients, Semmelweiss reduced the mortality of his patients to one tenth of the previous level. However, his ideas and results were not recognised during his lifetime. Semmelweiss, like Hewson and Kolletschka and many other pioneers, died prematurely of septicaemia after a minor surgical accident.

Robert Koch (1843-1910) studied the bacterial origins of wound infection, having served as a field surgeon during the Franco-Prussian war of 1870-71. Louis Pasteur (1822-1895) had already proven that fermentation and putrefaction were due to organisms, and the years that followed were notable for the number of organisms that were discovered and classified. Koch discovered the organisms of anthrax, tuberculosis and cholera, and advanced the science of bacteriology, being rewarded with a Nobel Prize in 1905.

Joseph Lister (1827-1912), whilst professor of Surgery in Glasgow, acted upon the discoveries of Pasteur by introducing a spray of dilute phenol to the operating theatre. He continued this work at King's College, London and by 1877 was successful in reducing infection rates and mortality due to surgery. His method of antisepsis was widely adopted by the end of the nineteenth century. The alternative strategy of asepsis - preventing contamination of a clean wound by the use of sterile instruments and aseptic technique - had by then begun to gain ground.

FACTORS AFFECTING THE CIRCULATING LEUCOCYTE COUNT

Following the discovery of white blood corpuscles by Jean Senac in the mid-18th century, methods of counting the blood cells were developed. By 1854, Moleschott had noted the greater variability of the white cell count as compared to the red cell count in any individual. The reasons for this variability in normal persons was not known, but digestion was suggested as a possible factor.

It became apparent that blood cell counting was subject to several sources of error, notably in the collection of samples (anticoagulants were not used) and in the counting apparatus. The following years saw improvements in the design of dilution pipettes and counting chambers (Potain, 1867: Thoma, 1881) and the introduction of oxalate as an anticoagulant (Keith, 1919).

Shaw (1927) was able to illustrate that there were no differences in the leucocyte counts of arterial and venous blood, and established that capillary blood obtained from a warmed, vasodilated earlobe had the same count as arterial blood.

Garrey and Bryan (1935) undertook a major review of all the literature relating to leucocyte counts, and were able to state.
"... as the count increases above 10,000 cells per cubic millimetre of blood, the chances of its occurrence in the normal individual decrease rapidly".

This conclusion has remained valid until the present day, although there is a great variability of leucocyte counts within any given population of individuals. This gives rise to a problem of interpretation, in that there are individuals with a normal leucocyte count of, for example, 5,000, in whom a count of 10,000 would be pathological.

A further consideration, recognised by Bayer (1881, quoted by Garrey), is that the normal range of leucocyte counts varies with age. Newborn infants have a leucocyte count of up to 28,000, a level which gradually declines throughout childhood to stabilise into the adult range following puberty. There is no evidence that normal aging produces an alteration in the reference range for leucocyte counts.

Sabin et al (1925) measured leucocyte counts in normal subjects at fifteen minute intervals throughout the day, and found an hourly rhythm of variation and a definite rise in the afternoon. This rise was independent of the effects of digestion. Shaw (1927) undertook a similar study and failed to find hourly variation, but confirmed Sabin's findings of an afternoon rise and detected a second peak, at about midnight. In both cases, the additional leucocytes were neutrophils. Cooke and Ponder (1927) attempted to establish the origin of these extra neutrophils and found that the ages of the cells, as determined by the Arneth count, were of identical mix to those in the normal circulation. They therefore drew the conclusion that the additional neutrophils had not come from the bone marrow, but from tissue spaces.

On the available evidence, Garrey and Bryan considered that there were identifiable depots of leucocytes within the viscera which could be redistributed promptly to cause a physiological leucocytosis (eg. during exercise), in addition to the source of new cells from the bone marrow. The idea that large amounts of leucocytes were sequestered within organs (particularly the spleen) from which they could be released became popular, particularly because of the associated observation that hypersplenic patients often had pancytopenia (Doan and Wright 1946, Kracke and Riser 1949). According to Chatterjea (1953) it was noted as early as 1903, by Loeper and Crouzon, that injection of adrenaline causes a leucocytosis of rapid onset. The observation that adrenaline also causes contraction of the spleen in dogs led to the assumption that the leucocytosis is of splenic origin.

This was accepted as being applicable to humans until Chatterjea (1953) established that the adrenaline-induced leucocyte rise also occurs in splenectomised patients. Chatterjea also expressed doubt about the value of using injected adrenaline as a haematological stimulation test, since his research revealed that the leucocytosis it produced was not characteristic of a bone marrow stimulation (being of mature neutrophils). Steel (1971), apparently unaware of Chatterjea's work, also established that adrenaline-induced leucocytosis occurs in splenectomised humans.

Athens et al (1963) used infusions of radio-labelled granulocytes to determine the distribution of leucocytes within the body. These determinations were carried out on 25 volunteers from Utah State Prison, groups of whom were subsequently given prednisone, adrenaline, bacterial endotoxin or exercise to assess the effect of these manipulations on leucocyte distribution. It was found that, in normal subjects, the circulating granulocyte pool was of approximately equal size to the marginal granulocyte pool (ie. those granulocytes adhering to the walls of blood vessels), and that a free interchange occurred between the two pools. Refinements in experimental technique, using the same radiolabelling agent $DF_{32}P$, have more recently revealed a storage pool of neutrophil granulocytes (both mature and band forms) within the bone marrow which is thirteen times the size of the total circulating pool (Goldman 1989).

Other physiological variations in leucocyte counts, all reviewed by Garrey in 1935, seem merely to reflect the lability of the resting count in a given individual. A change in posture from lying to standing or vice-versa may both produce a rise in leucocyte count. Emotional upset generally produces a rise, as does physical exercise. Smith and McDowell (1943) reported an increase due to mastication and swallowing, but the other evidence relating to digestion is conflicting. There is no firm evidence that starvation or climate has any effect on leucocyte count, but it is known that a natural neutropenia exists in some Africans (Shaper & Lewis 1971).

CHANGES IN THE LEUCOCYTE COUNT ASSOCIATED WITH INJURY

Research during the First World War using blood volume measurements led to a better understanding of the causes and effects of wound shock (Keith 1919). As part of these studies, the leucocyte counts of injured patients were measured, and it was noted that most had a leucocytosis, often within the range of 15-39,000. The highest counts were said to have been found in patients with wounds to the extremities which caused severe primary haemorrhage. Injury to the internal organs, even with haemorrhage, did not seem to produce the same response.

It is interesting that replacement of blood volume with 6% gum or whole blood at that time was extremely conservative, the average transfusion only amounting to 600 cc. This research provided the first realisation of the extent of blood volume changes following trauma.

Recognising that acute infection was one of the most serious complications following battle injury, Balch studied the phagocytic function of neutrophils in wounded soldiers during the Korean War (Balch 1955). These studies took place at the 46th Mobile Army Surgical Hospital (MASH) with full laboratory support. He found the leucocyte count to be elevated on the day of injury even after massive volume replacement with bank blood (range 7-23 litres). It was possible to prove that the bank blood contained no neutrophils and a very low count of other leucocytes.

A leucocytosis has been reported following injury to both the spleen and the liver (Brittain 1963, Corica and Powers 1975). This leucocytosis was reported to occur within one hour of trauma, to persist for approximately two hours and then rapidly resolve. Corica and Powers commented that this early time-course distinguished the leucocytosis from that seen 24 hours or more following haemorrhage.

Kirov et al (1979) identified a reduction in circulating lymphocyte count during and immediately after major abdominal surgery with a simultaneous rise in the neutrophil count. They explained these changes as being due to tissue injury, since they did not find similar changes in subjects donating blood or suffering the psychological stress of a university examination. Bolton et al (1979) undertook a similar study in mice, subjecting one group to nephrectomy and others to the minor stress of blood sampling. Both degrees of trauma triggered a 50-70% fall in circulating lymphocyte count. However, it was found that only B-lymphocytes were reduced in the minor trauma group, whereas both B- and T-cell lines were reduced in the nephrectomized mice. Adrenalectomy performed three days prior to the trauma completely abolished the fall in lymphocyte numbers and the authors concluded that adrenal cortical hormones were the mediators of the observed lymphopenia.

A delayed and persistent lymphopenia has been reported in patients with severe burns (Peterson et al 1988). This lymphopenia developed over 48 hours and appeared to be more pronounced in patients who did not survive the burn.

Thomassen et al (1986) reported an absolute lymphocytosis following major traumatic skeletal injury and after stab wounds to the trunk, and were the first to suggest that this phenomenon might be catecholamine-induced. A late lymphopenia occurred in all of these patients, which suggests a different mechanism to that causing the early lymphopenia in the abdominal surgery patients studied by Kirov. No lymphocytosis was noted after major surgery in the studies by Kirov and Bolton, despite frequent serial sampling. Thomassen's group found that atypical lymphocytes did not feature in the lymphocytosis they detected. A study by Teggatz et al (1987) of lymphocytosis in patients with emergency medical conditions found that atypical lymphocytes were the commonest form present. Five of the 73 patients studied were trauma patients but their features were not discussed separately.

Pinkerton et al (1989) considered that the lymphocytosis detected in their trauma patients was possibly indicative of a poor prognosis. The patient group was highly selected, chosen on the finding of an absolute lymphocytosis alone, and the control group was poorly matched in terms of injury severity.

CHANGES IN IMMUNE FUNCTION IN TRAUMATIC, SURGICAL AND THERMAL INJURY

Balch (1955), in his study of the effects of battle injury on resistance to infection, found that the neutrophils of severely injured patients showed a reduced capacity to phagocytose staphylococci within 24 hours of injury. This deficiency was not as marked on the second post-wound day and was corrected to control levels on subsequent days. A similar time-course has been found in surgical patients (Pietsch and Meakins 1979, Meakins 1988). In addition to the impairment of phagocytic capacity, the delivery of neutrophils to the site of bacterial challenge is impaired (Tchervenkov et al 1988). It is likely that this results from a depression of granulopoeisis at about 24 hours after surgical operation or thermal insult, since a reduction in granulocyte progenitor cells has been detected at this time (Philip et al 1980).

Miller et al (1973) analyzed the types of infection acquired by severely injured patients in Baltimore. Although the trauma unit patients accounted for 2.6% of the hospital admissions, they developed 18% of the hospital acquired infections, including 50% of all the bacteraemias. Half of these bacteraemias were of organisms of low virulence, suggesting that the patients had compromised immune defences. Saba and Scovill (1975) reviewed evidence that this failure of host defences might lie in the failure of the reticulo-endothelial system to clear foreign material, including bacteria. Like Balch (1955), they believed that this occurred because of the deficiency of a humoral factor or opsonin, whose function was to "mark" bacteria for clearance and killing by phagocytes.

Van Dijk et al (1982) demonstrated a significantly decreased level of opsonic activity in the serum of eleven patients after major surgical operations. All of the patients had normal levels of activity pre-operatively. The post-operative deficiency correlated with low serum IgG levels in seven cases but showed no relationship with complement activity. The phagocytic capacity and chemiluminescence of each patient's neutrophils remained normal.

Some of the early work on the function of neutrophils after trauma, burns and surgery is difficult to interpret, since it was not initially realised that the patient's plasma constituents affect neutrophil function (Howard and Simmons, 1974). Alexander et al (1971) identified a defect in the ability of the neutrophils of burned patients to ingest and kill Staphylococci. This defect appeared between 10 and 40 days post-burn and appeared to be independent of the plasma opsonic activity. Davis et al (1980) further found that neutrophil chemotactic activity and random migration were depressed in burned patients between 5 and 15 days post-burn. Sepsis and mortality were more frequent in those patients whose neutrophils had less than 65% of normal chemotaxis. These patients also showed a loss of neutrophil lysozyme content (from the specific granules) but no less of beta-glucuronidase (an azurophil granule enzyme). These findings suggest a selective degranulation may be taking place in vivo which impairs neutrophil function.

El-Maallem and Fletcher (1981) detected a different pattern of dysfunction occurring soon after abdominal hysterectomy. An impairment of candida killing ability in the neutrophils of their patients was evident within two hours of surgery and lasted for 8 days, although actual phagocytosis of the candida organisms was unaffected. The impairment of Candida killing was associated with a loss of the lysosomal enzyme myeloperoxidase from the neutrophils. This enzyme is normally found in the azurophil granules, which Alexander had found not to be degranulated in burned patients (although his study was examining changes occurring later).

El-Maallem and Fletcher also detected an increase in the average neutrophil alkaline phosphatase (NAP) content of the post-trauma neutrophils, and took this to indicate the presence of more immature forms than usual.

This assertion was based on the work of Williams (1975) who assessed neutrophil age on the basis of alkaline phosphatase content, finding that younger cells contained more. However, Fehr and Grossman (1979) produced opposite findings. A rather acrimonious debate took place in the letters column of the American Journal of Haematology on this topic (Mishler 1980, Fehr and Grossman 1980) without any firm conclusions being reached.

The number of studies which have examined neutrophil function in injured patients (ie. traumatic injury as distinct from thermal or surgical injury) is small. Several authors have assumed that the responses to surgical injury are identical to those of traumatic injury (Munster, 1973; Meakins et al 1977; Pietsch, 1979), although little comparative work has been presented to justify this conclusion. Virtually all studies of immune function in injured patients have commenced several hours after injury and most have a time course of days rather than hours. The only early studies performed have been in anaesthetized surgical patients undergoing operation. It would seem important, therefore, to consider whether factors such as anaesthesia have any effect upon immune function.

Anaesthesia clearly produces physiological changes within the cardiovascular system and it is possible that these changes could by themselves alter the numbers of circulating leucocytes (eg. by physical demargination) but there is also some evidence of a direct impairment of leucocyte function. Studies with ether anaesthesia (Graham, 1911) and chloroform anaesthesia (Hamburger 1916) suggested that some impairment of neutrophil phagocytic function results. Cullen (1974) found only minimal impairment of phagocytic function and Nitroblue Tetrazolium (NBT) reduction in normal neutrophils exposed to the same levels of nitrous oxide and halothane used in anaesthesia.

Local anaesthesia using lignocaine is known to suppress the adhesion of neutrophils at the wound site (Giddon, 1972) and could thus have a possible effect on the local response to infection. There is also evidence to suggest that, given systemically, lignocaine could have effects on granulocyte adherence and morphology, and that these effects are potentiated by the presence of acid-citrate-dextrose anticoagulant (Schiffer et al 1977).

Neutrophils carry specific adhesion molecules which show affinity for specific monoclonal antibodies against CG 11b and CD 18. The alpha sub-unit of the heterodimer adhesion molecules, CG 11b, is also thought to be important on phagocytosis of bacteria (White-Owen et al 1992)

THE EFFECT OF TRAUMA ON NEUTROPHIL FUNCTION

Apart from the studies by Balch (1955), none of the above research has been conducted on patients with traumatic injury. Three recent studies have examined the nature of the migration, locomotory and biochemical defects in the neutrophils of trauma patients.

Maderazo et al (1983) studied migratory function in the neutrophils of patients with serious blunt trauma (Injury Severity Score of 20 or above on a scale of 1-75), using a Boyden chamber and microspore filter. Neutrophils, isolated from the patients' blood, were placed in the upper compartment of the chamber and a chemotactic agent (opsonised zymosan) in the lower compartment. The depth of migration into the filter of the neutrophils was then measured. Six patients were studied at times commencing from five hours to three days post-injury, and both serum- and cell-associated locomotory abnormalities were detected. The serum-associated abnormality lasted for three days but the cell-associated one persisted for one week on average, but much longer in those patients developing infections. The dysfunction was not solely due to the presence of immature neutrophils, since cells of all ages were noted to be defective.

Further research by Maderazo et al (1986) discovered a possible mechanism for the locomotory defects. 46 patients with serious blunt trauma were tested as in the first study for neutrophil locomotory function, and in addition the neutrophils were tested for their ability to generate hydrogen peroxide and reduce oxidised glutathione. Both of the latter tests assess biochemical pathways necessary for intracellular killing. Their results suggested that an oxidative burst was taking place in some of the neutrophils tested between one and four days after injury.

Levels of hydrogen peroxide generation were raised in the neutrophils of the trauma patients to an extent which suggested the neutrophils were in a state of activation. Microtubular function was assessed using the concanalvin capping assay and found to be defective. The authors concluded that intracellular self-damage as a result of inappropriate activation might be the reason for the observed locomotory dysfunction.

The nature of the serum-associated inhibition of neutrophil migration is unclear. A reduction in opsonic activity in post-operative surgical patients has already been discussed above (Van Dijk 1982), and this appears to be related to changes in IgG levels rather than complement deficiency. Recent studies by Lanser et al (1986) have shown that serum from trauma patients suppresses one of the oxidative functions of normal donor neutrophils. Superoxide is a potent bactericidal agent produced by neutrophils by the reaction:

$$O_2 + NADPH \text{ ------} > \text{Superoxide } (O_2^-) + NADP$$

The formation of this radical was found to be reduced in normal neutrophils after incubation with the serum of seriously injured patients. The chemiluminescence response of normal neutrophils was also impaired by the trauma serum. Lanser's group were unable to discover the nature of the suppressive agent, but established that it was probably not a complement fragment, since C5a and C3b were tested and found to be non-inhibitory in vitro.

Hershman et al (1988) formulated an outcome predictive score based on trauma patients monocyte DR antigen expression, the injury severity score (ISS) and the degree of bacterial contamination of the patients' wounds or abdominal cavity following trauma. Good outcomes were limited to those patients who had little or no contamination, and the mean DR antigen expression in those patients with good outcome was significantly greater than that in patients developing major sepsis. The outcome predictive score using the three factors was reliable in predicting outcome when calculated within 24 hours of admission. No information was presented by the authors concerning which patients received major surgery in addition to their initial major injuries, or whether blood sampling took place before or after injury.

THE EFFECT OF TRAUMA ON LYMPHOCYTE FUNCTION

Most of the studies of lymphocyte function after trauma have examined the integrity of cell-mediated immunity (Meakins et al 1977, Pietsch et al 1977) which is found to be deficient in some patients.

However, these studies have tended not to differentiate clearly between types of trauma, nor do they convincingly relate the observed anergy to clinical events (Brown et al (1982).

Munster (1976) considered that all aspects of post-traumatic immunosuppression were due to activation of suppressor T-cells, although this was presented as an unproven hypothesis. A depression of T-lymphocyte activity was detected in multiple trauma patients by Bauer et al (1977) using a sheep red cell rosette assay. The depression occurred within 24-48 hours of trauma and persisted until the fifth day. Confirmation that this depression was due to suppressor T-cells was provided by Keane et al (1982) who established T-suppressor activity in three multiple trauma patients in mixed lymphocyte culture.

With the development of improved techniques in flow cytometry, using monoclonal antibodies to phenotype lymphocyte populations, it has been possible to illustrate that the T helper: T suppressor cell ratio is reversed in most trauma patients (Pinkerton et al 1989). Wood et al (1987) noted an increase in the numbers of $T6^+$ cells in moderately and severely injured patients, which they identified as immature T-lymphocytes. These cells were present in numbers which had otherwise only been seen in malignant disease.

NEUTROPHIL-MEDIATED TISSUE DAMAGE IN TRAUMA AND SHOCK

A loss of neutrophil lysosomal enzymes has been found to occur in patients following burns (Davis et al 1980) and immediately following abdominal surgery (El-Maallem and Fletcher 1981). This loss may have been the reason for the impairment of neutrophil function detected concurrently by the investigators, but the fate of the extruded enzymes was not considered. Myeloperoxidase and lysozyme are potent enzymes which are lethal not only to bacteria, but also to human cells (including the neutrophils carrying them) should the normal barriers preventing contact fail.

Neutrophils are able to use their killing mechanisms in two ways. The commonest is intracellular killing following phagocytosis, in which enzymes from the granules (lysosomes) are deposited into a phagocytic vacuole containing the ingested organism. However, when neutrophils encounter an object or surface too large to be ingested (eg. tissue coated with immune complexes), a second mechanism is initiated which has been termed "frustrated phagocytosis" (Henson 1971) or "reversed endocytosis" (Weissman et al 1972). The neutrophil first attempts to surround and ingest the surface and whilst doing so shows the usual burst of metabolic activity and increased oxygen consumption. When phagocytosis fails, the activated neutrophil discharges its granules by exocytosis, where the enzymes may damage host tissues (Weissman 1980). This mechanism is thought to be important in the auto-immune damage sustained in glomerulonephritis and some types of arthritis (Henson 1972).

This type of enzyme release also occurs after inappropriate activation of neutrophils in circulation (Hammerschmidt and Vercellotti 1987). Kaplow and Goffinet (1968) noted a marked neutropenia occurring during haemodialysis, which did not appear to be due to sequestration of neutrophils in the dialyser.

Incubation of normal plasma with the cellulose dialysis membrane, and subsequent infusion of this plasma into experimental subjects produced the same neutropenia. This was found to be due to activated complement in the plasma causing neutrophils to collect in the lungs (Craddock 1977), and a definite reduction in pulmonary function resulted from the aggregation.

Similar aggregation of neutrophils in lung, heart and muscle biopsy specimens has been detected during open-heart surgery (Ts'ao et al 1973), accompanied by evidence of local tissue injury. Complement activation, oxygen free radical generation and a marked increase in circulating elastase has been illustrated in patients on cardio-pulmonary by-pass (Westaby 1987). These events and the resulting lung damage is thought to lead to Adult Respiratory Distress syndrome (ARDS), a common condition seen in both trauma patients and those undergoing major surgery. Hammerschmidt et al (1980) were able to show that complement activation had occurred in polytrauma patients who developed ARDS, but rarely in those who did not develop ARDS. Nuytinck et al (1986) confirmed this finding in trauma patients, and established that both total haemolytic complement levels and elastase levels were predictive of the risk of ARDS developing.

Attempts have been made to find an inhibitor of neutrophil aggregation which might be used to stop the development of ARDS. As previously discussed, lignocaine inhibits neutrophil adhesion (Giddon 1972), but it appears to be ineffective in reducing the pulmonary leucocyte aggregation seen in shock ischaemia (Lewis 1987). Methylprednisolone is also ineffective in preventing the tissue damage seen in patients on cardiopulmonary by-pass (Ts'ao et al 1973).

However, it has been shown that another anti-inflammatory agent, ibuprofen, can reduce the aggregation of neutrophils in an experimental myocardial infarction and also reduce the infarct size (Romson et al, 1984).

Several studies have thus shown the damaging potential of neutrophils in different clinical conditions, some of which may be relevant to the events happening in the trauma patient.

ARDS, characterised by pulmonary oedema and reduced lung compliance, is second only to sepsis as a lethal complication in trauma victims. To some extent, the two complications are related, since both appear to involve aberrations of neutrophil function at the outset, and chest infection often accompanies ARDS once established. There is also a firmly established link between the events of sepsis, multiple organ failure and disseminated intravascular coagulation (DIC) in trauma patients (Effeney et al 1978), each of which may be initiated by derangements of neutrophil action. Far from always being the "friendly policeman" of the body, the neutrophil is capable of many violent and antisocial activities. It would seem important, therefore, to consider the mechanisms that keep neutrophils and their granules intact, allowing them to perform normally.

FACTORS MAINTAINING NEUTROPHIL GRANULE STABILITY AND THE MECHANISMS OF ENZYME RELEASE

Neutrophils contain two types of granules, which are both normally used for intracellular digestion of phagocytosed material. In this situation, the secondary (specific) granules fuse with the phagosome first and discharge their contents. The primary (azurophilic) granules then follow. The reason for this sequential degranulation is that the two types of granules contain different enzymes which have different optimum pH ranges (Bainton 1973). The specific granule enzymes lactoferrin, alkaline phosphatase and lysozyme work best at neutral pH, and the acid hydrolases of the azurophil granules act in the lower pH range.

Fusion and degranulation depend upon the activity of the microtubular system in the neutrophil. Microtubules are very thin cylinders, about 2-300 A in diameter, which are assembled and disassembled during both cellular motion and the internal movement of organelles within the cell (Novikoff and Holtzman 1970). Microtubules also play an important part in the adhesion of cells to each other and to foreign material.

The processes of assembly and disassembly of microtubules are mediated by the cyclic nucleotides, cyclic 3′, 5′ guanosine monophosphate (cGMP) and cyclic 3′, 5′ adenosine monophosphate (cAMP). The intracellular levels of these nucleotides are in turn controlled by receptors on the neutrophil's cell membrane which respond to external stimulation (Zurier et al 1974).

a) The assembly of microtubules

The external stimulus for the assembly of microtubules in degranulation, both during phagocytic digestion and exocytosis on enzymes, comes from complement fragments and immunoglobulin molecules (Weissman et al 1972). These may be either on the surface of the ingested object, or free in the circulation in the case of non-phagocytic release. The phagosome consists of invaginated neutrophil cell membrane, complete with its receptors, and the contents could be considered "external" to the neutrophil (an important feature in that the neutrophil protects itself against the digestive process).

Receptors for complement fragments (mainly C3b and C3-5a - according to Hammerschmidt and Vercellotti, 1987) on the neutrophil membrane respond to the stimulus by raising intracellular cGMP levels. This in turn promotes polymerisation of microtubule components into microtubules, which "pull" granules towards the membrane, bringing them into apposition ready for fusion and enzyme release (see figure 1, p37).

The membrane receptor mechanism is likely to have a cholinergic step, since acetylcholine markedly enhances the release of beta-glucuronidase from neutrophils stimulated by human IgG obtained from rheumatoid arthritis patients (Ignarro 1974). Conversely, the release of this enzyme is inhibited by the presence of atropine. Carbachol, another cholinergic stimulant, raises neutrophil intracellular cGMP and releases beta-glucuronidase when used on its own.

b) The disassembly of microtubules, or maintenance of the status quo.

Acting in a reciprocal manner to the cholinergic pathway, neutrophils have an adrenergic pathway responsible for maintaining the granules in a resting state.

Neutrophils have well-defined beta adrenergic receptors on their cell membrane. Stimulation of these receptors results in an activation of cyclic AMP pathways within the cell and an inhibition of microtubule assembly. Zurier et al (1974) found that adrenaline was effective in stimulating this chain, resulting in an inhibition of neutrophil degranulation after a zymosan challenge (zymosan consists of particles coated in complement fragments and is used as a phagocytic stimulant after opsonisation with normal serum). The effect of adrenaline was blocked by propranolol. Noradrenaline and the pure alpha-adrenergic agonist phenylephrine did not have any effect on enzyme release.

In advance of this discovery, the action of cAMP on microtubules had already been established by Zurier's group (Weissman 1972). At that time, another stimulant, prostaglandin E1, was found to raise intracellular cAMP levels and inhibit degranulation. This prostaglandin also acts on the cAMP pathway of platelets, inhibiting their adhesion (Kernoff 1989).

THE ROLE OF AUTONOMIC AGONISTS AND CYCLIC NUCLEOTIDES IN NEUTROPHIL ADHESION

The aggregation of neutrophils within the microcirculation is considered an important event in post-traumatic neutrophil mediated tissue injury (Ts'ao et al 1973, Nuytinck et al 1986, Lewis et al 1987).

IgG were needed to stimulate exocytosis of granule enzymes from neutrophils. Henson (1971) demonstrated that smaller quantities of adherent to non-phagocytosable surfaces (eg. endothelium or connective tissue) than in freely suspended neutrophils. The events of adhesion and degranulation are very closely related, and have in common the utilisation of microtubules.

Following the work of Athens et al (1961), which showed that the total blood granulocyte pool was double the size of the circulating pool, interest was stimulated in the phenomenon of margination - the sticking of neutrophils to blood vessel walls. Isotope studies revealed that the circulating and marginated pools were in a state of equilibrium, with free interchange of neutrophils between each. At that time, the bone marrow was thought to contribute only immature cells to the circulation.

MacGregor (1974) developed an in-vitro assay of neutrophil adhesion to investigate the factors affecting the interchange between the granulocyte pools. Using columns of standard nylon fibre packed into glass Pasteur pipettes, he was able to establish that a sample of normal blood percolating down the column would lose a predictable percentage of its neutrophils by adhesion to the fibre. This percentage was proportional to the weight of fibre used in the column, and the optimum weight was 50mg, for which a normal reference range was established. The assay appears to correlate well with the interaction of neutrophils with endothelial cells (MacGregor et al 1978).

MacGregor found that the addition of endotoxin to the blood sample increased the percentage adhesion and that adrenaline and glucocorticoids decreased it (MacGregor 1975, 1977). The inhibition of adhesion only took place in the presence of the original blood plasma, and did not occur when the neutrophils were separated in suspension and treated with adrenaline alone. Clotting of the plasma also removed the inhibiting factor.

Raising the intracellular levels of cyclic GMP and divalent cations (Mg^{2+}, Ca^{2+}) increased adhesion, whereas raising cyclic AMP levels reduced it. The adrenaline-induced reduction in adhesion was blocked by propranolol.

These features of the adhesion pathway appear to relate it closely to the degranulation pathway discussed above. However, the two events clearly do not always occur together, otherwise the entire marginated pool of neutrophils would be degranulated. The explanation for this situation probably lies in the observation that plasma factors are also required for receptor stimulation in addition to endotoxin or adrenaline. In the case of increased adhesion, this factor is almost certainly activated complement and the pathway cholinergic, acting via cGMP. For reduced adhesion, the factor acting with adrenaline on the beta-adrenergic pathway via cAMP is unknown. Boxer et al (1980) proposed the alternative explanation that the loss of neutrophil adhesion seen after subcutaneous adrenaline administration in humans could be due to an effect on the endothelial cells rather than the neutrophils.

MODULATION OF NEUTROPHIL AND LYMPHOCYTE ADRENERGIC FUNCTION BY NATURAL CORTICOSTEROIDS

Being readily available cells with defined adrenergic pathways, neutrophils and lymphocytes became the model for the study of the factors affecting beta-receptor density and responses (Galant et al 1978). Galant and his group detected an 85% reduction in receptor density on the neutrophils of asthmatic patients and controls undergoing terbutaline therapy, a response which started immediately after commencement of therapy and was maximal at 6 days. If the drug was stopped at 6 days, recovery started immediately and was achieved in the next 7 days. The results indicate a down-regulation of beta adrenergic receptor activity in response to therapy with beta-agonist.

Hetherington and Quie (1985) investigated the leucocytosis induced by adrenaline and found that the majority of the extra cells were mature neutrophils with normal granule protein content. They also found that hydrocortisone given intravenously to volunteers would produce a neutrophilia of mature and band forms.

Four hours after administration of hydrocortisone, the neutrophils showed decreased adherence, increased chemiluminescence response, increased myeloperoxidase release and decreased myeloperoxidase content. The authors considered that this effect might be due to a difference in function and enzyme content between band and mature neutrophils. Davies and Lefkowitz (1980) however, found an acute increase in beta-adrenergic receptor sites on the neutrophils of volunteers treated with cortisone acetate, using a specific radiolabelled beta-antagonist, ^{3}H dihydroalprenolol. These cells also exhibited an increase in adenylate cyclase activity (the enzyme responsible for creating cyclic AMP). Concurrently, the lymphocytes showed an acute decrease in beta receptor density, illustrating a differential regulation effect by the administered steroid.

The possible protective effect of steroids administered to trauma and shock patients has been a controversial topic since the work of Altura and Altura (1974) first suggested it. Their work established that massive doses of methylprednisolone and hydrocortisone, given intravenously to rats, inhibited the effect of catecholamines on the peripheral blood vessels in shock. There was a dose-dependent inhibition of catecholamine and vasopressin-induced arteriolar contractions, and an apparent enhancement of RE system function as evidenced by an increased clearance of injected carbon particles. However, extensive studies in trauma patients have shown no improvement in mortality from infection resulting from the administration of high dose steroids (Robertson, 1985). In vitro, cortisol does not affect neutrophil function but impairs lymphocyte blastogenesis in culture (Deitch, McIntyre 1987).

HORMONAL CHANGES FOLLOWING TRAUMA IN RELATION TO LEUCOCYTE FUNCTION

From the previous research outlined above, it would appear that high circulating levels of adrenaline and corticosteroids should be advantageous in maintaining the stability of neutrophil granules, at least until down-regulation of the beta-adrenoceptors becomes predominant. However, it would also follow that the effect on lymphocytes would be adverse, since high corticosteroid levels reduce the number of beta-adrenoceptors on lymphocytes and suppress their function.

Increases in the levels of adrenaline and noradrenaline are found in trauma patients in direct relationship to the severity of injury (Davies et al 1984, Frayn et al 1985). Cortisol levels are also consistently raised within the first 48 hours after injury (Barton and Passingham 1981, Frayn 1984) and gradually return to normal.

LEUCOCYTE MORPHOLOGY AND PHYSIOLOGY

To understand the tests of function which may be applied to the study of leucocytes, it is helpful to review the morphology and physiology of each member of the leucocyte family.

NEUTROPHILS

Neutrophilic polymorphonuclear leucocytes are so named because, when mature, they have multi-lobed nuclei and cytoplasmic granules which do not give a strong basophilic or eosinophilic staining reaction with standard Romanowsky stains.

These cells, in common with eosinophils and basophils, develop in the bone marrow from a stem cell, the myeloblast (Bessis 1977, Goldman 1989).

Maturation occurs within the bone marrow according to the following sequence:

Myeloblast	1 day
Promyelocyte	2 days
Myelocyte	4 days
Metamyelocyte	2 days
"Band form"	2 days

Mature neutrophils live for about five days, but it is thought that they spend only about 8 hours in the circulation. The major site of neutrophil loss and death in health is the gastro-intestinal tract, although some are cleared by the RE system. There are four pools of mature granulocytes - the largest situated within the bone marrow. This pool is about 10-20 times larger, at 9×10^9 cells/kg body weight, than the two intravascular pools combined. The circulating pool of granulocytes, the vast majority of which are neutrophils, is the one which is amenable to measurement by venous blood sampling. This pool contains approximately 32×10^7 cells/kg body weight (Athens et al 1961), or $2.5\text{-}7.5 \times 10^9$ cells/L of blood volume. There is much individual variation in the circulating counts.

A third pool, the marginated pool, is of approximately equal size to the circulating pool and consists of neutrophils adherent to the blood vessel walls. Factors affecting this adherence would also, therefore, affect the circulating neutrophil numbers.

A fourth pool of neutrophils lies in the tissues, which the neutrophils access by leaving the circulation. The neutrophils are able to deform during movement to allow them to traverse tight junctions between vascular endothelial cells (diapedesis). Once within the tissues, neutrophils probably do not re-enter the circulation and have a short life-span (Bessis 1977).

NEUTROPHIL MORPHOLOGY

Neutrophils in free circulation measure 10-12 microns in diameter. As each cell matures, the nucleus develops more lobes. The least mature cells, termed band forms or stab cells, have a bean-shaped nucleus with no appearance of segmentation. Arneth (1911) developed a classification system to describe neutrophil age based on nuclear segmentation. The relative percentages of each segmented type in normal blood (quoted by Bessis) are:

Arneth Type	Nuclear Lobes	Percent.
I	Band form	0-5
II	2	10-30
III	3	40-50
IV	4	10-20
V	5	0-5

Forms with more than 5 lobes are rare in health but may be seen in patients with megaloblastic anaemia. More band forms are seen in conditions associated with increased bone marrow output of neutrophils (eg. acute infections).

Figure 1

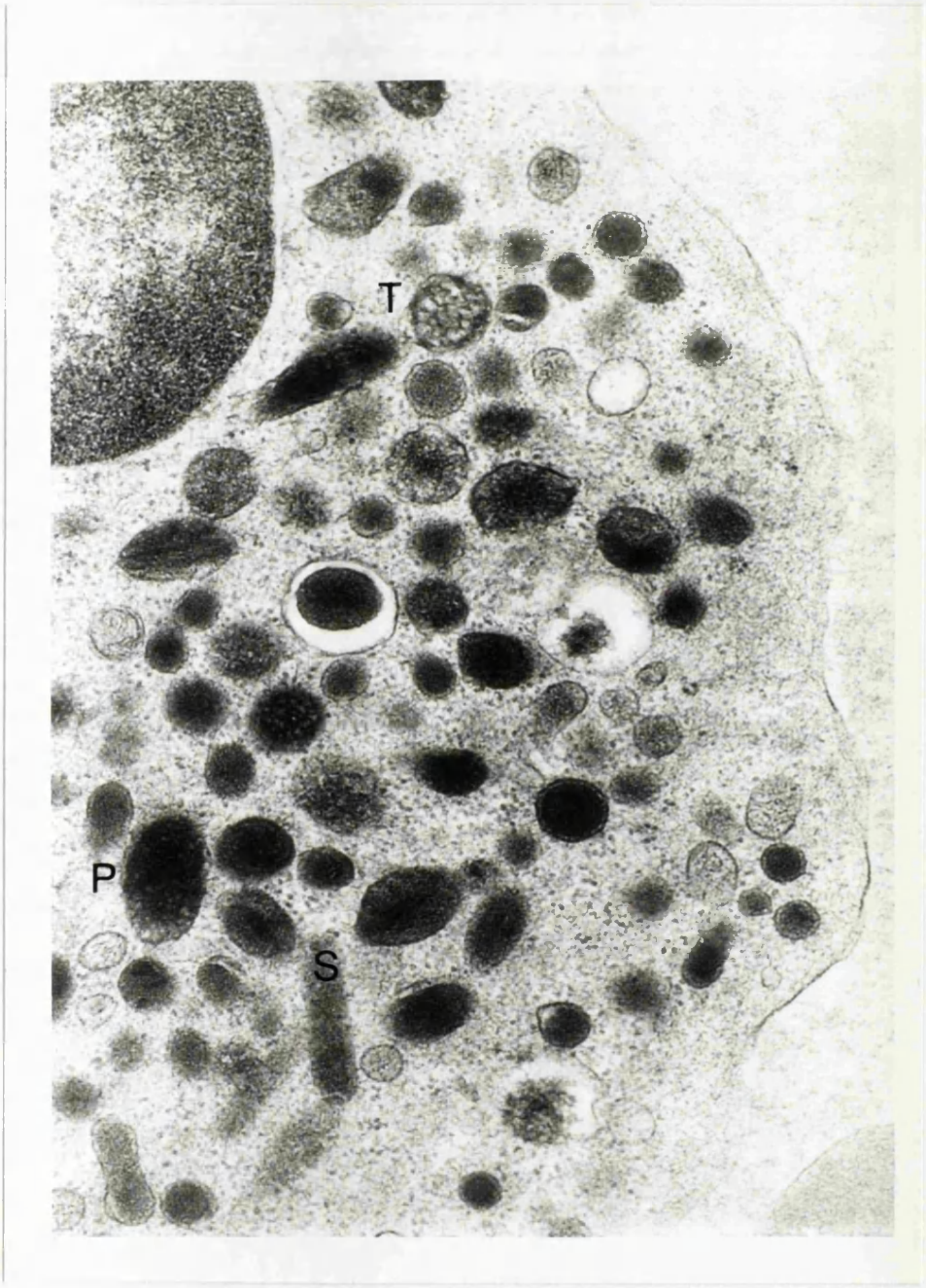


Figure 1: Neutrophil x 25,000 Transmission electron micrograph. Showing primary (P) and secondary (S) granules with parallel tubular arrays (T).

The cytoplasm of neutrophils contains granules which stain poorly. Since the resolution of the granules is at the limits of the light microscope, they are best studied using the transmission electron microscope (see figure 1, p 40). Using uranyl acetate and lead citrate stains, two types of granules are seen. The larger and more electron-dense type are termed primary or azurophil granules. These are round, about 350 nm in size, and contain the enzymes myeloperoxidase, elastase, collagenase, beta-glucuronidase and other proteolytic enzymes. The elongated and less electron dense variety are termed secondary granules: these measure about 300 x 600 nm and contain alkaline phosphatase, lysozyme, aminopeptidase and lactoferrin.

The granules may be more easily seen under the light microscope in neutrophils from patients suffering from acute bacterial infections, when the increased levels of proteolytic enzymes within the granules imparts an increased staining reaction, termed "toxic granulation". For both light and transmission electron microscopy, the primary granules can be enhanced by staining for myeloperoxidase, using a reaction between benzidine-based dyes and hydrogen peroxide. About one person in 2000 has a congenital lack of neutrophil myeloperoxidase (Goldman 1989).

NEUTROPHIL PHYSIOLOGY

Neutrophils are primarily phagocytic cells whose function is to locate, ingest and digest foreign material and organisms and also autogenous dead cells. They are attracted to the appropriate site by a variety of chemo-attractive substances, including bacterial proteins and toxins. Locally, when antibody binds to a foreign organism, there is a limited activation of the complement cascade in which C3a and C5a are generated at the site (Goldman 1989). These complement fragments have chemotactic activity and attract circulating neutrophils. The process by which an organism is thus marked for killing is known as opsonisation, and depends upon adequate levels of immunoglobulins in the circulation.

Neutrophils engulf particles by extending pseudopodia to surround them. In this way, the cell membrane invaginates to enclose the particle and the narrow neck of the pocket of cell membrane closes. This "phagosome" then fuses with granules, the enzymes of which are used to kill and/or digest the particle. At all times the toxic contents of the lysosomes are kept separate from the neutrophil's own cytoplasm.

The processes of ingestion and killing are associated with a burst of oxygen consumption by the neutrophil. Most of the killing mechanisms are oxygen dependent apart from those using lysozyme, lactoferrin and cationic proteins, and will be considered in the discussion of neutrophil function tests.

LYMPHOCYTES

MORPHOLOGY

Quite deceptively, only two types of lymphocyte may be reliably identified in Romanowski-stained blood films - small and large. Small lymphocytes are about 6-10 microns in diameter, and have scanty, pale blue cytoplasm. The nucleus stains uniformly dark blue. Large lymphocytes are about 10-16 microns in diameter and have more cytoplasm which may contain reddish granules.

Normally, small lymphocytes form over 90% of the lymphocytes seen in peripheral blood. This classification gives no useful information about the function or phenotype of the individual lymphocyte.

Electron microscopy may reveal additional information about the structure of both types. A small lymphocyte has a large nucleus with condensed chromatin and sparse cytoplasm. Often the nucleus is seen to have a cleft in it (figure 2). There are usually few organelles visible (Roitt 1988, Bessis 1977). Large lymphocytes have more cytoplasm and generally mitochondria, free ribosomes, endoplasmic reticulum and Golgi apparatus are easier to find. The nucleus contains less densely-packed chromatin.

Figure 2

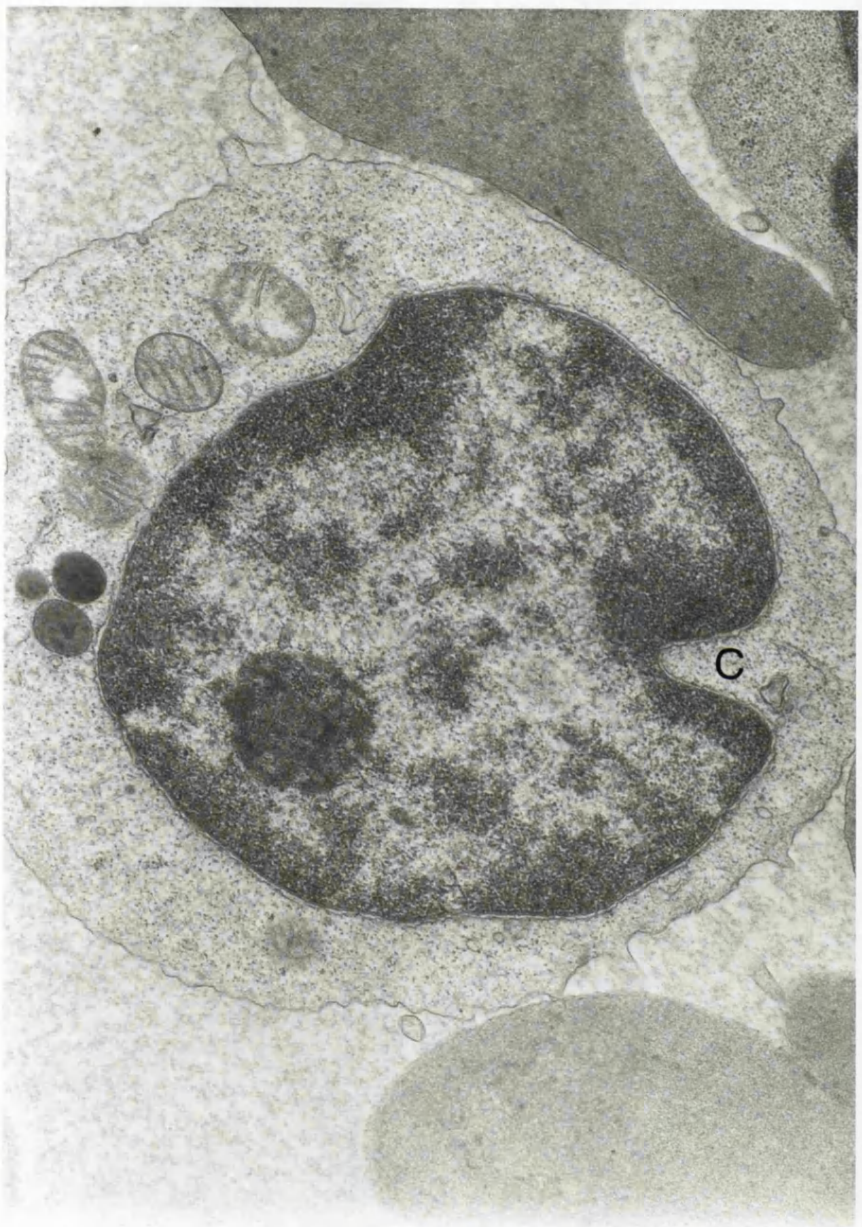


Figure 2: Small lymphocyte showing nuclear cleft (C). Patient H13B.

LYMPHOCYTE PHYSIOLOGY

Lymphocytes are functionally more heterogeneous than other blood cells. They develop from the stem cells of the foetal yolk sac, which have migrated to the liver, spleen, bone marrow and thymus between the 8th and 16th week of gestation (Brenner and Hoffbrand, 1989). Phenotypically, lymphocytes develop in two lines, thymus-derived and bursa-derived (the latter term being taken from the bursa of Fabricius, found in birds but not in man, whose equivalent site is in the bone marrow.

T-cells leave the marrow at an early stage in their development and complete their maturation in the thymus. Within the thymus they are in close contact with marrow-derived antigen-presenting cells. They are thus "educated" in the thymus. An important consequence of this is that all antigen receptors are pre-formed long before they are ever required to encounter antigen. Antigen selects for specific T-cells rather than causing adaptation in response to challenge (Roitt 1988). The survivors of the maturation process eventually leave to circulate in the peripheral blood and settle in the lymph nodes and spleen. Recirculation between nodes, blood, lymph and spleen occurs during an approximate ten-year lifespan. T-cells are programmed only to recognise antigen when it is encountered on the surface of a body cell. They require the simultaneous presence of surface markers of the major histocompatibility group to recognise the cell.

Different subsets of T-cells have a spectrum of functions. Some, termed T helper or T4 cells, detect macrophage-bound antigens and produce gamma-interferon to stimulate the macrophage. Others have natural killer (NK) activity and can destroy cells infected with viruses.

None produces antibodies, but all have the ability to clone themselves in response to their own matching antigen. T-cells activated by antigen produce Interleukin-2 (IL-2), a messenger (cytokine) which induces cell division in activated lymphocytes expressing IL-2 receptors, and promotes killer activity in NK cells.

B-cells differentiate within the foetal liver at 8-9 weeks gestation in man, the bone marrow taking over as the major site later when the marrow spaces develop (Roitt 1988). B-cells are programmed in the bone marrow to make only one type of antibody. They express this configuration on their cell membrane (about 10^5 immunoglobulin molecules) and are either transformed into terminally differentiated plasma cells on stimulation by antigen or into B memory cells. Both of these forms are capable of producing high concentrations of antibody. Normally , the initial antibody production is of IgM, in response to T-cell stimulation, and later of IgG. Some B cells are capable of antibody production by themselves although their range of products is limited to simple, repeating units.

B-cells and macrophages produce the lymphokine, interleukin-1 (IL-1), whose functions are widespread in developing the acute-phase response of inflammation. It induces IL-2 production by T-cells, stimulates bone marrow stromal cells to produce colony stimulating factors, and initiates C-reactive protein production by the liver.

TESTS OF LEUCOCYTE FUNCTION

NEUTROPHIL FUNCTION

Neutrophils possess the abilities to respond to stimuli, move in different ways, adhere to endothelial cells, ingest organisms and generate energy using their metabolic pathways in order to kill organisms. Each of these functions may be tested in vitro, although the conditions of the testing may not closely resemble those in vivo. It is, therefore, essential that each test should be validated to avoid misleading conclusions. This thesis is concerned with the identity and function of leucocytes found in the blood of injured patients soon after injury. As such, any abnormalities of function which may be detected should not be caused by established infection or anaesthesia, factors which have confounded previous research in surgical patients at various stages after operation.

1. **Neutrophil Chemotaxis**

   Chemotaxis, the movement of a cell towards or away from a stimulating substance, was first reported by Leber in 1888 (cited by McCutcheon 1945) as a result of his experiments on the rabbit cornea. Insoluble particles do not have chemotactic properties, and the most potent substances are natural proteins, but several other agents have been identified such as histamine and some carbohydrates.

   The movement of neutrophils during chemotaxis is no faster than when moving at random - the effect is purely on the direction of movement and not on its rate. Although many bacteria produce substances which stimulate positive chemotaxis, others generate toxic agents which immobilise neutrophils. Emys-Roberts and Cowell (1917) found that clostridium welchii organisms produce a toxin that paralyses neutrophils, accounting for the low numbers found in gas gangrene lesions. Similar findings have been noted in typhoid fever and virulent streptococcal infections (Stevenson and Reed 1940).

The two methods commonly used to measure chemotaxis are:

a) migration under agarose gel. Agarose gel is prepared on a petri dish and small wells are cut out of the gel using a template. A central well contains the chemo-attractive agent (eg. F-met-leu-phe) and neutrophil suspensions are placed in wells around this. The distance migrated by the neutrophils is then measured over a defined time, using a microscope.

b) migration through a microporous filter (Maderazo et al 1983). This method uses a chamber with two compartments (Boyden chamber) with a cellulose nitrate filter separating them. A chemo-attractive agent is placed in the lower compartment and the neutrophil suspension in the upper. Migration distance through the filter is measured after a known time.

2. **Neutrophil adhesion**

The methods of assessing neutrophil adhesion are discussed in detail in the methods section of this thesis, since this assay form an important part of the assessment of the leucocytosis of trauma.

The neutrophil adhesion assay using standard nylon fibre has been shown to correlate well with neutrophil adhesion to endothelial monolayers in vitro (MacGregor et al 1978).

3. **Neutrophil organism ingestion and killing**

Measurement of the ability of neutrophils to ingest and kill various microbes has been a popular method of assessing their function since phagocytosis was first discovered. The commonest organisms used have been Candida albicans and Staphylococcus aureus. All bioassays of this type depend on the presence of adequate opsonic activity in the medium, as previously discussed.

4. **Neutrophil oxidation/reduction functions**

Neutrophil granules contain enzymes whose principle function is to kill and digest organisms engulfed by the cell during phagocytosis. The chemical reactions resulting from the action of these enzymes can be detected and measured as a test of neutrophil function. The two assays used in the project are:

a) Neutrophil Chemiluminescence. Neutrophils actively using their enzyme pathways emit light, which can be measured using a scintillation counter. As a test of function, the neutrophil may be artificially stimulated with Phorbol myristate acetate (PMA) or zymosan to generate a maximal response. Details of the assay are given in chapter 3, study 3.

b) Nitroblue Tetrazolium (NBT) reduction. Neutrophil enzyme pathways may be harnessed to reduce the soluble pale yellow dye NBT to an insoluble black pigment, formazan. By studying neutrophils unstimulated and stimulated, both background states of activation and activation capacity may be measured. Details of the NBT reduction assays are given in chapter 3, study 3.

CHAPTER 2

GENERAL METHODS AND MATERIALS

ORGANISATION OF MATERIALS AND METHODS SECTIONS

For clarity and continuity, individual studies are described in full in chapter 3 with their materials and methods together with the results and statistics.

However, this chapter contains details of five general procedures which are applicable to more than one study:

1) Management of Blood Specimens.
2) Preparation of Blood Films.
3) Isolation of Living Neutrophils from Whole Blood.
4) Preparation of Blood Samples for Transmission Electron Microscopy.
5) The calculation of Injury Severity Scores.

1) MANAGEMENT OF BLOOD SAMPLES

Blood samples were taken as soon as possible after injury. Whenever possible, the initial sample was taken from the intravenous cannula prior to attaching the IV giving set and subsequent samples were withdrawn from the same cannula using a three-way tap.

The commonest sites chosen for cannulation were the forearm and antecubital fossa veins of an uninjured upper limb. The internal jugular vein and the femoral vein were occasionally cannulated in the more severely injured patients.

Precautions were taken to avoid dilution of samples taken from the IV cannula by discarding the first 1ml of blood. Studies were undertaken with different sizes of cannula to determine the dead space occupied by intravenous infusion fluid. Even using the largest bore of intravenous cannula (14 gauge), and including all of the blood drawn (i.e. including the first 1ml), the dilutional error was only 0.14ml. This represented a 0.7% error in a 20ml blood sample.

Each blood sample was divided into the following tubes:

1. 5ml Lithium heparin with 5mg reduced glutathione antioxidant, for catecholamine assay.
2. 5ml Potassium-EDTA for neutrophil separation.
3. 2.5ml Potassium EDTA for full blood count.
4. 2.5ml Potassium EDTA for electron microscopy.
5. 5ml Plain (clotted) for C-reactive protein.

Notes: Sample 1. This sample tube was pre-cooled in crushed ice and once collected, the sample was immediately returned to the ice. The plasma was separated by centrifugation in a refridgerated centrifuge (Damon/IEC DPR 6000) at 2500 G for 10 minutes. Plasma was placed in 4ml siliconised plastic tubes which were stored at minus 70 deg. C. until required for catecholamine assay.

Sample 5. This sample was allowed to stand at room temperature for 30 minutes to allow complete clotting, before centrifugation at 2500 G for 10 minutes. Serum was stored in 4ml siliconised plastic tubes at minus 20 deg. C.

FULL BLOOD COUNT

One 2.5ml potassium-EDTA sample was reserved for full blood counting and film preparation. The sample was maintained at room temperature on a Stirling mixer (50 rpm, 50 tilts/min.) until analyzed within one hour.

Blood counts were performed on one of two automated instruments. During the daytime, a Coulter model S was available which gave both total and differential leucocyte counts. At night, a counter was in use which gave total leucocyte counts only. For samples counted on the latter instrument, a differential count was obtained manually from the blood film using the method of Dacie and Lewis (1984).

2) **PREPARATION OF BLOOD FILMS**

Blood films were prepared in duplicate on all samples taken, irrespective of the instrument used for counting.

Method: Approximately 5 microlitres of K-EDTA anticoagulated blood was deposited at one end of a dry microscope slide (previously cleaned in absolute ethanol). A second slide chosen for the regularity of its spreading, was used to spread the film according to the technique of Dacie and Lewis. Films showing irregularities in spreading or other defects were rejected.

Films were rapidly air-dried and fixed with 100% methanol for one minute. After air-drying, a mixture of one part Wright's stain (BDH, England) and three parts of phosphate buffer pH 6.8 was added to the surface of the film.

After four minutes staining the mixture was washed away with excess phosphate buffer. The slide was then air-dried and stored in the dark until examined. One batch of stain was reserved for all slides.

3) ISOLATION OF LIVING NEUTROPHILS FROM WHOLE BLOOD

Two of the assays performed in this study (nitroblue tetrazolium reduction and neutrophil adhesion) required the preparation of leucocyte suspensions from whole blood. This procedure was performed according to a modification of the method of Cates (1981).

Materials

5 ml sample of whole blood in K-EDTA anticoagulant.
6% w/v Dextran 110 (Fluka, Switzerland) in 0.9% sodium chloride solution.
Phosphate-buffered saline (PBS), pH 7.4.
Sterile double-deionised water.
Tissue culture medium RPMI 1640 (Gibco Laboratories, Grand Island, New York) with 15 mmol/L Hepes buffer (Sigma).
10ml siliconised plastic tubes (Sarstedt).
10 ml tissue culture tubes.

Method

The 5ml blood sample was centrifuged at 600 G for 10 minutes and the supernatant plasma removed from the cell layers. 5ml of 6% Dextran 110 solution was then added to the cell layers and mixed thoroughly in a 10ml tube. The cell suspension was then incubated at 37 deg. C in a water bath for 30 minutes, after which time the majority of the red cells had formed rouleaux and sedimented to the bottom of the tube. The clear supernatant liquid, rich in leucocytes, was then pipetted into another 10ml tube and centrifuged at 600 G for 5 minutes. The cell pellet was resuspended in 1ml sterile deionised water to lyse any remaining red cells. After 45 seconds, isotonicity was restored by adding PBS to a volume of 10ml. Two steps of centrifugation and washing in PBS were then performed. The final cell pellet, which was white in colour, was suspended in Hepes-buffered RPMI 1640 tissue culture medium at 5 deg. C until the cells were used.

4) PREPARATION OF BLOOD SAMPLES FOR ELECTRON MICROSCOPIC STUDIES

Materials

2.5ml samples of K-EDTA blood for preparation.

4ml siliconised plastic tubes.

2.5% Glutaraldehyde buffered with 0.1 M sodium cacodylate

Osmium tetroxide (Sigma)

Absolute alcohol, pure (James Burroughs, Essex, UK)

1,2 Propylene oxide, EM purity (Merck)

EMIX resin (Emscope, Watford, Herts) *

Embedding rotor, vials and moulds (Taab)

Embedding oven

Reichert Ultracut ultramicrotome

Glass knives

EM grids 3mm, 200 mesh (Graticules Ltd, Tonbridge UK)

Electron microscope (Carl Zeiss, model 10CR)

Kodak EM film 4489 8.3 x 10.2 cm (Eastman Kodak Co., Rochester NY)

* EMIX resin was prepared freshly by adding equal quantities of resin and hardener together, mixing thoroughly, then adding 2% by volume of activator before final prolonged mixing.

Method

Specimens were fixed for electron microscopy according to the method of Anderson (1965). The 2.5ml blood sample was pipetted into a 4ml siliconised tube and centrifuged at 1000 G for 10 minutes. The blood at the end of centrifugation was separated into three layers - red cells, buffy coat and plasma. The plasma was carefully removed with a pasteur pipette, and 2.5% cacodylate-buffered glutaraldehyde was layered onto the buffy coat without disturbing it.

The tube was left for at least one hour to allow in situ fixation of the buffy coat, which was then removed from the red cells as a solid disc. The disc was washed in 0.1M cacodylate buffer to remove loosely adherent red cells, and stored in buffer until processed.

The buffy coat disc was sectioned with a scalpel under buffer in a Petri dish to produce blocks of less then 1mm^2 and 1.5mm length. The square face represented a cross section of the layers of the buffy coat, and the specimens were so shaped as to allow orientation in the moulds.

Specimens were post-fixed in 1% Osmium tetroxide for one hour and then washed in cacodylate buffer for 5 minutes.

Dehydration was accomplished by processing the specimens through the following sequence (each step including constant mixing on a rotor bed):

50% alcohol	10 minutes
50% "	10 "
70% "	10 "
70% "	10 "
95% "	10 "
95% "	10 "
100% "	10 "
100% "	15 "
100% "	15 "
Propylene oxide	15 "
Propylene oxide	15 "
50:50 PO:resin at 37 deg. C	1 hour
Pure EMIX resin	2 hours

After dehydration and impregnation the specimens were embedded in pure EMIX resin in moulds, orientated to present the buffy coat profile as the sectioning face. Blocks were polymerised at 60 deg. C in an oven overnight.

Sectioning

Blocks were trimmed on the ultramicrotome prior to cutting 1 micron thick sections. These "semi-thin" sections were stained with 1% toluidine blue in borax and examined under the light microscope (figure 3). The area representing the leucocyte layer was identified and the block-face trimmed to present this area for ultrathin sectioning. Ultrathin sections were made in the pale gold interference spectrum, and picked up as ribbons on EM grids.

Staining

Grids were stained suspended on drops of Uranyl acetate (saturated solution in 50% ethanol) for 10 minutes and Lead citrate (1%) for 10 minutes with triplicate washing after each stain.

Figure 3

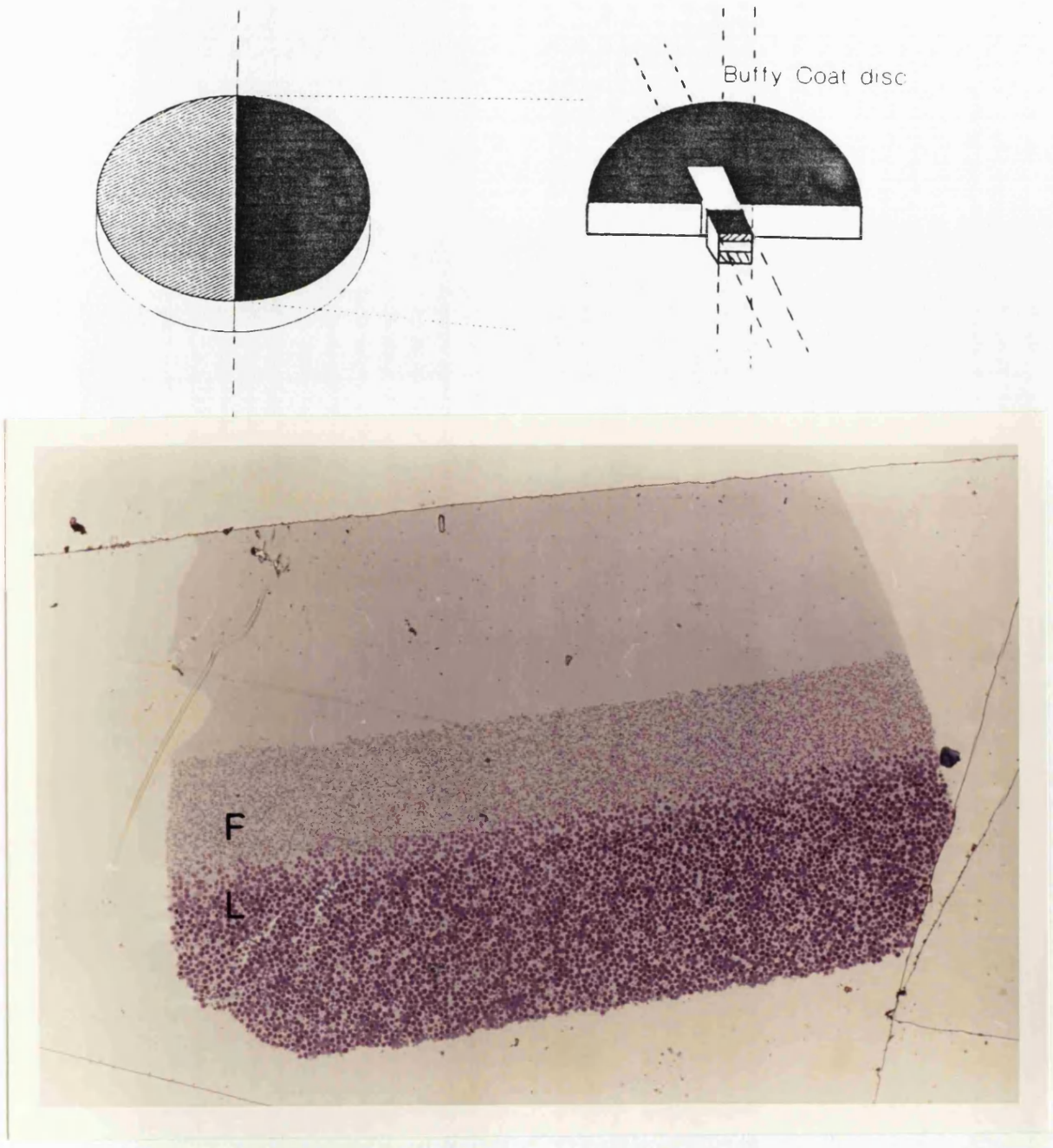


Figure 3: Semi-thin ultramicrotome section showing platelet (P) and leucocyte (L) cell layers.

5) CALCULATION OF INJURY SEVERITY

Injury severity was calculated for all patients using the Injury Severity Score (Baker et al, 1974). Anatomical injuries were recorded in detail and awarded a score between 1 and 5 using the Abbreviated Injury Scale (AAAM 1985). This scale contains a dictionary of all injuries by body region, each being allocated a severity of 1 (least severe) to 5 (most severe), severity being determined by an international panel of trauma surgeons.

Taking the three highest scores, counting one only from each body region (External, Head and Neck, Face, Chest, Abdominal/Pelvic contents, Extremities/Pelvic Girdle) each score was squared and the sum of the three squares calculated as the Injury Severity Score (range 1-75). For single injuries, the square of the single AIS score was used.

This method has been evaluated (Baker and O'Neill 1974, 1976) and found to be predictive of mortality. Mortality increases with ISS score but the linearity of this relationship is broken by peaks at 16 and 25. These points represent single, severe injuries at 4^2 and 5^2 respectively, which tend to carry an excess mortality (Cocks, 1987).

CHAPTER 3

DESCRIPTION OF STUDIES

INCLUDING RESULTS, STATISTICS AND DISCUSSION

CONTENTS

STUDY 1

COMPARISON OF THE LEUCOCYTE COUNTS OF 110 RECENTLY INJURED PATIENTS WITH THOSE OF A NORMAL CONTROL POPULATION.

STUDY 1

Comparison of the leucocyte counts of recently injured patients with those of a normal control population.

Introduction

Do trauma patients exhibit the phenomenon which I have called "the Leucocytosis of Trauma?" A suitable null hypothesis would be that trauma patients have white blood cell counts on admission to hospital which are not significantly different from those of a comparable normal control population. This question has not been answered in a formal way by previous research, and this preliminary study was designed to clarify the problem. In view of the conflicting evidence regarding the propensity for different injuries to produce a leucocytosis (Keith, 1919; Brittain, 1963), the results were also examined by body region.

Recognition of the phenomenon is important, since there is a danger that the very high leucocyte counts seen after injury may be misinterpreted. During a retrospective study of multiple trauma patients which led to this thesis, a haematology report was found which queried the presence of acute myeloid leukaemia in a recently injured patient. A bone marrow examination was being arranged when it was noted that a subsequent full blood count had returned to normal on the second day.

Protocol

Part 1) White blood cell counts were measured in 110 normal subjects taken from the laboratory staff and medical student population at the Prince of Wales Hospital, Shatin, Hong Kong. Ages of the subjects ranged from 18 to 50 years, and those suffering symptoms of any illness were excluded. Samples were generally taken during the morning and no restrictions were placed on dietary or fasting status.

Part 2) White blood cell counts were measured in 110 recently injured patients presenting to the Accident and Emergency departments at the Prince of Wales Hospital, Shatin, Hong Kong and at Hope Hospital, Salford, England. All of the patients had received their injuries less than three hours before sampling, and the samples were taken from the intravenous cannula inserted as part of the resuscitation.

The minimum severity of injury for admission into the study was ISS 9 and no upper limit was set.
The ages of the patients ranged from 15 to 75 years. All patients had a general medical examination to exclude pre-existing disease which might affect the leucocyte count.

RESULTS

Results for controls are given in table 1 and for patients in table 2, and graphically in figure 4. Tables 1 and 2 are located in Appendix 1.

1. Normal Controls - Table 1

As described above. Equal numbers of males and females were recruited.

Statistics:

a) Total WBC count: Mean 6.75
Standard deviation 1.62
Range 3.52 - 9.97
Variance 2.6

b) Neutrophil count: Mean 3.90
Standard deviation 1.3
Range 1.32 - 6.49

c) Lymphocyte count: Mean 2.21
Standard deviation 0.37
Range 1.47 - 2.95

d) Monocyte count: Mean 0.35
Standard deviation 0.13
Range 0.1 - 0.6

2. Patients - Table 2

62% of patients were male and 38% female. This reflects the general epidemological pattern of males being more frequently involved in accidents than females (Parliamentary Advisory Council for Transport Safety, 1992).

Statistics

Total WBC count: Mean 12.2
Standard deviation 3.73
Range 4.74 - 19.66
Variance 13.9

* Note: Garrey and Bryan (1935) found no evidence of sex differences influencing basal leucocyte counts, with the exception of a slight elevation in the later stages of pregnancy approaching term, and in labour.

Figure 4

Control and Trauma Populations Frequency of Leucocyte Counts

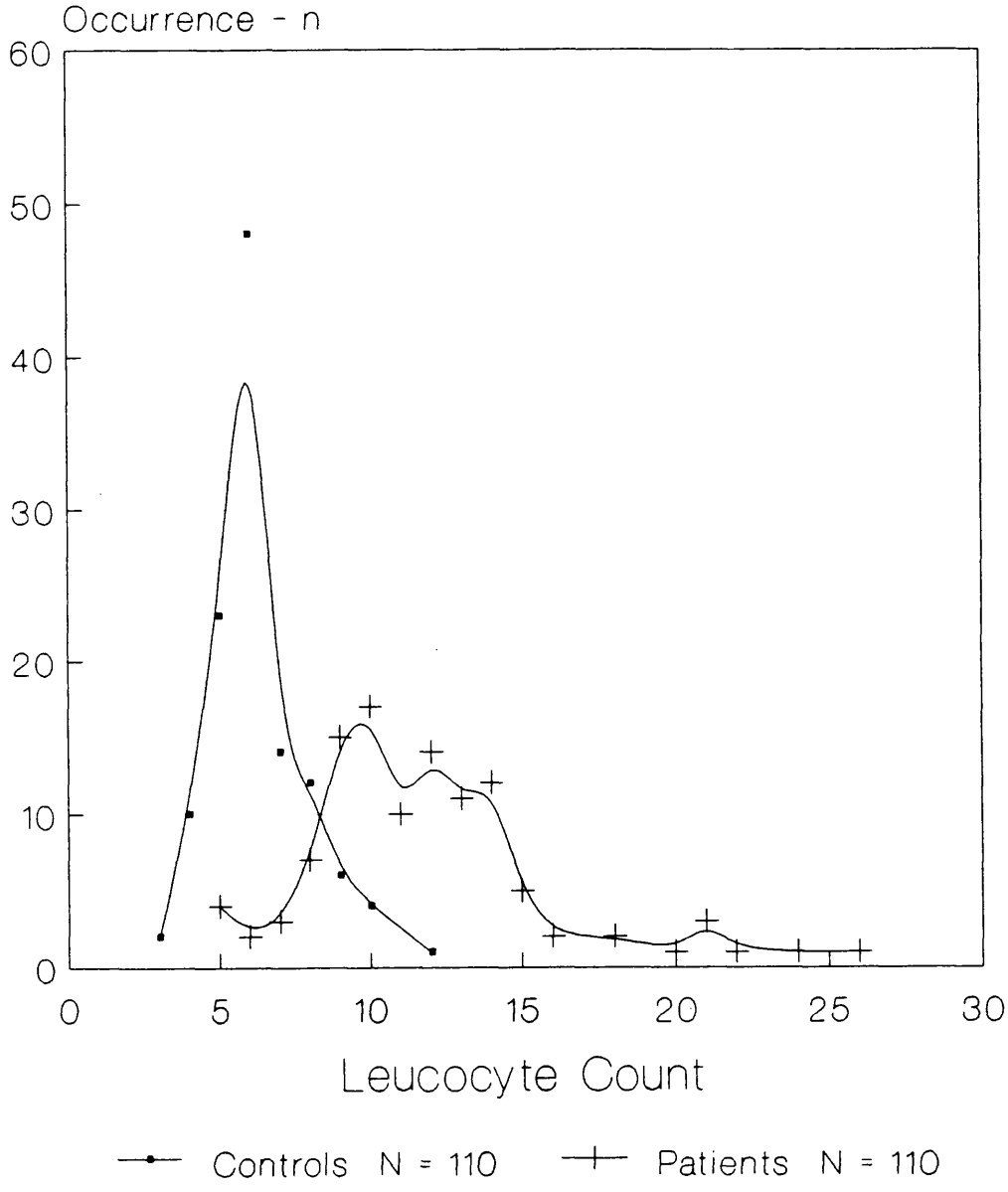


Figure 4: Distribution of leucocyte counts of 110 normal controls and 110 recently injured patients.

DISCUSSION

In this study, the total leucocyte counts of 110 normal controls (Table 1) were compared with those of 110 patients who had suffered moderate or severe traumatic injury (minimum ISS = 9, all sampled within 3 hours of injury) (Table 2).

Both populations were approximately normally distributed (figure 4). The distribution for patients had a larger variance (13.9 vs 2.6). For both populations, the mean (6.75 for normal controls and 12.2 for patients) and the median (6.42 for normal controls and 11.4 for patients) lay within the modal groups. The patient distribution was biased by a number of very high leucocyte counts (20 x 10^9 and above). This distribution was tested for goodness of fit using the Chi-square test and was found not to differ significantly from normality.

The null hypothesis that the sample mean of trauma patients is the same as that of normal controls, and that the trauma patient sample is drawn from the normal population is rejected. The chances of the trauma patients' results belonging to the control population are less than 1 in 10,000 ($p < 0.0001$, z value 15.36).

The mean for trauma patients (12.2) lies more than 3.09 Standard Deviations from the control mean of 6.75, offering additional evidence of the populations being different at a significance level of $p < 0.001$.

Attempts have been made to attach diagnostic and prognostic significance to the finding of a leucocytosis in emergency patients. Bouget et al (1990) found that hyper-leucocytosis was of poor value in the diagnosis of infection and inflammatory disease, giving an equal number of false positives and false negatives.

They commented that elevations of the total leucocyte and granulocyte populations were not exclusively related to inflammatory processes or infection but rather to a stress response, and advised particularly against making a diagnosis of the former conditions on the basis of an early sample. In addition, some patient groups (e.g. infants and pregnant mothers) have naturally elevated WBC counts which would complicate diagnosis of any emergency. Under such circumstances, examination of the differential count would become essential. On the basis of these results, it would appear that the finding of a leucocytosis following trauma (at least in the early phase) should not be taken to be relevant to the diagnosis of infection.

Analysis by Body Region

Only 39 of 110 patients had a single-region injury (29 extremities, 7 head, 1 chest and 2 abdomen). It is therefore difficult to assign any significance to minor differences between these small groups. The remaining 71 patients had multiple injuries, and when grouped by the predominant (i.e. most serious) injury by body region, differences were noted using the Wilcoxon rank sum test:

Comparison	WCC Magnitude	Significance
Head (14) v Chest (11)	No significant difference	Nil
Head (14) v Abdomen (5)	Abd. > Head	$p<0.01$
Chest (11) v Abdomen (5)	Abd. > Chest	$p<0.025$
Limbs (29) v Abdomen (5)	Abd. > Limbs	$p<0.025$
Head (14) v Limbs (29)	N/S	N/S

Mean ISS values were:	Head	17.7
	Chest	18.09
	Abdomen	14.0
	Limbs	10.0

In view of the confounding variable of unequal mean injury severity between the small groups, the conclusions which can be drawn from these results need to be treated with caution.

However, the finding that abdominal injury at lower mean severity perhaps produces a larger stimulus than head or chest injury would be worthy of further study. Corica and Powers (1975) considered that a high leucocyte count seen immediately after liver and spleen rupture was diagnostically significant, but did not compare their results with other body region injuries. Several years earlier, Keith (1919) concluded that visceral injury did not raise the leucocyte count, but rather that extremity injury did. Further work with larger groups of single-region injuries is needed.

STUDY SUMMARY

This study confirmed the existence of the leucocytosis of trauma, a phenomenon which is probably universal in injured patients early after injury. The distribution of the increased leucocyte counts approximates to a normal distribuion, confirmed by the chi-square test for goodness of fit.

Analysis of results by body region suggests that abdominal injury may be a particularly potent stimulus which should be further investigated.

STUDY 2

DETERMINATION OF THE CHARACTERISTICS AND TIME-COURSE OF THE LEUCOCYTOSIS OF TRAUMA.

STUDY 2

Determination of the characteristics and time course of the Leucocytosis of Trauma.

Introduction

Previous studies of changes in circulating leucocyte numbers following surgical and traumatic injury have produced conflicting results, particularly with respect to lymphocyte numbers (Kirov, 1979: Teggatz, 1987; Thomassen, 1986; Pinkerton, 1989). The apparent conflict may be due to differences in the timing of the samples taken.

To investigate further the rise in white blood cell count seen in recently injured patients, data from 117 patients at varying times after injury were analysed. In a further 42 patients, differential counts and detailed blood film examinations were performed. 28 of these counts were performed serially to assess changes occurring at different times after injury within the individual.

Protocol

Total leucocyte counts were measured prospectively on 117 trauma patients for whom the timing of the blood sampling was known with certainty. Patients with pre-existing disease were excluded.

Detailed differential leucocyte counts were performed on 42 patients as soon as possible after injury, with careful account being taken of the timing of each sample.

28 patients were studied serially. Minimum injury severity for inclusion in the study was ISS 9, with no set upper limit.

In addition to the standard differential count, films were examined to determine the numbers of neutrophil band forms and atypical lymphocytes present.

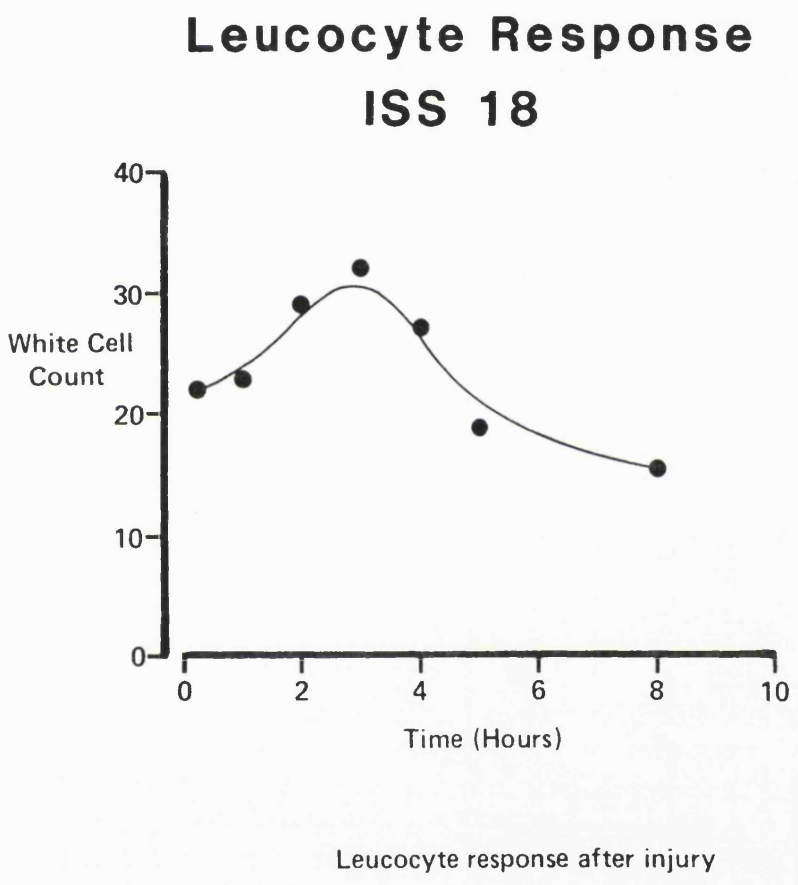
Results

Differential leucocyte counts for 42 patients are shown in table 3 (Appendix 2), band form counts in table 4 and atypical lymphocyte counts in table 5.

The pattern of response in serially-sampled patients is shown graphically in figures 5 and 6. Changes in total leucocyte counts and lymphocyte counts over time are shown in figures 7 and 8.

To avoid repetition, a detailed narrative on these results is included with the discussion section.

Figure 5



Leucocyte response after injury

Figure 5: Serial leucocyte measurements in a single patient with major injuries.

Figure 6

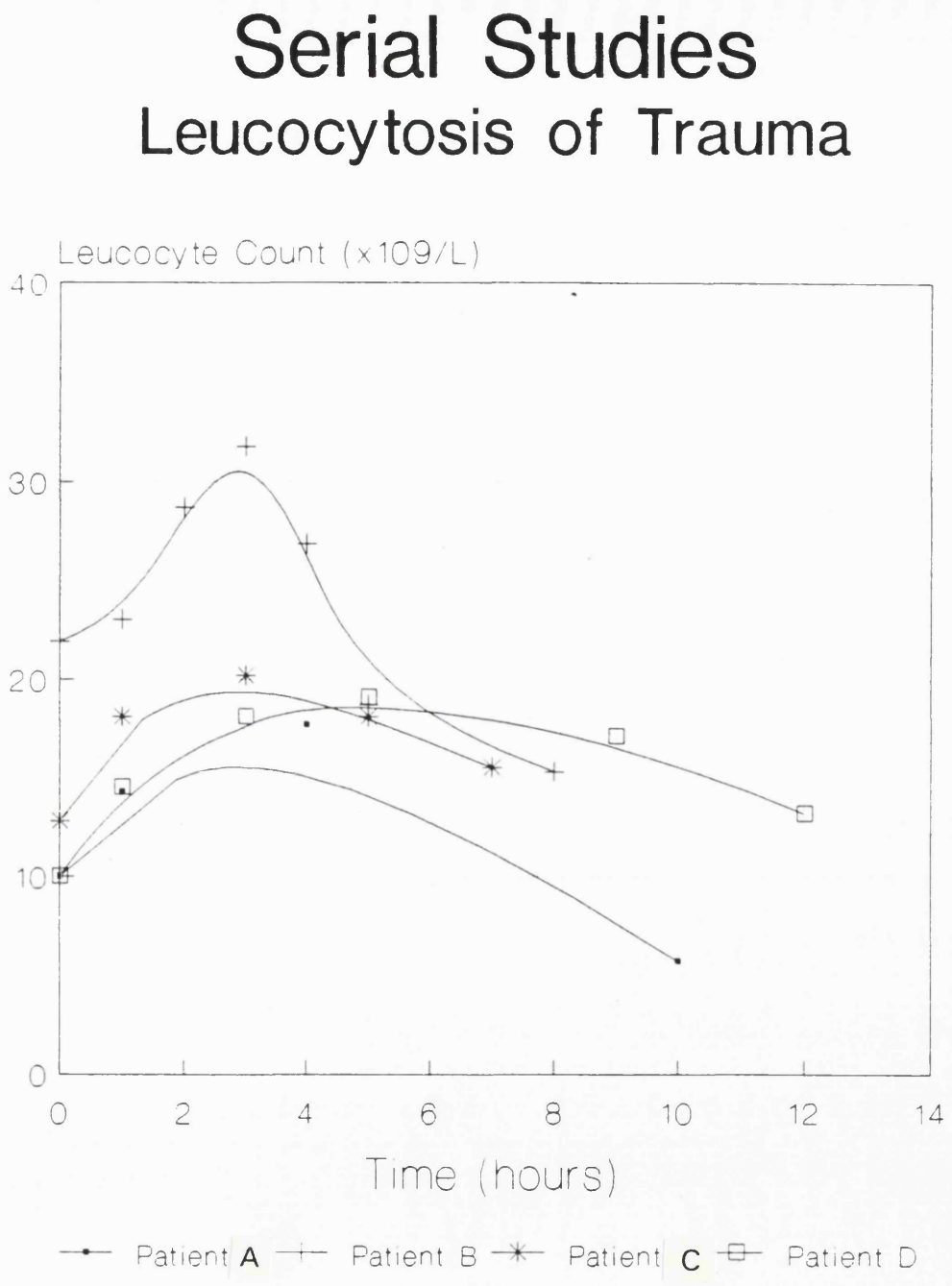


Figure 6: Composition graph of serial studies in 4 patients with major injuries.

Figure 7

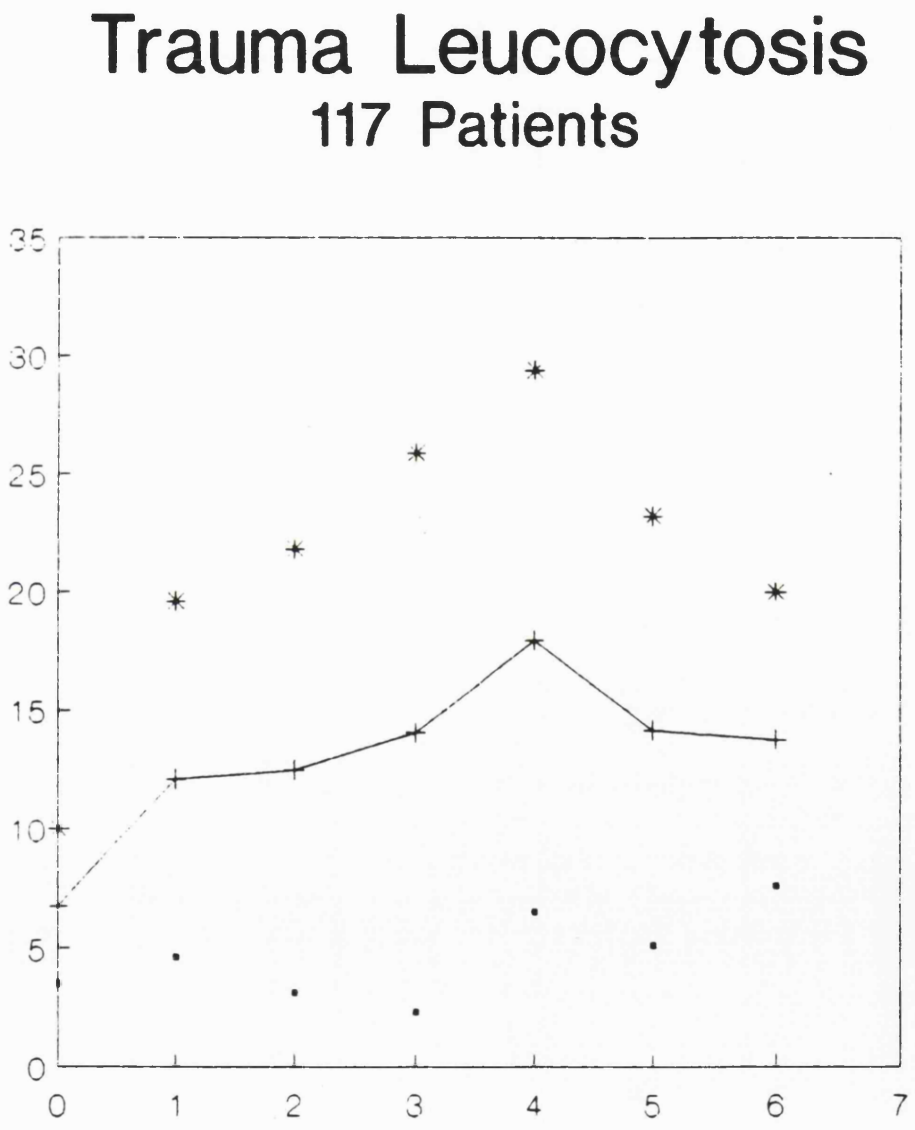


Figure 7: + mean and * confidence intervals for 117 patients' total leucocyte counts by sample time.

DISCUSSION

1. Study of 110 trauma patients.

Incidence

Of 110 patients, 80 had leucocyte counts more than 2 SD above the normal range mean within 3 hours of injury, and a further 26 were in the right-hand half of the distribution, above the mean. Only four results lay below the mean. Two of these were AIS 3/ ISS 9 isolated lower limb injuries and two were major trauma patients, one with an isolated AIS 4/ISS 16 Head Injury and the other with a major chest injury and moderate abdominal and limb injuries (ISS 17).

These results would suggest that leucocytosis in injured patients is virtually universal. It is not possible to determine whether those patients whose results lay in the upper half of the normal range sustained a rise from a lower resting level, but the normal range is so broad that such an effect is likely. These results are probably the left-hand tail of the trauma normal distribution rather than "normal" results drawn from the general population.

Examining the differential counts of the 42 trauma patients studied in detail, 3 had neutrophil counts below the upper end of the normal range (+2 SD = 6.5) and there were no results below the mean at all.

The definition of lymphocytosis differs between research papers and text books. In the normal control study contained in this thesis, the upper end of the normal range approximates to 3 (2.95). Teggatz et al (1987) define "lymphocytosis" to be a count of 4 and above, and Pinkerton et al (1989) together with some haematology texts adopt 5 as the level, although these same authorities quote ranges which suggest 3.5 to be the upper limit of normal.

	Number (+2SD) above range	Number above mean	(-2SD) Number below range*
Normal control this study >3	17	8	5
Lymphocytosis = >4 (Teggatz)	11	11	4
Lymphocytosis = >5 (Pinkerton)	6	11	4

* **Initial sample only considered - see study 6 for discussion of later lymphopenia.**

9 patients exhibited a monocytosis following trauma, with no evidence of monocytopenia in the series, although some monocyte counts were not obtainable.

An illustration of the light microscopic features is given in figure 8.

Characteristics

A study of the 42 patients on whom differential counts were available demonstrated a leucocytosis involving three cell lines during the first 3 hours - Granulocytes, Lymphocytes and Monocytes.

i) **Granulocytes** - Mean granulocyte count in the early samples was 11.22 (S D 5.29) compared with a control mean of 3.9 (S D 1.29). This cell line showed the greatest magnitude of change, the sample mean lying 5.67 S D away from the control mean. Calculating Z for this sample population, $p < 0.0001$ that the sample came from the normal control distribution.

ii) **Lymphocytes** - Mean lymphocyte count in the early samples was 2.93 (S D 1.64) with a control mean of 2.2 (S D 0.37). Calculation of Z suggests a probability of $p < 0.004$ that the lymphocyte counts were part of the normal control distribution. Recognising the rapid changes in lymphocyte count with time, however, 3 hours could be considered too broad a time span for the study. Examining the very early counts (within 0.5 hours of injury) in nine patients, mean lymphocyte count was 4.65 however, reflecting the transient nature of the trauma lymphocytosis. This phenomenon is further examined in Study 6.

iii) **Monocytes** - Monocyte counts were available in 34 patients. These data had a mean of 0.536 (S D 0.42) against a normal control range of 0.095 - 0.595 (mean 0.3452) Z for this sample was 2.65, indicating separate populations at a probability level of $p = 0.004$. Data taken from later samples showed a slightly elevated mean at 0.41 which was not statistically significant.

Examination of Blood Films

Neutrophils

Neutrophilia in many conditions is associated with a "left shift" - i.e. an increase in the number of immature neutrophils seen on the blood film. These are distinguished by a smaller number of nuclear lobes (two lobes or a single, kidney-bean nucleus, termed a band form). This picture is particularly prevalent in infective conditions.

Of 17 trauma patients studied in detail, 16 showed raised band counts above the expected normal maximum of 5% (See table 4) with 5 having counts greater than 20% (the usual maximum of all immature counts).

Lymphocytes

In normal subjects, large granular lymphocytes make up no more than 10% of the total seen on a blood film. A study of the blood films of the 17 trauma patients showed that 7 exceeded this proportion at some stage following injury, predominantly in the first sample taken (Table 5).

Teggatz et al (1987) found a high proportion of atypical lymphocytes in emergency medical patients, most of whom had suffered cardiac arrest. All of the cardiac arrest patients had been given intravenous bolus injections of adrenaline. Further examination of these cells is discussed in study 6.

<u>Time Course</u>

Serial samples obtained in 4 patients gave findings of:

1) A very early rise in both neutrophil and lymphocyte numbers, already present in samples as little as 15-20 minutes after injury.
2) A further rise, predominantly in neutrophil numbers, peaking at about 3-4 hours after injury.
3) A fall in neutrophil and lymphocyte numbers following the peak. The neutrophil numbers usually remained high with the lymphocyte numbers falling back into the normal range.

These features are summarised in figure 9.

A study of a larger number (n = 117) of patients with known sampling times showed statistically significant differences between the total leucocyte counts obtained at less than one hour compared to those taken at 3-4 hours (Wilcoxon Rank Sum Test $p < 0.005$) and those between 3-4 hours and 6-7 hours ($p < 0.005$).

These results support the observation by Corica and Powers (1975) of an increase in white blood cell count within one hour of rupture of the spleen or liver, persisting for approximately two hours and then falling rapidly (the latter fall noted by Brittain, 1963). In this series, the pattern was not found unique to viceral injuries, but occurred in each case in a patient with skeletal injuries.

Figure 8

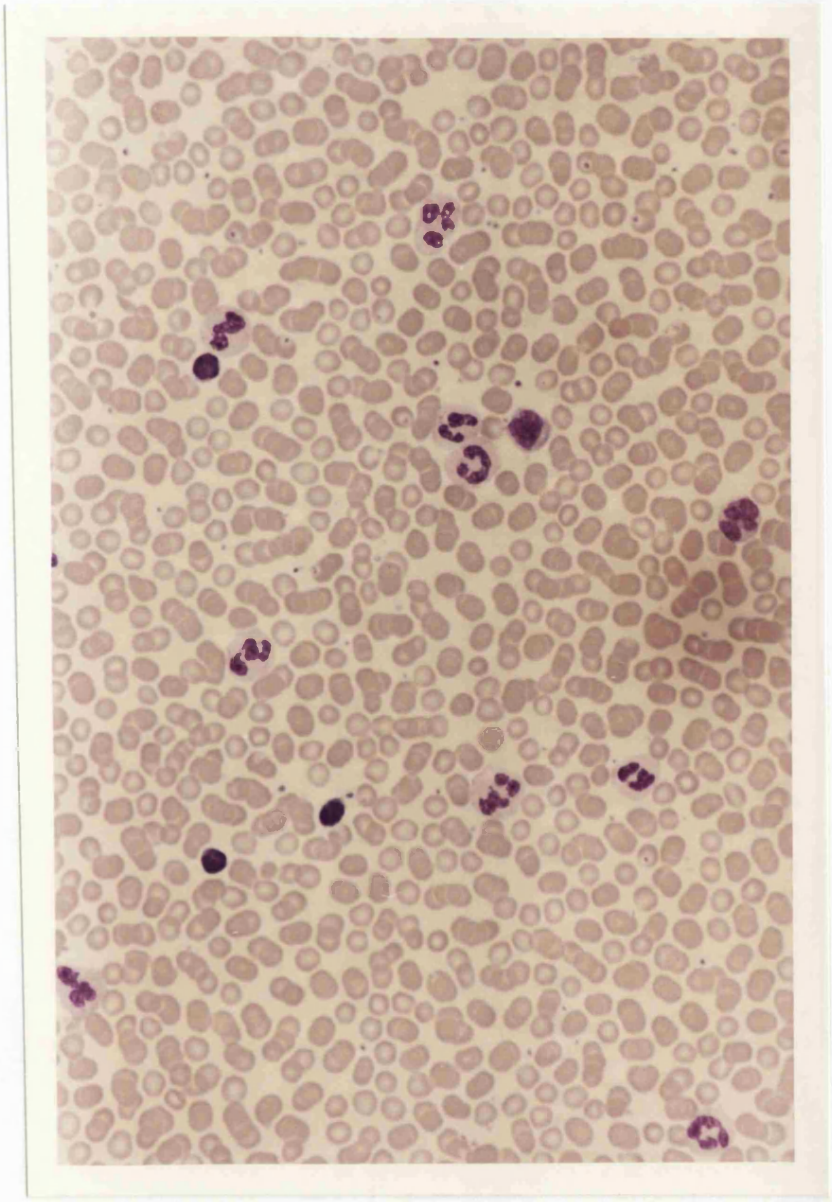


Figure 8: The leucocytosis of trauma.

Figure 9

Leucocytosis of Trauma
Schematic Representation

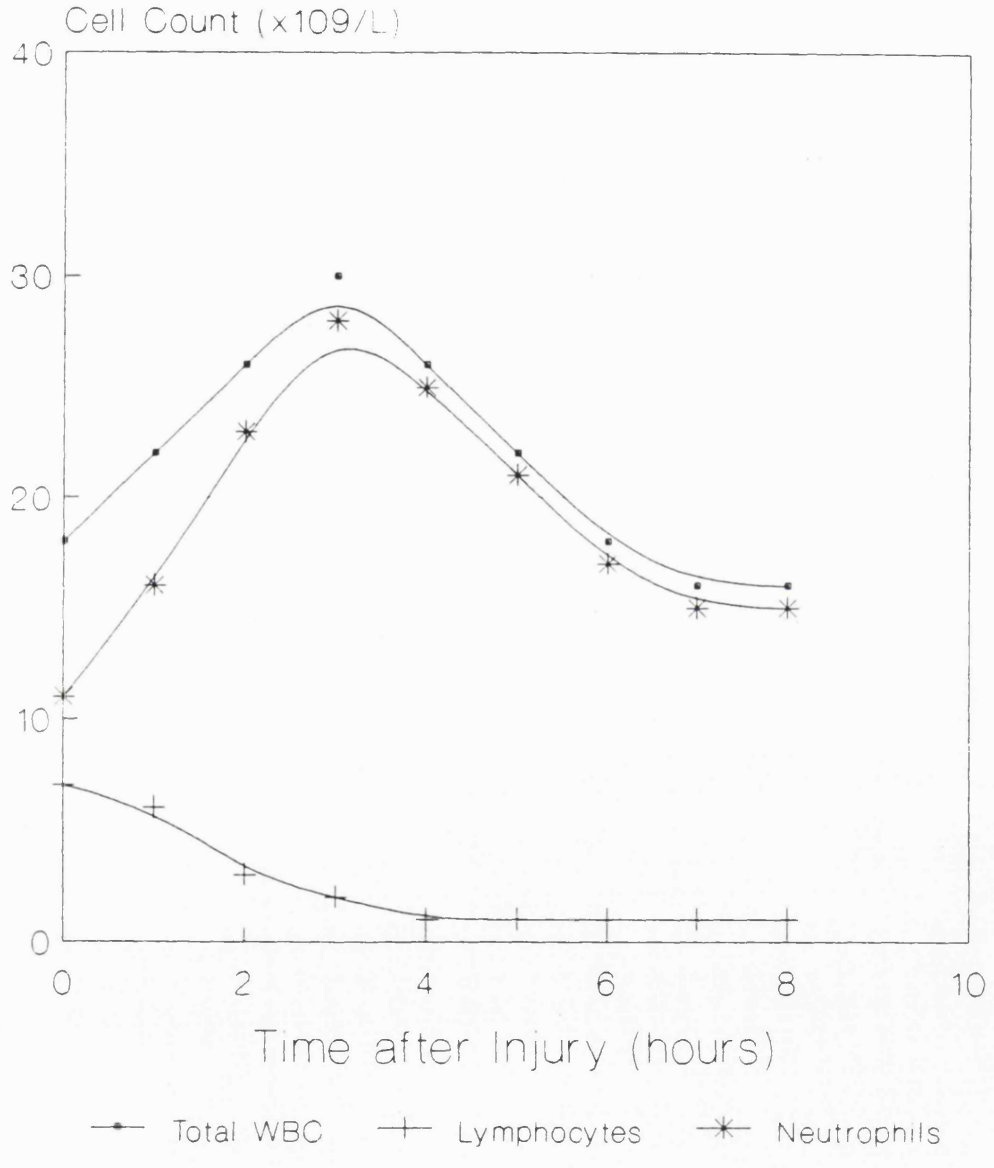


Figure 9: Schematic representation of the leucocytosis of trauma.

The Effect of Intravenous Fluid Administration - A Confounding Variable?

Cases were examined closely to ensure that the observed changes were not being influenced unduly by the administration of intravenous fluid. In all four cases where serial samples were taken (see figure 6), the leucocyte count continued to rise during the period of resuscitation (1-4 hours) during which the maximum amount of fluid administration was taking place.

Stored blood does not contain any neutrophils which would have contributed to the rise. It is concluded that, if any effect is likely, that the rise seen in the four patients was possibly blunted, but that the shape of the response curve would not be affected. Even in patients who went on to receive blood or IV fluids over many hours, the neutrophils persisted. The lymphopenia seen in study 6 occurred whether or not the patient had received IV fluid replacement and is not considered to be due to dilutional effects (see page 146). These results and conclusions are supported by evidence contained in the papers by Keith (1919) and Balch (1955). In particular, Balch provides a table of leucocyte counts immediately following large blood transfusions on the first day after battle injury. The range of volumes given was 5.5-26.0 litres of whole blood plus 0.5-1.0L Dextran and in 8 out of 10 patients a leucocytosis was still present (between 11.85 and 16.8).

The marrow pool of neutrophils is vast (it is thought to contain about 10-20 times as many neutrophils as the circulating pool and marginated pool) and contains enough neutrophils - both mature and new - to maintain a leucocytosis for 24 hours or more after injury.

STUDY SUMMARY

This study further describes the characteristics of the leucocytosis of trauma.

An early rise (within 30 minutes) in neutrophil and lymphocyte numbers has been detected, which is followed by a rapid fall in lymphocycte numbers, the neutrophilia peaking at about 3-4 hours and resolving more slowly.

The early neutrophilia is made up on both mature and band-form neutrophils, and examination of the microscopic features of the lymphocyctes showed an increase in large granular lymphocytes in just under half of the patients.

STUDY 3

INVESTIGATION OF THE FUNCTIONAL STATE OF THE MICROBICIDAL PATHWAYS OF CIRCULATING NEUTROPHILS IN RECENTLY INJURED PATIENTS.

STUDY 3

Investigation of the functional state of the microbicidal pathways of circulating neutrophils in recently injured patients.

Introduction

The reasons why injured patients are prone to serious and sometimes fatal infections remain unclear. No studies have been performed in the very early post-trauma period in man, but neutrophil locomotory defects have been observed later which may be due to inappropriate activation and self-damage (Maderazo et al, 1986). In this study, the state of the oxidation/reduction pathways within the neutrophils was determined using two methods - chemiluminescence response and nitroblue tetrazolium reduction.

Protocol

1) Nitroblue tetrazolium reduction was assessed in 20 recently injured patients, again with moderate or severe injuries. 4 patients were studied serially to assess changes over a time period. Results and details of timings and injuries are given in table 7. A preliminary study of five injured patients presenting to Hope Hospital, Salford, was followed by a larger study of 15 patients at the Prince of Wales Hospital, Shatin, Hong Kong.

2) Neutrophil chemiluminescence response was assessed in 7 patients soon after moderate or severe injury and again after an interval of 1-3 hours in 6 of the patients. One patient was studied three times at two hourly intervals. Details of the results, timings and injury severity are give graphically in figures 10 to 18. All patients were studied following admission to the Accident and Emergency department at Hope Hospital, Salford.

Materials and Methods

1) Nitroblue-tetrazolium Reduction

Neutrophil function was assessed by using the stimulated nitroblue tetrazolium (NBT) test first described by Park and Fikrig (1968). The method measures the ability of the neutrophil's redox system to reduce a pale yellow dye, nitroblue tetrazolium, to the black pigment formazan. This pigment can be seen in the cell cytoplasm by light microscopy. The mechanism of NBT reduction depends on an oxygen-dependent oxidase which generates O_2 (superoxide) radicals. The first part of the test measures the state of activation of the neutrophils in a sample. The second part utilises a stimulant to assess the capacity of the neutrophils to respond by increasing their reduction pathways. The stimulant, phorbol myristate acetate (PMA), induces 95-100% stimulation in normal neutrophils.

Materials

Neutrophil suspension in RPMI to be tested.

Nitroblue tetrazolium (Sigma). prepare 0.2% solution in 0.34% aqueous sucrose, filter and store in the dark at 0-4 deg. C. Phorbol myristate acetate (Sigma). Prepare stock solution of 2mg/ml to be stored at -20 deg. C. Dilute to 2.5mcg/ml as working solution. CAUTION-TOXIC AND CARCINOGENIC SUBSTANCE.

200 microlitre adjustable Gilson pipette.

4ml siliconised plastic tubes.

37 deg. C water bath.

Haemocytometer with heavy cover glass.

Method

Part 1. 200 microlitres of neutrophil suspension was incubated with 100 microlitres of NBT solution for 15 minutes. The percentage of neutrophils containing definite blue/black formazan deposits was determined using a haemocytometer chamber.

Part 2. 200 microlitres of neutrophil suspension was incubated with 200 microlitres of PMA and 100 microlitres of NBT solution for 15 minutes. The percentage of formazan-positive neutrophils was determined in a similar manner.

2) Neutrophil Chemiluminescence Assay

Neutrophils emit light during the oxygen burst phase of phagocytosis. This effect, termed chemiluminescence, also occurs during the artificially induced oxygen burst caused by PMA. Chemiluminescence is impaired in myeloperoxidase-deficient neutrophils (Rosen and Klebnoff 1976), indicating that the mechanism is dependent upon the generation of superoxide anions.

The chemiluminescence capacity of neutrophils from trauma patients was tested with the help of Dr M Potter of the Immunology department at Hope Hospital.

Materials and Method

The LKB 1251 luminometer is a fully automatic scintillation counter capable of providing instantaneous readings of chemiluminescence within its reaction chamber. For this assay, a computer programme was used to drive readings at 3 minute intervals over 30 minutes. Reagents were added automatically to the reaction vials by the machine, which was set up in a similar manner to that used in the NBT test above. Opsonised zymosan was occasionally used as an alternative stimulant to PMA.

Results

The data obtained for stimulated nitroblue-tetrazolium (NBT) assays in 20 trauma patients are contained in table 7, appendix 3. The stimulated chemiluminescence response data of eight further patients are contained within table 8, appendix 3, and are expressed graphically on the following pages in figures 10-18. The pattern of response to the stimulants phorbol myristate acetate (PMA) and opsonised zymozan are also depicted here.* Because the data require individual consideration, the detailed narrative on these results is included within the discussion section, which follows the figures on the next nine pages.

* controls

Figure 10

Patient JM
Tibial Shaft Fracture

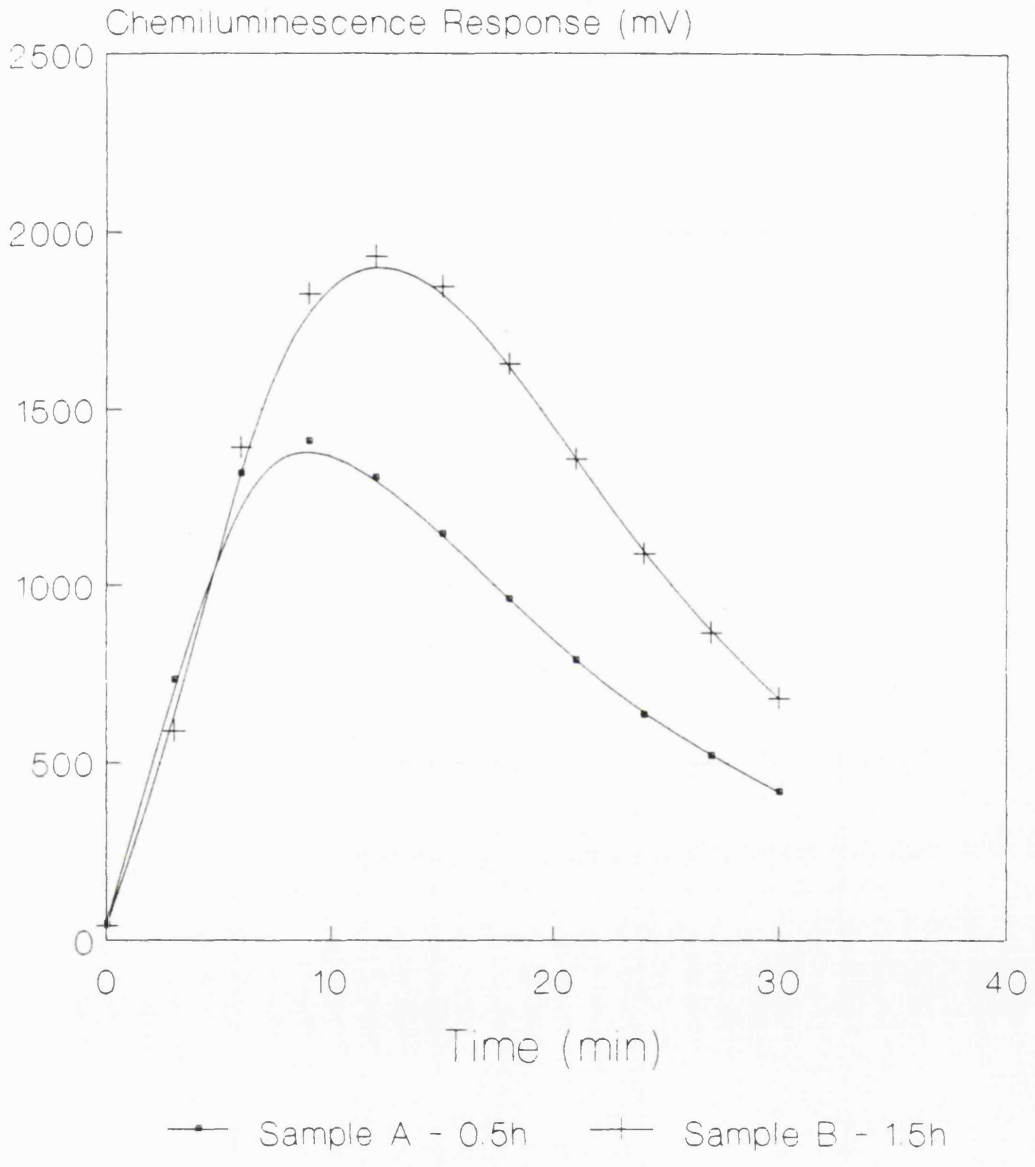


Figure 11

Patient JD
Tibial Shaft Fracture

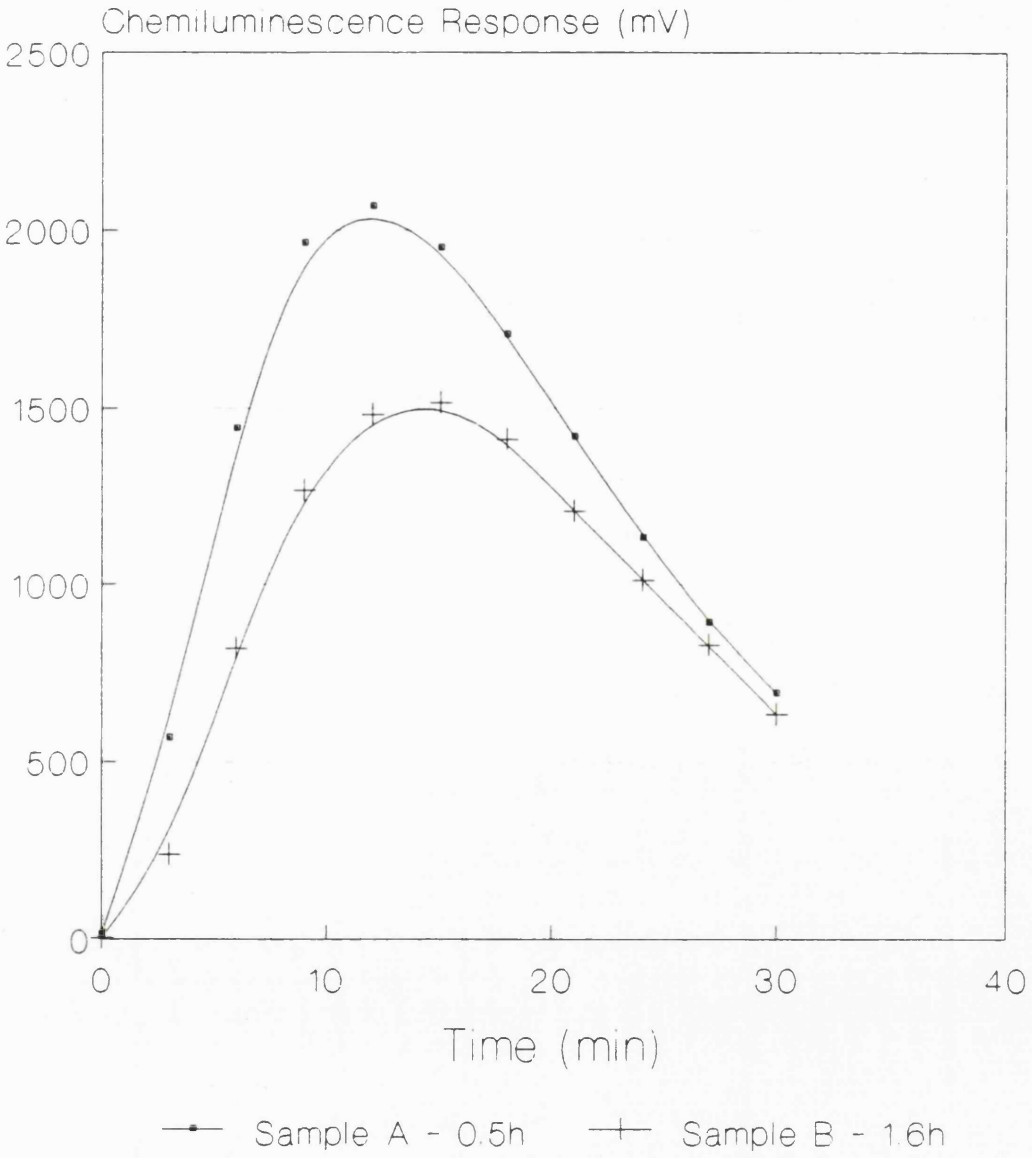


Figure 12

Patient TT
Tibial Shaft Fracture

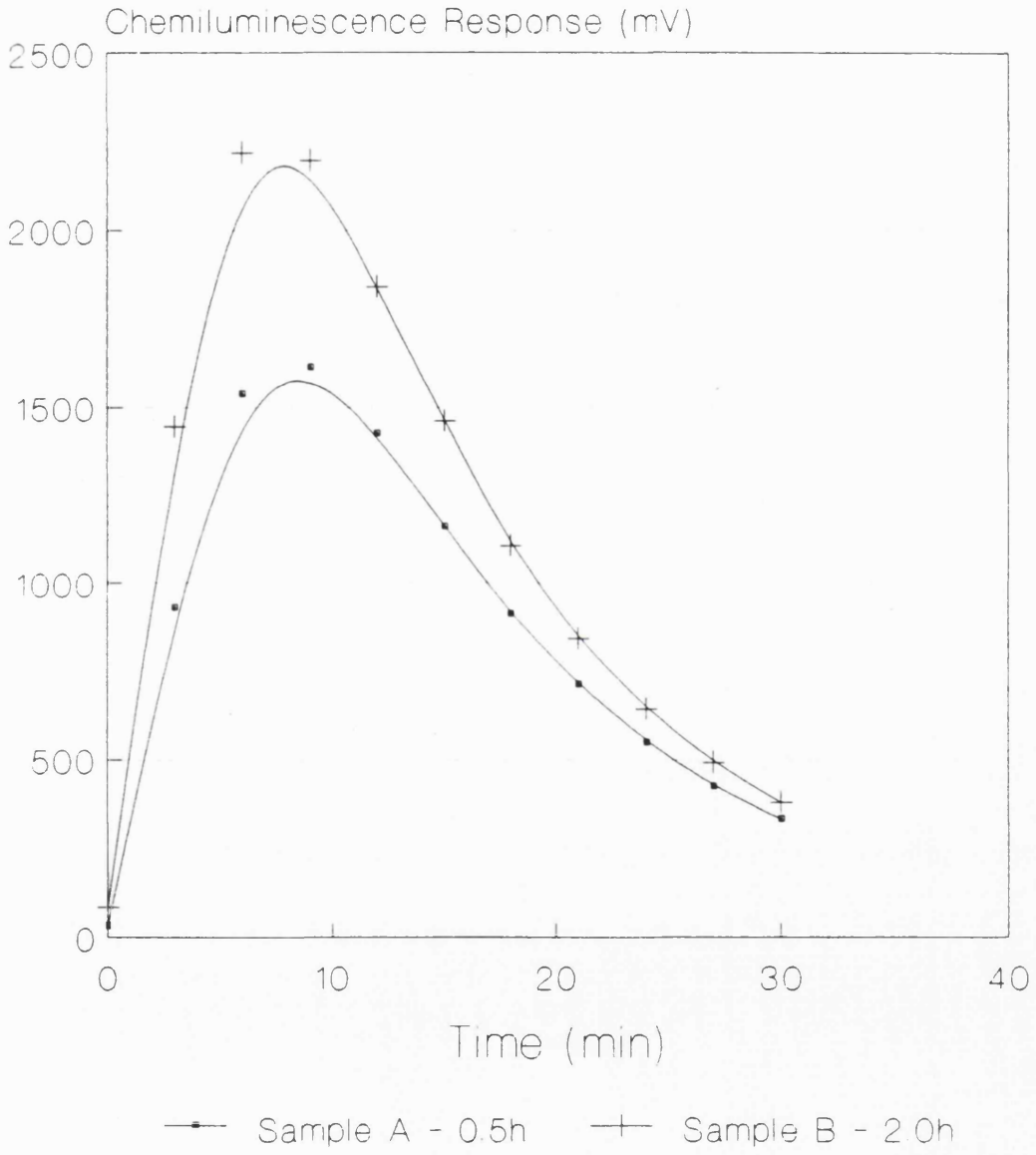


Figure 13

Patient FT
Femoral Shaft Fracture

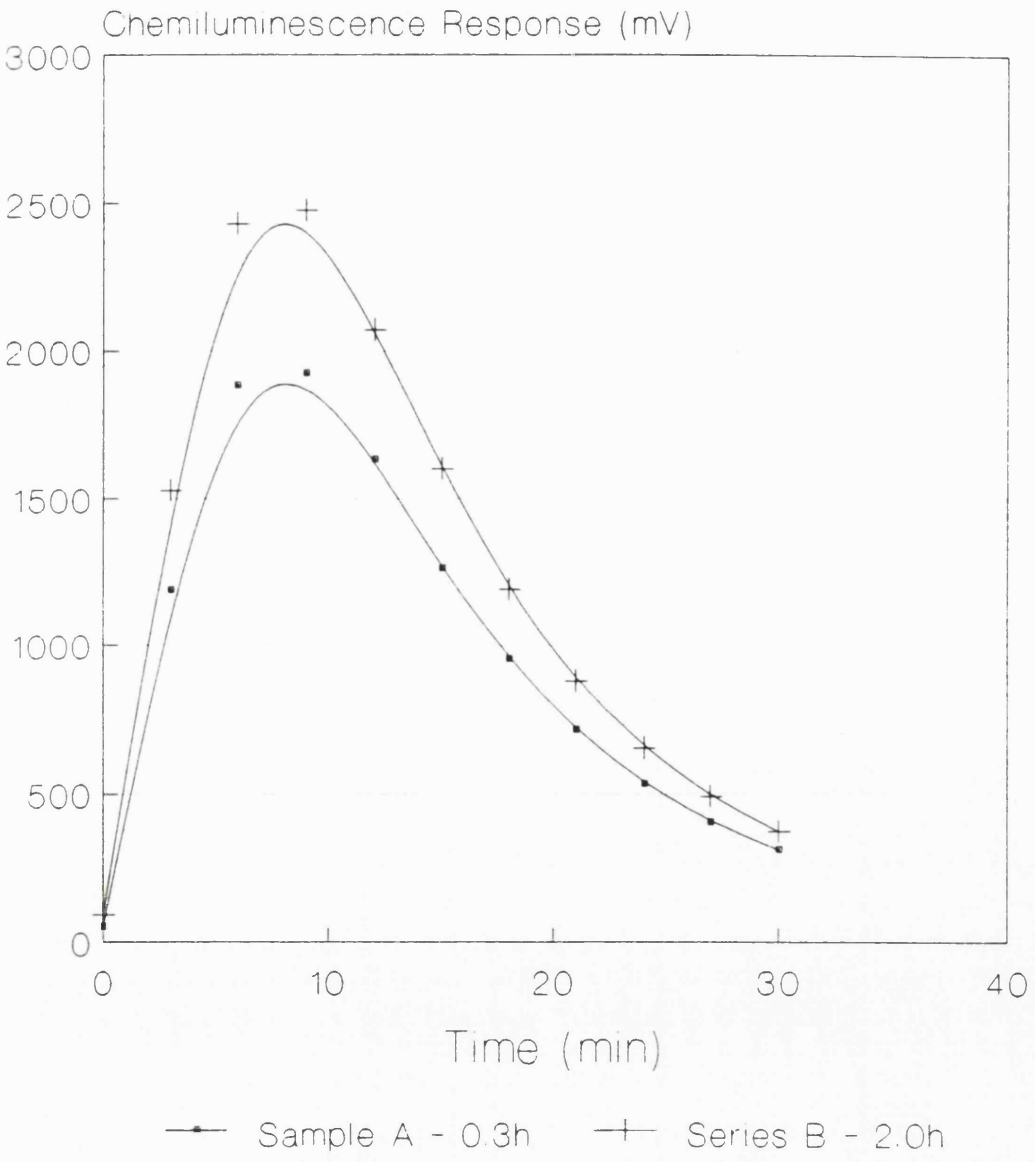


Figure 14

Patient IW
Multiple Injuries

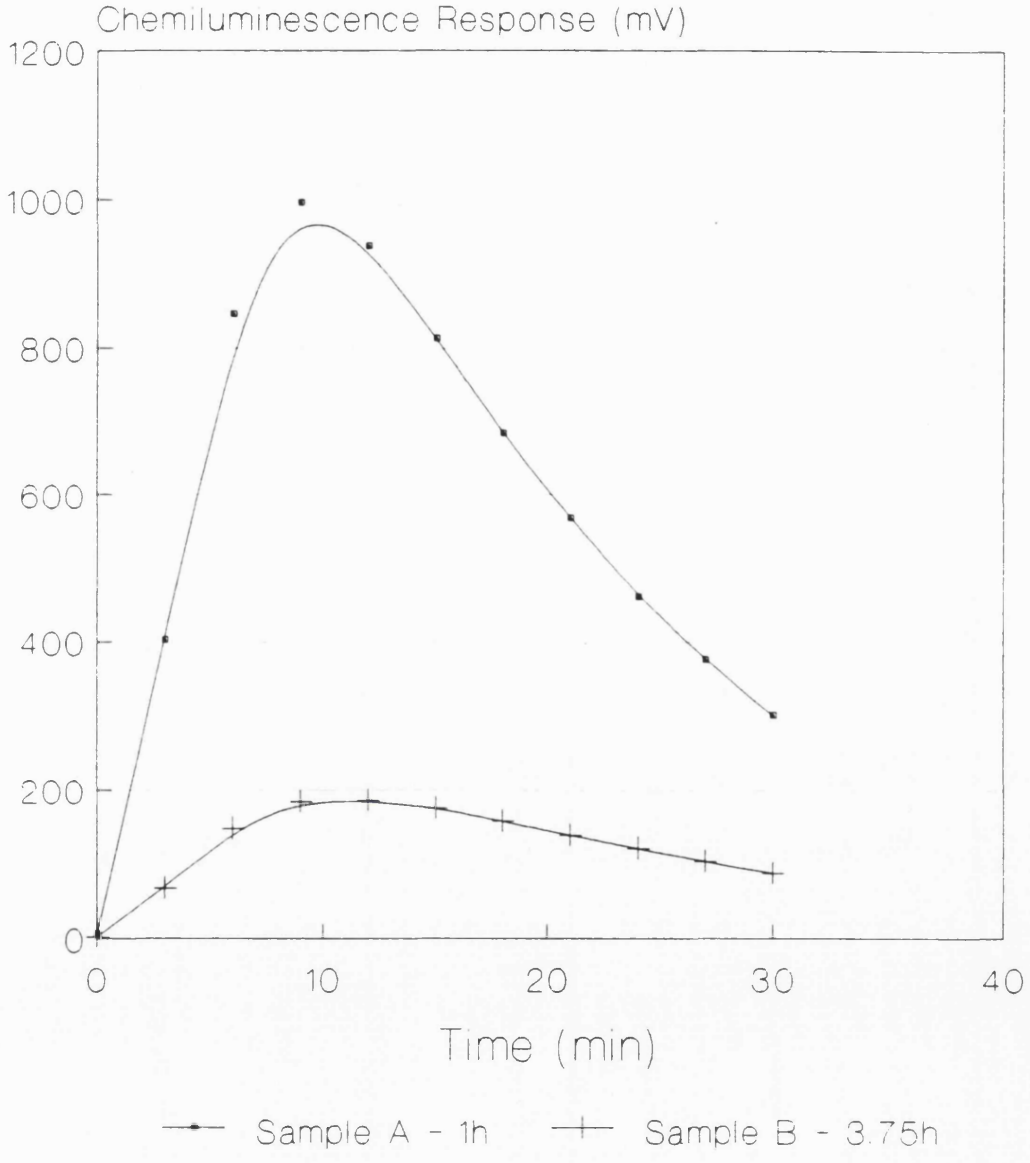
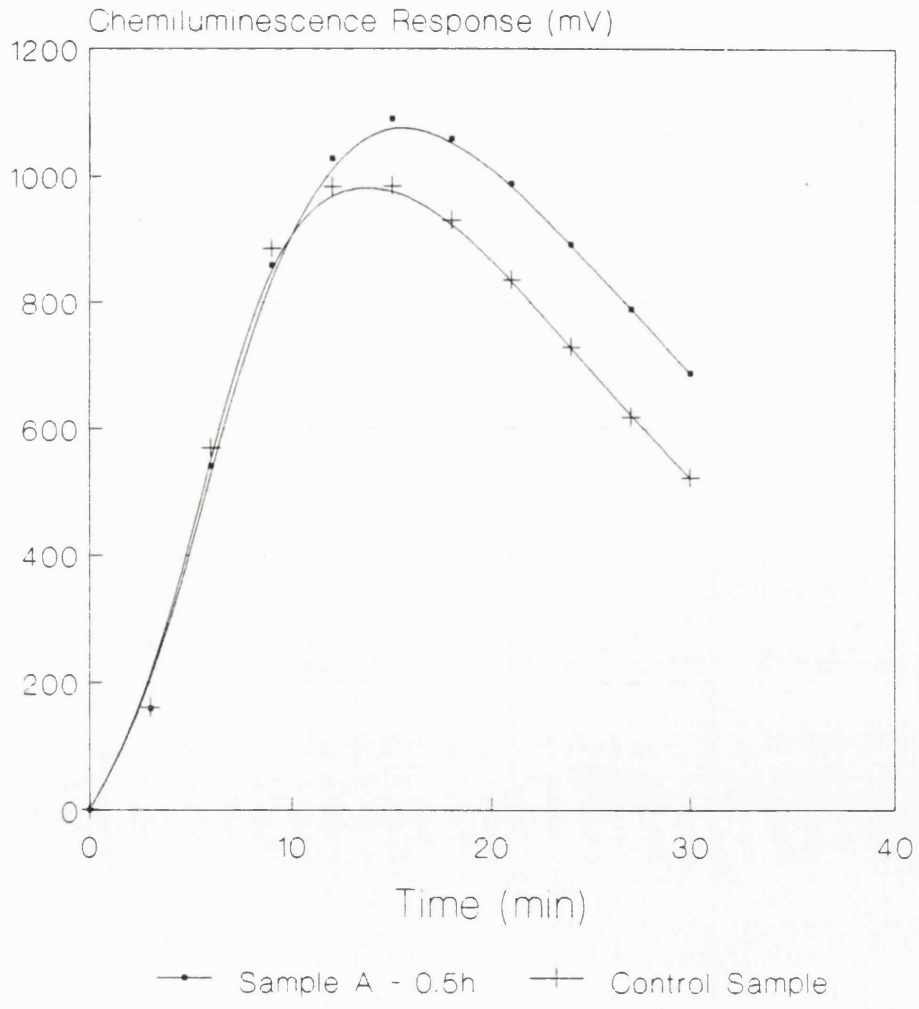


Figure 15

Patient ED
Multiple Injuries



One sample only - patient died

Figure 16

Patient CF
Multiple Injuries

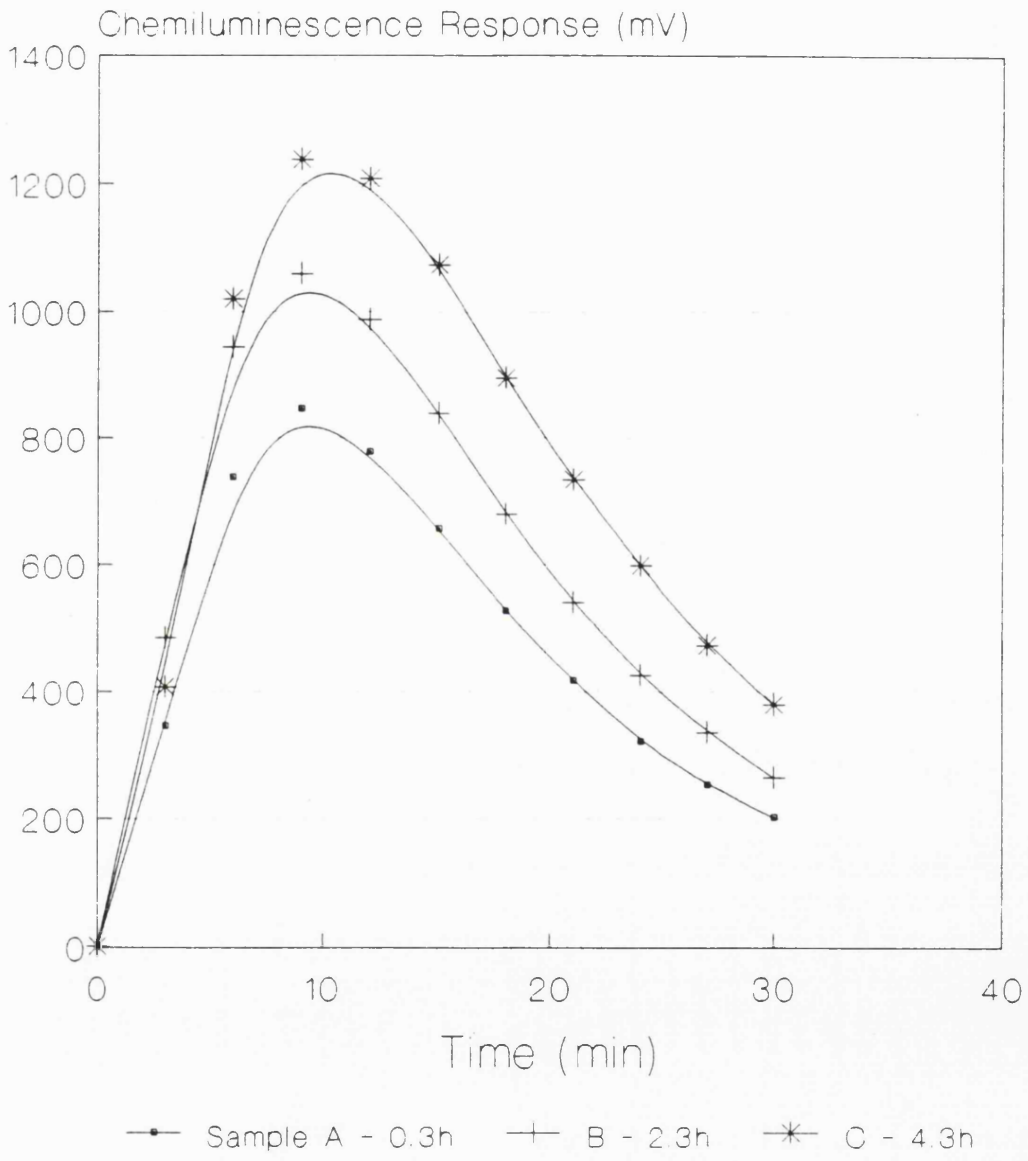
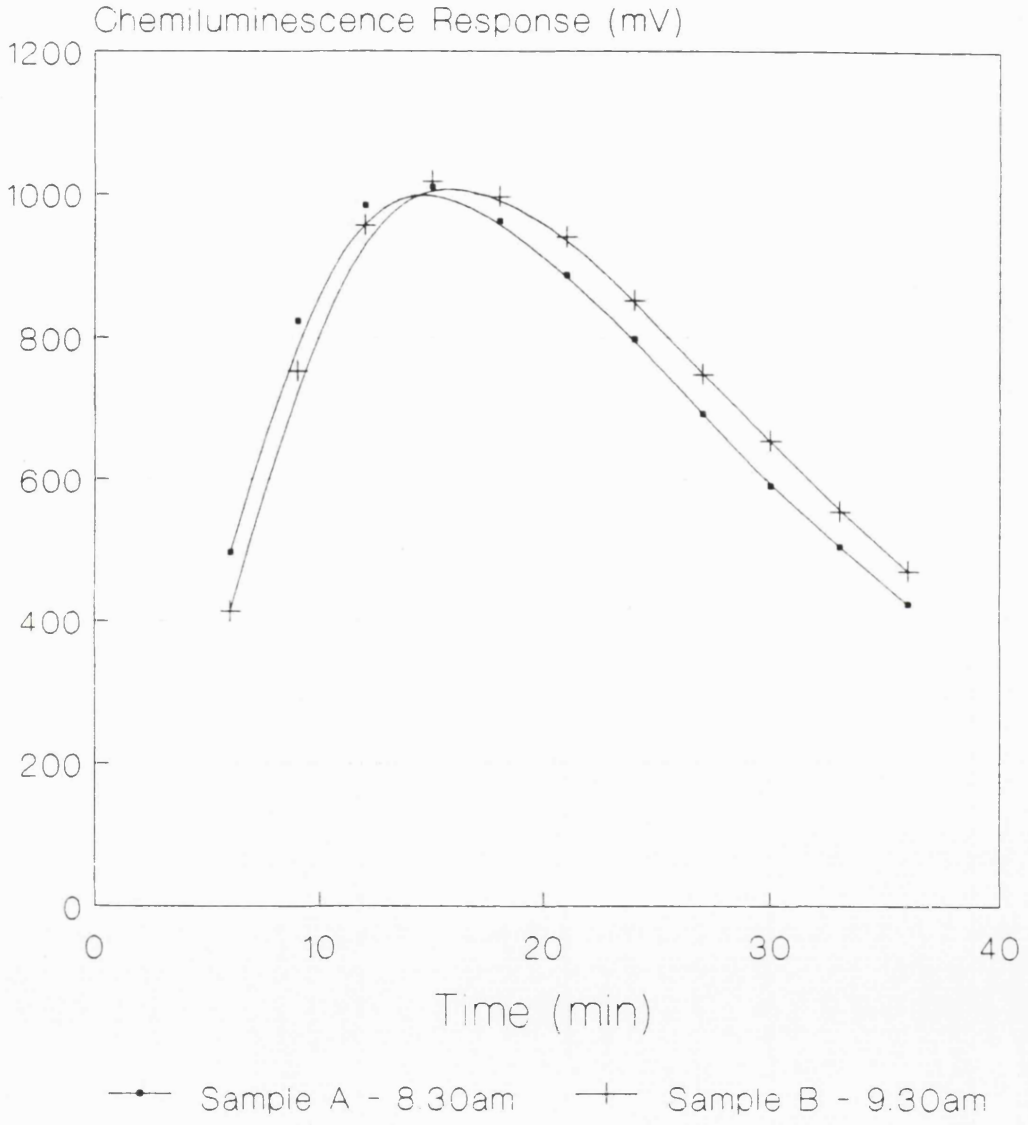


Figure 17

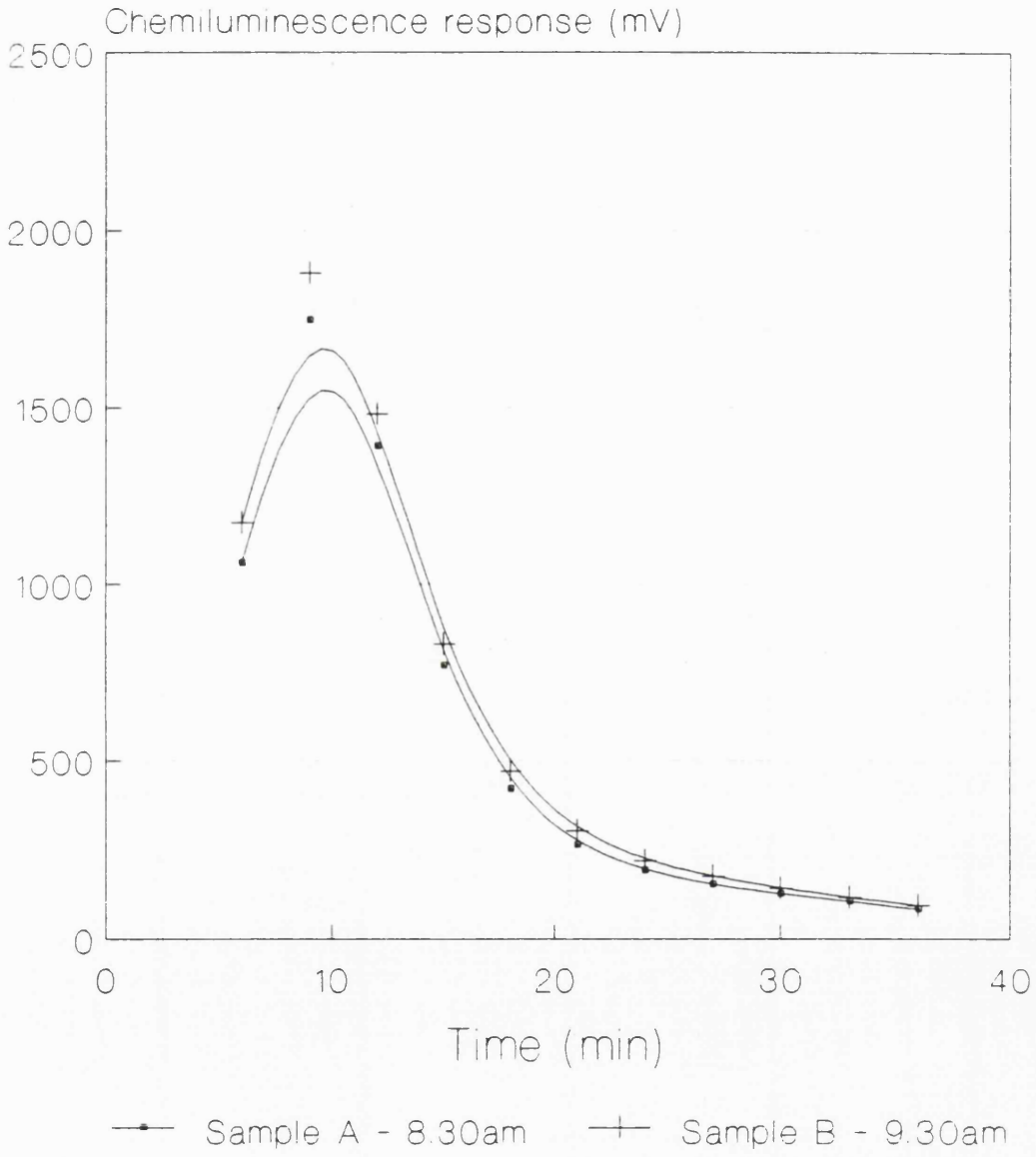
Control
Zymosan-Stimulated



Serial samples - same subject

Figure 18

Control
PMA-Stimulated



DISCUSSION

Nitroblue - Tetrazolium Reduction

This study was performed in two phases:

1) The preliminary study was of five injured patients, three of whom had sustained major trauma (ISS>16) and two of whom had sustained minor or moderate trauma (ISS 4-9). The timing of sampling in this study was opportunistic rather than controlled. Two patients with major injuries studied at 8 hours showed normal resting levels of under 10% NBT positive cells, but the stimulated phase of the test revealed sub-normal activation of neutrophils (76.2 and 50.6% respectively versus normal of 95% +). A further multiply injured patient studied at 4 hours returned normal results in both phases of the test. The final patient, an elderly lady with a minor (AIS 4) head injury, showed a slightly depressed response to the stimulated test, although this may be partly due to a delay of 12 hours in testing the blood sample. The sample was drawn into an EDTA anticoagulant tube, and EDTA is known to denature neutrophils after prolonged exposure (Dacie and Lewis 1984).

2) 15 patients were studied as soon as possible after injury. 13 patients, all studied within 6 hours, gave a greater than 90% response (6 patients greater than 95%) on the stimulated NBT test. Of those patients scoring less than 90%, one (P9) had an abnormally low yield of neutrophils and the test may not be valid for this reason. Of the remaining two, P5 returned a result of 83.9 at 1.5 hours and P12 returned a result of 55.8 at 8 hours. The great majority of patients studied at 6 hours or less returned normal findings.

Chemiluminescence Response

None of the 7 patients studied showed any evidence of neutrophil activation in the unstimulated sample, the background level of chemiluminescence continuing throughout the study. On stimulation, 4 patients showed normal responses in samples taken at less than 0.5 hours after injury, and increased responses in the second (and in one case, the third) samples taken 1.5 - 4.3 hours after injury. The results indicated an enhanced neutrophil activation and killing pathway response following injury. In a fifth patient (ED), studied once, the response at 0.5 hours was better than the control. In the sixth patient (JD), responses at 0.5 and 1.6 hours were both supra-normal, with the second sample being somewhat lower (the peak still reacting double the minimum normal response).

The remaining patient (IW), gave unusual results. A sample taken at 1 hour showed normal stimulated responses, but a second sample at 3.75 hours showed depressed activity well below the normal range. This patient was a young insulin-dependent diabetic who experienced a hypoglycaemic attack at the wheel of his van on the motorway. He veered off onto the hard shoulder and struck the rear of a stationary van. He sustained fractures of the right clavicle, bruising over the sternum and two fractures of the left lower ribs in the seatbelt distribution, and a fracture of the shaft of his right tibia. His blood sugar on admission was normal, presumably due to the hyperglycaemic effect of trauma (see Frayn 1986). He recovered from his injuries rapidly without any infective sequelae. The low response of the second (3.75 hours) sample is interesting. Sager (1990) investigated the effect of insulin on human lymphocyte B-adrenoceptor function and found an enhanced response to isoprenaline after 1 minute exposure, but a depressed response after 35 minutes exposure. Although brief exposure increased the B-adrenoceptor density, the cells after 35 minutes had a receptor density equal to control values.

It is known that diabetics have a normal beta-receptor complement on both neutrophils and lymphocytes (Maki, 1990). As in the normal subject, hormones such as adrenaline, insulin and corticosteroids can modulate the receptor activity. It is possible that patient ID had a modified response due to complex hormonal changes rather than an orthodox response to trauma.

Examining the results for both NBT reaction and chemiluminescence, it would appear that abnormalities in the neutrophil activation and killing pathways are uncommon within the first 6 hours after injury (21 normal out of 23 studied). 8 patients were studied at 8 hours or later, and 7 of their results were abnormally low (<80%).

Similarly, no evidence of inappropriate activation was seen during the first six hours. Maderazo et al (1990) found evidence of depression of neutrophil chemotactic responses "soon" after blunt trauma, but it is clear from their results that the time course was being measured in days rather than hours. Maderazo and his group detected a loss of natural antioxidants during the days following trauma and postulated that this represented additional evidence for auto-oxidation being the mechanism for neutrophil locomotory dysfunction.

The finding of normal neutrophil function early after trauma is important. Patients who develop shock following traumatic injury usually do so within the first few hours. It is known that, during shock, bacterial translocation occurs from the gut into the bloodstream in many patients. Moore et al (1992) established that 11% of trauma victims without shock and 32% with shock develop an early bacteraemia. Staphylococci were isolated in 13% of shocked patients but were not associated with clinical deterioration when found alone. However, 18% of patients with shock had enteric bacteraemias (bacteroides, clostridium and enterobacter) and all died.

The authors comment that these patients would have died of their injuries anyway, and concluded that cause and effect were difficult to unravel. In those patients who do stand a chance of survival, early aggressive therapy of shock and an intact neutrophil defence system would appear to offer the best chance of a successful outcome.

Trauma patients are also known to be at risk from candida albicans infections. Sweeney et al (1993) established that neutrophil abnormalities associated with impaired anti-candida function in injured adults could be corrected with cytokine "therapy". This therapy was investigated in vitro by the addition of Granulocyte macrophage colony stimulating factor (GM-CSF), interferon-gamma and interleukin-8 (IL-8) individually to isolates of neutrophils from trauma patients who developed evidence of susceptibility to candida invasion. 75% of these patients developed their rise in candida antigen titre within 1 week of injury. The order of effectiveness of cytokine therapy was GM-CSF > interferon-gamma > IL-8. These cytokines either individually or in combination could be exploited for administration in the early phase after injury before neutrophil abnormalities become established.

Other recent work mapping the development of neutrophil abnormalities (Rothe et al 1990, White-Owen et al 1992) has not included study of the admission blood sample or any sample taken early after admission. The latter study reported a reduced expression of CD11b and CD16 receptors on neutrophils following severe traumatic injury. However, the handling of the blood samples gives cause for concern. Neutrophils deteriorate rapidly in EDTA anticoagulant, and the White-Owen paper mentions that samples were "stored at room temperature for no more than 24 hours". The development of abnormalities detectable by flow cytometry are well known even after shorter periods than 12 hours storage under these conditions. Notwithstanding these reservations, differences were found between moderately and severely-injured patients.

The former had 68% of normal CD11b/CD 18 activity and a septic complication rate of 0.8 per patient. The severely injured group had 35% of normal activity and a complication rate of 3.3 late septic events per patient.

In view of the latter findings, a study to repeat the CD11b/CD 18assays under controlled conditions and including the very early post-trauma phase would be valuable.

STUDY SUMMARY

In this study, 21 out of 23 patients (91%) studied within 6 hours of injury had normal neutrophil microbicidal pathways. After 8 hours, only 1 of 8 patients (12.5%) showed normal findings.

The early phase after trauma would appear to be a window of opportunity for the prevention of subsequent abnormalities in the circulating neutrophil phagocytes if suitable therapies could be developed.

STUDY 4

INVESTIGATION OF THE FACTORS AFFECTING NEUTROPHIL ADHESION IN RECENTLY INJURED PATIENTS.

STUDY 4

Investigation of the factors affecting neutrophil adhesion in recently injured patients.

Introduction

A rise in the number of circulating neutrophils must involve a redistribution of neutrophils between either of the marrow or marginated pools and the circulating pool. One of the most potent factors causing such a redistribution in healthy man is an increase in the circulating adrenaline level (Samuels 1951, Athens 1961, McCarthy 1987). Although it does not directly affect neutrophils, it is possible that an increase in noradrenaline level could also displace marginated neutrophils into the circulating pool by constricting peripheral vessels.

This study was designed to measure the adhesion of neutrophils in samples taken from patients as early as possible after injury, and to relate the results to the plasma catecholamine levels.

Protocol

Blood samples were taken from 12 patients who had suffered injuries of ISS 9 or greater, and the neutrophils and plasma were separated as described in the methods section. The determinations of neutrophil adhesion were performed in four groups:

a) Patient neutrophils suspended in tissue culture medium.

b) Patient neutrophils suspended in their original plasma.

c) Normal donor neutrophils suspended in patient trauma plasma.

d) Normal donor neutrophils, pre-treated with 10^{-5} M propranolol, suspended in patient plasma ("trauma plasma").

Methods

1) Determination of Plasma Catecholamine Levels

Adrenaline and noradrenaline levels were measured with the kind assistance of Dr J M Low and Miss P Tan of the Department of Anaesthesia, Chinese University of Hong Kong.

The preparation and storage of plasma samples is detailed in Chapter 2, section 1. The method used for catecholamine estimation was by high performance liquid chromatography (HPLC) using an electrochemical column as described by Low et al (1984).

2) Neutrophil Adhesion Assay

Neutrophil adhesion was measured by the method of MacGregor (1974), as modified by Griswold (1988).

Assays were performed at room temperature using isolated neutrophils resuspended in autologous plasma. Since it was essential that the trauma plasma samples should retain their levels of adrenaline, they were only defrosted from minus 70 deg. C immediately prior to resuspension, by warming in a 37 deg. C water bath.

As recommended by MacGregor (1975), the higher wool weight of 80mg applicable for assays in which decreased adherence is expected, was used in all the determinations.

Materials

Frozen trauma plasma samples.
1ml Insulin syringes (M-S Surgical, Tokyo).
Scrubbed nylon fibre (3 denier, 3.81cm, type 200, Fenwal, Illinois).
4ml siliconised tubes (Sarstedt).
1000 microlitre pipette (Gilson pipetman).

Haemocytometer with heavy cover glass (Improved Neubauer, bright line).
Microscope (Leitz laborlux 12).
Precision chemical balance (Mettler AE 160).

Method

80 mg portions of Nylon fibre were weighed out on a balance to an accuracy of 1mg. The fibre was cut before weighing to a length of 6cm and adjustment of weight was done by removing whole fibres rather than shortening the bundle.

The weighed fibre was bent double over the end of an orange stick and pushed into the barrel of a 1ml insulin syringe until the fibre reached the luer end. With a 6cm fibre length, the fibre reached from the 0ml to the 0.5ml markings on the graduated scale, with minor adjustments using the orange stick, which was then withdrawn.

For the assay, barrels prepared as above were placed vertically inside 4ml siliconised plastic tubes held in a test-tube rack. 1ml of a suspension of neutrophils in plasma was carefully dropped onto the upper end of the fibre column using a Gilson pipette, ensuring that no suspension contacted the barrel above the column. The suspension was left to elute for 10 minutes at room temperature and the barrel was then discarded.

The original suspension and the eluted sample were both counted for neutrophil numbers using an improved Neubauer haemocytometer with heavy cover glass, the average of four large grids being taken. The percentage adhesion to the column was calculated as follows:-

$$\text{Adhesion (\%)} = 100 - \frac{[\text{Neutrophils in eluted sample}] \times 100}{[\text{Neutrophils in original sample}]}$$

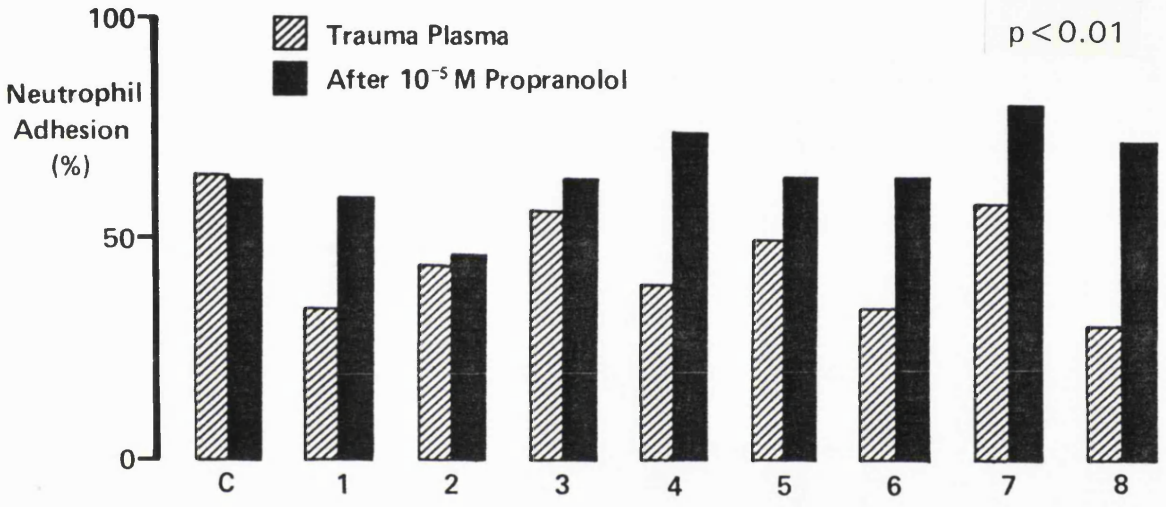
Control samples were treated in the same manner as the patient samples (i.e. the plasma was similarly separated and frozen).

Adhesion inhibition studies

In an attempt to discover whether beta-adrenergic receptor blockade would inhibit the reduction in adhesion produced by trauma plasma, identical samples of donor neutrophil suspension were prepared. This was achieved by taking 50ml of fresh donor blood and separating the neutrophils in the manner previously discussed. A stock suspension of these neutrophils in Hepes-buffered RPMI tissue culture medium was prepared (5×10^6 /ml). 1 ml aliquots of this suspension were placed into 4ml siliconised plastic tubes in duplicate ranks. Propranolol solution (MW = 259) was added to one rank of tubes to achieve a final concentration of propranolol of 10^{-5} M (after Ignarro, 1974) and the neutrophils were incubated in this medium for 15 minutes at 37°C.

The supernatants were removed from both ranks of tubes after 10 minutes centrifugation at 600 G and 1ml of trauma plasma added to each pair of tubes. One pair (one propranolol pretreated, one untreated) of tubes was used for each trauma plasma assay, and autologous control plasma was added to the control pair. Adhesion assays were performed after incubation for 10 minutes at room temperature.

Figure 19



Adhesion of donor neutrophils in trauma plasma with and without beta-blockade (one control and eight patients)

Figure 19: Adhesion of donor neutrophils in trauma plasma with and without beta-blockade (one control and eight patients).

Figure 20

Lymphocyte Counts
vs Adrenaline Levels

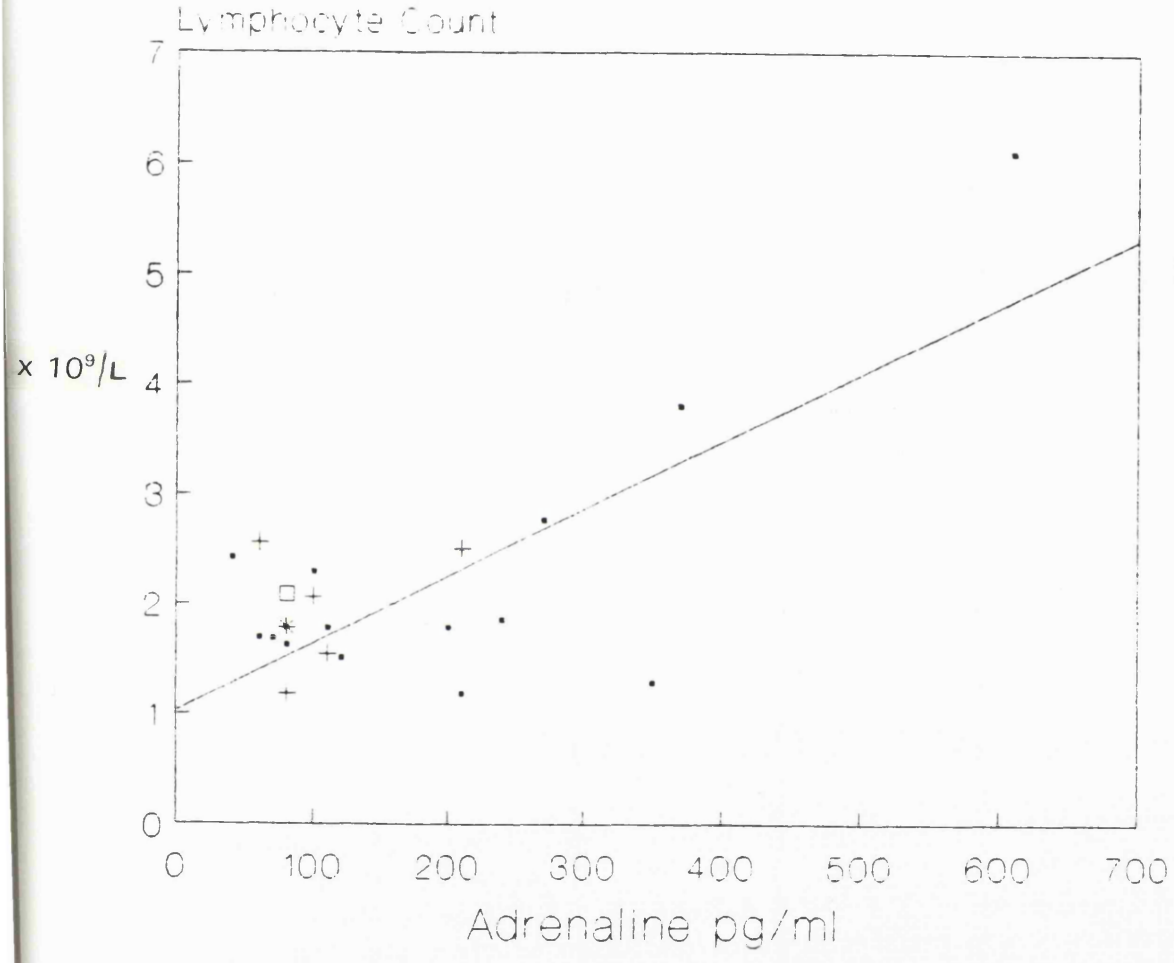
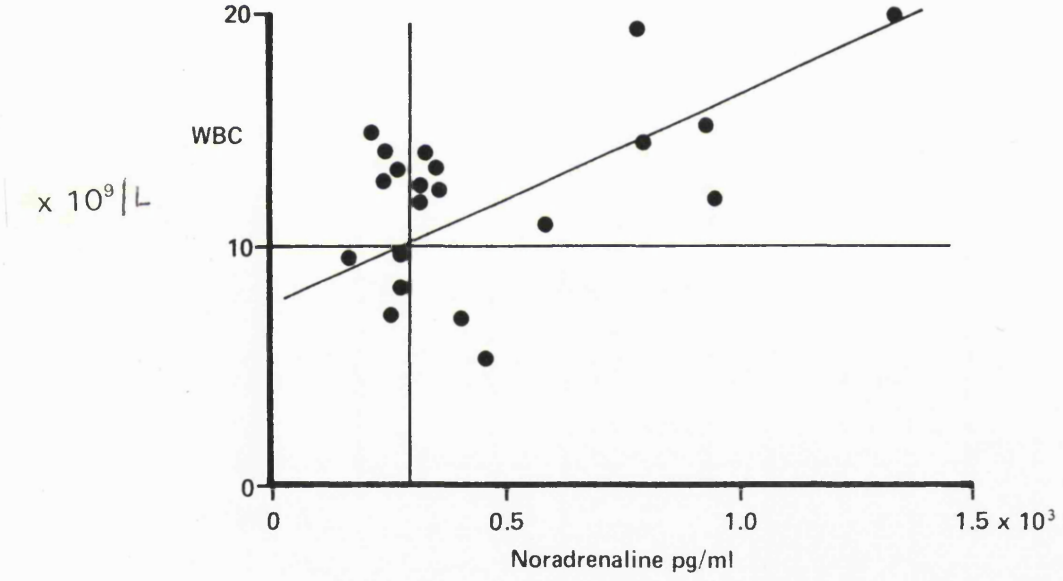


Figure 20: Relationship between lymphocyte counts and adrenaline levels in recently injured patients.

Figure 21



Relationship between leucocyte count and noradrenaline levels following trauma

Figure 21: Relationship between total leucocyte counts and noradrenaline levels in recently injured patients.

Figure 22

Neutrophil Counts
vs Noradrenaline Levels

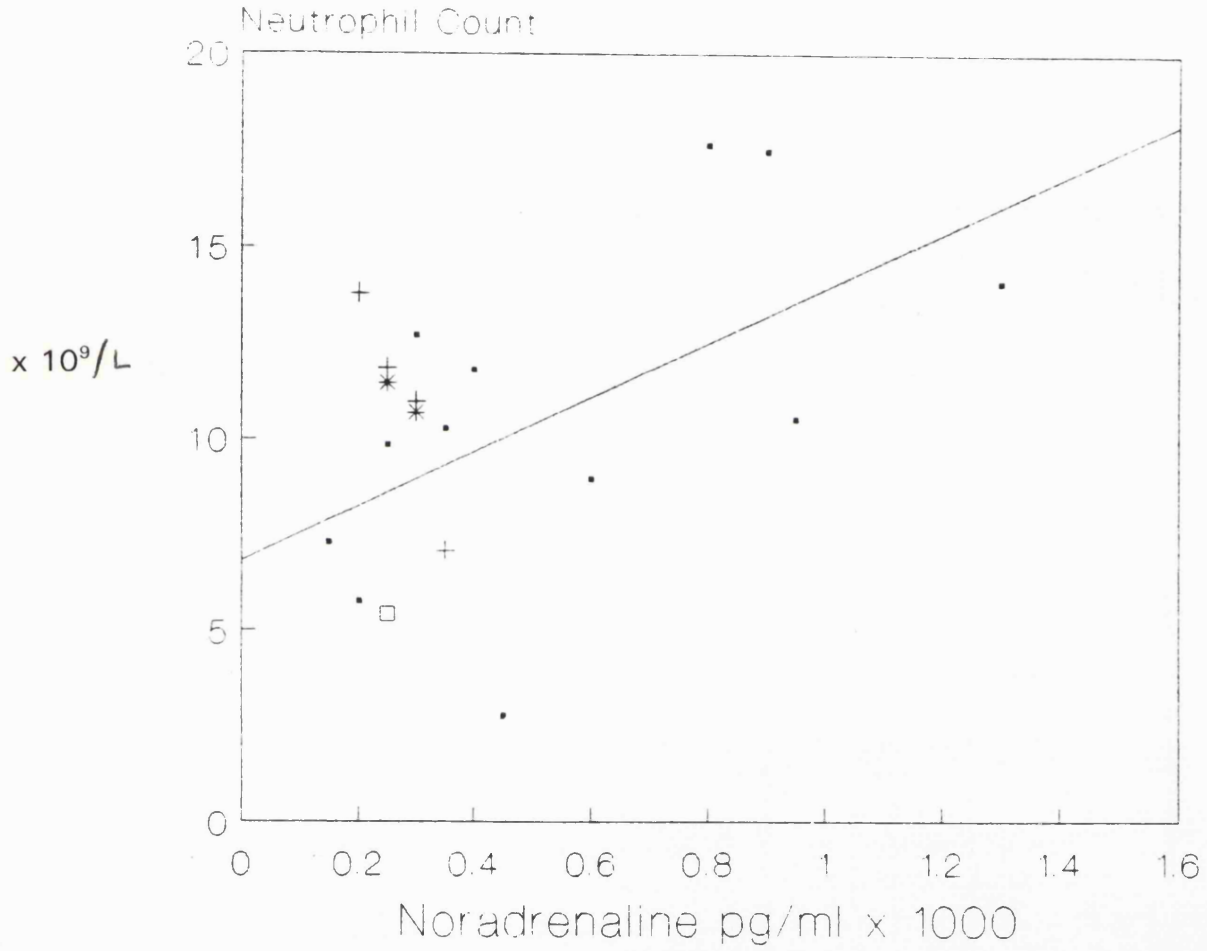


Figure 22: Relationship between neutrophil counts and noradrenaline levels in recently injured patients.

RESULTS

1. Control Studies

Duplicate estimations of neutrophil adherence to 80mg nylon fibre gave results in my hands which parallel those of the original study by MacGregor et al (1974). The limited sample size gave a tighter control range, and for the purposes of analysis, MacGregor's own range was adopted to avoid unnecessary presumption of abnormalities.

2. Studies of Patient Neutrophils in Tissue Culture Medium

Normal adhesion results were obtained for patient neutrophils suspended in RPMI 1640 tissue culture medium, confirming that adhesion properties of the cells themselves were normal. MacGregor (1974) obtained similar findings and made the assertion that plasma factors were required for any reduced adhesion.

3. Patient Neutrophils in Autologous Trauma Plasma.

Patient neutrophils showed reduced adhesion when recombined with their own plasma. Two patients (16 and 17) were studied serially and illustrated a restitution of adhesion back into the normal range at 5-6 hours following injury.

4. Adrenergic Blocking Studies

In all but one case (p12) trauma patient plasma induced a reduction in adhesion in normal donor neutrophils, although the magnitude of this reduction was less than in the previous study, recombining patients' neutrophils with their own plasma. This may have been due to the difference in the handling of plasma samples, the first study being performed with "fresh" plasma and the latter with frozen plasma.

Incubation with 10^{-5}M propranolol restored the adhesion of normal control neutrophils to the normal range, except for the one case (p12) which did not respond in the first phase of the experiment. $(p<0.01)$

DISCUSSION

This study gives evidence of factors being present in trauma plasma which induce a reduction in adhesive properties to both patient and normal donor neutrophils. The effect of this factor or combination of factors is abolished by pre-incubation with 10^{-5} propranolol, a B-adrenergic blocking agent.

All of the initial trauma plasma samples studied were subsequently analysed for catecholamine levels and found to have adrenaline level 1.5 - 10 times the upper limit of normal.

The non-responder (p12) was first sampled at 8 hours post-injury, although the adrenaline level was raised at 210 (upper limit of normal range = 60).

Comparison of leucocyte population changes with changes in catecholamine levels suggests that the magnitude of the rise is multifactorial. 2 patients with a raised total WBC count did not have a concurrently raised adrenaline level. However, one of these patients had a raised noradrenaline level and the other had a level near to the upper limit of the normal range.

Neither total WBC counts nor neutrophil counts bore a direct relationship to the actual levels of Adrenaline. The rise in WBC count appears to be of a threshold nature rather than being dose-dependent. There was a positive correlation ($r = 0.74$) between lymphocyte counts and adrenaline levels in patients sampled within 4 hours (figure 20). In this case, the correlation coefficient was 0.74 which is statistically significant at $P=0.01$.

There was a correlation between Noradrenaline levels and total WBC count, with coefficient of determination $r^2 = 0.237$. This indicated that Noradrenaline accounts for 23% of the variation in WBC counts with a probability of $P=0.05$ (figure 21). It appears that this variation involves the neutrophil fraction (figure 22).

Figures 23 and 24 illustrate possible mechanisms for an increase in the WBC count due to the effects of catecholamines.

1) Adrenaline

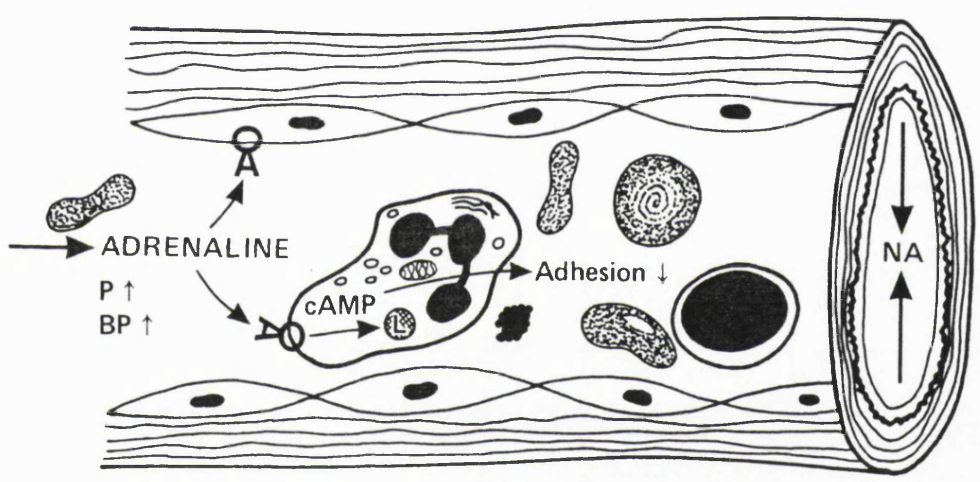
a) Mechanical Mechanisms

The physiological effect of adrenaline is to raise pulse rate, raise both systolic and diastolic blood pressure, and to increase cardiac output. These gross effects could in themselves account for an increase in the circulating granulocyte pool by "washing off" some of the marginated granulocytes from vessel walls. Violent shaking of the limbs, as in epileptic convulsions, is known to raise the WBC count, and the trauma leucocytosis could be partly due to mechanical factors. However, the effect is prolonged in trauma patients and is present in some patients after haemodynamic stabilisation. A preliminary comparison of pulse rate versus WBC count failed to show any clear correlation between the two.

b) A Reduction in Neutrophil Adhesion

A reduction in neutrophil adhesiveness in the presence of plasma factors after trauma has been illustrated in the current study. It is likely that this accounts for the largest portion of the observed increase.

Figure 23

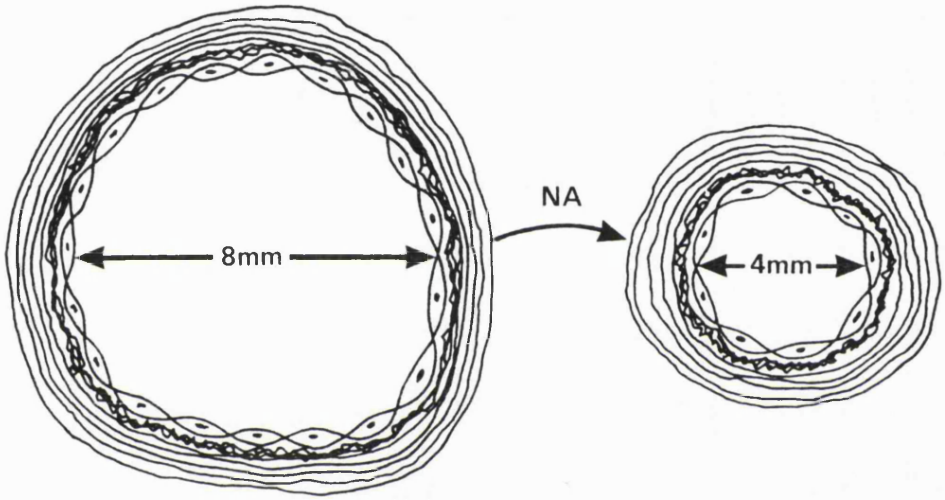


A – Adrenaline
O – Beta Adrenoceptor
L – Lysosome

Summary of the effects of catecholamines on blood vessels and neutrophils

Figure 23: Summary of the effects of catecholamines on blood vessels and neutrophils.

Figure 24



Possible effect of noradrenaline on the internal surface area of a peripheral blood vessel

Figure 24: Possible effect of noradrenaline on the internal surface area of a peripheral blood vessel.

c) A Reduction in Lymphocyte Adhesion

Lymphocytes are known to have well-developed adrenergic pathways which are modulated by various other naturally occurring hormones, e.g. steroids. It is likely that lymphocyte adhesion is influenced by plasma adrenaline levels since a good correlation was noted in this study, although no direct experimental proof has been obtained. The early rise (within 30 min) in lymphocyte numbers bears a resemblance to the pattern of the leucocytosis noted by Steel et al (1971). This will be further discussed in study 6.

d) Effects on the Endothelium

Boxer et al (1980) proposed that the leucocytosis induced by injection of adrenaline was mainly a function of reduced adhesion by endothelial cells. Although nothing in the current investigations contradicts this, the findings of a decreased neutrophil adhesion to nylon fibre (which is corrected by pre-incubation of Neutrophils with propranolol) suggests that the endothelium does not dominate the effect. Since similar pathways, modulated by cAMP, exist in both neutrophils and endothelial cells, it is likely that a two-sided reduction in adhesion occurs.

2) Noradrenaline

Although noradrenaline infusion has been shown to inhibit neutrophil chemotaxis (Deitch and McIntyre Bridges 1987), there is no evidence of a direct effect upon adhesion properties of isolated cells. However, in vivo noradrenaline does induce an increase in circulating neutrophils (Gader and Cash, 1975). IV noradrenaline has no effect on lymphocyte counts. Noradrenaline does have weak actions on beta-adrenergic receptors (Sanders and Murson, 1985).

Noradrenaline exerts profound effects upon peripheral blood vessels, acting to vasoconstrict arteries and raise blood pressure by increasing Systemic Vascular Resistance. In doing this, the internal surface area of the blood vessel is inevitably reduced (fig. 24).
It could be postulated that this reduction is internal surface area would displace some of the marginated neutrophil pool, thus raising the WBC count. The current studies suggest that this effect of noradrenaline might account for about a quarter of the variation seen in the leucocyte counts of trauma patients.

Although none of the patients in Hong Kong in Study 4 had evidence of alcohol ingestion, it has been found that ethanol consumption in the trauma patient leads to a significant accentuation of noradrenaline response (Newsome, 1988).

Whilst both adrenaline and noradrenaline levels are raised in chronic alcoholics, alcoholism leads to depressed plasma cyclic AMP levels and reduced lymphocyte cAMP production (Maki, et at 1990). Withdrawal of alcohol leads to a rapid elevation of cAMP and an increase in B-receptor density on lymphocytes, but a slower resolution of catecholamine levels.

Theoretically, an alcoholic suffering enforced abstinence due to hospital admission following traumatic injury would manifest a persistently high neutrophil count (loss of adhesion) and gain protection from ARDS due to the stabilisation of his neutrophils. Recent work by Bazzoni et al (1991) confirms the suppressant action of adrenaline and cAMP on the activation of neutrophils.

As discussed in Study 3, cytokines may modulate cAMP - mediated cellular activities. However, there is evidence that the effects of cyokines are delayed. Van Oosterhuit (1992) found no alteration in cAMP - mediated activities in peripheral blood monocytes immediatedly after incubation with interleukin 1, 2, 3, or 4, GM-CSF or interferon-gamma, but a marked reduction in activities 20 hours after incubation.

Individually, noradrenaline, adrenaline, glucagon and cortisol have been found to inhibit neutrophil chemotaxis, but acting together produce an enhancement in neutrophil metabolic activity (Deitch and McIntyre 1987). Neither of the last two research studies made comment about neutrophil and lymphocyte adhesion in relationship to these hormonal effects, but it would appear likely that some adhesion reduction should occur due to the shared mechanisms (via cyclic nucleotide pathways) of catecholamine action on these cells.

STUDY SUMMARY

In this study, the neutrophils of trauma patients showed a reduction in adhesion to nylon fibre when studied in their own plasma, but not when studied in tissue culture medium. Plasma from trauma patients also induced a reduction in adhesion in normal control neutrophils, and this effect was reversed by pre-incubation of the neutrophils with the beta-adrenoceptor blocking agent, propranolol.

The factors in trauma plasma which resulted in the reduction in adhesion are therefore likely to act upon the beta-adrenergic receptors of the neutrophil, which in turn affect adhesion by a cyclic-AMP (cAMP) pathway. Levels of adrenaline in trauma patients were found to correlate with lymphocyte counts during the first four hours after injury. Levels of noradrenaline correlated with both total WBC counts and neutrophil counts.

The mechanism of the leucocytosis of trauma is probably complex, involving physiological changes in the vascular system and also changes in the adhesion between vascular endothelium and leucocytes.

STUDY 5

STUDY OF THE ULTRASTRUCTURE AND GRANULE ENZYMES OF THE CIRCULATING NEUTROPHILS IN RECENTLY INJURED PATIENTS.

STUDY 5

Study of the ultrastructure and granule enzymes of the circulating neutrophils in recently injured patients.

Introduction

It has been suggested that a unifying cause for the development of infection, ARDS and DIC in injured patients may be found in the inappropriate activity of neutrophils (Lewis et al 1987, Efferney et al 1978). Studies of the micro-circulation in shock have established that sluggish blood-flow in capillaries, particularly in the lung, occurs and leads to the aggregation of neutrophils. Damage due to neutrophil proteolytic enzymes has been demonstrated in lung, heart and skeletal muscle following similar aggregation in patients on cardiopulmonary by-pass (Ts'ao et al 1973, Westaby 1987). Ts'ao et al detected evidence of neutrophil degranulation in some of their tissue sections under the electron microscope, but did not study the circulating neutrophils.

Maderazo et al (1986) suggested that the defects observed in neutrophil locomotion after trauma might be due to inappropriate neutrophil activation and consequent auto-oxidative damage. The locomotory defect was present in the circulating neutrophils, suggesting that evidence of degranulation and auto-oxidative damage should be detectable by electron microscopy of peripheral blood neutrophils. This type of study has never been performed in trauma patients at any stage in the time-course after injury. The purpose of study 5 was to examine the morphology and granule compliment of peripheral blood neutrophils in recently injured patients.

Protocol

Blood samples were drawn and prepared for electron microscopy on 17 injured patients presenting to the Accident and Emergency department at the Prince of Wales Hospital, Shatin, Hong Kong.

The minimum Injury Severity Score (ISS) for admission to the study was 9, with no set upper limit. All patients were medically examined to exclude pre-existing infection or other disease capable of affecting the leucocytes.

Under the electron microscope, all of the neutrophils presenting their full diameter on each section were examined for morphological abnormalities and primary granule content. Neutrophils which had been sliced other than through their full diameter were not considered (since certain parts of their structure and granule content may not have shown).

Materials and Methods

Please see Chapter 2 - General Methods and Materials - for details of the preparation of specimens for electron microscopy.

Details of the assay for myeloperoxidase (MPO) follow on the next page.

DETERMINATION OF PLASMA MYELOPEROXIDASE LEVELS

Plasma myeloperoxidase was measured according to the method of Klebanoff (1965), with minor modifications to allow adaptation to the Gilford response spectrophotometer. Plasma samples for this assay were stored at -70 deg. C., having been collected and prepared in the same way as the samples for catecholamine estimation. Samples were thawed immediately before use to avoid loss of enzyme activity.

Materials

Frozen plasma samples for assay
0.01 M Hydrogen Peroxide (BDH)
0.2 M O-Diansidine (Sigma) in Methanol
0.01 M Phosphate buffer, pH 6.0
Gilford response recording spectrophotometer

Method

0.5ml plasma samples were diluted 1:1 with phosphate buffer in a 10ml plastic tube and cooled in crushed ice. 300 microlitres of 0.01 M hydrogen peroxide were added followed by 50 microlitres of 0.2 M o-diansidine. The contents of the tube were rapidly mixed on a vortex mixer and immediately aspirated into the spectrophotometer, which was set to read absorbency at 460 nm, path length 1cm, at 25 deg. C. The enzyme kinetics software allowed a choice of measurement frequencies, but a frequency of 1 reading per second was chosen throughout, over a total time of 15 minutes. The blank contained all of the reagents except for the plasma sample, and two control specimens were assayed at the start of the run (these specimens were treated and prepared in exactly the same way as patient specimens). All assays were performed on the same day, using freshly prepared reagents. The o-diansidine was kept in the dark to avoid photochemical degradation.

Results were calculated from the initial reaction rate (the straight portion of the slope - see fig. 25). One unit of myeloperoxidase activity was taken as that producing a change in absorbency of 0.001 per minute. Since the plasma samples were diluted 1:1, the result was doubled.

RESULTS

Results of investigations are found in Appendix 5. Table II details the electron microscopic appearance of neutrophil granule content in 17 trauma patients and examples of normal and abnormal cells are shown in figures 26 and 27.

Tables 12, 13 and 14 give the results of myeloperoxidase, alkaline phosphatase and c-reactive protein levels respectively.

Electron Microscopy

No evidence was found to suggest the presence of widespread systemic degranulation or auto-oxidative damage in this study (figure 26). 1017 neutrophils in 17 trauma patients were examined and only 12 were found to have low granule counts, 5 of these being immature (band) forms. 8 of the abnormal neutrophils were found in just two patients, at 6 hours and 8 hours following injury (figure 27).

Primary Granule Contents

Measurement of plasma myeloperoxidase levels failed to show any obvious excess over normal controls in the early phase after trauma. One patient (H17) was sampled three times and a rising level was found (figure 25).

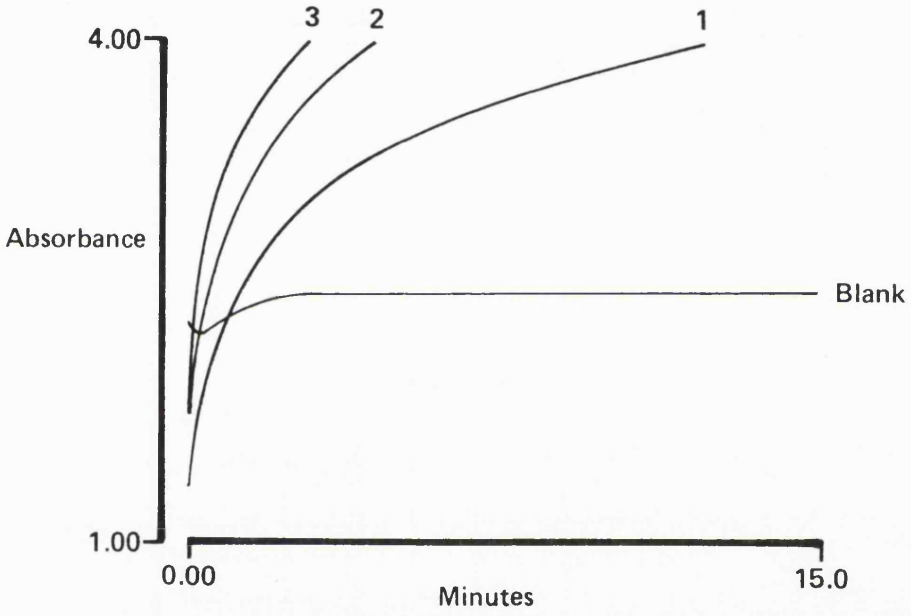
Secondary Granule Contents

No raised levels of alkaline phosphatase were detected in any of the patients studied at any stage in time following trauma.

Serum C-Reactive Protein

Serum C-reactive protein was at normal levels in all patients studied at times less than 3 hours after injury (N = 7). One patient studied at 3 - 5 hours had a mildly raised CRP level, but the greatest elevation was detected at 12 hours after injury in patient No.10. All other patients returned normal results.

Figure 25



① 1 hour
② 6 hours } After injury
③ 9 hours

Determination of myeloperoxidase levels
Serial samples in patient 17

Figure 25: Determination of myeloperoxidase levels - serial samples in a single patient (H17).

Figure 26

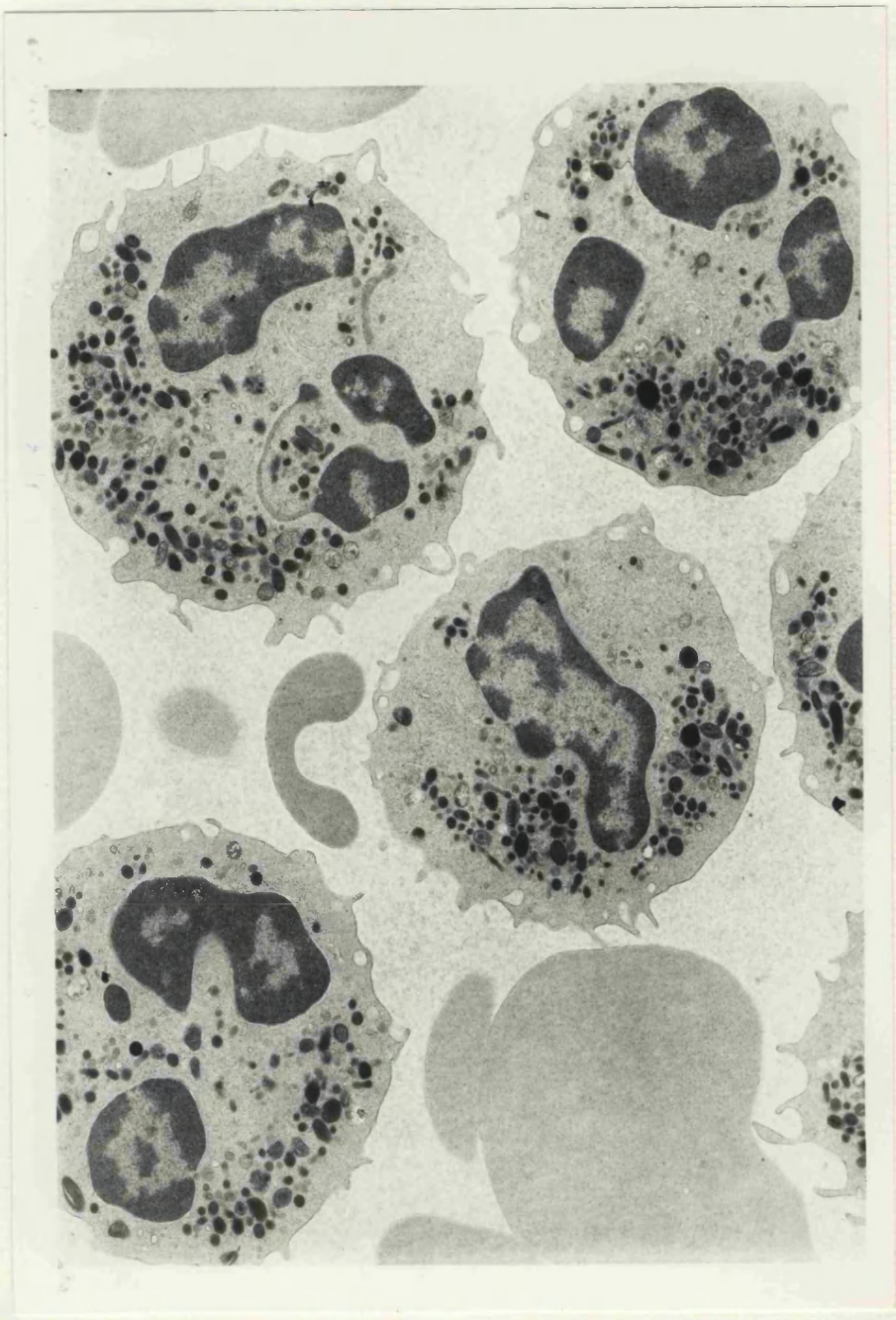


Figure 26: The leucocytosis of trauma.

Electron micrograph showing a group of neutrophils with normal granule complements.

Figure 27

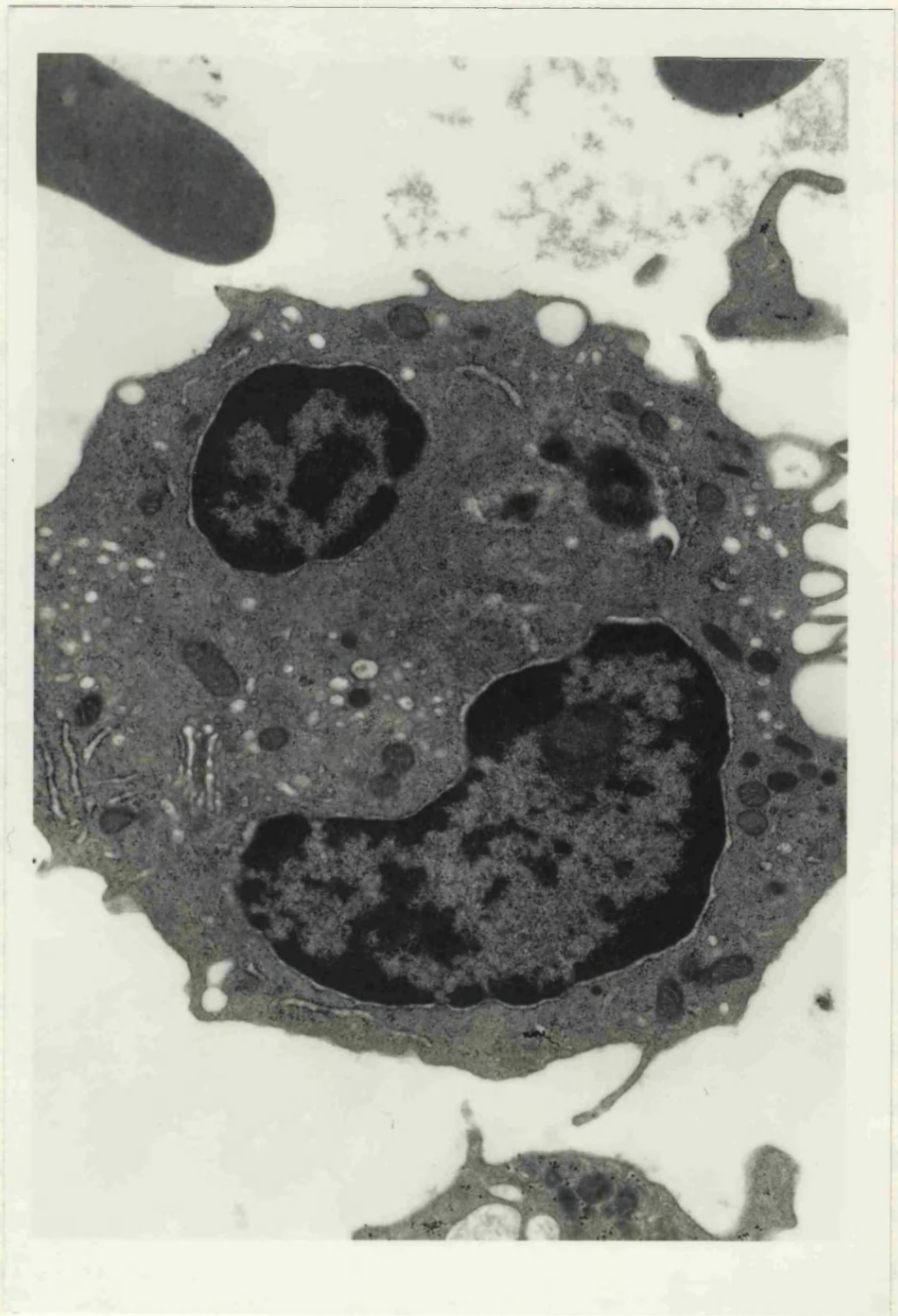


Figure 27: Electron micrograph showing a degranulated neutrophil from a patient with a sternal fracture (6 hours post-injury).

DISCUSSION

Taken together, these results do not offer any evidence of an early widespread activation of neutrophils in the circulating blood. The few abnormalities detected tended to occur 6 hours or more following injury. Normal levels of CRP in the early phase would suggest that the leucocytosis of trauma is unlikely to be due to an accelerated form of inflammation. Serum CRP normally rises in inflammatory processes after 8 hours (Fleck et al, 1985).

Donnelly et al (1992) sought evidence of increased neutrophil elastase levels in the plasma as a possible indicator of the risk of patients developing ARDS. Most patients showed such a rise within two hours of trauma, but the current study of myeloperoxidase levels in 8 patients did not show a similar increase over controls. However, there are no published reference levels for plasma myeloperoxidase and it is difficult to assess the meaning of the results in this small sample. The absence of other indicators of systemic degranulation would tend towards the conclusion that any increase in plasma levels of neutrophil granule products found early after trauma would be likely to have arisen from neutrophils bound to injury sites or sequestered within the lung or liver. At the injury site, bound neutrophils could be activated by inflammatory cascade pathways and release their enzymes. Similarly, sequestration of neutrophils in the lung and liver (Thorne et al 1989) has been illustrated, together with evidence of potential adverse effects of inappropriate activation and enzyme release (Hammerschimdt and Vercellotti 1987, Westaby 1987). Recent work by Nagata et al (1992) has shown a suppression of C5a-induced superoxide formation, inhibition of aggregation and enzyme release in the presence of cyclic AMP activators. The effects of high levels of circulating adrenaline following trauma would be to augment intracellular trauma cAMP levels in neutrophils, thereby giving protection against inappropriate activation and enzyme release.

STUDY SUMMARY

This study provides further evidence that the neutrophils in circulation in the early phase after trauma are structurally and physiologically intact. Other authors have demonstrated later instability, and research into preventative therapy during the early phase, possibly to augment cyclic AMP pathways, might offer the chance to avoid septic sequelae and multiple organ failure.

STUDY 6

CHANGES IN CIRCULATING LYMPHOCYTE POPULATIONS IN RECENTLY INJURED PATIENTS.

STUDY 6

Changes in circulating lymphocyte populations in recently injured patients.

Introduction

The significance of changes in circulating lymphocyte numbers following trauma remains unclear. Pinkerton et al (1989) claimed an adverse prognostic significance for the lymphocytosis they observed, but Conlan (1989) found a highly significant association between lymphopenia and mortality in patients with femoral neck fractures. It is likely that both lymphocytosis and lymphopenia occur in sequence after trauma (Study 2), but careful account must be taken of the sample timing. Teggatz et al (1987) found that atypical lymphocytes were common in the emergency room patients they studied, some of whom were injured. However, Thomassen et al (1986) did not find that these atypical cells were a feature of the lymphocytosis they observed in patients with major skeletal injury and stab wounds of the trunk.

This study was undertaken to clarify the features of the circulating lymphocytes in recently injured patients, using flow cytometry to follow changes in the T-cell population and electron microscopy to study morphology of the cell types.

Protocol

Flow Cytometry Study

Venous blood was collected from 31 patients, 11 with moderate injuries (ISS 9-15) and 19 with major injuries (ISS 16 or above), the first sample being taken within four hours of injury and the second 12-24 hours after injury. Total T-cells (CD3), T-helper (CD4) and T-suppressor (CD8) cells were measured in all samples using a Coulter Epics Profile II flow cytometer with specific monoclonal antibody markers for each of the phenotypes studied.

Method

100 microlitres of EDTA - anti-coagulated whole blood was incubated with 10 microlitres of fluorescent-labelled monoclonal antibody (separate tubes for CD3, CD4 and CD8) at room temperature for 15 minutes. Following incubation, specimens were lysed using an automatic Q-prep processor (Coulter Ltd, Luton UK) and left for a further 15 minutes at room temperature before being aspirated into the flow cytometer (Coulter Ltd, Luton UK). Following entry of sample haematological data (Total WBC and differential counts) automatic measurements of absolute CD3, CD4 and CD8 counts were read and calculated by the flow cytometer.

Electron Microscopic Study

TEM studies were performed on the same 17 patients who had grids prepared for the neutrophil study (Study 5). A further two patients who showed definite populations of low-density CD8 cells on flow cytometry were also examined.

RESULTS

Flow Cytometry Study

The results for 31 patients studied are given numerically in table 15 and aspects of individual population expressed graphically in figures 28 to 32. Results were incomplete in patients 3 and 16 - the former because of failure of two automated cell counts and the latter due to the death of the patient before a second sample could be obtained. Therefore, the results of 29 patients were used in statistical analysis.

Total WBC Counts

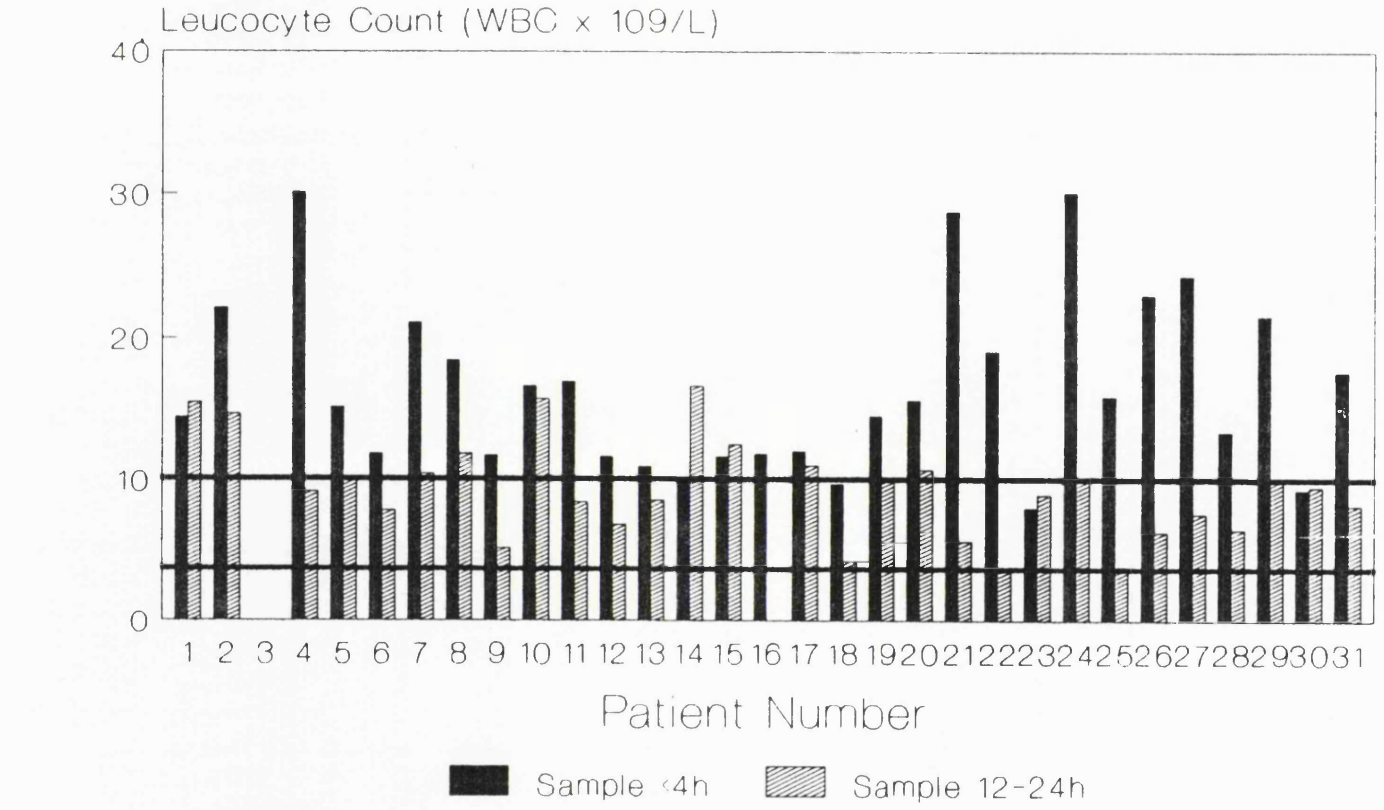
Similar rises in total leucocyte counts were seen in the early sample comparable with those in Study 2. 26 patients out of 30 had counts above the upper limit of the normal range, with a mean count of 16.7. The later series of samples showed 13 raised counts with a lower mean of 9.37.

TABLE 15 - Blood results for 31 patients - Study 6

		SAMPLE 1						SAMPLE 2					
PATIENT NO.	ISS	TIME (min)	WBC	NEUT	LYMPH	CD4	CD8	TIME (h)	WBC	NEUT	LYMPH	CD4	CD8
N1	16	45	14.5	7.8	6.10	0.95	0.78	18	15.5	12.8	2.17	0.459	0.548
N2	14	60	22.1	16.4	5.13	2.16	1.05	12	14.7	12.6	1.51	0.58	0.19
N3	17	100	-	-	-	-	-	23	-	-	2.1	0.79	0.41
N4	13	30	30.1	25.6	3.5	1.029	0.413	18	9.2	8.0	0.5	0.161	0.167
N5	9	105	15.2	-	1.9	0.75	0.49	21	10.2	8.6	1.1	0.36	0.25
N6	22	75	11.9	8.8	2.4	0.86	1.05	17	7.9	6.7	1.0	0.18	0.37
N7	14	250	21.1	18.9	1.0	0.19	0.193	23	10.5	8.1	1.6	0.61	0.51
N8	27	30	18.5	11.6	6.11	1.20	1.23	24	11.9	10.2	1.2	0.39	0.18
N9	34	100	11.8	9.3	2.3	0.63	0.76	18	5.2	4.2	0.7	0.26	0.27
N10	13	60	16.7	-	5.6	2.20	1.30	21	15.8	-	3.8	1.84	0.66
N11	22	60	17.0	12.1	3.6	1.54	0.77	19	8.5	7.3	1.0	0.416	0.188
N12	34	60	11.7	6.9	4.5	1.37	0.92	16	6.9	5.8	0.8	0.41	0.10
N13	17	60	11.0	7.7	3.6	1.15	1.14	16	8.6	6.8	1.6	0.566	0.560
N14	18	20	9.9	5.1	4.3	1.57	0.95	24	16.7	14.9	0.9	0.36	0.20
N15	21	60	11.7	7.4	3.9	1.19	0.862	15	12.6	11.0	1.0	0.374	0.159
N16	59	70	11.9	8.3	2.7	0.899	0.807	DIED	-	-	-	-	-
N17	9	75	12.1	9.8	1.7	0.377	0.605	27	11.1	9.8	0.9	0.258	0.441
N18	16	35	9.8	5.41	3.75	1.22	1.08	19	4.3	3.2	0.9	0.359	0.159
N19	27	50	14.6	13.7	0.8	0.27	0.23	24	10.0	8.5	1.0	0.24	0.22
N20	16	55	15.7	13.7	1.4	0.48	0.40	24	10.8	9.2	1.0	0.43	0.274
N21	38	20	28.8	20.4	7.7	2.18	2.89	12	5.7	4.9	0.7	0.309	0.215
N22	9	80	19.1	16.4	2.02	0.802	0.455	21	3.8	2.98	0.51	0.218	0.10
N23	10	30	8.1	4.2	3.3	1.102	0.33	20	9.0	7.6	0.9	0.362	0.05
N24	9	55	30.1	25.3	2.5	0.705	0.688	18	10.3	8.0	1.6	0.386	0.597
N25	17	75	16.0	12.1	3.5	1.12	1.21	18	3.9	3.2	0.5	0.189	0.139
N26	10	100	23.0	19.4	2.83	0.928	0.843	21	6.4	5.0	0.87	0.316	0.207
N27	19	115	24.3	20.0	3.52	0.74	0.78	24	7.7	6.51	0.80	0.40	0.19
N28	14	60	13.5	9.5	2.8	0.88	0.52	19	6.6	5.2	1.0	0.35	0.26
N29	17	75	21.6	19.0	1.9	0.304	0.306	15	10.1	8.0	1.3	0.406	0.25
N30	25	210	9.4	7.4	1.3	0.339	0.316	22	9.6	8.8	0.6	0.215	0.095
N31	20	150	17.7	14.9	2.2	1.016	0.543	21	8.3	7.0	1.0	0.538	0.249
MEAN	20	76	16.6	12.7	3.26	1.00	0.80	19.6	9.37	7.67	1.15	0.42	0.27
SD	10	50	6.1	6.0	1.64	0.54	0.51	3.7	3.45	2.93	0.65	0.30	0.16

Figure 28

Total Leucocyte Counts
Moderate/Major Trauma



Horizontal bars = Normal range

Figure 28: Total leucocyte counts - Study 6.

Figure 29

Neutrophil Populations
Moderate/Major Trauma

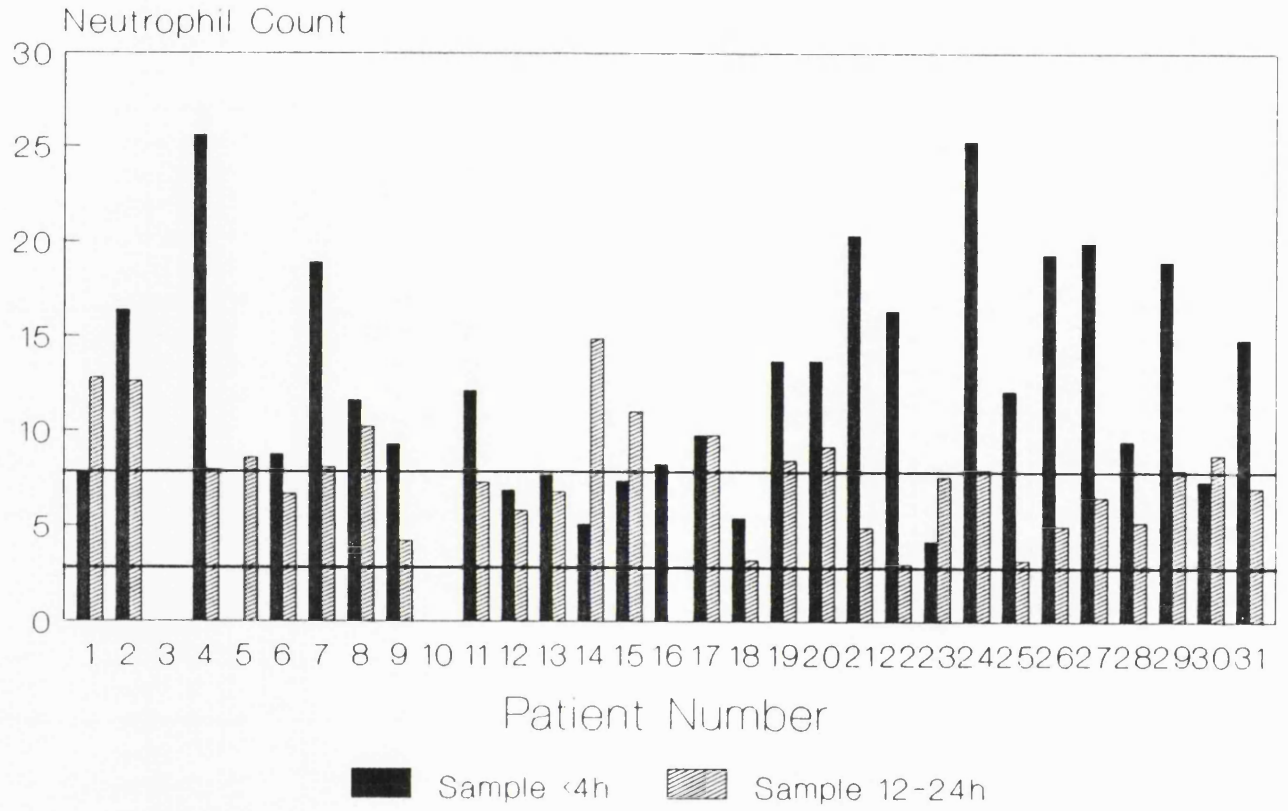


Figure 29: Neutrophil counts - Study 6.

Lymphocyte Populations
Moderate/Major Trauma

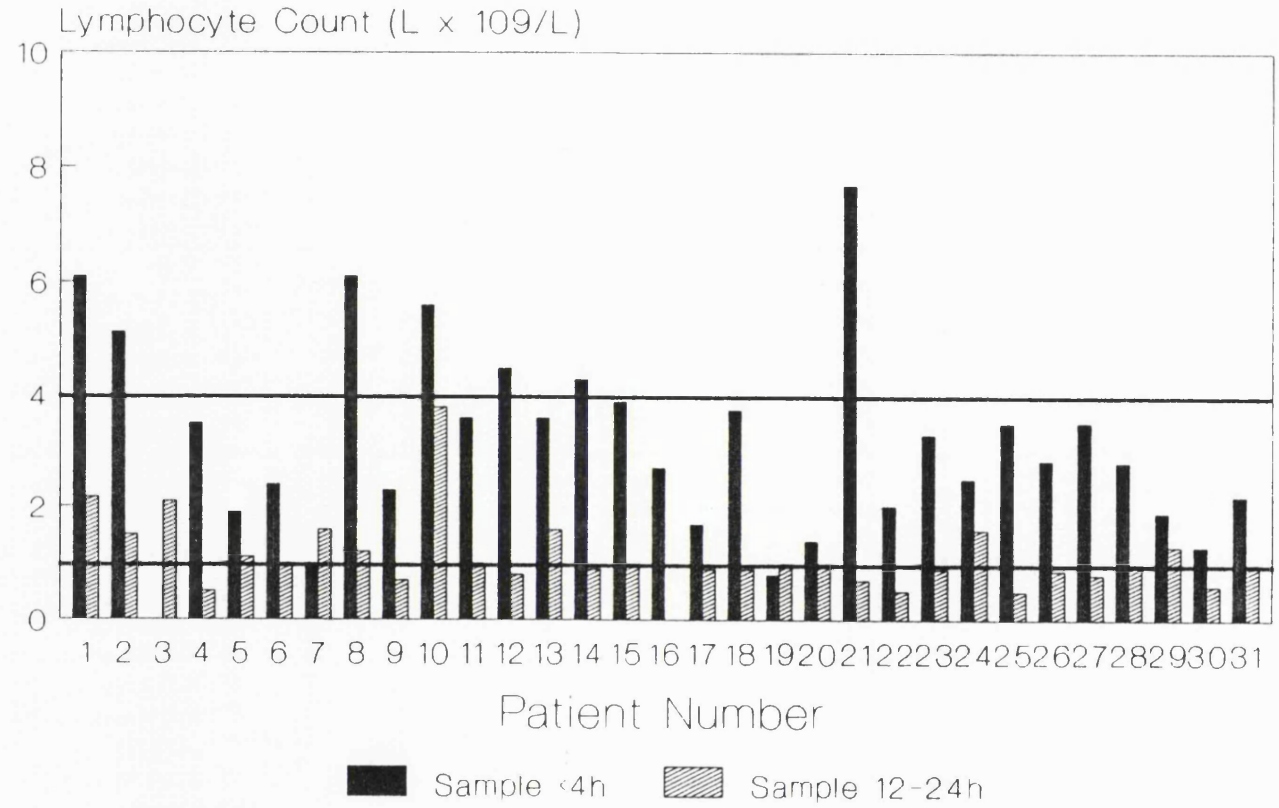
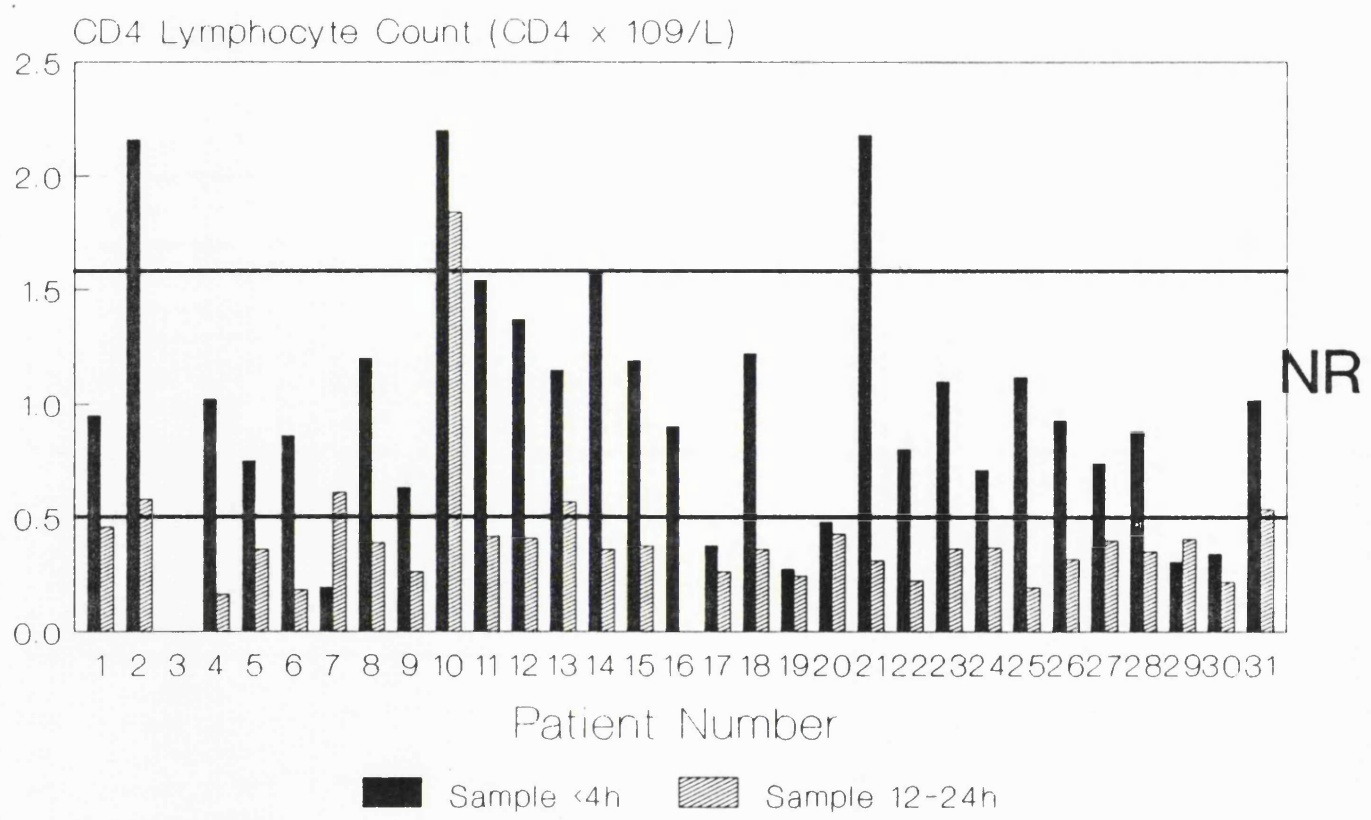


Figure 30: Total lymphocyte counts - Study 6.

Figure 31

CD4 Lymphocyte Populations
Moderate/Major Trauma

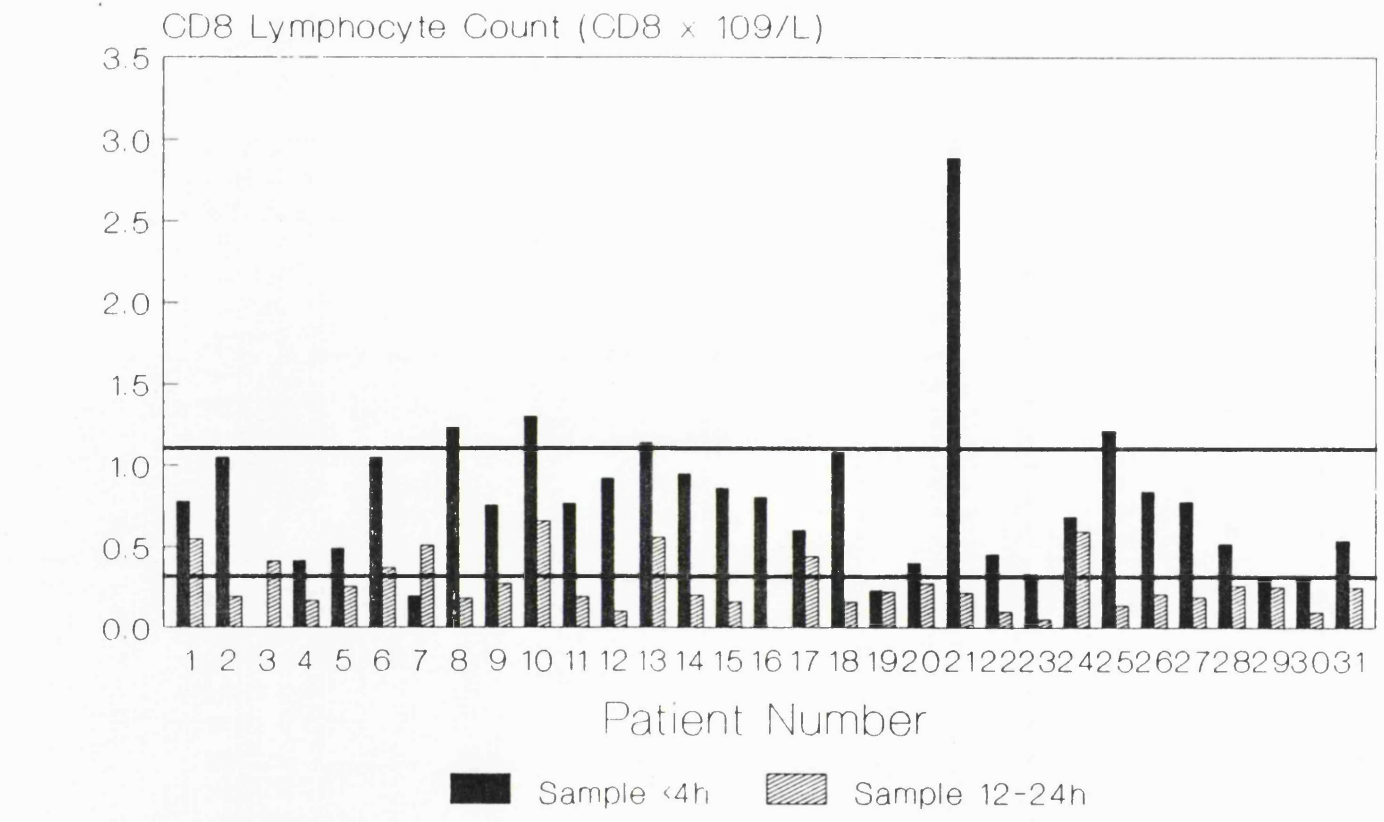


Horizontal bars = Normal range

Figure 31: CD4 lymphocyte counts - Study 6.

Figure 32

CD8 Lymphocyte Populations
Moderate/Major Trauma



Horizontal bars = Normal range

Figure 32: CD8 lymphocyte counts - Study 6.

Neutrophil Counts

The leucocytosis observed in most of the patients was predominantly due to an early neutrophilia. 25 of the 28 patients on whom precise neutrophil counts were available had a count above 6.5 (mean 12.8). Raised levels of neutrophils also tended to persist until the later samples, 19 patients still having a neutrophilia (mean 7.7). Neutropenia was not found in any of the samples studied.

Lymphocyte Counts

1) Early Samples <4h: 7 patients showed an early lymphocytosis (L/count > 4) and one showed a marginal lymphopenia with a lymphocyte count of 0.8 (N=>1). 21 patients had lymphocyte counts above the control mean (2.2).

   Examination of the CD4 counts revealed 6 cases (20%) with a CD4 count below the 5th percentile (0.52). Four of these also had CD8 counts below the 5th percentile (0.37).

   One patient (No. 23) had a low CD8 count in the presence of a normal CD4 count. All other patients had normal or raised CD4 counts, 6 also having raised CD8 counts. 3 patients had a reversed CD4:CD8 ratio (N=>1 0.82, 0.62, 0.75).

2) Follow-up samples 12-24h

   No patient showed a lymphocytosis on the follow up sample. In contrast, 13 had lymphopenia (43%) and all but one patient had lymphocyte counts below the normal mean (2.2).

   Examination of flow cytometry results revealed 24 cases (80%) with low CD4 counts (<0.52) and 22 cases (73%) with low CD8 counts (<0.37). 3 patients had a reversed CD4:CD8 ratio, 2 of these being the same patients as those found to have a reversed ratio in the early samples.

Applying the Wilcoxon signed rank test to each of the lymphocyte subsets and total lymphocyte counts, a significant fall ($p<0.01$) occurred in all parameters between the early and follow-up samples.

Four patients developed infections. All of these patients had sustained major trauma:

1. Patient 9 - Major chest injuries, mesenteric tear, perinephric haematoma. Fractures of right femur and tibia, left upper femur. Developed E coli urinary tract infection. Did not receive antibiotics in A&E at initial presentation.

   CD4 level on second sample (18 hrs) was low at 0.26×10^9. CD8 count also low at 0.27×10^9.

2. Patient 12 - Compound comminuted fracture of right femur. Sternal fracture. Left pulmonary contusion underlying flail segment (left 2nd-5th ribs). Facial lacerations. Developed E coli chest infection - prolonged. Had received prophylactic antibiotics (Co-Amoxiclav) in A&E at presentation.

   CD4 level on second sample (16hrs) was low at 0.4×10^9. CD8 count also low at 0.1×10^9.

3. Patient 18 - Compound fractures of left femur, right tibia. Crush injury to left femoral artery (occluded). Developed pseudomonas wound infection. No record of prophylactic antibiotics given at presentation in A&E..

   CD4 level on second sample (19hrs) was low at 0.359×10^9. CD8 count also low at 0.159×10^9.

4. Patient 20 - Dislocation right shoulder, fracture of cricoid cartilage with surgical emphysema of neck and chest tissues. Pulmonary contusions. Developed chest infection. Did not receive prophylactic antibiotics in A&E.

   CD4 level on second sample (24hrs) was low at 0.43×10^9. CD8 count also low at 0.274.

Six patients had critically low CD4 lymphocyte counts on the second (follow-up) samples (patients 4, 6, 19, 22, 25 and 30). All except for patient 6 also had low CD8 counts. This patient had a reversed CD4:CD8 ratio of 0.48. All of these patients had received prophylactic antibiotics in the resuscitation phase of treatment in A&E, mostly for the indication of a compound fracture. Two had moderate and four major trauma. All of these patients developed pyrexia within 24 hours of injury. None developed infections and all survived.

Four patients (7, 10, 15 and 23) were notable in that they showed a high proportion (20-30%) of low density CD8 cells in the first sample which had markedly dropped by the follow-up sample.

DISCUSSION

Total Lymphocyte Counts

All patients except one had adequate total lymphocyte counts on admission, seven showing a lymphocytosis. However, a significant fall in lymphocyte counts occurred by the time the second, follow-up sample was taken 12-24 hours later. At that time, 13 patients manifested lymphopenia (43%) and all but one had lymphocyte counts below the mean. These results clearly demonstrate the need for precision in timing of blood samples when interpreting lymphocyte counts following injury. Previous studies looking at total lymphocyte counts (Conlan 1989, Thomassen et al 1986, Teggatz et al 1987, Pinkerton et al 1989) drew conclusions from untimed samples, leading to a confusion concerning the adverse prognostic significance of low and high lymphocyte counts. The present results show that both lymphocytosis and lymphopenia may occur in the same patient within 12 hours. Patient 21 returned an initial lymphocyte count 20 minutes after injury of 7.7×10^9/L, the highest in the study, but within 12 hours this count had fallen to 0.7×10^9/L. Only small amounts of intravenous fluid were given during the intervening period, the major injuries being to the head and face.

CD4 (T-helper Lymphocyte) Counts

Whilst most patients had adequate levels of CD4 lymphocytes on admission, 20% already had CD4 counts below the lower end of the normal range, in one case (p19) within 50 minutes of injury. This was the only patient who showed a lymphopenia (0.8) on the initial sample and the only one whose lymphocyte count rose (1.0) by the follow-up sample. However, the CD4 count fell to a critically low level (0.24) during this time. CD4 lymphocytes coordinate the activities of many other cell lines involved in mounting an immune response. It is clear from these results that 84% of patients with major injuries (ISS>16) developed low CD4 counts within 12-24 hours of injury, including 21% whose CD4 counts become critically low.

These patients, with CD4 counts of less than 0.25 x 10^9/L, have T-helper cell levels similar to those of patients in the critical phase of Acquired Immune Deficiency Syndrome (AIDS). However, all of the patients in the study with such critically low levels, despite developing pyrexia, survived without evidence of overwhelming sepsis. Each patient had received antibiotic prophylaxis immediately upon reception into the Accident and Emergency department. In contrast, four patients with low (0.26-0.52) CD4 counts developed infections. One of these had been given antibiotic prophylaxis. Although these patient numbers are very small the role of intravenous prophylactic antibiotics in mitigating the effects and risks of immunosuppression after trauma would be worthy of further investigation.

There appears to be considerable biological variation amongst individual patients who have sustained trauma with respect to their risk of developing sepsis post-injury (Guillou, 1993). Even when variables such as adequacy of resuscitation, antibiotic prophylaxis and degree of surgical skill of the attending staff are taken into account in a group of similar patients, it is difficult to predict which patients will develop sepsis. Acute measurement of CD4 lymphocyte levels might offer some indication of which patients are at special risk, even if a reliable means of prevention is not yet available.

CD8 (T-suppressor Lymphocyte) Counts

The CD8 phenotype contains not only cells with T-suppressor activity but also lymphocytes with natural killer (NK) activity. The latter group are characterised by low density on flow cytometry. Four patients showed high levels of low density CD8 cells and these were likely to be of the NK variety.

Trauma did not only affect the CD4 population. 5 patients had a low CD8 count ($<0.37 \times 10^9$) on the initial sample, the earliest within 30 minutes of moderate injury (patient 23). In this patient, CD8 activity fell to 0.05 $\times 10^9$/L at 20 hours, the lowest figure recorded in the study. This patient had sustained head injuries and did not receive significant quantities of IV fluids, so the effect on cell counts could not have been due to dilution.

By 12-24 hours, 79% of patients with major injuries had developed low CD8 counts, including 42% with critically low ($<0.19 \times 10^9$/L) results. Of patients with moderate (ISS 9-15) injuries, 63% developed low counts, including 27% critically low.

CD4:CD8 Ratio

Although significance has been attached to the T-helper:T-suppressor ratio (Fosse et al 1987, Groom et al 1990), the results in this study indicate that CD8 populations are at least as depressed by traumatic injury as CD4 levels. Under these circumstances, the ratio becomes less significant. Phenotypes measured by flow cytometry merely indicate CD4 and CD8 activity, not pure cell lines. It is thought that some lymphocytes may exhibit several phenotypes and the terms "helper" and "suppressor" are less applicable as more is known about lymphocyte populations. Gough et al (1992) recently advised against the use of helper:suppressor ratios in measuring immunosuppression after thermal injury.

Low Density CD8 Populations

Electron microscopy was performed on buffy coat samples from patient 10 and 15, and large numbers of lymphoctyes showing abundant cytoplasm and large mitochondria were seen like those in samples from the index patient (patient 17 in the Hong Kong study). Figure 33 shows one of the cells from this patient with typical features also seen in patients 10 and 15.

Teggatz et al (1987) saw similar lymphocytes in one of their patients with an acute medical condition. The exact details of this patient are not given, but over 85% had been given intravenous adrenaline for cardiac arrest (due to cardiac conditions or traumatic injury) and the remainder had suffered epileptic convulsions.

Returning to study 2, table 5, it can be seen that large numbers of large granular lymphocytes were also seen in some of the trauma patients studied earlier. In these patients, the highest levels were detected in the very early samples. Examining the catecholamine results again, it can be seen that the highest levels of adrenaline occur immediately after trauma. The relationship between adrenaline levels and lymphocyte counts has been established in study 4.

The large granular lymphocytes seen during the very early phase following injury may be released by the high levels of adrenaline present, and may manifest CD8 and natural killer activity. Figure 34 shows a further large lymphocyte with azurophil granules in patient 14.

Figure 33

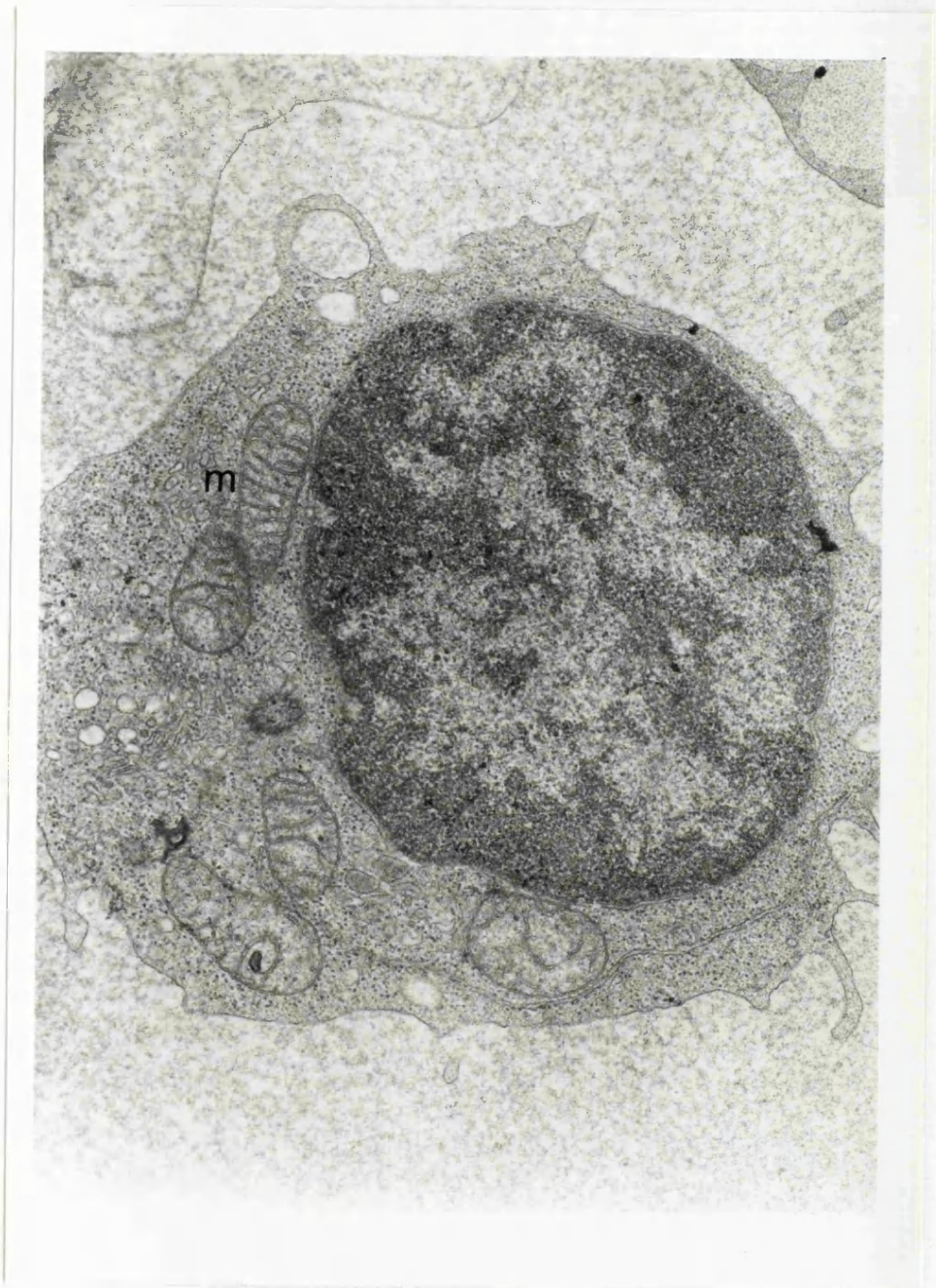


Figure 33: Large lymphocyte showing abundant cytoplasm and large mitochondria (m).

Figure 34

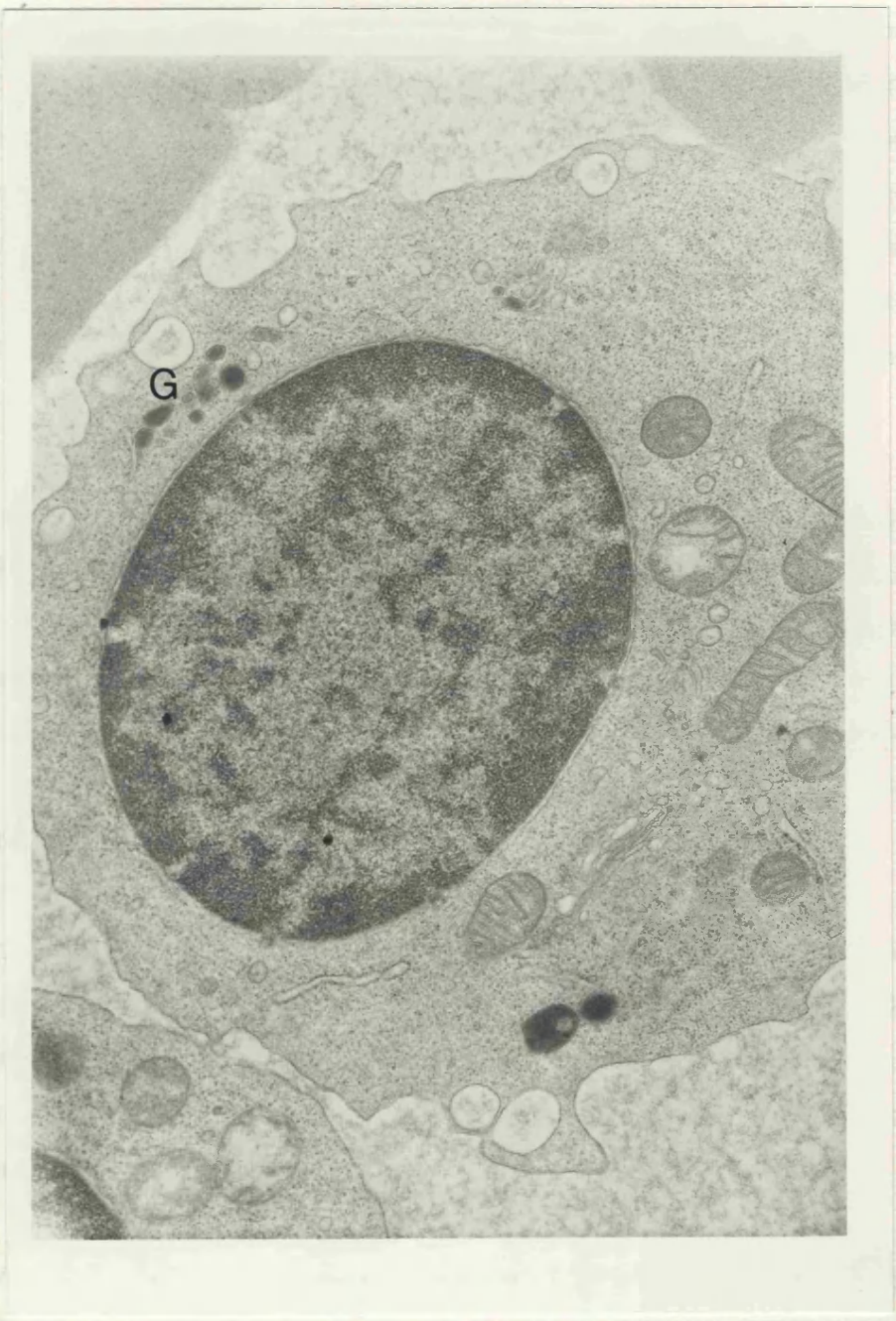


Figure 34: Large lymphocyte showing azurophil granules (G) Patient H14.

B-cells

B-cell populations were not specifically sought although special monoclonal antibodies are available to detect B and activated B lymphocytes.

During electron microscopic examination of samples from patient H17, the index case to study 6, cells with large amounts of endoplasmic reticulum were seen (figure 35). From the appearance of some of this seen approximately 24 hours after injury, it resembles a plasmablast (resulting from an activated B-cell). These cells are virtually never seen in the circulating peripheral blood in the healthy individual. Their presence following trauma would be consistent with mobilisation of the immune system.

B-cell proliferation has been found to be enhanced in the early phase following burn injury (Schulter et al, 1990) but suppression occurred four weeks post-burn. Miller-Graziano et al (1990) used increased levels of Interleukin-6 (IL-6) similar to those seen after trauma to investigate its effect on B-cells and monocytes in trauma patients. They found high levels of non-specific IgG, polyclonal B-cell activation and monocyte activation. IL-4 produced a suppression of monocyte activity.

McRitchie et al (1990) studied severely injured patients and found depressed IgM and IgG production in peripheral blood mononuclear cells stimulated with pokeweed mitogen. They suggested a failure of T-cell mediated help as the cause, a conclusion which is supported by the CD4+ deficiency found in this study.

Richter et al (1990) similarly found impaired IgG synthesis by B-cells in injured patients but not in elective surgical patients. They considered this to be a true difference between the two groups.

Figure 35

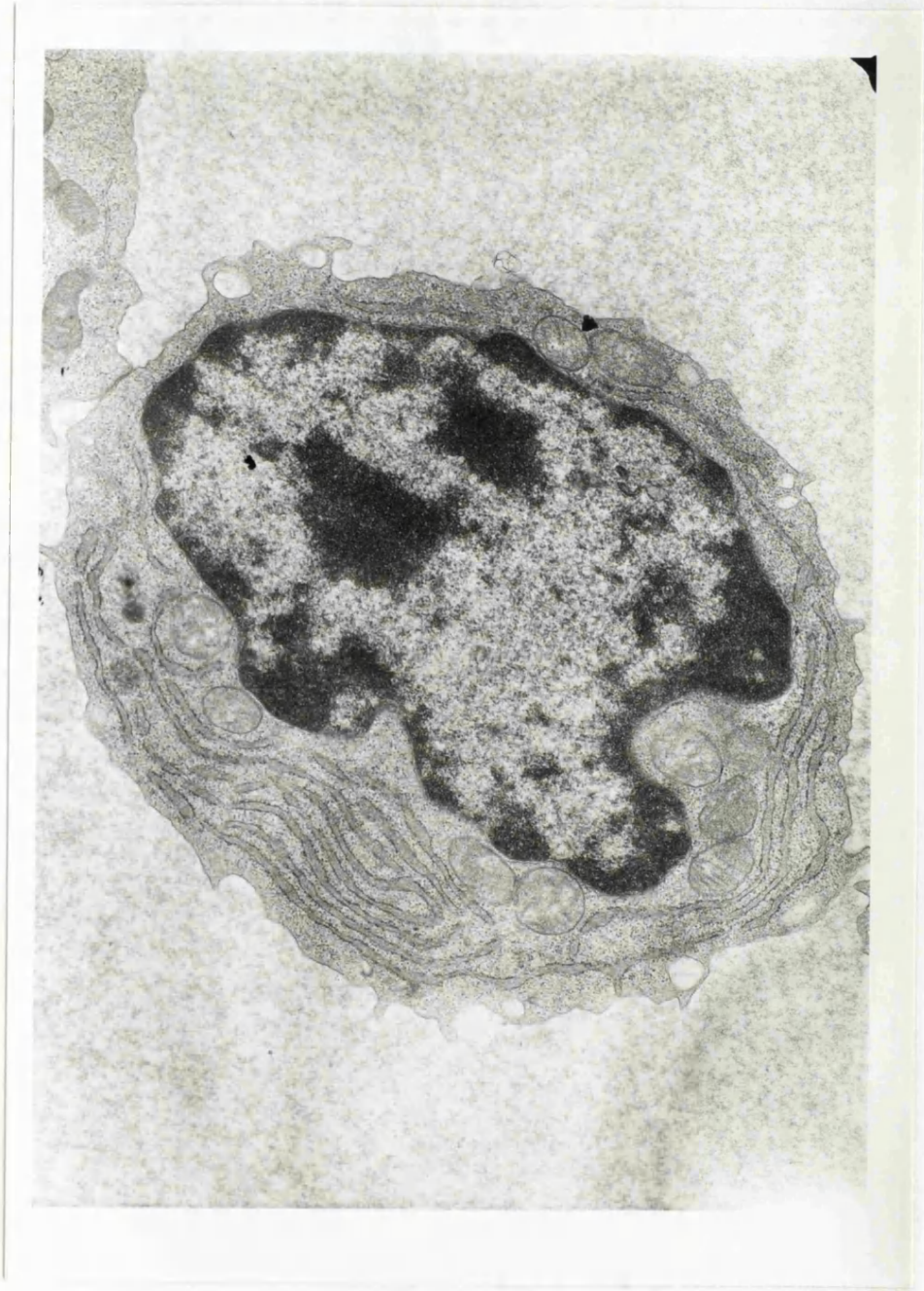


Figure 35: Plasmablast seen in patient H17(E).

CONCLUSIONS

In the United Kingdom, injury kills more people under the age of 40 than all of the other causes of death combined. Some injuries are so severe that the victim dies immediately and is beyond medical help. The only way to save a small proportion of these lives would be to ensure a high level of first-aid skill in the general public and to improve the speed of response of highly trained Paramedics. Significant reductions in the number of early deaths - i.e. those in the first few hours - may be achieved by the speedy application of Advanced Trauma Life Support (ATLS) techniques.

In the United States of America, improvements in mortality have been achieved by concentrating trauma cases in regional specialist units - trauma centres. However, despite excellent and optimal initial care, complications of injury continue to arise. Chief amongst these are infection and multiple organ failure. In both conditions, functional abnormalities in neutrophil leucocytes have been implicated. A failure of immune control by the T-lymphocytes, which not only control their own cell line but also neutrophils and virtually all other aspects of the immune response, has also been demonstrated by previous work. However, most studies have commenced many hours or even days following the traumatic insult, and insufficient distinction has been made between different types and magnitudes of trauma.

In 1975, Saba and Scovill described the situation thus "This multi-potential nature of the RES (Reticulo-endothelial System), coupled with the variable distribution of RE cells in the body ..... precludes a knowledgable grasp of the literature".

In these circumstances, a study of the changes occurring immediately following injury (an area which has not previously been specifically considered important) seemed to be needed.

The studies undertaken in this thesis have only begun to unravel the changes occurring just after injury. However, an important finding is that the neutrophil cell line remains stable in the circulation for many hours after injury, with minimal signs of functional deficit (Study 3). The immunosuppression seen following trauma seems more likely to be due to a failure of coordination, consequent upon a deficiency of CD4+ (T-helper) cells (Study 6), and an inability to mount defences against opportunistic pathogens. Some active self-damage due to the sequestration of neutrophils in the lung may also occur, aggravated by activation of the complement cascade, and therapeutic options need to be directed towards prevention of this complication. The high levels of catecholamines found after injury are likely to both increase the numbers of circulating neutrophils and lymphocytes, and also to stabilise circulating neutrophils via a well-defined cyclic-AMP mechanism. Study 4 in this thesis has demonstrated the role of beta-adrenoceptors in the reduction of neutrophil adhesion induced by samples of plasma from trauma patients.

Studies 1 and 2 illustrated the existence of a "leucocytosis of trauma" and defined its characteristics. The pattern discovered has never before been described in trauma patients, and consists of a very early rise (within 15 minutes) of both neutrophil and lymphocyte cell lines. The lymphocyte rise is short - lived, and is replaced within a few hours by a lymphopenia. The neutrophilia tends to peak at 3-4 hours post-injury, but may persist for 24 hours or more after injury. The high levels of neutrophils may be maintained by the onset of inflammatory and healing processes, although the initial rise in the two cell lines is almost certainly due to the effects of stress hormones.

Experience has shown that patient survival is increased if resuscitation is commenced within "the golden hour". It may be that long-term survival and the avoidance of infective complications may hinge on treatment given within the next few hours afterwards, whilst the therapeutic window of opportunity is still open.

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APPENDIX 1 (STUDY 1)

Contents

*NOTE: Throughout the appendices, all cell counts are expressed in the units: CELLS X 10^9 PER LITRE unless otherwise specifed.

Appendix 1

TABLE 1 - CONTROL HAEMATOLOGICAL VALUES

Control	Total WC	Neutro	Lymphs	Monos
H1	6.73	3.80	2.31	0.41
H2	5.86	3.43	1.67	0.49
H3	6.58	3.98	2.04	0.30
H4	9.63	5.95	2.65	0.71
H5	6.05	2.49	2.98	0.35
H6	8.18	5.71	1.94	0.39
H7	6.04	3.88	1.71	0.29
H8	5.14	2.54	2.18	0.20
H9	6.86	4.02	2.09	0.45
H10	6.28	3.65	2.05	0.38
H11	7.19	4.00	2.49	0.29
H12	11.32	4.92	2.62	0.24
H13	5.22	3.23	1.58	0.27
H14	6.70	4.46	1.59	0.42
H15	4.16	2.32	1.50	0.13
H16	13.50	9.88	2.60	0.73
H17	6.72	3.40	2.76	0.39
H18	8.37	4.10	3.15	0.54
H19	6.36	3.22	2.13	0.25
H20	7.06	4.19	2.21	0.39
H21	6.72	4.10	2.04	0.28
H22	10.76	7.59	2.10	0.70
H23	6.58	3.82	1.97	0.44
H24	6.59	3.54	2.45	0.40
H25	8.08	4.87	2.45	0.52
H26	6.11	3.25	2.13	0.49
H27	6.85	3.74	2.52	0.36
H28	6.66	3.11	3.04	0.19

Control	Total WC	Neutro	Lymphs	Monos
H29	3.93	1.78	1.75	0.17
H30	6.11	3.21	2.30	0.36
H31	9.75	5.91	3.06	0.40
H32	8.42	4.74	2.96	0.45
H33	8.83	5.21	2.66	0.59
H34	7.08	4.26	2.00	0.52
H35	7.68	4.29	2.70	0.39
H36	7.95	5.03	2.01	0.51
H37	6.43	3.29	2.50	0.37
H38	6.37	4.25	1.70	0.29
H39	6.30	3.74	2.14	0.24
H40	10.23	7.29	2.02	0.65
H41	10.27	6.50	2.78	0.55
H42	6.07	3.34	1.98	0.36
H43	6.41	3.42	2.37	0.39
H44	5.22	2.69	2.01	0.21
H45	5.06	2.37	2.26	0.22
H46	8.56	5.09	2.86	0.28
H47	6.65	3.96	2.28	0.22
H48	6.59	3.55	2.41	0.39
H49	7.23	3.96	2.76	0.26
H50	5.42	2.63	2.26	0.23
H51	5.77	3.15	1.93	0.19
H52	6.59	4.42	1.65	0.27
H53	6.60	4.09	2.02	0.21
H54	4.83	2.02	2.38	0.30
H55	4.82	2.47	1.89	0.22
H56	6.22	3.26	2.40	0.23
H57	5.47	3.07	1.85	0.31
H58	6.17	3.39	2.17	0.26
H59	8.10	5.12	2.28	0.51

Control	Total WC	Neutro	Lymphs	Monos
H60	5.72	3.12	2.01	0.32
H61	7.73	4.31	2.42	0.51
H62	5.86	2.80	2.53	0.28
H63	5.93	2.96	2.47	0.23
H64	5.84	3.84	1.87	0.32
H65	5.77	3.37	1.91	0.29
H66	8.69	6.22	1.78	0.42
H67	6.64	3.72	2.45	0.25
H68	3.86	1.61	1.72	0.23
H69	6.62	3.34	2.72	0.29
H70	5.88	3.04	2.44	0.27
H71	6.05	3.70	1.79	0.36
H72	6.24	3.80	2.06	0.24
H73	4.42	2.32	1.74	0.22
H74	8.26	6.00	1.69	0.36
H75	5.02	2.74	1.88	0.23
H76	6.95	3.79	2.46	0.42
H77	4.59	2.56	1.76	0.13
H78	9.69	6.84	2.24	0.39
H79	6.31	3.66	2.04	0.32
H80	8.00	5.32	2.07	0.43
H81	5.18	2.59	2.10	0.28
H82	6.77	4.27	1.94	0.32
H83	9.04	5.35	2.57	0.63
H84	6.68	3.76	2.44	0.31
H85	7.02	4.35	2.06	0.40
H86	7.63	4.29	2.43	0.52
H87	6.88	4.03	1.82	0.32
H88	8.58	5.38	2.48	0.43
H89	8.49	5.20	2.74	0.36
H90	7.51	4.38	2.56	0.38

Control	Total WC	Neutro	Lymphs	Monos
H91	7.05	4.38	1.98	0.42
H92	8.60	5.13	2.60	0.39
H93	5.09	2.16	2.41	0.31
H94	4.86	2.45	2.03	0.24
H95	5.80	3.20	2.15	0.26
H96	6.63	3.93	2.18	0.26
H97	5.47	3.07	1.74	0.42
H98	5.46	3.00	1.95	0.30
H99	6.06	3.82	1.88	0.19
H100	5.53	3.13	2.03	0.24
H101	5.66	2.81	2.09	0.36
H102	4.88	2.32	2.20	0.14
H103	7.03	3.92	2.52	0.28
H104	5.87	3.29	1.99	0.44
H105	7.89	5.22	2.24	0.21
H106	4.88	2.48	1.91	0.27
H107	6.82	3.91	2.39	0.36
H108	8.19	4.80	2.59	0.46
H109	4.21	2.52	1.36	0.21
H110	5.20	2.52	2.24	0.20

Statistics

TOTAL WHITE CELL COUNT:

Sample Standard Deviation = 1.62 Mean = 6.75

Range (+/- 2 Standard Deviations) 3.52 - 9.97

NEUTROPHIL COUNT:

Sample Standard Deviation = 1.3 Mean = 3.90

Range (+/- 2 Standard Deviations) 1.32 - 6.49

LYMPHOCYTE COUNT:

Sample Standard Deviation = 0.37 Mean = 2.21

Range (+/- 2 Standard Deviations) = 1.47 - 2.95

MONOCYTE COUNT:

Sample Standard Deviation = 0.13 Mean = 0.35

Range (+/- 2 Standard Deviations) = 0.1 - 0.6

TABLE 2

LEUCOCYTE COUNTS IN 110 RECENTLY INJURED PATIENTS WITH BODY REGION AND SEVERITY OF INJURY

Abbreviations for Body Regions:

E = Extremities including bony pelvis

C = Chest

A = Abdominal and pelvic contents

H = Head and neck

F = Face

S = External (Skin)

NB: In the calculation of the Injury Severity Score, only the highest AIS scores for three body regions are considered. In this table, the worst injuries for each body region are given for completeness.

Patient	Injuries by Body Region and AIS Score	ISS	Leucocyte Count
H1	E3	9	5.2
H2	H3 F2 S1	14	10.5
H3	E3	9	7.6
H4	C3 E2 S2	17	20.4
H5	A3 E3 H2	22	9.1
H6	E3 A1	10	10.4
H7	E3 F2 S1	14	11.4
H8	H5 E3 A3 C3	43	21.2
H9	E4 A1	17	14.6
H10	E3	9	11.2
H11	E3 H2 C2	17	11.3
H12	H5 F2 E2	33	10.4
H13	H5	25	26
H14	E3	9	13.5

Patient	Injuries/AIS	ISS	WCC
H15	C3 E3 H2	20	6.9
H16	H5 F3 E3	43	9.5
H17	H4 E3	25	11.7
H18	H3 A2 C2 S2	17	11.1
H19	H5 C3 E3	43	9.7
H20	H3 C3 F2 E2	22	12.6
H21	E3	9	10.8
H22	A3 C2 S1	14	16.4
H23	E3 A2	13	14.2
H24	E3 H2	13	15.1
H25	H4 F1 S1	18	9.1
H26	E3	9	9.1
H27	A4 E3 S1	26	10.2
H28	C3	9	13.7
H29	H3 E3 S2	22	10.1
H30	C3 H2 E2	17	13.2
H31	C3 H2 E2	17	9.5
H32	C3 A3	18	13.3
H33	H4 E2	20	12.2
H34	E3 H2	13	9.4
H35	H4 C3 A3 E2	34	8.7
H36	E3 S2 C1	14	9.4
H37	E3	9	15.2
H38	H5	25	8.4
H39	E3	9	8.7
H40	E3 S1	10	13.0
H41	A4 C2 E1	21	18.0
H42	H4 A3 E3 S2	34	9.7
H43	H4 C3 F2 S1	29	7.7
H44	E3	9	13.4
H45	E3 C2 S1	14	9.0

Patient	Injuries/AIS	ISS	WCC
H46	H3 S1	10	14.3
H47	H3 E3 S1	19	12.4
H48	E3	9	14.7
H49	A3	9	13.1
H50	E3	9	10.3
H51	H3 F2	13	10.9
H52	C4 H3	25	10.1
H53	E3 H2	13	8.6
H54	E3 S2	13	10.1
H55	H3 E2	13	11.7
H56	H4 E3	25	24.1
H57	A5 C4	41	14.4
H58	H3 E3	18	9.9
H59	E3	9	9.7
H60	E3	9	16.3
H61	E3	9	10.1
H62	E3 H2	13	21.5
H63	H3	9	9.5
H64	C4 S2	20	13.6
H65	E3 H2	13	13.9
H66	E3	9	15.0
H67	A3	9	14.8
H68	E3	9	9.9
H69	E3	9	10.1
H70	E3 S1	10	14.3
H71	E3 A2	13	10.9
H72	C3 A3 E3	27	8.6
H73	E3	9	15.3
H74	C3 A2	13	12.9
H75	E3	9	10.0
H76	C4 E3	25	9.0

Patient	Injuries/AIS	ISS	WCC
H77	C4 A4 E3	41	10.3
H78	F3 H3 S2	22	14.5
H79	E3	9	10.7
H80	E3 H2	13	12.0
H81	E3	9	12.7
H82	E3 H2 S1	14	14.4
H83	H3 E3	18	12.2
H84	H5 S1	26	12.1
H85	H4 E4	32	12.2
H86	E3	9	11.2
H87	H4 E3	25	11.9
H88	E3	9	5.6
H89	H3 E3 C2	22	7.8
H90	E3	9	11.3
H91	H4 E3 A2	29	8.9
H92	C3 A2 E2	17	5.8
H93	C4 E3	25	12.7
H94	H4 E3	25	22.7
H95	H3	9	12.9
H96	H3 E3	18	10.2
H97	E3 A3 H2	22	18.1
H98	E3 A3	18	21.9
H99	E3 H2 S2	17	12.4
H100	H4	16	14.5
H101	E3	9	12.6
H102	A3 F2 H2 S1	17	14.4
H103	C3 E2 F1	14	13.5
H104	E3	9	11.1
H105	E3	9	8.4
H106	E3	9	14.3
H107	F3 H2 S1	14	15.1

Patient	Injuries/AIS	ISS	WCC
H108	E3	9	12.0
H109	H3	9	13.5
H110	H4	16	6.7

APPENDIX 2 (STUDY 2)

Contents

Appendix 2

TABLE 3

DIFFERENTIAL LEUCOCYTE COUNTS OF 42 TRAUMA PATIENTS

	Time	WCC	Neut	Lymph	Mono
H1	4h	12.6	9.83	2.42	0.34
H2	4h	19.5	17.06	1.77	0.66
H3	6h	12.2	10.52	1.54	0.14
H4	6h	11.1	9.77	1.03	0.21
H5	1.5h	9.7	7.28	2.29	0.13
H6	2h	14.4	12.7	1.68	0.24
H7	2h	13.5	11.81	1.69	0.16
H8 A)	2.5h	11.1	8.94	2.06	0.21
B)	4.5h	12.5	10.26	2.08	0.16
H9	3h	8.4	5.63	2.56	0.18
H10	12h	10	7.07	2.76	0.17
H11	2.5h	14.3	11.86	1.85	0.46
H12	8h	7.1	5.75	1.18	0.17
H13 A)	3.5h	15.1	13.8	1.18	0.24
B)	8h	12.9	10.98	1.78	0.14
H14	3.5h	12.0	10.7	1.28	-
H15	2.5h	5.4	2.76	2.5	0.13
H16 A)	2h	13.5	11.46	1.78	0.25
B)	5h	7.3	5.42	1.5	0.37
H17 A)	0.75h	14.5	7.8	6.1	0.56
B)	3h	18.1	14.1	3.8	0.27
C)	6h	19.1	17.5	1.62	-
D)	10h	17.1	15.0	2.06	-
E)	18h	15.5	13.2	1.88	0.41
F)	23h	16.0	14.6	1.33	0.14
G)	47h	17.8	15.2	2.29	0.25
H)	72h	9.9	7.5	2.02	0.33

	Time	WCC	Neut.	Lymph.	Mono.
S18 A)	0.5h	12.4	6.3	5.72	0.38
B)	2h	16.63	14.1	2.19	0.31
C)	3.5h	14.9	12.38	2.30	0.20
S19 A)	0.25h	21.9	17.5	4.3	-
B)	1h	23.0	19.9	2.49	0.55
C)	2h	28.7	24.6	3.58	0.51
D)	3h	31.8	27.4	3.53	0.88
E)	4h	26.9	22.2	3.62	1.03
F)	5h	18.7	16.6	1.48	0.59
G)	8h	15.3	12.9	1.96	0.39
S20 A)	0.3h	12.8	9.3	3.2	0.26
B)	1h	18.1	-	2.1	-
C)	3h	20.2	15.3	3.62	1.2
D)	5h	18.1	16.6	1.06	0.35
E)	6.5h	15.5	12.5	2.35	0.67
N21 A)	1h	22.1	16.4	5.13	0.60
B)	12h	14.7	12.6	1.51	0.63
N22 A)	0.5h	30.1	25.6	3.5	1.0
B)	18h	9.2	8.0	0.5	0.7
N23 A)	1.7h	15.2	-	1.9	-
B)	21h	10.2	8.6	1.1	8.5
N24 A)	1.25h	11.9	8.8	2.4	0.7
B)	17h	7.9	6.7	1.0	0.2
N25 A)	4.2h	21.1	18.9	1.0	1.2
B)	23h	10.5	8.1	1.6	0.8
N26 A)	0.5h	18.5	11.6	6.11	0.83
B)	24h	11.9	10.2	1.2	0.5
N27 A)	1.6h	11.8	9.3	2.3	0.2
B)	18h	5.2	4.2	0.7	0.3
N28 A)	1h	16.7	-	5.6	-
B)	21h	15.8	-	3.8	-

	Time	WCC	Neut.	Lymph.	Mono.
N29 A)	1h	17.0	12.1	3.6	1.2
B)	19h	8.5	7.3	1.0	0.3
N30 A)	1h	11.7	6.9	4.5	0.3
B)	16h	6.9	5.8	0.8	0.3
N31 A)	1h	11.0	7.7	3.6	-
B)	16h	8.6	6.8	1.6	0.2
N32 A)	0.3h	9.9	5.1	4.3	0.5
B)	24h	16.7	14.9	0.9	0.9
N33 A)	1h	11.7	7.4	3.9	0.5
B)	15h	12.6	11.0	1.0	0.6
N34 A)	1.25h	12.1	9.8	1.7	0.6
B)	27h	11.1	9.8	0.9	0.5
N35 A)	0.5h	9.8	5.41	3.75	0.64
B)	19h	4.3	3.2	0.9	0.2
N36 A)	0.8h	14.6	13.7	0.8	0.1
B)	24h	10.0	8.5	1.0	0.5
N37 A)	0.9h	15.7	13.7	1.4	0.6
B)	24h	10.8	9.2	1.0	0.6
N38 A)	0.3h	28.8	20.4	7.7	0.6
B)	12h	5.7	4.9	0.7	0.1
N39 A)	1.3h	19.1	16.4	2.02	0.69
B)	21h	3.8	2.98	0.51	0.31
N40 A)	0.5h	8.1	4.2	3.3	0.6
B)	20h	9.0	7.6	0.9	0.5
N41 A)	0.9h	30.1	25.3	2.5	2.3
B)	18h	10.3	8.0	1.6	0.7
N42 A)	1.25h	16.0	12.1	3.5	0.4
B)	18h	3.9	3.2	0.5	0.2

TABLE 4

NEUTROPHIL BAND FORM COUNTS OF 17 TRAUMA PATIENTS

Patient	Time after Injury	TOTAL WCC	Neutrophil Count	Percentage Band Forms
H1	4h	12.6	9.83	32.6
H2	4h	19.5	17.06	33.8
H3	6h	12.2	10.52	53.1
H4	6h	11.1	9.77	23
H5	1.5h	9.7	7.28	20
H6	2h	14.4	12.7	35.8
H7	2h	13.5	11.81	7.1
H8 A)	2.5h	11.1	8.94	6.8
B)	4.5h	12.5	10.26	10.9
N9	3h	8.4	5.63	12.1
N10	12h	10	7.07	7.1
N11	2.5	14.3	11.86	19.4
N12	8h	7.1	5.75	22
N13 A)	3.5h	15.1	13.8	5.1
B)	8h	12.9	10.98	18.8
N14	3.5h	12.0	10.7	20
N15	2.5h	5.4	2.76	14.2
N16 A)	2h	13.5	11.46	15.5
B)	5h	7.3	5.42	13.7
N17 A)	0.75h	14.5	7.8	7.1
B)	3h	18.1	14.1	42
C)	6h	19.1	17.5	35
D)	10h	7.1	15.0	17.6

TABLE 5

LARGE GRANULAR LYMPHOCYTE COUNTS OF 17 TRAUMA PATIENTS

Patient	Time	Total Lymphocyte Count	Atypical Lymphocytes (Percentage)
H1	4h	2.42	7.6
H2	4h	1.77	12.5
H3	6h	1.54	22.2
H4	6h	1.03	0
H5	1.5h	2.29	7.7
H6	2h	1.68	14.2
H7	2h	1.69	8.3
H8 A)	2.5h	2.06	5.2
B)	4.5h	2.08	15.3
H9	3h	2.56	7.2
H10	12h	2.76	0
H11	2.5h	2.98	0
H12	8h	1.18	14.3
H13 A)	3.5h	1.18	10.0
B)	8h	1.78	0
H14	3.5h	1.28	0
H15	2.5h	2.50	5.2
H16 A)	2h	1.78	37.5
B)	5h	1.50	0
H17 A)	0.75h	6.10	36.3
B)	3h	4.00	15.4
C)	6h	1.45	40.0
D)	10h	1.87	14.0

TABLE 6

STUDIES OF TOTAL LEUCOCYTE COUNT IN 117 TRAUMA PATIENTS AT KNOWN TIMES AFTER INJURY

Time (hrs)	N	Mean	+2 SD
< 1	64	12.11	19.6
1 - 2	24	12.46	21.8
2 - 3	14	14.07	25.9
3 - 4	7	17.94	29.4
4 - 5	4	14.15	23.2
5 - 6	4	13.75	19.9
TOTAL	**117**		

These results are depicted in figure 7.

APPENDIX 3 (STUDY 3)

Contents

TABLE 7

1) STIMULATED NITROBLUE - TETRAZOLIUM REDUCTION ASSAY

1.1 PRELIMINARY STUDY

Patient	Time After Injury (hrs)	Injuries	Neutrophils NBT+ (%) Unstimulated	Stimulated
HH	8	{# Pelvis {# 2 Ribs	4.4	76.2
DG	8	{# L Femur {Rupt. Spleen	2.2	50.6
AT	1	{# R Tibia	3.4	98.5
AN	4	{Head Injury {# R Tibia {Rupt. Spleen	3.4	100
NT	3	{Head Injury	4.7	81.5

Notes: Patients HH and DG developed pyrexia on the day of admission and subsequently developed Adult Respiratory Distress Syndrome. Cultures of blood, urine and tracheal aspirate were negative.

1.2 SECOND STUDY

Patient	Time After Injury (hrs)	Injuries	Neutrophils NBT+ (%) Unstimulated	Stimulated
H3	6	{# NOF	4	98
H4	6	{# NOF	7.2	99.4
H5	1.5	{Head Injury {# Olecranon	3.9	83.9
H6	2	{HI, # Mandible {# L3, Multiple	1.0	98.8
H7	2	{# Clavicle {Mediastinal {Haematoma	1.4	96.4

Patient	Time After Injury (hrs)	Injuries	Neutrophils NBT+ (%) Unstimulated	Stimulated
H8 A)	2.5	{Severe Crush Injury	4.2	92
B)	4.5	{R. Foot	4.3	96.9
H9	3	{Compound # {R. Tibia	0.0	69.9*
H10	12	{Multiple Fractures	7.2	92.2
H11	2.5	{# NOF	1.4	94.9
H12	8	{# NOF	6.3	55.8
H13 A)	3.5	{HI, # Nose Le Fort II	1.9	98.5
B)	8		3.8	45.3
H14	3.5	{Multiple Lac.	1.6	91.8
H15	2.5	{NOF	1.5	94.7
H16 A)	2	{HI	0.0	90.1
B)	5		0.9	70.9
H17 A)	0.75	{HI	7.9	89.6
B)	3		0.9	91.7
C)	10		1.9	45.8
D)	18		3.5	29.0
E)	47		1.4	48.5

Abbreviations:

NOF = Fracture of Femoral Neck

HI = Head Injury

TABLE 8
STIMULATED CHEMILUMINESCENCE RESPONSE DATA FOR 7 TRAUMA PATIENTS AND FOR PMA AND ZYMOSAN-STIMULATED CONTROLS

1) PMA Stimulated Control: 2 Samples taken 1 hour apart (8.30am and 9.30am). All readings in millivolts (mV).

Time	08.30 Sample	09.30 Sample
0	-	-
3	-	-
6	1059	1173
9	1748	1882
12	1390	1482
15	771	832
18	420	471
21	264	303
24	191	219
27	152	176
30	126	142
33	104	116
36	83	93

2) Zymosan Stimulated Control: Same samples as PMA control.

Time (min)	08.30 Sample	09.30 Sample
0	-	-
3	-	-
6	496	413
9	823	753
12	985	957
15	1010	1018
18	962	997
21	887	941
24	798	852
27	692	748
30	591	655
33	504	555
36	424	470

3) Patient IW: Multiple injuries.

Time (min)	1hr Sample	3.75hr Sample
0	5	2
3	403	68
6	846	148
9	996	184
12	938	186
15	813	176
18	684	158
21	568	139
24	461	121
27	376	104
30	301	88

4) Patient JM: Tibial shaft fracture.

Time (min)	0.5hr Sample	1.5hr Sample
0	43	40
3	733	587
6	1317	1390
9	1408	1826
12	1304	1932
15	1145	1847
18	961	1630
21	789	1357
24	635	1089
27	519	865
30	418	681

5) Patient JD: Tibial shaft fracture.

Time (min)	0.5hr Sample	1.6hr Sample
0	14	3
3	567	238
6	1443	819
9	1965	1267
12	2068	1481
15	1952	1516
18	1710	1411
21	1419	1207
24	1132	1011
27	892	829
30	692	633

6) Patient TT: Tibial shaft fracture.

Time (min)	0.5hr Sample	2.0hr Sample
0	33	85
3	929	1445
6	1538	2217
9	1615	2196
12	1425	1842
15	1161	1460
18	912	1106
21	711	841
24	548	640
27	425	491
30	334	380

7) Patient FT: Femoral shaft fracture.

Time (min)	0.3hr Sample	2.0hr Sample
0	53	93
3	1188	1572
6	1884	2430
9	1924	2477
12	1633	2072
15	1263	1603
18	953	1192
21	715	878
24	533	653
27	405	491
30	313	374

8) Patient CF: Multiple injuries. Three serial samples.

Time (min)	0.3hr Sample	2.3hr Sample	4.3hr Sample
0	2	2	2
3	345	486	407
6	738	944	1019
9	846	1059	1238
12	778	987	1208
15	656	839	1072
18	528	680	895
21	417	541	734
24	321	426	598
27	253	335	473
30	202	265	379

9) Patient ED: Multiple injuries. Single sample only possible - patient died. Control shown for comparison.

Time (min)	0.5hr Sample	Control
0	1	1
3	161	159
6	570	541
9	885	858
12	984	1028
15	985	1090
18	930	1059
21	835	989
24	729	891
27	619	789
30	523	687

APPENDIX 4 (STUDY 4)

Contents

TABLE 9

NEUTROPHIL ADHESION TO 80mg STANDARD NYLON FIBRE

1. Control Studies

Duplicate determinations using the neutrophils of 5 normal donors resuspended in their own (autologous) plasma:

Control	Estimation	Adhesion (%)
H1	A	75.0
	B	72.5
H2	A	77.8
	B	75.0
H3	A	72.8
	B	77.3
H4	A	73.1
	B	77.0
H5	A	80.0
	B	73.4

Mean adhesion of Control neutrophils using 80mg standard nylon fibre = 75.4 % (range $\pm$1.96 SD = 70.4 - 80.4 %).

Normal control range as published by MacGregor et al (1974) = 76 $\pm$ 12 % (64 - 88 %).

2. Patient Neutrophils in RPMI 1640 medium:

Patient	Estimation	Adhesion (%)
H5	A	69.7
	B	61.2
H6	A	65.4
	B	68.4
H7	A	66.0
	B	67.9
H9	A	83.1
	B	84.2
H10	A	62.4
	B	64.6
H11	A	67.6
	B	67.6
H12	A	81.4
	B	83.4

3. Patient Neutrophils in Autologous Trauma Plasma

Patient	Estimation	Adhesion (%)
H13	A	23.4
	B	22.8
H14	A	12.5
	B	10.0
H15	A	16.2
	B	20.3
H16	At 2 hrs	15.5
	At 5 hrs	67.3
H17	At 0.75 hrs	21.5
	At 6 hrs	66.7

4. Adrenergic Blocking Studies

Normal Donor Neutrophils in Patient Trauma Plasma

Patient	Adhesion (%)	Adhesion post-incubation with 10^{-5} M Propranolol
Control	66	64
H8	34	60
H12	44	46
H14	56	64
H15	40	74
H16 (A)	50	64
(B)	34	64
H17 (A)	58	80
(B)	30	72

These results are expressed graphically in figure 19.

TABLE 10

PLASMA CATECHOLAMINE LEVELS AND CIRCULATING LEUCOCYTE COUNTS

Patient	Time after Injury (hrs)	WCC	Noradrenaline (pg/ml)	Adrenaline (pg/ml)
H1	4	12.6	269.5	40
H2	4	19.5	773.9	110
H3	6	12.2	946.7	110
H5	1.5	9.7	172.75	100
H6	2	14.4	324.8	70
H7	2	13.5	345.5	60
H8 (A)	2.5	11.1	580.4	100
(B)	4.5	12.5	359.3	85
H9	3	8.4	283.3	60
H10	12	10	283.3	270
H11	2.5	14.3	248.8	240
H12	8	7.1	400.8	210
H13 (A)	3.5	15.1	221.1	85
(B)	8	12.9	248.8	80
H14	3.5	12	317.9	350
H15	2.5	5.4	456.0	210
H16 (A)	2	13.5	269.5	200
(B)	5	7.3	262.6	120
H17 (A)	0.75	14.5	787.7	610
(B)	3	18.1	1340.5	370
(C)	10	17.1	912.1	80

Upper limits of normal ranges:

Noradrenaline - 300 pg/ml

Adrenaline - 60 pg/ml

(Ref. Dr J Low, Department of Anaesthesia, Chinese University of Hong Kong. Ranges relate to the electrochemical method used by the department).

APPENDIX 5 (STUDY 5)

Contents

TABLE 11

NEUTROPHIL PRIMARY GRANULE CONTENT IN 17 TRAUMA PATIENTS

Transmission electron microscopic study. Neutrophils with a normal complement of primary granules (40 - 120) as counted under TEM are given as a proportion of the total number counted (third column). Neutrophils with counts of less than 40 primary granules are listed individually (fourth column) with their actual count. Asterisks in this column denote that the neutrophil was a band form.

Patient	Time After Injury (hrs)	Normal Neutrophils	Low Granule Counts (N)
H1	4	37/37	-
H2	4	37/38	20
H3	6	30/31	29
H4	6	45/50	26,12*,8,19,9
H5	1.5	33/33	-
H6	2	37/37	-
H7	2	22/24	19*,21*
H8 A)	2.5	54/54	-
B)	4.5	50/50	-
H9	3	52/52	-
H10	12	57/57	-
H11	2.5	47/47	-
H12	8	44/44	-
H13 A)	3.5	37/37	-
B)	8	39/42	17,27*,24*
H14	3.5	60/60	-
H15	2.5	42/42	-
H16 A)	2	46/46	-
B)	5	40/40	-
H17 A)	0.75	55/55	-
B)	3	72/72	-
D)	10	69/69	-

Total number of neutrophils examined - 1017.

Total number with low granule counts - 12 (1.1%) 5/12 neutrophils with low granule counts were band forms.

TABLE 12

PLASMA MYELOPEROXIDASE (MPO) LEVELS IN 8 TRAUMA PATIENTS

Patient	Time After Injury (hrs)	Initial Rate of Reaction	Plasma MPO (Units)
Blank	-	0.0350	-
Control 1	-	0.2611	452
Control 2	-	0.2636	456
H8 A)	2.5	0.1300	452
H9	3	0.3047	538
H12	8	0.3026	534
H13 A)	3.5	0.3130	556
B)	8	0.2482	426
H14	3.5	0.2612	452
H15	2.5	0.2943	518
H16 A)	2	0.2826	494
B)	5	0.3063	542
H17 A)	0.75	0.3027	534
B)	6	0.3122	554
C)	9	0.4790	958

TABLE 13

PLASMA ALKALINE PHOSPHATASE LEVELS IN 15 TRAUMA PATIENTS

Patient	Time after Injury (hours)	Plasma Alkaline Phosphatase (IU/L)
H2	4	87
H3	6	56
H4	6	62
H5	1.5	59
H6	2	49
H7	2	64
H8	2.5	93
H9	3	23
H10	12	89
H12	8	45
H13	3.5	77
H14	3.5	50
H15	2.5	47
H16	2	111
H17	0.75	65

NORMAL REFERENCE RANGE: 40 - 136 IU/L

TABLE 14

SERUM C-REACTIVE PROTEIN LEVELS IN 13 TRAUMA PATIENTS

Patient	Time after Injury (hours)	Serum CRP (mg/dL)
H2	4	< 0.6
H4	6	< 0.6
H5	1.5	< 0.6
H7	2	< 0.6
H8	2.5	< 0.6
H9	3	< 0.6
H10	12	8.5
H12	8	< 0.6
H13	3.5	< 0.6
H14	3.5	2.3
H15	2.5	< 0.6
H16	2	< 0.6
H17	0.75	< 0.6

NORMAL REFERENCE RANGE: 0 - 0.99 mg/dL