INFLAMMATORY RESPONSES TO PLATELET-ACTIVATING FACTOR IN HUMAN SKIN

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ABSTRACT

Since its discovery over 20 years ago, platelet-activating factor (PAF) has been the subject of intensive research. In particular, its role in pathophysiology has provoked as much controversy as research interest. The pharmacological profile and ubiquity of PAF have made it a candidate for involvement in a number of inflammatory processes. No clear evidence has been demonstrated however to establish a role in disease in man, although clinical caution about its potential toxic effects has limited the number of available experimental human models.

One particular tissue which lends itself to examination of the effects of PAF is the skin. A series of studies has been undertaken to define accurately the characteristics of the acute responses to PAF in human skin and to study the role of histamine in these responses. By way of comparison, the acute responses to other inflammatory mediators (PGE_2, substance P, and CGRP) has also been investigated. The effects of histamine antagonism, and depletion on the PAF responses have been examined together with evaluation of local histamine release from the skin after PAF treatment. Additionally, a comparison of the PAF-induced responses in atopic and non-atopic subjects has been undertaken.

As a consequence of these studies, quantitative methods have been validated as sensitive and reproducible techniques for evaluating hyperalgesia and histamine-type flare and weal responses in the skin. Using the hyperalgesia model, intradermally injected PAF failed to cause hyperalgesia in the skin. Conversely intradermal PGE_2 was shown to provoke dose-related hyperalgesia, which was associated with spreading erythema, lasting for 2-3 hours. Apart from at high doses where the effect was marginal when compared with PGE_2, the neuropeptides substance P and CGRP also failed to produce hyperalgesia in the skin.

Using the flare and weal model, PAF, substance P and PGE_2 were associated with dose-related histamine-like weal and flare responses, although the PGE_2 responses were considerably weaker. CGRP did not produce these responses and instead caused a local intense erythema, similar to the later effect of PGE_2.

Evaluation of the effects of H_1-antagonism and histamine depletion, and the quantification of local histamine release have demonstrated that the PAF responses in the skin are largely mediated by histamine. Indomethacin was also found to have a small inhibitory effect on the PAF responses implicating secondary prostaglandin release.

The acute inflammatory responses to intradermal PAF have been shown to be similar in atopic subjects compared with non-atopics using the techniques described above. Baseline plasma histamine, however, was found to be higher in atopic subjects.

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CHAPTER I

INTRODUCTION

A. Historical perspective

Platelet-activating factor (PAF) is a membrane derived phospholipid. Over the ten years since its structure was confirmed, PAF has become recognised as an important inflammatory mediator while creating much controversy over its role in pathophysiology.

Its original discovery some 20 years ago was the result of observations by Henson (1970). Leukocytes drawn from rabbits sensitised with either bovine serum albumin (BSA) or horse spleen ferritin (HSF) were found to be capable of releasing a 'soluble factor' when incubated with antigen (BSA or HSF). This 'soluble factor' provoked the release of histamine from platelets, a reaction which was found to be reproducible and was formally termed the 'leukocyte-dependent release of histamine' (LDHR) test. The test was used to screen different populations of white cells for the response and was found to be independent of complement.

It was established that generation of the soluble factor presumed to mediate the LDHR was relatively confined to the white cells of the 'buffy coat' or layer above the red cells after light centrifugation of whole blood. Furthermore, it appeared that, in the rabbit, basophils were primarily implicated (Benveniste et al,1972). Benveniste and his colleagues, including Henson, termed the 'soluble factor' as

platelet-activating factor (PAF) in deference to its experimental effects on platelets. Suspicions concerning the phospholipid nature of PAF were eventually confirmed when Hanahan's group (Demopoulos et al,1979) reported on an alkylacetyl glycerophosphocholine (AGEPC), which mimicked the biological activity attributed to PAF. Subsequently, some ten years after the original observations of its effects, the structure of the naturally occurring compound produced by sensitised rabbit basophils in response to exposure to antigen was established (see later section on Chemistry for structure). Thus the first phospholipid with potent pharmacological activity was characterised.

Insight into the structure of PAF enabled proliferation of research on the mediator, particularly with regard to molecular sub-species. It became clear that the great majority of naturally occurring PAF fell into two types having a sixteen or an eighteen carbon chain at the sn1 position (Oda et al,1985).

Alongside developments in the understanding of the chemistry of PAF, were major advances in elucidating its biosynthesis and pharmacology. As described earlier, the basophil was the first cell type to be positively identified as a source of PAF (Benveniste et al,1972) and, as methods of detection for the mediator became more sensitive and precise, the list of cellular sources grew rapidly.

Initially, detection was based upon biological activity alone (eg platelet aggregation or release of other mediators

such as histamine and serotonin), but chromatographic techniques have enabled a more specific and reproducible approach to identification (Pinckard et al,1984). From the original basophil source, the capacity for PAF production has now been identified in several cell types and it is likely that methodological artefacts have prevented identification in more cell types.

It is now understood that certain cells which have the capacity to generate PAF, do not actually release the PAF; measurement of supernatant in such a circumstance would give the impression that the cell lacked the ability to synthesise PAF. Such observations have led to considerable interest in the role of PAF as a putative intracellular messenger which can modify cell-cell behaviour (Sisson et al,1987).

Although a considerable amount of information concerning the pharmacological activity of PAF was collected in the 10 years following its discovery, the first demonstration of specific receptors for PAF (Valone et al,1982) opened up new doors to the understanding of the phospholipid's role in pathophysiology. Naturally the first receptors were described for platelets but just as the list of cells capable of synthesizing PAF has grown, so have the cells types bearing receptors. Undoubtedly the discovery of novel potent and selective antagonists has greatly enhanced this process. It is interesting to note that following early forays using analogues of PAF, much progress has been made with natural products from which active antagonists have been isolated.

Such molecules include the ginkgolides (Foldes-Filep et al,1987) and pseudolignans (Hwang et al,1985).

As stated earlier, the original effect attributed to PAF was the LDHR response and current knowledge of the pharmacology of the mediator is based essentially on this stimulation/release phenomenon.

Since the first observations of the effects of PAF on platelets, PAF has been found to activate neutrophils (O'Flaherty et al,1983), eosinophils (Wardlaw et al,1986), basophils (Bochner et al,1988), monocytes, T-lymphocytes (Rola-Pleszczynski et al,1988), smooth muscle (Stimler-Gerard,1986) and vascular endothelium (Northover,1989). In many cases, the action of PAF on cells results in the production or release of other inflammatory mediators, including both histamine and leukotrienes (Voelkel et al,1982). It is not surprising therefore that such an ubiquitous mediator as PAF has been implicated in a wide variety of pathophysiology.

While the main thrust of PAF research has derived from a hypothesis that it may have a role in asthma (Barnes,1988), there has also been interest directed towards a role for PAF in psoriasis (Mallet et al,1985), renal disease (Camussi,1986), inflammatory bowel disease (Wallace et al,1986), cardiovascular disease (Hampel et al,1989) and even fertility (O'Neill et al,1989).

Perhaps the most important clinical event in recent years for PAF was the finding some human volunteers, following inhalation of PAF, became more sensitive to inhaled methacholine, a characteristic of asthma (Cuss et al,1986). This report led to renewed enthusiasm for PAF in asthma research even though antagonists were in short supply for testing the hypothesis.

Since this report, however, only two studies have supported the original findings (Rubin et al,1987;Chung et al,1989) while more recent evidence appeared to refute the original findings (Stenton et al,1990;Lai et al,1990;Spencer et al,1990). Despite an early clinical lead, therefore, it remains to be established whether PAF has any important role in clinical asthma.

B. Chemistry and Biochemistry

1. Structure

PAF is a white, hygroscopic powder which is sparingly soluble in water and extensively soluble in organic solvents such as ethanol and chloroform. Although a polar molecule, it is extremely lipophilic.

In 1979, nearly a decade after the discovery of the 'soluble factor', Demopoulos and his colleagues described a glyceryl ether containing phosphoglyceride which was shown to have a pharmacological profile so similar to naturally occurring PAF that it was proposed that they were the same compound. The synthetic phospholipid, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC) was prepared from the ether-containing phospholipids of fresh beef heart. The choline containing fraction was further purified by thin layer chromatography (TLC) and then subjected to catalytic hydrogenation followed by base-catalysed methanolysis. The resulting phospholipid, AGEPC, was then finally isolated by TLC.

Although the unavailability of more sophisticated analytical techniques of chemical identification (high pressure liquid chromatography/mass-spectrometry) did not permit absolute verification that natural PAF was AGEPC, several critical pieces of evidence were supportive. The potency of AGEPC was virtually identical to natural PAF in producing release of serotonin from, and irreversible

aggregation of, rabbit platelets. A calcium-independent shape change was also shown to be associated with similar concentrations of native PAF and AGEPC.

Using this model it was further demonstrated that cross-desensitisation occurred for PAF and AGEPC by prior exposure to one compound in the absence of calcium followed by exposure, in the presence of calcium, to the alternative compound at concentrations which would normally cause aggregation. This cross-desensitisation to PAF was not shown for other mediators eg collagen or thrombin.

If either native PAF or AGEPC (which were found to co-chromatograph on TLC) was treated with base, resulting in complete loss of biological activity, both products also co-chromatographed. Additionally it was found that treatment with acetic anhydride restored activity to both compounds still co-eluting on TLC.

Nearly a year later, the same group (Hanahan et al, 1980) were able to confirm the identity of PAF using a combination of gas-liquid chromatography and mass spectrometry. Three techniques of degradation were deployed to facilitate the analysis: acid hydrolysis, base hydrolysis, and acetolysis. Results confirmed a glyceryl ether structure, the choline and phosphate groups and the acetic acid side chain at the sn2 position. In establishing the glyceryl ether group, a mixture of octadecyl (>90%) and hexadecyl (<10%) carbon chains were observed.

These results were obtained in washed rabbit platelets but the C16/C18 mix has been also demonstrated in man (Pinckard et al,1984) although the ratio appears to favour the C16 homologue in man. Thus the structure of PAF was known; with this knowledge concerted effort was directed toward investigation of the relative pharmacological importance of the various parts of the PAF molecule.

The structure of PAF is shown in Figure 1 and may be split into three sections for discussion purposes:

1. The long chain glyceryl ether (sn1).
2. The short chain acetate (sn2).
3. The phosphorylcholine group (sn3).

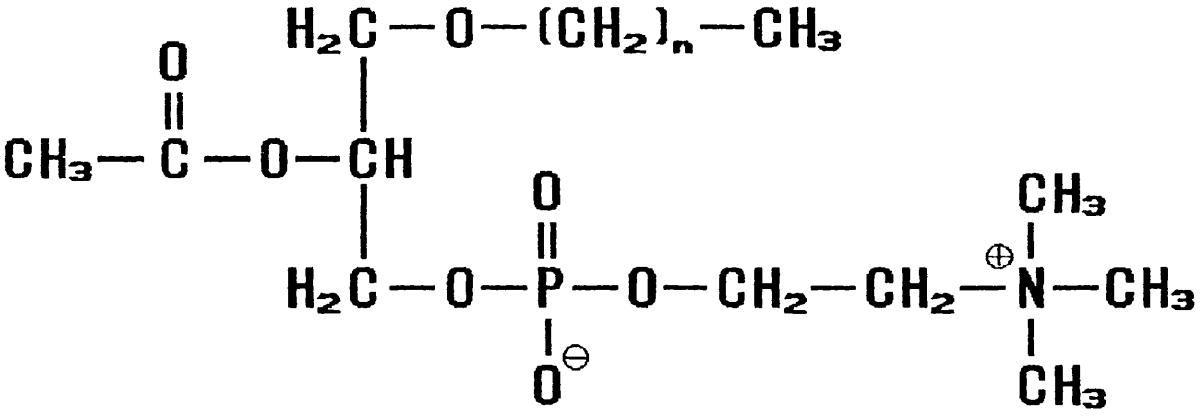

Figure 1.1 Molecular structure of platelet-activating factor, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine

When the structure of PAF was first investigated by Demopoulos et al (1979), it was noted that both the presence and the length of the short chain fatty acid group at the sn2 position was critical in determining the pharmacological potency of the molecule. Propionyl and butyryl substitution of the acetyl group resulted in significant reduction in functional activity on rabbit platelets. Hydrolysis of the group rendered the molecule biologically inactive.

It has since been shown that the natural metabolic pathway for PAF is de-acetylation (see Synthesis/Metabolism). If the ether group is modified, activity is also affected (see Table 1.1). Again the length of the carbon chain appears to be critical with the C16 and C18 homologues being by far the most potent. If an acyl group is substituted for the alkyl group, a dramatic loss of activity is observed demonstrating the importance of the ether group. Finally the choline group is important. If a methylethanolamine group is substituted, again a reduction of function results.

Molecular species	Relative activity*
1-O-alkyl	
C12:0-AGEPC	8
C14:0-AGEPC	11
C16:0-AGEPC	100
C18:0-AGEPC	27
C18:1-AGEPC	50
C16:0/C18:0-AGEPDME	35
C16:0/C18:0-AGEPMME	4
C16:0-AGEPE	0.04
1-O-acyl	
C12:0-AGPC	0.003
C16:0-AGPC	0.3
C18:0-AGPC	0.02

Table 1.1 Biological activity of PAF homologues and analogues; * activity is expressed as a percentage relative to the platelet stimulating activity of C16:0-AGEPC on washed rabbit platelets (serotonin release).
AGEPDME: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphodimethylethanolamine;
AGEPMME: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphomonomethylethanolamine;
AGEPE: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoethanolamine;
AGPC: 1-O-acyl-2-acetyl-sn-glycero-3-phosphocholine (Ludwig et al,1987)

2. Synthesis

Once the structure of PAF was known, elucidation of the routes of synthesis and enzymes involved were not long following. The major biosynthetic route for PAF is achieved by remodelling of alkylacyl-glycero-phosphocholine (alkylacyl-GPC, see Figure 1.2). This polar phospholipid is of importance not only as a precursor pool for PAF but also as a structural membrane constituent in many cells (Lee et al,1989). The alkyl-arachidonoyl form is thought to be the main source and also acts as a precursor pool for another biologically active lipid, arachidonic acid. It is hardly

surprising therefore that the biology of PAF appears inextricably bound up with that of arachidonic acid and, in particular its metabolites e.g. prostaglandins and leukotrienes. For example, it has been proposed that PAF and eicosanoids act synergistically as pro-inflammatory agents in the pathogenesis of psoriasis (Greaves et al,1988).

The first step in the synthetic route involves hydrolysis of the sn2 acyl group (usually arachidonic acid) from alkylacyl-GPC and involves the enzyme phospholipase A_2 (PLA_2). Little is known about the specific phospholipase involved in this reaction although there is evidence that lipoxygenase products (eg LTB_4) modulate PAF synthesis in human neutrophils (PMN) by inhibition of PLA_2 (Billah et al,1985).

The second step in PAF synthesis is better characterised and consists of the incorporation of an acetyl group at the sn2 position through the action of the enzyme alkyllyso-GPC:acetyl-CoA acetyltransferase. After its first discovery in rat liver microsomes at the beginning of the decade (Wykle et al,1980), information is now available on the substrate specificity and location of this enzyme (Lee et al,1986).

There does exist a second but less well investigated route for PAF biosynthesis which involves de novo synthesis rather than remodelling of cellular stores of ether lipid (Lee et al,1986). This pathway has the same point of origin (alkylglycero-phosphate, AGP) as the synthetic route for the ether lipids forming the membrane precursor stores for PAF

synthesis via the remodelling route. In the de novo pathway, the sn2 position of AGP is acetylated to the intermediate, alkylacetylglycero-phosphate (AAcGP), which, in turn, loses a phosphate group hydrolytically to form alkylacetylglycerol (AAG), the immediate precursor for PAF (see Figure 1.2). In the remodelling route, AGP gains a long chain fatty acid (eg arachidonic acid) to subsequently form the ether lipid alkylacyl-GPC, the precursor for lyso-PAF.

The final step in the de novo route is catalysed by dithiothreitol-insensitive cholinephosphotransferase (DTT-CPT) and involves the incorporation of a polar phosphocholine group at the sn3 position. This enzyme has important differences from the acetyl-transferase of the remodelling route. The specific activity of DTT-CPT (and other de novo route enzymes) is high in tissues such as rat kidney (Woodard et al,1987) which maintain a basal production of PAF while activity of acetyl-transferase is low; moreover inflammatory stimuli enhance the activity of the latter while DTT-CPT remains unaffected. Such observations have led to the proposal that the de novo route supplies physiologically necessary levels of intracellular PAF while the remodelling route is the 'rapid response' pathway capable of producing extra PAF in the face of an inflammatory insult (Lee et al,1989).

3. Catabolism

Catabolism of PAF is rapid and involves hydrolytic cleavage of the sn2 acetyl group. The enzyme involved, alkylacetyl-GPC (PAF) acetylhydrolase, produces alkyllyso-GPC or lyso-PAF which is biologically inactive and is also the precursor for PAF (see figure 2). Acetylhydrolase is found both intracellularly and in plasma (Blank et al,1981; Wardlow et al,1986) and is probably quite distinct from phospholipase A_2 (Lee et al,1989). The enzyme is strongly specific for the sn2 position and the length of carbon chain appears to be important since a single carbon increase reduces activity by 18-fold (Wardlow et al,1986). The activity of plasma-bound acetylhydrolase is reported to be associated with low density lipoproteins (LDL), although high density lipoprotein (HDL) may contain a temporarily inactive form (Stafforini et al,1987).

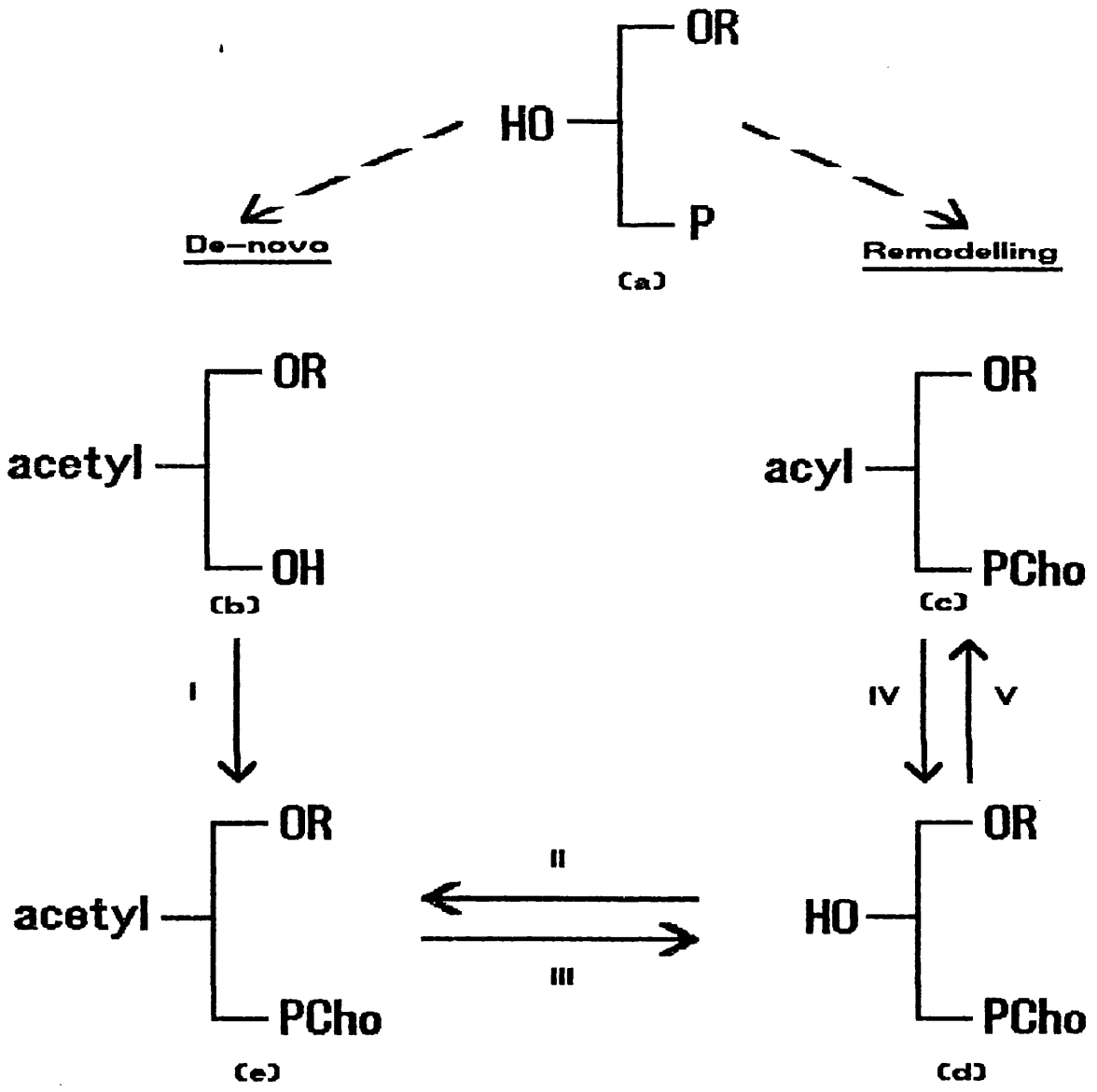

Figure 1.2 Diagrammatic representation of PAF synthesis and catabolism showing immediate precursors in the 2 known synthetic routes.
(a) alkylglycerophosphate; (b) alkylacetylglycerol;
(c) Alkylacylglycero-phosphocholine; (d) Lyso-PAF; (e) PAF.
I 1-alkyl-2-acetyl-sn-glycerol DTT-insensitive cholinephosphotransferase;
II 1-alkyl-2-lyso-sn-glycero-3-phosphocholine acetyltransferase;
III 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine acetylhydrolase;
IV phospholipase A2; V polyenoic acid transacylase

4. Cellular sources

PAF is produced de-novo by cells and not stored pre-formed as is the case with histamine. Early research into the cellular origins of PAF was hampered by the fact that some cells which are capable of synthesising PAF, transiently retain the phospholipid intracellularly. Insight into this phenomenon has led to a much better understanding of the possible role of PAF as a secondary messenger (Leyravaud et al,1989;Sisson et al,1987).

It is established that PAF synthesis takes place in human neutrophils (Sisson et al,1987) monocytes, basophils (Cammussi et al,1981), platelets (Touqui et al,1985), eosinophils (Lee et al,1984), skin fibroblasts (Michel et al,1988), lung mast cells (Schleimer et al,1986), renal tissue (Pirotzky et al,1984), vascular endothelium (Zimmerman et al,1987), and in human embryo tissue (O'Neill et al,1987). In terms of comparative abilities to synthesize PAF, neutrophils and endothelial cells are potentially the most active with a measured production of 100 and 150pmol/10^6 cells respectively in response to A23187 calcium ionophore (Jouvin-Marche et al,1984;Bussolino et al,1987). The monocyte is capable of producing 44 pmol/10^6 cells.

None of these cells however releases all of the synthesized PAF and various theories have been put forward to explain this finding. Leyravaud and Benveniste (1989) have recently demonstrated that the pH of the extracellular medium

and number of cells present in the experimental preparation heavily influence the release of PAF from neutrophils.

In neutrophils it appears that PAF production correlates with adhesion of these cells but there is no correlation between degree of adhesion and amounts of PAF released (Sisson et al,1987). It has been proposed therefore that newly synthesised PAF (which is rapidly translocated to the cell surface where it may remain, Record et al,1986) may act as an intracellular messenger or as an intercellular mediator being strategically placed to take part in the cell-cell interactions during an inflammatory response without the requirement for release into the extracellular medium.

A number of factors regulate PAF production and probably the most important is extracellular Ca^{2+} (Ludwig et al,1984). Membrane bound phospholipase A_2 is known to have an absolute requirement for Ca^{2+} (van Den Bosch,1980) and this enzyme plays a critical role in the remodelling synthetic pathway (see Figure 1.2); additionally, acetyltransferase activity is enhanced by increased levels of intracellular Ca^{2+} (Ludwig et al,1984). It is not surprising therefore to find published evidence that agents which enhance transmembrane Ca^{2+} flux such as N-formyl-methionyl-leucyl-phenylalanine (FMLP) or A23187-calcium ionophore (Sisson et al,1987) also stimulate PAF production.

Conversely, calmodulin antagonists such as trifluoperazine have been found to inhibit PAF synthesis

(Billah et al,1986), as has the Ca^{2+} channel blocker, nifedipine (Jouvin-Marche et al,1983).

C. Pathophysiology

PAF is a potent phospholipid autacoid. It has profound pharmacological effects in a wide range of biological situations. At the cellular level PAF causes degranulation and stimulates secondary production of mediators (McManus et al,1981;Dahl,1985) while at the tissue level it provokes bronchoconstriction (Vargaftig et al,1980), increased vascular permeability (Humphrey et al,1982;Sirois et al,1988) and cellular infiltration (Archer et al,1985;Michel et al,1987).

In many respects, the effects of PAF parallel the effects of histamine with the ability to provoke oedema formation following increased vascular permeability and chemotactic effects on eosinophils (Wardlaw et al,1986;Clark et al,1975).

There is substantial evidence that PAF exerts its effects via specific cell-surface receptors and recently, using functional expression techniques, Honda et al (1991) reported the cloning of a complementary DNA for a PAF receptor from guinea-pig lung. The evidence indicated that the receptor belongs to the superfamily of G protein-coupled receptors.

1. Cellular aggregation and activation

The fact that PAF causes cellular aggregation was fundamental to its original discovery and indeed its name. It was found subsequently that PAF not only caused aggregation of but also activated platelets, and that these two phenomena could be separated. Workers found that ADP scavengers or indomethacin prevented irreversible platelet aggregation but had no effect on platelet secretion of serotonin in response to PAF-challenge (McManus et al,1981).

The mechanism by which PAF provokes the aggregation of platelets is dependent upon extracellular Ca^{2+}. It has been found that chelating agents such as EDTA prevent the aggregation due to PAF in human platelets (McManus et al,1981). Furthermore the process is receptor mediated and has been shown to be inhibited by specific PAF antagonists eg L-653,731 (Hwang et al,1985) and L-659,989 (Hwang et al,1988).

It is thought that the Ca^{2+} complexes with glycoproteins IIb and IIIa (Hagen et al,1982) and ,in turn, forms a receptor for fibrinogen, a potent pro-aggregatory molecule. PAF-induced intracellular Ca^{2+} increases are probably due to both mobilisation of intracellular stores together with promotion of cellular influx (Valone et al,1985). PAF is known to enhance phosphodiesterase activity and hence reduce cAMP levels (Miller et al,1982); this effectively would remove an inhibitory influence on cytosolic Ca^{2+} and hence promote a tonic increase.

Activation and degranulation of platelets is probably mediated by an entirely different process. As stated earlier, chelating agents and eicosanoid synthesis inhibition prevents aggregation without influencing activation of vasoactive amine release or TXB_2 by platelets. Furthermore, Ca^{2+} uptake is not necessary for PAF-induced primary aggregation of platelets (Clare et al,1984). It could be that PAF mediates these responses by mobilising intracellular stores of Ca^{2+}.

There are species differences in platelet responsiveness to PAF. While rabbit platelets are the most sensitive to PAF, platelets from rats and some non-human primates are more or less refractory to PAF (Chignard et al,1987). Human platelets appear to be somewhat less sensitive than rabbit platelets but will aggregate irreversibly to appropriately high PAF concentrations.

PAF produces aggregation, activation and degranulation in human PMN (Dahl,1985). There is evidence of an inter-relationship with LTB_4 (Chilton et al,1982) in these effects which differentiates PAF's effect on PMN from that on platelets. Although it has been suggested that LTB_4 may mediate PAF's effects, the situation is by no means clear. Both PAF and LTB_4 have been found to desensitize PMN to subsequent respective challenge but no cross-desensitisation was found; additionally PAF and LTB_4 were found to act synergistically (O'Flaherty,1985).

It could be argued that, in terms of biosynthesis, both molecules have a common origin since arachidonic acid, the precursor for LTB_4, is formed as a by-product of PAF synthesis (see section 2B). Indeed, PAF stimulates PMN to produce leukotrienes (Tou,1985) probably through an effect on PLA_2. It is interesting to note that PAF also promotes its own further synthesis by activating acetyltransferase (Doebber et al,1987). PAF has also been found to 'prime' cells to the actions of other mediators; in particular the effect of FMLP is dramatically enhanced by pre-incubation with PAF (Vercellotti et al,1988). It is evident that cell exposure to PAF initiates a train of metabolic events aimed at amplifying the overall reaction, as is the biological custom for any response to pathogen invasion or physical damage to tissue.

2. Chemotaxis and eosinophils

Chemotaxis is phenomenon whereby a chemical causes a cell or cells to move along an increasing (positive) or decreasing (negative) concentration gradient of the chemical. In many cases a chemotactic chemical may also induce chemokinesis which is simply an increased rate of locomotion of cells. Leucocytes have a primarily defensive role to play in the maintenance of homeostasis; hence it would be expected that these cells should recognise toxic or noxious stimuli and respond by accumulating at the site of highest concentration of the stimulus.

FMLP, for instance, is a synthetic peptide (similar to those released by bacteria) which exerts chemotactic effects on eosinophils and neutrophils (Morita et al,1989). PAF, however, is considerably more potent than FMLP as a chemoattractant for eosinophils and has a similar potency to the anaphylotoxin complement fragment C5a. By way of contrast, it exerts only a weak chemotactic effect on neutrophils compared with C5a and LTB_4.

Czarnetzki et al (1989) found that, in contrast to other reports, LTB_4 appeared to be a more potent chemoattractant than PAF for eosinophils. It should be noted, however, that the cells were taken from patients with various types of allergic and viral disease which may have altered specific responsiveness to various mediators. Other workers have noted that the density of the eosinophil population influences the chemotactic response (Sigal et al,1987) and it appears that PAF-induced chemotaxis is dependent upon the state of the target cell.

There are a number of pathological conditions which tend to be associated with a raised eosinophil count e.g. atopic disease, parasitic infection etc. It has been found that eosinophils from such individuals may be morphologically and functionally different from those in normal subjects (Bass et al,1980). Furthermore these differences appeared to be related to the density of the eosinophil. It is now commonly accepted that eosinophils may be classified as "normodense" or

"hypodense" (Fukuda et al,1989) with the split point being 1.081 g/ml on an isotonic Percoll gradient (Shult et al,1988).

In normal individuals, approximately 10% of eosinophils are hypodense. In diseases associated with eosinophilia such as asthma, however, there tends to be an increase in this percentage. Asthma for instance is associated with increased numbers of hypodense eosinophils (41%-Shult et al,1988;65%-Kloprogge et al,1989).

In a study of allergic rhinitis patients (Frick et al,1988), eosinophil morphology was studied before and during the ragweed pollen season. Before the season started there was a normal profile of normodense to hypodense cells; as the season progressed the percentage of hypodense eosinophils increased. In a more acute model, the same workers found that the proportion of hypodense eosinophils increased during the late asthmatic response to inhaled antigen (Frick et al,1988). The evidence seems to point to a inverse link between eosinophil density and disease activity.

Hypodense eosinophils characteristically display a number of properties consistent with activation. Such cells demonstrate higher oxygen metabolism (Winqvist et al,1982), and greater LTC_4 (Shaw et al,1985) and PAF (Fukuda et al,1989) production in response to stimuli such as A23187. It has been found that the hypodense eosinophil possesses greater cytotoxic potential than their normodense counterparts (increased IgG Fc receptors, Winqvist et al,1982; significant release of eosinophil peroxidase, Khalife et al,1986). The

main reason for the reduced density in this type of eosinophil appears to be significantly smaller granules together with reduced protein content e.g. major basic protein (MBP). Figure 1.3 illustrates a possible explanation for the generation of hypodense eosinophils.

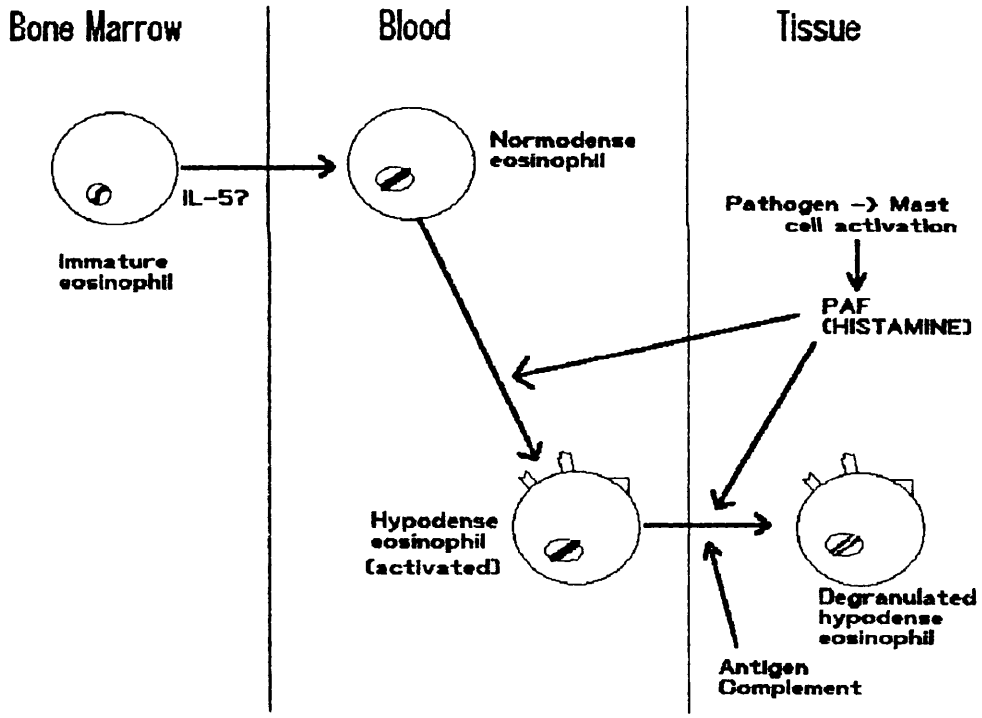

Figure 1.3 Schematic representation of the generation of hypodense eosinophils through the action of PAF.

The chemotactic response of eosinophils to PAF is greatest in normodense cells (Sigal et al,1987). It was stated earlier that the hypodense eosinophils appear to have more potent cytotoxic properties so Sigal's observations seem inconsistent with a hypothesis where PAF initiates the host response to pathogen invasion.

In explaining this paradox, one should perhaps consider a cycle of events where PAF actually stimulates production of the hypodense cells and, during this metamorphosis, the normodense cells, having been enticed into the target area by the action of PAF, release some of their granule contents and, in particular, major basic protein while assuming a more appropriate metabolic state to deal with a pathogen. It has been shown that incubation with PAF results in such a morphological and metabolic transformation (Kloprogge et al,1989).

That the chemotactic effects of PAF are receptor mediated has been shown by a number of different studies. The ginkgolide, BN 52021, inhibited the chemotactic effect of PAF in a dose dependent manner (Tamura et al 1987). It was also found that incubation with PAF rendered eosinophils refractory to further chemotactic responses to PAF.

Cetirizine, a potent anti-allergic compound with antihistamine activity, has also been shown to inhibit PAF-induced chemotaxis. The action was non-specific however since the drug also inhibited FMLP effects (De Vos et al,1989).

Bruijnzeel et al (1989) found that the mast cell stabilising drugs sodium cromoglycate (DSCG) and nedocromil sodium, as well as BN 52021, inhibited PAF-induced chemotaxis. Both the former drugs were also effective against FMLP, but BN 52021 was considerably less potent. When PAF-induced Ca^{2+} mobilisation was measured however, only BN 52021 was found to be an effective inhibitor.

Kurihara et al (1989) found that BN 52021 was significantly more potent in inhibiting the PAF-induced chemotactic effects on eosinophils than neutrophils. Furthermore, it was found that specific binding to eosinophils by ^{3}H-PAF was inhibited in a dose dependent fashion. These workers were unable to demonstrate any inhibitory activity for nedocromil or DSCG on PAF-induced chemotaxis.

3. PAF and endothelium

In an interesting recent publication, Zimmerman et al (1990) demonstrated an important role for PAF in regulating the behaviour of leucocytes in relation to vascular endothelium. It was shown that agonists capable of promoting PAF synthesis in endothelium (thrombin, LTC_4, H_2O_2) also promote endothelium-dependent adhesion of neutrophils. Tight temporal coupling was found between accumulation and degradation of PAF and the development and disappearance of the cellular adhesion. Together with the association of desensitisation of neutrophils by PAF pre-incubation with inhibition of the neutrophil-endothelium binding, these

observations make a strong case for the role of PAF as a cell-bound intercellular signal for adhesion. Furthermore it was found that endothelial cells do not possess the capacity for the de novo pathway of PAF synthesis; instead PAF is synthesised via the rapid remodelling pathway which is thought to be the pro-inflammatory pathway (see section B.2).

Similar observations have been made for eosinophils (Kimani et al,1988). Binding to endothelium was stimulated by exogenous PAF in this case. Expression of a surface glycoprotein complex, Mac-1, which has been implicated in leucocyte adhesion, was enhanced by PAF.

CHAPTER II

PAF AND DISEASE

A. Asthma

Establishing a role for PAF in asthma has captivated major research interest for several years. The basis of this interest has been that PAF has a number of biological properties relevant to allergic disease and, in particular, the pathogenesis of asthma.

1. Vascular permeability

Vascular permeability is increased by PAF and Table 2.1 summarises supportive published data. Generally, the evidence suggests that the potency of PAF on vascular permeability is many times greater than conventional pro-inflammatory mediators such as histamine and prostaglandins. Investigation of potential interactions between PAF and other such mediators has produced conflicting results. It has been reported that neither H_1-antagonism nor cyclooxygenase inhibition influence the PAF response (Archer et al,1985), which led the authors to conclude that the response was independent of histamine and prostaglandins. There is, however, considerable evidence to the contrary (Hwang et al,1985;Pirotzky et al,1984;Oh-ishi et al,1986; see also Table 2.1).

SPECIES	METHOD	REMARKS
Rat -skin	^{125}I-HSA	1000x potency of H, independent of cell infiltrate (Gerdin et al,1985)
	^{125}I-BSA	Lyso-PAF ineffective, independent of cyclooxygenase, H_1-antagonists, neutrophils, 100x potency of 5-HT, 1000x H (Pirotzky et al,1984)
	Evans blue	9000x potency of H, independent of H_1-antagonism (Humphrey et al,1982)
		Immediate onset (Hwang et al,1985)
	Carbon label	Permeability increases limited to post-capillary venules (Humphrey et al,1984)
	Pontamine blue	Independent of 5-HT + H, partially suppressed by indomethacin, significantly suppressed by dexamethasone (Oh-ishi et al,1986)
Guinea pig-skin	Evans blue	10,000x potency of H (Humphrey et al,1982)
		>1000x potency of H and BK, 5-10min delay in onset, inhibited by cyclooxygenase inhibitors (Hwang et al,1985)
	Carbon label + histology	(Dewar et al,1984)
Rabbit -skin	Evans blue	1000x potency of H (Humphrey et al,1982)
	Carbon label	Probably direct action on venular cells, independent of neutrophil and mast cell activation (Humphrey et al,1984)
Sheep -lung	Lymph: plasma ratio	Independent of cyclooxygenase metabolites and vascular surface area alterations (Burhop et al,1986)

Table 2.1 Evidence of PAF-induced vascular permeability changes in various species. Legend: H = histamine, BK = bradykinin.

Overall, the published data appear to indicate a direct effect of PAF on vascular permeability, which may be enhanced by secondary release of eicosanoids, but which has been shown to be uninfluenced by prior platelet depletion (Paul et al,1984).

In man, intradermal injection of PAF results in rapid flare and weal formation within 10 to 15 minutes of treatment (Archer et al,1984). The appearance and pharmacology of this phenomenon bears similarities to the effects of histamine in the skin. In fact, it has been shown that H_1-antagonists inhibit the PAF induced responses in the skin although effects on flare are more marked than for weal (Archer et al,1985). This observation is in contrast to experiments in animal models where H_1-antagonists have not been shown to influence the effects of PAF.

As has been shown in animal studies, prostaglandins act synergistically with PAF in promoting weal reactions in human skin (Archer et al,1984). Michel et al (1987), using a skin window technique, demonstrated that PGE_2 enhanced protein diffusion induced by PAF.

2. Cell Recruitment

The chemotactic effects of PAF have already been discussed (see Chapter 1). A useful clinical model for investigating the cellular events initiated by PAF is that of intradermal skin injection. It has been shown that PAF in human forearm skin induces an intravascular accumulation of

neutrophils together with a predominantly neutrophilic perivascular cellular infiltrate which occurred at around 4 hours post injection (Archer et al,1985). Lyso-PAF was found to be ineffective in promoting cellular recruitment in this study. In another study conducted in rat skin, the dose-dependent induction of plasma protein extravasation by PAF was accompanied by platelet accumulation, although the extravasation of protein was shown to be independent of either platelets or neutrophils (Pirotzky et al,1984). Prior intravenous injection of PAF completely abolished this response and once again lyso-PAF was found to be ineffective. Moreover, the extravasation was not inhibited by indomethacin, methylsergide or pyrilamine-maleate thus excluding a role for platelet-release products.

More recently, using a skin window technique in atopic subjects, intradermal injection of PAF has also been found to produce an eosinophilic skin infiltrate (Henocq et al,1988). This cellular response was found after 24 hours and contrasted with a predominantly neutrophilic infiltrate in normal subjects. A clinically observable 'late phase' response which had been earlier reported (Archer et al,1984), was not evident in this study. Michel et al (1985) found PAF to be a weaker chemotactic agent than LTB_4 for neutrophils in human skin (10^{-8} vs 10^{-7}M). In the same study, the presence of albumin was found to be imperative for PAF activity. It has also been shown in normal subjects that while PGE_2 enhanced neutrophil diffusion into a skin chamber, the synergistic activity of

PGE_2 was more marked with LTB_4; this may reflect the relative potencies of the two inflammatory mediators.

3. Bronchoconstriction and hyperreactivity

When given intravenously to guinea-pigs, PAF produced increased bronchial resistance to inflation (Vargaftig et al,1980). This phenomenon was accompanied by hypotension and thrombocytopaenia. It was found that the bronchoconstriction was inhibited by prostacyclin and prior platelet depletion, indicating an intermediary role for a platelet derived substance. Interestingly, the effects of PAF on platelets in this study (ATP release, aggregation) were independent of cyclooxygenase and no evidence of TXA_2 release was found. Under direct in vitro exposure to PAF, guinea-pig lung parenchymal strips contracted in a manner independent of platelets (Stimler et al,1983). The concentrations required for this latter effect were however noticeably higher than those required in the i.v. experiment; in addition, it appeared that the effect of PAF could have been via parasympathetic stimulation since it was abolished by atropine.

In rat lung tissue, PGE_2, which stimulates adenylate cyclase in airways, inhibited the PAF-induced formation of LTC_4 and D_4 while $PGF_{1\alpha}$, which does not inhibit adenylate cyclase, had no effect on the response (Di Marzo et al,1987). In the same study cAMP inhibited the response to PAF indicating that the PG effect is mediated by the nucleotide.

Vasoactive intestinal polypeptide has also been shown to inhibit PAF-induced leukotriene release in rat lung (Beaubien et al,1984). β-receptors are known to stimulate adenylate cyclase and it is interesting to note that PAF has been shown to reduce the density of such receptors in human lung tissue without changing the affinity (Agrawal et al, 1987); thus the potency of isoprenaline in reversing histamine- or methacholine-induced constriction was significantly reduced by PAF. This effect of PAF was inhibited by the ginkgolide, BN 52021. Oliver et al (1985) demonstrated that 5-lipoxygenase inhibitors significantly inhibited the bronchoconstrictive effects of PAF in rat lung.

Vargaftig et al (1980) reported that prostacyclin (PGI_2) pre-treatment inhibited PAF-induced increases in bronchial resistance although the hypotensive effects of the phospholipid were unaffected. In man, however, prostacyclin was not shown to influence PAF-induced bronchoconstriction (Lammers et al,1990). It should nevertheless be noted that the doses of PGI_2 used in this study were not sufficient to inhibit ADP-induced platelet aggregation; consequently it could be argued that the experimental design was flawed.

The effect of PAF on human lung tissue was reported by Schellenberg (1987) to be dependent upon platelets as had been reported for guinea pigs (see above). PAF (10^{-9} to 10^{-6}M) failed to cause contraction in isolated human lung smooth muscle strips but when PAF activated platelets were added, contraction was observed. The contractile response was

thought to be due to a mediator other than TXA_2, leukotriene, 5-HT or ADP since these factors did not provoke contraction directly in isolated human airways. Conversely, more recent work has demonstrated a direct effect of PAF on isolated human airways (Johnson et al,1990). The discrepancy was thought to be linked to the use of albumin in the latter study and not in the former. Nevertheless, inhaled PAF has been demonstrated to provoke acute bronchoconstriction in human subjects (Cuss et al,1986; Rubin et al,1987), together with, at high doses, thrombocytopaenia (Gateau et al,1984). PAF-induced bronchoconstriction has also been shown to be subject to rapid tachyphylaxis (Cuss et al,1986;Chung et al,1989) making dose response studies extremely difficult. There appears to be no significant difference in the acute PAF-induced bronchoconstriction between normal and asthmatic subjects (Rubin et al,1987;Chung et al,1989). Transient neutropaenia following PAF inhalation has been observed, and this phenomenon also shows tachyphylaxis (Chung et al,1989).

The relationship between histamine and PAF has been investigated in man. Ketotifen, primarily an antihistamine, inhibited the cutaneous but not the airway responses to PAF in normal volunteers (Chung et al,1988) indicating at least a role for the amine in the dermal responses to PAF. Other workers have concluded that such a relationship is minimal (Archer et al,1985).

Probably one of the most significant findings in recent years of PAF research has been the demonstration that PAF pre-

treatment increases responsiveness of lung tissue to histamine or methacholine. Christman et al (1987) showed in an awake sheep model that the PAF-induced changes in dynamic compliance closely correlated with increases in responsiveness to histamine. In a mixture of normal and atopic subjects, Cuss et al (1986) observed a significant increase in responsiveness to methacholine (although not in all the subjects studied) following PAF inhalation, which was most marked at three days post PAF challenge and lasted from one to four weeks. Lyso-PAF was found to be inactive.

A subsequent study by Rubin et al (1987) confirmed that PAF inhalation was capable of increasing the responsiveness of normal subjects to methacholine but did not show similar effects in asthmatics. This intriguing finding was confounded by observations that PAF inhalation provoked increased methacholine responsiveness in mild asthmatics but not in more severely affected patients (Kim et al,1988). More recently, Chung et al (1989) were able to show further evidence of PAF-induced hyperreactivity in normals.

Antigen challenge is used frequently as a predictive model for potential anti-asthma drugs. Many atopic subjects demonstrate a so-called 'late response' (secondary reduction in airway function) following antigen challenge. Whilst it is tacitly proposed by some that PAF inhalation may also offer an in vivo laboratory model for asthma, no such late response following PAF-inhalation has yet been reported. Furthermore, no convincing explanation has yet been forwarded to explain

the hyperreactivity phenomenon, although the in-vitro findings by Agrawal et al (1987, see above) indicate that PAF-induced down-regulation of β-receptors could contribute. No further strong evidence has emerged to support the hyperreactivity action of PAF; in fact recent evidence appears to refute the finding (Spencer et al,1990;Lai et al,1990). The rapid tachyphylaxis to the phospholipid has undoubtedly hampered controlled studies.

4. PAF antagonists and asthma

There is a conspicuous absence of published data on the effect of PAF antagonists in clinical asthma. Studies examining the effects in putative models of asthma are more forthcoming. The gingkolide mixture, BN 52063, was found to significantly inhibit the bronchoconstriction due to PAF inhalation in normal man (Roberts et al,1988). Again, a neutropaenic effect was noted which was not inhibited by BN 52063. After 3 days of treatment, the same compound significantly inhibited antigen-induced acute bronchoconstriction when compared with placebo in atopic asthmatic subjects (Guinot et al,1987). No conclusive evidence of an effect on late hyperreactivity (6h post antigen, acetylcholine challenge) was demonstrated. A more recent study of the effect of BN 52063 on cutaneous responses to antigen in man (Roberts et al,1988) demonstrated inhibition of the late onset response to antigen, but no significant effect on flare and weal. Conversely, the PAF-induced flare and weal response was significantly inhibited, indicating that

PAF may not be involved in the acute response to antigen. Histamine-induced responses were unaffected by the PAF antagonist.

A more potent PAF antagonist, WEB 2086, has been found to inhibit PAF-induced hyperresponsiveness to histamine in isolated human lung tissue (Johnson et al,1990), as well as inhibiting the direct PAF-induced bronchoconstriction both in vitro and in vivo (Adamus et al,1990). These results indicate that both the acute constriction and the provoked hyperreactivity due to PAF are receptor mediated in man.

B. Renal Disease

Infusion of PAF via the renal artery in anaesthetised dogs has been shown to result in a dose-dependent decrease in blood flow, glomerular filtration rate (GFR), urinary flow and sodium excretion (Scherf et al,1986;Hebert et al,1987). That these effects have been seen without any measurable systemic changes indicates that the renal effects of PAF are direct and not a result of other haemodynamic alterations. It has been proposed that the effects of PAF on GFR are explained by a post-glomerular vasodilatation in conjunction with contraction of mesangial cells leading to increased permeability (Camussi et al,1989). Consistent with these observations, PAF has been shown to increase urinary protein excretion in isolated perfused rabbit kidney (Perico et al,1988). Bolus injections of PAF, again in isolated perfused rat kidney, were associated with histological damage at the proximal tubular level

together with significant histamine release (Alessandri et al,1988).

BN 52021 has been found to inhibit the renal effects of PAF in dogs in a dose-dependent manner (Hebert et al,1987) indicating that the effects in the kidney are receptor mediated. The renal changes associated with PAF are essentially the same as would occur in an acute shock situation. Terashita et al (1985) found that the structurally related antagonist to PAF, CV-3988, completely inhibited endotoxin-induced hypotension. More recently, rat glomerular mesangial cells were shown to produce and release PAF in response to E. Coli endotoxin in a dose and time-related fashion.

PAF has been implicated in studies of renal graft rejection. In a rabbit model of hyper-acute rejection, a rare but serious condition in man characterised by vasoconstriction accompanied by intravascular accumulation and aggregation of platelets and leucocytes resulting in ischaemic necrosis, release of PAF into venous effluent has been observed followed by the cellular recruitment phase which leads to the pathological changes (Camussi et al,1987). Prior treatment with BN 52021, together with cyclosporin A, has resulted in prolongation of cardiac graft survival time in rats (Foegh et al,1986).

C. Cardiovascular disease

When PAF is administered systemically, a marked hypotensive response is the most acute effect and has been noted in a wide variety of species including guinea pig, rabbit and man (Vargaftig et al,1980;McManus et al,1980;Gateau et al,1984). In vitro studies of the isolated rat heart have demonstrated that PAF exposure results in a dose-dependent coronary artery vasoconstriction and decreased cardiac contractility (Piper et al,1986). Additionally, release of LTC_4, LTB_4, PGE_2 and TXB_2 have been measured in the cardiac effluent. The coronary vasoconstriction was inhibited by both indomethacin and FPL 55712 (a 5-lipoxygenase inhibitor) whereas the contractility changes were only modified by indomethacin. Thus it has been concluded that both cyclooxygenase and 5-lipoxygenase metabolites mediate the coronary effects of PAF. In guinea pigs, PAF was found to reduce the threshold concentration for ouabain-induced arrhythmias (Riedel et al,1987). Inhibition of 5-lipoxygenase abolished the arrhythmogenic action of PAF, while aspirin was not found to have any significant effect.

D. Pregnancy

Recently, studies have implicated a possible role for PAF in embryo implantation and foetal development. O'Neill et al (1987) found that production of embryo-derived PAF correlated with subsequent embryo quality and viability. Successful embryos, i.e. those resulting in pregnancy,

produced significantly higher levels of PAF in vitro than those that failed. In a subsequent study, supplementation of culture medium with PAF during in vitro fertilisation procedures was associated with a higher rate of pregnancy as compared with a control group (O'Neill et al,1989). PAF supplementation did not, however, influence the outcome of pregnancy. The conclusion of the study was that PAF may mediate pre-embryo development.

E. Skin

The relationship between PAF and the pathophysiology of the skin is one of particular interest. The skin offers a useful model for investigating the reactions to PAF. Administration can be relatively confined while the effects can be seen easily and evaluated rapidly. In such circumstances it might be expected that a clear picture should emerge of the exact mechanisms involved in the PAF-induced changes in the skin. There is, however, controversy over certain attributed effects and further investigation of this has formed the basis of the present thesis.

The acute responses to intradermal PAF are flare and weal (Archer et al,1984). The reaction is similar in appearance to that of histamine and a transient itch has been reported (Fjellner et al,1985). The time course of the flare response is more acute than that of weal (Archer et al,1984); the maximal flare was reported to occur at around 5 min post intradermal injection and to have disappeared by 25 min. The

times for weal were 15 min and 90 min respectively. Over the dose range 30 ng (50 pmol) to 200 ng (350 pmol) per site, a clear dose response relationship was evident for weal but not so marked in the case of flare. Lyso PAF was found to be inactive.

A late onset response was also reported comprising an area of erythema occuring at 3-6 hours after injection and associated, on occasion, with hyperalgesia. The area was described as indurated and was repeatable, although only occuring at all in certain subjects. Histological examination of the responses to PAF in the skin revealed an extravascular infiltration of neutrophils by 4 to 12 hours followed by a predominantly lymphocyte infiltrate at 24 hours (Archer et al,1985). The conclusion of this study was that, by virtue of its ability to induce inflammatory cell accumulation, PAF should be considered as a potential mediator in such inflammatory conditions as psoriasis. In fact, PAF has been measured in psoriatic plaque at nanogram levels per 100 mg scale (Mallet et al,1985).

When the effects of various pharmacological agents were studied in relation to PAF responses, it was found that while H_1-antagonism by chlorpheniramine inhibited flare and weal slightly, indomethacin was found to have no effect (Archer et al,1985). Ketotifen has also been found to inhibit PAF-induced responses in the skin (Chung et al,1988). Serum albumin was found to exert a significant potentiating influence on the PAF responses (Archer et al,1985). PGE_2 has

also been found to have a synergistic effect on the PAF responses (Archer et al,1984). Fjellner et al (1985) found corroberative evidence that histamine antagonism (mepyramine) inhibited PAF-induced flare. Weal response was not measured in this latter study; however it was also found that PAF-induced itch was inhibited by antihistamine treatment thus further implicating histamine as a secondary mediator to PAF. When the effects of C_{16} and C_{18} were compared in the skin (Archer et al,1988), no significant difference was found in the dose response curves.

More recently, workers have studied the cellular effects of intradermal PAF using skin window techniques. Henocq et al (1988) found no clinical evidence of a late response, but were able to show a late phase response histologically. In normal subjects, PAF injection resulted in a largely neutrophilic infiltrate, while in atopic subjects the infiltrate was largely eosinophilic with 30 to 40% degranulation. The maximal cell response was measured at around 24 h post injection. The flare and weal response to PAF was not found to differ quantitatively between the atopic and the normal group.

BN 52063 has been shown to inhibit the flare and weal response to PAF in a dose related manner (Chung et al,1987;Roberts et al,1988). Cetirizine, a potent H_1-antagonist, was reported to have not only inhibited the acute flare and weal response to PAF, but also the eosinophil infiltrate response to allergen.

In summary there appears to be controversy in the literature as to the nature of the late response to PAF. Additionally there is conflicting evidence regarding the role of histamine in PAF-induced responses in the skin.

A series of studies has been undertaken to define accurately the characteristics of the acute responses to PAF in the skin and to study the role of histamine in these responses. By way of comparison, the acute responses to other inflammatory mediators (PGE_2, substance P, and CGRP) has also been investigated. The effects of histamine antagonism, and depletion on the PAF responses have been examined together with evaluation of local histamine release from the skin after PAF treatment. Additionally, a comparison of the PAF-induced responses in atopic and non-atopic subjects has been undertaken.

Each of the following five chapters will describe practical work together with a summary of experimental results. Presentation of each individual study will contain a brief section detailing conclusions while full discussion of the results will be found in Chapter 8 together with a general conclusions section.

CHAPTER III

HYPERALGESIA

A. Introduction

Inflammation is the reaction of living tissue to injury. It comprises the series of changes in the terminal vascular bed and perivascular tissue tending to eliminate the injurious agent and to repair the damaged tissue. One of the most characteristic features of inflammation is hyperalgesia. The phenomenon of hyperalgesia may be defined as reduced threshold to pain and, as such, has been the subject of scientific investigation for many years.

An informative review on the subject by Bilisoly et al (1954) described how hyperalgesia was a component of a complex series of inflammatory events involving both vascular and neural changes. For instance, in urticarial reactions under experimental conditions, vasodilatation and oedema was accompanied by increases in 'vulnerability' of the skin to a variety of different stimuli. Since the 'triple response' phenomenon, first described by Lewis (1924), was known to have a neural component, attempts were made to establish a relationship between the vasodilatation, pain threshold and axon reflex. The experimental results led to the conclusion that pain threshold appeared to be reduced within the flare zone of the axon reflex during the time period of flare. Lewis also showed that this area of hyperalgesia was associated with an increased susceptibility to tissue damage,

and concluded that while the local inflammation protects the whole organism, it does so at the cost of the integrity of a part.

In the seminal studies of the triple response, Lewis found that vascular occlusion of the study site resulted in prolongation of the reaction, and thus inferred that the response was primarily due to a vasodilator substance which could not dissipate under conditions of vascular occlusion.

Considerable progress has been made over the last 20-30 years in elucidating the mediators of the acute inflammatory response. Much of the research effort has been directed toward elucidating the role of arachidonic acid metabolites after membrane disruption. The response of mammalian cells to potentially disruptive stimuli involves a specific, self-amplifying sequence of responses, initiated usually by membrane receptor stimulation in a suitable cell type (eg mast cell via cross-linkage of receptors) followed by increased adenyl cyclase activity and resultant enhanced cAMP and phospholipase C activity. Inositol triphosphate is released which raises cytosolic Ca^{2+} and, consequentially, phospholipase C. As described in the introductory chapter, this latter enzyme is associated with the 'remodelling' or 'rapid response' route of PAF synthesis as well as arachidonic acid and hence prostaglandin production.

Early studies in human skin with PAF reported hyperalgesia as a phenomenon (Archer et al,1984). This was demonstrated using subjective ratings of "tenderness" from

visual analogue scales. It was therefore decided to investigate whether PAF could be a mediator of hyperalgesia in tissue damage. Since a methodology more objective than visual analogue scales would be desirable, consideration was given to the various models reported in the literature.

B. Hyperalgesia models

There is much electrophysiological evidence to support the hypothesis that afferent, unmyelinated (or c-) fibre nerves can promulgate the sensation of pain in response to physical stimuli in mammalian skin (Zotterman,1939; Beck et al,1974); furthermore these fibres have been shown to serve receptors which, in the cat, characteristically respond to multiple stimuli (Bessou et al,1969). Hence the term 'polymodal' has been coined to describe functionally these receptors. A further type of fibre which, although myelinated, has similar multiple functional modality has also been shown to contribute to pain sensation; these fibres tend, however, to have high thresholds to physical stimuli compared with c-fibres (Campbell et al,1979) Such receptors have been identified in human skin (Torebjork et al,1984).

Hyperalgesia is characterised by a decrease in the threshold for eliciting pain and/or enhanced pain produced by a suprathreshold stimulus. In the skin, as in other tissues, it is the perceptual component of inflammation. Typically, this inflammation is the result of physical injury such as burning, crushing or freezing. Probably the best

characterised provoking agent is temperature. In a study of the responses evoked by a 53°C 30-sec stimulus on the glabrous skin of human volunteers, a resultant blister was observed in 50% of subjects after several hours (Meyer et al,1985). Furthermore, a marked decrease in pain threshold to either heat or pressure was demonstrated in the affected area within minutes. For example, a 41°C stimulus was not painful before the burn, but was rated as painful as a 49°C stimulus had scored before burn. Similarly a mechanically stimulated (Von Frey probe) pain threshold decreased from 12.6 bars to 5.4 bars as a result of the experimental burn.

It has been shown, therefore, how a physical stimulus may, in sufficient degree, cause hyperalgesia. It has also been demonstrated that the same stimulus, at a reduced intensity, may be used to detect the presence of hyperalgesia. Hence a clear distinction should be made between the two ways in which a stimulus may be used; the former may be termed hyperalgesic agent or HA while the latter may be referred to as the pain stimulus or PS.

Several pain models have been used experimentally, not merely to demonstrate hyperalgesia but also to evaluate potential analgesics. Two measures of perception are most often used in such studies: the point of pain threshold and that of pain tolerance or 'ceiling' pain. Both of these indices have arguments for and against. Pain threshold has the significant advantage that it is unlikely to cause significant tissue damage and is therefore convenient for

repeated site application; threshold has however been the subject of criticism from various sources as both a relatively poor index of clinical pain (Wolff,1978) and likely to produce equivocal results when used in analgesic studies (Kutscher et al,1957). Equally, pain tolerance has been reported to be a more reliable measure of pain (Wolff,1978), but appears to be more susceptible to manipulation by the subject and its validity has been drawn into question in cross-modal studies (eg electrical vs pressure pain).

Measurement of pain in the human is clearly fraught with difficulty since one is dealing with a perception rather than a directly estimable variable. Physiological correlates of pain have been tried without significant success (Chapman et al,1985) It is therefore imperative that any experimental design for algesimetry should attempt to control for psychological variables such as environment, training of the subject in how to make known response etc. Mood and degree of potential for distraction should also be carefully controlled. Cognitive or 'attitudinal' variables were identified by Clark et al (1956) as probably largely responsible for inter-individual differences in pain threshold.

Broadly speaking, the majority of paradigms involving measurements of pain in the skin of human volunteers fall into two main categories.

1. Temperature

Both heat and cold have been used; as discussed above varying degrees of heat may be applied to the skin under closely controlled conditions to act as either a HA or PS. Radiant heat is probably the more commonly used technique and forms the basis for the ubiquitous 'tail-flick' test used in animals for assessing analgesics.

The traditional apparatus consists of two main components: a "dolorimeter" to control the electrical output and the duration of the PS, and a projection lamp which, through lenses, focuses light and heat onto a fixed surface area. The skin is usually blackened and areas often used are the forehead, forearm and back.

LaMotte et al (1984) report that in normal skin (forearm), the pain threshold was generally between 41-43°C for short duration stimuli and this is consistent with other published data (Meyer et al,1985).

The reproducibility of cold water as a PS was evaluated in a study by Harris et al (1983); the authors found that coefficients of variation for both pain threshold and tolerance exceeded 100%. The results did show that cold as a PS correlated well with pressure and electrical shock for both threshold and tolerance.

2. Pressure

The use of pressure to elicit pain response is well established in animal research (Randall et al,1957). In human subjects it may be the oldest of methods for quantifying pain perception with reports dating back to Victorian times (Keele,1954).

A simple and relatively easy to administer procedure was described by Merskey et al (1964); the so-called 'pressure algometer' consisted of a plunger mounted on a calibrated spring. The flat end of the plunger could easily be applied to bony surfaces such as the forehead or tibia. Although the description of the technique is rather sketchy, the authors report that by applying a pressure of between 0 and 7.75 Kg, assessment of pain threshold (when 'pain began') and an estimate of pain tolerance (when it 'hurt a lot') was possible in various groups of volunteers. Pressure was increased at approximately 1 Kg/sec. Results were relatively more consistent for threshold than for tolerance with coefficients of variation ranging between 22-33% for the former and 19-46% for the latter.

A more sophisticated instrument for assessing the skin response to pressure was described by Handwerker (1984). Using a set of motorised calipers, a controlled pressure could be applied to a skinfold for 2 min periods and subjects could then be asked to assess their pain using visual analogue scales.

Using this technique, it was shown that subjects demonstrated a tendency to rate pain lower from session to session. The author concluded that a prolonged period of training was required to overcome this effect. The same author also investigated the effect of aspirin on pain threshold with placebo control and found that a subjects guess as to which treatment he was receiving heavily influenced pain ratings. Subjects believing themselves to have received aspirin rated a stimulus as much as 20% lower than those assuming placebo to be the treatment. Hence the need for careful blinding and randomisation of controlled studies with adequate pre-study training was clear.

After considering the two approaches, it was decided to use pressure as the PS for responses to intradermal PAF, as it was more likely to be reproducible, simple to apply, quantifiable and non-injurious.

C. Pressure algesimeter

The instrument used to administer the pressure stimulus was designed with the following important pre-requisites:

(a) Safe, non-injurious

The instrument should be able to operate without inflicting any significant degree of tissue damage - certainly it should not be capable of breaking the skin. Additionally there should be a mechanical limit to the operating ability of the instrument.

(b) Pressure precisely controlled

The instrument should be capable of precise and reproducible calibration and able to deliver a controlled pressure to a fixed area of skin.

(c) Robust yet flexible

The instrument should be stable during operation to prevent confounding influences such as tremor to have any effect, while allowing ease of manoeuvre during application at different sites.

The algesimeter equipment is shown in figure 3.1 and consists of the algesimeter itself (figure 3.2), an electronics control box and a hand held release device. A set of standard industrial thickness calipers was modified to form the algesimeter so that the movable jaw is motorised while the

stationary jaw has a sensitive built-in strain guage/pressure transducer.

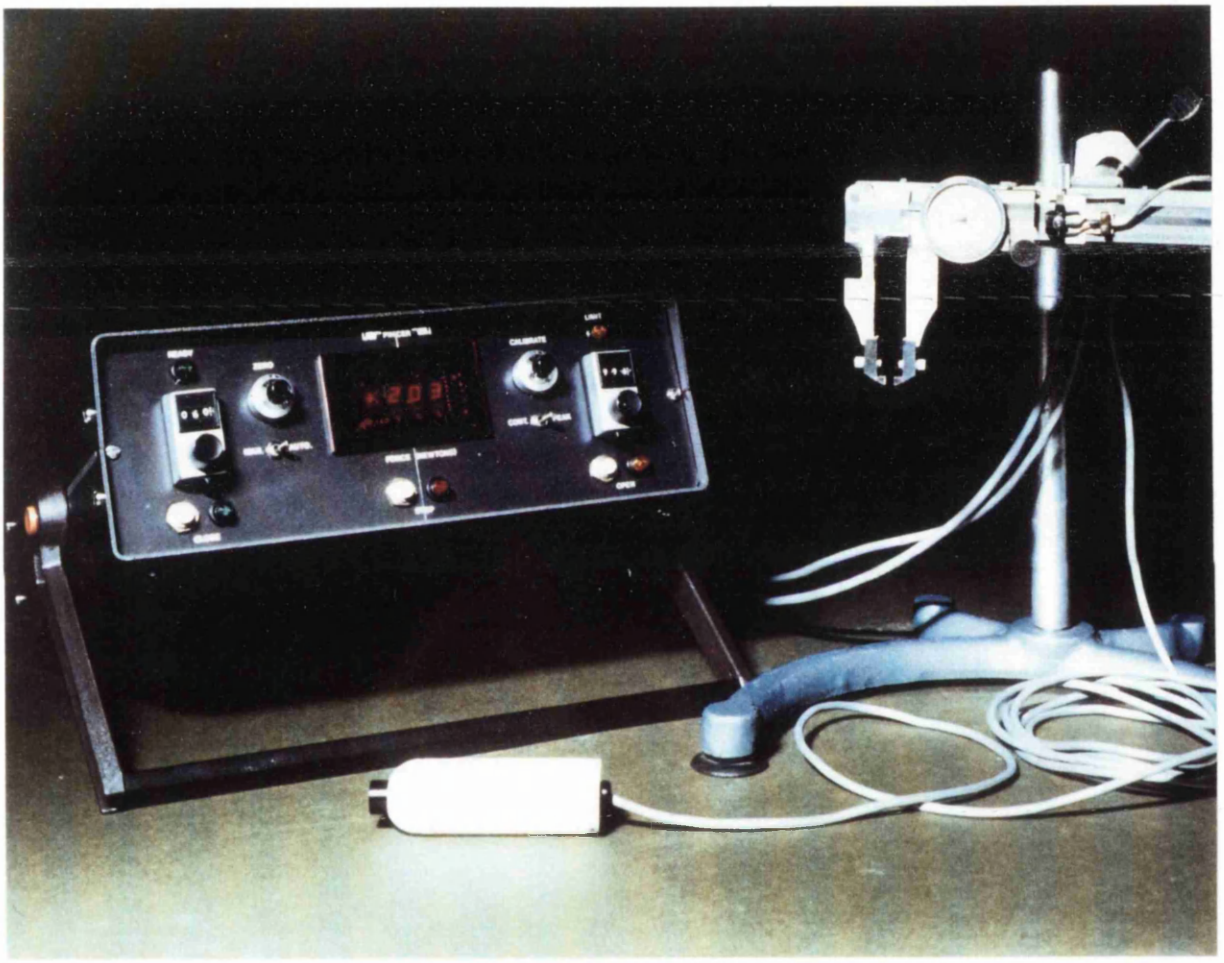

Figure 3.1 The algesimeter equipment consisting of the modified thickness calipers held on a retort stand, the hand held release button (in the foreground) and the electronics box to record pressure (on the left of the photograph).

In operation, pressure detected on the stationary jaw by the sensor was converted to an electrical signal which, in turn, was converted by the electronics box into a digital readout. The electronics box controlled the speed of the motorised jaw, both for closing and opening, so as to provide a variably selectable constant speed. Under normal circumstances the speed of opening would be set at maximum (9 mm/sec) while the speed of closing was set at a considerably slower speed (0.6 mm/sec).

The algesimeter was fixed to a standard retort stand by means of adjustable clamps which allowed full 360° rotation of the algesimeter in any plane together with vertical movement. Hence the 'business end' of the instrument could be moved easily around a locus so that testing various points along a subject's arm could be accomplished without requiring the subject to move his arm. Calibration of the instrument was acheived by suspending a 1 kg weight from the stationary jaw in its vertical position and setting the readout on the electronics box to 9.81; thus the algesimeter would then read pressure applied to a fixed area in Newtons (N) by virtue of the following rationale:

Force (N) = Mass (kg) x Acceleration (gravity, m/s^2)

i.e. 9.81 N = 1 kg x 9.81 m/s^2

The surface area of the point of contact for each jaw was approximately 16 mm^2. The maximum pressure that the instrument was capable of applying was set at 20 N. This

pressure did not cause any sustained bruising or break the skin. All studies were performed on the volar surface of a subject's forearm.

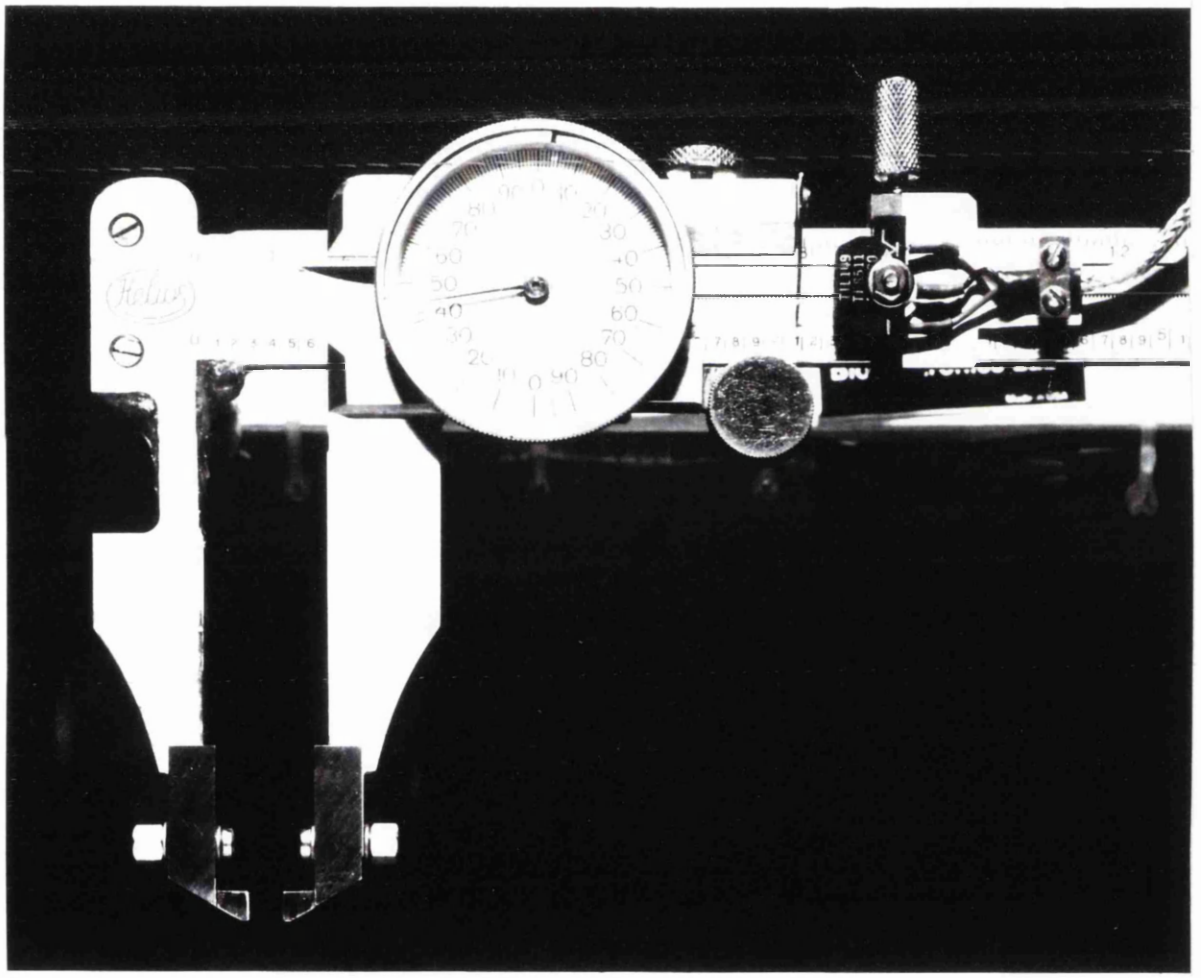

Figure 3.2 The modified thickness calipers; the stationary jaw (left) has a strain-guage incorporated while the moving jaw (right) has been motorised allowing independently controlled opening and closing speeds

During a stimulus cycle, the skin site was held gently between the jaws of the algesimeter by the operator (see figure 3.3) with the jaws having been closed at fast speed and halted upon reaching a gap that would not require much further travel before applying pressure to the skin. The automatic mode was selected on the electronics box which initiated closure of the jaws at slow speed, pinching the site with increasing pressure. The subject would be asked to hold the release device with the button held down. When the button was released by the subject, the jaws would instantly reverse direction and open at the selected faster speed. The electronics box would display the pressure applied at the moment of release for recording purposes, and this pressure was regarded as the pain threshold (PT).

In order to control for environmental and subjective factors, subjects were blindfolded and studies were conducted in a quiet room with only the subject and operator present. Instruction on what was expected of the subject was carefully worded with respect to definition of end points:

> 'As soon as you feel the pinch pressure becoming uncomfortable, you should release the button. It is not necessary that you think too hard about whether the stimulus is painful or not in the true sense of the word, just set in your mind what you regard as an arbitrary point of discomfort and try to reproduce that in each pinch test. Please remember that this is not an endurance test so please try to be honest. During the

test try to relax your mind so that all you are thinking about is concentrating on the skin being squeezed'

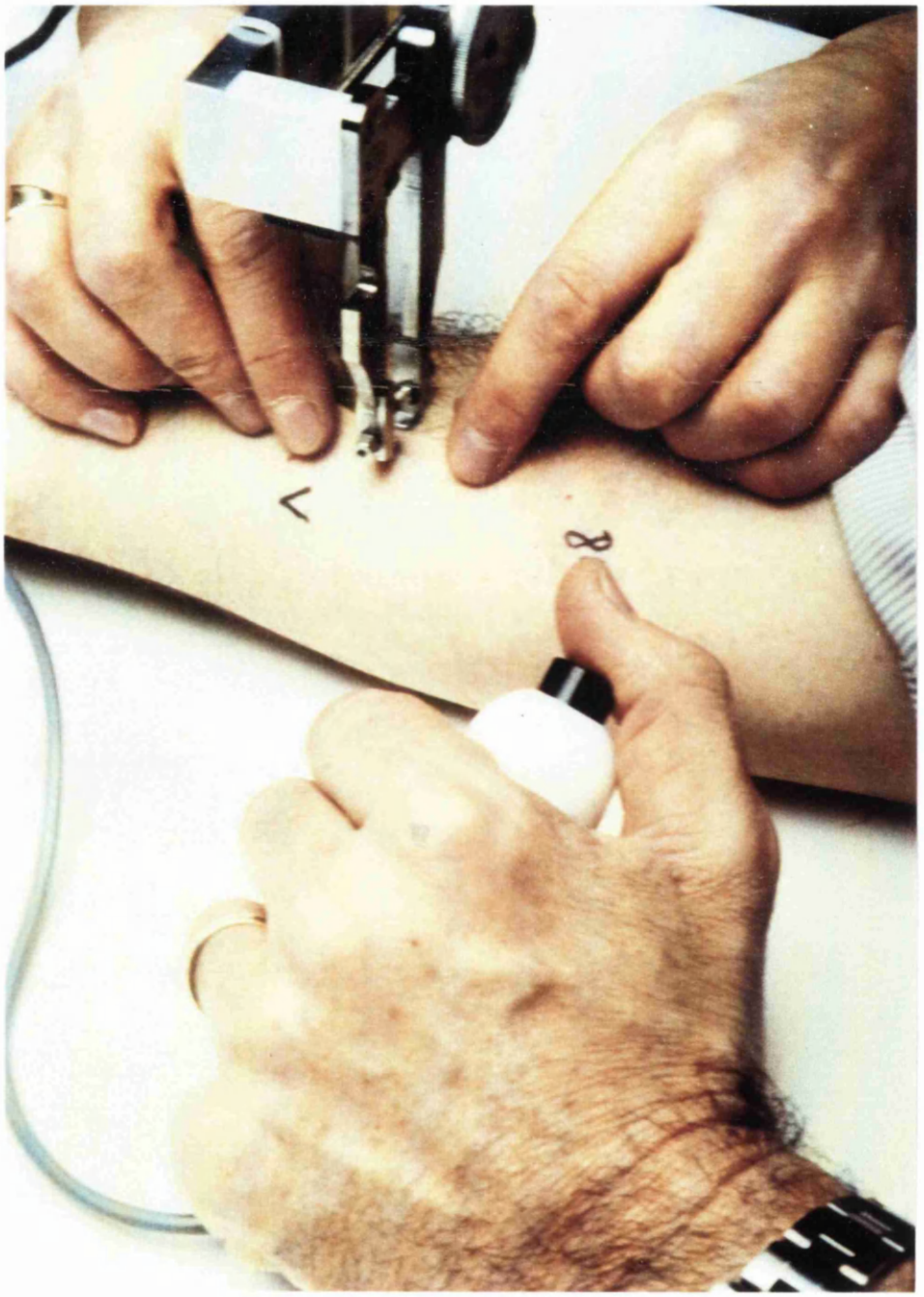

Figure 3.3 Demonstration of the use of the algesimeter equipment at a site on the volar surface of the forearm. The skin is gently held between the jaws of the calipers by the experimenter while the subject holds the release button.

In every experiment, enrolled subjects took part in a pre study screening period where they could get used to the instrument and its operation (training) thus enabling more consistent responses. Certain individuals did not respond before the 20 N limit and were thus excluded during this screening. Other subjects who were not able to give consistent responses were also excluded at this stage.

D. Validation

Before embarking upon formal study of PAF and hyperalgesia, it was important to establish that the methodology was valid. A study was undertaken to examine the nature of the PT response in healthy male adults.

1. Study hypotheses

a. The pressure algesimeter gives measurable pain threshold measurements in healthy volunteers.

b. There are no significant differences between different anatomical sites in pain threshold measurements.

2. Design and methods

PT's were determined in 8 subjects (age range 22-45) on 2 separate days. On day 1, PT was measured in 2 sites per arm (proximal and distal) on 6 occasions separated by 2 minute intervals. The order of testing of sites in any individual was randomised. On day 2, PT was measured in similar fashion

except that instead of 6 assessments at 2 minute intervals, 4 PT measurements were taken on 2 occasions separated by 1 hour.

The rationale was that day 1 results should give an estimate of variability of response at a given site together with variability of response between sites. Day 2 results should allow evaluation of whether there is any 'late onset' carryover effect of the test. This would be important if, in later studies, any PAF-induced hyperalgesic effects lasted for a number of hours and duration of effect needed to be assessed.

3. Results

Results of analysis of day 1 findings are summarised in Table 3.1. Statistical analysis was performed using analysis of variance (ANOVA, SAS statistical software). It appeared that the right arm was slightly more sensitive than the left and the distal site appeared less sensitive than the proximal. Six consecutive pinches at 2 minute intervals yielded negligible, if any,change in sensitivity from the first to last pinch. An interval of 1 hour between tests revealed increased sensitivity at the proximal site but no difference at the distal site.

4. Conclusions

The main conclusion of this study was that randomisation of treatment vs site would be important in a formal study in order to control for the observed inter-site differences.

Otherwise the procedure appeared sensitive enough to allow the study of a putative hyperalgesic agent; the pooled results indicated that a study of 8 subjects under controlled conditions would yield 80% power to detect (at α=0.05, one tailed) approximately 37% increase in sensitivity at a skin site.

Day 1			**PT**	**ANOVA result**
Arm	Left		6.4	p=0.03, difference
	Right		7.0	between arms
Site	Distal		7.0	p=0.01, difference
	Proximal		6.4	between sites
Time		1	6.5	p>0.20, no difference
(at 2 min		2	6.6	for repeated assessment
intervals)		3	6.7	
		4	6.9	
		5	6.8	
		6	6.5	
Day 2				
Site	Proximal	0h	7.6	1h result differs from
		1h	5.4	baseline (0h), p=0.01
	Distal	0h	7.4	No sig differences
		1h	7.5	
Arm	Left		6.7	No sig differences
	Right		7.2	

Table 3.1 Summary of analysis of interactions in algesimeter pain threshold (PT) measurements. Overall mean PT results are given in Newtons; pooled SD within subjects was 1.74N (Day 1) and 1.89N (Day 2).

E. Effects of PGE_2, Substance P and CGRP

Having shown the algesimeter capable of sensitive PT measurements at untreated sites, it was important to evaluate whether the instrument could detect alterations in skin sensitivity using a known hyperalgesic agent. It has been shown that PGE_2 induces hyperalgesia (Ferreira,1972; Ferreira et al,1981; Lorenzetti et al,1985) although the objectivity of published studies is limited. Although PGE_2 has been shown to act synergistically with other mediators of inflammation in the skin to produce an inflammatory response (Williams et al,1977), it has not been found to provoke flare and weal responses to the same extent as histamine when used alone. Nevertheless it was decided to investigate this lipid mediator together with 2 other putative peptide mediators of painful skin responses, substance P and calcitonin gene related peptide (CGRP). Both these peptides produce inflammatory responses when injected intradermally. Substance P produces a flare and weal response similar to histamine (Barnes et al,1986; Devillier et al,1986). CGRP does not cause weal, but does produce a long lasting erythema (Brain et al,1986).

1. Study hypotheses

a. PGE_2, substance P and CGRP reduce pain threshold at skin sites when injected intradermally.

b. The effects of the three mediators are dose-related.

2. Design and methods

The study was conducted in 4 healthy male volunteers (all age <40). Two injection sites were chosen on the volar surface of each subjects forearm. All subjects took part in training sessions with the algesimeter to control for training effects and to check for reproducibility of response. All solutions were made up in normal saline (BP) and ID injections were 50 μl. Substance P (mol. wt.=1348) was prepared at concentrations of 0.27 and 2.7 μg/ml. CGRP was made up to 0.76 and 7.6 μg/ml. PGE_2 was prepared from an ethanolic stock at 1 mg/ml by evaporation of aliquots under nitrogen.

The study was conducted in two parts, 3 of the subjects (PH, TC and JP) took part in both. In the first part low doses (10 pmol per skin site) of CGRP and substance P were used; in the second part a 10 fold higher dose of neuropeptide was used (100 pmol/site). The dose of PGE_2 was the same on both occasions (1 nmol/site). Intradermal injection of treatment was made according to a randomised schedule for site. Saline was used as a vehicle control and injections were performed using 1 ml sterilin syringes and 25 guage steel needles. Coding of solutions allowed a double blind design to be used. Statistical analysis was performed using BMDP software. Specifically, ANOVA (program 2V) was performed on the results for drug and time with log-transformation of the data to allow for unequal variability. The values in the tables are shown as untransformed data.

In addition to pain threshold measurements made at 90, 150 and 210 mins after injection, flare and weal responses were measured according to a standard protocol (see Chapter 4).

3. Results

The algesimeter results of the low dose study are shown in Table 3.2; a significant reduction in PT, compared with saline, was associated with PGE_2 treatment ($p<0.05$). This increased sensitivity appeared to last for at least 3.5 h, although there was approximately 50% decline in the effect between 2.5 and 3.5 h. Neither of the neuropeptides showed evidence of a significant hyperalgesic effect.

Table 3.3 shows the PT results in the low dose study expressed as % saline control. PGE_2 clearly reduced pain threshold as compared with saline by approximately 65%. Similarly, the part 2 results (Tables 3.4 and 3.5), where higher doses of the neuropeptides were tested, revealed an hyperalgesic effect of PGE_2 ($p<0.05$, same dose as part 1). Neither neuropeptide produced a statistically significant reduction in pain threshold.

Subject	Site	Treatment	90min	150min	210min
PC	rprox	PGE2/10nmol	8.70	7.57	11.67
	rdist	CGRP/10pmol	20.00	17.69	18.58
	lprox	SUBP/10pmol	13.46	12.44	16.63
	ldist	Vehicle	19.87	18.99	19.89
PH	rprox	Vehicle	7.93	8.90	8.83
	rdist	SUBP/10pmol	12.90	7.20	12.53
	lprox	CGRP/10pmol	8.56	6.21	7.52
	ldist	PGE2/10nmol	3.26	3.82	3.56
TC	rprox	SUBP/10pmol	3.14	2.43	2.87
	rdist	PGE2/10nmol	1.35	1.67	3.14
	lprox	Vehicle	3.38	4.06	3.99
	ldist	CGRP/10pmol	2.46	4.70	3.94
JP	rprox	CGRP/10pmol	8.23	4.16	8.73
	rdist	Vehicle	7.50	4.16	5.91
	lprox	PGE2/10nmol	1.14	2.32	6.46
	ldist	SUBP/10pmol	8.51	9.86	8.09
Mean		PGE2	3.61	3.84	6.21
SEM			1.76	1.32	1.96
Mean		CGRP	9.82	8.19	9.69
SEM			3.67	3.20	3.13
Mean		SUBP	9.50	7.98	10.03
SEM			2.39	2.14	2.96
Mean		Vehicle	9.67	9.03	9.65
SEM			3.55	3.51	3.55

Table 3.2 Pain threshold (PT, N) data from low dose neuropeptide study in 4 subjects at 90,150,210 min post injection.

Subject	Treatment	90min	150min	210min
PC	PGE2/10nmol	43.78	39.86	58.67
	CGRP/10pmol	100.65	93.15	93.41
	SUBP/10pmol	67.74	65.51	83.61
PH	SUBP/10pmol	162.67	80.90	141.90
	CGRP/10pmol	108.20	69.78	85.16
	PGE2/10nmol	41.11	42.92	40.32
TC	SUBP/10pmol	92.90	59.85	71.93
	PGE2/10nmol	39.94	41.13	78.70
	CGRP/10pmol	72.78	115.76	98.75
JP	CGRP/10pmol	109.73	100.00	147.72
	PGE2/10nmol	15.20	55.77	109.31
	SUBP/10pmol	113.47	237.02	136.89
Mean	PGE2	35.01	44.92	71.75
SEM		6.65	3.67	14.77
Mean	CGRP	97.84	94.67	106.26
SEM		8.59	9.55	14.10
Mean	SUBP	109.19	110.82	108.58
SEM		20.13	42.30	17.98

Table 3.3 Pain threshold (PT, N) data from low dose neuropeptide study in 4 subjects at 90,150, and 210 min post injection. Results expressed as % vehicle.

Subject	Site	Treatment	90min	150min	210min
PH	rprox	PGE2/10nmol	3.37	5.91	4.37
	rdist	CGRP/100pmol	8.42	11.27	6.34
	lprox	SUBP/100pmol	4.42	8.06	6.07
	ldist	Vehicle	9.60	7.85	10.54
TC	rprox	Vehicle	3.66	7.34	2.51
	rdist	SUBP/100pmol	6.43	6.89	3.64
	lprox	CGRP/100pmol	1.74	2.24	1.97
	ldist	PGE2/10nmol	1.40	0.46	1.15
JP	rprox	SUBP/100pmol	5.17	6.27	7.69
	rdist	PGE2/10nmol	3.58	2.81	6.88
	lprox	Vehicle	9.45	8.62	6.07
	ldist	CGRP/100pmol	9.38	10.57	5.35
DM	rprox	CGRP/100pmol	9.85	9.40	9.29
	rdist	Vehicle	13.83	14.34	13.82
	lprox	PGE2/10nmol	4.68	5.48	7.67
	ldist	SUBP/100pmol	6.71	9.50	6.15
Mean		PGE2	3.26	3.66	5.02
SEM			0.68	1.27	1.47
Mean		CGRP	7.35	8.37	5.74
SEM			1.89	2.08	1.51
Mean		SUBP	5.68	7.68	5.89
SEM			0.54	0.71	0.84
Mean		Vehicle	9.13	9.54	8.24
SEM			2.09	1.62	2.48

Table 3.4 Pain threshold (PT, N) data from high dose neuropeptide study in 4 subjects at 90,150,210 min post injection.

Subject	Treatment	90min	150min	210min
PH	PGE2/10nmol	35.10	75.29	41.46
	CGRP/100pmol	87.71	143.57	60.15
	SUBP/100pmol	46.04	102.68	57.59
TC	SUBP/100pmol	175.68	93.87	145.02
	CGRP/100pmol	47.54	30.52	78.49
	PGE2/10nmol	38.25	6.27	45.82
JP	SUBP/100pmol	54.71	72.74	126.69
	PGE2/10nmol	37.88	32.60	113.34
	CGRP/100pmol	99.26	122.62	88.14
DM	CGRP/100pmol	71.22	65.55	67.22
	PGE2/10nmol	33.84	38.21	55.50
	SUBP/100pmol	48.52	66.25	44.50
Mean	PGE2	36.27	38.09	64.03
SEM		1.07	14.22	16.70
Mean	CGRP	76.43	90.56	73.50
SEM		11.22	25.93	6.17
Mean	SUBP	81.24	83.88	93.45
SEM		31.53	8.60	24.91

Table 3.5 Pain threshold (PT, N) data from high dose neuropeptide study in 4 subjects at 90,150,210 min post injection. Results are expressed as % vehicle.

Scrutiny of the data expressed as % vehicle shows that once again PGE_2 produced a 64% decrease in PT which had declined to 34% by 3.5 h post injection. Substance P was associated with a 19% increase in sensitivity which also declined by 3.5 h. CGRP induced hyperalgesia (24% decrease in PT) appeared to wane at 2.5 h post injection but then returned at the 3.5 h assessment. Neither of these effects were however significant.

4. Conclusions

It was shown that PGE_2-induced hyperalgesia in the skin was detectable by the algesimeter and that the effect was reproducible for a given dose over two experiments. It is interesting to note that there were considerable inter-individual differences in PT response but the response to PGE_2 in relation to vehicle (i.e. PGE_2 response expressed as % vehicle response) was similar for all individuals. The data for those subjects who took part in both studies (PH, TC and JP) bear this out particularly well.

F. Effects of PAF

The effects of intradermal PAF on PT were now studied using doses similar to those quoted in the literature as producing hyperalgesia. PGE_2 was used as a positive control.

1. Study hypotheses

a. PAF and PGE_2 reduce pain threshold at skin sites when injected intradermally.

b. The effects of the two mediators are dose-related.

2. Design and methods

Eight volunteers were recruited for study. The protocol for assessing hyperalgesia differed from that described above only in that assessment was timed at 1, 2 and 3 h after injection. Injection were made at 3 sites per arm in each subject. Furthermore, reproducibility was assessed by repeating the experiment after an interval of 1 week. Randomisation of treatment vs site took account the 2-part design. Treatment was as follows:

PAF vehicle: human serum albumen (HSA), 0.25%, in normal saline

PAF doses: 0.7 and 1.4 nmol/site

PGE_2 vehicle: normal saline

PGE_2 doses: 0.01, 1 and 100 nmol/site

Choice of doses was based on the literature. PAF had been shown to provoke a hyperalgesic response at both the above concentrations in an open study (Archer et al,1986). The range of doses of PGE_2 was chosen also using the literature as a guide (Ferreira,1972) together with preliminary pilot studies. The wide choice of doses was made on the assumption that PGE_2 was a positive control and therefore a more rigorous examination of dose-response would be desirable. The PAF dose was chosen with the prime intention being to assess whether the mediator caused hyperalgesia per se, and so two relatively high doses were used. Safety was monitored by measuring peak expiratory flow rate (Mini-Wright Peak Flow Meter) and taking blood for routine haematology and chemistry before and after treatment.

HSA was supplied by Immuno UK Ltd and passed as heat treated and free of viral contamination. PAF was made up by dilution of an ethanolic stock solution with normal saline. The final concentration of ethanol did not exceed 0.2%. All injection solutions were made up on the day of study in polypropylene vials (Eppendorf).

Human serum albumen was used in the PAF vehicle as previous work had shown that it facilitated solubilisation of PAF (Ludwig et al,1986). Statistical analysis of the data was performed using parametric ANOVA. Computerised statistical software (SAS) was utilised, specifically the GLM procedure (Type III sum of squares).

3. Results

No subjects withdrew from the study because of excessive side effects. Complaints were limited to anticipated effects of intradermal injection of an inflammatory agent, such as itching and tenderness. No clinically significant effects on either haematological or biochemical parameters were seen, nor were there any effects on lung function.

The mean PT measurements following PAF injection are shown in Figure 3.4. There was no detectable effect of PAF on skin sensitivity at either of the two concentrations used. There were no significant interactions of site, time or week on the data. There was, however, a significantly increased sensitivity in the left arm than in the right arm at 2h post injection ($p<0.05$). No differences in PT were found between any of the time points which could be attributed to treatment.

PGE_2 produced a dose dependent hyperalgesic response (Figure 3.5). There was a consistent decrease in mean PT with increasing dose of PGE_2 at all 3 timepoints and PT responses to all doses were significantly different from vehicle. The difference between successive doses of PGE_2 declined between the 1 h and 3 h assessments (cf. earlier study of PGE_2 effects on PT).

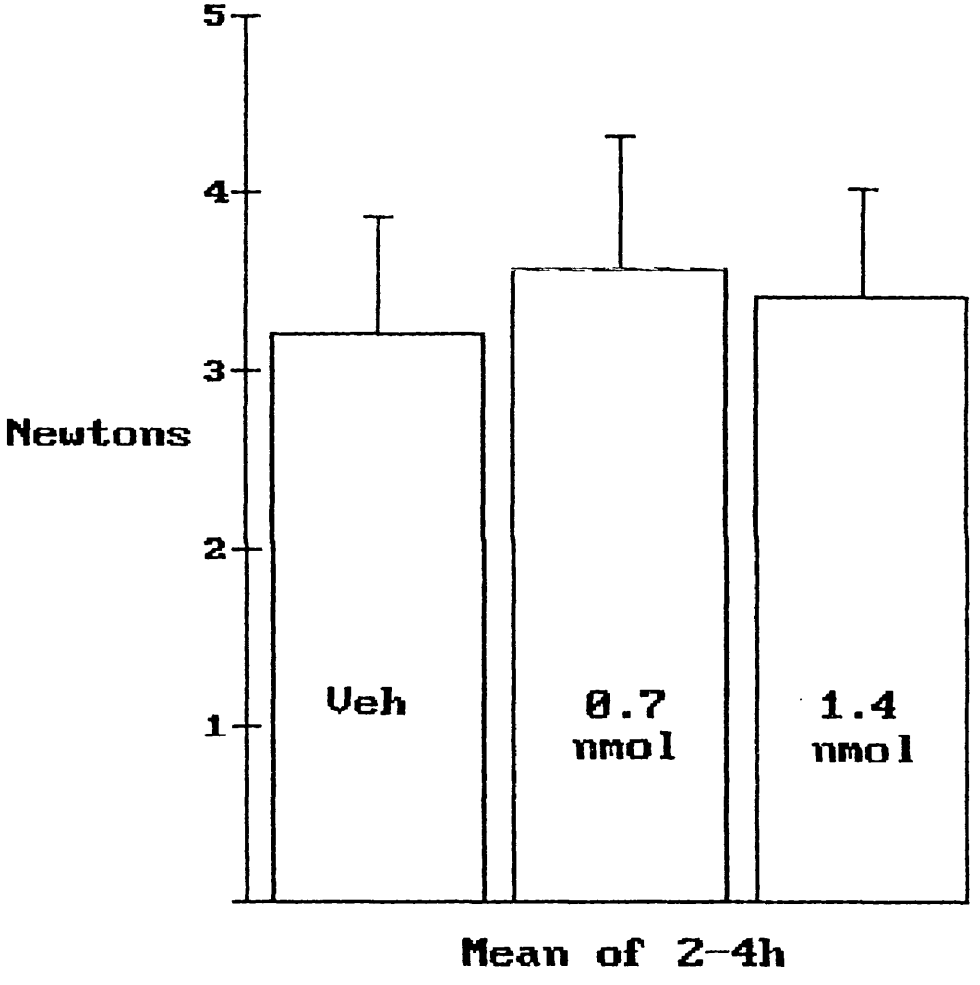

Figure 3.4 Mean pain thresholds estimated as pressure (Newtons) applied at hourly intervals following intradermal PAF injection at the volar forearm surface. Results are expressed as mean of 2 weeks (n = 16 observations in eight subjects + s.e. mean)

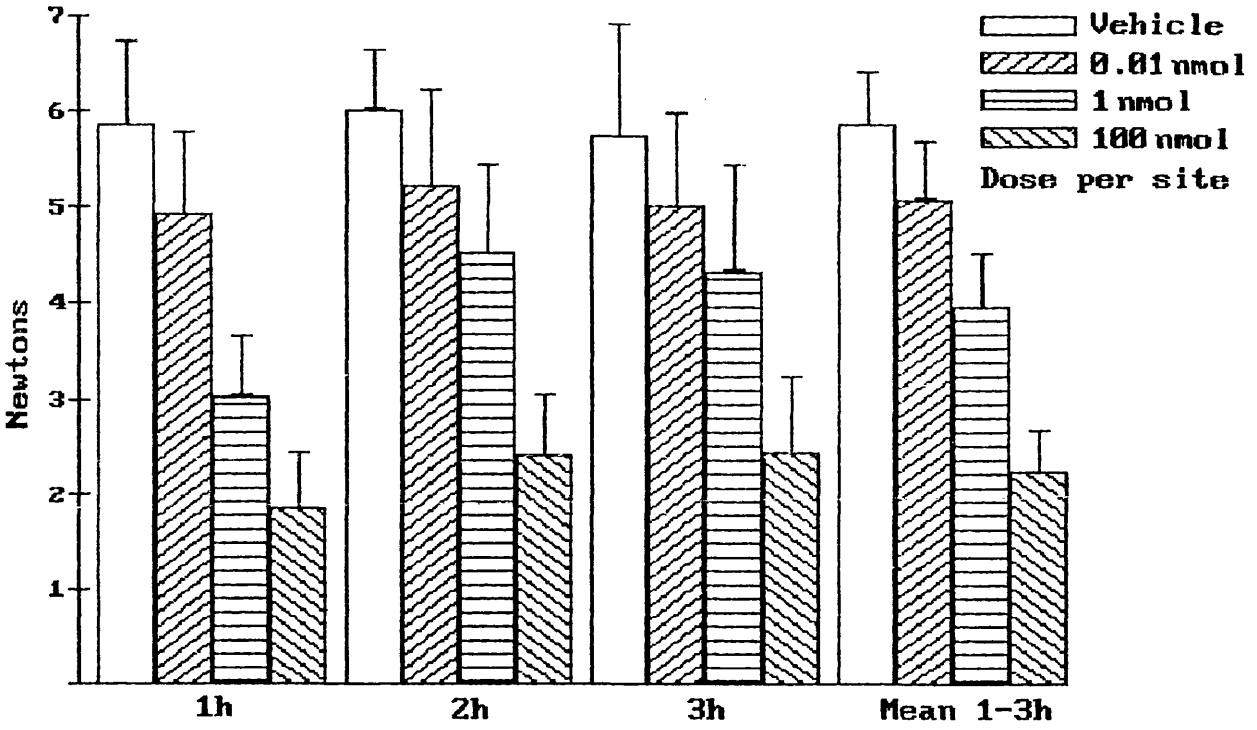

Table of statistical significances (P values)

Time post injection	Comparison	Vehicle	0.01nmol	1nmol
1h	0.01nmol	=0.04		
	1nmol	<0.01	<0.01	
	100nmol	<0.01	<0.01	<0.01
2h	0.01nmol	NS		
	1nmol	<0.01	NS	
	100nmol	<0.01	<0.01	<0.01
3h	0.01nmol	NS		
	1nmol	=0.01	NS	
	100nmol	<0.01	<0.01	<0.01
Mean 1-3h	0.01nmol	<0.01		
	1nmol	<0.01	<0.01	
	100nmol	<0.01	<0.01	<0.01

Figure 3.5 Mean pain thresholds estimated as pressure (Newtons) applied following intradermal PGE_2 injection at the volar forearm surface. Results are expressed as mean of 2 weeks (n = 16 observations in eight subjects + s.e. mean)

All pairwise comparisons between doses at 1 h after injection were statistically significant, but by the 2 and 3 h the significant difference between vehicle and the the low dose of PGE_2 and between the low and middle doses of PGE_2 had disappeared.

Mean PT at the distal site appeared to be lower than proximal site ($p<0.01$) at 1 h and this was reflected in the mean regardless of timepoint. Only the between-week comparisons at the distal site in the first hour were significant at the 5% level. The distal site appeared to be more sensitive on week 1. All other between-week comparisons were not significant.

4. Conclusions

The objectives of this study were to assess quantitatively the effects on pain threshold of intradermally injected PAF and PGE_2. The reproducibility and dose-relationship of observed responses were also addressed.

A hyperalgesic response to PAF assessed as change in pain threshold to skin pressure could not be demonstrated. The effect of 0.7 and 1.4 nmol could not be distinguished from vehicle. PGE_2, conversely, was associated with a clear dose-related and significant hyperalgesia. Comparison of responses to vehicle between studies indicated that PAF vehicle was associated with greater sensitivity than PGE_2 vehicle. It should be pointed out, however, that in the earlier neuropeptide study, differences in individual sensitivity were

quite large but did not influence the overall % reduction in PT caused by PGE_2. Hence valid comparisons for the subjective pain threshold responses must be made within each experiment. Different subjects were used in each study.

A separate study was conducted to compare the effect of the two vehicles on PT using a within subject, double-blind design. Saline was compared with saline plus HSA. No significant differences were found between the two vehicles (Table 3.6)

Treatment	PT 1h	PT 2h	PT 3h
H	6.52±0.73	6.16±0.59	7.74±0.67
S	5.90±0.60	6.43±0.75	6.49±0.53

Table 3.6 Pain threshold (PT) estimated as pressure (Newtons) applied at hourly intervals following intradermal injection of saline (S) or 0.25% HSA/saline (H) in healthy male volunteers (n = 4, means ± s.e. mean). PT was assessed at 1, 2 and 3h after injection.

The PGE_2 results enabled validation of the algesimeter as a sensitive and reproducible method for measuring changes in pain threshold and hence hyperalgesia. Intradermal PGE_2 was shown to produce a dose-related and prolonged hyperalgesia unlike PAF which was not associated with hyperalgesia.

CHAPTER IV

FLARE AND WEAL

A. Introduction

Lewis (1924) first described the inflammatory changes which result from cutaneous trauma. In a series of classic experiments, he demonstrated how vigorous rubbing of the skin in healthy subjects (or merely stroking in urticarial patients) resulted in redness, followed by a flush and subsequently wealing of the skin. A typical response pattern would be as follows:

> Within seconds, a central and sharply defined red line, following the course of the stroke or rub, and deduced to be due to a 'local dilatation of the superficial capillaries, venules and terminal arterioles'; within the first minute or so, a flush began to spread in an irregular fashion over the surrounding skin to an average diameter of up to 3 or 4 centimetres; finally, after one to two minutes, a weal began to develop as a raised area of oedema over a considerably smaller area than the flush, but still centered around the location of the initial trauma. The reaction had substantially diminished by 40 minutes post trauma.

This characteristic series of events later came to be known as the 'triple response': redness, flushing (or flare) and wealing. Lewis was fascinated by the similarity between the skin response to trauma and the consequences of

intradermal application of histamine to the skin. He used a variety of manoeuvres to overcome the technical difficulties of administering the histamine without the convenience of modern fine-guage needles.

In one method the skin was scarified and then painted with a dilute solution of histamine. In another, the sharp hairs of mucuna were rubbed onto the skin so that subsequently applied histamine could enter through the resulting perforations. This method was later refined by substituting finely spun glass for the hairs. Both of the above methods were somewhat less than reproducible as well as being intrinsically inflammatory; they were thus abandoned by Lewis (no doubt to the relief of his subjects). Probably the most reliable method found was that of 'drawing' capillary glass tubing to an extremely fine point which could then painlessly pierce the skin and deliver a controlled dose of histamine. Further investigations showed how the temperature of the skin affected by the flare rose during the response. Additionally, if small amounts of fluid withdrawn from wealed skin were applied to normal skin and the skin punctured through the fluid, a further weal response occured.

Apart from similarity of the inflammatory changes to those following histamine, perhaps the most significant observation concerned the role of nervous tissue. If the skin was pre-treated with a local anaesthetic, the flare response was substantially diminished. Conversely, central nerve anaesthesia or section eg blockade of the ulnar nerve trunk at

the elbow, did not reduce the triple response to trauma or to histamine at all.

Lewis concluded that the physiology of the triple response involved release of a chemical substance in the skin which had a histamine-like action on the blood vessels and nerves. The initial redness was associated with a local vasodilatation independent of nervous tissue; the flare was caused by a widespread dilatation of the arterioles, dependent upon a local reflex (shown in Figure 4.1) and increased permeability of local vascular tissue which, like the initial redness, was independent of nervous tissue.

Over the next 40 or so years, evidence grew to suggest that while histamine was implicated in the initiation of the triple response and causation of the local wealing, the true perpetrator of the antidromic reflex was another mediator entirely. Specifically, a number of groups were able to show that antihistamine treatment did not diminish vasodilatation resulting from antidromic stimulation (Holton et al,1951; Frumin et al,1953). Parrot (1954) concluded from his studies that trauma-induced histamine release 'is liable to stimulate peripheral nerve endings of the vasodilator fibres and elicit a vasodilator response' thus acting 'not as a mediator at the end of the axon reflex, but as the stimulus'. Various alternative molecules came under suspicion; e.g. acetylcholine (Dale,1934) and ATP (Holton,1959).

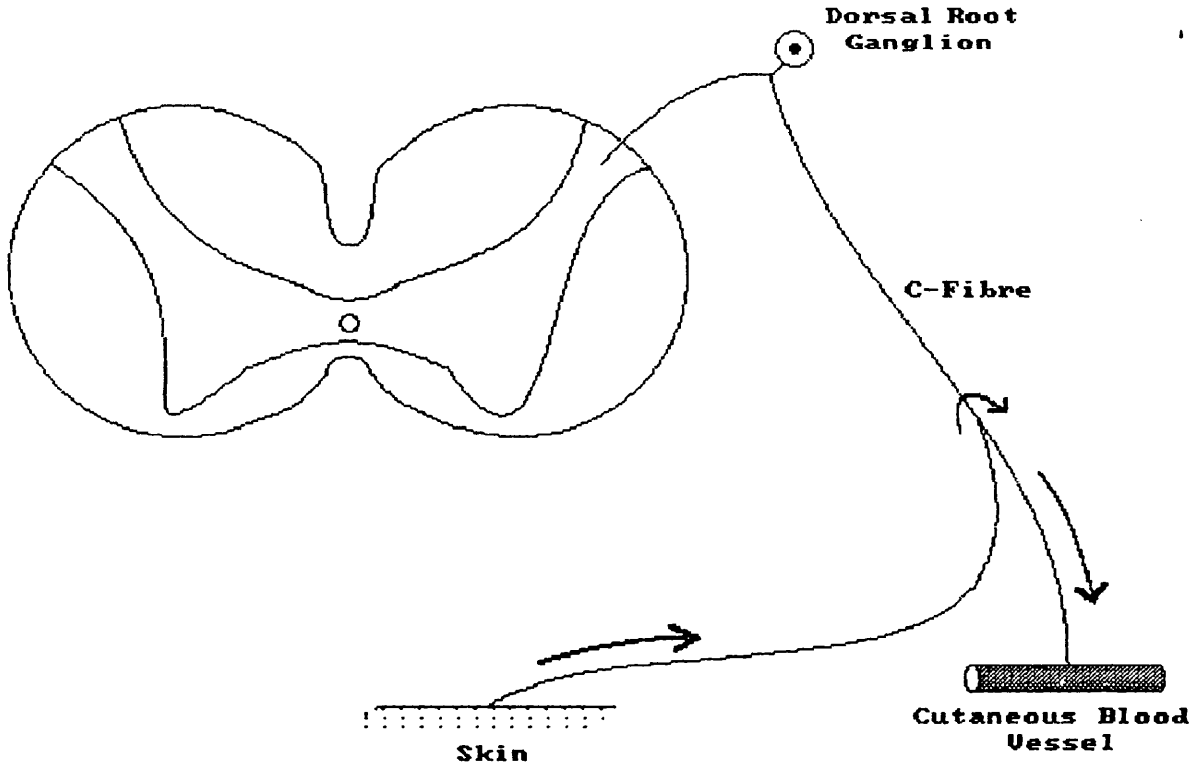

Figure 4.1 Diagrammatic representation of the antidromic reflex involving a C-fibre with an intact dorsal root ganglion. The action potential originates in the skin terminal and, instead of travelling solely to the central ganglion, loops back via a local branch to a terminal located around local superficial vascular tissue also in the skin

Some 40 years later, a series of experiments were conducted by Wolff's group examining the effects of various kinds of neural lesion on the axon reflex (Chapman et al,1961). In addition they were able to collect a subcutaneous perfusate during vasodilatation evoked by skin trauma. The results confirmed Lewis' findings in that while ganglionectomy abolished the axon reflex, section of the dorsal root fibres between the ganglia and the spinal cord (leaving the ganglion intact) did not affect the capacity for

the axon reflex. Perfusate collected during vasodilatation and flare bore many pharmacological similarities to polypeptides such as bradykinin or oxytocin. As a result of its association with neurogenic vasodilatation, the term 'neurokinin' was coined for the mediator.

Jancso et al (1968) confirmed the independance of the neurogenic (flare) and local vascular permeability (weal) responses. It was shown, in rats, that local leakage of systemically administered Evans blue dye, as a result of capsaicin administration, remained undiminished by lignocaine-induced local anaesthesiae. When, however, the irritant was administered to skin in which local enervation had been destroyed (eg saphenous severing), the denervated skin remained uncoloured.

In human subjects, capsaicin similarly failed to produce any sign of an inflammatory response in denervated skin (subjects with nerve injuries). Conversely, compound 48/80, a polyamine with potent mast cell degranulating properties, and histamine were found to be produce weal without a flare in denervated skin. Lignocaine, likewise, virtually abolished the flare component. Repeated treatment with capsaicin was found to desensitise skin to its own effects.

Using direct stimulation of the saphenous nerve, Lembeck et al (1979) were able to further elucidate neurogenic vasodilatation. Substance P, a polypeptide occuring in sensory fibres of the saphenous nerve, was implicated as the 'neurokinin' released antidromically by sensory nerve

terminals. This deduction was based upon a close correlation between the pharmacology of the peptide and that of direct antidromic stimulation, together with the circumstantial evidence of its presence at the effector site.

The new hypothesis was that substance P, released from nerve terminals provoked the release of histamine from mast cells. This attractive theory gave a plausible functional explanation for the ubiquitous anatomical proximity of mast cells and sensory nerve terminals. More recently however, Barnes et al (1986) demonstrated that, while substance P may well induce histamine release from skin mast cells, terfenadine had no effect on the flare response to capsaicin; these data effectively contradicted the substance P/histamine hypothesis for neurogenic inflammation.

Calcitonin gene-related peptide (CGRP) has, like substance P, been shown to reside in sensory nerve terminals (Rosenfeld et al,1983). The peptide has been shown to produce prolonged vasodilatation which was unaffected by either H_1-antagonism (chlorpheniramine) or local anaesthesia (prilocaine, Piotrowski et al,1986). Unlike other inflammatory mediators such as substance P, histamine etc, CGRP is not associated with weal or conventional flare upon intradermal injection (Brain et al,1986). As a result of its closer pharmacological fit with the evidence, CGRP is currently under investigation as the most likely contender for the title of the elusive 'neurokinin' of neurogenic vasodilatation.

As has already been discussed, intradermally injected PAF is reported to be associated with a flare and weal response similar to that of histamine. It has been shown that both flare and weal components of the PAF-induced triple response may be reduced by H_1-antagonism (Chung et al,1988), although some believe that weal is unaffected (Archer et al,1985). Before examining the pharmacology of the PAF response and attempting to resolve the conflict, the dose relationship and variability of the PAF-induced flare and weal methodology was investigated. After evaluation of the technique, attention was then given to the importance of human serum albumin (HSA) in the vehicle for PAF and, subsequently, what alterations might be made.

B. Methodology

A model for evaluating flare and weal should primarily have the following characteristics:

Practical: a site should be chosen where experiments may be performed conveniently and repeatedly.

Measurable: with the inherent difficulties of measuring area on a curved surface accurately, the recording technique should be precise and not affect the response itself.

In animal studies, a number of elegant techniques are used such as Evans blue, radiolabelling albumen and punching out skin samples. Clearly none of these are practical for

human studies. Lewis' original work (1924) was based upon planimetry of the skin and this has formed the basis of the vast majority of such research since. A modification of the planimetry technique was described by Basran et al (1982) where perpendicular diameters were used to estimate the area of response while thickness calipers were also used to estimate the degree of change in skin thickness as a result of oedema.

In the present studies, further refinements were made to this methodology. Instead of using diameters, flare and weal areas were traced onto transparent acetate paper with an indelible marker. The area of the resulting permanent tracings could conveniently be measured later using computerised image analysis. Additionally, hand-held, spring-loaded calipers were used to take rapid skinfold thickness measurements. A commercially available portable thickness guage (Mitutoyo^{R}) comprised of a stationary, fixed jaw and a sliding jaw (see Figure 4.2). A finger-operated button opened the sliding jaw (against a low tension spring) enabling a skinfold to be introduced between the jaws. Normal skin gave very consistent and robust readings. When the button was released, however, onto a weal-containing skinfold, there was a momentary point when the thickness reading remained constant, after which the reading began to decline, presumably as the tension of the spring caused a redistribution of the underlying oedema. This meant that measurements had to be taken quickly at post-injection sites and the manner in which the guage was applied needed to be consistent.

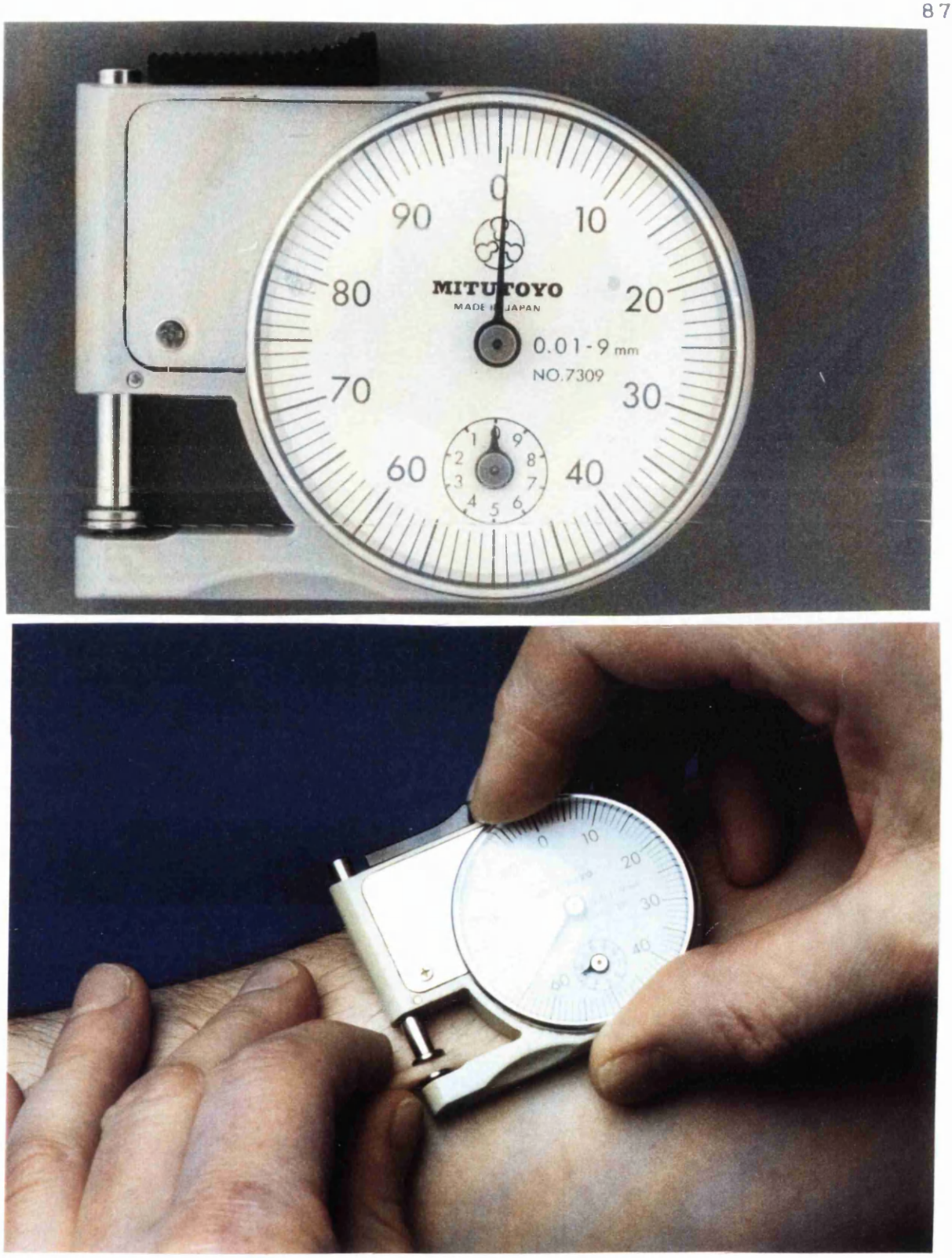

Figure 4.2 Mitutoyo spring-loaded calipers used to measure skinfold thickness at injection sites. The finger-operated button opens the upper, low-tension spring loaded jaw of the instrument; a skinfold around the injection site is inserted between this jaw and the stationary jaw and the button released.

1. Intradermal injection (see also Appendix 1)

All intradermal treatments were administered using 1ml sterilin syringes together with 25-guage needles. Injection solutions were always prepared fresh on the morning of a study day and all equipment used was sterilised. No needles were ever used more than once. Evidence in the literature indicated that a protein carrier such as HSA is important for the PAF-induced responses (Archer et al,1985, see also Chapter 2, section B). All injection solutions were therefore prepared using 0.25% HSA in normal saline for the first dose response study described below. The source of HSA was Immuno (UK) and the material was guaranteed in the product license to be heat-treated as well as having been screened for viral contamination.

The volar forearm surface was used in all studies for convenience. Intradermal injections were 50μl and generally took less than 5 secs to administer. Care was always taken to avoid areas of skin dyspigmentation and superficial veins. Except where specifically stated, 3 sites per arm were always used, proximal, medial and distal, each separated by >5 cm to avoid overlap in response. Allocation of site to treatment was always randomised and double-blind with respect to observer and subject. Injections were always made with the needle

inserted in a proximal direction along the axis of the arm.

2. Flare

Evidence in the literature suggested that the flare response is maximal at around 5 min post-injection (Archer et al,1984). Observations in the current studies were consistent with previous work hence all measurements of flare were made at 5 min post-injection. The observer placed a piece of transparent acetate paper on the arm over the injection site and the area of observed flare was traced using an indelible, fine, black pen. The irregular margin of the flush was often not clearly defined, tending to 'fade-out' with the perimeter often resembling more of a fine mosaic of discrete red spots than a uniform erythema; frequently small patches appeared to be isolated from the main bulk of the flare. The flare usually had several pseudopodia-like 'limbs' stretching out from the central area. In tracing the flare the observer needed to be consistent about the level of intensity where the line should literally be drawn. No isolated patches of the kind described above were included in the trace. Flare area (FA, mm^2) was later estimated directly from the acetate using an image analyser (Quantimet 920, Cambridge Instruments).

3. Weal

In similar fashion to the flare response, the timing of the measurement of weal was consistent with previous literature (Archer et al,1984) where 15 min post injection was shown to be the time of maximum response. A tracing of the weal area (WA) was made again on acetate paper. The weal was far more clearly defined than the flare, being an area of raised, pale skin identical to the standard histamine weal (see Figure 4.4). As with the FA, use was made of an image analyser for calculation of the area (mm^2). A typical trace of the response is seen in Figure 4.3.

In addition to measuring area, an estimate of weal volume was made by measuring skinfold thickness (T) at the site of injection immediately before (T_0) and at 15 min post-injection (T_{15}). The skinfold thickness measurements (mm) were made using commercially available spring-loaded calipers (Mitutoyo, see Figure 4.2). An estimate of weal volume (WV) was made by the following calculation (nb : δT was halved since a fold of skin was used to estimate thickness):

Change in skin thickness (δT) = ($T_{15}-T_0$)

$$WV\ (\mu l) = WA \times (\delta T/2)$$

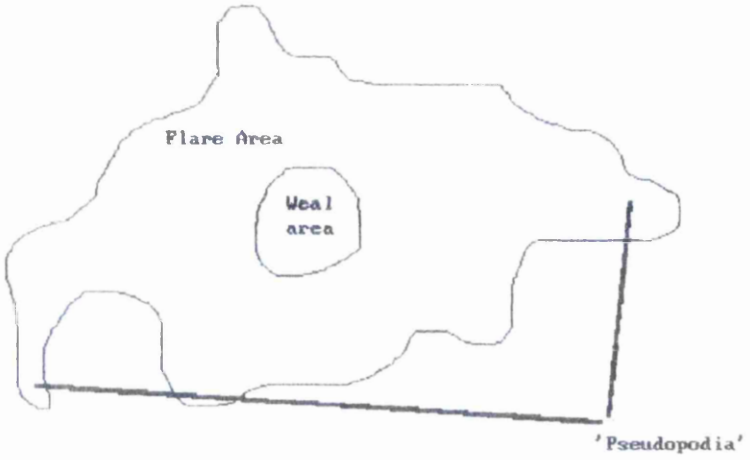

Figure 4.3 A typical trace of the flare and weal response onto acetate paper. The two areas were estimated by planimetry.

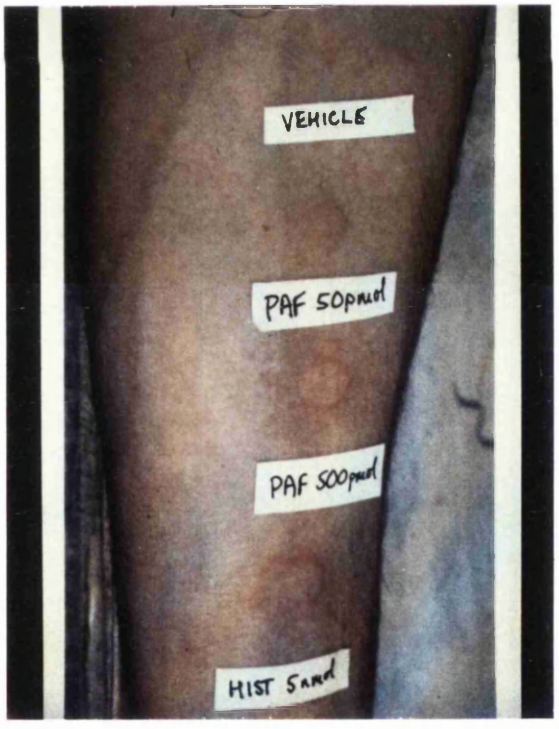

Figure 4.4 Comparative appearance of the PAF and histamine-induced flare and weal response in human forearm skin; vehicle response is also shown.

C. Dose relationship and variability of flare and weal

1. Study hypotheses

a. The flare and weal responses to intradermal PAF are dose-related over the range 20 to 500 pmol.

b. The administration of PAF intradermally over the 20 to 500 pmol dose range is safe and well tolerated by healthy subjects.

2. Design and methods

The study was a single day study in eight healthy male volunteers. On the study day 3 sites were chosen on both the left and right forearms of each subject. Following baseline skin thickness measurements at each site (T_0), each subject received intradermal injection of vehicle (0.25% HSA/saline), or 20, 50, 200 or 500 pmol PAF. Histamine (5 nmol) was used as a positive control for comparative purposes in the study. The methods for flare and weal measurement were as described above and in Appendix 1.

In order to establish the safety of the procedure, special attention was payed to monitoring each subjects lung function, blood biochemistry and haematology. Assessments were made of peak expiratory flow rate (PEFR, Mini-Wright peak flow meter) before and at 2 and 4 hours after treatment.

Blood samples were taken pre-injection and 4 hours after treatment for routine haematology and biochemistry.

Statistical analysis of the results was by use of parametric analysis of variance (ANOVA, specifically procedure GLM, Type III sum of squares in SAS statistical software). Allowance was made in the model used for subject, arm, site, and dose. The normality assumptions of ANOVA were tested using the Wilk-Shapiro statistic and was accepted for all parameters. The homogeneity of variance assumption was tested using the Hartley Fmax statistic which did not appear to have been violated for any response variable. All tests of significance were 2-tailed at $\alpha = 0.05$ and all p values were rounded off to 2 decimal places (see also Appendix 1).

3. Results

No significant effects were found on lung function as evidenced by PEFR (Pre:662±48, 2h post:683±46, 4h post:673±49 l/min). No clinically significant changes were noted in either the haematology or biochemistry safety data. No subjects were withdrawn from the study because of adverse experiences and, apart from reports of 'tenderness' and 'itching' at and around the injection site, no other adverse effects were noted. ANOVA results are displayed in Table 4.1 (excluding the histamine results).

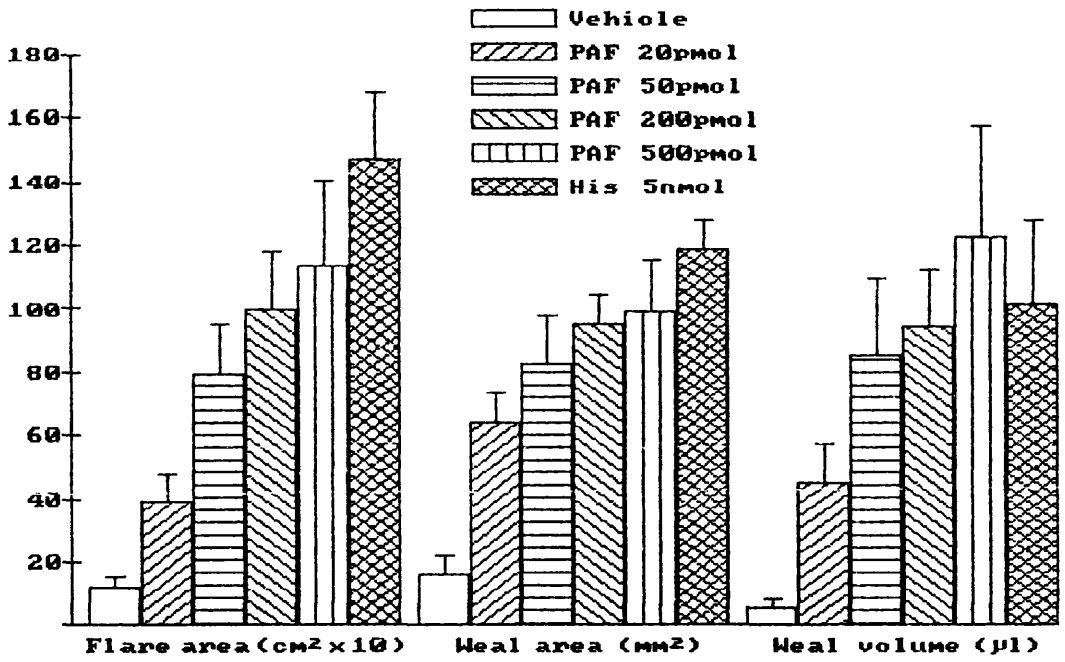

Figure 4.5 Flare (FA) and weal responses (WA,WV) to intradermal injection of PAF or histamine (mean + sem, n = 8). FA was measured at 5 mins post injection while WA and WV was measured at 15 mins post injection. Vehicle was 0.25% HSA in normal saline.

Dose means (including histamine results) and pairwise comparisons are shown in Tables 4.2 and 4.3, and site means and pairwise comparisons in Table 4.4. Finally Table 4.5 shows arm means and pairwise comparisons.

Dose comparisons (Tables 4.2 and 4.3) The mean response of FA, WA and WV after injection of vehicle was consistently lower than after any other treatment and was always significantly lower, except after the 20 pmol/site dose of PAF, for FA and WV. After injection of histamine, the mean responses of WA and FA and of WV were higher than after any other dose except after the 500

pmol/site dose of PAF for WV. The mean response after injection of histamine was significantly higher versus the reponse after vehicle or 20 pmol PAF for all three variables (pairwise p-values <0.01). With respect to FA, the mean response was also significantly higher after histamine than after 50 pmol or 200 pmol PAF (pairwise p-values <0.01 and 0.02 respectively), but not significantly different from the 500 pmol dose of PAF (pairwise p-value 0.09). With respect to WA and WV the differences in mean response after histamine versus that after 50, 200 or 500 pmol PAF were not significant (pairwise p-values ≥ 0.08).

For all three variables, tests for linear trends for the response across the PAF range were significant (Table 4.1). Comparison of the sums of squares for the linear trend with the total sums of squares for dose indicated that 60 to 90% of the variation in the dose-response could be explained with a linear equation. The rest of the variation appeared to be explained by a cubic trend i.e. there appeared to be some curvature in the response across dose. Examination of the histogram of response across doses (Figure 4.5) reveals that the curvature appears to derive from a plateauing of the response at the higher doses of PAF, particularly for FA and WA.

Response	FACTORS: Subject	Arm	Site	Dose	[1]SD	Linear [2]DR
Flare area	<0.01	>0.20	>0.20	<0.01	3.18	<0.01
Weal area	<0.01	>0.20	<0.01	<0.01	0.15	<0.01
Weal volume	<0.01	>0.20	<0.01	<0.01	0.35	<0.01
Skin Thickness (T_0)	<0.01	>0.20	>0.20	>0.02	0.23	>0.02

[1]Within subject (pooled)
[2]Dose response trend

Table 4.1 Analysis of variance results from flare and weal dose response study, showing level of significance (or otherwise) of each of the factors included in the ANOVA model. Mean pre-injection skinfold thickness (T_0) data is also shown for comparison.

Response	PAF: Veh	[1]20	50	200	500	Hist [2]5
FA(cm^2)	1.23	3.92	7.94	9.99	11.36	14.7
WA(mm^2)	16.4	64.4	82.8	95.3	99.0	119.2
WV(μl)	5.69	45.5	85.6	94.8	123.1	102.5
[3]ST (mm, T_0)	2.75	2.74	2.73	2.82	2.77	2.99
[4]STC (mm, $\delta T/2$)	0.36	0.65	0.66	0.75	0.90	0.88

Table 4.2 Mean response data for each dose of PAF and for histamine. Also shown is the mean pre-injection and 15 min post-injection skin thickness data (change in thickness divided by 2). [1]pmol, [2]nmol, [3]ST-skin thickness, [4]STC-change in skin thickness.

Response	Comparison	Vehicle	20pmol	50pmol	200pmol
FA	20pmol PAF	NS			
	50pmol PAF	<0.01	NS		
	200pmol PAF	<0.01	<0.01	NS	
	500pmol PAF	<0.01	<0.01	NS	NS
	5nmol HIS	<0.01	<0.01	<0.01	<0.05
WA	20pmol PAF	<0.01			
	50pmol PAF	<0.01	NS		
	200pmol PAF	<0.01	<0.01	NS	
	500pmol PAF	<0.01	<0.01	NS	NS
	5nmol HIS	<0.01	<0.01	NS	NS
WV	20pmol PAF	<0.01			
	50pmol PAF	<0.01	=0.04		
	200pmol PAF	<0.01	=0.01	NS	
	500pmol PAF	<0.01	<0.01	NS	NS
	5nmol HIS	<0.01	<0.01	NS	NS

Table 4.3 Flare and weal statistics (ANOVA results) for each dose of PAF and for histamine allowing for site, arm and inter-subject effects. Responses are flare area (FA), weal area (WA) and weal volume (WV).

Site comparisons (Table 4.4) The mean response at the proximal site was significantly less than either the medial or distal sites with respect to WA and WV ($p<0.01$, see Table 4.4). There was no significant difference in the mean response between the medial and distal sites ($p>0.20$).

Arm comparisons (Table 4.5) No significant differences were noted between the mean response of the left versus the right arm with respect to FA, WA or WV ($p\geq0.17$). Although no significant differences were found, the mean response was consistently higher in the right arm than in the left arm for all three variables.

Baseline skin thickness (Tables 4.2, 4.4 and 4.5) No significant differences were observed in the mean skinfold thickness prior to injection (T_0) with respect to site or arm ($p>0.20$).

Response	Mean response Prox (P)	Med (M)	Dist (D)	Pairwise p-values P vs M	P vs D	M vs D
Flare area (cm^2)	7.47	8.74	8.38	>0.20	>0.20	>0.20
Weal area (mm^2)	0.47	0.89	0.95	<0.01	<0.01	>0.02
Weal volume (μl)	0.27	0.94	1.08	<0.01	<0.01	>0.20
Skin thickness (mm, T_0)	2.84	2.76	2.80	>0.20	>0.20	>0.20

Table 4.4 Statistics for mean response comparisons between sites including pre-injection skin thickness values.

Response	Mean response Left arm	Right arm	p-value
Flare area (cm^2)	7.36	9.03	=0.17
Weal area (mm^2)	0.74	0.80	>0.20
Weal volume (μl)	0.73	0.80	>0.20
Skin thickness (mm, T_0)	2.84	2.76	>0.20

Table 4.5 Statistics for mean response comparisons between arms including pre-injection skin thickness values.

4. Conclusions

Generally there appeared to be a trend towards increased weal response with the distal site. This was not the case for

flare. A possible explanation for this phenomenon might be that that the skin is under more tension higher up the arm due to increased muscle mass. This might create a back-pressure which would serve to limit the potential for vascular leakage. The underlying mechanism for flare propogation does not involve vascular leakage which may explain why this component of the response was not site-related.

The significant interactions of both site and subject indicates the importance of carefully balanced randomisation of treatment to site in any experiments using flare and weal in the forearm. The overall dose relationship, acceptably limited variability and good subject tolerance of the model supports its use as a convenient and practical method for exploring the underlying pharmacology of the PAF response.

D. Effects of PAF using saline and Haemaccel as vehicle

Use of the natural blood product, HSA, is not without theoretical immunological risk. In order to address the necessity of its inclusion in the PAF vehicle, a study was conducted to evaluate the model without HSA. The literature indicates that the dose-relationship between WV and PAF is weakened and significantly shifted to the right in the absence of HSA (Archer et al,1985). Given the ethical considerations and previous consistent results with the model including HSA, it was decided not to include an HSA limb in the study but instead to investigate solely whether PAF responses using a normal saline vehicle still showed a dose-relationship.

Retrospective comparisons may be made with previous data based on HSA-containing vehicle.

Additionally, a further study was conducted using the synthetic colloid, Haemaccel^{R} (Hoechst), as a substitute for HSA. Haemaccel is a degraded and modified gelatine (polygeline) of molecular weight ca. 35,000. The product is used as a plasma volume substitute in cases of hypovolaemic shock and as a carrier for insulin. It contains no preservatives. The opportunity was taken with this latter study to observe whether the upper back could be used as an alternative to the forearm. In situations where more than 6 doses/treatments were required to be given (i.e. exceeding the the maximum number of sites in the forearm), the upper back might be used. In this study PAF was solubilised in neat Haemaccel.

1. Study hypotheses

a. The flare and weal responses to intradermal PAF using saline as a vehicle are dose-related over the dose range 5 to 500 pmol.

b. The flare and weal responses to intradermal PAF using Haemaccel as a vehicle are dose-related over the dose range 50 to 500 pmol.

2. Design and methods

A similar protocol was followed as for the earlier dose response study reported above except that in the case of the saline vehicle study, the experiment was repeated after a period of 1 week. Again eight subjects were recruited for both studies, but flare and weal responses were accidentally omitted in one subject on day 1 of the saline vehicle study hence data are reported for n = 7 on this day. Saline was used as a vehicle.

Since histamine was not strictly required in order to test the hypothesis of either study, a lower dose of PAF (5 pmol/site) was substituted in the saline study in order to retain a balanced randomisation with respect to site/arm (i.e. 3 sites per arm: 6 treatments required). The lower dose was opted for since the earlier dose response study indicated that the dose-relationship waned with the higher doses of PAF. In the Haemaccel study, histamine was included but only 3 doses of PAF were used:

Treatments:	Saline study	saline, 5, 20, 50, 200, 500pmol PAF.
	Haemaccel study	Haemaccel, 50, 200, 500pmol PAF, 5nmol histamine

3. Results

Saline study: Responses to PAF (FA and WV) on each day are shown in Figure 4.6. Mean responses across the two days are shown in Figure 4.7. It is clear from the bargraphs that, although the response to the highest dose (500 pmol) is consistent with previous data (see Figure 4.5) for both FA and WV, the lower doses and particularly the 20 pmol dose are associated with a much reduced response compared with previous data. Furthermore the dose relationship is weak below 200 pmol.

Haemaccel study: Responses to PAF in Haemaccel vehicle are shown in Figure 4.8. Both FA and WV data are similar to the responses to respective doses of histamine and PAF in the earlier dose-response study (see figure 4.5). Clearly the effect of the lowest dose of PAF (50 pmol) is maintained compared with the effect of this dose in the study with saline. Furthermore, the dose-relationship is also restored by the inclusion of Haemaccel in the vehicle.

4. Conclusions

It is concluded that Haemaccel is a viable alternative to HSA in the vehicle for PAF intradermal studies. As a result, the use of HSA was avoided in all subsequent experiments and Haemaccel included in the vehicle. The study with Haemaccel also demonstrated the back as a useful alternative site for intradermal PAF studies.

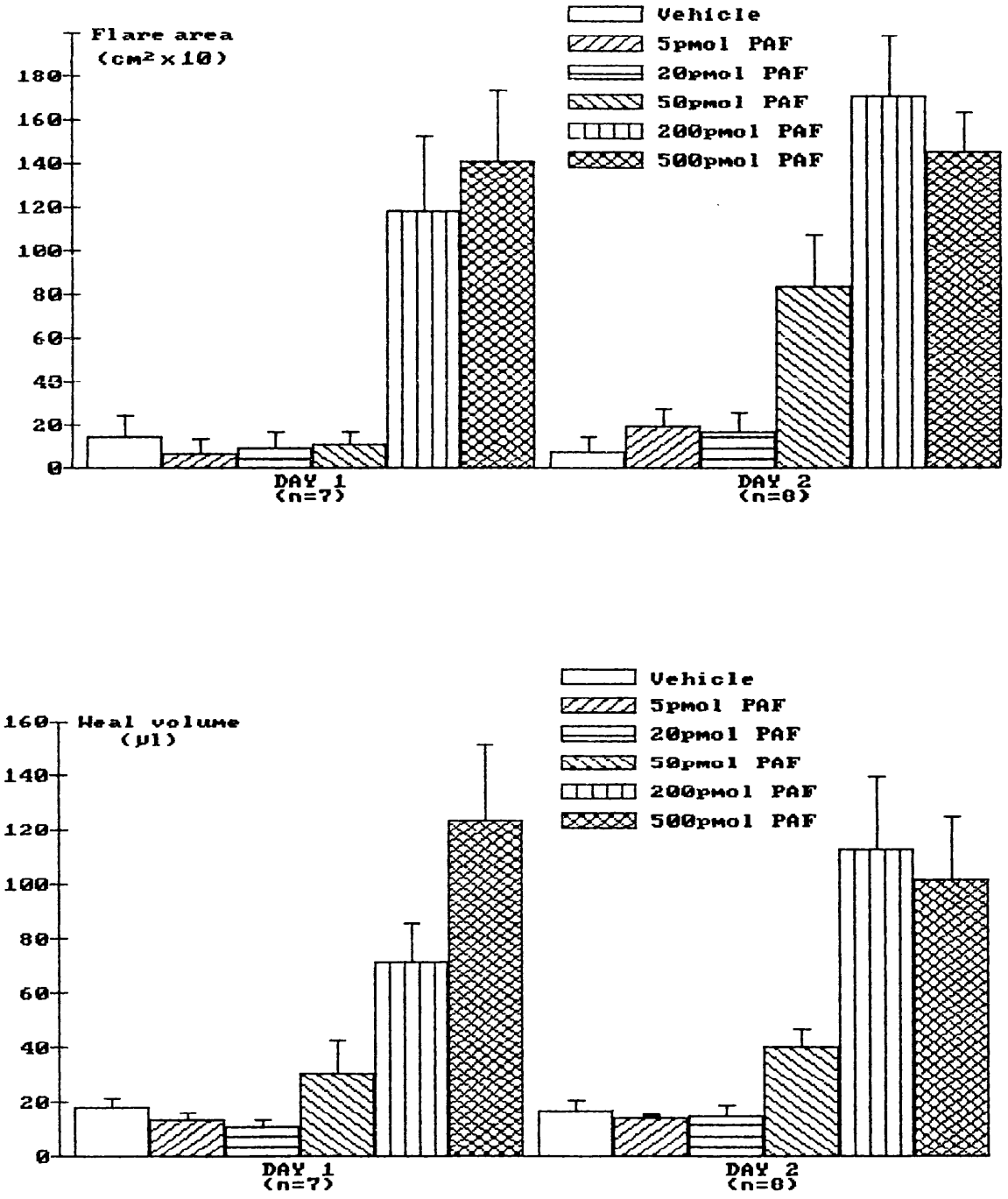

Figure 4.6 Mean flare and weal response on each of two days (separated by one week) from study of PAF in saline vehicle (mean + sem).

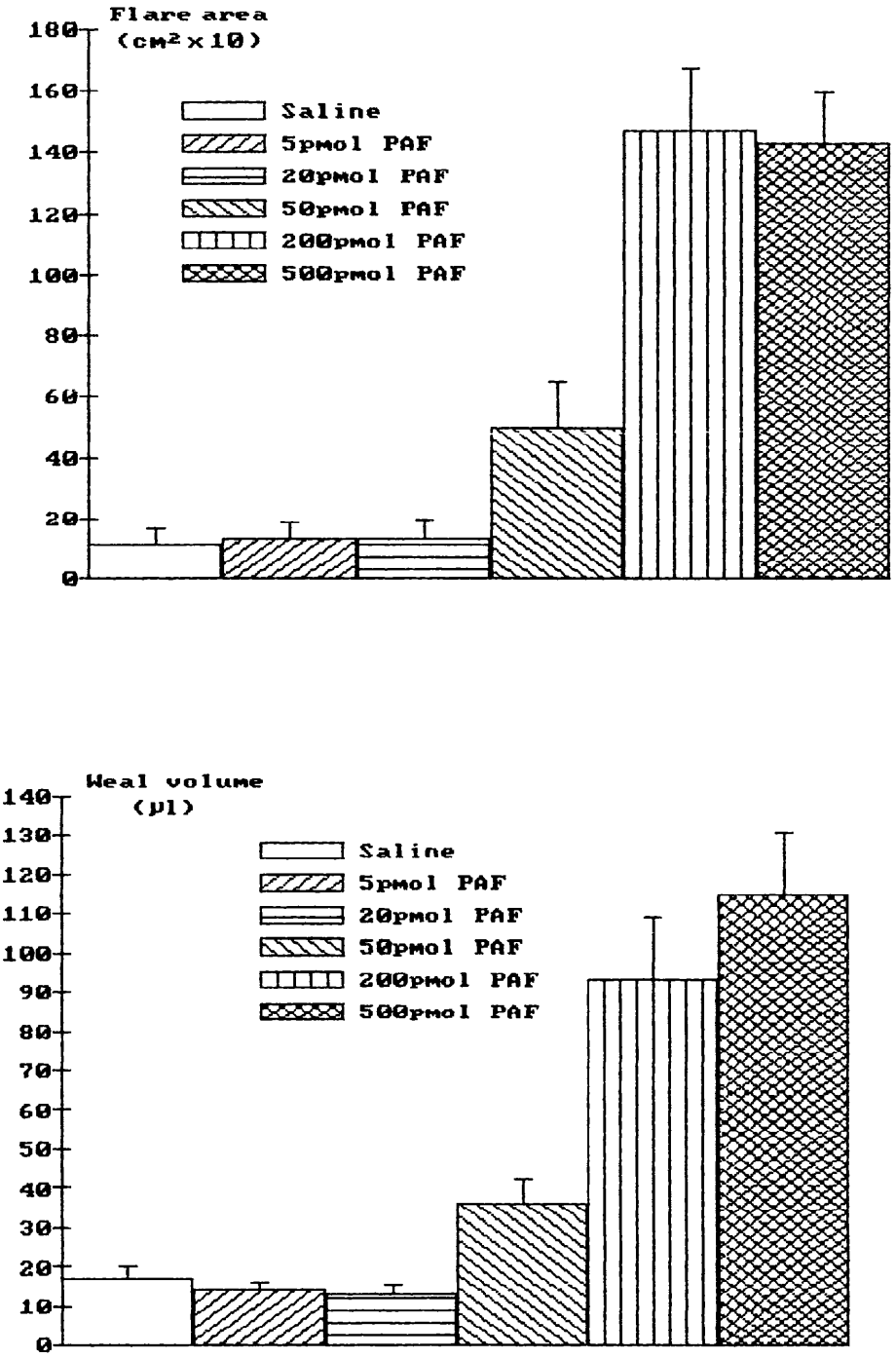

Figure 4.7 Mean flare and weal response across the two days from study of PAF in saline vehicle (mean + sem).

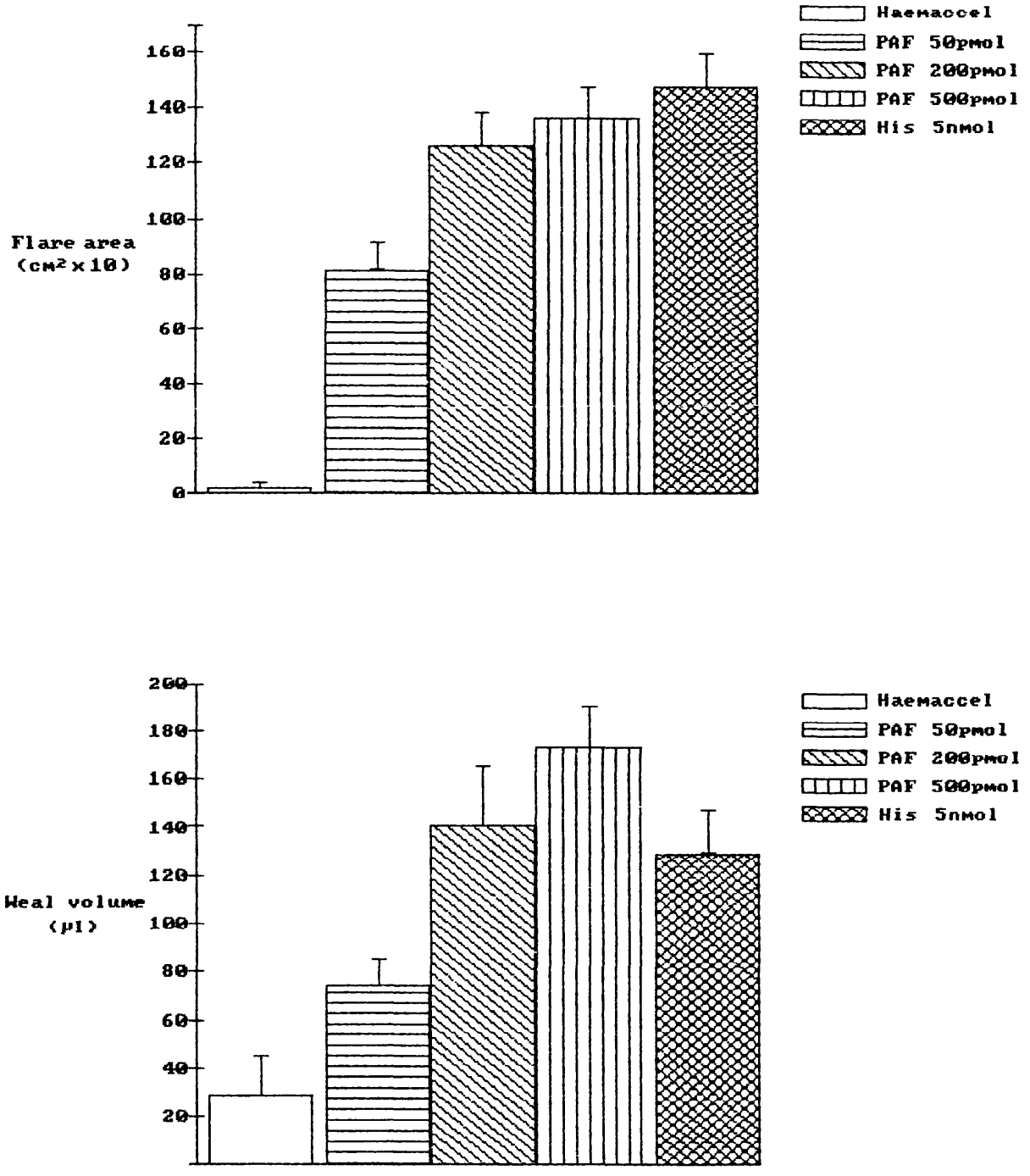

Figure 4.8 Flare (FA) and weal (WV) responses to PAF and histamine using Haemaccel as a vehicle (mean + sem, n = 8). The skin of the upper back was used for this study.

E. Effects of intradermal PGE_2, substance P and CGRP

In the experiments described in Chapter 2 where the hyperalgesic potential of substance P and CGRP were investigated, observations were also made of the flare and weal responses. Also reported here are the flare and weal results for PGE_2 from the hyperalgesia study comparing with the responses to PAF (see page 71).

PGE_2 (Archer et al,1984) and substance P (Foreman et al,1983) produced a flare and weal response similar to that of intradermal histamine while CGRP was not associated with such effects except at high doses (100 pmol; Brain et al,1986).

1. Study hypotheses

a. PGE_2, substance P and CGRP when given intradermally are associated with flare and weal responses.

b. The flare and weal responses to the three mediators are dose related.

2. Design and methods

The standard protocol was followed for the investigation of the flare and weal responses of intradermal injections of each of the mediators in the volar surface of the forearm (see Appendix 1). In the PGE_2 study, 8 subjects were used and studied at three dose levels; in the neuropeptide studies, observations were made in four subjects at two dose levels. Saline was used as a vehicle for all three mediators.

Doses: PGE_2 - 0.01, 1 and 100 nmol.

Substance P and CGRP - 10 and 100 pmol

3. Results

PGE_2 was associated with a flare and weal response. Although PAF appeared to be more potent than PGE_2 in provoking a weal (see Figures 4.5 and 4.9), the two studies were carried out in different subjects; since there is a quite marked intersubject variability, comparison of the two agonists in the same subjects is needed to confirm that PAF is the more potent agonist. The time course of acute response was similar to that of PAF and histamine; after the initial flare had disappeared (20-30 mins), a striking erythema was observed which spread progressively and was reported by subjects to be associated with 'tenderness'. One subject reported tenderness around the sites of injection for 36 h. Injection of PGE_2 was reported to be more painful than PAF.

The PGE_2-associated erythema was different from a triple-response flare, being a deeper red colour and having a more clearly defined border. 'Tracking' of this erythema in a proximal direction along the arm was noted at the high dose. This effect had disappeared by 2 h after injection while the erythema persisted for several hours although fading significantly after 1.5 to 2 h.

Substance P produced a flare and weal response following a similar time course to either PAF or histamine (see Figures 4.9 and 4.10). No persistent erythema-like effects were noted and the acute effects had disappeared within 30-40 mins. Comparisons of potency between substance P and PAF may be inappropriate since only 4 subjects were used compared with 8 in the PAF study. Furthermore there was no histamine control in the neuropeptide study. The FA response to 100 pmol was only marginally greater than to 10 pmol whereas WV responses were clearly dose-related at the doses used.

CGRP produced a tight ring of erythema quite unlike the usual triple-response flare and resembling the PGE_2-associated erythema in consistency but not in degree of spread. The erythema appeared gradually over the 20 to 40 mins after injection and persisted for several hours. Just as had been seen with PGE_2-associated erythema, pseudopodia-like 'tracking' along the arm often developed especially with the higher dose. There was however no associated tenderness nor was there a weal response any more than would be expected from injection of vehicle.

4. Conclusions

Both PGE_2 and substance P were associated with histamine-like flare and weal response, although PGE_2 was probably the least potent of mediators studied in the series of studies so far. PGE_2 was also associated with a spreading erythema which persisted long after the flare and weal had

disappeared. CGRP was not associated with a flare and weal response; it did however produce an intense erythema response which persisted for several hours.

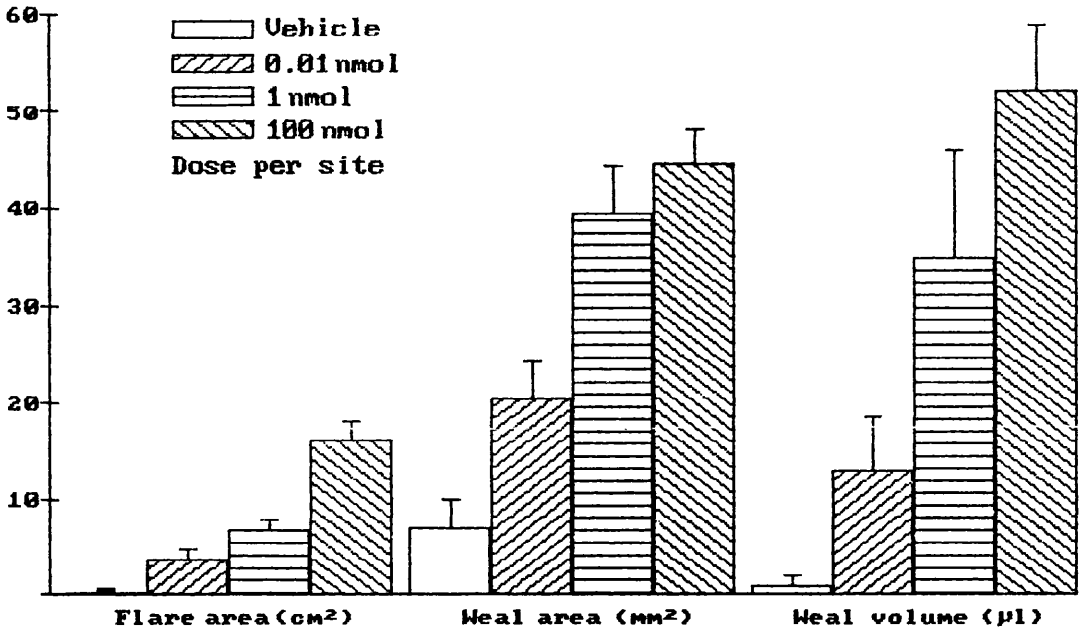

Figure 4.9 Flare and weal responses to PGE_2 (mean + sem, n = 8).

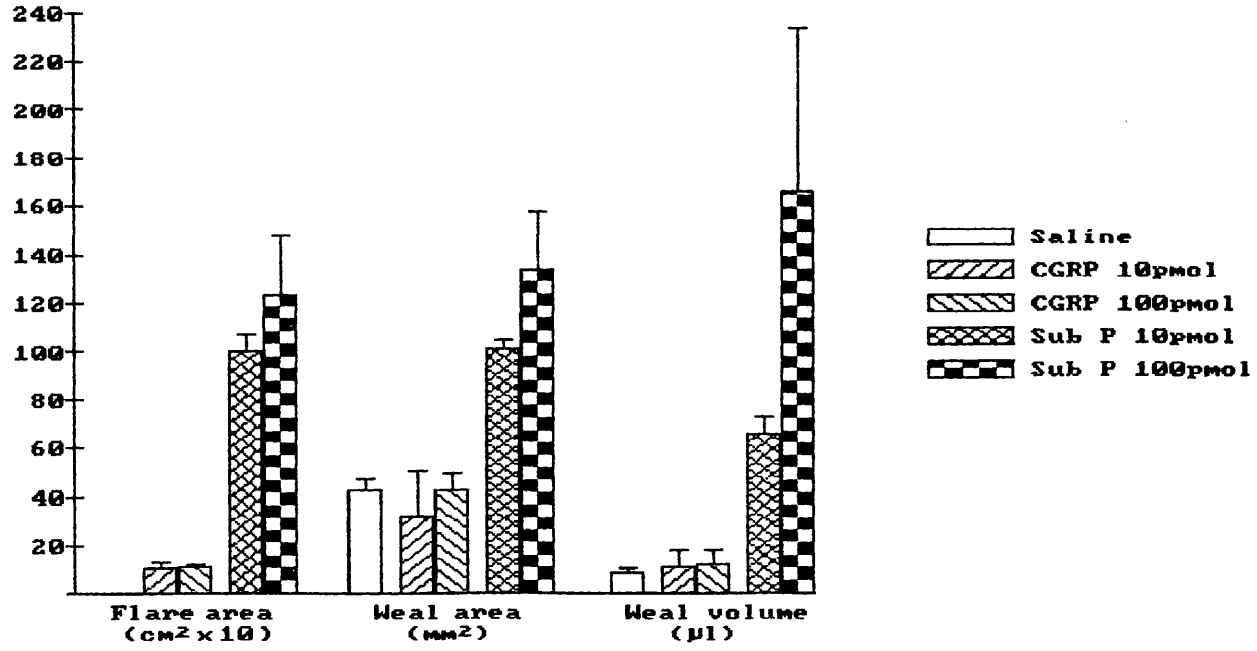

Figure 4.10 Flare and weal responses to CGRP and substance P (mean + sem, n = 4).

CHAPTER V

HISTAMINE ANTAGONISM AND CYCLOOXYGENASE INHIBITION

A. Introduction

It has been shown that H_1-antagonists such as mepyramine and chlorpheniramine can significantly attenuate the flare but not the weal response to intradermal PAF in man (Archer et al,1985;Fjellner et al,1985). Furthermore, ketotifen, an H_1-antagonist, has been shown to inhibit PAF-induced flare and weal response in the skin without influencing the bronchoconstriction following inhaled PAF (Chung et al,1988). Pre-treatment with a cyclooxygenase inhibitor, indomethacin, has been shown to have no effect on either the flare or the weal response (Archer et al,1985).

Much of the current interest in PAF stems from early observations of its effects in the skin bearing close similarities to the response to intradermal antigen (Archer et al,1984;Basran et al,1984). If the effects of PAF are indeed immunological in nature then one would expect involvement of skin mast cells. It would follow that release of pre-formed mediators such as histamine should occur together with activation of mast cell production of the de-novo mediator PGD_2.

Inflammatory mediators acting on skin mast cells may not necessarily provoke PGD_2 production however; neuropeptides such as substance P, for instance, are associated with a much

higher ratio of histamine to PGD_2 release compared with anti-IgE (Church et al,1989) when human skin mast cells are incubated with each. It might be suggested that this differentiation may reflect a neuro-immune role of skin mast cells unlike other mast cell types (e.g. lung or intestinal) which do not respond to substance P.

Thus it seems reasonable to expect mast cell-derived histamine and possibly PGD_2 involvement in PAF-induced responses in the skin. The published data are indicative rather than conclusive and merit further investigation with a more potent and selective H_1-antagonist, terfenadine (Woodward et al,1982).

B. Effect of Terfenadine

Terfenadine is a potent H_1-antagonist which was originally synthesised in the early 1970's and developed as a drug for clinical use after it had been found to be essentially devoid of significant central effects (Brandon et al,1980). Historically, antihistamines have been dogged by problems of non-specificity with pronounced CNS and anti-cholinergic effects which effectively limited the acceptable oral dose. The advantage of terfenadine over previous H_1-antagonists is the fact that it does not readily penetrate the blood-brain barrier; additionally, terfenadine has has no demonstrable anti-cholinergic, anti-serotonergic or anti-adrenergic activity (Woodward et al,1982).

In man, terfenadine appears to be subject to almost complete biotransformation to two main metabolites, one of which, a carboxylic acid derivative, has approximately one-third of the activity of the parent compound and may account for a portion of the overall antihistamine effect (Sorkin et al,1985). ^{14}C radio-label studies have indicated a plasma half-life of approximately 4.5 h which roughly corresponds with the time of peak inhibition of histamine-induced weal, suggesting that the metabolite makes a significant contribution to the clinical effect. Terfenadine is excreted via the urine (40%) and faeces (60%, Okerholm et al,1981).

In studies of the effect of terfenadine on histamine induced weal in man, single doses of 20, 60 and 200 mg have been shown to produce dose-related decreases (Huther et al,1977). The most convincing data however have come from studies where the drug has been given over a period of up to three days. Ryan et al (1988) compared 60 mg bid with 120 mg given either bid or qid for three days in a placebo controlled study against histamine-induced weal in man. All three doses significantly decreased weal at 12 and 24 h versus placebo. The 120 mg dose was found to be the most effective, however, especially on day 3.

Single 180 mg doses of terfenadine have been studied for effects on antigen (Curzen et al,1987) and adenosine 5'-monophosphate-induced bronchoconstriction with the intention of saturating the H_1-receptors as far as possible. In both of these studies the dose of terfenadine was given 3 h before the

allergen challenge. The BNF recommendation for terfenadine is 60 mg bid, however a 180 mg dose would be expected to result in plasma levels of ca. 1.1 x 10^{-8} mol/l (Okerholm et al,1981) which would be within the the range for competitive antagonism for the drug (3.16 x 10^{-8} - 1 x 10^{-7} mol/l) where no anticholinergic, antiadrenergic or antiserotonergic effects have been shown (Woodward et al,1982).

1. Study hypothesis

Terfenadine treatment significantly reduces the responses to intradermal PAF.

2. Design and methods

8 healthy, non-atopic, male volunteers were recruited for study with the customary provisions for ethical committee approval and informed consent. Atopy status was investigated by skin prick testing in each potential recruit using a proprietary range of standard allergens (Bencard^{R}). In a two-period crossover double-blind design, with seven days between each treatment, flare and weal responses to intradermal injection of PAF in ascending dose (20, 50, 200, 500 pmol per site) and histamine (5 nmol per site) were investigated; vehicle in this study was 0.25% HSA in normal saline (study conducted before the change to Haemaccel). Preparation of injection solutions were as described in Appendix 1. For the two days preceding each study day, volunteers received either terfenadine or matching placebo in randomised fashion. The

dose of terfenadine was 60 mg bid. On the third day (the study day) each volunteer took a single dose of 180 mg of terfenadine or placebo. Four hours after this dose, PAF, histamine or vehicle was injected intradermally at sites on the volar surface of the forearm. The flare and weal response was then measured according to the usual protocol (see Chapter IV B).

Statistical analysis was performed using SAS procedure GLM type III sums of squares. Parametric analysis of variance (ANOVA) was used to analyse all response variables where the normality assumption was confirmed, otherwise ANOVA was performed on the ranks of the data. Pairwise comparisons were made using SAS LSmeans. The normality assumption was tested using the Kolmogorov D statistic while the homogeneity of variance assumption was tested with Hartley's Fmax statistic on the variance of the residuals by dose from the ANOVA, and by inspection of residual plots. All tests of significance were two-tailed at $\alpha = 0.05$ and all p-values were rounded to two decimal places.

3. Results

Summary statistics and pairwise comparisons for FA, WA, WV and skin thickness pre-injection (T_0) are given in Table 5.1. The placebo results showed a significant linear trend in the dose relationship ($p<0.01$). The results resembled the data from the reproducibility study (see Figures 5.1 and 4.5). The terfenadine treated sites had significantly lower adjusted

mean FA and WA after injection of 20 (FA only), 50, 200 and 500 pmol PAF and 5 nmol histamine ($p<0.01$ in each case, see Table 5.1). WV responses were significantly lower after the 500 pmol dose only ($p<0.01$), although the response after 50 and 200 pmol approached significance ($p=0.09$). There was, however, a significant overall effect of terfenadine treatment on PAF response regardless of dose ($p<0.05$). The histamine WV was significantly reduced after terfenadine treatment. There was no significant difference between placebo and terfenadine treatment with regard to skinfold thickness before injection ($p>0.10$).

4. Conclusions

Terfenadine significantly inhibited the intradermal flare response to PAF across the full dose range. Inhibition in terms of WA revealed significant inhibition by terfenadine at all doses except the lowest, although the level of significance diminished for the 50 ($p=0.03$) and 200 ($p=0.01$) pmol doses. In contrast, terfenadine significantly inhibited the weal response to PAF in terms of WV only at the highest dose of PAF used. The inhibition of WV at 50 and 200 pmol did approach significance however ($p=0.09$) and the terfenadine WV responses were all lower than for placebo.

It appears that flare is more sensitive than weal to terfenadine treatment. The disparate sensitivity to H_1-antagonism may reflect the difference in mechanisms between the two manifestations of the triple-response. Alternatively,

it may be evidence of a direct vascular effect of PAF itself which is independent of histamine.

Response		Terfenadine		Placebo		
	Dose	Mean*	S.D.	Mean*	S.D.	p-value
FA	Veh	0	0	0.5	0.8	=0.05
(cm^2)	20	0.6	0.7	3.4	2.2	<0.01
	50	0.1	0	4.8	3.7	<0.01
	200	0.1	0.5	8.5	3.7	<0.01
	500	0.4	0.9	9.8	5.2	<0.01
	Hist	1.1	1.0	14.7	6.7	<0.01
WA	Veh	67	18	63	12	>0.10
(mm^2)	20	69	20	86	24	>0.10
	50	67	31	95	33	=0.03
	200	94	33	120	14	=0.01
	500	90	26	137	23	<0.01
	Hist	72	39	126	19	<0.01
WV	Veh	16	13	22	22	>0.10
(μl)	20	36	14	55	19	>0.10
	50	41	25	67	43	=0.09
	200	63	44	90	17	=0.09
	500	75	51	128	68	<0.01
	Hist	38	35	111	37	<0.01
$^1T_0$	Veh	2.9	0.3	2.8	0.4	>0.10
(mm)	20	3.0	0.3	2.9	0.4	>0.10
	50	2.9	0.2	2.8	0.3	>0.10
	200	2.9	0.4	3.0	0.5	>0.10
	500	2.9	0.4	3.0	0.4	>0.10
	Hist	2.9	0.3	3.0	0.4	>0.10

Table 5.1 Summary statistics for flare (FA) and weal (WA, WV) responses after terfenadine or matching placebo. *Mean data from adjusted SAS LSMEANS in ANOVA model. All p-values refer to terfenadine versus respective placebo data (N = 8). Vehicle was 0.25% HSA/normal saline; PAF doses were 20, 50, 200 and 500 pmol per site; histamine dose was 5 nmol per site.
$^1T_0$ - Baseline skinfold thickness

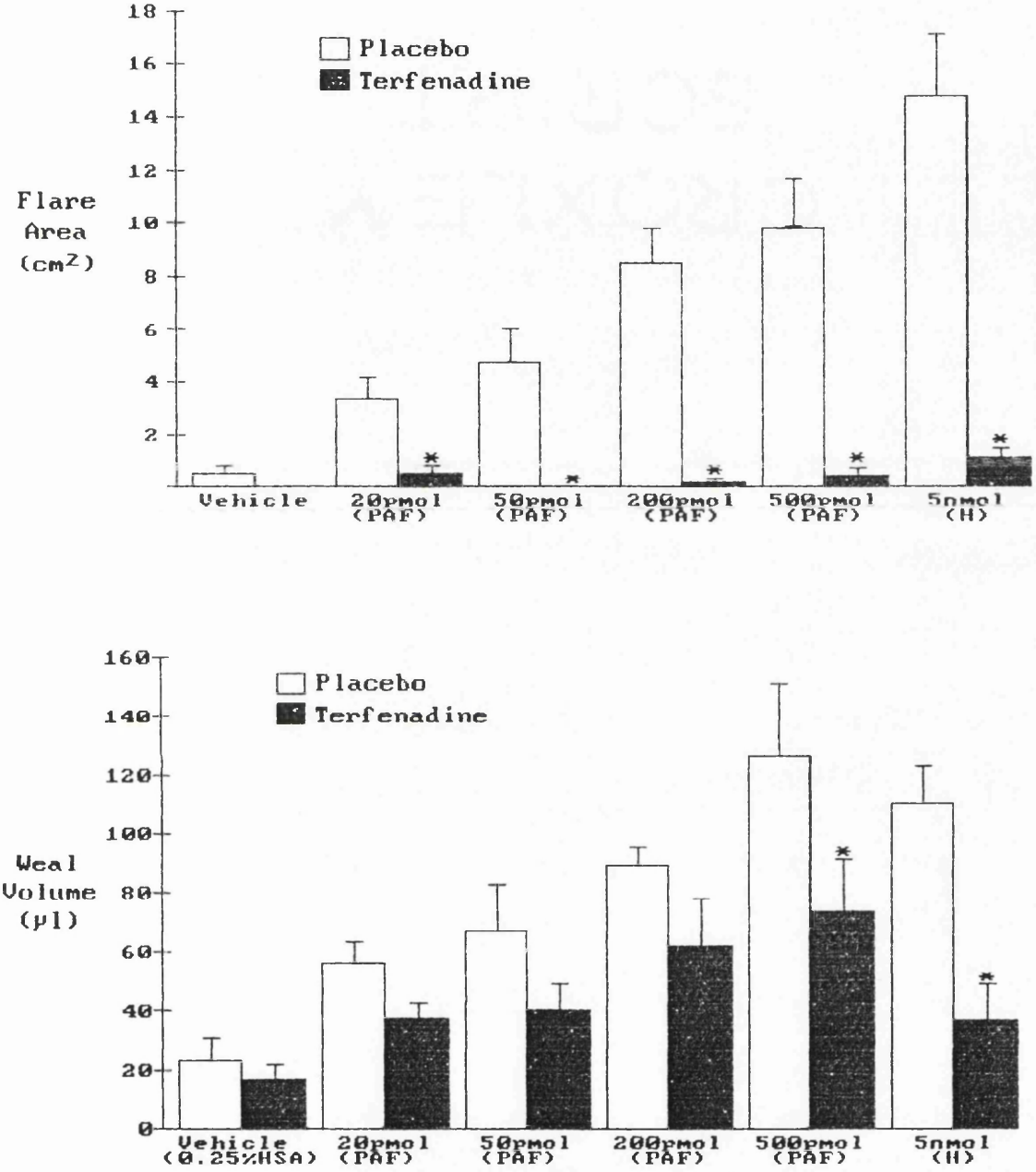

Figure 5.1 Flare and weal responses (FA and WV) after terfenadine (60 mg bid for 2 days, then 180 mg sd on 3rd day 4h before PAF/histamine challenge) or matching placebo (N = 8, + SE Mean, *p<0.01 versus placebo, H - histamine).

C. Effect of Indomethacin

There is evidence in studies in guinea-pig and rat skin that PAF effects may be cyclooxygenase-dependent (see Table 2.1, Chapter II). In man, it has been reported that cyclooxygenase inhibition has no effect on the acute PAF responses in the skin (Archer et al,1985). The same authors also reported that there was no effect of cyclooxygenase metabolites in guinea pig skin, data which are at odds with other published evidence.

It has been discussed in Chapter I that PAF, leukotrienes and prostaglandins share a similar source material. It has been reported, however, that PAF can actually stimulate release of arachidonic acid, the leukotriene and prostaglandin precursor, under certain conditions in guinea-pig neutrophils (Tou,1985). Furthermore, it has already been shown that PGE_2 can produce a weak flare and weal response in human skin. It would be reasonable to hypothesize that cyclooxygenase played some role in the PAF response. A study was conducted therefore to examine the effect of cyclooxygenase inhibition on the PAF responses.

1. Study hypothesis

Indomethacin treatment will significantly reduce the responses to intradermal PAF.

2. Design and methods

A similar design to the terfenadine study, in all but oral treatment, was used for this study. Instead of terfenadine taken for three days, indomethacin was taken at a dose of 25 mg tid for two days with a single final dose of 50 mg on the morning of the third day. The crossover comparative treatment was matching placebo. As usual, the study was double-blind, with the customary procedures followed for ethics approval and informed consent. Eight healthy, non-atopic volunteers were used; intradermal injections were given 1 hour after the final dose. The doses of PAF were 20, 50, 200 and 500 pmol with a 5 nmol dose of histamine for comparison.

3. Results

The flare results to increasing doses of PAF following placebo did not show as close a linear dose-relationship as for previous studies. Graphical representation of the results are shown in Figure 5.2.

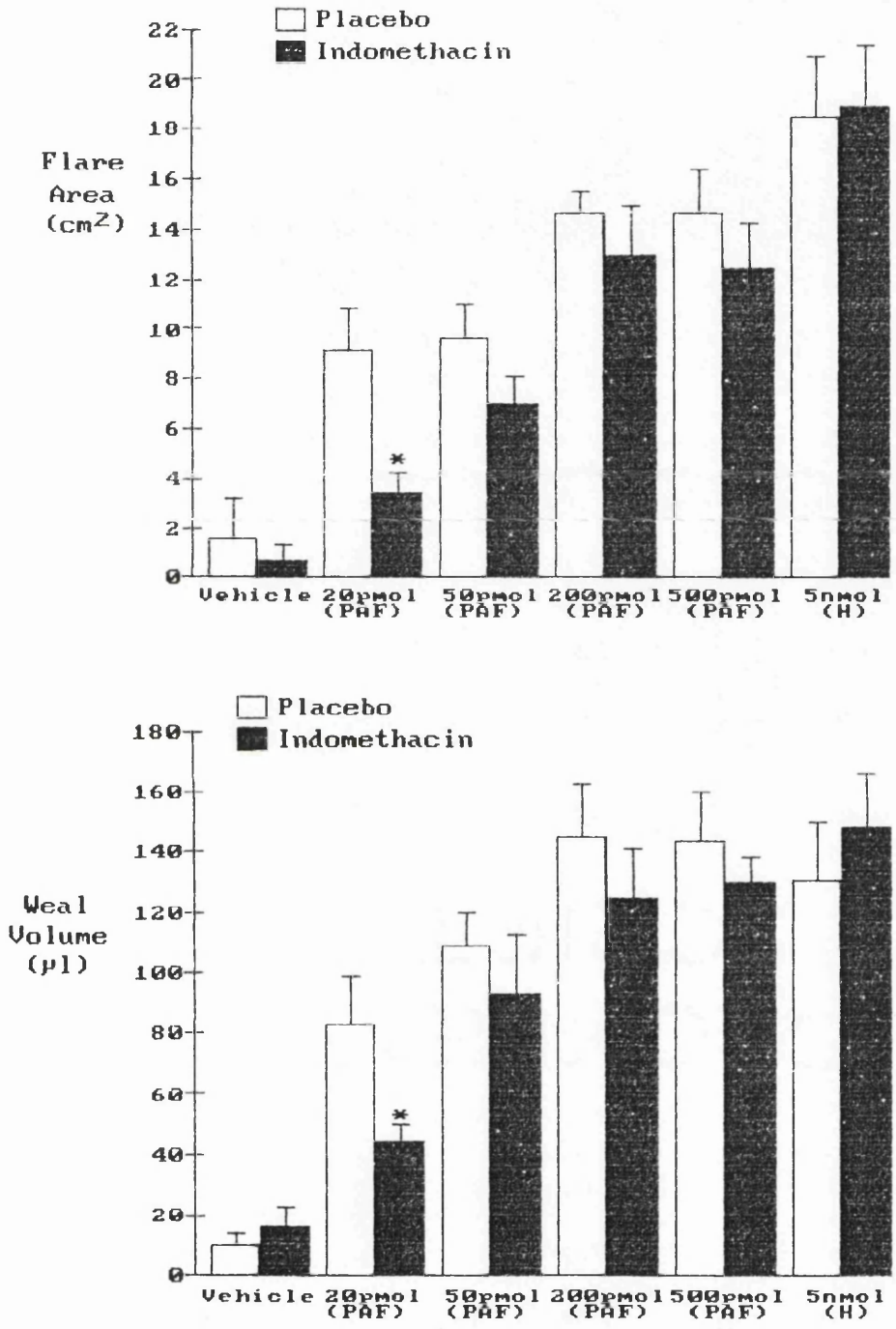

Figure 5.2 Effects of indomethacin vs matched placebo on the flare and weal responses to intradermal PAF and histamine. Indomethacin was given 25 mg tid for two days and then a final morning dose of 50 mg 1 hour before injection (N = 8 + SEMean, * - $p<0.05$ vs placebo). Histamine dose was 5 nmol.

Indomethacin significantly reduced both FA and WV following PAF only at the lowest dose (20 pmol; $p=0.01$, FA; $p<0.05$, WV). At all other doses there was no significant difference between drug and placebo, although both PAF-induced responses were always lower following indomethacin than after placebo. There was also a significant overall effect of indomethacin on PAF-induced FA ($p<0.01$). Indomethacin had no overall effect on WV or histamine responses.

4. Conclusions

Indomethacin was found to reduce significantly both flare and weal responses to PAF although only at the lowest dose of PAF. Indomethacin was also found to have a significant overall effect on flare but not on weal.

CHAPTER VI

EFFECTS OF HISTAMINE DEPLETION

A. Introduction

In previous experiments, it has been shown that PAF-induced responses in the skin resemble those following intradermal histamine and are inhibited by antihistamine pre-treatment. It would be reasonable to deduce from this evidence that intradermal PAF exerts it's effects at least in part through histamine release.

The major source of histamine in the skin is the mast cell (Eady et al,1979) where the histamine is stored preformed in cytoplasmic granules. The mast cell was first described by Ehrlich who coined the name 'mastzellen' meaning well-fed cells in reference to the cytoplasm which appeared packed with granules. In human mast cells, these granules are a proteoglycan matrix consisting of both heparin and chondroitin sulphate.

1. Rat mast cells

Much of the literature concerning the anatomical and functional classification of mast cells is based on rat tissue. In the rat, mast cells have conventionally been divided into two general types: 'mucosal mast cell' (MMC) which is the most common type found in intestinal lamina propria and 'connective tissue mast cell' (CTMC) which comprises the most common form found in connective tissue,

lung, skin and serosal surfaces such as the peritoneal cavity. The two rodent mast cell types may also be discriminated using staining techniques: the MMC characteristically stains blue with alcian blue and the CTMC red with safranin. There is convincing evidence that the rodent MMC is T-lymphocyte-dependent (Bienenstock,1988), probably via IL-3 release which has been shown to promote growth in vitro. More recently, it has been demonstrated that IL-4 has similar effects on CTMC growth.

A wide variety of immunological and non-immunological stimuli activate mast cells to release inflammatory mediators of which the most important in pathophysiological terms is anti-IgE.

The primary events in the initiation of an allergic response to pathogen invasion is the reaction between antigen and lymphocytes (B type or precursors) resulting in the secretion of IgE from differentiated plasma cells. IgE is known to bind almost exclusively to mast cells (and basophils) at the Fc receptor. When specific antigen binds to the cell fixed IgE there results a chain of events of which the consequence is mast cell activation. Monovalent antigen does not give rise to secretion; the anti-IgE must be di- or poly-valent since cross-linking of the Fc receptors must occur. Table 6.1 shows some of the events which may follow this cross-linkage.

Secretagogues

Calcium ionophore
- A23187

Anaphylotoxins
- C3a
- C5a

Neurokinins
- Substance P
- Somatostatin

Drugs
- Opioids
- Antibiotics
- Muscle relaxants
- Compound 48/80

ATP

Anti-IgE

Mast cell mediators

Preformed:	De novo synthesis:	Granule matrix:
Histamine	Superoxide	Heparin
ECF[1]	LTC_4	Tryptase
Kininogenase	PGD_2	Chymotryptase
Serotonin	PAF	Peroxidase
	Adenosine	Superoxide-dismutase
	TXA_2	

Intracellular events following cross linking

Calcium mobilisation:	extracellular calcium flow into the cell is facilitated
Phospholipid metabolism:	Increased phosphatidyl inositol turnover Increased phospholipase A activity
Cyclic nucleotides:	Decreased cAMP levels

Table 6.1 Mast cell secretagogues, mediators and mechanisms involved in mediator release following cross-linkage of Fc receptors. [1]-Eosinophil chemotactic factor.

Although a wide variety of agents may promote secretion and a number of intracellular events have been demonstrated in conjunction with mediator release, it appears that normal cellular respiration and free extracellular calcium are mandatory requirements (Church et al,1982). The role of cAMP is somewhat less clear, although studies have shown that β_2 agonists, which cause increased levels of cAMP, inhibit dispersed human lung mast cell secretion (Church et al,1986) and phosphodiesterase inhibitors (which also increase cAMP) such as theophylline are also known to inhibit histamine release.

Rat mast cells have differing granule contents; MMC proteoglycan is exclusively chondroitin sulphate while the CTMC type is heparin. Similarly the response to various stimuli often differs depending upon the cell type. Most striking of these differences is the marked degranulation response to compound 48/80 (C48/80), a low molecular weight polymer of *p*-methoxy-N-methylphenethylamine seen in the CTMC type but not in MMC type. However the evidence is not clear cut; when CTMC from peritoneum are compared with cells derived from tissues such as the skin, heart and lung in terms of response to compound 48/80, widely differing potencies are found for the polymer (see table 6.2). This observation serves to emphasise that histochemical classification does not correlate functionally with anatomical source.

Source	Concentration (μg/ml)
Peritoneum	0.3
Pleura	0.3
Mesentery	0.8
Skin	20
Lung	20
Heart	>100
Intestine	>100

Table 6.2 Relative concentration of compound required to produce 20% of maximum histamine release in rodent mast cells from various anatomical locations (Pearce et al,1985).

In addition to proteoglycan and histamine, rodent mast cells also contain protease enzymes. Two types of this enzyme have been characterised and each appears to be present in only one of the mast cell types: rat mast cell protease (RMCP) I is found only in the peritoneal or CTMC type while RMCP II is present in the intestine or MMC type (Beinenstock,1988). A further discriminating feature of the two types of rodent mast cell relates to immunological stimulation of the cell to synthesize non-preformed inflammatory mediators. The CTMC type has been shown to produce mainly PGD_2 while the MMC appears largely to generate LTC_4.

An area of considerable research interest is whether mast cells and nervous tissue have a structural association rather than merely being coincidentally adjacent in many tissues. Mast cells predominantly reside in tissues interfacing with the external environment and hence are strategically placed for early response to pathogen invasion.

Discussion concerning the implication of histamine, and hence mast cells, in the antidromic response is detailed in Chapter IV. While the evidence in man indicates a substance or substances other than histamine as the end-effector, the role of mast cells (and indeed of histamine) cannot be excluded either from the initiation stage of this reflex mechanism or from the vascular permeability component of the triple response. It is not surprising to find that nerve growth factor (NGF) enhances growth of both CTMC and MMC in neonatal rats (Tomioka et al,1988), lending further supportive evidence to the hypothesis that the skin mast cell and nociceptive c-fibres have an intimate functional relationship.

2. Human mast cells

Human mast cells are not as easily classified as the rodent cells. Staining does allow differentiation of two types of cell, but it seems that all human mast cells may contain heparin. Biochemically, there appears to be a correlation with the RCMP I and II classification. Human mast cells located in the intestinal lamina propria contain mainly tryptase (T, 98%) while cells from the intestinal submucosa and skin contain chymotryptase (CT, 87-88%; Irani et al,1986). While the lung has a more mixed population, the majority of cells in this organ are of the T type, i.e. similar to the rodent MMC (Schwartz et al,1987).

Functional differences between human mast cells from various anatomical origins have been studied using

immunological and non-immunological stimuli by Church and co-workers (Holgate et al,1988;Church et al,1989a;Church et al,1989b). A summary of the evidence is shown in Table 6.3.

	Lung[1]	Skin[2]
Compound 48/80	-	+
Anti-IgE	++	++[3]
Substance P	-	+

Table 6.3 Effects of immunological and non-immunological stimuli on mast cells from the skin and lung. + - histamine release only, ++ - lipoxygenase (LTC_4 release) and cyclooxygenase (PGD_2 release) activation.
[1] From bronchoalveolar lavage.
[2] From foreskin tissue.
[3] Only small amounts of LTC_4 released.

Close examination of the time course of histamine release from dispersed skin mast cells revealed that the neuropeptide- and C48/80-induced release was rapid in onset (peak <1min) unlike the anti-IgE response which was more gradual, reaching a plateau at about 10 min. All three stimuli caused similar maximal release.

B. Effect of compound 48/80 and terfenadine

As stated earlier, it has been shown that intradermal administration of PAF results in a dose-related flare and weal reminiscent of the histamine triple response. Moreover, H_1-antagonism using terfenadine results in a significant reduction of this response (almost abolition in the case of the flare component); it was thus decided to investigate the effects of prior histamine depletion on the responses to PAF.

In order to accomplish this, C48/80 was used to repeatedly degranulate skin mast cells until the flare and weal response was virtually abolished. The effects of intradermal PAF could then be evaluated under histamine-free conditions.

A pilot study was conducted so as to identify the extent of depletion after successive injections of C48/80. In two healthy male volunteers, single daily intradermal injections of C48/80 (10μg per site) were given at pre-selected volar forearm sites for 3 days in total. The vehicle for C48/80, normal saline, was injected at alternative volar forearm sites as a control. It was demonstrated that the flare and weal responses after the third set of C48/80 injections were reduced by 87% and 94% respectively compared with the responses after the first injection. Thus it appeared reasonable to study the effect of PAF at sites which had been pre-treated with 2 prior injections of C48/80. As a precaution, an extra site per arm would be used for assessing the effect of a third injection of C48/80 or vehicle in order to confirm the results of the pilot study in terms of histamine depletion.

In order to control for the possibility of residual endogenous histamine presence, terfenadine was included in the design. Inclusion of exogenous histamine in the treatment schedule allowed control for possible desensitisation to the effects of histamine which could lead to a false conclusion concerning histamine depletion.

1. Study hypotheses

a. Prior depletion of histamine from skin using C48/80 will significantly reduce the responses to intradermal PAF.

b. Addition of terfenadine to C48/80 treatment will not cause any overall increase in effect on responses to intradermal PAF.

2. Design and methods

Once again a 2-period crossover design was used and 8 healthy non-atopic male volunteers were recruited for study. Terfenadine or matching placebo was given in similar fashion to an earlier study (see chapter V). Four sites were chosen on the volar surface of each arm. Preparation of injection solutions were as described in Appendix 1. For the two days prior to each study day, all four sites in one arm (the three sites chosen for PAF challenge in one arm) were injected with C48/80, while those in the contralateral arm were injected with saline vehicle. In the control sites, C48/80 or saline vehicle were injected on both pre-study days and on the challenge study day. On study days 1 and 2, injection was given immediately prior to the first dose of oral treatment for that day.

On the 3rd day (challenge study day) the final dose of terfenadine or placebo was taken. Four hours later the flare and weal responses to intradermal injections of PAF (500 pmol

per site), histamine (5 nmol per site) and haemaccel vehicle at the pretreated challenge sites, and to C48/80 or saline vehicle in the control sites, were evaluated in each arm (see schedule below, Table 6.4).

Phase:	Day 1 Pre study day	Day 2 Pre study day	Day 3 Challenge study day
I.D. Treatment:			
Challenge sites (n = 3/arm)	C48/80 or vehicle[1]	C48/80 or vehicle[1]	PAF, hist or vehicle[2]
Control sites (n = 1/arm)	C48/80 or vehicle[1]	C48/80 or vehicle[1]	C48/80 or vehicle[1]
Oral therapy:	------Terf/plac------ ------60 mg bid------		Terf/plac 180 mg sd

Table 6.4 Intradermal and oral treatment schedule from histamine depletion study. Oral treatment was given 4 hours before injection on Day 3.
[1] Saline, [2] Haemaccel; Terf-terfenadine, Plac-placebo.

FA and WV were measured as in previous studies, 5 and 15 mins after intradermal injection of all treatments on all study days. Analysis of the results was performed using analysis of variance (ANOVA) techniques within SAS statistical software (GLM, Type III Sum of Squares and LS means). Pairwise comparisons were made by t-tests. Assumptions of the

ANOVA were tested with the Kolmogorov Goodness-of-Fit test and Hartleys Fmax test. When they were not met, i.e. either normality and/or homogeneity of residual errors were rejected at $\alpha=0.05$, a normalising and/or homogenising transformation was sought. Natural log transformations were used for flare area and weal volume. Raw untransformed data are however used for presentation purposes in this report. All p values were rounded up to 2 decimal places and significance has been attributed if $p \leq 0.05$.

3. Results

One volunteer dropped out of the study for personal reasons unrelated to the study procedures or therapy; results from this individual were excluded from analysis, thus results are presented for N=7

a) Responses to C48/80

Flare and weal responses at the control sites were used to determine the extent of C48/80-induced mast cell depletion. The results from each subjects placebo period are shown in Figure 6.1.

Both flare and weal were statistically significantly reduced ($p<0.01$) on days 2 and 3 compared with day 1. The response after the 3rd injection was <25% of that following the 1st injection and since the skin response to exogenous histamine was unaffected (see below) these observations confirmed comprehensive depletion of local histamine on the challenge study day (Day 3). Comparison of the flare and weal results following first injection (Day 1) in each of the two crossover periods revealed no differences in response indicating that histamine stores had therefore been regenerated during the inter-experiment period (two weeks). As expected, terfenadine treatment significantly reduced both the FA and WV responses to C48/80 on day 2 compared with placebo (<0.01). There was no significant difference between

placebo and terfenadine on the C48/80 responses on Day 3, providing confirmation of the histamine depletion by C48/80.

b) Effect of histamine depletion on PAF responses

The results of histamine depletion on the PAF response either with or without concurrent terfenadine therapy are shown in Figure 6.2. In the placebo therapy group, PAF-induced flare was virtually abolished by compound 48/80 pre-treatment (97% inhibition) compared with vehicle pre-treatment (see placebo responses in Figure 6.2, $p<0.01$). Histamine-induced flare was unaffected. The weal response to PAF, after placebo treatment, was significantly inhibited by compound 48/80 (63%, $p<0.01$) whilst the histamine response was not significantly affected. The combination of terfenadine therapy with compound 48/80 pre-treatment did not produce any significant enhancement of the effect of each treatment alone. Terfenadine did, however, significantly inhibit PAF-induced flare by 90% ($p<0.01$) and weal by 70% ($p<0.01$) at the sites pre-treated with saline (vehicle for compound 48/80).

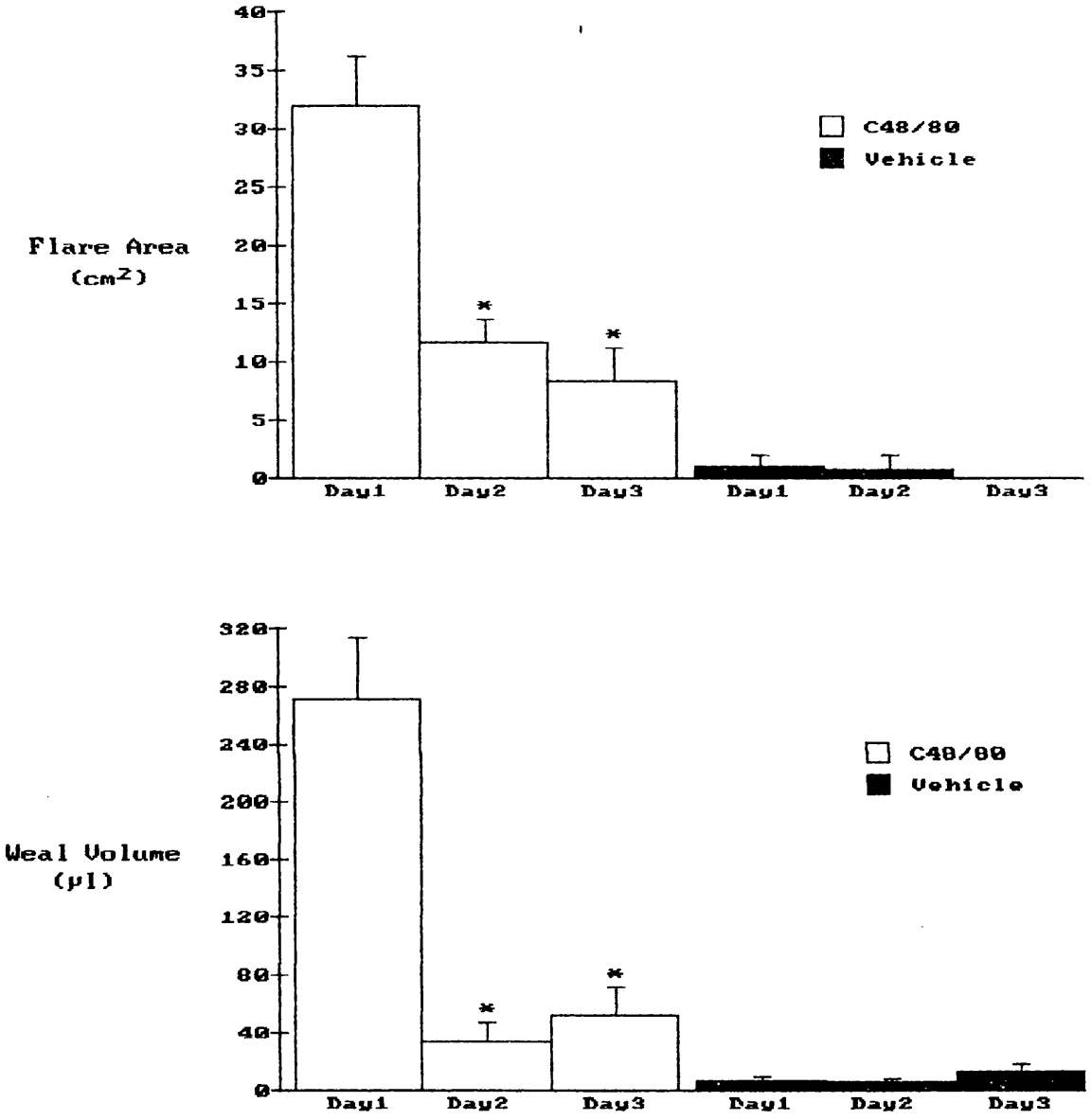

Figure 6.1 Flare (FA) and weal (WV) responses after each of three days repeated C48/80 intradermal injection during placebo treatment. Vehicle was normal saline (mean + sem, N=7, * - $p<0.01$ vs Day 1 response).

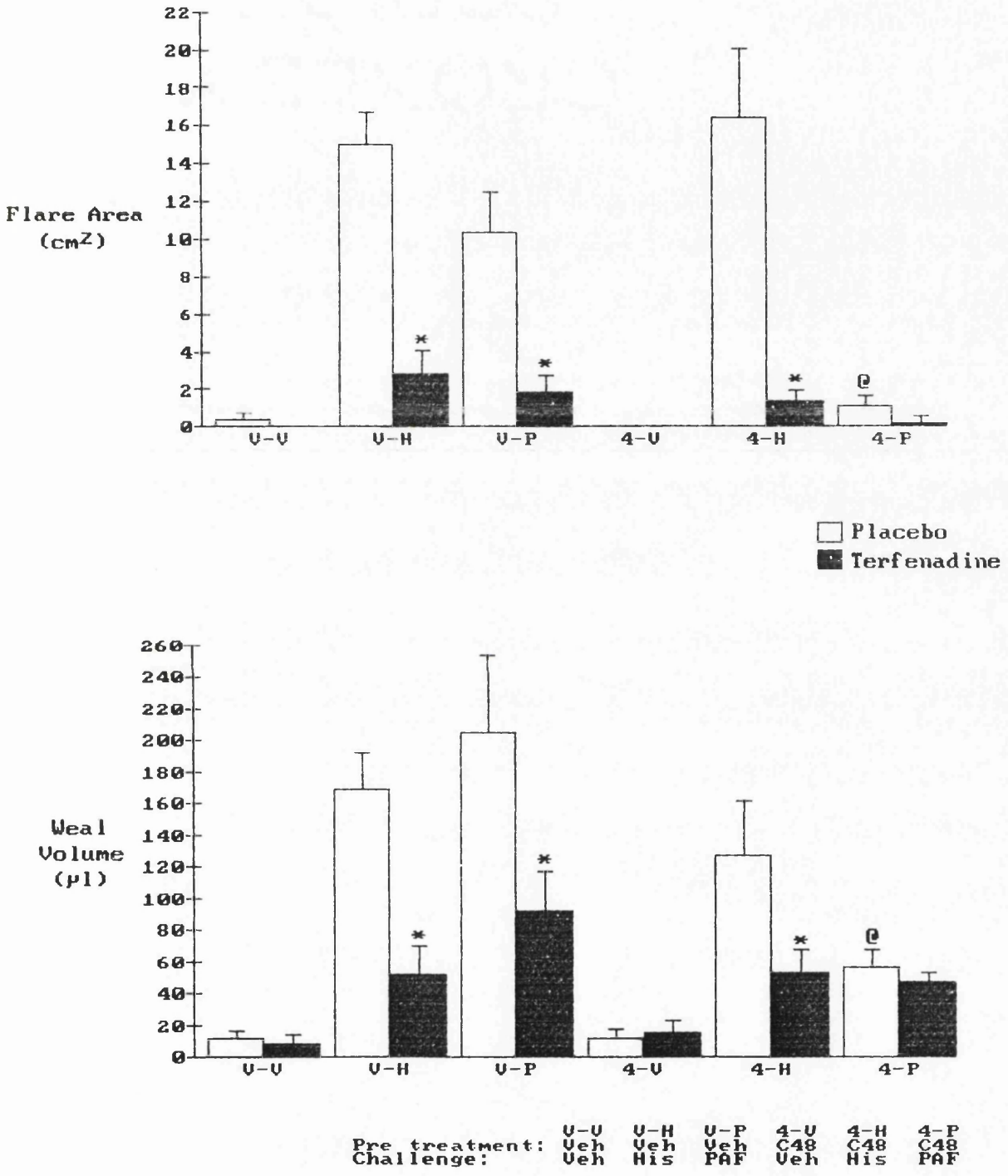

Figure 6.2 Flare and weal responses after intradermal challenge with PAF, histamine or vehicle (Haemaccel) on Day 3 following 2 days of prior treatment with either C48/80 or saline (pre-treatment) and oral therapy with either terfenadine or placebo (mean + sem). * - $p<0.01$ versus placebo, @ - $p<0.01$ versus corresponding saline pre-treatment.

4. Conclusions

Two consecutive intradermal injections of C48/80 separated by 24 hours have been demonstrated to produce comprehensive depletion of skin mast cell histamine. Furthermore, mast cell histamine complement appears to regenerate in <2 weeks. Using this model of histamine depletion, it has been shown that the responses to PAF are significantly reduced to a similar extent as has already been shown for H_1-antagonism using terfenadine. In this design, terfenadine had no additional effect over pre-treatment with C48/80. Thus it is concluded that mast cell histamine is necessary for PAF to exert provoke flare and weal responses in the skin.

CHAPTER VII

EFFECT OF PAF IN ATOPICS

A. Introduction

The term 'atopic' may be defined as the adjective used to describe a type I or 'immediate hypersensitivity' form of allergy. An atopic individual commonly has raised IgE levels, often in conjunction with an elevated eosinophil count, during active disease. More simply, the term may apply when an 'immediate-type' skin reaction (flare and weal) is demonstrated following exposure (usually i.d. injection) to a common allergen such as pollen-extract. Such skin reactions are usually associated with periodic clinical disease such as dermatitis, rhinitis, or asthma.

1. IgE and Mast Cells

The structure and function of mast cells have already been summarised in Chapter 6. The contribution of the mast cell to the Type I immediate hypersensitivity is fundamentally linked with the IgE class of antibody. Prausnitz and Kustner found, nearly three quarters of a century ago, that intradermal exposure of a normal, unaffected individual to the serum from an actively allergic patient conferred, in the normal subject, an immediate-type allergic responsiveness to the original allergen.

Although the nature of the substance mediating this phenomenon was not elucidated for many years, this was one of

the first and most important observations of the properties of the IgE class of antibodies. The presence, in the serum of the allergic individual, of elevated levels of the IgE type antibody, born of the interaction between allergen and B-lymphocyte, enables priming of the otherwise dormant mast cells of the non-allergic individual (see section on cross-linking of F_c receptors). Subsequent exposure of these mast cells to the allergen (e.g. intradermal injection of grass pollen extract) results in an allergic flare and weal response atypical for the subject; the allergen would not provoke this response in any other skin site in this subject.

Comparisons have been made between the responses following intradermal PAF and those following allergen in atopic individuals (Basran et al,1984). If PAF is indeed a primary mediator of the allergic response, it would seem reasonable to propose that the effect of PAF on the 'primed' mast cells in atopic individuals should be greater than the corresponding effect in normal subjects. Furthermore, it has already been shown that PAF effects in the skin are largely histamine dependent from experiments using histamine blockade and depletion.

In order to explore this hypothesis it was decided to look for substantive evidence from release of histamine into the local circulation following intradermal PAF. Hence, a two part experiment was undertaken where the acute inflammatory responses to intradermal PAF were evaluated in atopic and non-

atopic individuals and local venous histamine was measured after PAF challenge.

B. Comparison of atopic and non-atopic responses

1. Study hypotheses

a. The acute inflammatory responses following intradermal PAF are quantitatively different in atopic subjects when compared with non-atopic subjects.

b. Intradermal injection of PAF results in an increase in local plasma histamine.

c. Release of histamine after PAF is quantitatively different in atopic subjects compared with non-atopic subjects.

2. Methods

The study consisted of two distinct study days separated by a period of one week. 12 healthy male volunteers were recruited of whom six were atopic and six non-atopic. Atopic status was confirmed or refuted according to the following criteria:

a. Group 1 (Non-atopic): No history of allergic disease and any weal response must be <5mm diameter after a skin prick test with a range of standard allergen extracts (Bencard^{R}).

b. Group 2 (Atopic): History of allergic disease such as atopic rhinitis, hayfever, atopic dermatitis etc (asthma was excluded) together with a response of >6mm diameter to one or more allergens after the skin prick test.

On the first study day the flare and weal responses were assessed in all 12 volunteers to the same doses of PAF and histamine as in earlier studies: 20, 50, 200 and 500 pmol PAF and 5 nmol histamine per site. Preparation and administration of intradermal injections was as described in Appendix 1. Methods for measuring flare and weal are described in Chapter 4.

On the second study day, 11 subjects were studied (6 atopic, 5 non-atopic). Bilateral cannulae were inserted into a superficial vein in the antecubital fossa and blood samples drawn immediately prior to and at 1, 3, 5, 10, 15 and 30 mins following intradermal PAF (500 pmol) or vehicle (see Figure 7.1). Each 5ml blood sample, collected into freshly prepared EDTA solution (final conc 5mmol/l), was immediately put on to ice pending prompt centrifugation and separation of resulting plasma which was in turn kept frozen at -20°C until histamine assay was performed. All volunteers were left to rest supine for at least 20 minutes after cannula insertion to allow for any traumatic effects of cannulation to wear off. Cannulae were kept patent using freshly prepared heparin/saline (hepsal). The first 1ml of any drawn sample was discarded to avoid hepsal contamination.

a) Histamine Assay

Plasma histamine was assayed by Dr. D. Gill of the Royal Free Chemical Pathology Dept using a commercially available radioimmuno assay (RIA) kit from Immunotech SA. This RIA is based on the acylation of histamine in samples in order to confer maximum affinity for the mouse monoclonal antibody. The technique was recently reviewed favourably by Kaliners group (McBride et al,1988). The assay is very specific and does not significantly cross-react with the usual contaminants:

Methylhistamine	<0.006%
Histidine	<0.0004%
Serotonin	<0.0001%

A preliminary validation study was conducted using histamine-spiked plasma samples. Sensitivity of the assay was 10pg/ml (0.1nM) histamine in plasma and the coefficients of variation for inter (9.4%) and intra (8%) assay were within acceptable limits (N = 18).

b) Statistical Analysis

Analysis of results was performed using a combination of SAS, BMDP and RS/1 statistical software. Analysis of variance was used including, where appropriate, factors for group, subject within group, period, treatment, arm, site and dose. All tests of significance were two-tailed and differences were referred to as significant if $p<0.05$ or less.

3. Results

A highly significant dose-relationship for flare and weal response was observed in both the atopic and the non-atopic group (see Figure 7.2, $p<0.0001$). There was no statistically significant difference found in either flare or weal between the two groups.

A sharp rise in plasma histamine levels was found within 5 minutes of PAF treatment in both groups (see Figure 7.2). Mean peak plasma histamine levels during the 5 minutes following injection were statistically significantly greater following PAF compared with vehicle treatment (see Figure 7.3) although there were no differences found between groups for the effect of PAF. There was, however, a significant difference found in the baseline plasma histamine level in that the atopic group had approximately twice the level found in the non-atopic group (see Figure 7.2 and 7.3). Furthermore, the time to peak plasma histamine following PAF appeared to be shorter in the atopic group.

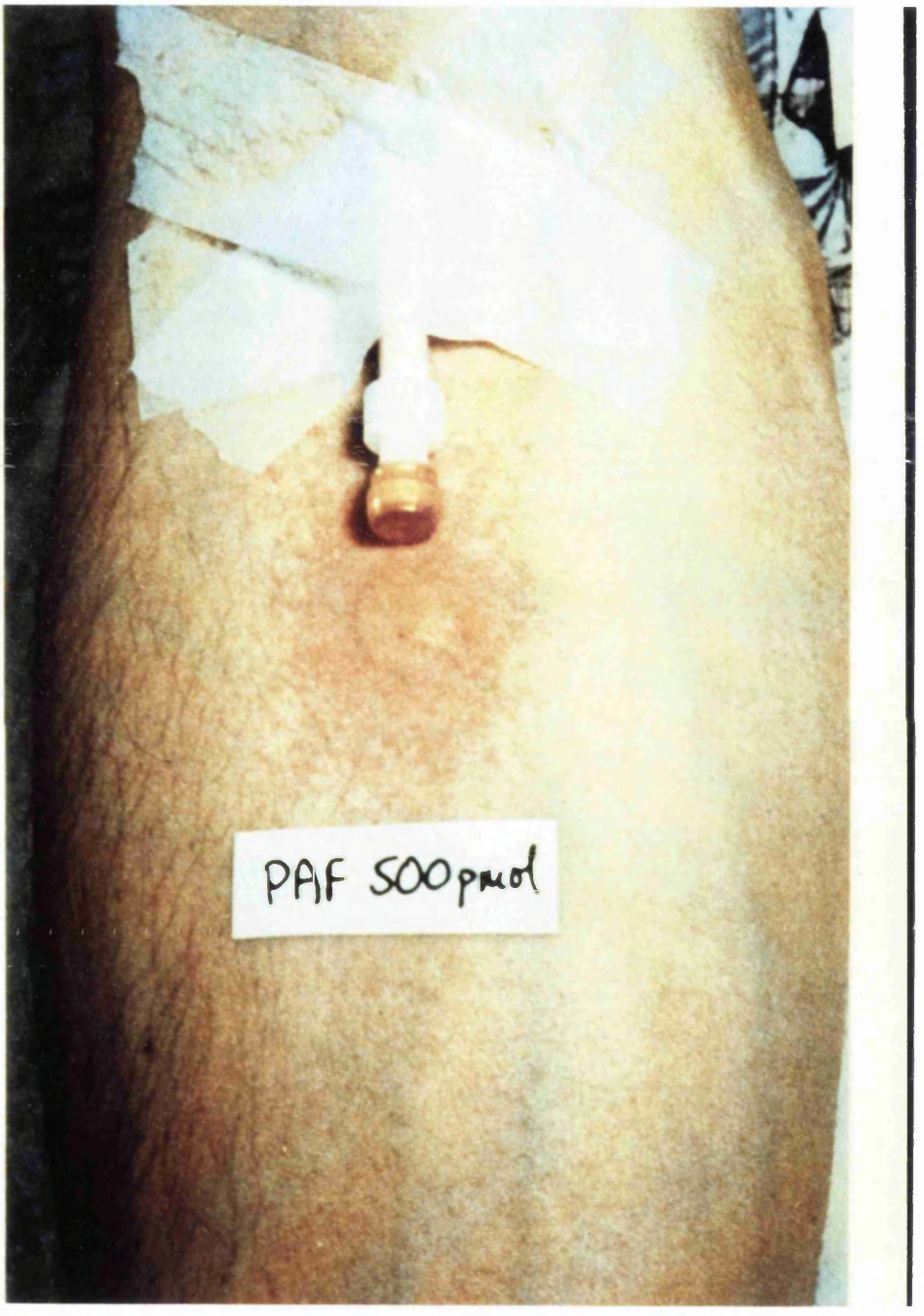

Figure 7.1 Arrangement of intradermal injection and superficial venous cannula enabling collection of plasma for subsequent histamine assay.

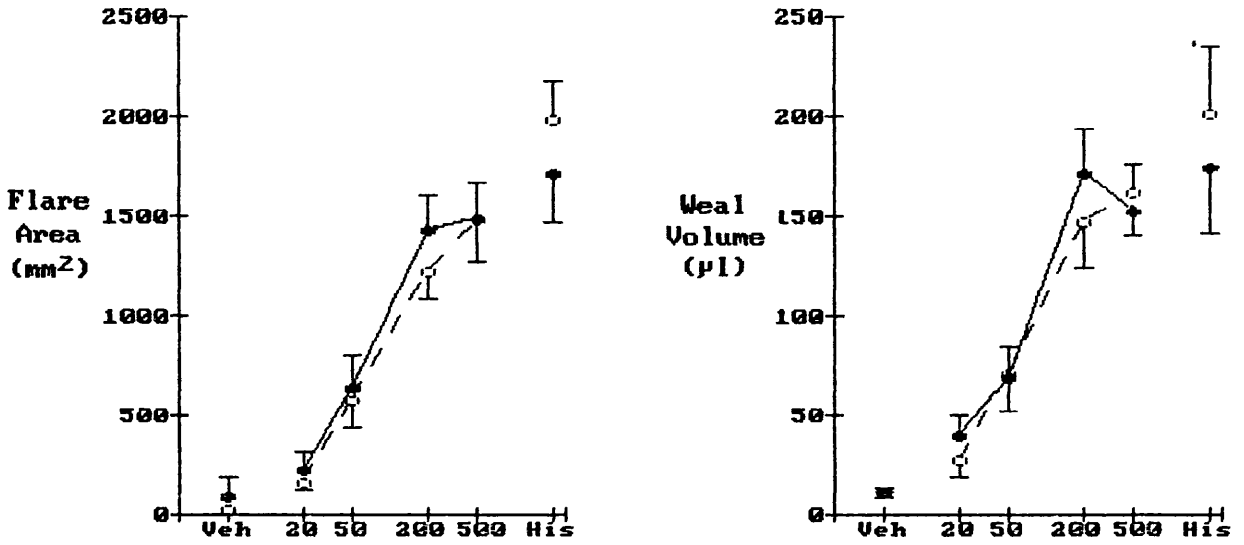

Figure 7.2 Mean flare (FA) and weal (WV) responses to a range of doses of PAF (pmol), histamine (His, 5 nmol) and vehicle (Veh) in atopic (closed circles) and non-atopic (open circles) subjects (N=6 per group, +/- S.E. Mean).

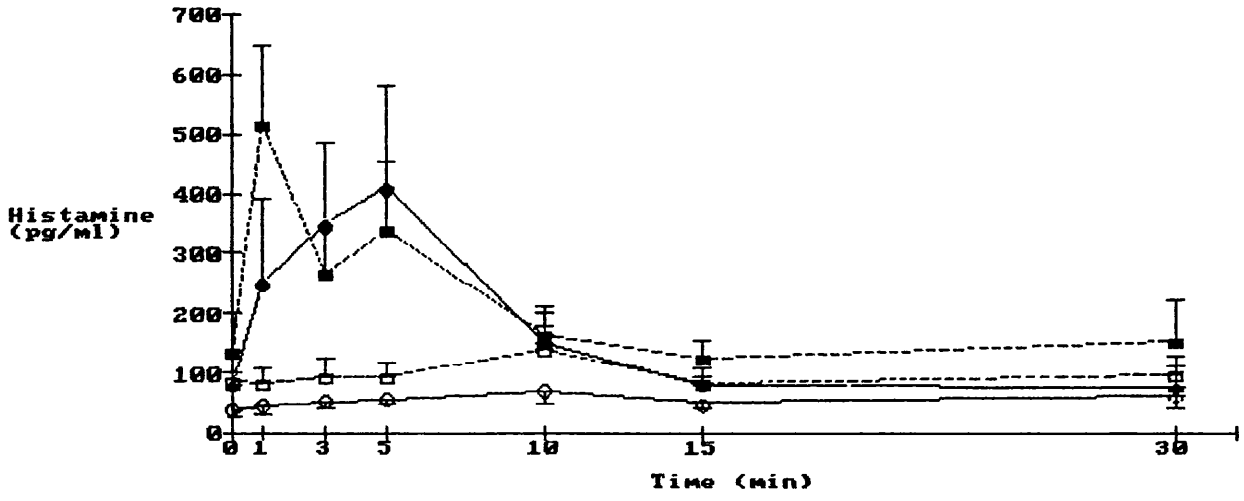

Figure 7.3 Mean plasma histamine following intradermal injection of either PAF (500 pmol, closed symbols) or vehicle (Haemaccel, open symbols) in atopic (square, dashed lines) and non-atopic (circle) subjects (N=6 atopics and 5 non-atopics, +/- S.E. Mean).

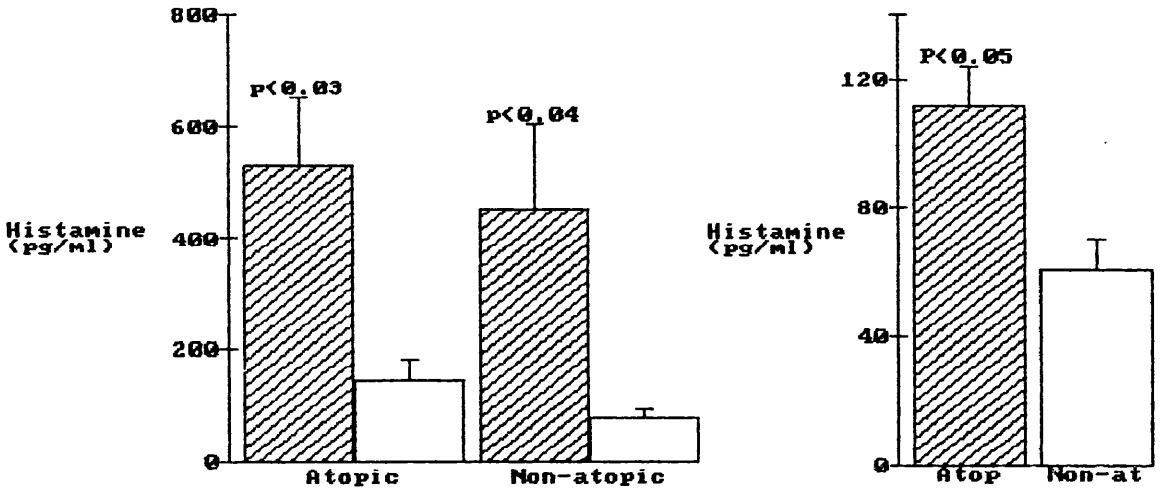

Figure 7.4 Mean plasma histamine levels following either 500 pmol PAF (shaded bars) or vehicle (open bars, p vs vehicle) in left hand bargraph and a comparison of basal plasma histamine levels in atopic (shaded bars) and non-atopic (open bars, p vs non-atopic) subjects in right hand bargraph (N=6 atopic and 5 non-atopic, +S.E.Mean).

4. Conclusions

It has been demonstrated that atopic and non-atopic subjects respond in both a qualitatively and quantitatively similar fashion to intradermal PAF. Furthermore it has been shown that local histamine release follows intradermal injection of PAF. The histamine response is similar in both non-atopic and atopic subjects.

Basal plasma histamine levels in atopic individuals appear to be significantly higher than in non-atopic subjects.

CHAPTER VIII

DISCUSSION AND CONCLUSIONS

Since its discovery, PAF has caused much excitement in the field of inflammatory research largely because its pharmacological profile and ubiquity make it a candidate for involvement in a number of inflammatory processes. A capacity for causing vasoconstriction, vascular permeability, chemotaxis, cell degranulation and activation, bronchoconstriction and bronchial hyperreactivity is a unique portfolio amongst conventional inflammatory mediators.

All of the attributed properties of PAF have been extensively researched in animal models; hard evidence to support its pathophysiological role in man, however, remains controversial. There are two major reasons for this. In view of the potentially extensive toxic effects of PAF, there has been appropriate clinical caution in attempting to reproduce animal model effects in man (e.g. bronchial provocation). Early studies of PAF in man were limited to the skin where systemic absorption would be limited and hence severe adverse events unlikely.

The first observations of systemic effects of PAF appeared in an article recounting changes seen after intratracheal administration of PAF to patients qualified as having 'cerebral death' (Gateau et al,1984). The main effects were marked deterioration in lung function (increased peak inspiratory pressure and mean airway pressure together with increased $PaCO_2$ and reduced PaO_2), decreased platelet and

leucocyte count and a 'dramatic' fall in cardiac index. It is hardly surprising that a subsequent major publication on the in-vivo effects of PAF in human lung did not appear for another two years (Cuss et al,1986).

Since this Lancet publication declaring that PAF inhalation provoked chronic, nonspecific bronchial hyperreactivity in normal man, there have been only two other studies supporting these findings (Rubin et al,1987;Chung et al,1989). Three studies conducted more recently however have either found the phenomenon unreproducible (Stenton et al,1990), short-lived (Hopp et al,1990), or have not been able to demonstrate this effect of PAF at all (Spencer et al,1990;Lai et al,1990;). Without a more clearly defined role for PAF in asthma, it is highly likely that interested major pharmaceutical companies have been reluctant to commit resources to a PAF antagonist and rather rely on more established asthma leads e.g. leukotrienes.

There are methodological difficulties associated with the use of PAF in clinical pharmacology of which the most important is a tendency to stick to surfaces and sequester out of solutions. Chapter 4 contains details of experiments showing how the stability of PAF in solution is dependent upon the presence of a carrier such as albumen or Haemaccel. Studies where PAF is delivered in a vehicle lacking such a carrier are subject to unpredictable results which could be interpreted as a negative outcome.

The skin provides a useful clinical model for the study of inflammatory mediators such as PAF and cautious parallels may be drawn between effects seen in the skin and effects in other tissues. Such inferences must however rely on a clear understanding of the underlying pharmacological mechanisms. It has been discussed earlier in the thesis how there is controversy concerning the involvement of histamine in PAF-induced skin responses (see Chapter 2.E).

A series of studies were undertaken in man to investigate the clinical and biochemical consequences of intradermal PAF injection. Any such effects were examined for reproducibility and, where relevant, compared with analogous effects of other inflammatory mediators. Each set of experimental results from the preceding chapters will now be discussed.

A. Hyperalgesia (Chapter III)

A hyperalgesic response to PAF assessed as change in pain threshold to skin pressure could not be demonstrated in this study. The effect of 0.7 and 1.4 nmol PAF per site could not be distinguished from vehicle. Detectable hyperalgesia in response to PAF, using similar concentrations as in the present study, was referred to in an open study (Archer et al,1986). The hyperalgesia was assessed using a 'dermographometer' - a pen-like instrument which allows a spring-loaded tip to be pressed against the skin; subjects were then asked to record their experience of pain on a 10 cm

visual analogue scale. The authors stated that the methodology was of 'low sensitivity' which casts doubt on the validity of the methodology. A so-called 'late-response' consisting of both erythema as well as the purported hyperalgesia was reported to occur in 8 of 10 subjects on two occasions separated by seven days. The 'late-response' had been referred to in earlier published literature (Archer et al,1985;Basran et al,1984;Archer et al,1984).

More recently, however, it has been reported in two separate studies that no such late response could be distinguished clinically. Henocq et al (1988) referred to 'the absence of macroscopic LPR' (late phase response) in a study of the effects of PAF at 0.7 nmol/site in atopic and non-atopic subjects. Furthermore, Guinot et al (1986), in a study of the effects of PAF at the same dose in normal volunteers, reported that a late cutaneous reaction was not observed in any of the subjects. As far as the author is aware, the studies of hyperalgesia reported in this thesis are the first to be objective in measurement and conducted under double-blind conditions; earlier studies have relied upon subjective opinion or at best visual analogue scales. The studies of PGE_2 clearly demonstrated a hyperalgesic response and enabled positive validation of the algesimeter which, together with the unstimulated reproducibility data, showed the use of the instrument to be a sensitive and reproducible methodology for monitoring pain threshold changes. Additionally, this study was able to demonstrate a dose-response in the PGE_2-associated hyperalgesia.

The absence of an hyperalgesic effect (compared with saline) of the neuropeptides substance P and CGRP is of interest. Substance P has been associated with hyperalgesia to pressure-induced pain when injected intrathecally in rats, although tachyphylaxis was also observed (Sweeney et al,1986). In a study of the hyperalgesic potential of intradermal substance P in man, the neuropeptide was injected into the anterior temporal muscle (Pedersen-Bjergaard et al,1989). Substance P was found to provoke cutaneous pain as elicited by visual analogue scales while the use of a quantitative 'pressure algometer' did not enable distinction between active and vehicle injections. Once again, therefore, a quantitative test did not confirm subjective opinion. The doses used were 50 and 200 pmol per site which are comparable to those used in the present studies. Lembeck et al (1977) were also unable to demonstrate an 'algesic' effect of substance P in a model utilising reflex falls in systemic blood pressure in response to injections of pain-producing substances in anaesthetised rabbits.

CGRP appears to co-reside with substance P in the polymodal c-fibre type nerves occuring close to vascular tissue (Brain et al,1986). Its primary function appears to be as a potent and long-lasting vasodilator.

Just as was found in the PAF hyperalgesia study, PGE_2 was shown to produce hyperalgesia. It is important to draw attention to the considerable inter-individual baseline pain threshold differences seen in these studies. If the PGE_2

responses are normalised as a % of vehicle, the pattern of response becomes considerably more uniform with all subjects demonstrating an approximate 60% reduction in pain threshold after injection of the prostaglandin. It has been hypothesized that the effect prostaglandins exert on neuronal tissue (eg c-fibres) is mediated by an elevation of cAMP levels (Ferreira et al,1981). Nevertheless it is of interest to reflect on evidence of the multiple role of c-fibres; release by peripheral nerve fibres of substance P and/or CGRP together with altered function in response to local release of prostaglandins undoubtedly play an important role in the global inflammatory response to pathogen invasion.

B. Flare and Weal (Chapter IV)

The flare and weal responses to PAF have been reported by a number of workers. In a study where doses of up to 0.4 nmol/site were used (Archer et al,1984), the authors were able to demonstrate a dose-related response for weal while the dose-relationship for flare was less clear. Other workers (Fjellner et al,1985) have reported a dose-relationship for flare response following injection of 0.02 to 0.2 nmol per site.

More recently, in studies of the effects of ketotifen (Chung et al,1988) and BN 52063 (a ginkgolide PAF antagonist; Chung et al,1987) upon the cutaneous responses to doses of 0.13 to 1.0 nmol PAF, a clear dose-relationship was found for both flare and weal responses. In the present studies, observations of the acute inflammatory responses to PAF have demonstrated dose-related effects for the phospholipid mediator within a similar dose range to that used in the work quoted above. A number of methodological aspects of the evaluation of flare and weal response are worthy of critical examination.

Flare and weal responses have traditionally been measured using orthoganol or maximal diameters which were then used to calculate area (Cook et al,1980;Archer et al,1984). Such a methodological approach does not take into account the irregular shape of both weal and, in particular, flare since

circular form is assumed. By tracing the margin of each response onto transparent acetate sheets and subsequently measuring area accurately using computerised image analysis, pseudopodia were included in the calculation of area in the present studies. This should have aided both the precision and objectivity of the methodology. In fact, several groups have now used this approach in their studies (Shall et al,1987;Humphreys et al,1987;Chung et al,1987).

In order to interpret more accurately the weal response, skinfold thickness measurements were taken at pre-injection and 15 minutes post injection. Cook et al (1980) examined the use of Harpenden calipers to estimate skin thickness as an aid to weal volume evaluation. It was found that the mean untreated skin thickness in 25 normal volunteers was 2.6mm (S.E.Mean 0.1mm, volar forearm surface). In the present studies, the Mitutoyo spring-loaded thickness guage was preferred for reasons of portability and ease of use. A set of mean pre-treatment skinfold thickness values are shown in Table 4.2 (Chapter 4) which compares favourably with the Cook et al data. A criticism of the use of calipers to measure skin thickness has been that the measurement procedure compresses the weal and therefore causes a shift in weal oedema which could influence the data. Cook et al found that this produced an error which was limited to approximately 6% and hence unlikely to influence results in antagonist studies where one would expect greater than 20-30% shifts.

A-scan pulsed ultrasound devices have been used for skin thickness estimation (Shall et al,1987). This method purported to have the advantage of avoiding the compression flaw. The mean readings from normal volunteers in this study were 0.77mm (S.D. 0.12mm) which were rather low in comparison with readings from the currently reported studies and those of Cook et al. Furthermore, the A-scan generated skin thickness change ($\delta T/2$) data were also dissimilar to those reported using Harpenden calipers (2.22mm - A-scan, 225 nmol histamine vs approximately 1.4mm - calipers, 240 nmol histamine). It appeared however that the caliper method detected less change than the ultrasound method for comparable histamine doses. A skin thickness change of approximately 0.70mm (reading taken from graph in paper) was reported by Cook et al for a dose of 9 nmol histamine, which compares favourably with the data reported in Table 4.2 (Chapter 4) for 5 nmol histamine (0.88mm). It appears therefore that the A-scan technique and the caliper method may not be measuring exactly the same thing. Lawrence et al (1985) compared the two methodologies in the same study and concluded that the the caliper method had a greater precision, reproducibility and reliability than ultrasound for measurement of skin thickness in inflamed skin.

Scrutiny of the Cook et al paper with respect to time course of histamine weal response compares well with thesis data in that the time of maximum weal was 15 to 20 minutes. Flare was found to be maximal at around 5 minutes as with presently reported studies for both PAF and histamine.

In the studies addressing the effect of vehicle on PAF responses, a protein (HSA) or artificial polygeline (Haemaccel) carrier appeared to be important in determining the reliability of these responses. Ludwig et al (1986) reported that albumin significantly enhanced the aqueous solubility of PAF using radiolabelling techniques. Furthermore, the presence of extracellular albumin was shown to increase the synthesis of intracellular PAF in neutrophils, an effect which was presumed to be due to enhancement of PAF release from the cell removing a negative feedback effect. While there is no published data quantifying the uptake of PAF by albumin, given two aqueous solutions of PAF with identical concentrations but one containing albumin, one would expect the albumin-free solution to exert a greater biological effect. In fact, carrier-free solutions produced similar, if not reduced, flare and weal (notwithstanding the lack of consistent dose-response) in experiments described in Chapter 4.D; these data emphasise the importance of carrier proteins in modulating the effects of PAF in biological systems.

PGE_2 was also shown to produce dose-related flare and weal responses. Although PAF appeared to be more effective than PGE_2 in producing weal, the two mediators were studied in different experiments and so this finding requires confirmation. Comparison showed that the phospholipid was approximately 50 times as effective as PGE_2.

There is little published data on the flare and weal effects of PGE_2 in human skin, although the eicosanoid has been shown to act synergistically with not only PAF (Archer et al,1984) but also with other mediators (Williams,1977). It is clear however that the dose-responses in the present studies were relatively shallow compared with PAF further demonstrating that the phospholipid was more potent than the eicosanoid. A clear difference in the responses to the two mediators was that PGE_2 produced a deep red erythema which gradually appeared after the flare had virtually disappeared (20-30 min). This erythema spread outwards at the higher doses, with 'pseudopodia' tracking up the arm. A possible explanation for this phenomenon was that the erythema followed diffusion of the prostaglandin via lymphatic vessels. The erythema lasted for 2-4 hours and, in some subjects, was reported to be associated with extended hyperalgesia. PGE_2 may have an important role to play in both enhancing the effect of other mediators and mediating the appearance of hyperalgesia at sites of physical injury or pathogen invasion.

The effects of intradermal substance P and CGRP were clearly dissimilar. Substance P was shown to provoke a histamine-like flare and weal response, while CGRP produced a sharply defined, small area of persistent erythema without any visible signs of wealing. Similar observations were made by Brain et al (1986). Piotrowski et al, however, reported that CGRP produced a dose-related weal response at doses of 12.5 to 50 pmol, i.e. of the same order of magnitude as the presently reported studies.

Although methodologies differed for quantifying weal (weal area estimated by orthogonal diameters by Piotrowski as opposed to the method specified in Chapter 3), both relied upon the clinical interpretation of skin response in order to define extent of wealing. In the present studies, even vehicle response was often interpreted as weal, under blind conditions, even though it was probably only a function of physical skin displacement as a result of injection volume; CGRP produced a response which was indistinguishable from vehicle, regardless of dose, in terms of wealing. Since the associated erythema did not appear to any great extent until some 30-40 minutes post injection, the study-blind was not compromised in this respect.

It is unlikely therefore that the conflicting interpretations of response to CGRP may be explained by methodological differences although it should be said that the methodology of planimetry together with estimates of skin thickness change would be more likely to detect a dose-related effect than orthoganol diameters alone, an issue which is discussed earlier in this section. Piotrowski et al (1986) reported lack of effect of chlorpheniramine on substance P-induced weal. Substance P is known to be a potent mast cell degranulator (see Chapter 6), and it would be expected that substance P effects would, in part, be a function of histamine release. Hence this casts further doubt upon the validity of their studies.

The respective roles of substance P and CGRP in propogating the inflammatory response are thus highlighted. Substance P appears to be involved with promotion of vascular permeability changes and in this respect may act in parallel with other mediators to enhance leucocyte infiltration at a site of pathogen invasion while also augmenting mast cell activity.

CGRP could well be the much sought after end-mediator of the axon reflex, with its release producing a mosaic of discrete areas of vasodilatation coinciding with end-terminals of c-fibres located close to superficial microvasculature. The global view of this effect would be the histamine-type flare. Since CGRP does not appear to have any intrinsic capacity either to cause mast cell degranulation or increase vasular permeability, no weal response is associated with intradermal injection. The neuropeptide may rather oppose and hence modulate the vasoconstrictive effects of other released inflammatory mediators (e.g. PAF), as does PGE_2 (Williams,1977), to enhance extravasation and thus assist leucocyte infiltration. Compared with PGE_2, however, the neuropeptides were only weakly associated with tenderness or hyperalgesia, and only then at high doses.

These findings, together with those addressing hyperalgesia, give insight to the differential roles played by lipid and neuropeptide mediators in the global inflammatory response.

C. Effect of Indomethacin (Chapter V)

Indomethacin was shown to inhibit significantly the overall flare response to PAF, irrespective of dose. Scrutiny of data by individual dose revealed a specific effect only at the lowest dose of PAF. By way of contrast, there was no overall effect upon weal although, as for flare, there was significant inhibition at the lowest dose only. These findings are at odds with those of Archer et al (1985) who found no effect of indomethacin on either weal or flare. The differing results may be a function of methodology sensitivity; Archer et al used orthogonal diameters as opposed to planimetry.

Human skin challenged with antigen mainly releases PGD_2 together with some PGE_2 (Barr et al,1988). Mast cells are undoubtedly the source of this prostaglandin material (see Chapter 6.A). It has been proposed that the PAF response may mimic that to allergen both in the skin (Basran et al,1984) and elsewhere (Barnes et al,1988). If PAF were to act on mast cells in a similar way to antigen, it is likely that prostaglandin release would ensue (Church et al,1989a & b) which would go some way to providing a possible explanation for the apparent effect of indomethacin.

Intradermal PGD_2 in man has been shown to provoke a similar effect to that reported above for PGE_2 (Flower et al,1976), an extended erythema with some small degree of wealing, although this latter was not quantified and was only reported as 'oedema'. Nevertheless, PGD_2 was shown to produce

permeability changes in rat skin using albumin extravasation techniques. It would be easy under blind conditions to confuse the prostaglandin-type erythema with the histamine type flare; furthermore cyclooxygenase inhibition would be expected to inhibit PGD_2 synthesis in mast cells such that the end result would be an apparent inhibitory effect on PAF-induced flare.

PGE_2 was shown to be much less effective in producing weal than flare. In reported studies (Flower et al,1976), however, PGE_2 was more potent than PGD_2 in provoking extravasation. It would therefore be expected that inhibition of cyclooxygenase-mediated PGD_2 synthesis would have a significantly less marked effect on weal when compared with flare/erythema. The results of the present study appear to support this hypothesis, as do earlier studies of the vascular permeability effects of PAF in animal models where the data suggested the involvement of secondary release of cyclooxygenase products (see Chapter II.A.1).

D. Histamine Antagonism, Depletion and Release (Chapters V & VI)

When PAF was first described by Henson (1970) it was as a soluble factor released from rabbit basophils causing aggregation of platelets and release of histamine. Since then other workers have examined the relationship between PAF and histamine. Archer et al (1985) reported that the H_1-receptor antagonists mepyramine and chlorpheniramine slightly modified acute inflammatory responses in human and guinea pig skin. The authors concluded that histamine release was not extensive enough to induce oedema since only the flare results were significantly inhibited by locally administered chlorpheniramine.

Hwang et al (1985) were not able to demonstrate any effect of H_1 or H_2-antagonism in either guinea pigs or rats on PAF-induced acute cutaneous vascular permeability changes; nor were the authors able to demonstrate any histamine accumulation in the cutaneous injection site. Humphrey et al (1982) concluded that the lack of any apparent effect of chlorpheniramine upon PAF-induced vascular permeability changes in rabbits and guinea pigs indicated a histamine-independent mechanism not involving H_1-receptors.

Fjellner and Hagermark (1985) not only found that mepyramine significantly inhibited the intradermal PAF-induced Flare and itch responses, but also showed that prior histamine

depletion using C48/80 significantly inhibited the PAF-induced responses. Furthermore, ketotifen (Chung et al,1988), primarily an H_1-receptor antagonist, was found to significantly inhibit PAF-induced flare and weal responses in human skin to the extent that it was concluded that these responses were probably histamine dependent.

In the present study it has been shown that H_1-antagonism, by systemically administered terfenadine, significantly inhibits both the flare and weal components of the acute inflammatory response to intradermal PAF. It has also been demonstrated that prior histamine depletion has a similar effect to H_1-antagonism. Further confirmation of the role of histamine was provided by detecting significant increases in local plasma levels following cutaneous PAF administration.

It appears however that PAF-induced flare is more sensitive than weal to histamine manipulation. This may reflect a direct, histamine-independent mechanism by which PAF induces vascular permeability changes. Alternatively, it is worth considering that histamine-induced weal was not entirely blocked by H_1-antagonism. Cook et al (1980) found that the disappearance of histamine-induced weal was too slow to be explained by simple absorption since saline weals were found to be absorbed 10 times as fast. They therefore suggested that histamine itself may liberate vasoactive substances in the skin (possibly histamine itself although unlikely as histamine-type tachyphylaxis was not found with repeated

dosing). This was also assumed to be a plausible explanation for the observed limited effect of H_1-antagonism on histamine-weal compared with flare. Shall et al (1987) found that terfenadine, given in a similar dosing regimen to that reported in Chapter 5, produced a 33% decrease in weal thickness and a 69% decrease in weal area. In another study, terfenadine at a dose of 120 mg bid for 5 doses was found to inhibit weal formation by 68%.

In the studies reported in Chapter 5, histamine-induced weal area was reduced by 43%, while weal volume was reduced by 66%. This was in the face of a 93% reduction in flare area. Weal volume due to PAF at the 500 pmol dose was reduced by 41% after terfenadine treatment. In the histamine depletion study however, terfenadine reduced weal volume due to 500 pmol PAF by 70%. The discrepancy is difficult to explain, but the overall indication is that there is a component of the weal response to both PAF and histamine which is resistant to H_1-antagonism in man. This is confirmed by the results for C48/80 depletion which are similar to the effects of terfenadine in that same study. C48/80-induced mast cell depletion reduced weal by 63%.

These data confirm the hypothesis that PAF-induced responses in the skin are mainly mediated by histamine, and the measurable PAF-induced histamine release gives further corroberation. The spillover effect seen in residual weal following both H_1-antagonism and mast cell depletion could well be explained by release of vasoactive substances either

directly by PAF or as a result of secondary histamine release. The fact that indomethacin was found to have a small but significant effect on the PAF responses may give insight to the nature of these secondary mediators. Potential direct effects of PAF on the local microvasculature are likely and should not be overlooked; such effects may only be evaluated, however, by the use of specific PAF-antagonists, ideally in combination and compared with H_1-antagonism.

E. PAF and Atopy (Chapter VII)

It has been shown that atopic and non-atopic individuals respond identically, in terms of acute response, to intradermally administered PAF. Similar data have been reported by Henocq et al (1988) although only at one dose of PAF (these data were not yet published when the thesis experiments were being designed and conducted). In the presented studies, a comparison was made over a range of doses of PAF and the resulting dose-response curves virtually overlay each other. Henocq et al also reported that there was a difference in the cellular response to PAF between atopic and non-atopic subjects at 24h after administration; the atopics had a prominent eosinophilic infiltration compared with the normal group. It was not possible for cellular infiltration to be addressed in the present thesis work.

It is interesting to note that the peak histamine levels were of the same order of magnitude and occurred around the same time as has been reported following intradermal antigen (Heavey et al,1984). McBride et al (1989) also monitored peak plasma histamine levels after intradermal injection of antigen and although a similar time of occurrence to the study described in Chapter VII was observed, maximum concentrations were substantially higher in their study. The authors did, however, note a high degree of between-subject variability in overall plasma levels, as with the study presented in Chapter VII, which was concluded by the authors to be related to site

of cannula insertion. Intra-subject reproducibility was lower. The time course for return to baseline was significantly longer following antigen in both of the above published studies compared with that observed for PAF. These differences may well reflect the contrast between a true immunological activation of mast cells and simple degranulation. Although there are extensive similarities between the inflammatory effects of PAF and those of antigen in the skin, there is inadequate evidence to support the view that PAF may account entirely for the events following intradermal antigen.

The finding that baseline plasma histamine was significantly higher in atopics than in non-atopics is of potential importance to the understanding of allergy. While it may have been a function of response to cannula insertion, it should be pointed out that all volunteers were allowed to rest for at least 20 minutes after insertion; furthermore all subsequent blood samples were collected using small volume syringes with as little suction applied as possible. McBride et al (1988) did not demonstrate any difference in basal plasma histamine between atopics and non-atopics. Samples were, however, collected in vacutainers in their study and mean plasma histamine levels in both groups were approximately similar to levels found in atopics in the present study. The levels may therefore have reflected an artefact of cellular degranulation (eg basophils) due to physical trauma in collection procedure.

F. Conclusions

a) Quantitative methods have been validated as a sensitive technique for evaluating hyperalgesia and histamine-type flare and weal responses in the skin.

b) Using the hyperalgesia model, intradermally injected PAF failed to cause hyperalgesia in the skin. Conversely intradermal PGE_2 was shown to provoke dose-related hyperalgesia, which was associated with spreading erythema, lasting for 2-3 hours. The neuropeptides substance P and CGRP also failed to produce hyperalgesia.

c) Using the flare and weal model, PAF, substance P and PGE_2 were associated with dose-related histamine-like flare and weal responses, although the PGE_2 responses were considerably weaker. CGRP did not produce these responses and instead caused a local intense erythema, similar to the later effect of PGE_2.

d) Evaluation of the effects of H_1-antagonism, histamine depletion and quantification of local histamine release has demonstrated that the PAF responses in the skin are largely mediated by histamine. Indomethacin was also found to have a small inhibitory effect on the PAF responses implicating secondary prostaglandin release.

e) The acute inflammatory responses to intradermal PAF have been shown to be similar in atopic subjects compared with non-atopics using the techniques described above. Baseline plasma histamine, however, was found to be higher in atopic subjects.

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APPENDIX 1

A. Volunteer considerations

A number of mandatory protocol requirements were implemented in every study in accordance with the RCOP and ABPI guidelines on human volunteer research:

Informed consent In line with the Declaration of Helsinki, potential recruits for studies were given full explanations of the aims, methods, anticipated benefits and potential hazards of each experiment together with any discomfort it may entail. The potential subject was also informed of his right to refuse to participate or to withdraw from the study at any time. Documentation of each subjects informed decision to participate in studies was always provided by their signature on a formal consent sheet. This was always available for scrutiny as appropriate by the local ethics committee (see below).

Ethics committee A copy of the protocol was submitted to the Royal Free Ethics committee for consideration prior to any study. Each study was only commenced when written approval from the committee had been received and any queries answered as appropriate.

Confidentiality Each subjects data in any study were regarded as confidential information. Steps taken to safeguard this were to give each recruit a unique allocation number on entry into study and to use this number together with initials only as far as possible.

Adverse experiences Untoward effects in study subjects were always looked for throughout any study and such events were recorded as part of the subjects written record of study results. Where appropriate, further action was taken to follow up on the event with suggestions or action to assist in resolving the condition. In any event, all adverse experiences were followed up as to eventual outcome.

All study protocols contained a section on inclusion/exclusion criteria which generally consisted of the following requirements:

Subjects should be healthy on basis of full physical examination, haematology and biochemistry screen.

All subjects should be male and between 18 and 45 years of age.

Subjects should not be taking any regular medication which could influence the outcome of study eg. aspirin, NSAID's, steroids.

Subjects should not drink alcohol within 24 hours of a study day.

Subjects should avoid food containing hot spices (e.g. curry) on the day before study (capsaicin, the 'hot' ingredient of curry is known to produce flare via a neurogenic mechanism).

Subject is participating or has participated within the last 3 months in any other volunteer study. Submission of names of intended subjects to the Medical School Dean helped to uphold this rule.

Subject should not have any history of clinically significant allergic disease, eg. atopic eczema, allergic rhinitis, hay fever, asthma. In the case of the atopic study described earlier, however, only significant asthma was excluded.

Generally, the subjects safety was held to be of primary importance and checks were made of clinical status at the beginning and end of each study with a full physical examination and blood biochemistry and haematology. Although no clinically significant adverse events thought to be associated with the study treatment occurred, all unexpected observations (eg biochemistry value outside normal range) were fully discussed with the head of department (Dr. Ian James) before any appropriate action was taken. Adverse events were limited to isolated reports of headache and complaints of itching/discomfort at intradermal injection sites.

B. Drug supplies

The following sources were used for the various treatments used in studies:

PAF Baachem (Switzerland) supplied the PAF used in all studies in anhydrous powder form. All supplies were routinely accompanied by a guarantee of purity together with an HPLC trace of the batch. PAF was kept as 1mM and 10mM ethanolic stock solutions at -80°C.

Histamine Acid phosphate, 1mg/ml (BP).

Normal saline 0.9% (BP).

Human Serum Albumin Immuno (UK) Ltd. All supplies were guaranteed to be free of viral contamination by virtue of 3 separate safety checks:

1. Screening of donors.
2. Heat treatment of material.
3. Assay of batch samples of the final material for HIV and Australia antigen.

In all studies where HSA was used the final concentration in vehicle was 0.25% in normal saline.

Haemaccel[R] A synthetic polygeline colloid supplied by Hoechst.

PGE_2 Sigma chemicals. The chemical was stored as an ethanolic stock solution (1mM) at -80°C.

Substance P and CGRP These were gifts from Dr. Sue Brain, Vascular Biology, CRC, Northwick Park.

Compound 48/80 Sigma chemicals. The solutions were made up fresh from original chemical on the day of study in normal saline.

Terfenadine/placebo Drug and matching placebo were kindly supplied by Merrell-Dow.

C. Intradermal injection preparation and administration

All injection solutions were prepared with sterile materials. Generally, solutions were prepared on the day of a study from ethanolic stock solutions of PAF, substance P, CGRP and PGE_2, and commercially available injectable saline solutions of histamine (BP). Where an ethanolic stock solution was used, an appropriate aliquot of the ethanolic solution was evaporated under nitrogen and redissolved in sterile normal (0.7%) saline (BP) or Haemaccel^R (Hoechst) depending upon choice of vehicle (in early studies 0.25% human serum albumin in normal saline was used in place of Haemaccel). Saline or Haemaccel was also used to dilute aqueous solutions (e.g. histamine). Polypropylene Eppendorf tubes (1.5 ml) were used to contain the solutions to reduce risk of active chemicals (particularly PAF) 'sticking' to the container.

Intradermal injection of treatment was made according to a randomised schedule for site. All injections were 50 μl in volume and were performed using 1 ml sterilin syringes and 25 guage steel needles. Coding of solutions allowed a double blind design to be used in all experiments although it was recognised that presence of flare and weal did tend to weaken the blind nature of studies. The volar forearm surface was used in all studies for convenience. Intradermal injections were 50μl and generally took less than 5 secs to administer. Care was always taken to avoid areas of skin dyspigmentation and superficial veins. Except where specifically stated, 3

sites per arm were always used, proximal, medial and distal, each separated by >5 cm to avoid overlap in response. Allocation of site to treatment was always randomised and double blind with respect to observer and subject. Injections were always made with the needle inserted in a proximal direction along the axis of the arm.

D. Statistical analysis

All statistical analysis was performed using a combination of RS/1, SAS and BMDP statistical software. For the early studies (PAF hyperalgesia, dose response studies, effects of terfenadine and compound 48/80) help was given by Dr. J. Bolognese of Merck, Sharp and Dohme Biostatistics Dept. Subsequently, analysis was performed by the author, although review of statistical interpretation was made by the above-mentioned Dept.

APPENDIX 2

Abbreviations

AGEPC: Alkylacetyl-glycerophosphocholine

BSF: Bovine serum ferritin

cAMP: Cyclic adenosine mono-phosphate

C48/80: Compound 48/80

CGRP: Calcitonin gene-related peptide

DTT-CPT: Dithiothreitol-insensitive cholinephosphotransferase

FA: Flare area

FMLP: N-formyl-methionyl-leucyl-phenylalanine

HA: Hyperalgesic agent

HDL: High density lipoprotein

HSA: Human serum albumin

HSF: Horse serum ferritin

LDHR: Leucocyte dependent histamine release

LDL: Low density lipoprotein

MBP: Major basic protein

PAF: Platelet-activating factor

PLA_2: Phospholipase A_2

PS: Pain stimulus

WA: Weal area

WV: Weal volume

APPENDIX 3

Associated published material

1. Sciberras, D.G., Fox, J., James, I. & Baber, N.S. (1987). The intradermal responses to injection of platelet activating factor (PAF) in man. Br. J. clin. Pharmacol., 23, 116P.

2. Sciberras, D.G., Drake, K., Dyer, T., Goldenberg, M.M., James, I. & Baber, N.S. (1987). Effects of H_1-antagonism on the inflammatory responses to PAF-acether and histamine. Br. J. Clin. Pharmacol., 24, 257P-258P.

3. Sciberras, D.G., Goldenberg, M.M., Kath, G. & Baber, N.S. (7-10th June 1987). Detection of hyperalgesia in the skin by an automated pressure algesimeter. Sixth International meeting of Pharmaceutical Physicians (Abstract booklet), 18.

4. Sciberras, D.G., Goldenberg, M.M., Bolognese, J.A., James, I. & Baber, N.S. (1987). Inflammatory responses to intradermal injection of platelet activating factor, histamine and prostaglandin E_2 in healthy volunteers: a double blind investigation. Br. J. clin. Pharmacol., 24, 753-761.

5. Sciberras, D.G., Bolognese, J.A., Goldenberg, M.M., James, I. & Baber, N.S. (1988). Platelet activating factor induced inflammatory responses in the skin. Prostaglandins, 35 (5), 833.

6. Sciberras, D.G., Bolognese, J.A., Wilson, S., James, I. & Baber, N.S. (1988). Histamine mediation of the inflammatory responses to intradermally injected platelet activating factor. Br. J. clin. Pharmacol., 26 (2), 209P-210P.

7. Sciberras, D.G., Gill, D.S., Jordan, S., James, I. & Baber, N.S. (1989). Histamine release following intradermal PAF in atopic and non-atopic subjects. Br. J. clin. Pharmacol., 28(2), 213P.

8. Sciberras, D.G., Jordan, S., Gill, D., Baber, N.S. & James, I. (1991). The role of histamine in the acute inflammatory responses to intradermal platelet activating factor. Br. J. clin. Pharmacol., (In Press).