GLUCOCORTICOIDS AND THE SKIN

by ·

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Doctor of Philosophy University of Edinburgh 1995



UNIVERSITY OF EDINBURGH ABSTRACT OF THESIS (Regulation 3.5.10)

Address		100	
Degree Ph.D.			Date September 1994.
Title of Thesis	GLUCOCORTICOIDS	AND THE SKIN	

Glucocorticoids are the most effective anti-inflammatory agents currently available, but a variety of adverse effects limit their clinical usefulness. This work explores further two facets of the interaction between glucocorticoids and the skin, with the aim of identifying means of reducing glucocorticoid toxicity.

- (a) Metabolism of glucocorticoids by skin: Human skin is active in the terminal metabolism of cortisol to cortisone, but the biological implications of this process in skin are uncertain. Because there are technical difficulties in dealing with human skin, an animal model, the nude mouse, has been evaluated for its suitability to the study of the metabolism of corticosterone to 11β-dehydrocorticosterone (the homologous reaction in rodents of cortisol to cortisone conversion in man); a process mediated by 11β-hydroxysteroid dehydrogenase. The skin of the nude mouse has previously been shown to be appropriate for pharmacokinetic and pharmacodynamic studies of glucocorticoids. In this model, skin 11β-hydroxysteroid dehydrogenase had an apparent Km for corticosterone of 37 μM. Skin 11β-hydroxysteroid dehydrogenase was up-regulated, in-vivo, by active glucocorticoids and was NADP dependent. By comparison, kidney 11β-hydroxysteroid dehydrogenase had a higher apparent Km (120 μM) for corticosterone, used NAD and NADP with equal facility and was not regulated in-vivo by glucocorticoids. These data suggest that the skin may possess an isoform distinct from that of the kidney. Immunohistochemical studies demonstrated that 11β-hydroxysteroid dehydrogenase was most abundant in the epidermis. In-vitro, this enzyme was markedly inhibited by glycyrrhetinic acid, the active principle in liquorice. Using the classic bioassay of glucocorticoid activity (skin vasoconstrictor assay), it was found that co-application of glycyrrhetinic acid and hydrocortisone résulted in potentiation of skin vasoconstrictor activity of hydrocortisone. This suggests that inhibition of hydrocortisone metabolism might explain the long recognised but poorly understood anti-inflammatory action of liquorice and its congeners and may represent a novel means of targeting glucocorticoid therapy.
- (b) Skin vasoconstrictor response (blanching) to topical glucocorticoids: Glucocorticoids applied topically to human skin produce vasoconstriction in dermal vessels, the degree of which correlates closely with the potency and clinically efficacy of these compounds. Although previous workers had noted heterogeneity in blanching responses to glucocorticoids, this was never systematically studied. In qualitative studies, it was shown that skin blanching was inducible by RU-28362, a specific glucocorticoid receptor (type II) agonist and blocked by RU-38486, a glucocorticoid antagonist. Moreover, aldosterone (type I receptor agonist) failed to produce blanching. In addition blanching was observed in an individual with clinical and biochemical features of aldosterone receptor deficiency. These data therefore suggest that blanching is a glucocorticoid specific phenomenon mediated via the classical glucocorticoid receptor. To test whether skin vasoconstrictor response might reflect glucocorticoid sensitivity, blanching responses was tested in a clinical model of glucocorticoid resistance. In patients with glucocorticoid resistant asthma, skin responsiveness was also found to be diminished. Responsiveness was also somewhat diminished in a cohort of asthmatics on long term prednisolone. Skin vasoconstrictor responsiveness might therefore reflect systemic sensitivity to glucocorticoid and previous glucocorticoid use might reduce skin responsiveness. When tested against other parameter indicating systemic glucocorticoid effects, acute systemic glucocorticoid exposure over 10 days did not affect skin responsiveness. It is possible that resistance to the anti-inflammatory effects of glucocorticoids might accrue from long term exposure and it might therefore be possible to use the skin vasoconstrictor assay a marker for glucocorticoid sensitivity a novel purpose for this long used assay.

DECLARATION:

I hereby verify that this thesis has been composed by myself and that where others have contributed that their contributions have been duly acknowledged on page (iii) or indicated in the text.

ACKNOWLEDGEMENTS:

The financial support of the Government of the Republic of Trinidad and Tobago, the Association of Commonwealth Universities, the Department of Medicine, University of Edinburgh and Fisons Pharmaceuticals is gratefully acknowledged.

I am indebted to my colleagues at the Western and Northern General Hospitals and the Royal Infirmary, Edinburgh for their stimulation, support and assistance with the studies described in this work.

I thank Ms. Jill Campbell for help in performing the cortisol, 11-deoxycortisol and corticosterone assays, Ms. June Noble for assistance with the animal experiments, Dr. Laila Tibi for performing the osteocalcin assays, Mr. Lawrence Brett for help with immunohistochemistry and Dr. Rokiah Pendek for assistance with thin layer chromatography. Sister Pauline Burnet and her staff of the Metabolic Unit ensured that I never went without tea and were invaluable assistants with my human volunteer experiments. I also wish to kindly acknowledge my colleagues who co-authored the publications which arose out of this work.

It is with great pleasure that I express special gratitude to Dr Paul Padfield, Dr Jonathan Seckl and Professor Christopher Edwards, clinicians, scientists and teachers with whom it was an honour and privilege to work and from whom it was a thrill to learn.

I acknowledge with sincere thanks the kind help of Ms. Bernadine Diaz in preparing the typescript and Dr. Dan Ramdath for his many helpful suggestions in the final preparation of this work. Dr. Robin Antoine and Mr. Kevin Gough provided expert advice on statistics.

DEDICATION:

to Sabita for making this possible (and all else seem possible!),
to Siana, Siara, Shravan and Sachi for making it worthwhile,
to my father and brother for support,

with fond memories of my mother

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CHAPTER 1

INTRODUCTION

AIMS AND OBJECTIVES

CHAPTER 1.1: GLUCOCORTICOIDS

1.1a GENERAL:

Cortisol and corticosterone are steroid hormones derived from the adrenal cortex. One of the earliest understood and demonstrable actions was a profound influence upon glucose metabolism (Melby, 1974; Bowes *et al.*, 1991) and hence the term glucocorticoid is used to describe these compounds and their congeners. Cortisol is the principal naturally occurring glucocorticoid in man. In some lower species e.g. rodents, corticosterone is the homologue.

Glucocorticoids have a variety of effects and because glucocorticoid receptors are ubiquitous (Ballard, etal., 1974; La Pointe and Baxter, 1989) they influence virtually all tissues. The physiologic effects vary depending on the target tissue and may be anabolic or catabolic. Catabolic actions are predominant in lymphoid tissues whereas in the liver the overall effects are anabolic. Other effects include maintenance of blood pressure, salt and water balance, and interactions with other hormones (La Pointe and Baxter, 1989). These influences are crucial to normal homeostasis. Under periods of stress or illness their value in this regard becomes obvious such that when there is glucocorticoid deficiency, simple illness can become life-threatening. There are also

important effects on foetal growth (Reinisch, et al., 1978) and tissue differentiation (Torday, 1980; Murphy, 1981). In the foetal lung for example glucocorticoids are crucial to maturational processes (Torday, 1980). Effects on growth persist long after delivery (Loeb, 1976).

The effects of glucocorticoids on intermediary metabolism are complex. In respect of glucose metabolism, the net effect of glucocorticoid (action) is "glucose-sparing" i.e. reduced glucose uptake by peripheral tissues as a result of insulin antagonism, increased hepatic glycogenolysis as well as increased gluconeogenesis (Baxter and Tyrrell, 1986). There are other important effects upon intermediary metabolism. Thus protein catabolism is affected by their action releasing amino acids that can be channelled into gluconeogenesis (Baxter and Tyrrell, 1986). The effects upon lipids are site dependent such that although lipolysis is the dominant feature, there is the tendency for centripetal deposition of fat. This occurs because glucocorticoids sensitize subcutaneous tissue in the arms and legs to the lipolytic effects of catecholamines (Melby 1974). On the other hand, subcutaneous tissue of the trunk exhibit lipogenesis in response to glucocorticoids (Melby, 1974).

It is the anti-inflammatory and immunomodulatory properties of these

compounds, however, that make them important therapeutically. In response to physical, chemical or immunologic stimuli the host mounts an inflammatory response. Glucocorticoids act at almost all phases (Tsurufuji and Ohuchi, 1989) to muffle the inflammatory response which if left unchecked could itself threaten homeostasis (Munck *et al.*, 1984). In this regard glucocorticoids have been shown to alter the white cell count (Parrillo and Fauci, 1979), to stabilize lysosomal membranes, reduce capillary permeability, and decrease phagocytosis (Fauci and Dale, 1974; Fauci *et al.*, 1976). In addition, they suppress synthesis of pro-inflammatory proteins e.g. interleukin 2 (Goodwin *et al.*, 1986) as well as stimulate other proteins (lipocortins) which are inhibitory to pro-inflammatory eicosanoids (Peers and Flower, 1990).

Glucocorticoids are today vital weapons in our therapeutic armamentarium. They are by far the most effective anti- inflammatory agents in current use and a diverse range of human diseases is amenable to treatment with them. Millions of prescriptions are written for them annually in the United States alone (Christy, 1971). Yet as recently as forty five years ago the benefits of this group of drugs were unknown.

1.1b HISTORICAL:

Although the adrenals were recognized anatomically as far back as the Renaissance (Murray, 1989), their physiological role remained obscure for four centuries. It was the observations of Thomas Addison that were to spark interest in this area. In 1855 Addison recorded that disease of the "suprarenal capsules" was associated with weakness, fatigue, hyperpigmentation, anaemia, feeble heart action, hypotension, and gastrointestinal irritability; a symptom-complex which today bears his name (Thorn, 1949). Soon thereafter, Brown-Sequard demonstrated the deleterious effects of bilateral adrenalectomy in experimental animals (Murray, 1989), establishing the crucial physiological role of the adrenals. Almost another hundred years were to elapse before the pharmacologic effects of extracts of the adrenal cortex were appreciated. The discovery that was to revolutionize scientific therapeutics was the consequence of perseverance, scientific collaboration and mere chance (Kendall, 1949).

The symptoms of rheumatoid disease as well as asthma and psoriasis were known to be ameliorated in some patients by concurrence of pregnancy or the appearance of jaundice, a marker of liver disease. Hench, a clinician and rheumatologist, surmised that bile, bilirubin, or steroids might have a therapeutic role

in these diseases. Bile and bilirubin proved ineffective (Hench et al., 1949). In the meantime, Reichstein and Kendall (reviewed in Kendall, 1949) had devised means of synthesizing adrenal steroids, interestingly enough, from bile acids. The stage was set for a revolutionary discovery.

On September 21, 1948, Hench prescribed the first dose of Kendall's Substance E (cortisone) (Hench *et al.*, 1949). Days later, patients who were virtually crippled by rheumatoid arthritis had displayed a dramatic response to this new treatment. The era of glucocorticoid therapy for inflammatory disease had begun. Following this discovery, glucocorticoid therapy was applied to any disease in which inflammation was known to or be suspected of playing a role in the pathogenesis. Current uses of glucocorticoids are summarized in Table 1.1, according to Thomas and Keenan, (1986).

Replacement therapy: adrenocortical insufficiency:

Addison's disease

Adrenalectomy

Congenital adrenal hyperplasia

Palliative therapy: anti-inflammatory and immune suppression:

Arthritis and tendinitis

Allergy and asthma

Dermatological disorders

Organ transplantation

Hematological disorders

Cerebral edema

Shock

Gastrointestinal disorders

Hypercalcemia

Collagen vascular disorders

Neuromuscular disorders (myasthenia gravis)

Specialized therapy:

Fetal respiratory distress syndrome

Idiopathic nephrotic syndrome

1.1c ADVERSE EFFECTS:

It was not long after the introduction of glucocorticoids to therapeutics that the two-edged nature of this form of therapy was revealed. Undesirable effects of cortisone were soon described (Boland and Headley, 1950). Hench himself had reported signs of mild hypercortisolism eg. water retention and weight gain and in a few cases more marked clinical features of Cushing's syndrome (Hench et al., 1949). Soon thereafter, Fraser et al., (1952) reported adrenal atrophy and irreversible shock in a patient with rheumatoid arthritis who had been on cortisone treatment for eight months. Adrenal insufficiency still remains a feared complication for those on long term therapy. Other effects also soon became apparent. Harvey Cushing had noted a tendency for bony fracture in his description of the state of endogenous glucocorticoid excess (Cushing, 1932). Likewise, Curtess (et al., 1954) were among the first to recognize that exogenous glucocorticoids had similar deleterious effects upon the skeleton. The adverse effects of glucocorticoids are numerous but broadly, they can be classified into two categories.

- (A) Withdrawal effects associated with sudden cessation of prolonged therapy with glucocorticoids can be associated with:-
 - (i) Fever, myalgia, arthralgia and malaise which may simulate symptoms of the actual condition being treated e.g. rheumatoid disease or rheumatic fever;

- (ii) Pseudotumour-cerebri;
- (iii) Secondary adrenal insufficiency: This depends on duration and dose of treatment. Recovery of the hypothalamic-pituitary-adrenal axis can be protracted (Streck and Lockwood, 1979; Harrison et al., 1982) and incomplete for many ensuing months (Graber et al., 1965). Adrenal insufficiency may be precipitated by intercurrent illness up to twelve months after discontinuing therapy, even if supra-physiological dosages of glucocorticoids were administered for only a period of weeks (Melby, 1974; Axelrod 1989).
- (B) The adverse effects that accrue from prolonged therapy especially if used in high dosage are numerous and are summarized in the Table 1.2 (according to Axelrod, 1989). There does not appear to be any particular complication of glucocorticoid therapy that is unique to any one agent and all have potential for similar adverse effects. However, the more potent glucocorticoids (and usually the more efficacious) are more likely to produce such unwanted effects.

Ophthalmic

Posterior subcapsular cataracts, increased intraocular pressure and glaucoma, exophthalmos

Cardiovascular

Hypertension

Congestive heart failure in predisposed patients

Gastrointestinal

Peptic ulcer disease, pancreatitis

Endocrine-metabolic

Truncal obesity, moon facies, supraclavicular fat deposition, posterior cervical fat deposition (buffalo hump), mediastinal widening (lipomatosis), hepatomegaly due to fatty liver (rare)

Acne, hirsutism or virilism, impotence, menstrual irregularities

Suppression of growth in children

Hyperglycemia; diabetic ketoacidosis; hyperosmolar, nonketotic diabetic coma;

hyperlipoproteinemia

Negative balance of nitrogen, potassium, and calcium

Sodium retention, hypokalemia, metabolic alkalosis

Secondary adrenal insufficiency

Musculoskeletal

Myopathy

Osteoporosis, vertebral compression fractures, spontaneous fractures

Aseptic necrosis of femoral and humeral heads and other bones

Neuropsychiatric

Convulsions

Benign intracranial hypertension (pseudotumor cerebri)

Alterations in mood or behavior, such as euphoria, insomnia, increased appetite, depression Psychosis

Dermatologic

Facial erythema; thin, fragile skin; petechiae and ecchymoses; violaceous striae; impaired wound healing

Panniculitis (following withdrawal)

Immune, infectious

Suppression of delayed hypersensitivity

Neutrophilia, monocytopenia, lymphocytopenia, decreased inflammatory responses Susceptibility to infections. Whereas the magnitude of adverse effects is related to total dose and duration of therapy (Messer *et al.*, 1983), some patients respond to and develop side effects from glucocorticoids more readily than others when comparable doses are used (Axelrod, 1989). Differences in pharmacokinetic handling of drugs among individuals (Kozower *et al.*, 1974) might be responsible in part for heterogeneity in propensity to adverse effects but inherent differences in sensitivity mediated by cellular mechanisms (Becker 1965; Bigger *et al.*, 1972; Becker *et al.*, 1973, 1976) might also contribute to this. This has been a relatively ignored area and further work along these lines might reap rich rewards.

The dilemma remains to separate the anti-inflammatory/immunosuppressive (desirable) effects of glucocorticoid therapy from its adverse effects. The initial approach to this problem put reliance upon manipulation of structure - function relationship of the steroid molecule.

1.1d CHEMISTRY:

The chemical structure of cortisol is depicted in Figure 1.1. Biological activity is dependent upon the presence of the C-4, 5-double bond and a ketone group at C-3. The presence of the OH group at C-11 is crucial for glucocorticoid (anti-inflammatory) activity. This conclusion was drawn after years of study by several laboratories including those of Liddle and Bush (Brownie, 1992), stalwarts in the early studies of glucocorticoids. In fact, this conclusion might have been deduced from the observation that cortisone was devoid of anti-inflammatory effects on the skin despite appreciable absorption, whereas cortisol was highly effective when used in this way (Sulzberger and Witten, 1952). This was probably the earliest (overlooked!) lesson that the skin might be targeted/protected with regard to glucocorticoid action. Cortisone (chemical structure shown in Figure 1.2) differs from cortisol in having a keto group (=0) instead of an hydroxyl (-OH) at C-11. The conversion of cortisol to cortisone is a biological process with which many mammalian tissues, including skin, are empowered (Monder and White, 1992) via the enzyme complex, 11ß-hydroxysteroid dehydrogenase. If this enzyme system can be modulated locally then it might be possible to target glucocorticoid therapy with the potential of reducing the risks commonly associated with systemic glucocorticoid exposure. Exploring this novel path is worthwhile because most attempts thus far at optimizing glucocorticoid action have left room for improvement.

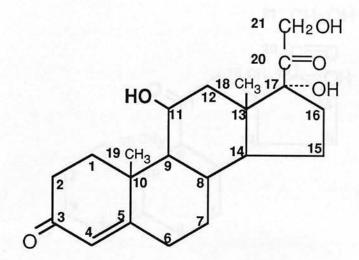


Figure 1.1: Chemical Structure of Cortisol

Figure 1.2: Chemical Structure of Cortisone

clause viscont restormed corpsul (but for 1579). There was still however, no send

1.1e OPTIMIZING GLUCOCORTICOID ACTION:

Modification of the basic cortisol molecule (Figure 1.3) has resulted in an array of glucocorticoid agents with widely varying properties and clinical efficacy. Early studies were focussed on improving the anti-inflammatory effects i.e. glucocorticoid activity of the endogenous hormone, cortisol (hydrocortisone). Such modifications involved both the ring structure and the side-chains as indicated in Figure 1.3 (Thalen et al., 1989). Thus the insertion of a double bond at the C-1,2-position yielded prednisolone with four times the potency of cortisol. The introduction of a fluorine atom at the C-9-α position of cortisol produced fludrocortisone with ten times the glucocorticoid potency of the parent compound as well as a profoundly enhanced (x 125) mineralocorticoid action (Szefler, 1989). Methyl substitution at the C-16-position of fludrocortisone largely eliminated mineralocorticoid activity resulting in dexamethasone (C-16-α) or betamethasone (C-16-β) both of which have twenty five times the glucocorticoid potency of cortisol (Szefler, 1989). There was still, however, no real separation of anti-inflammatory activity from the undesirable effects of glucocorticoids. Adrenal suppression, osteoporosis, and propensity to infection can be serious complications of prolonged use of these agents (Thalen et al., 1989).

R = H: Hydrocortisone R = CO(CH₂)₂ CH₃: Hydrocortisone 17α-butyrate

CH₂OH

X = Y = H: 16α -Hydroxyprednisolone X = F; Y = H: Triamcinolone X = Y = F: Fluocinolone

......ОСОСН₂СН₃

CH 20COCH2 CH3

CH2CH2CH3CH3

CH2 OH CH2 OH

e X=Y=H: Desonide

X = F; Y = H: Triamcinolone acetonide X = Y = F: Fluocinolone acetonide

Budesonide

$$X = F$$
: Betamethasone 17 α , 21-dipropionate $X = Y = F$
 $X = CI$: Beclomethasone 17 α , 21-dipropionate $X = F$; $Y = F$

Figure 1.3: Modifications of cortisol molecule to produce a variety of congeners for clinical use (Thalen et al 1989).

The next advance was topical application to the site of disease and it seemed reasonable that such an approach might limit unwanted systemic effects. Sulzberger and Witten (1952) had already introduced this concept when hydrocortisone was used topically to treat skin disease. Indeed the introduction of topical hydrocortisone therapy to skin represented a major step towards overcoming the problem of systemic toxicity. Such effects have not been described with topical hydrocortisone except in the very young with severe skin disease (Turpeinen et al., 1986; Turpeinen, 1988). Although topical hydrocortisone proved an enormous breakthrough, its relatively low potency and poor transcutaneous bioavailability limited its clinical usefulness. The subsequent use of derivatives with enhanced lipophilicity resulted in increased topical efficacy. Thus masking the -OH groups in the C- 16α -, C- 17α - and C-21-positions produced compounds which were highly efficacious (Phillips, 1976; Brattsand et al., 1981). Unfortunately, the bogey of adverse systemic effects remains a problem even within recommended dosages (Staughton and August, 1975; Himathongkam et al., 1978) but especially when used injudiciously (Teelucksingh et al., 1993).

Subsequent work showed, however, that better topical to systemic activity therapeutic ratios might yet be achieved. Such studies demonstrated that the introduction of C-16-, C-17-acetal substitutions was superior to C-6- and C-9- position halogenation in this regard (Dahlberg et al., 1983). Beclomethasone dipropionate is the prototype of the former and its introduction made topical therapy applicable to the lung i.e. inhaled glucocorticoid for asthma (Brown et al., 1972; Gaddie et al., 1973 a, b). Previous attempts to treat asthma with aerosolized glucocorticoid proved disappointing. Hydrocortisone was not sufficiently potent (Gelfand, 1951) and its more potent congeners e.g. dexamethasone, though effective, provided little advantage over oral treatment because the dose required for equivalent control of symptoms was similar by both routes (Linder, 1963). Undoubtedly, beclomethasone dipropionate and more recently budesonide, have been a major advance in reducing the risk benefit ratio of topical therapy (Thalen et al., 1989). Nevertheless, systemic exposure does occur with this form of therapy (Toogood et al., 1977; Toogood, 1989, 1990; Ali et al., 1991) although whether enough to be clinically relevant is still debatable (Reid et al., 1986; Hollman and Allen, 1988, Littlewood et al., 1988; Stead and Cooke, 1989; Capewell et al., 1990; Meeran et al., 1991; Pouw et al., 1991; Toogood, 1990; Wolthers, 1991).

Herein lies the challenge with glucocorticoid therapy; the need to dissociate the

anti-inflammatory/immunomodulatory effects of glucocorticoids from their undesirable effects. Current clinical approaches to minimize adverse effects of glucocorticoids include:-

- 1. Use in diseases only where they are known to be of benefit
 - 2. Use of minimum maintenance doses for the shortest possible time
- 3. Use of topical therapy where possible
- 4. Use of alternate-day therapy (Bengtsson and Malmvall, 1981; Axelrod, 1989).

More recent elucidation of the mechanism of action of these compounds have opened up some exciting possibilities with respect to enhancing therapeutic index (Flower, 1988 a, b).

1.1f MECHANISM OF ACTION:

Despite the diverse and apparently contradictory effects of glucocorticoids on different tissues a unifying hypothesis for such manifold effects has long been considered (Selye, 1946; Ingle, 1952; Schayer, 1964, 1967). The permissive action upon homeostasis of basal glucocorticoid production was recognised (Ingle, 1952) but the role of increased levels of circulating glucocorticoids accompanying stress (Selye, 1946) would not be accommodated by this hypothesis. The microcirculatory hypothesis as proposed by Schayer (1964, 1967) suggested that glucocorticoids induced vasoconstriction by antagonism of a putative vasodilator of the microcirculation. The latter would not have explained the contrasting effects of enhanced protein synthesis in liver with simultaneous lympholytic effects.

The unifying hypothesis came not from debates regarding the observed functions and effects of glucocorticoids but from a search for their mechanism of action. Karlson's hypothesis that hormones may act at the level of gene transcription (Karlson, 1963) together with Edelman's observation of the accumulation of radio-labelled aldosterone in the nuclear and perinuclear areas of epithelial cells in the toad bladder (quoted in Cope, 1972) set the scene for what was later elucidated about glucocorticoid action.

Glucocorticoids enter target cells by passive diffusion and bind to specific receptors. This steroid-receptor complex then translocates to the nucleus and binds to glucocorticoid responsive elements of specific genes. In so doing, a change in transcription rate ensues resulting in altered messenger RNA production and ultimately protein synthesis (reviewed in Moudgil, 1985).

With the discovery of the glucocorticoid receptor came the understanding and the final unifying concept that the diverse effects of glucocorticoids emerge from a single molecular mechanism (Munck *et al.*, 1984; Munck and Guyre, 1989). It was an earlier (and erroneous) concept that repression or depression of genetic material was responsible solely for the 'physiological actions' of glucocorticoids, and the anti-inflammatory effect (pharmacological action) of glucocorticoids was a peculiarity associated with high doses, the effects of which did not accrue from gene activation (in Munck *et al.*, 1984). Evidence from a number of quarters however, emerged to dispel this hypothesis. The anti-inflammatory actions of glucocorticoids were clearly shown to require de-novo protein synthesis implying that gene expression was also crucial to anti-inflammatory actions (Tsurufuji and Ohuchi, 1989).

Weissmann and Thomas had proposed that the anti-inflammatory actions of

glucocorticoids arose out of the ability to stabilize lysosomal membranes (Stevenson, 1977). However, pharmacologic concentrations of glucocorticoids failed to produce such an effect in purified preparations of lysosomes indicating that stabilisation of lysosomal membranes was not a direct action but necessitated a second messenger (Stevenson, 1977). The search for and discovery of such a mediator was achieved almost simultaneously by three independent groups of workers (Flower, 1988 b).

Following the discovery that aspirin and other non-steroidal anti-inflammatory drugs mediated their action via inhibition of the cyclo-oxygenase enzyme (and hence prostaglandin synthesis) the possibility that glucocorticoids (with their known anti-inflammatory action) might also be involved in prostaglandin metabolism was pursued (Flower, 1988 b). Whereas there was no apparent direct effect of glucocorticoids on cyclo-oxygenase, hydrocortisone and dexamethasone were shown to prevent prostaglandin generation in a variety of systems (reviewed in Flower 1988 b). Using the guinea-pig isolated perfused lung in connection with bioassays sensitive to prostaglandin endoperoxides and thromboxane A_2 a series of experiments by Flower's group elucidated this area. Thus, the site of activity of glucocorticoids in this pathway was localized to inhibition of arachidonic acid release from membrane phospholipids (Nijkamp *et al.*, 1976), presumably by an effect on phospholipase A_2 . Subsequent work

showed that glucocorticoid-induced phospholipase inhibition was mediated by the classic steroid pathway involving protein biosynthesis (Flower and Blackwell, 1979; Russo-Marie *et al.*, 1979). Extracts containing such a glucocorticoid-induced product were shown to possess anti-inflammatory effects in a model of acute inflammation (Blackwell *et al.*, 1982).

Many of the anti-inflammatory effects of glucocorticoids have now been shown to be mediated by this family of proteins, the lipocortins (or annexins) (Duncan et al., 1993). Increased levels of lipocortin 1 have been demonstrated both in vitro (Hirata, 1989) and in vivo (Goulding et al., 1990) following treatment with glucocorticoids. The sequencing of the amino acid structure of this protein, cloning of the gene and expression of the recombinant protein (Wallner et al., 1986) provided further evidence for its role in glucocorticoid action. Human recombinant lipocortin 1 has been shown to mimic the activity of glucocorticoids in inhibiting thromboxane release in the guineapig isolated perfused lung bioassay system (Cirino et al., 1987) and this protein or an N-terminal peptide fragment has been shown to have anti-inflammatory effects in many models of acute inflammation (Cirino et al., 1989, 1993). In addition the antiinflammatory action of dexamethasone can be reversed by anti-serum to lipocortin-1 (Duncan et al., 1993).

If lipocortins are the "second messenger" for the anti-inflammatory signals (alone) then there must be great optimism about the future of anti-inflammatory therapy. However, lipocortins may have effects outside of their recognized anti-inflammatory role. Thus lipocortin-1 has been shown to have potential for effects on the hypothalamic-pituitary-adrenal axis (Taylor *et al.*, 1993) as well as growth and differentiation (Croxtall and Flower, 1992; Croxtall *et al.*, 1993 a,b). This implies that analogues of this protein might well retain these properties (and lead to unwanted side effects) when used therapeutically.

A common mechanism for both 'physiologic' and 'pharmacologic' effects of glucocorticoids would therefore explain the difficulty in separating the benefits of glucocorticoids from their adverse effects. Indeed, the distinction between pharmacologic and physiologic dosages is quite arbitrary, since it has been found that supra-physiologic replacement dosages of glucocorticoids offer no benefit over 'physiologic' replacement dosages in adrenalectomized primates undergoing surgical stress (Udelsman *et al.*, 1986).

Separating the benefits of glucocorticoids from their side effects therefore continues to be a clinical and pharmacological challenge. This work turns attention to what further lessons the skin may yet reveal with respect to glucocorticoid therapy.

1.2 SKIN AND GLUCOCORTICOID:

1.2a SKIN: GENERAL

Skin is the major interface between the body and the environment and is well-adapted to offering protection against physical, chemical, and microbiological assault. Its appearance, texture, and smell are important elements in social and sexual communication. The receipt of signals by it from the outside world can critically alter behaviour leading to actions that can defend the internal milieu. The reverse is also true. An endless variety of lesions on its surface may give clues to significant internal turmoil. A classic example is that of vitiligo, especially in the setting of adjacent increasing pigmentation, which might suggest disease as remote as the adrenal glande.g. Addison's disease. Can the skin reveal deeper secrets of adrenal/glucocorticoid activity? This work aims to answer just this question.

1.2b SKIN: STRUCTURE AND FUNCTION

The skin is arguably the largest and, perhaps, heaviest organ in the body. The average surface area in an adult is 1.5 - 2.0 square metres and has an estimated weight of 20 kg (Nasemann *et al.*, 1983). The epidermis and its appendages (hair, sebaceous glands, and sweat glands) are derived from ectoderm. Together they account for only about 0.5 kg and represent the outer horny layer. The underlying dermis is derived

from mesoderm and averages 3.5 kg (Nasemann *et al.*, 1983). The balance is made up of subcutaneous fat. The epidermis is avascular and must depend for its nutrition on the underlying dermis which has a rich vascular supply. Homeothermia is advantageous in that metabolic processes can function independent of ambient temperature. The skin plays a major role in thermoregulation, facilitated by its dermal vasculature and subcutaneous insulating fat.

Skin is compressible as well as distensible providing a flexible coat ready to accommodate physical stresses. Because the skin is at the front line of defences it is a veritable immunologic organ equipped with antigen-processing Langerhan's cells and keratinocytes with the capacity to secrete a variety of immunomodulatory cytokines (Shimada and Katz, 1988). Unfortunately this can sometimes work against it and the skin can frequently be the site of immunologically-mediated disease e.g. eczema, pemphigoid, and erythema multiforme.

Skin is not just a simple, passive protective coat. Indeed it exhibits high metabolic activity (Lorincz and Stoughton, 1958) and is well furnished with a repertoire of enzymes (Ellis, 1964) to support its many important functions. Its role in lipid synthesis has been long recognized (Hsia *et al.*, 1970). Indeed, the elaboration of

cholecalciferol from 7-dehydrocholesterol represents a unique photoendocrine system (Holick, 1981). Another important and perhaps overlooked function is the ability to biotransform various endogenous and exogenous substrates which are traditionally the domain of liver and kidney; for example, the skin has been shown to participate in the metabolism of a variety of drugs (Bickers *et al.*, 1982 a, b; Bickers, 1983).

The skin is the target for a wide range of hormones. The effects of steroid hormones, in particular, have long been of interest. Hippocrates, for example, noted that eunuchs never developed secondary sexual characteristics (Medvei, 1982). Indeed it is now clear that several human skin disorders are related to abnormalities in the terminal metabolism of androgens (Price, 1975). These include acne vulgaris, hidradenitis suppurativa, and male-pattern alopecia. Importantly, the skin is not a passive recipient but actively participates in the activation of androgens (Wotiz *et al.*, 1956; Jenkins and Ash, 1971) and oestrogens (Weinstein *et al.*, 1968) via a network of enzymes which have been extensively studied (Berliner *et al.*, 1968; Berliner, 1972; Longcope, 1980). The role of skin 5α -reductase, for example, in defining secondary sexual differentiation is perhaps the best known (Imperato-McGinley *et al.*, 1974).

The metabolism of glucocorticoids by skin has been less well studied.

1.2c SKIN - A GLUCOCORTICOID TARGET TISSUE:

Glucocorticoid receptors have only recently been demonstrated in skin of both man (Ponec *et al.*, 1980; Epstein, 1983) and rodents (Epstein and Munderloh, 1981; Smith and Shuster, 1984). In man, these receptors have been demonstrated in cultured fibroblasts (Ponec *et al.*, 1980), cultured keratinocytes (Ponec *et al.*, 1981), as well as homogenates of separated dermis (Smith and Shuster, 1987) and epidermis (Epstein and Bonifas, 1982; Smith and Shuster, 1987). The presence of glucocorticoid receptors in skin might have been deduced on clinical grounds alone. Dermatological features e.g. skin thinning and purpura have long been recognized and are common in patients with Cushing's syndrome (Ross and Linch, 1982; Walker and Edwards, 1992) and occur early in the course of treatment with glucocorticoids (Singleton *et al.*, 1979).

There is considerable data to support a physiologic role for glucocorticoids in skin. In human keratinocyte cultures, for example, the absence of hydrocortisone from the culture medium results in poor proliferation and terminal maturation which can be prevented by the addition of hydrocortisone (Ponec *et al.*, 1988). Similar effects had previously been demonstrated, *in vivo*, in both birds and rodents (Robertson and Maibach, 1989). Keratinization and inhibition of collagenase and gelatinase activity are processes intimately associated with normal skin integrity. These processes have also

been shown to be under glucocorticoid influence (Robertson and Maibach, 1989).

These physiological effects may have all been overlooked were it not for the responsiveness of dermatological disease to glucocorticoid therapy. Soon after Hench et al., (1949) introduced cortisone into the therapy of rheumatoid disease, dermatologists were quick to pick up the trail. Cortisone proved useless but when hydrocortisone became available, its value to treat skin pathology was immediately obvious (Sulzberger and Witten, 1952). Today, glucocorticoids remain the single most important therapeutic option in a variety of dermatoses (Garden and Freinkel, 1986).

1.2d METABOLISM OF GLUCOCORTICOIDS BY SKIN:

Malkinson *et al.*, (1959) were the first to demonstrate that human skin could metabolize hydrocortisone. Incubation of human skin slices with cortisol produced cortisone as indicated by the chromatographic evidence. Hsia (Hsia *et al.*, 1964; Hsia and Hao, 1966; Hsia, 1971) later confirmed and extended this work in a series of *in vitro* experiments. Both the epidermis and dermis were shown to be capable of this conversion. Although other substances were identified cortisone accounted for > 80%

of the products (Hsia *et al.*, 1964). The reverse reaction, cortisone to cortisol, was also demonstrated (Hsia and Hao, 1967). At the same time, Berliner and Ruhmann, (1966) not only demonstrated cortisol-cortisone conversion in skin connective tissue but also highlighted the contrasting effects of cortisol and cortisone on fibroblast growth in culture; cortisol suppressed growth whereas cortisone was without effect. This led Berliner and Ruhmann, (1966) to speculate that cortisol to cortisone conversion might be of biological significance in influencing growth and maturation of skin elements. As is the case with the sex steroids, it therefore seems that the terminal metabolism of cortisol might be important in normal skin physiology. The conversion of cortisol to cortisone is mediated by the enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD). This enzyme is ubiquitous in mammalian tissues (Table 1.3 modified from Monder and White, 1992).

Table 1.3: Distribution of 11 B-hydroxysteroid dehydrogenase activity in mammalian tissues (Modified from Monder and White 1992)

Tissue or organ	11 B-dehydrogenase	11 -oxoreductase
Liver	1,2,3,4,5,6,7,9,11	2,3,4,5,7,11
Kidney	1,2,3,4,5,6	1,2,110
Lung	2,3,4,5,6	1,2,3,4,50
Spleen	3,5,6	3
Heart	2	
Blood Vessels	4	4
Lymphocytes	2,3	2
Thymocytes	2,3	3
Testes	2,3,4,7,11	2
Ovary	20	70
Uterus	1	1
Amniotic membrane	1	1
Chorion	ī	i
Placenta	1,2,8	1,8
Diaphragm	2,6	
Brain	2,3,6,8	2
Pituitary	2	
Stomach	2	
Small intestine	1,2	
Large intestine	2	
Skeletal muscle	2,3,7	20,70
Adrenal Cortex	1,2,4,6,9,10,12	10
Adipose tissue	1	10
Gingivaltissue	1	
Mammary gland	1,2	20
Salivary gland	2	
Dermis	1	
Skin fibroblasts	1	1
Wholeskin	ĺ	

^{1,} Human; 2, rat; 3, mouse; 4, rabbit; 5, guinea pig; 6, dog; 7, cattle; 8, baboon; 9, sheep; 10, meadow vole, 11, pig; 12, cat; 0, none detected in designated tissue.

Important physiologic roles have been defined for 11B-hydroxysteroid dehydrogenase in many organs in which this has been studied. In the kidney for example, 11ß-hydroxysteroid dehydrogenase has been shown to protect the non-specific mineralocorticoid receptor from occupancy by physiologic glucocorticoids (Stewart et al 1987, 1988, 1991, Edwards et al., 1988). Likewise, placental 11B-hydroxysteroid dehydrogenase provides a barrier to the transfer of maternal glucocorticoids to the foetus (Monder and White, 1992) and in the foetal lung (Abramovitz et al., 1982, 1984) and gastrointestinal tract (Smith et al., 1981) changes in the activity of this enzyme crucially influence glucocorticoid-dependent maturation. The role of this enzyme in skin is speculative but from a pharmacological viewpoint, if it was possible to inhibit skin 11ß-hydroxysteroid dehydrogenase this might potentiate the action of cortisol. Cortisol is itself a weak glucocorticoid if systematically absorbed (Turpeinen et al., 1988) so that local inhibition of metabolism might confer site-specific action.

11ß-hydroxysteroid dehydrogenase has received much attention in other organs but has been somewhat overlooked in the skin.

1.2e SKIN - A MARKER OF GLUCOCORTICOID ACTIVITY:

Skin has proved to be an excellent window for evaluating glucocorticoid activity.

Thirty years ago, it was observed that in the course of treating skin diseases with topical glucocorticoids, pallor of the surrounding skin occurred (McKenzie and Stoughton, 1962). It was hypothesized that vasoconstriction in the dermal vascular bed was responsible for this phenomenon and that this might serve as an index of potency of the glucocorticoid agent. Early studies confirmed that this was indeed the case. Thus the physical measure of plastic occlusion, which is known to enhance percutaneous absorption, was shown to result in increased vasoconstrictor responses, verifying the hypothesis (McKenzie and Stoughton, 1962). Later on, it was shown that this bioassay could reflect intrinsic activity of glucocorticoids, a conclusion drawn on the basis of penetration studies. Thus agents that exhibited far less penetration than others were capable of inducing greater vasoconstrictor responses (Stoughton, 1969), implying greater inherent glucocorticoid activity. Stoughton went on further to demonstrate that apart from predicting potency of glucocorticoids, skin vasoconstriction was an accurate predictor of likely clinical efficacy (Cornell and Stoughton, 1985). Thus agents that produce intense pallor were likely to be more efficacious in clinical practice. The skin vasoconstrictor bioassay therefore became an important screening tool for testing new glucocorticoid agents allowing potency standardization of such agents. After thirty years of use, there is still no other bioassay for glucocorticoids that is simpler or cheaper

and which has the reliability and predictability for detecting such potential for clinical efficacy (Stoughton, 1992).

Previous authors have noted that responsiveness to topically applied glucocorticoids is heterogeneous (Burdick, 1974; Barry and Woodford, 1978; Stoughton, 1992). There are those individuals who respond with an intense degree of pallor and others who only barely do so or not at all. Indeed for the purposes intended, subjects were screened and then used in experiments to evaluate glucocorticoids only if they were found to be good responders (Burdick, 1974). These experiments were designed to ascertain the potency of glucocorticoids and the significance of skin unresponsiveness in individuals was not explored.

A negative response could presumably arise as a result of poor bioavailability across the skin barrier. It is also possible that individuals who do not demonstrate a skin response to highly bioavailable topically applied glucocorticoid may be exhibiting some form of glucocorticoid resistance. In clinical practice, it is well recognized that there is heterogeneity in systemic responsiveness to glucocorticoids administered in equivalent doses (Axelrod, 1989). The question that arises therefore is whether the skin could be used as a marker of glucocorticoid sensitivity.

1.3 AIMS AND OBJECTIVES:

HYPOTHESIS 1:

The skin is involved in the terminal metabolism of the natural glucocorticoids and that inhibition of this metabolic process could have pharmacologic potential.

AIMS:

- (a) To demonstrate the presence or absence of the enzyme(s) that inactivate cortisol/corticosterone in skin.
- (b) To define an inhibitor for this enzyme system in skin.
- (c) To demonstrate that inhibition of this enzyme system in skin could lead to potentiation of glucocorticoid action with consequent therapeutic possibilities.

HYPOTHESIS 2:

The skin vasoconstrictor response may be used as a marker of glucocorticoid sensitivity.

AIMS:

- (a) To evaluate skin vasoconstrictor responsiveness/unresponsiveness longitudinally in a group of subjects and the effects of glucocorticoid agonists and antagonists.
- (b) To compare skin vasoconstrictor responsiveness in individuals with known sensitivity to glucocorticoids.

In so doing it is hoped that such information might provide a means of reducing systemic and/or local toxicity associated with glucocorticoid therapy.

CHAPTER 2 MATERIALS AND GENERAL METHODS

CHAPTER 2: MATERIALS AND METHODS

CHEMICALS:

[1,2,6,7- ³H]-corticosterone (sp.act. 86 Ci/mmol), iodinated insulin ¹²⁵I (Cat No: IM38), iodinated cortisol (Cat No: IM129): Amersham International Plc. Lincoln Place, Green End, Aylesbury, Buckinghamshire. HP20 2TP.

Bovine serum albumin (Miles, fraction V, reagent grade): ICN Flow (formerly ICN Biochemicals), Eagle House, Peregrine Business Park, Gomm Road, High Wycombe, Buckinghamshire, HP13 7DL.

Reagent grade salts, analytical grade solvents, Cocktail T "Scintran" scintillation fluid, Merck (5553) thin-layer chromatography plates, high vacuum silicone grease. Merck Limited (formerly BDH), Burnefield Avenue, Thorneback, Glasgow G46 7TP.

Bio-Rad protein assay kit: Biorad Laboratories Ltd., Maylands Avenue, Hemel Hempstead, Herts. HP2 7TD.

Aldosterone, hydrocortisone acetate, hydrocortisone: NAD, NADP, Tris: Sigma London Co. Ltd., Fancy Road, Poole, Dorset BH17 7NH.

Beclomethasone dipropionate: Steraloids Ltd., 31 Radcliffe Road, Croydon, Surrey.

Beclomethasone dipropionate metered dose inhaler: Allen and Hanburys Ltd.,

Horsenden House, Oldfield Land North, Greenford, Middlesex UB6 0HE.

RU-28362 and RU-38486: Gifts from Roussel Uclaf, France.

ß-glycyrrhetinic acid: Aldrich Chemical Co, Ltd., The Old Brickyard, New Road, Gillingham, Dorset SP8 4JL.

Antiserum to 11ß-hydroxysteroid dehydrogenase - a gift from Dr. Carl Monder. (Population Council, N.Y.).

OTHER MATERIALS:

Polyester film (Melinex, 12 μ m), DPX: ICI, U.K.

Tubigrip: Seton Healthcare Group, Oldham, England.

Double-sided adhesive tape (Scotch TM): 3M.

7 x 7 mm square rubber stamp: Thomas H. Peck Ltd., York Place, Edinburgh EH1.

Tissue homogenizer: Ystral homogenizer, Scottish Scientific Instruments Centre,

Limited.

Double-beam spectrophotometer: Shimadzu UV-210A.

Radioactivity counter: Packard 4430 series beta counter.

BUFFERS:

Glass distilled deionized water was used for all aqueous solutions.

Krebs-Ringer bicarbonate buffer (gassed with 95% 0₂ and 5% CO₂ for 1 hour) had the following composition:

118 mM NaC1

3.8 mM KC1

1.19 mM KH₂PO₄

2.54 mM Ca C1₂. 2H₂0

25 mM NaHCO₃

Glucose and bovine serum albumin (final concentrations of 0.2% w/vol) were added prior to use.

Tris-HC1 buffer (pH 8.5) had the following composition:

0.1 M-tris 50 ml

0.1 M-HCI 14.7 ml

H₂0 35.3 ml

ANIMAL STUDIES:

These studies were performed under an Animal Licence obtained from the Home Office and had the approval of the Animal Research Studies Ethics Committee.

EXPERIMENTAL ANIMALS:

- (1) Hairless mice strain MF1-hr/Ola/Hsd. Harlan Olac, Oxon, U.K.
- (2) Nude mice (nu/nu, mixed genetic background), Imperial Cancer Research Fund.

HUMAN STUDIES:

Subjects were recruited either from;

(1) hospital personnel (volunteers) or

(2) hospital clinics (patients) for asthma study

All studies in humans had the approval of the Local Ethics Committee. For studies involving the use of systemic drugs, the conduct of experiments were in keeping with the declaration of Helsinki as to the recommendations guiding medical doctors in treatment involving human subjects (Appendix 1).

GENERAL METHODS:

OSTEOCALCIN: Plasma osteocalcin was measured by an in-house radioimmunoassay set up as described by Price and Nishimoto (1980), using antiserum, raised in rabbits, against purified bovine osteocalcin. Purified bovine osteocalcin was also used to prepare the tracer (125 I- labelled osteocalcin) and the standards. Precipitated second antibody was used in the separation system. Intra and inter-assay coefficients of variation were 5% and 12% respectively. The reference range was up to 12 μ g/L. (This assay was performed by Dr. Laila Tibi, Department of Clinical Chemistry, Royal Infirmary, Edinburgh).

PLASMA GLUCOSE: This was measured on Beckman CX3 autoanalyser using a glucose oxidase specific electrode. Intra and inter-assay coefficients of variation were

PLASMA INSULIN: An in-house single antibody radioimmunoassay was used to measure plasma insulin. Bound and unbound fractions were separated with charcoal. Antibody to porcine insulin, raised in guinea pig, was obtained from Scottish Antibody Production Unit (Lanarkshire, Scotland) and ¹²⁵I insulin from Amersham International (Aylesbury, Bucks, England). Standards were prepared from human insulin (1st International Rf Prep.) obtained from National Biological Standards Board (Potters Bar, Herts, England). Intra and inter-assay coefficients of variation were < 10% throughout the range.

PLASMA C-PEPTIDE: This was measured by radioimmunoassay using reagents obtained from Navo Biolabs. Intra and inter-assay coefficients of variation were < 10% throughout the range.

PLASMA CORTICOSTERONE AND CORTISOL: These were measured by inhouse radioimmunoassays (courtesy of Dr. Brent Williams, Department of Medicine, Western General Hospital, Edinburgh) employing a double antibody precipitation technique to separate bound from free glucocorticoid. The intra- and inter-assay coefficients of variation were < 10%.

PLASMA 11-DEOXYCORTISOL: This was measured using the method of Perry (Perry et al., 1982).

PLASMA TRIGLYCERIDES AND CHOLESTEROL: These were measured using standard enzymatic procedures (Kodak Ektachem). Intra and inter-assay coefficients of variation were < 2% throughout the ranges of these assays.

NEUTROPHIL COUNT: This was measured using a Coulter counter (Luton, Bedfordshire, U.K.).

CHAPTER 3 SKIN VASOCONSTRICTOR ASSAY

3.1 INTRODUCTION:

This bio-assay evolved from a simple clinical observation. The treatment of human skin with topical corticosteroids was shown to result in pallor (McKenzie and Stoughton, 1962), which was presumed to occur on the basis of vasoconstriction in the dermal vascular bed. This led to the idea that the visible cutaneous effect of a glucocorticoid might be a reflection of its potency. Early work verified this and the human skin vasoconstrictor assay evolved. The assay was found to be robust and could predict penetrability and activity of new corticosteroid agents (McKenzie, 1962, McKenzie and Atkinson, 1964). Later on, studies demonstrated excellent correlation between the vasoconstrictor assay and clinical efficacy (Barry and Woodford, 1976, 1978; Gibson et al., 1984; Cornell and Stoughton, 1985). This made it appropriate and relevant to clinical practice through the predictable relationship between a glucocorticoid's vasoconstrictor ability and its clinical efficacy and it has been the standard method of testing new glucocorticoid agents for clinical use (Stoughton, 1992).

3.1a CURRENT USES OF SKIN VASOCONSTRICTOR BIOASSAY:

(i) Screening new synthetic glucocorticoids:

The initial use of the skin vasoconstrictor bioassay was to evaluate percutaneous absorption and later, potency of glucocorticoids. This bioassay has fulfilled its role so well and for so long that it is now the basis on which new agents are tested and ranked for potency.

(ii) Development of topical formulations:

In the initial stages potency of different agents in the same base was evaluated. Its use has since been extended to compare the effects of different vehicle formulations of the same drug. It was on this basis, for example, that foams were found to be superior to ointment, gel or cream (Barry and Woodford, 1986).

(iii) Study of penetration enhancers:

Using blanching as an end-point it has been possible to study the effects of substances that could increase drug delivery across the skin e.g. propylene glycol.

(iv) Determining dose and time regimens:

The application of dermatological formulations in the past has been largely

empiric with respect to both frequency and dosing. The repetitive application of topical glucocorticoid can lead to attenuation of blanching responses. Blanching in response to potent agents will disappear within 48 hours if applied twice a day (Du Vivier and Stoughton, 1975). The response will return in 3-4 days if there is no intervening exposure. When less potent glucocorticoids are used, more frequent application is necessary to induce this tachyphylactic response (Du Vivier and Stoughton, 1975; Du Vivier, 1976). In this way it may be possible to design therapeutic regimens for any given agent.

A great value of this assay is its simplicity and low cost. With thirty years experience of its use, the following points regarding its precision have emerged.

3.1b FACTORS AFFECTING PRECISION OF ASSAY

(i) Occlusion: In some cases investigators have performed the assay without polythene occlusion (Christie and Moore-Robinson, 1970; Coldman *et al.*, 1971). The effect of occlusion, by its hydrating effect on the epidermis is to enhance penetration (Robertson and Maibach, 1989). This effect could mask intrinsic differences that exist among different agents (Burdick, 1974). Without occlusion, however, lower blanching scores and greater intra- and inter- subject variation can be expected (Barry and

- (ii) Site: The human forearm has been the traditional site for skin testing although the back has also been used (Burdick, 1974). The accessibility of the forearm is an obvious advantage. The disadvantages are that blanching is inconsistent in volunteers with very narrow or very short forearms (Burdick, 1974, Barry and Woodford, 1978). Inconsistent blanching scores also occur if application sites are too near the elbow or wrist (Burdick, 1974). In individuals who during the period of application indulge in activities involving sustained action of the forearm muscles (Barry and Woodford 1978; Burdick, 1974) blanching can also prove erratic.
- (iii) Duration of occlusion: The duration of occlusion has also been a variable with considerable influence on results. It is known that a period of about 6 hours exposure is required before blanching becomes obvious (Barry and Woodford, 1978). Some studies have utilized this period of exposure and yet others have allowed exposure for up to 72 hours. In evaluating the effect of duration of exposure upon blanching scores, Barry and Woodford, (1978) measured blanching at intervals ranging from 6 to 96 hours after application. They found that blanching scores increased with duration of application up to a maximum at 18 hours and decreased exponentially after this time.

Most workers will therefore now utilize a period of exposure of 16 to 18 hours before assessing the blanching response.

- (iv) Single or multiple readings? The use of a single or multiple readings across an assay provides yet another variable. The precision of the assay is undoubtedly improved by multiple measurements (Gibson *et al.*, 1984). Here a series of readings are taken over a specified time and a time-response curve plotted from which the area under the blanching curve can be determined. Nonetheless, it has been found that a single reading technique yields substantial information (Gibson *et al.*, 1984). Using a single reading after 16 h exposure, the blanching produced by a series of agents was known to correlate closely with the known clinical efficacy of such agents (Cornell and Stoughton, 1985).
- (v) Scoring blanching: The method of assessing the response has also been variable. Originally, although different patterns/intensities of blanching were observed, no attempt was made to grade these and response was expressed qualitatively, as "present" or "absent" (McKenzie, 1962). It was not long before the mathematical advantage of assigning a numeric value to the blanching response became evident (Place et al., 1970). This amounted to no more than a visual analogue scale where normal skin

in relation to test sites served as a negative control and was rated "0". Depending on the intensity of blanching for any given test-substance a scale of 0 - 3 or 0 - 4 could then be employed.

Scoring by instrumentation: The evaluation of responses by the human eye has (vi) been the time-honoured means of assessing the skin vasoconstrictor response to topically applied glucocorticoids. A considerable amount of work has gone into utilizing more objective means of evaluating this response. Laser-doppler velocimetry (Amantea et al., 1983, Bisgaard et al., 1986), reflectance spectrophotometry (Feather et al., 1982), surface thermography and xenon wash-out methods have all been employed and are still under investigation (Shah et al., 1989). Initial results with some of these have been disappointing (Amantea et al., 1983). Many of the biophysical principles while excellent for measuring increases in cutaneous blood flow e.g. erythema appear not to be sensitive to reduced blood flow. Blanching readily discerned by the eye may, therefore, not be detected using biophysical methods (Amantea et al., 1983). Thus the human eye is currently the most sensitive and versatile tool for evaluating the vasoconstrictor response and continues to be the preferred method in the current literature (Stoughton, 1992).

(vii) Lighting: Appropriate lighting is crucial to proper interpretation of responses.

Direct or bright sunlight attenuates visual assessment of blanching responses (Barry and Woodford, 1978). Responses are best read in artificial light provided by fluorescent tubes (Barry and Woodford, 1978).

3.2 METHODOLOGY 1: COMPARISON OF TEST SUBSTANCES WITHIN A GROUP OF SUBJECTS.

When the aim of the study was to examine the efficacy of various test-substances, the Stoughton-McKenzie vasoconstrictor assay as modified by Place et al (1970) was used. Here a rubber stamp imprinted with the design of 7 x 7 mm squares in 4 vertical columns and 9 horizontal rows was used. The print surface was lightly coated with silicone grease and when impressed upon the mid-forearm served as a template for test-sites. The columns were oriented in the long axis of the forearm (Plate 3.1). This made best use of the skin area of the forearm and avoided getting too close to the elbow or wrist where blanching tends to be erratic.



PLATE 3.1

Rubber stamp template of 7 x 7 mm squares oriented in long axis of forearm

It was important to use the minimum of silicone grease as excessive amounts smudged and obscured the symmetry of the outline. Moreover the aim was to outline the margins of the squares and not to coat the area of the square with the grease. The latter can influence bioavailability of test-substances and prejudice outcome. In practice best results were achieved by lightly coating grease-proof paper with the silicone grease, applying the rubber stamp on to the paper and then to apply the stamp on to the subject's forearm. The square-pattern reduced the problem of recognition of test sites (Place *et al.*, 1970). The grid-pattern was marked with indelible ink to assist in the identification of individual sites when these were subsequently being scored. Plate 3.2 shows the typical appearance of a grid-pattern blanching test using this methodology.

Using this grid-system it was possible to assess up to 36 test-substances simultaneously. Test-substances were assigned positions on the grid at random and using a pipette $10~\mu l$ aliquots were transferred to their designated sites. With the forearm kept horizontal the test solution remained confined to its site by the borders of silicone grease. Test-substances were made up in a vehicle of 95% ethanol. When the alcohol evaporated the test-substance was left deposited in its ascribed test-site.



PLATE 3.2

Typical appearance of a grid-pattern blanching test

After evaporation of the alcohol was complete, usually within 3-5 minutes, the sites were occluded with polythene. This was kept in place by tape and further secured by covering with tubular gauze.

3.3 METHODOLOGY 2: COMPARISON OF SUBJECTS ACCORDING TO CLINICAL GROUPS.

Where qualitative differences were sought or in evaluating the effects of a limited range of test-substances the skin vasoconstrictor test was performed using a technique similar to that originally described (McKenzie and Stoughton, 1962). Here double-sided adhesive tape was used as the template. A cork-borer was used to cut the outline of a circle of 2 cm diameter. Up to 5 such circles could be cut upon a strip of double-sided adhesive tape. Such a strip was readily accommodated by most forearms. Having applied the template, test-solutions were spread via a pipette with circular movements over the surface of the skin. In this way test-substances were distributed uniformly across the test-areas. Sites were occluded with polythene after the alcohol had evaporated. The sticky nature of the upper surface of the tape facilitated polythene occlusion without need for further dressing (Plate 3.3). Using this methodology, dose-response relations for up to 5 concentrations of a particular substance could be evaluated. The use of a larger area of application facilitated recognition and scoring of blanching. Plate 3.4 shows the typical appearance of a blanching test using this



PLATE 3.3

Double-sided adhesive tape method for skin vasoconstrictor assay



PLATE 3.4

Typical appearance of skin blanching using the adhesive tape method

methodology.

For both methods, test-substances were applied between 1600 h and 1700 h, occluded overnight and all dressings removed the following morning at 0800 h (about 16 h occlusion). Removal of the occlusive dressing revealed hyperaemia of the underlying skin due to the adhesive tape. This was allowed to resolve and usually did so within an hour. Only then was any attempt made to read the results. Using such a protocol one took advantage of the observation that 16-18 h application time yielded maximal blanching responses (Barry and Woodford, 1978; Stoughton, 1992).

3.4 SCORING OF SKIN VASOCONSTRICTOR RESPONSE:

The ease of reading of skin vasoconstrictor responses was critically dependent upon ambient lighting. Direct sunlight attenuated the intensity of the response. Responses were best observed in artificial light. For all experiments responses were read under fluorescent lighting. Sites were first evaluated for the presence or absence of blanching. Absent blanching implied that the skin at the test site was similar to contiguous normal skin and was scored '0'. When blanching was present this was rated according to intensity as '3', '2' or '1', where:-

- 3 = intense blanching
- 2 = definite blanching

1 = mild blanching

Where the double-sided adhesive tape method (method 2) was used, four categories of blanching could be recognized:-

- 4 = intense blanching extending beyond the area of application of test substance
- 3 = intense blanching
- 2 = definite blanching
- 1 = mild blanching

3.5 PREPARATION OF TEST SUBSTANCES:

Steroids obtained as pure dried compounds from their manufacturers were dissolved in 95% ethanol. Strength of solutions is indicated for each experiment and expressed as weight of substance per unit volume of solvent (e.g. μ g/ml). In situations where glycyrrhetinic acid had been added to steroid preparations, final concentrations are indicated.

Solutions were stored in light-tight containers between 2 - 8 °C and utilized

within two weeks. When glycyrrhetinic acid was added these solutions were used within 24 hours.

The skin vasoconstrictor bioassay will be referred to in further chapters of this work. It will be used in the traditional role of defining glucocorticoid efficacy. It was also evaluated for a novel purpose - to define glucocorticoid sensitivity.

CHAPTER 4

AN ANIMAL MODEL FOR STUDY

OF .

11B-HYDROXYSTEROID DEHYDROGENASE IN SKIN

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AN ANIMAL MODEL FOR STUDY

OF

11B-HYDROXYSTEROID DEHYDROGENASE IN SKIN:

4.1 THE ENZYME: 11B-HYDROXYSTEROID DEHYDROGENASE:

The enzyme 11ß-hydroxysteroid dehydrogenase catalyses the conversion of the native glucocorticoid in man (cortisol) and rodents (corticosterone) to their metabolically inactive metabolites cortisone and 11-dehydrocorticosterone, respectively.

The presence of this enzyme was first described in tissues in the 1950s (Amelung et al., 1953; Ganis et al., 1955). Enzyme from the liver received most of the early attention and has been extensively investigated and characterized. Its subcellular localization was found to reside in the microsomal fractions (Hurlock and Talalay, 1959; Bush et al., 1968). Purification of the enzyme from rat liver was eventually achieved and it was found to be a glycoprotein with molecular weight 34 KD and NADP-dependent (Lakshmi and Monder, 1985a, 1988). Initial studies indicated that this might be an enzyme complex comprising two enzymes facilitating opposite reactions; an 11-dehydrogenase converting cortisol (and corticosterone) to cortisone

(and 11-dehydrocorticosterone) with the second enzyme, an 11-oxoreductase, mediating the reverse reaction (Lakshmi and Monder, 1985 b). This was further supported when the purified enzyme was found to exhibit dehydrogenase but no significant reductase activity (Lakshmi and Monder, 1988). Later work with the cloned enzyme, however, expressed in transfected chinese hamster ovary cells and osteosarcoma cells demonstrated that both dehydrogenase and reductase activities could be detected (Agarwal et al., 1989). The latter group (Agarwal et al., 1990) showed subsequently that the predominant direction of the enzyme could be influenced both by redox state of coenzymes and by the extent of post-translational processing (level of glycosylation).

though both the level of activity and direction (dehydrogenase or reductase) can vary greatly (Monder and White, 1992). Whereas experimental conditions do undoubtedly influence the direction of the reaction (Monder and Shackleton, 1984) net dehydrogenation or reduction is, for some part, tissue dependent. For example, the kidney is largely a site of dehydrogenation (11ß-HSD₂) whereas in the liver reductase activity (11ß-HSD₁) is dominant (Rundle *et al.*, 1989; Walker *et al.*, 1992 b). There is also great variation in the properties of the enzyme between species and even in the same species (and same organ) properties differed depending on the stage of the life cycle

(Monder and Shackleton, 1984). Moreover, the relative direction of metabolism in an organ can change during development (Monder and Shackleton, 1984). The factors regulating such transformation in net direction of metabolism are complex and probably hormonally related (Zumoff *et al.*, 1983; Koerner and Hellman, 1964; Pepe *et al.*, 1988; Tye and Burton, 1980).

Recent data, however, suggest that the existence of different proteins might explain the known biological and biochemical differences among tissues. The evidence in favour of different isoenzymes include the following. Firstly, kinetically distinct forms in the liver (Monder and Lakshmi, 1989) as well as differing species of messenger RNA for 11B-hydroxysteroid dehydrogenase could be identified in the kidney (Krozowski et al., 1990). In addition, immunohistochemical studies using an antibody raised against liver-type protein failed to demonstrate staining for enzyme in some tissues although in these tissues enzyme activity could be readily demonstrated (Edwards et al., 1988; Brown et al., 1993). Moreover, defects in a gene encoding a protein with 11ß-hydroxysteroid dehydrogenase activity could not be detected in patients exhibiting clinical biochemical features of diseases known to be associated with either 11-dehydrogenase or 11-oxoreductase activity (Nikkila et al., 1993). All the foregoing with the latter, in particular, indicated that at least another gene (and perhaps other/several proteins) encodes for 11ß-hydroxysteroid dehydrogenase activity. Studies involving coenzyme preferences further support this view. Thus NAD-dependent 11ß-hydroxysteroid dehydrogenase activity (cf NADP-dependent liver isoform) has been identified in placenta (Brown *et al.*, 1993) and renal cortical distal convoluted tubule (Mercer and Krozowski, 1992).

Current knowledge favours the existence of at least two isoforms of the enzyme of which the liver isoform designated (11ß-HSD₁) and placental isoform (11ß-HSD₂) are prototypes (Brown *et al.*, 1993). 11ß-HSD₂ is NAD-preferring, has an apparent molecular weight of 40 KD, has a higher affinity for glucocorticoids than 11ß-HSD₁ and acts predominantly as a dehydrogenase. By comparison 11ß-HSD₁ is NADP dependent, has a molecular weight of 34 KD, and acts predominantly as a reductase. Whether the NAD-dependent activity detectable in the distal nephron represents yet another isoform is not yet clear.

Knowledge of the high degree of conservation and broad tissue distribution have long suggested that the enzyme might have some important physiologic role. Evidence to support this has only recently been forthcoming.

The crucial role that this enzyme plays in the kidney has now been elucidated. Here this enzyme has been shown to protect the non-specific distal tubular mineralocorticoid receptor from glucocorticoids. This conclusion has been drawn from a series of studies involving a patient with congenital deficiency of the enzyme (Stewart et al., 1988) and in metabolic-balance studies in subjects exposed to liquorice, an inhibitor of the enzyme (Stewart et al., 1987). These studies explained the following paradox. In vitro, both the purified (Krozowski and Funder, 1983) and cloned mineralocorticoid receptor expressed in a cell line (Arriza et al., 1987; Arriza, 1991) were non-specific and bound aldosterone, cortisol and corticosterone with similar affinity. Yet, in vivo, despite the fact that circulating free cortisol levels may be as much as 100-fold higher than those of aldosterone, renal mineralocorticoid receptors will selectively bind aldosterone (Edwards, 1990). By converting cortisol to cortisone (which has no affinity for the mineralocorticoid receptor) the enzyme therefore allows the preferential access of aldosterone (which is not metabolized by the enzyme (Edwards and Hayman, 1991), to the mineralocorticoid receptor. Thus when the enzyme is either congenitally absent or there is acquired deficiency secondary to liquorice or carbenoxolone ingestion, aldosterone specificity is lost and mineralocorticoid receptors become exposed to physiological levels of cortisol. Because the binding of cortisol to the mineralocorticoid receptor results in a sequelae indistinguishable from that when aldosterone interacts with the mineralocorticoid receptor (Cato et al., 1991), the result is that there are not only the classical features of mineralocorticoid excess; hypertension, hypokalaemic alkalosis and suppressed plasma renin activity, but also suppressed aldosterone levels (Edwards et al., 1988).

The expression of enzyme activity in tissues without mineral ocorticoid receptors e.g. liver and testis (Walker et al., 1992b), organs in which glucocorticoid receptors and 11ß-hydroxysteroid dehydrogenase colocalize (Phillips et al., 1989; Edwards, 1991; Monder and White, 1992), suggests that the role of this enzyme extends beyond protection of mineralocorticoid receptor and it might also serve to protect tissues from glucocorticoid excess. Placental enzyme is thought to play such a role. As normal pregnancy progresses both total and free maternal cortisol levels increase exponentially (Burke and Roulet, 1970; Rees et al., 1975). Placental enzyme inactivates cortisol (Burton and Jeyes, 1968; Pasqualini et al., 1970; Levitz et al., 1978; Waddell et al., 1988) thereby protecting the developing foetus from the potentially growth inhibitory and teratogenic effects of this hormone. Indeed, the importance of this barrier to the fetus may be even more far reaching. Thus the administration of dexamethasone (which is not metabolized by the enzyme) to pregnant rats has been shown to adversely affect birth weight and predispose to hypertension in adult life (Benediktsson et al., 1993;

Edwards *et al.*, 1993). In a similar fashion, the enzyme is present in testis where studies on the ontogeny of the enzyme suggest that its temporal expression is closely linked to the developmental rise in testosterone at puberty (Phillips *et al.*, 1989). Glucocorticoid excess is detrimental to testicular function (Bambino and Hseuh, 1981) and the enzyme seems well-placed anatomically and functionally to protect against such deleterious effects (Phillips *et al.*, 1989).

Indeed, wherever the enzyme has been studied it appears to modulate local glucocorticoid exposure. This usually impacts positively on some local physiologic processes (Monder and White, 1992).

4.2 11B-HYDROXYSTEROID DEHYDROGENASE IN SKIN:

That corticosteroids are actively metabolized by human skin was first shown by Malkinson *et al.*, (1959) who demonstrated that slices of human skin converted cortisol to cortisone. Subsequent work by Hsia (Hsia *et al.*, 1964; Hsia and Hao, 1966, 1967; Hsia, 1971) confirmed and extended these findings. Hsia noted the formation of other substances notably C-20-OH compounds but cortisone accounted for greater than 80% of the products. Skin (Hsia and Hao, 1967) or skin fibroblasts (Hammami and Siiteri, 1991) were also shown to be capable of performing the reverse reaction: cortisone to

cortisol. These findings implied that 11ß-hydroxysteroid dehydrogenase was present in skin.

The earlier studies were essentially qualitative though a number of characteristics were determined. Firstly, the direction of the reaction favoured dehydrogenation (Malkinson et al., 1959; Hsia et al., 1964). Secondly, NADP seemed to be the preferred co-enzyme (Hsia et al., 1964). More recently, Hammami and Siiteri, (1991) studied this enzyme in greater detail using cultured human skin fibroblasts. They established a Km value of 200-300 nM for cortisol using whole cells. They also found that active glucocorticoids, cortisol and dexamethasone, increased 11ß-hydroxysteroid activity whereas other steroid hormones had no appreciable effect. Contrary to what had been previously reported for whole skin (Hsia et al., 1964) Hammami and Siiteri, (1991) found that fibroblasts exhibited greater reductase than dehydrogenase activity and concluded that skin was mainly a site of reduction of cortisone. This may have however, been an effect of the cell type studied. Fibroblasts from other tissues have also been found to exhibit greater reductase activity. Thus, Abramovitz et al., (1982) had previously demonstrated that human fetal lung fibroblasts exhibited greater reductase activity, whereas lung epithelial elements were superior in ability to perform the reverse reaction. The dominance of reductase activity in fibroblasts is, however, not always consistent (Smith and Giroud, 1975). The significance of reductase activity (cortisone to cortisol conversion) in human skin remains dubious given the greater metabolic activity of epidermal keratinocytes (Ellis, 1964) compared with dermis (where fibroblasts are located). As previously discussed experimental conditions (and perhaps choice of tissue) do influence the direction of reaction but the most compelling indication of the predominant direction of metabolism of cortisol in skin comes from clinical observations. Thus, when cortisone is applied to the skin it is well-absorbed (Robertson and Maibach, 1989) but without any biological effect (implying lack of significant conversion to cortisol in skin). It is for this reason that the thrust of current work was to focus upon the dehydrogenase activity (cortisol to cortisone) of skin 11ß-hydroxysteroid dehydrogenase.

Cortisol (and its congeners) produce beneficial and often dramatic therapeutic effects in a wide variety of dermatoses. These include not only inflammatory conditions (eg. eczema) where glucocorticoid benefits are self-evident but, in addition, in conditions where there are features of abnormal epidermal proliferation and maturation are features eg. psoriasis. The latter can probably be explained on what has been observed *in-vitro*. Thus it has been shown in keratinocyte culture, that cortisol promotes terminal differentiation (Ponec *et al.*, 1988), emphasizing the importance of glucocorticoid in cell

maturation. Is the role of 11ß-hydroxysteroid dehydrogenase in the skin to modulate local glucocorticoid action? Berliner and Ruhmann, (1966) had previously noted that cortisol but not cortisone inhibited skin fibroblast proliferation in culture, thus implying a potential role for the enzyme in regulating skin differentiation.

Given the important physiological role defined for this enzyme in other tissues and the known benefits of glucocorticoid therapy in skin diseases, it seems plausible that the enzyme might also be important in skin. One possible way of defining a role for the enzyme in skin includes evaluating the factors influencing its activity (control). Another is to study the possible impact of its deficiency. It was the latter situation that led to the elucidation of the enzyme's role in the kidney (Stewart *et al.*, 1988). Thus the study of this enzyme in skin diseases might define possible pathophysiologic mechanisms of particular dermatoses. Finally, there is an obvious therapeutic implication. If this enzyme could be inhibited locally then the action of physiologic levels of glucocorticoid at that site could be potentiated. Although one of the primary objectives of this thesis was the latter, it became necessary to seek out a model that might be suitable for all of the above.

4.3 THE NEED FOR AN ANIMAL MODEL:

It was important to find an appropriate animal model for the study of the skin enzyme because of some difficulties posed by human skin. Firstly homogenization of human skin is extremely difficult (Voigt *et al.*, 1970). Fibrous proteins such as keratin and collagen are important for the structural integrity of skin but these very proteins, however, make it extremely difficult to homogenize human skin using methods that are excellent for visceral organs like liver and kidney.

Secondly, it was not always possible to obtain readily human skin for study. Moreover the use of cultured keratinocytes or skin fibroblasts while representing fair surrogates may mislead as to what exists *in vivo*, because of changes in cell characteristics upon culture (Priestley, 1986). Finally, there are some experiments that can only be performed in animals.

4.4 WHY THE HAIRLESS MOUSE?

Rodents seem a particularly suitable model in which to elucidate the biochemical and immunologic effects of glucocorticoids (Spearman and Jarrett, 1975; Back and Egelrud, 1985; Cohen, 1989). Hairless mice, in particular, have skin that bears many similarities to human skin in physicochemical handling of glucocorticoids and this

species has previously been used as a model system for evaluating pharmacodynamics and pharmacokinetics of topical glucocorticoids (Du Vivier *et al.*, 1978; Marks *et al.*, 1973). In addition, because they are congenitally athymic, hairless mice have been shown to accept skin grafts from several species, including human skin - normal and diseased (Briggaman, 1985). Human skin thus transplanted retains its features for months without alterations (Reed and Manning, 1973; Lane *et al.*, 1989). The characteristics of 11ß-hydroxysteroid dehydrogenase in the skin of hairless mice was therefore investigated.

4.5 METHODOLOGY:

4.5a Preparation of a skin homogenate.

Dorsal skin was obtained from freshly killed hairless mice (usually nu/nu or for some experiments as specifically indicated, MF1-hr/ola/Hsd) and placed immediately in universal containers kept on ice. Subcutaneous fat was removed with a pair of forceps. Then 1.0 g of skin was weighed out and finely minced with a scalpel. Minced tissue was then homogenized in 10 ml Krebs-Ringer buffer using an Ystral homogenizer (Scottish Scientific Instruments Ltd) at maximum setting for periods of 20 seconds with intervals of 30 seconds for cooling. Four or five cycles of homogenization followed by cooling produced a dense suspension with small fragments of residual skin. To prevent

excessive rise in temperature, tubes containing the tissue were surrounded in ice during the procedure.

The resulting homogenate was then centrifuged at 750 g for 5 minutes at 4°C to sediment unbroken cells and debris. The supernatant was withdrawn by suction using a plastic transfer pipette.

4.5b Preparation of a kidney homogenate: A positive control for 11B-hydroxysteroid dehydrogenase activity.

Most of the impetus to recent research into 11ß-hydroxysteroid dehydrogenase came from the important physiologic role defined for the enzyme in kidney. Although different isozymes may reside in proximal and distal tubules, the kidney is similar to the skin (Malkinson *et al.*, 1959; Hsia *et al.*, 1964) in having dehydrogenation as the dominant reaction (Walker *et al.*, 1992 b). It was therefore thought that the kidney would be useful for comparative studies with the skin. Kidneys were simultaneously removed from animals and kept in ice-cold 0.9% saline. Perinephric fat was peeled away with forceps and both kidneys (combined wet weight was usually about 0.5 g) minced and homogenized in 10 ml Krebs-Ringer buffer using a Dounce tissue grinder (Wheaton Scientific, New Jersey) for about 20 strokes.

4.5c Measurement of protein concentration of homogenates.

This was performed using the Bio-Rad protein assay kit, a dye binding assay based on the differential colour change in response to various concentrations of protein. The Bio-Rad assay employs an acidic solution of the dye Coomassie Brilliant Blue G-250 which when bound to protein shifts its absorbance maximum from 465 nm to 595 nm (Biorad Laboratories). A Shimadzu UV-210-A double beam spectrophotometer was used to measure results. Using a range of known concentrations of bovine serum albumin a standard curve was produced. Skin homogenate diluted 1:7 and kidney homogenate diluted 1:8 with Krebs-Ringer buffer were then assayed and protein concentration determined from the standard curve.

4.5d 11B-hydroxysteroid dehydrogenase enzyme assay.

(i) Incubation Procedure: A volume of homogenate yielding the desired protein content (usually 0.5 mg for both skin and kidney) made up to 1 ml with Krebs-Ringer buffer (with added 0.2% glucose and 0.2% bovine serum albumin) was incubated with $10 \mu l$ (equivalent to 37 KBQ) ³H-corticosterone for a specified time, usually one hour, (unless otherwise stated) in a shaking water bath at 37°C. The final concentration of ³H-corticosterone in each incubate was 12 nM. Where indicated other substances e.g. NAD/NADP or glycyrrhetinic acid were added in volumes of $10 \mu l$ to achieve final

concentrations as stated.

All incubations were performed in triplicate in disposable borosilicate glass culture tubes (Corning Glassware, N.Y.). Blank samples comprising buffer and ³H-corticosterone but no homogenate were run with each experiment. At the end of the incubation period tubes were transferred to ice and immediately centrifuged at 1500 g for 15 minutes at 4°C. The supernatant was removed by suction and stored at -70°C until required for steroid extraction and identification.

- (ii) Extraction: Samples were extracted with 2.5 ml ethyl acetate by agitating in a motor-driven shaker (Griffin and George, U.K.) for 5 minutes and then allowed to stand for 5 minutes to ensure separation of the organic and aqueous phases. With a pipette 2 ml of the organic phase (upper) was transferred to glass tubes for drying. This was undertaken under air in a sample concentrator (Dri-Block DB 3, Jencons Scientific, U.K.). Radioactivity was then reconstituted in 100 μ l of ethanol prior to thin-layer chromatography.
- (iii) Identification of metabolic product: Separation of substrate and products of the incubation procedure was performed by thin-layer chromatography. Unlabelled

corticosterone and 11-dehydrocorticosterone in ethanolic solution (10μ l, 2 mg/ml) were admixed with extracted steroids and spotted on silica gel plates [Merck (5553)]. These were developed in a solvent system containing chloroform: 95% ethanol (92:8) to a distance of 15 cm usually taking 1 hour to do so. Spots corresponding to the added standards were identified under an ultraviolet light and scraped into vials. After the addition of 4 ml of scintillation fluid (Scintran, BDH Chemicals) radioactivity was counted using a Packard 4430 series beta counter.

- (iv) Determining enzyme activity: Using this procedure the substrate 3 H-corticosterone was separated from the product 3 H-11-dehydrocorticosterone. Enzyme activity was expressed as the percentage conversion of substrate to product which was determined from the radioactivity (counts per minute) corresponding to each. i.e. Activity = counts per minute (3 H-11-dehydrocorticosterone) / counts per minute (3 H-11-dehydrocorticosterone + 3 H-corticosterone). Blank incubations (i.e. tubes not containing homogenates) usually showed a percent conversion $\leq 2\%$ and this factor would be deducted from the values for actual experimental samples.
- (v) Recovery of radioactivity: Recovery of radioactivity after chromatography was compared with analogous aliquots which were mixed with liquid scintillant and

subjected to radioactivity counting after the extraction stage. This showed that recovery following TLC was always greater than 90%. The combined counts from ³H-corticosterone and ³H-11-dehydrocorticosterone accounted for greater than 95% of recoverable radioactivity implying that other metabolites of corticosterone were relatively unimportant in this animal tissue.

4.6 RESULTS:

4.6a Relationship between protein content of homogenates and 11B-hydroxysteroid dehydrogenase activity:

Figure 4.1 depicts the relationship between varying protein content and percentage conversion (mean ± SEM) of ³H-corticosterone to ³H dehydrocorticosterone for 9 homogenates. For skin homogenates, there was a linear relation up to 0.75 mg protein per incubate and percentage conversion. Thereafter the concentration - conversion response curve was flat. On the basis of this, protein content of 0.5 mg was chosen for incubation in most subsequent experiments.

For incubations with whole kidney homogenates, the results were similar but kidney exhibited higher activity (Figure 4.1). For most subsequent experiments with kidney, 0.5 mg protein content was also chosen for incubation.

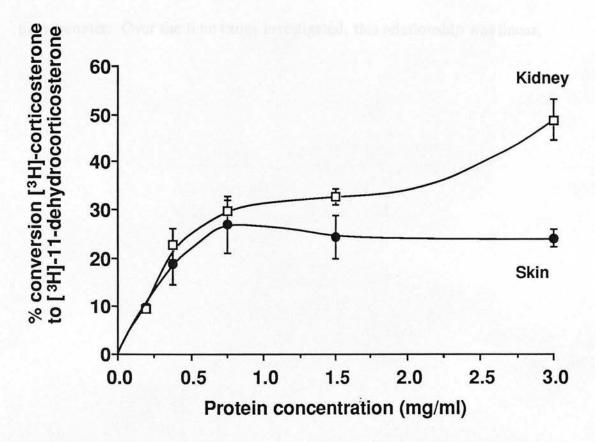


Figure 4.1: The relationship between protein content of homogenates and activity of skin (•) and kidney (□) 11 beta-hydroxysteroid dehydrogenase.

Results shown as mean \pm SEM, n=3 for both tissues with triplicate incubations.

4.6b Relationship between incubation time and 11B-hydroxysteroid dehydrogenase activity:

Figure 4.2 depicts the mean (± SEM) percentage conversion of ³H-corticosterone to ³H-dehydrocorticosterone for 0.5 mg protein equivalent of kidney and skin homogenates. Over the time range investigated, this relationship was linear.

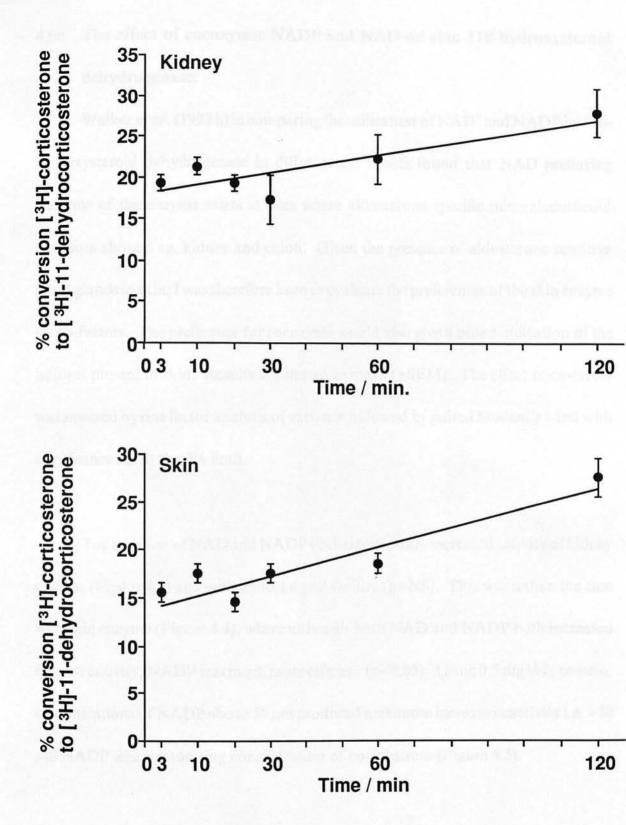


Fig. 4.2: The relationship of 11 beta-hydroxysteroid dehydrogenase activity (mean \pm SEM, n=3 with triplicate incubations) with time for kidney and skin homogenates of nude mouse

4.6c The effect of coenzymes NADP and NAD on skin 11B-hydroxysteroid dehydrogenase:

Walker *et al.*, (1992 b) in comparing the utilization of NAD⁺ and NADP by 11ß-hydroxysteroid dehydrogenase in different rat tissues found that NAD preferring isoforms of the enzyme exists at sites where aldosterone specific mineralocorticoid receptors abound eg. kidney and colon. Given the presence of aldosterone-sensitive sweat glands in skin, I was therefore keen to evaluate the preferences of the skin enzyme for co-factors. The preference for coenzyme would also give a broad indication of the isoform present in skin. Results are shown as mean (±SEM). The effect of co-factor was assessed by one factor analysis of variance followed by paired Student's t-test with significance set at the 5% limit.

The addition of NAD and NADP both significantly increased activity of kidney enzyme (Figure 4.3) and with almost equal facility (p=NS). This was unlike the case with skin enzyme (Figure 4.4), where although both NAD and NADP both increased enzyme activity, NADP was much more efficient (p<0.05). Using 0.5 mg skin protein, concentrations of NADP above 50 μ m produced maximum increase in activity i.e. >50 μ m NADP was a saturating concentration of co-substrate (Figure 4.5).

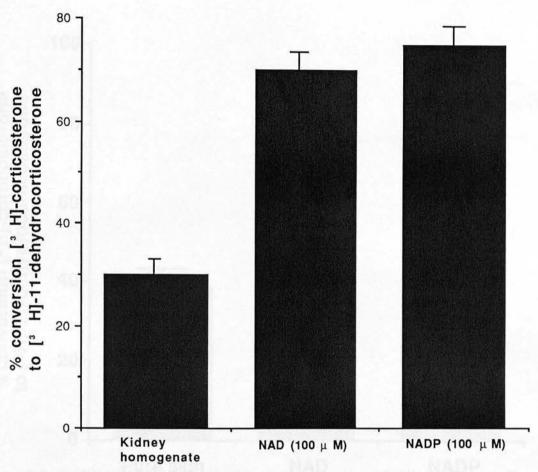


Figure 4.3: The activity of 11 beta-hydroxysteroid dehydrogenase in kidney homogenate (mean \pm SEM, n=3) without cofactor, with NAD and NADF

NAD vs NADP = NS

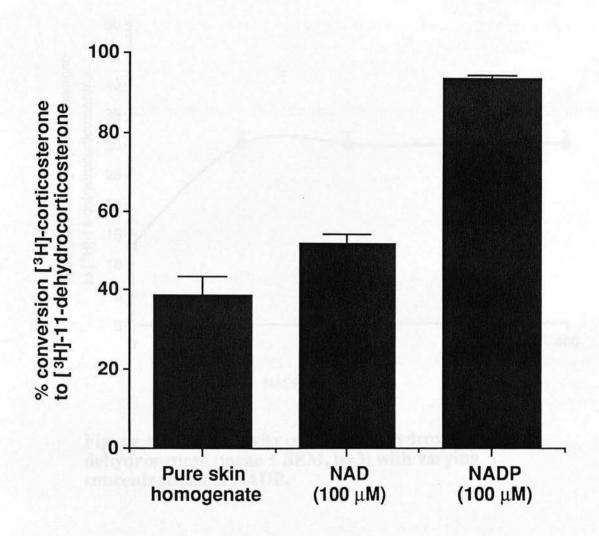


Figure 4.4: The activity of skin 11 beta-hydroxysteroid dehydrogenase (mean \pm SEM, n=3 with triplicate incubations) without co-factor and with NAD and NADP.

NAD vs NADP p < 0.05.

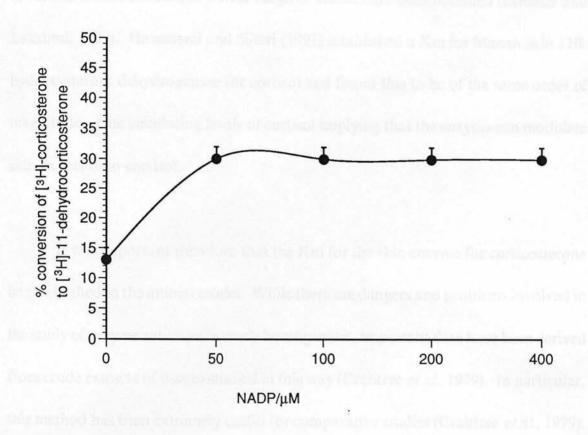


Figure 4.5: The activity of skin 11 β -hydroxysteroid dehydrogenase (mean \pm SEM, n=3) with varying concentrations of NADP.

4.6d ESTABLISHING A KM FOR SKIN 11B-HYDROXYSTEROID DEHYDROGENASE FOR CORTICOSTERONE:

Previous investigations have determined the Michaelis constants of the enzymes in various tissues in rats and a wide range of values have been obtained (Monder and Lakshmi, 1989). Hammami and Siiteri (1991) established a Km for human skin 11ß-hydroxysteroid dehydrogenase for cortisol and found this to be of the same order of magnitude as the circulating levels of cortisol implying that the enzyme can modulate skin exposure to cortisol.

It was important therefore that the Km for the skin enzyme for corticosterone be established in the animal model. While there are dangers and problems involved in the study of enzyme activities in crude homogenates, important data have been derived from crude extracts of tissues studied in this way (Crabtree *et al.*, 1979). In particular, this method has been extremely useful for comparative studies (Crabtree *et al.*, 1979). Thus the Km for kidney enzyme in these animals could also be easily established for comparison.

Incubations were performed in tris-HCl buffer (pH 8.5) with NADP 250 μ M at 37°C for 10 minutes. Tissue protein concentrations (0.38 mg for kidney, 0.5 mg for

skin) were chosen so that reaction velocities were linear with time and percent conversion of substrate was ≤ 25 %.

A fixed amount of 3 H-corticosterone (107 μ M) was added to each incubate along with increasing amounts of cold corticosterone. Substrate concentrations [S] ranged from 107 - 1070 μ M. From the percentage conversion of 3 H-corticosterone to 3 H-dehydrocorticosterone at each substrate concentration the mass of product [V] was calculated as depicted in Tables 4.1 and 4.2. The results are the mean of nine incubations at each concentration.

Table 4.1: Substrate concentration[S] and
% conversion of corticosterone by homogenates of mouse skin

[S] x 10 ⁻⁴	Conversion (%) * [V]	1	1
(mol/L)		Mass converted	:2	
		x 10 ⁻⁵ (mol/l)	v	S
			(10 ⁴)	(10^3)
1.07	12.1	1.3	7.7	9.35
1.20	12.4	1.4	7.0	8.33
1.50	10.0	1.5	6.7	6.67
2.10	7.3	1.5	6.7	4.76
3.60	4.5	1.6	6.3	2.78
5.40	3.0	1.6	6.3	1.85
10.70	1.7	1.8	5.6	0.94

^{*} Mass converted = [S] x % conversion

Results are the mean of 9 incubations at each concentration.

Table 4.2: Substrate concentration[S] and
% conversion of corticosterone by homogenates of mouse kidney

[S] x 10 ⁻⁴ (mol/L)	Conversio	n (%) * [V] Mass converted x 10 ⁻⁵ (mol/l)	1 V (10 ⁴)	1 - S (10 ³)
1.07	20.7	2.2	4.5	9.35
1.20	16.3	1.9	5.3	8.33
1.50	16.2	2.4	4.2	6.67
2.10	13.3	2.7	3.7	4.76
3.60	8.3	2.9	3.4	2.78
5.40	5.9	3.2	3.1	1.85
10.70	3.3	3.2	3.1	0.94

Results are the mean of 9 incubations at each concentration.

^{*} Mass converted = [S] x % conversion

Using the Lineweaver-Burk transformation of the Michaelis-Menten equation a plot of 1/[V] versus 1/[S] was then made and Km determined for skin and kidney 11ß-hydroxysteroid dehydrogenase for corticosteroid in the mouse. The apparent Km for skin enzyme was 37×10^{-6} M (Figure 4.6), and that for the kidney enzyme was 120×10^{-6} M (Figure 4.7).

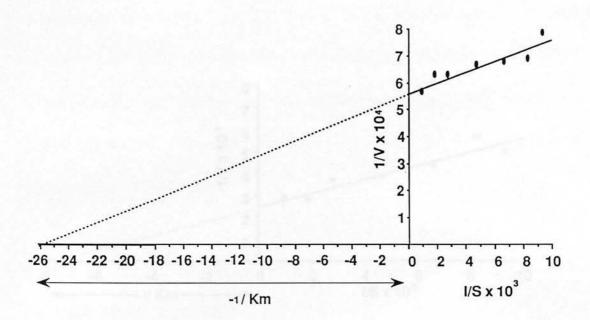


Figure 4.6: Lineweaver-Burk plot for skin 11 β-hydroxysteroid hydrogenase using corticosterone as substrate

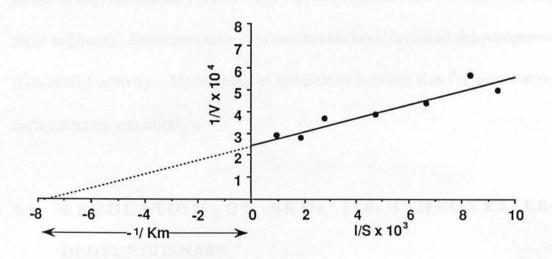


Figure 4.7: Lineweaver-Burk plot for kidney 11 β-hydroxysteroid hydrogenase using corticosterone as substrate

Conclusions:

The apparent estimated Michaelis constant for kidney 11 β -hydroxysteroid dehydrogenase for corticosterone in this experiment was similar to that described for rat kidney enzyme for corticosterone (160 μ M) by Stewart (M.D. Thesis, University of Edinburgh, 1989). The apparent Km for skin 11 β -hydroxysteroid dehydrogenase corticosterone has not been previously studied. It is known that there are at least two species of enzyme in kidney (11 β -HSD₁, in proximal tubule and probably 11 β -HSD₂ in distal nephron). Experimental conditions in this work favoured dehydrogenase (11 β -HSD₂) activity. Under parallel conditions a lower Km for skin enzyme for corticosterone was obtained.

4.7 REGULATION OF SKIN 11B-HYDROXYSTEROID DEHYDROGENASE

4.7a Introduction:

11ß-hydroxysteroid dehydrogenase is known to be influenced by a number of hormones (Monder and Shackleton, 1984). Skin 11ß-hydroxysteroid dehydrogenase was shown to be regulated by glucocorticoids *in vitro* (Hammami and Siiteri, 1991). It was therefore important to establish the influence of glucocorticoids on the skin enzyme *in vivo*.

4.7b METHODS:

Six week old male hairless mice weighing 20-25 g were used for this experiment. A control group was sham-operated and all other animals underwent bilateral adrenalectomy under halothane anaesthesia. Adrenalectomized animals were randomly allocated to one of four groups; no replacement, corticosterone replaced (5 μ g/g day⁻¹), dexamethasone replaced (0.5 µg/g day 1) (Haigh et al., 1990), aldosterone replaced (0.05 μ g/g day 1). All drugs were administered by subcutaneous injection using olive oil as vehicle. The sham-operated group received vehicle only. All animals were fed similar diets and adrenalectomized animals (unreplaced group) were maintained on 0.9% NaCl. Treatment was of 10 days duration, commencing 24h after operation and finishing 2h prior to removal of tissues. Animals were killed by decapitation, blood collected for plasma corticosterone measurement and dorsal skin and kidneys were removed. Spleens were also removed and weighed. Homogenates of skin and kidney were prepared and assayed for 11ß-hydroxysteroid dehydrogenase activity as previously described in Methods of this chapter. Protein content of each homogenate was 0.5 mg with NADP at a final concentration of 100 μ M.

4.7c Statistical analysis:

The values are shown as mean $(\pm SEM)$ for triplicate incubates from between 5

and 8 animals in each group. The differences among groups was tested by one factor analysis of variance (ANOVA) with significance set at the 5% limit.

4.7d Results:

Plasma corticosterone measured by radioimmunoassay was below the limit of detection of the assay (<1.1 nmol/l) in all groups, except in the sham-operated (median 24 nmol/l, range 8 - 54) and the corticosterone-replaced groups (median 4.3 nmol/l, range 2.3-9). Post mortem examinations confirmed bilateral adrenalectomy in the respective groups except shams.

Sham-operated (107.5 ± 9.6) mg and corticosterone-treated groups (102.5 ± 10.3) mg had similar splenic weights. Compared with shams, splenic weight was markedly reduced in the dexamethasone treated group (47.5 ± 2.5) mg, p<0.005. Splenic weight rose in the adrenalectomy (150.0 ± 15.3) mg p<0.05 and aldosterone-treated groups (165.0 ± 21.8)mg, p<0.05 compared with shams. There was no significant difference in the change in body weight of the various groups. The changes in splenic weights were consistent with glucocorticoid deficiency in the adrenalectomized and aldosterone-treated animals and with glucocorticoid excess in the dexamethasone treated group.

The activity of kidney enzyme was similar in all five groups of animals (Figure 4.8). This was in contrast to the findings for the skin enzyme (Figure 4.9). When compared with the sham-operated group, the unreplaced group and aldosterone replaced group exhibited significantly (p<0.05) reduced skin enzyme activity. In both glucocorticoid-replaced groups, skin enzyme activity was similar to that of the sham operated group (Figure 4.9).

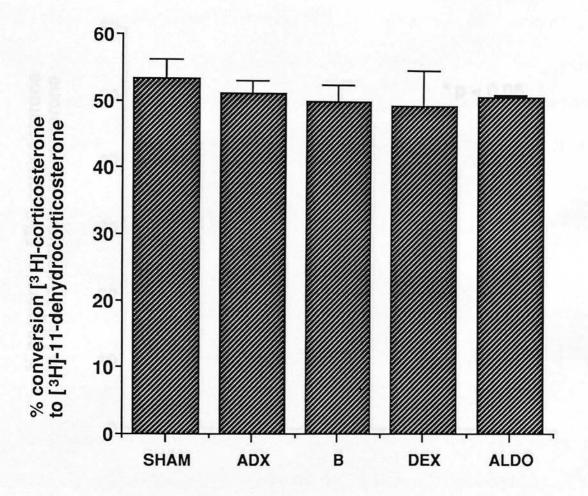


Figure 4.8: The regulation of kidney 11 beta-hydroxysteroid dehydrogenase: the effects of adrenalectomy (ADX) and replacement with corticosterone (B), dexamethasone (DEX) and aldosterone (ALDO).

Results shown as mean \pm SEM, n=8 for sham and 5 for all other groups.

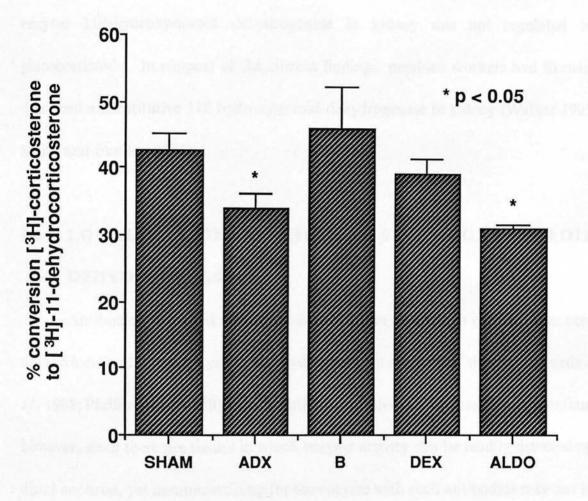


Figure 4.9: The regulation of skin 11 beta-hydroxysteroid dehydrogenase: the effects of adrenalectomy (ADX) and replacement with corticosterone (B), dexamethasone (DEX) and aldosterone (ALDO).

Results shown as mean \pm SEM, n=8 for sham and 5 for all other groups.

These findings suggest that similar to what has been demonstrated with cultured human skin fibroblasts *in vitro* (Hammami and Siiteri, 1991), the skin enzyme *in vivo* is positively regulated by glucocorticoids (with mineralocorticoid replacement in adrenalectomized animals not exhibiting any influence). Unlike the findings for skin enzyme 11ß-hydroxysteroid dehydrogenase in kidney was not regulated by glucocorticoids. In support of the current findings, previous workers had likewise described a constitutive 11ß-hydroxysteroid dehydrogenase in kidney (Walker 1993; Smith and Funder, 1991).

4.8 LOCALIZATION OF SKIN 11B-HYDROXYSTEROID DEHYDROGENASE:

Antibodies generated against the enzyme from rat liver, in rabbits, have been used to localize 11ß-hydroxysteroid dehydrogenase in a variety of tissues (Edwards *et al.*, 1988; Phillips *et al.*, 1989). Using antibodies to liver type protein has limitations however, since there are tissues in which enzyme activity can be readily detected eg. distal nephron, yet immunostaining for the enzyme with such antibodies may not be obtained (Edwards *et al.*, 1988; Rundle *et al.*, 1989). Bearing this shortcoming in mind an attempt to localise 11ß-hydroxysteroid dehydrogenase in both mouse and human skin was undertaken.

Dorsal skin from freshly killed nude mice and normal human skin obtained at laparotomy from the anterior abdominal wall were used. Immunohistochemical localization of the enzyme was undertaken using an antibody (Monder and Lakshmi, 1990) raised in rabbit against a purified hepatic microsomal preparation of rat 11ß-hydroxysteroid dehydrogenase. As negative controls pre-immune rabbit serum and inappropriate antibody (to 1-antitrypsin) were also performed.

Tissue samples were placed immediately in Bouin's fixative for 24-48 h and then subjected to routine paraffin embedding. Sections were cut at $4\,\mu\text{m}$, deparaffinised and then rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by 10 min treatment with 3% hydrogen peroxide. To minimize background staining slides were pre-incubated with 20% donkey serum. Primary antibody, pre-immune serum or inappropriate antibody, at dilutions of 1:100 or 1:200, were then incubated with sections of tissue for 30 min at room temperature. This was followed by labelled avidin-biotin immunodetection (Guesdon *et al.*, 1979) with biotinylated donkey antibody to rabbit immunoglobulin and peroxidase-conjugated streptavidin. The peroxidase substrate chromogen was 3, 3,-diaminobenzidine tetrachloride. The substrate was hydrogen peroxide. Slides were then counterstained with Mayer's haematoxylin and mounted in D.P.X.

Plate 4.1 shows positive staining for 11ß-hydroxysteroid dehydrogenase in mouse skin. This was largely confined to the epidermis. There was no significant staining with pre-immune serum (Plate 4.2).

Sections from normal human skin revealed strong positive cytoplasmic staining for 11B-hydroxysteroid dehydrogenase in the epidermis with the exception of the basal cell layer (Plate 4.3). There was moderate positivity on the luminal border of sweat ducts and some staining in the cytoplasm of sweat glands (Plate 4.3). For comparison, Plates 4.4 and 4.5 show the staining pattern with pre-immune serum and inappropriate antibody (α_1 -antitrypsin), respectively.

The localization of the enzyme to the epidermis, predominantly, favours the notion that inhibition might be achieved with topical therapy.

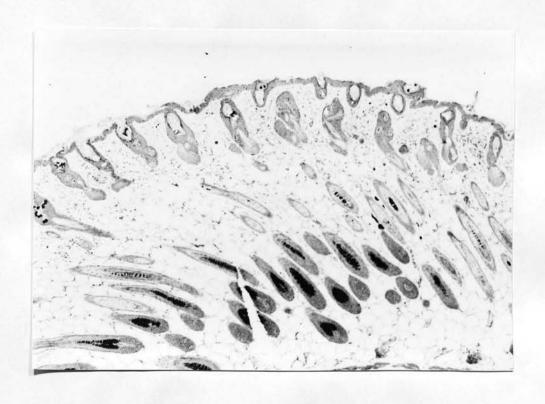


PLATE 4.1

Immunohistochemical staining of mouse skin with antiserum to 11B-hydroxysteroid dehydrogenase

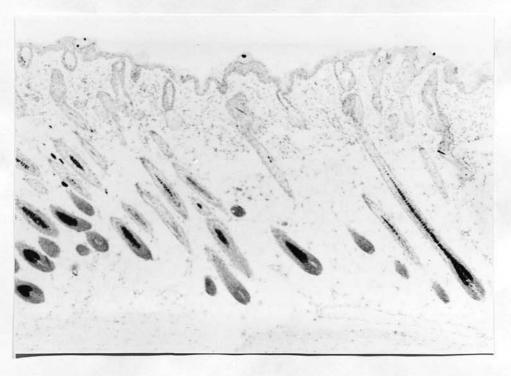


PLATE 4.2

Immunohistochemical staining of mouse skin with pre-immune serum



PLATE 4.3

Section of human skin stained for 11B-hydroxysteriod dehydrogenase using anti-serum to rat liver 11B-hydroxysteriod dehydrogenase



PLATE 4.4
Section of human skin stained with pre-immune serum

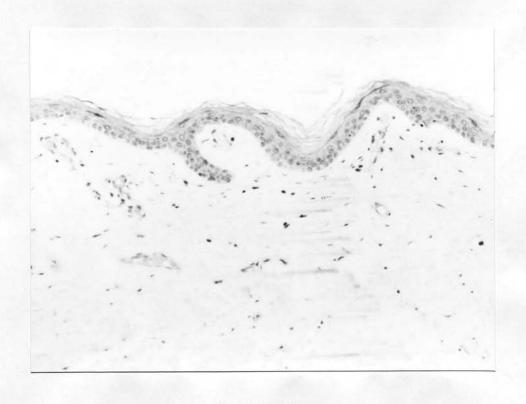


PLATE 4.5

Section of human skin stained with inappropriate antibody (α_1 -antitrypsin)

4.9 CONCLUSIONS:

For the first time, skin 11ß-hydroxysteroid dehydrogenase has been characterized and localized in an animal model. It is also the first time that the regulation of this enzyme has been studied *in vivo*. The hairless mouse seems a suitable model for further studies involving 11ß-hydroxysteroid dehydrogenase in skin.

Using this model, the whole skin enzyme activity differs from that of whole kidney in several respects. Skin 11ß-hydroxysteroid dehydrogenase activity is regulated by glucocorticoids whereas kidney enzyme is unaffected. The skin enzyme utilizes NADP preferentially. This biochemical characteristic favours the existence of 11ß-HSD₁ in skin (liver-type protein). This is further supported by immunohistochemical studies which have localized liver-type protein to the epidermis.

These findings however, do not preclude the presence of other isoforms in the skin or indeed an isoform unique to the skin. In particular, the absence of staining of the dermis with antiserum to liver-type protein and the findings of previous studies indicating cortisone to cortisol conversion by fibroblasts (Hammami and Siiteri, 1989) indicate that dermis might well be a site of 11\(\textit{B}\)-HSD₁ activity. Unlike the skin, kidney utilizes both NAD and NADP reflecting the presence of both 11\(\textit{B}\)-HSD₁ and 11\(\textit{B}\)-HSD₂

in this organ.

Kinetic studies and enzyme regulation experiments further indicate differences between skin and kidney 11ß-hydroxysteroid dehydrogenase. The differences demonstrated *in vitro* are consistent with clinical experience. A patient shown to be deficient in kidney enzyme (Stewart *et al.*, 1988) did not exhibit clinical cutaneous stigmata of glucocorticoid excess. In this patient (G.B.) the roof of an epidermal suction blister was incubated with ³H-cortisol, up to 15% was recovered as ³H-cortisone, implying presence of 11ß-hydroxysteroid dehydrogenase activity in skin despite evidence for its absence in kidneys (presumably absent from distal nephron).

Whether deficiency (or excessive activity) of the skin enzyme is important in the pathogenesis of skin diseases or susceptibility to glucocorticoid effects remains to be elucidated. The nude mouse provides a model in which these hypotheses may be tested.

The localization of 11ß-hydroxysteroid dehydrogenase to the epidermis would facilitate attempts to inhibit it by topical means.

CHAPTER 5

POTENTIATION OF HYDROCORTISONE ACTIVITY IN SKIN

CHAPTER 5

POTENTIATION OF HYDROCORTISONE ACTIVITY IN SKIN

5.1 INTRODUCTION:

Glucocorticoids play an important role in the treatment of many skin diseases. Modifications of the cortisol structure have resulted in a wide variety of clinically efficacious agents. The more potent, however, often carry a risk of skin thinning and systemic toxicity (Editorial 1977 Lancet); this is not too surprising given the high intrinsic potency of such agents. There are theoretical advantages of targeting the glucocorticoid receptor by achieving high local levels of an intrinsically weak steroid. In this way one need not compromise local efficacy but may significantly reduce systemic toxicity. Thus systemic side-effects from topical glucocorticoids are well recognized in the setting of chronic or relapsing disease when more potent, highly bioavailable agents are used (Himathongkam 1978; Teelucksingh et al., 1993) but such effects have not been described with topical hydrocortisone in adults (Turpeinen, 1988). On the whole, the systemic absorption of hydrocortisone from skin is very low (Wester et al., 1983).

Human skin has the capacity to metabolize and inactivate hydrocortisone to cortisone (Malkinson *et al.*, 1959, Hsia and Hao, 1966). If it was possible to inhibit skin metabolism of hydrocortisone then this might result in enhanced local biologic action with minimal systemic toxicity. Previous work had demonstrated that liquorice and its extracts were potent inhibitors of 11ß-hydroxysteroid dehydrogenase in liver and kidney (Valentino *et al.*, 1987, Monder *et al.*, 1989). The question that follows is whether local inhibition of this enzyme could enhance hydrocortisone action?

5.2 LIQUORICE:

The latter question is particularly interesting because of what has been previously described with glycyrrhetinic acid and carbenoxolone, derivatives of liquorice. These substances had long been thought to possess glucocorticoid (anti-inflammatory) activity (Finney and Somers, 1958; Finney and Tarnoky, 1960; Khan *et al.*, 1967; Parke, 1967). In fact, the clinical use of 2% carbenoxolone and glycyrrhetinic acid has been reported to benefit a variety of inflammatory disorders both in man (Csonka and Murray, 1971; Annan, 1957; Adamson and Tillman, 1955; Somerville, 1957; Colin-Jones, 1957) and animals (Halpin, 1956). It was also shown that the administration of glycyrrhetinic acid to normal human volunteers or patients with adrenal disease could influence ACTH (as evaluated by decreases in urinary excretion of 17-ketosteroids) and MSH (Louis and

Conn, 1956), further implying glucocorticoid-like action. The anti-inflammatory effect was, however, unpredictable (Warin and Evans, 1956; Donaldson and Duthie, 1956) and it was noted that carbenoxolone possessed no intrinsic anti-inflammatory potency in adrenalectomized animals (Finney and Somers, 1958). The latter suggested that these substances did have a direct glucocorticoid action but that such an effect was perhaps mediated by reduced metabolism/clearance of endogenous glucocorticoids. To support this latter view, Kumagai (Kumagai *et al.*, 1957) found that the concomitant use of liquorice-extract produced greater efficacy when combined with glucocorticoids in patients with rheumatologic diseases than when these patients were treated with glucocorticoids alone. To further elucidate this I turned to the animal model for the study of skin 11ß-hydroxysteroid dehydrogenase.

5.3 INHIBITION OF SKIN 11B-HYDROXYSTEROID DEHYDROGENASE:

Dorsal skin obtained from freshly killed female nude mice (nu/nu, mixed genetic background) was homogenized in 10 ml Krebs-Ringer buffer as described in Methods. Incubation of homogenized skin (1.5 mg) with ³H- corticosterone was undertaken (see Methods, Chapter 4). To determine the possible effects of glycyrrhetinic acid on skin enzyme, incubations were also performed with glycyrrhetinic acid at final

concentrations ranging from 10^{-4} - 10^{-8} M. Solutions of glycyrrhetinic acid were prepared fresh in absolute ethanol. Control samples containing either no homogenate or $10 \ \mu l$ ethanol were compared with incubations containing glycyrrhetinic acid.

5.3a Results:

The *in vitro* experiments using skin from nude mice showed that 11ß-hydroxysteroid dehydrogenase activity as assessed by the conversion of corticosterone to dehydrocorticosterone was high (Figure 5.1). This was slightly reduced by ethanol alone but markedly inhibited in a dose dependent manner by all concentrations of glycyrrhetinic acid used.

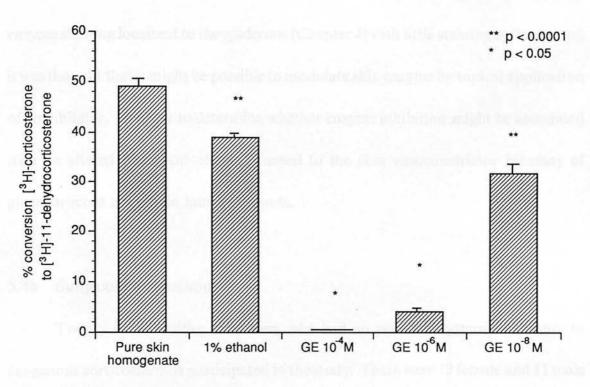


Figure 5.1: The activity of 11 beta-hydroxysteroid dehydrogenase in skin homogenate and the effects of ethanol and various concentrations of glycyrrhetinic acid (GE).

Results shown as mean \pm SEM, n=5. p values were obtained after comparison by ANOVA followed by paired Student's t test.

5.4 TOPICAL GLYCYRRHETINIC ACID ENHANCES SKIN VASOCONSTRICTOR ACTIVITY OF HYDROCORTISONE.

Having demonstrated that skin 11ß-hydroxysteroid dehydrogenase was susceptible to inhibition of glycerrhetinic acid *in vitro* and having found that most enzyme staining localized to the epidermis (Chapter 4) with little staining in the dermis, it was thought that it might be possible to modulate skin enzyme by topical application of an inhibitor. In order to determine whether enzyme inhibition might be associated with an altered biological effect, I turned to the skin vasoconstrictor bioassay of glucocorticoid activity in human subjects.

5.4a Subjects and methods:

Twenty three healthy volunteers who had no previous systemic exposure to exogenous corticosteroids participated in the study. There were 12 female and 11 male subjects of mean age 29 years (range 21-50 years). The skin vasoconstrictor bioassay as modified by Place (1970) and described in Methodology 1, Chapter 3 was used to determine the potency of test substances. Two subjects were tested twice after an interval two months so that a total of twenty five tests were performed taking groups of 4 to 6 subjects at a time. Test solutions comprised hydrocortisone acetate, beclomethasone dipropionate, glycyrrhetinic acid and glycyrrhetinic acid with

hydrocortisone acetate (HCA) all made up fresh in 95% ethanol in the following concentrations:-

Hydrocortisone acetate: 10, 3, 1, 0.3, 0.1 mg/ml

Beclomethasone dipropionate: 10, 3, 1, 0.3, 0.1 μ g/ml

Glycyrrhetinic acid: 20 mg/ml

Hydrocortisone acetate and glycyrrhetinic acid together:

10 mg HCA and 20 mg GE/ml,

3 mg HCA + 20 mg GE/ml,

1 mg HCA + 20 mg GE/ml,

0.3 mg HCA + 20 mg GE/ml

0.1 mg HCA + 20 mg GE/ml.

Test substances were kept in coded vials and used within twenty four hours of preparation. Ten μ l of each solution were applied in random order to 7x7 mm sites demarcated by silicone grease on the flexor aspect of the forearm. After having been allowed to dry the test area was occluded with polyester film (Melinex 12 μ M, ICI) for 16-18 hours. The degree of blanching was assessed 1, 2, 3, and 6 hours after removal of the occlusive dressing and scored independently by two observers using a scale where 0 = no blanching, 1 = mild, 2 = definite, and 3 = intense blanching. All solutions were

applied and test sites subsequently read using double-blind procedure. Time-effect curves for each test substance were plotted and area under the curve determined using the trapezoidal rule.

5.4b Statistical analysis:

Comparison between groups was by analysis of variance (ANOVA), followed by paired Student's t-test. Significance was set at p<0.05, and results expressed as mean ±SEM. Linear regression analysis was used to plot the dose-response relationship.

5.4c Results:

The dose-response relationship of test substances on the skin vasoconstrictor response in normal volunteers is shown in Figure 5.2.

Hydrocortisone acetate alone produced a flat response at the lower limit of detection of the assay consistent with what has been previously reported (Place *et al.*, 1970). The addition of glycyrrhetinic acid to hydrocortisone acetate, however, produced significant potentiation of cutaneous vasoconstrictor effect at concentrations of hydrocortisone acetate 10 mg/ml (p<0.01), 3 mg/ml (p<0.001) and 1 mg/ml (p<0.001). Glycyrrhetinic acid alone and ethanol alone produced no effect. Beclomethasone dipropionate produced a steep dose-response consistent with previous assays (Johansson *et al.*, 1982).

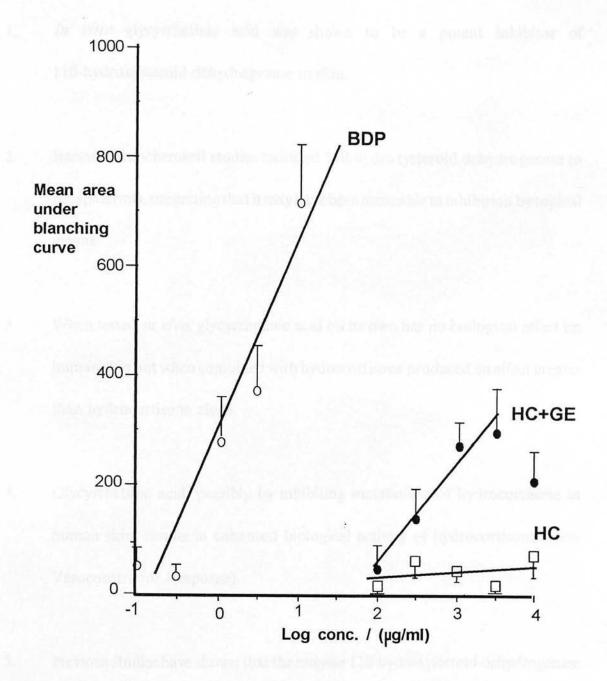


Fig 5.2:
Relation of human vasoconstrictor response (area under blanching curve in arbitrary units x h), to dose of beclomethasone dipropionate (BDP), hydrocortisone plus glycyrrhetinic acid (HC+GE) and hydrocortisone (HC) alone.

Results shown as mean (\pm SEM), n=25.

5.5 CONCLUSIONS:

- In vitro glycyrrhetinic acid was shown to be a potent inhibitor of 11β-hydroxysteroid dehydrogenase in skin.
- 2. Immunohistochemical studies localized 11ß-hydroxysteroid dehydrogenase to the epidermis, suggesting that it may have been amenable to inhibition by topical means.
- 3. When tested *in vivo*, glycyrrhetinic acid on its own has no biological effect on human skin but when combined with hydrocortisone produced an effect greater than hydrocortisone alone.
- Glycyrrhetinic acid, possibly by inhibiting metabolism of hydrocortisone in human skin, results in enhanced biological activity of hydrocortisone (Skin Vasoconstrictor Response).
- 5. Previous studies have shown that the enzyme 11ß-hydroxysteroid dehydrogenase controls access to the mineralocorticoid receptor. The current evidence, by virtue of the fact that the inhibition of the enzyme results in an enhanced

glucocorticoid response, suggests that the enzyme might also regulate access to the glucocorticoid receptor.

6. Local glycyrrhetinic acid-inhibition of cortisol metabolism provides a possible mechanism of increasing glucocorticoid anti-inflammatory action in the skin. This principle of local enzyme inhibition producing enhanced glucocorticoid activity may well be applicable to other sites where there is local glucocorticoid metabolism e.g. gastrointestinal tract and bronchial tree.

CHAPTER 6

MECHANISM OF ANTI-INFLAMMATORY ACTION OF LIQUORICE

CHAPTER 6

MECHANISM OF ANTI-INFLAMMATORY ACTION OF LIQUORICE 6.1 INTRODUCTION

Liquorice and its derivatives have long been known to possess both mineralocorticoid (Borst et al., 1953; Elmadjian et al., 1956) as well as glucocorticoid properties (Kumagai et al., 1957; Annan, 1957; Adamson and Tillman, 1955; Somerville, 1957; Colin-Jones, 1957; Finney and Tarnoky, 1960). The mechanism of these actions was poorly understood, although the presence of an intact adrenal gland or cortisol replacement in patients with adrenal insufficiency was known to be essential (Borst et al., 1953; Elmadjian et al., 1956). Glycyrrhetinic acid, the active principle in liquorice was shown to be a potent inhibitor of 11ß-hydroxysteroid dehydrogenase from liver and kidney in vitro (Valentino et al., 1987; Monder et al., 1989) and that such inhibition, in vivo, could explain the mineralocorticoid effects of liquorice (Stewart et al., 1988).

In Chapter 5, it was demonstrated, like that from liver and kidney, skin 11ß-hydroxysteroid dehydrogenase was also inhibited by glycyrrhetinic acid *in vitro*, and that such inhibition *in vivo* may have led to potentiation of skin vasoconstrictor response - an apparent glucocorticoid-mediated phenomenon. Thus inhibition of the enzyme might also account for the glucocorticoid action of liquorice.

6.2 LIQUORICE: AN INHIBITOR OF STEROID AND PROSTAGLANDIN DEHYDROGENASES.

There is, however, structural homology between 11 β -hydroxysteroid dehydrogenase and several other hydroxysteroid dehydrogenases, including 3 α -, 17 β - and 20 β - hydroxysteroid dehydrogenase in both prokaryotes and eukaryotes (Monder and White, 1992). Because of the small contribution of the latter enzymes to the metabolism of cortisol in human skin (Hsia, *et al.*, 1964, 1966) it seems unlikely that inhibition of these enzymes could be responsible for the potentiation of hydrocortisone action by glycyrrhetinic acid.

On the other hand, there is considerable structural homology between prostaglandin metabolizing enzymes and 11ß-hydroxysteroid dehydrogenase (Baker and Fanestil 1991; Krook et al., 1990). On the basis of the latter there was speculation that the anti-inflammatory capacity and mineralocorticoid action of liquorice and its derivatives could be in part the result of altered prostaglandin metabolism. Indeed Peskar (Peskar et al., 1976) had suggested that the inhibition of metabolism of prostaglandin was the possible mechanism by which carbenoxolone healed peptic ulcers. This conclusion was drawn from the finding that gastric prostaglandin metabolism was inhibited by carbenoxolone at fairly high concentrations (Peskar et al., 1976; Rask-

Madsen *et al.*, 1983). To determine whether the anti-inflammatory effects of liquorice derivatives (glycyrrhetinic acid and carbenoxolone) demonstrated in human subjects might arise from some influence on prostaglandin metabolism, the comparative effects of these liquorice-derived compounds upon the prostaglandin metabolizing enzyme, 15-hydroxyprostaglandin dehydrogenase and 11ß-hydroxysteroid dehydrogenase was examined *in vitro*. Human placental tissue was chosen because it is a rich source of both 11ß-hydroxysteroid dehydrogenase (Monder and White, 1992) and 15-hydroxyprostaglandin dehydrogenase (Krook *et al.*, 1990).

6.3 METHODS:

Chorionic homogenate (protein content 1.5 mg) was incubated with 500 ng PGE₂ and 1 mM NAD at 37° C for 15 min in a phosphate buffer containing 20% glycerol (pH 8.4). Prostaglandin dehydrogenase activity was measured by radioimmunoassay of the quantity of 15-keto PGE₂ produced after transformation to its methyl oxime (this assay was performed by Cheng Li Nan, MRC Reproductive Biology Unit, Edinburgh). The experiment was completed with glycyrrhetinic acid, carbenoxolone and 5-(4-fluorobenzoyl) 2-hydroxybenzene acetic acid (FHBA), a known prostaglandin dehydrogenase inhibitor, all at concentrations up to 40 μ M.

The effects of similar concentrations of the latter substances on human placental 11ß-hydroxysteroid dehydrogenase were evaluated as described for the skin (Methodology, Chapter 4). Briefly, 0.3 mg protein from fresh placental homogenate was incubated with 12 μ M ³H-cortisol and 200 μ M NAD in Krebs-Ringer buffer at 37°C for 1h. Steroids were extracted with ethyl acetate and separated by thin-layer chromatography. Activity of 11ß-hydroxysteroid dehydrogenase was expressed as a percentage conversion percentage ³H-cortisol to ³H-cortisone.

6.4 RESULTS:

The results show that glycyrrhetinic acid and carbenoxolone had no effect on 15-hydroxyprostaglandin dehydrogenase activity (Figure 6.1, top), in contrast to the inhibition produced by FHBA. On the other hand, FHBA had no affect on 11ß-hydroxysteroid dehydrogenase while both carbenoxolone and glycyrrhetinic acid produced marked inhibition (Figure 6.1, bottom).

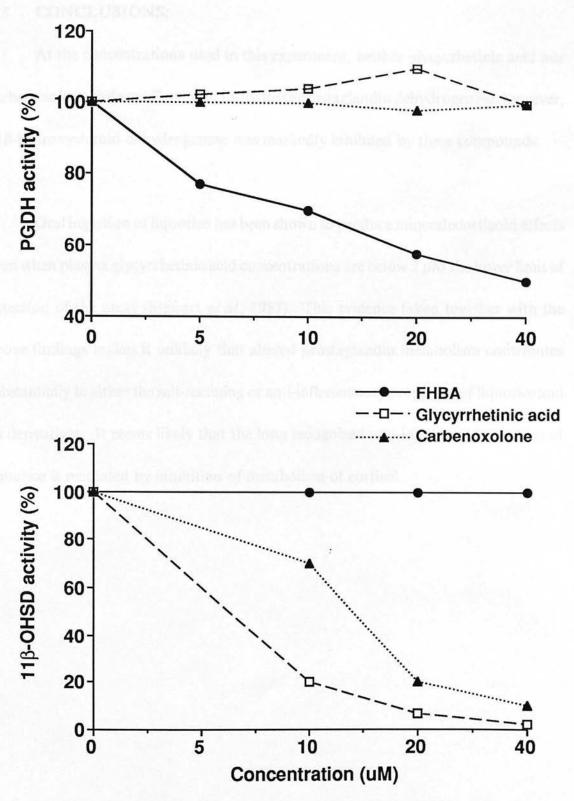


Figure 6.1: The comparative effects of FHBA, glycyrrhetinic acid and carbenoxolone on prostaglandin dehydrogenase activity (top) and 11 beta-hydroxysteroid dehydrogenase activity (bottom), expressed as percentage of control.

6.5 CONCLUSIONS:

At the concentrations used in this experiment, neither glycyrrhetinic acid nor carbenoxolone had anyeffect upon 15-hydroxyprostaglandin dehydrogenase, however, 11B-hydroxysteroid dehydrogenase was markedly inhibited by these compounds.

Oral ingestion of liquorice has been shown to produce mineral corticoid effects even when plasma glycyrrhetinic acid concentrations are below 2 μ M the lower limit of detection of the assay (Stewart *et al.*, 1987). This evidence taken together with the above findings makes it unlikely that altered prostaglandin metabolism contributes substantially to either the salt-retaining or anti-inflammatory properties of liquorice and its derivatives. It seems likely that the long recognized anti-inflammatory action of liquorice is mediated by inhibition of metabolism of cortisol.

CHAPTER 7

VARIATION IN SENSITIVITY TO GLUCOCORTICOIDS

CHAPTER 7: VARIATION IN SENSITIVITY TO GLUCOCORTICOIDS

7.1 BACKGROUND

A possible shortcoming in the approach to glucocorticoid therapy has been the relative disregard for what has, in fact, been a long held clinical perception; that sensitivity to glucocorticoid therapy can be strikingly variable. It is known for example, that glucocorticoid regimes will be successful in preventing acute renal transplant rejection in only about 50% of cases (Dumble et al., 1981; Langhoff and Ladefoged, 1987). Similar heterogeneous corticosteroid sensitivity has also been described in the treatment of myasthenia gravis (Willcox et al., 1989; Tindall, 1980). Though recognized in a number of clinical settings, the phenomenon of glucocorticoid resistance has probably been best documented in asthma. Thus, although most patients with asthma will benefit from glucocorticoids, bronchospasm in some patients proves refractory to such therapy (Dwyer et al., 1967; Schwartz et al., 1968; Carmichael et al., 1981). That this phenomenon is not confined to any particular disease state suggests that inherent biological factors rather than specific diseases might be dictating this.

There are a number of possible factors that could contribute to reduced sensitivity to glucocorticoids:

- (i) Severity of the disease being treated is an obvious factor. Thus patients with more severe disease may require larger dosages or more prolonged treatment to derive an optimal response.
- (ii) Differences in metabolic clearance could also contribute to such a phenomenon. It has been shown, for example, that the metabolic clearance rate and the volume of distribution of prednisolone are lower and the half-life longer in patients with an increased tendency to toxicity (Axelrod, 1989). Likewise, co-existing diseases may significantly reduce clearance. Thus patients with impaired hepatic and renal function will clear glucocorticoids less effectively (Axelrod 1989). In patients on enzyme inducing drugs like phenytoin and rifampicin, the reverse is seen. These patients can exhibit "resistance" (Becker *et al.*, 1973; Edwards *et al.*, 1974; Lofdahl *et al.*, 1984) to glucocorticoids because of increased metabolic clearance.
- (iii) Many of the anti-inflammatory actions of glucocorticoids are mediated by the anti-inflammatory proteins, the lipocortins or annexins (Peers *et al.*, 1993). The development of antibodies to lipocortin-1 has been shown to be associated with attenuated responsiveness to glucocorticoid therapy in patients with rheumatoid

However, there is another scenario which will not be explained by the above. There are those patients who seem exquisitely sensitive to development of glucocorticoid associated side-effects. Thus Becker (Becker 1965; Becker et al., 1973) had observed, for example, that the topical administration of glucocorticoids resulted in greater rises of intra-ocular pressure in patients with primary open-angle glaucoma compared with non-glaucomatous controls. Moreover, this ocular sensitivity was paralleled by an increased sensitivity to adrenal suppression with dexamethasone (Becker et al., 1973). Lymphocytes from such patients, likewise, exhibited greater suppression of phytohaemagglutinin-induced transformation under the influence of glucocorticoids (Becker et al., 1973). This implied, that in these patients this enhanced glucocorticoid sensitivity was not tissue-specific but might be a generalized phenomenon. More suggestive evidence for heterogeneity in response to glucocorticoid had, in fact, first come from animal studies.

It has been recognized for some time now that there are marked inter-species differences in the response to glucocorticoids. Thus hamster, mouse, rat and rabbit are glucocorticoid-sensitive whereas guinea pigs, monkey and man are glucocorticoid-resistant species (Claman, 1972). This differentiation is based on the

relative ease of producing lymphoid depletion after systemic glucocorticoid exposure (Claman, 1972). Such differences in sensitivity can be explained by the finding that there are differences in affinity among glucocorticoid receptors in different species, for the same ligand (Kraft *et al.*, 1979). Interestingly, it was shown that even within a single species there might be differences in sensitivity and this sensitivity may be genetically determined. Thus, the thymus cells of mice with H2a haplotype were shown to be much more sensitive to the lytic action of glucocorticoids both *in vivo* and *in vitro* than those from mice with the H2b haplotype (Becker *et al.*, 1976).

Moving upwards in the animal kingdom, there are marked differences in glucocorticoid sensitivity among primates. New World monkeys, for example, marmoset and squirrel are glucocorticoid resistant compared with Old World species eg. chimpanzee (Lipsett *et al.*, 1985). Despite the 10-fold or so greater circulating levels of cortisol in resistant species, life expectancy, biochemistry and metabolism are similar to that of Old World species (Lipsett, *et al.*, 1985). The affinity of the glucocorticoid receptor for dexamethasone, however, is markedly reduced in New World primates.

Evidence that a similar situation might also exist in man came with the report by Vingerhoeds (Vingerhoeds et al., 1976) of a "possibly genetically determined

hyposensitivity to the glucocorticoid action of cortisol". In this description, two patients, a father and son, exhibited gross, sustained hypercortisolism without the clinical stigmata of Cushing's syndrome. This paradox was explained by the finding of qualitative abnormalities of glucocorticoid receptor in lymphocytes and skin fibroblasts (Chrousos *et al.*, 1982). Since this description there have been subsequent reports indicating a variety of abnormalities of the glucocorticoid receptor. These include a decreased number of glucocorticoid receptors (Iida *et al.*, 1985), decreased binding of the activated receptor to DNA (Nawata *et al.*, 1987) and decreased stability of the receptor at higher temperatures (Bronnegard *et al.*, 1986).

Interestingly, a cortisol hyperactive syndrome in which there are features of Cushing's syndrome associated with subnormal levels of serum cortisol, free cortisol and 24-h urinary cortisol excretion, has also been described (Iida *et al.*, 1990, 1991). The latter might represent the prototype of those patients who are particularly prone to glucocorticoid side-effects.

Extensive studies in the evaluation of such patients and their extended kinship showed that there was, however, no association between the transmission of the condition and HLA haplotypes (Lipsett et al., 1985). This implied, unfortunately, that

it was not possible to utilize a very convenient marker, HLA haplotypes, to screen wider populations for such abnormalities.

Further study of such inherent variability in responsiveness to glucocorticoids might provide insight as to how it might be possible to tailor glucocorticoid therapy to individual needs rather than through empiricism. The value of some clinical or biochemical marker that might predict the outcome to glucocorticoid therapy for inflammatory or neoplastic conditions would be immense.

The skin vasoconstrictor bioassay has served as a useful method to assess glucocorticoid potency. Can it be utilized to define glucocorticoid sensitivity?

7.2 RATIONALE FOR USE OF SKIN VASOCONSTRICTOR ASSAY AS A MARKER OF GLUCOCORTICOID SENSITIVITY

That the skin vasoconstrictor response in an individual might reflect inherent glucocorticoid sensitivity is suggested from what is understood to be the mechanism of blanching. Although the precise mechanism of the blanching phenomenon is not known a number of possibilities has been proposed (Marks and Sawyer, 1986). Glucocorticoids are known to reduce the erythema induced by a variety of vasodilators.

Thus topical arachidonic acid will induce skin erythema but prior treatment of the skin with glucocorticoid will substantially reduce this erythematous response (Marks and Sawyer, 1986). Similarly, histamine characteristically induces a wheal and flare cutaneous response which can be subdued by prior glucocorticoid application (Marks and Sawyer, 1986). Glucocorticoids are known to inhibit both histamine release from basophils (Schleimer et al., 1981) as well as to prevent arachidonic acid release from membrane phospholipids (Blackwell et al., 1980). It is possible that inhibition of phospholipase A2 is the final common pathway to both these processes since both histamine as well as exogenous arachidonic acid can stimulate endogenous phospholipase A2 activity (Flower, 1988 b). Furthermore glucocorticoid sensitizes blood vessels to the vasoconstrictive influence of endogenous catecholamines (Ginsburg and Duff, 1958; Haigh et al., 1990), a process that can likewise be linked to inhibition of endogenous phospholipase A2 activity (Rascher et al., 1980). Glucocorticoids are known to inhibit phospholipase by way of the anti-inflammatory proteins - lipocortins (Flower, 1988 a,b). Direct evidence for a role for lipocortins in this vasoconstrictor process is, however, not currently available. Even so, this would not necessarily be exclusive of other mechanisms (Walker et al., 1992 c; Walker and Williams, 1992) especially those involving endothelium-dependent factors (Brenner et al., 1989).

7.3 SKIN VASOCONSTRICTION - A GLUCOCORTICOID-SPECIFIC PHENOMENON

Although the precise cellular mechanism of skin blanching is not known, it does seem to arise as a result of interaction between glucocorticoid and glucocorticoid receptors in the skin vasculature (Marks et al., 1982; Kornel et al., 1982; Gaillard et al., 1985). Thus neither progesterone, deoxycorticosterone, estradiol nor testosterone, even at high concentrations, induce blanching when applied on their own (Marks et al., 1982). However, when co-applied with potent glucocorticoids, progesterone and deoxycorticosterone but not estradiol or testosterone antagonize the vasoconstrictor response in dose-dependent fashion (Marks et al., 1982). Progesterone and deoxycorticosterone behave as glucocorticoid antagonists in most glucocorticoid-responsive systems whereas estradiol and testosterone are without effects (Funder, 1985).

More direct evidence to suggest that blanching is mediated by occupancy of glucocorticoid receptors comes from a study that showed attenuation of blanching responses to topical glucocorticoids following the systemic administration of RU 38486 (Gaillard *et al.*, 1985). This compound exhibits potent anti-glucocorticoid properties which correlate with strong binding to the glucocorticoid receptor leading to its

7.4 SKIN VASOCONSTRICTOR ASSAY IN A POPULATION: INITIAL STUDIES VALIDATING THE SKIN VASOCONSTRICTOR RESPONSE AS A POTENTIAL MARKER OF GLUCOCORTICOID SENSITIVITY.

An inherent value of the skin vasoconstrictor bioassay is the built-in control of the subject's normal skin for comparison. Quantitative validation was therefore unnecessary. Initial qualitative blanching assays were therefore performed.

7.4a Subjects and methods

Fifty eight healthy adult subjects (mostly Caucasians) of both sexes and mean age 35 years (range 16-50) were used as a pool of volunteers. All were tested initially with solutions of beclomethasone dipropionate, a potent topically active glucocorticoid of high topical bioavailability at concentrations of 100 and 10 μ g/ml using the skin vasoconstrictor assay as described in Methodology 2, chapter 3.

7.4b Results:

A single reading of vasoconstrictor response after 16 hours of occlusion revealed

that 52 (90%) had a positive response to 10 μ g/ml. Fifty five (95%) had a positive response to 100 μ g/ml. Three individuals remained negative on serial testing over the ensuing three months.

Serial qualitative testing of the latter three individuals over the ensuing three months at intervals of one month showed they remained consistently unresponsive to topically applied beclomethasone dipropionate. Two patients were otherwise normal and one had type I diabetes.

Results very similar to these have been reported by previous assayists. Burdick (1974) reported 90% positive responders to fluosinolone acetonide out of 24 subjects. Place (1970), likewise, reported 92% responsiveness using triamcinolone acetonide. Both fluosinolone acetonide and triamcinolone acetonide, like beclomethasone dipropionate (Johansson *et al.*, 1982) exhibit high topical activity.

Previous users of this assay have recognized that there is "biologic-variation" in responsiveness, (Burdick, 1974; Barry and Woodford, 1978; Stoughton, 1992) and thus selected only those individuals who were good responders (Burdick, 1974). The purpose of these studies was to determine bioavailability and potency of topical agents however,

and the significance of unresponsiveness in individuals was not explored.

A negative response could presumably arise as a result of impaired bioavailability across the skin-barrier. However, beclomethasone dipropionate is highly lipophilic and exhibits high bioavailability across the skin (Johansson *et al.*, 1982). It is therefore possible that individuals who do not demonstrate a skin response with such an agent may have some defect involving glucocorticoid-glucocorticoid receptor interaction.

7.5 EVIDENCETHATSKIN VASOCONSTRICTION IS MEDIATED VIA CLASSICAL (TYPE II) GLUCOCORTICOID RECEPTORS.

The systemic administration of a known glucocorticoid receptor blocking agent RU 38486, has been found to attenuate the skin blanching phenomenon (Gaillard *et al.*, 1985). The compound RU 38486 also blocks progesterone receptors (Kelly, 1992) but the application of progesterone to the skin does not result in blanching (Marks *et al.*, 1982). This suggested that the blanching phenomenon was the likely result of interaction of glucocorticoid with its receptor in skin. A negative skin response could, therefore, reflect some abnormality of glucocorticoid: glucocorticoid-receptor interaction i.e. glucocorticoid resistance.

It is noteworthy that route of administration of RU-38486 (and perhaps dose) can influence observed effects of this compound (Laue et al., 1989). Thus oral administration of RU-38486 failed to stimulate vasopressin release in monkeys, whereas intramuscular administration had produced such an effect. The inhibitory effect of systemic administration of this compound on skin vasoconstriction had previously been shown (Gaillard et al., 1985). The effect of topically applied RU-38486 on the blanching response was, therefore, tested.

RU-28362 is another synthetic glucocorticoid of high potency with negligible affinity for renal mineralocorticoid (Type I glucocorticoid) receptors (Morris and Souness, 1992; Tempel *et al.*, 1993). It was therefore also important to study the effect of this compound.

7.5a Methods:

The skin vasoconstrictor assay was conducted as described in Methodology 2 (Chapter 3). Beclomethasone dipropionate, $10 \mu g/ml$, was applied alone or was coapplied with RU-38486, $10\mu g/ml$, and the effect compared with RU-38486 applied alone. RU-28362, $10\mu g/ml$, was also applied alone.

7.5b Results:

Six initially positive responders were tested serially over three months at monthly intervals. These subjects retained the ability to exhibit a blanching response to becomethasone dipropionate. This pool of six subjects was used for the above tests.

Beclomethasone dipropionate 10 μ g/ml, produced intense blanching when applied alone. When, however, the glucocorticoid antagonist, RU 38486 (10 μ g/ml), was co-applied with 10 μ g/ml beclomethasone dipropionate solution, there was complete attenuation of the blanching response. RU-28362 produced intense blanching in all subjects tested.

It is unlikely that suppression of blanching arose out of impaired local bioavailability of beclomethasone dipropionate when co-applied with RU-38486 since similar findings have been previously obtained following systemic administration of RU-38486 (Gaillard *et al.*, 1985). RU-38486 applied on its own also showed no blanching response.

That blanching can be attenuated by a glucocorticoid receptor blocker supports the central role of the glucocorticoid receptor in this process and because blanching

occurs with RU-28362, a substance with little affinity for Type I glucocorticoid receptors, strongly supports that vasoconstriction is mediated via classic (Type II) glucocorticoid receptors.

A lack of cutaneous response to topically applied glucocorticoids might also therefore reflect insensitivity (resistance) to glucocorticoids; representing some defect in type II glucocorticoid receptor number or function.

7.6 SKIN VASOCONSTRICTOR AND ALDOSTERONE (TYPE I GLUCOCORTICOID) RECEPTORS.

Using the methods described above, aldosterone in concentrations of 10-100 μ g/ml produced no visible blanching in three subjects who were repeatedly responsive to beclomethasone dipropionate. This suggested that aldosterone receptors were not important in producing blanching. To further support this, other evidence was obtained.

Pseudohypoaldosteronism is a condition presenting in early childhood with salt wasting and failure to thrive and is characterized by high urinary levels of sodium despite hyponatraemia, hyperkalaemia and hyperreninemia occurring in the face of

elevated aldosterone levels indicating renal tubular insensitivity to mineralocorticoids (Dillon et al., 1980). Aldosterone receptor deficiency have been demonstrated in mononuclear leucocytes in patients with this syndrome (Armanini et al., 1985) suggesting that mineralocorticoid receptor deficiency is a generalized phenomenon in some patients. One such patient, an eighteen month old boy (A.D., a patient of Ninewell Hospital, Dundee) with clinical and biochemical features of this syndrome was tested for skin vasoconstrictor responses to beclomethasone dipropionate.

The application of beclomethasone dipropionate (100, 30, 10, 1 μ g/ml) to the skin of this boy resulted in blanching at all doses.

All of this evidence points to the lack of contribution of Type I glucocorticoid receptors to the blanching phenomenon.

7.7 CONCLUSIONS:

The blanching phenomenon represents a specific glucocorticoid receptormediated effect which from the current data is mediated via classical (Type 11) glucocorticoid receptors. Like previous workers, I was struck by the marked heterogeneity in responsiveness to topically applied glucocorticoids. There is a small subset of apparently normal individuals in a population who do not respond to topically applied glucocorticoid or those who will only respond when high topically doses are applied.

Variable skin sensitivity could reflect variable glucocorticoid sensitivity. It might therefore be possible to apply this principle to evaluate glucocorticoid sensitivity in other systems where responses to glucocorticoids have been otherwise defined e.g. glucocorticoid resistant and sensitive asthma.

CHAPTER 8

THE EFFECTS OF ACUTE SYSTEMIC GLUCOCORTICOID

EXPOSURE ON SKIN VASOCONSTRICTOR RESPONSIVENESS

CHAPTER 8 THE EFFECTS OF ACUTE SYSTEMIC GLUCOCORTICOID EXPOSURE ON SKIN VASOCONSTRICTOR RESPONSIVENESS

8.1 BACKGROUND

There is abundant data regarding the reproducibility of the skin vasoconstrictor assay (Barry and Woodford, 1976; Smith *et al.*, 1992). There are, however, no data on the behaviour of this assay in respect of systemic glucocorticoid exposure. Can systemic glucocorticoid exposure alter skin vasoconstrictor responsiveness to topically applied glucocorticoids? Since the blanching response correlates so closely with the anti-inflammatory actions of glucocorticoids it would be important to determine whether systemic exposure impacts on cutaneous responsiveness.

Preliminary data suggest that previous glucocorticoid exposure may lead to altered glucocorticoid sensitivity. This comes principally from the work of Du Vivier (Du Vivier and Stoughton, 1975; Du Vivier, 1976). Thus acute tolerance to the vasoconstrictor effect of topically applied glucocorticoid has been described (Du Vivier and Stoughton, 1975). A glucocorticoid applied topically will cause blanching within 6 to 8 hours. This vasoconstrictor response will then fade within the ensuing 24-48

hours. With repeated applications over several days the vasoconstrictor response becomes no longer elicitable. If left untreated for 72 to 96 hours, the vasoconstrictor response will once more become elicitable. When compared with other known effects of glucocorticoids on the skin, tolerance to the vasoconstrictor response was not an isolated finding; other biological effects of glucocorticoids were similarly affected. Thus it was demonstrated (Singh and Singh, 1986) that suppression of histamine induced erythema and wheal in skin by glucocorticoids underwent tachyphylaxis with continued glucocorticoid use. Similarly, the initial application of glucocorticoid to mouse skin leads to marked inhibition of epidermal mitosis. With repeated application of glucocorticoid, acute tolerance to its anti-mitotic effects can also be demonstrated (Du Vivier, 1976).

Evidence for the diminution of glucocorticoid sensitivity following systemic exposure also comes from other studies (Goulding *et al.*, 1989; and Podgorski *et al.*, 1992). In these studies, patients maintained on higher dosages of oral prednisolone (> 7.5 mg/day) for rheumatoid arthritis exhibited reduced sensitivity to subsequent glucocorticoid therapy. It was postulated that the generation of anti-lipocortin antibodies following oral administration of glucocorticoids might be responsible for such reduced responsiveness.

Glucocorticoid receptor levels in target tissues are subject to up-regulation and down-regulation (Smith and Shuster, 1984; Rosewicz et al., 1988). In most tissues there is an inverse relationship between hormone and glucocorticoid receptor levels (Reul et al., 1989; Frey and Frey, 1992). This might represent a possible explanation for the tachyphylaxis to topically applied glucocortcoids.

It is also possible that induction of enzyme/s that metabolize glucocorticoids might be relevant. There is data to implicate the latter mechanism in glucocorticoid tachyphylaxis. Thus B lymphocytes which metabolize cortisol more rapidly than T lymphocytes are less sensitive to the lytic effects of glucocortcoids (Frey and Frey, 1992). In Chapter 4 it was shown that 11ß-hydroxysteroid dehydrogenase enzyme activity in skin is inducible; reduced in adrenalectomized animals and normal (compared with sham-operated animals) once glucocorticoids are replaced. This would lend support to the notion that steroid enhanced metabolism might be a mechanism of such tachyphylaxis in the skin.

Whatever the mechanism it seems evident that variation in sensitivity might influence glucocorticoid toxicity since dosages are prescribed empirically without regard to such differences in individual sensitivity. Can systemic glucocorticoid exposure lead to an alteration in peripheral sensitivity to glucocorticoid in the manner that topical

therapy does? If this is the case then extinction of the vasoconstrictor response might serve as a guide to systemic glucocorticoid exposure. This study was designed to assess whether systemic exposure to low dose glucocorticoid altered the skin vasoconstrictor response. Since it was the eventual aim to study skin blanching in glucocorticoid resistant asthmatics, it was decided to carry out a detailed study in normal subjects of the effects of taking varying doses of inhaled glucocorticoid. Normal subjects rather than asthmatic patients were chosen to eliminate the possible effects of intercurrent or previous glucocorticoid therapy.

8.2 SKIN VASOCONSTRICTOR RESPONSES FOLLOWING SYSTEMIC EXPOSURE TO GLUCOCORTICOIDS

8.2a Methods:

Sixteen subjects who were initially found to be positive responders were followed prospectively for six months. Over this period all sixteen subjects were exposed in random order to be clomethas one dipropionate administered via standard metered-dose inhaler in dosages of 0 (placebo), 400, 800, 1400 and 2000 μ g/day in four divided doses for ten days. Wash-out intervals of 18 days separated treatments which were assigned in random order using double-blind approach in a protocol summarized in Figures 8.1 and 8.2.

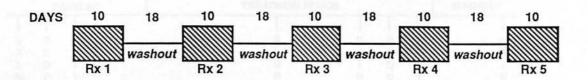


Figure 8.1: The design of experiment to determine the effects of varying doses of beclomethasone dipropionate.

Figure 8.2: The sequence of tests to evaluate the systemic effects of BDP

Sand Jens Charger 2

Skin sensitivity to topically applied beclomethasone dipropionate (10, 3, 1, 0.1 μ g/ml test solutions) was determined after 10 days of treatment at each dose as described in Methodology 2, (Chapter 3). Test substances were applied on the ninth day of treatment and readings were taken the following day after 16 h of occlusion. The presence or absence of blanching at each topically applied dose of beclomethasone was first ascertained and then graded on a four-point scale as previously described in Chapter 3.

It was important to ascertain, using other parameters, the degree of systemic glucocorticoid effect induced by each treatment. Because the time-course of systemic glucocorticoid effects are tissue-dependent (Axelrod, 1989) it was necessary to test a variety of glucocorticoid end-points. The following were therefore also tested; circulating neutrophils, bone metabolism, and intermediary metabolism. Methods of measurement of all these parameters are outlined in Chapter 2. Testing the hypothalamic-pituitary-adrenal axis is the traditional yardstick of both the degree of systemic glucocorticoid exposure and whether there is recovery from chronic treatment with such (Harrison et al., 1982; Byyny, 1976; Feek et al., 1981). The hypothalamic-pituitary-adrenal axis across each treatment period was therefore also tested.

0900 h plasma cortisol has been used to indicate basal hypothalamic-pituitaryadrenal function (Mygind and Hansen, 1973; Sherman et al., 1982; Bisgaard et al., 1988). Likewise, 24h urinary free cortisol has also been used to test basal hypothalamicpituitary-adrenal function (Prahl et al., 1987, Smith and Hodson, 1983). Dynamic testing of this axis would provide a more sensitive index of systemic glucocorticoid exposure. While there are a number of ways of evaluating the hypothalamic-pituitaryadrenal axis by dynamic tests (Feek et al., 1981; Harrison et al., 1982; Cunningham 1983), the short metyrapone test (Dickstein et al., 1986, 1991) represent a simple, convenient and sensitive way to do this (Feek et al., 1981; Holt et al., 1990). The short metyrapone test was performed according to the method of Dickstein (Dickstein et al., 1986). Briefly, metyrapone 1500 mg was administered with 300 ml milk at 0600h and blood taken at 0800h, 1200h and 1400h. Plasma samples were analysed for cortisol and 11-deoxycortisol by radioimmunoassay. Plasma cortisol at 0800h established the efficacy of the block to cortisol biosynthesis. The mean value of 11-deoxycortisol obtained at 1200 and 1400h was taken as a measure of pituitary-adrenal responsiveness.

This study had the approval of the Lothian Region Ethics Committee and was conducted in accordance with the Declaration of Helsinki (Appendix I) as to guiding

medical doctors in research involving drug treatment of human subjects.

8.2b Statistical analysis:

The effects of each treatment was analyzed by three way analysis of variance (subject, dose and phase). Least squares estimates of effects relative to placebo were obtained with their standard error. The hypothesis of no treatment effect was tested by t-tests at each dose and significance set at the 5% limit.

The proportion of patients demonstrating a positive response to various doses of topical beclomethasone dipropionate was compared across each treatment of systemically administered dose by chi-squared tests. Significance was also set at the 5% limit.

8.2c Results

(i) Skin vasoconstrictor responses:

The mean blanching score produced by application of 10, 3, 1 and 0.1 μ g/ml of beclomethasone dipropionate to the skin of sixteen positive responders were similar for placebo and all doses of inhaled beclomethasone dipropionate administered to the airways (Figure 8.3). Similarly, the number of individuals having a positive blanching

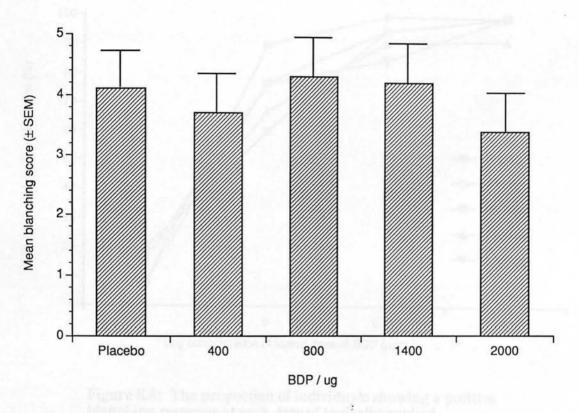


Figure 8.3: The mean blanching scores after placebo and following treatment with varying doses of beclomethasone dipropionate (BDP). Error bars represent SEM, n=16.

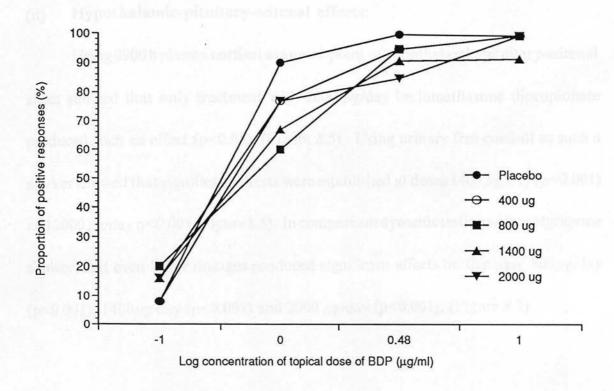


Figure 8.4: The proportion of individuals showing a positive blanching response at each dose of topically applied beclomethasone dipropionate after placebo treatment and varying doses of inhaled beclomethasone dipropionate (BDP).

response at each of these dosages (Figure 8.4) did not differ significantly irrespective of the systemically administered dose.

(ii) Hypothalamic-pituitary-adrenal effects:

Using 0900 h plasma cortisol as an end-point of hypothalamic-pituitary-adrenal effect showed that only treatment with 2000 μ g/day beclomethasone dipropionate produced such an effect (p<0.05), (Figure 8.5). Using urinary free cortisol as such a marker showed that significant effects were established at doses 1400 μ g/day (p<0.001) and 2000 μ g/day p<0.001 (Figure 8.6). In comparison dynamic testing with metyrapone showed that even lower dosages produced significant effects on this axis: 800 μ g/day (p<0.001), 1400 μ g/day (p<0.001) and 2000 μ g/day (p<0.001), (Figure 8.7).

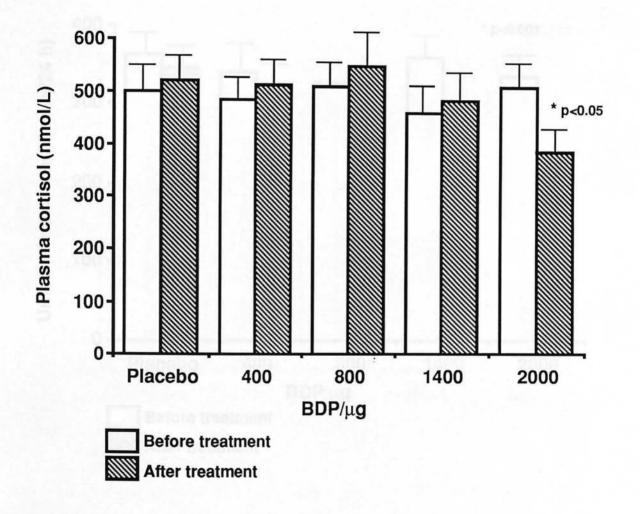


Figure 8.5: Effects of varying doses of beclomethasone dipropionate (BDP) on the hypothalamic-pituitary-adrenal axis - 0900h PLASMA CORTISOL.

Results shown as mean \pm SEM, n=16.

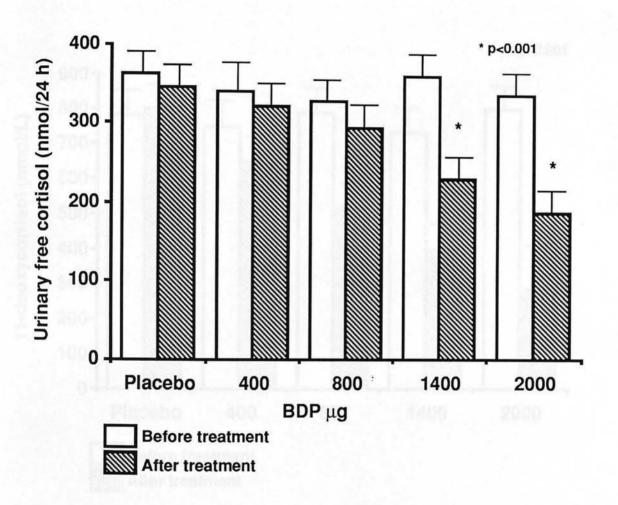


Figure 8.6: Effects of varying doses of beclomethasone dipropionate (BDP) on hypothalamic-pituitary-adrenal axis - 24h urinary free cortisol.

Results shown as mean \pm SEM, n=16.

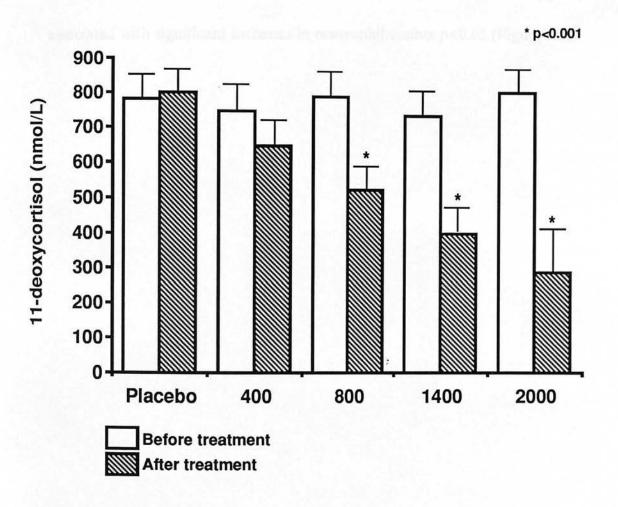


Figure 8.7: Effects of varying doses of beclomethasone dipropionate (BDP) on the hypothalamic-pituitary-adrenal axis - 11-deoxycortisol response to metyrapone.

Results shown as mean \pm SEM, n=16.

(iii) Circulating neutrophils

Glucocorticoid exposure is known to induce changes in circulating leucocytes (Fauci *et al.*, 1976). In the current experiment, doses greater than 800 μ g/day were associated with significant increases in neutrophil counts p<0.05 (Figure 8.8).

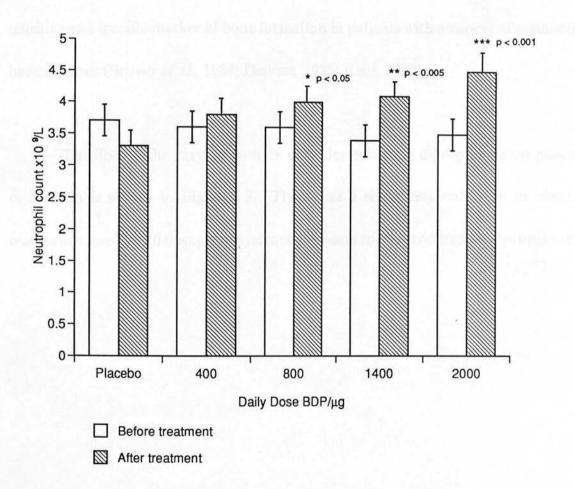


Fig. 8.8: Effects of varying doses of beclomethasone dipropionate (BDP) on circulating neutrophils.

Results shown as mean \pm SEM, n=16.

(iv) Bone metabolism:

Glucocorticoids have many effects on bone metabolism (Baylink, 1983; Reid, 1989) and osteoporosis is a frequent complication of glucocorticoid therapy irrespective of the disease being treated (Adinoff and Hollister, 1983). Plasma osteocalcin is a sensitive and specific marker of bone formation in patients with a variety of metabolic bone diseases (Brown *et al.*, 1984; Delmas, 1985; Reid, 1989).

The effect of the varying dosages of beclomethasone dipropionate on plasma osteocalcin is shown in Figure 8.9. There was a significant reduction in plasma osteocalcin levels at all dosages of systemic exposure to beclomethasone dipropionate.

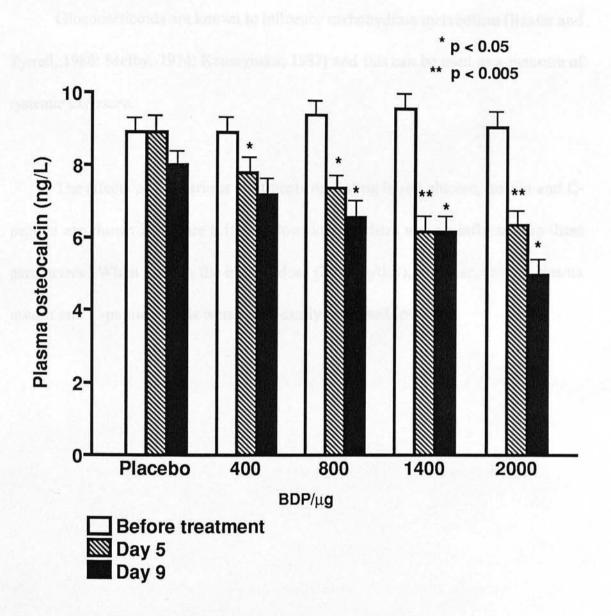


Figure 8.9: Effects of varying doses of beclomethasone dipropionate (BDP) on plasma osteocalcin after 5 days (SS) and 9 days (SS) of treatment.

Results shown as mean \pm SEM, n=16.

(vi) Intermediary metabolism:

Glucocorticoids are known to influence carbohydrate metabolism (Baxter and Tyrrell, 1986; Melby, 1974; Kruszynska, 1987) and this can be used as a measure of systemic exposure.

The effects of the various treatments on fasting blood glucose, insulin and C-peptide are shown in Figure 8.10. At low dosages there was no influence on these parameters. When used at the highest dose (2000 μ g/day), however, fasting plasma insulin and C-peptide levels were significantly increased (p<0.05).



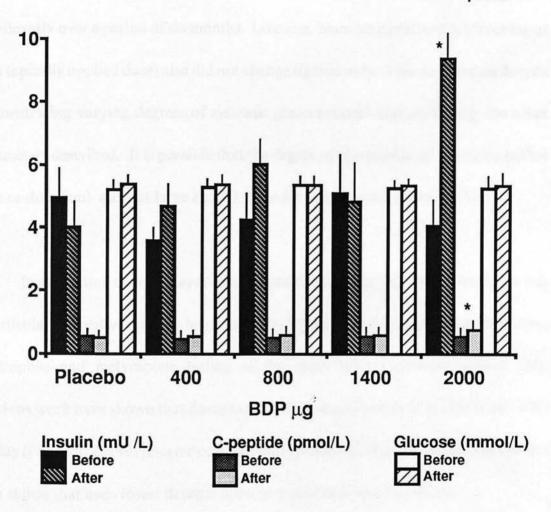


Figure 8.10: Effects of varying doses of beclomethasone dipropionate (BDP) on glucose homeostasis.

Results shown as mean \pm SEM, n=16.

8.3 CONCLUSIONS:

Mean blanching score in a cohort of positive responders did not change significantly over a period of six months. Likewise, blanching profiles (% blanching at each topically applied dose) also did not change significantly. This was the case despite demonstrating varying degrees of systemic glucocorticoid exposure using the other parameters described. It is possible that the degree of glucocorticoid exposure (either dose or duration) was not large enough to induce such a change in the skin.

In evaluating degree of systemic glucocorticoid effects, plasma osteocalcin was a particularly sensitive marker; being more readily affected than changes in circulating neutrophils and metyrapone testing of the hypothalamic-pituitary-adrenal axis. Previous work have shown that dosages of $1000 \,\mu\text{g/day}$ (Meeran *et al.*, 1991) and 2000 $\mu\text{g/day}$ (Pouw *et al.*, 1991) can reduce circulating plasma osteocalcin levels. The current data shows that even lower dosages are also associated with this effect.

Despite detecting changes in the mentioned variables, there was no apparent change in skin vasoconstrictor responsiveness at any dosage of treatment with

beclomethasone dipropionate compared with placebo treatment. Short term but significant systemic exposure to glucocorticoids therefore does not appear to affect skin vasoconstrictor responses.

This is the first time that the skin vasoconstrictor response has been compared systematically with other end-points of systemic glucocorticoid effect. Although 10-day systemic exposure was not associated with change in skin vasoconstrictor responsiveness, it is important to determine whether changes in skin vasoconstrictor responsiveness do occur after more prolonged or higher dose systemic exposure.

For obvious reasons it is difficult to carry out these studies in normal subjects. It would be appropriate to carefully evaluate the skin vasoconstrictor response in patients treated chronically with glucocorticoid therapy especially if these patients could be evaluated for other parameters indicative of systemic glucocorticoid sensitivity. In this way correlation between skin responsiveness and systemic glucocorticoid sensitivity could be established. Glucocorticoid sensitivity in bronchial asthma provides the best defined and most available model in which to pursue this.

CHAPTER 9

TESTING SKIN VASOCONSTRICTOR RESPONSES IN GLUCOCORTICOID-RESISTANT ASTHMA

CHAPTER 9: TESTING SKIN VASOCONSTRICTOR RESPONSES IN GLUCOCORTICOID RESISTANT ASTHMA

9.1 GLUCOCORTICOID RESISTANT ASTHMA

The phenomenon of glucocorticoid resistance has probably been best studied in asthmatics. Schwartz demonstrated a decreased eosinopenic response to cortisol and an accelerated plasma cortisol clearance in patients who were clinically resistant to cortisol's anti-asthmatic effect (Schwartz et al., 1968). Further study of severe steroid-dependent asthmatics (Rose et al., 1980) suggested that altered binding, distribution and clearance of prednisolone were not responsible for the large requirement of these patients. On the basis of this finding Rose deduced that differences in severity or pathophysiology or that some intrinsic difference in glucocorticoid receptor sensitivity might be operational (Rose et al., 1980).

Carmichael, continuing this theme studied the clinical characteristics of patients defined as either sensitive or resistant to glucocorticoids after therapeutic trials (Carmichael et al., 1981). It was found that patients who responded poorly had suffered

longer, had more severe airways disease and had a family history of asthma more commonly. In further studies of these patients, it was also found that there was an apparent inability of oral prednisolone to reduce the level of monocyte complement receptors in resistant compared to sensitive patients (Carmichael et al., 1981; Kay et al., 1981). A further series of in vitro studies demonstrated that colony growth of mononuclear cells from glucocorticoid-resistant asthmatics was less susceptible to inhibition with glucocorticoids when compared with glucocorticoid-sensitive patients (Poznansky et al., 1984, 1985). More recently, monocytes from asthmatics have been shown to release a cytokine which primes neutrophils and eosinophils for enhanced leukotriene generation when subsequently stimulated by ionophore. In glucocorticoid sensitive asthmatics low concentrations of hydrocortisone completely inhibited the production of this cytokine whereas 1000-fold higher concentrations did not suppress it in glucocorticoid-resistant patients (Wilkinson et al., 1989, 1990).

Even more recently, the role of anti-lipocortin-1 antibodies has also been invoked as a possible explanation for glucocorticoid resistance. Many of the anti-inflammatory action of glucocorticoids are mediated by lipocortins and the presence of antibodies to lipocortin-1 has been associated with lack of effect of glucocorticoids in rheumatoid patients (Podgorski *et al.*, 1992). When however, the presence of anti-

lipocortin antibodies in glucocorticoid-resistant asthmatics was investigated (Wilkinson et al., 1990) the findings were inconclusive (Busse, 1990).

As previously described, the skin vasoconstrictor response is glucocorticoid-mediated. It was hypothesized that it might be possible to distinguish between a specific lung glucocorticoid resistance and a more generalised defect, by testing whether there was also abnormal responsiveness in the skin of patients whose disease was resistant to glucocorticoid therapy. Glucocorticoid-sensitivity in bronchial asthma provided the best clinically defined and the most available model in which to test this hypothesis.

The aim of this experiment was therefore to determine whether skin responsiveness to topically applied glucocorticoid could indicate clinical sensitivity of asthma to systemic glucocorticoid therapy.

9.2 METHODS

9.2a Criteria for selection of groups:

Asthmatic patients were drawn from the outpatients' department at the Northern General Hospital, Edinburgh. Informed consent was obtained from all patients and the

study had the approval of the Local Ethical Committee. All patients had a typical history of variable dyspnoea and wheeze. To be included these patients must have demonstrated > 20% diurnal variation in peak expiratory flow as well as > 15% improvement in peak expiratory flow or forced expired volume in 1 second (with minimum improvement of 200 ml) after exposure to inhaled B_2 -agonists (modifications of previously detailed criteria, Carmichael *et al.*, 1981).

Patients were classified according to the following sub-groups:

- a. Mild asthmatics, n = 26. These patients had few symptoms and relatively normal lung function. Some of these were on inhaled B2-agonists alone while others were on, in addition, inhaled glucocorticoids (up to 2000 μ g of either beclomethasone dipropionate or budesonide daily). This group was to serve as a control for possible influences of inhaled B_2 -agonist or inhaled glucocorticoid.
- b. Asthmatics on long term systemic glucocorticoids, n = 13. Median dose of prednisolone was 7.5 mg daily, with a median duration of treatment 10 years.
 These patients had mild airways obstruction and were taking long-term prednisolone. Most were suffering from allergic bronchopulmonary aspergillosis. This group was to serve as control for the possible effect of long

term oral glucocorticoid therapy.

- c. Asthmatics who were glucocorticoid-sensitive, n=31. All were on regular inhaled glucocorticoids, range 400-2000 μ g/day, median dose was 800 μ g/day. Four of these patients were also taking oral prednisolone (5-10 mg daily), all for less than 5 years.
- d. Asthmatics who were glucocorticoid-resistant, n = 15. All were on regular inhaled glucocorticoids, range 400- 2000 μ g/day. 8 were also on oral prednisolone, median 7.5 daily (range 5-10) mg with, median duration of treatment 8 years.

The latter two groups (c and d) were defined according to the following criteria (modification of previously used criteria, Carmichael *et al.*, 1981). Asthmatics with significant airways obstruction (Forced expired volume in 1 second < 70% predicted for age and sex) seen at the outpatients' clinic underwent a two-week trial of oral prednisolone to determine whether their asthma was responsive to glucocorticoid. Before commencing the glucocorticoid-trial, peak expiratory flow rate was measured three times a day for a week while the patient continued whatever treatment that was

already being used. Patients were then given oral prednisolone 20 mg daily for one week, followed by 40 mg daily for a second week. Baseline forced expired volume was measured prior to commencing treatment and this was repeated at the end of each treatment week. The patient continued to monitor peak expiratory flow throughout treatment. Mean peak expiratory flow during the last two days of the pre-treatment week was compared with that of the last two days of each treatment week.

Patients were deemed glucocorticoid resistant if there was failure of initial forced expiratory volume in 1 second and mean peak expiratory flow to improve by at least 15 % after two weeks of treatment.

For all groups the following were also noted: atopy, smoking history, family history of asthma, duration of asthma symptoms and highest achieved forced expiratory volume in the preceding two years. Atopy was defined as two or more reactions with a weal diameter more than 2 mm greater than the control test when skin-prick tests to a range of common allergens including house-dust mite, pollen, animal danders and Aspergillus fumigatus were performed. These data were usually extracted from the case notes and are summarized in Table 9.1.

Missin-V	п	*Age (Range)	Sex (M/F)	*FEV ₁ % predicted (CI)	Current smokers(%)	No. atopic (%)	+ve family history (%)	*Years with asthma (range)
Mild asthma (inhaled b_2 agonist alone)	12	43 (21-63)	7//5	94 (87.3-100.7)	1 (8.3)	8 (66.7)	5 (41.7)	11.7 (1-50)
Mild asthma (inhaled steroid)	14	44.1 (19-72)	4/10	91.1 (81.7-100.5)	1(7.)	10 (71.4)	8 (57.1)	13.2 (2-30)
Asthma - long term Prednisolone	13	54.7 (29-71)	8/9	77 (57.5-96.5)	3 (23.1)	9 (69.2)	4 (30.8)	29.8 (8-55)
Asthma-corticosteroidsensitive	31	49.1 (18-69)	15/16	81.7 (76.7-86.7)	8 (25.8)	25 (80.6)	14 (45.2)	23.2 (1-54)
Asthma-corticosteroidresistant	15	45.6 (27-66)	2/8	53.7 (43.7-63.7)	4 (26.7)	11 (73.3)	7 (46.7)	32.9 (3-57)

* Mean values (CI) 95% confidence intervals

9.2b Skin vasoconstrictor bioassay

This was performed as described in Methodology 2 (Chapter 3). Beclomethasone dipropionate was dissolved in 95% ethanol to concentrations between 3 and 100 μ g/ml. Test solutions were applied in random order to the forearm of subjects whose asthma responsiveness was unknown to the skin tester.

Blanching responses were evaluated qualitatively (i.e presence or absence of blanching) as well as quantitatively (scored 0 - 4) on the visual analogue scale, at each concentration of topically applied beclomethasone dipropionate after 16 hours of occlusion.

9.2c Statistical analysis

Chi-square tests with Yates' correction were used to compare groups for differences with respect to atopy, smoking, family history and proportion showing blanching responses at different doses of topical beclomethasone dipropionate. Two-sample t-tests were used for comparison of lung function and duration of asthma. Mann-Whitney U tests were used for comparing intensity of blanching responses among

the groups.

9.2d RESULTS:

All groups were fairly well-matched for age, sex, smoking, atopy and family history of asthma (Table 9.1). The duration of symptoms in the mild asthma group was, however, somewhat shorter and there was a smaller proportion of smokers in this group.

Mean FEV₁% predicted was slightly reduced in glucocorticoid-sensitive (CS) asthmatics (82%) and those taking long term prednisolone (77%). Airway obstruction was marked in glucocorticoid-resistant (CR) asthmatics (mean 54% predicted, range 20 - 80%) and significantly greater (p<0.001) than in those patients who were corticosteroid sensitive.

At the start of the trial of corticosteroids, mean FEV₁% predicted was 50% (95% confidence intervals (CI) 44 - 56%) in CS asthmatics and 41% (95% CI 31 - 51%) in CR patients. Following the trial, FEV₁% predicted improved to 73% (95% CI 65 - 80%) in CS but was unchanged (39%, 95% CI 29 - 49%) in CR asthmatics.

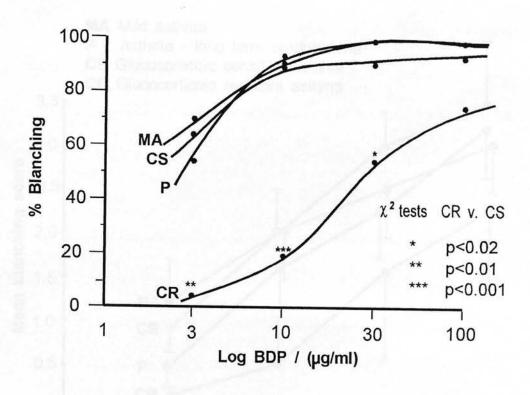
There were no significant differences between the patient groups with respect to

atopy, smoking or family history of asthma (Table 9.1). The duration of symptoms in the mild asthmatics was shorter (mean 12.3y, 95% CI, 7.6-17y) than in the other groups (p<0.01). There were no significant differences between these groups though a trend (p=0.063) towards longer duration of asthma was observed in CR patients (mean 32.9y, 95% CI 24.9-40.9y) compared with CS patients (mean 23.2y, 95% CI 16.5-29.5y). However, within the latter group those who required two weeks of prednisolone to show improved airway calibre (n=10) had suffered from asthma longer (mean 33y, 95% CI 18.5-47.5y) than those who responded (n=21) after one week (mean 18.5y, 95% CI 12.2-24.8y; p<0.05).

Figure 9.1 depicts the proportion of individuals from each group showing a positive blanching response to cutaneous beclomethasone dipropionate at any given concentration. Patients with glucocorticoid resistant asthma showed blanching that was significantly lower at all concentrations of beclomethasone dipropionate (p<0.02 at 30 μ g/ml, p<0.001 at 10 μ g/ml, p<0.01 at 3 μ g/ml) except 100 μ g/ml. The blanching curves for mild asthmatics, glucocorticoid sensitive and the prednisolone groups were quite similar.

Figure 9.2 illustrates the intensity of blanching at each cutaneous concentration

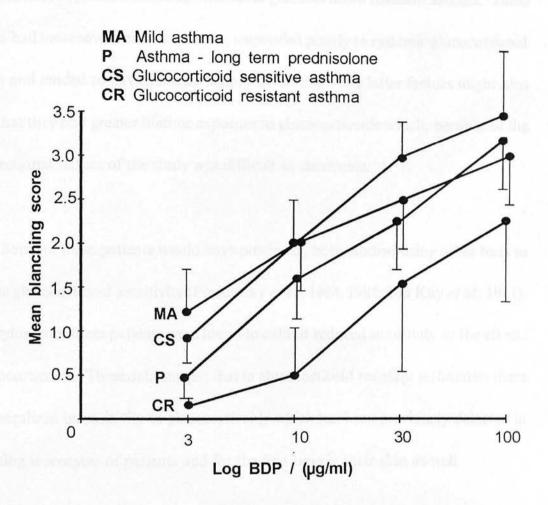
in the different groups. There was a linear relationship between mean blanching score and topical dose of glucocorticoid. Compared with the glucocorticoid sensitive group the dose-response relationship for the glucocorticoid resistant group was shifted markedly to the right with significantly reduced blanching scores at all doses (p<0.001 at 3 μ g/ml, p<0.001 at 10 μ g/ml, p<0.05 at 30 μ g/ml) except 100 μ g/ml. The dose-response relationships for glucocorticoid responsive and prednisolone treated patients were very similar with no significant difference in blanching scores at any dose. Likewise there was no significant differences in the response between mild asthmatics and glucocorticoid responsive patients. Interestingly, the dose-response curve for the prednisolone treated group was also shifted to the right compared with mild asthmatics but this shift only achieved significance at 30 μ g/ml point (p<0.01).



MA Mild asthma
P Asthma - long term prednisolone
CS Glucocorticoid sensitive asthma
CR Glucocorticoid resistant asthma

Fig 9.1:

Proportion of asthmatics showing any degree of blanching to beclomethasone dipropionate (BDP).



Error bars indicate 95% confidence intervals.

Fig 9.2 :
Intensity of Cutaneous Vasoconstrictor Response to Beclomethasone Dipropionate (BDP) in Asthmatics

9.3 CONCLUSIONS

The skin vasoconstrictor response was reduced (both qualitatively and quantitatively) in patients fulfilling criteria for glucocorticoid resistant asthma. These patients had more severe airways disease, responded poorly to systemic glucocorticoid therapy and tended to have a longer history of asthma. The latter factors might also imply that they had greater lifetime exposure to glucocorticoids which, because of the cross-sectional nature of the study was difficult to determine.

Some of these patients would have previously been studied using other tests to evaluate glucocorticoid sensitivity (Poznansky *et al.*, 1984, 1985; and Kay *et al.*, 1981). Leucocytes from these patients were found to exhibit reduced sensitivity to the effects of glucocorticoids. These data suggest that in glucocorticoid resistant asthmatics there is a generalized insensitivity to glucocorticoids which has been previously detected in circulating leucocytes of patients and for the first time in their skin as well.

There was some indication that the prolonged use of oral prednisolone might have attenuated skin vasoconstrictor responsiveness. Thus, there was an apparent shift of the blanching curve to the right i.e. reduced skin sensitivity in asthmatics on long term prednisolone compared with mild asthmatics. This was however, only a trend as

the differences between those on prednisolone and mild asthmatics were not uniformly significant. Indeed, there is apparent ordering of the blanching intensity curves for mild asthma, glucocorticoid sensitive, prednisolone-treated and glucocorticoid resistant asthmatics from left to right. This is interesting, since previous glucocorticoid use has also been associated with reduced clinical responsiveness in other settings. Thus it has been shown that previous therapy with glucocorticoids was associated with reduced clinical and leucocyte responsiveness in rheumatoid patients (Podgorski *et al.*, 1992). Even more supportive of this hypothesis is the findings from a study by Linder (Linder *et al.*, 1993) who upon my suggestion tested skin vasoconstrictor responsiveness in various groups of elderly patients. These workers found that compared with healthy elderly, age-matched patients receiving prednisolone for up to one year exhibited reduced blanching responses.

The skin vasoconstrictor response may be used to predict systemic sensitivity to glucocorticoid therapy. It would be important to test this hypothesis in other situations of glucocorticoid resistance eg. familial glucocorticoid resistance syndromes (Chrousos et al., 1982). Apparent glucocorticoid resistance has also been described in depression (Whalley et al., 1986) and anorexia nervosa (Kontula et al., 1982). It would be interesting to test skin sensitivity in these clinical situations.

Whereas the cause of reduced skin responsiveness (and indeed reduced asthma responsiveness) to glucocorticoids remain speculative, the current data and that of Linder *et al.*, (1993) suggests that previous systemic glucocorticoid exposure might be relevant.

CHAPTER 10: CONCLUSIONS AND FURTHER WORK

at both pharmacological and biochemical levels. Most importantly, a number of cutaneous diseases, both inflammatory and non-inflammatory, is amenable to treatment with glucocorticoids, the introduction of which revolutionized the practice of dermatology (Stoughton and Cornell, 1988). Secondly, skin blanching, the pharmacodynamic response to topically applied glucocorticoids, has been utilized for over thirty years to predict efficacy (and potency) of new topical glucocorticoids. Finally, the ability of the skin to metabolise cortisol has been fairly well documented (Malkinson *et al.*, 1959; Hsia *et al.*, 1964; Hammami and Siiteri, 1991) even though the biological significance of this metabolic process is speculative. In this work I have explored the latter two facets of the interaction between glucocorticoids and the skin.

METABOLISM OF CORTISOL BY SKIN:

It is known that the metabolic fate of cortisol can be diverse including products of both A-ring reduction as well as side chain oxidation (Bodor, 1988), but C-11\(\beta\)-dehydrogenation has been shown to be most crucial (Brownie, 1992). The latter process is mediated by 11\(\beta\)-hydroxysteroid dehydrogenase (Monder and Shackleton,

1984), an enzyme with vital physiologic functions in many tissues (Monder and White, 1992). In skin 11ß-hydroxysteroid dehydrogenase is not only active but is also the dominant metabolic pathway (Hsia et al., 1964) for cortisol. The spin-offs from this aspect of the current work are two-fold. Firstly, an animal model suitable to the study of this enzyme in skin has been developed eliminating the technical and other practical difficulties inherent in studies with human skin. Using this model, it might therefore now be possible to study the biological impact of this enzyme upon skin integrity. The use of inhibitors and enhancers, for example, might well illustrate such effects. Glycyrrhetinic acid, for example, was shown to be an effective inhibitor of 11ß-hydroxysteroid dehydrogenase activity both in vivo (Chapter 5) and in vitro (Chapters 5, 6,) I have also derived data (not shown) that 11B-hydroxysteroid activity is enhanced, in vitro, in the presence of dithranol (1,8-dihydroxy-9[10H]-anthracenone), a compound known to be efficacious in psoriasis. Important lessons might be learnt from study of inhibition or enhancement of enzyme activity using glycyrrhetinic acid or dithranol in the animal mode.

There are preliminary data from a number of quarters which suggest that altered enzyme activity / change in glucocorticoid flux might be relevant to the pathogenesis of skin disease. To begin with, "stress" is a frequent precipitant of many dermatoses

(Fitzpatrick et al., 1987). Stress is known to activate the hypothalamic-pituitary-adrenal axis, the consequences of which have been a source of great debate (Selye, 1946; Ingle, 1952; Schayer, 1964, 1967; Munck et al., 1984). More recently, Walker (Walker et al., 1992 a) added a further dimension to this when it was demonstrated that there was defective 11ß-hydroxysteroid dehydrogenase (11ß HSD) activity in clinical situations associated with ACTH excess. Thus, during stress these effects on the hypothalmicpituitary- adrenal-cortisol-11B HSD axis could possibly influence terminal metabolism of cortisol and impact upon pathogenesis of skin disease. The following points would support such a view. Alcohol, for example, is a known risk factor for psoriasis (Poikolainen et al., 1990) and alcohol was shown in this work (Chapter 6) to inhibit skin 11B-hydroxysteroid dehydrogenase. Likewise, the hypothesis of alteration in skin 11ß-hydroxysteroid dehydrogenase activity in dermatoses would be supported by the findings of Buhles (Buhles et al., 1987) which revealed that despite normal circulating cortisol levels, urinary metabolites of cortisol was markedly diminished in untreated patients with eczema and psoriasis compared with normal individuals. As a preliminary step in testing this hypothesis I evaluated psoriatic and eczematous skin for 11ß-hydroxysteroid dehydrogenase using immunohistochemistry as described for normal skin in Chapter 6. It was found that because of the increased thickness of the epidermis in both eczema and psoriasis, that immunostaining for 11ß-hydroxysteroid

dehydrogenase appeared increased (data not shown). This may not be contrary to the foregoing, however, since bioactivity and not immunoactivity in the diseased state is what might prove relevant. It is possible that the enhanced immunostaining in disease states could represent the product of variant mRNA species without the potential to produce active 11ß-hydroxysteroid dehydrogenase, a situation known to exist in other organ systems (Brown *et al.*, 1993). The current work provides the basis for further exploration of the role of skin 11ß-hydroxysteroid dehydrogenase for which the nude mouse model seems appropriate.

An even more significant consequence of the current work was the finding of potentially novel means of targeting glucocorticoid therapy. It was shown that by combining hydrocortisone, an intrinsically weak glucocorticoid, with glycyrrhetinic acid the action of hydrocortisone was enhanced in producing skin blanching, the classic bioassay of glucocorticoid efficacy. The ideal drug should have a high therapeutic index i.e. one with a low risk of adverse effects compared with any given desired effect (Bodor, 1988). A major objective of topical glucocorticoid therapy has been to enhance therapeutic activity but the use of high potency agents have led to a concomitant risk of systemic toxicity (Editorial Lancet, 1973, 1977). Indeed, hypothalamic-pituitary-adrenal axis suppression has been shown to occur with most topical

glucocorticoids in current use and to be proportional to the potency of the formulation (Stoughton and Cornell, 1988). Such effects have not been described with topical hydrocortisone (Turpeinen, 1988) except in the isolated group of the very young with severe and extensive disease (Turpeinen et al., 1986). On the whole, the systemic absorption of hydrocortisone from human skin is low (Wester et al., 1983). The increased biologic effect observed when hydrocortisone is co-applied with glycyrrhetinic acid could be due to enhanced topical bioavailability but Walker (Walker et al., 1992 c, 1993) has demonstrated similar enhancement of hydrocortisone action even after systemic administration of liquorice (active principle of which is glycyrrhetinic acid). Nonetheless, increased skin blanching implies increased access to the dermal vasculature (Barry and Woodford, 1978) and perhaps the systemic circulation but given the weak intrinsic activity of hydrocortisone and its short half-life it seems plausible that this particular combination might prove to be associated with less risk than currently available potent agents. This principle of local inhibition of cortisol to derive an enhanced site specific action might also be applicable to other sites where 11ßhydroxysteroid dehydrogenase is active, for example, tracheobronchial tree and gastrointestinal tract. The nude mouse would be an attractive model to test whether such local inhibition of cortisol metabolism is indeed devoid of systemic effects. Du Vivier (Du Vivier et al., 1978) had previously used this model for this purpose using epidermal DNA synthesis remote from the area of application as an end-point to evaluate systemic effects.

Finally, study of skin 11ß-hydroxysteroid dehydrogenase in the nude mouse model indicates biochemical characteristics which suggest that a distinct isoform exists in skin. This would explain why patients with deficient renal 11ß-hydroxysteroid dehydrogenase do not exhibit skin signs.

11B-hydroxysteroid dehydrogenase: Protector of the glucocorticoid receptor?

There is strong association between the anti-inflammatory activity of glucocorticoids which results from gene activation (Blackwell *et al.*, 1982; Tsurufuji and Ohuchi, 1989) and blanching. In addition, it usually takes about 6 hours before blanching becomes manifest. Such a definite time lag is generally seen in whole animal experiments for the manifestations of anti-inflammatory effects of glucocorticoids (Tsurufuji and Ohuchi, 1989). Moreover, blanching is inducible by active glucocorticoids, blocked by glucocorticoid antagonists (Marks *et al.*, 1982; Gaillard *et al.*, 1985), and from the current work, arises when glucocorticoids interact with classical (type II) glucocorticoid receptors. Thus the skin blanching response most likely depends upon receptor activation as well as transcriptional and translational events presumably

arising in the blood vessels of skin. That the concomitant use of glycyrrhetinic acid, an inhibitor of 11ß-hydroxysteroid dehydrogenase, led to an enhanced blanching response induced by hydrocortisone suggests that 11B-hydroxysteroid dehydrogenase might normally act to protect the glucocorticoid receptor from cortisol exposure, thereby limiting glucocorticoid responsive gene activation. There are data from a number of other sources to support the conclusions from the current work that 11B-hydroxysteroid dehydrogenase might be a protector of the glucocorticoid receptor. The wide tissue distribution of this enzyme has long invoked such a teleological argument (Monder and Shackleton, 1984; Monder and White, 1992). From experimental studies too, there are supportive data. Thus, glucocorticoids are known to adversely affect testicular function (Phillips et al., 1989). Ontogenic studies in the rat have suggested that 11ß-hydroxysteroid dehydrogenase of Leydig cells protects against the deleterious effects of glucocorticoid excess in the testis, an organ where glucocorticoid receptors abound (Phillips et al., 1989). Similarly, if the foetus is exposed to glucocorticoid excess, there are adverse influences on birth-weight (Reinisch et al., 1978). The foetus is normally protected from the exponential increases in maternal glucocorticoid levels as pregnancy progresses (Burke and Roulet, 1970; Rees et al., 1975) by the placental 11ß-hydroxysteroid dehydrogenase barrier. When this glucocorticoid barrier is lacking e.g by administering dexamethasone (which is not metabolized by 11ß-hydroxysteroid

dehydrogenase) offspring with low birth-weight are produced (Benediktsson et al., 1993; Similarly, the findings in the colon, also a site of Edwards et al., 1993). 11ß-hydroxysteroid dehydrogenase activity, that glucocorticoid responsive gene expression could be derepressed by liquorice derivatives (Fuller and Verity, 1990) provides more supportive evidence for the protective role of this enzyme over the glucocorticoid receptor. In addition, the halogenation of the C-9-α position of cortisol is known to produce agents resistant to the metabolic action of 11B-hydroxysteroid dehydrogenase (Oelkers et al., 1994). When cortisol is converted to 9-α-flurocortisol it has been found that not only is its mineralocorticoid action potentiated (due to increased type I receptor activation) but that in addition there is a 10-fold increase of glucocorticoid (anti-inflammatory) action (Szefler, 1989; Oelkers et al., 1994) providing further proof for the influence of this enzyme on glucocorticoid-mediated effects. Moreover, a detailed localization study of 11ß-hydroxysteroid dehydrogenase gene expression (and enzyme activity) in various rat tissues and the presence of glucocorticoid receptors in those tissues revealed close co-localization (Whorwood et al., 1992).

Thus the conclusion from the current work that 11ß-hydroxysteroid dehydrogenase might influence access to the glucocorticoid receptor receives support

An explanation of the glucocorticoid-activity of liquorice.

Since the 1940's the literature has been replete with references to the mineralocorticoid properties of liquorice and its derivatives (reviewed in Edwards 1991; Farese et al., 1991). It was thought for a long time that liquorice produced its mineralocorticoid actions via direct interaction of its active principle, glycyrrhetinic acid, with mineralocorticoid receptors. This hypothesis was incorrect for two reasons. Firstly, glycyrrhetinic acid had 1/100 the affinity of aldosterone for mineralocorticoid receptors (Arriza et al., 1987). Secondly, liquorice and its derivatives did not display such properties unless the adrenals were intact or cortisol was replaced when there was adrenal insufficiency (Borst et al., 1953; Elmadjian et al., 1956). Recent work (Stewart et al., 1987; Edwards et al., 1988) have shed new light on this subject and it is now known that liquorice mediates its mineralocorticoid action via inhibition of the cortisol metabolism by 11ß-hydroxysteroid dehydrogenase. Cortisol has been shown to have similar affinity to aldosterone for mineralocorticoid receptors, in vitro (Arriza et al., 1987). By inhibiting 11ß-hydroxysteroid dehydrogenase, high levels of a cortisol are generated locally with the result that mineralocorticoid receptors become activated in excess.

Although mineralocorticoid properties were first recognised, liquorice wassome time later also shown to possess glucocorticoid activity (Kumagai *et al.*, 1957; Finney and Somers, 1958; Finney and Tarnoky, 1960; Fletcher *et al.*, 1967). Using a variety of models of inflammation, glycyrrhetinic acid was shown to consistently demonstrate anti-inflammatory activity (Finney and Somers, 1958). When it came to clinical trials in man, the results were less predictable (Warin and Evans, 1956, Donaldson and Duthie, 1956). Within recent times there has been renewed interest in this area following a series of reports which suggests a role for traditional Chinese herbal remedies in treating skin diseases (Sheehan *et al.*, 1992; Harper *et al.*, 1990; Galloway *et al.*, 1991). Although the active ingredient/s is not known, glycyrrhetinic acid was found to be a component of such decoctions (Galloway *et al.*, 1991).

Despite the foregoing, the mechanism of the anti-inflammatory actions of liquorice has remained speculative. The similarity in chemical structure between glycyrrhetinic acid and hydrocortisone was notable (Finney and Somers, 1958) but as was the case with mineralocorticoid actions, an intact adrenal (Khan and Sullivan, 1967) or exogenous cortisol (Kumagai *et al.*, 1957) was also important for glucocorticoid activity.

In this work, the hypothesis that the anti-inflammatory action of liquorice resided in the inhibition of cortisol metabolism was tested. The liquorice/glucocorticoid interaction was elucidated using the classic bioassay of glucocorticoid action - skin vasoconstrictor bioassay. The results showed that glycyrrhetinic acid was ineffective on its own but in the presence of hydrocortisone the glucocorticoid effect was potentiated. *In vitro* skin 11ß-hydroxysteroid dehydrogenase was shown to be markedly inhibited by glycyrrhetinic acid.

There has been speculation that glycyrrhetinic acid could mediate anti-inflammatory activity by mechanisms related to altered prostaglandin metabolism (Peskar, 1976; Baker and Fanestil, 1991); mechanisms independent of glucocorticoid influences (Baker and Fanestil, 1991). It is known that there is structural homology between some steroid metabolising and prostaglandin metabolising enzymes (Baker and Fanestil, 1991; Monder and White, 1992), so that direct effects on prostaglandin metabolism are indeed possible. When, however, the inhibitory effects of glycyrrhetinic upon 11ß-hydroxysteroid dehydrogenase (cortisol metabolism) and 15-hydroxyprostaglandin dehydrogenase (most structurally homologous to the steroid metabolising dehydrogenase above), were compared, there was no apparent inhibition of the prostaglandin dehydrogenase at any concentration used in the experiment

whereas there was marked inhibition of steroid metabolism (ID $_{50} = 5 \times 10^{-6}$ M). However, this data relate purely to a lack of effect on prostaglandin degradation but effects on synthesis are still possible. The latter possibility has in fact, been previously investigated (Inoue *et al.*, 1986) and it was found that even at high concentrations glycyrrhizin, glycyrrhetinic acid and carbenoxolone produced little or no effect on either prostaglandin or leukotriene production, implying lack of any significant effect on synthetic pathways.

There are other important biological effects of glycyrrhetinic acid apart from its glucocorticoid-like action, e.g. bacteriostatic (Benigni and Franco, 1958) and anti-viral properties (Pompei *et al.*, 1979; Inoue *et al.*, 1986) but it does seem that the long recognised anti-inflammatory action of glycyrrhetinic acid accrues from inhibition of cortisol metabolism, a mechanism similar to that explaining its mineralocorticoid action (Edwards *et al.*, 1988).

SKIN VASOCONSTRICTOR ACTIVITY OF GLUCOCORTICOIDS

Glucocorticoids when applied topically on human skin undergo absorption resulting in apparent vasoconstriction (Shah et al 1989) in the dermal vascular bed (Barry and Woodford 1978) to produce skin blanching. For more than thirty years, this

blanching effect has been used as a measure of percutaneous bioavailability and potency of glucocorticoids from topical formulations and has proven invaluable in selecting optimum formulations from the skin vasoconstrictor bioassay (Barry and Woodford 1976, 1978, 1986). In the current work this bioassay was utilised for this traditional purpose and in the conventional manner to evaluate the effects of liquorice derivatives on hydrocortisone action but in addition, it was also tested for a novel purpose - to ascertain glucocorticoid sensitivity.

While the precise mechanism of blanching has not been fully elucidated, the use of a glucocorticoid receptor blocking agent was known to ablate the blanching response (Gaillard et al 1985). Blanching is therefore the likely pharmacodynamic response of glucocorticoid-receptor interaction and subsequent gene activation. It was an hypothesis to be tested in the current work that skin blanching might be possible marker of such interaction and could therefore be used to indicate sensitivity to glucocorticoids. The finding of reduced skin blanching in asthmatics whose airways responsiveness to systemic glucocorticoid therapy was reduced and of higher blanching scores in asthmatics whose disease was amenable to glucocorticoid therapy supports this hypothesis. The heterogeneity of skin blanching responses in a population (Burdick 1974, Stoughton 1992) might therefore be a reflection of the mechanism whereby some

patients respond to or develop side-effects from glucocorticoids more readily when comparable doses are used (Axelrod 1989).

It is noteworthy that there is a small subset of individuals who fail to blanch despite the application of high concentrations of highly bioavailable glucocorticoids. It is tempting to suggest that these individuals may have some form of glucocorticoid-resistance syndrome which has been well characterised in lower primates (Lipsett et al 1985) and though initially assumed to be rare in humans (Malchoff et al 1990), may, in fact, be more common in man than previously thought (Lamberts et al 1992). Indeed there might be grades of glucocorticoid resistance that are subtle. Whereas in its most extreme form glucocorticoid resistance is characterised by hypertension and hypokalaemic alkalosis secondary to mineralocorticoid excess (Vingerhoeds et al 1976) there is some evidence that hypertension without biochemical features of mineralocorticoid excess can also occur in this syndrome (Lamberts et al 1992). It is interesting that, using the skin vasoconstrictor assay, Walker demonstrated that in patients with essential hypertension, blanching responses were more intense compared with normal control subjects (Walker 1993). This implies that increased sensitivity of the vasculature to circulating glucocorticoids might be contributory to the hypertensive process and that the skin vasoconstrictor response reflects this. It seems

that 11 β -hydroxysteroid dehydrogenase activity might not be relevant to this supersensitivity however, since plasma half-life or $(11\alpha^{-3}H)$ cortisol, a measure of 11 β -hydroxysteroid dehydrogenase activity, was not different from normal controls (Walker 1993). This increased sensitivity in hypertensives was also non-specific for glucocorticoids and was shown to occur both with cortisol (which is metabolised by 11 β -hydroxysteroid dehydrogenase) and beclomethasone dipropionate - a glucocorticoid with a protected C-11-OH group (Walker 1993, Oelkers et al 1994).

It would be interesting to utilise the skin vasoconstrictor bioassay in order to determine whether glucocorticoid receptor structure/function might be abnormal in common clinical situations. Such studies might help to explain why persistent hypercortisolaemia in not always associated with clinical features of Cushing's syndrome. Thus increased activity of the hypothalamic-pituitary-adrenal axis is found in a substantial proportion of patients with primary depression (Schlesser et al 1979, Schlechte et al 1985, Whalley et al 1986), anorexia nervosa (Kontula et al 1980,1982) and in pregnancy (Rees et al 1975), yet clinical stigmata of Cushing's syndrome are lacking. Altered glucocorticoid sensitivity has been demonstrated both in the ability to suppress serum cortisol levels and in the ability of dexamethasone to suppress lymphoproliferative responses in depressed patients (Lowy et al 1984). Reduced levels

of glucocorticoid receptors have been found in mononuclear cells from patients with both depression (Whalley et al 1986) and anorexia nervosa (Kontula et al 1982). Using the skin vasoconstrictor assay it might be possible to determine in a variety of such clinical states whether abnormal glucocorticoid action/sensitivity might be contributory to the pathophysiology and whether altered glucocorticoid sensitivity might be contributory to decompensated states in pregnancy eg. pregnancy induced hypertension and gestational diabetes.

In an attempt to rank the blanching response against traditional markers of glucocorticoid action skin vasoconstrictor response was evaluated following varying degrees of systemic glucocorticoid exposure and compared with the observed effects of such treatments on the hypothalamic-pituitary-adrenal axis, intermediary and bone metabolism and hematopoietic effects. The results showed that, at least in the short term, changes in the skin blanching responsiveness were not sensitive to systemic glucocorticoid exposure. This is not too surprising given the number of factors which are known to influence the precision of the skin bioassay and the high level of variance inherent in the assay. It is because of this high variance that skin blanching responses might be inadequate to define sensitivity in individuals though group effects (as in glucocorticoid resistant asthma) may be readily identified. With refinement,

particularly if less subjective means of evaluating blanching are developed (Shah et al 1989) then the sensitivity of the assay might improve sufficiently to overcome this problem. Another inherent weakness of the assay is its sigmoidal dose-response relationship. Thus it has been shown (and reproduced in this work) that increasing concentrations of the same glucocorticoid in the same vehicle will produce an increasing blanching effect (McKenzie and Stoughton 1962) but with larger amounts of glucocorticoids further increase in the blanching effect may not occur despite 10-fold increase in concentration (Stoughton 1987, Stoughton and Wullich 1989). Hence the flattening of the dose-response curve could mask real differences in sensitivity. However, the absence of blanching at high doses of topically applied glucocorticoids indicate, with high specificity, lack of glucocorticoid responsiveness.

It is possible that intrinsic abnormalities of glucocorticoid receptor number or function (Chrousos et al 1982, Iida et al 1985, Bronnegard et al 1986, Nawata et al 1987) could dictate an individuals response to glucocorticoid therapy. It also seems that altered responsiveness can arise as a result of previous glucocorticoid exposure. Thus, in the cross-sectional study of skin vasoconstrictor responsiveness in asthmatic patients there emerged an ordering of the results which suggests that previous glucocorticoid therapy might have influenced skin vasoconstrictor responsiveness. The question

therefore arises as to whether in the subset of asthmatics who proved refractory to anti-asthma effect of glucocorticoids might represent a group who might have been selected out by their own long term use of glucocorticoids. Alternatively, it is possible that these individuals exhibited tachyphylaxis to glucocorticoid therapy. The first reported tachyphylaxis to glucocorticoids was observed using the skin vasoconstrictor assay (Du Vivier and Stoughton 1975). It is not just blanching that undergoes this tachyphylactic phenomenon. Indeed tachyphylaxis to glucocorticoids has also been shown for DNA synthesis which is readily suppressed in both normal and diseased skin following application of topical glucocorticoids. Continuous glucocorticoid application over ensuing days will lead to subsequent increase in DNA synthesis above normal levels even while glucocorticoid is still being applied (Du Vivier et al 1982). Similarly, the histamine induced "wheal and flare" response in skin that is normally suppressed by glucocorticoids will undergo tachyphylaxis with continued use of glucocorticoid (Singh and Singh 1986). Whether systemic glucocorticoid therapy is associated with similar induction of tachyphylaxis is speculative but the findings from the study of asthmatics undertaken in this work encourages this form of speculation.

There are however, data from the literature to support such a view. Thus basophils from patients, receiving long term glucocorticoid therapy for connective tissue

diseases and asthma, were found unresponsive to the effects of dexamethasone in inhibiting histamine release when these cells were subsequently challenged in vitro by anti-IgE (Lampl et al 1985). Similarly, patients treated with higher doses of glucocorticoids for rheumatic diseases have been shown to exhibit reduced clinical responses when subsequently challenged with glucocorticoid (Podgorski et al 1992). These data suggest that tachyphylaxis to the anti-inflammatory actions of systemic glucocorticoid therapy can in fact, occur. This does not necessarily imply that all effects of glucocorticoids exhibit tachyphylaxis to the same degree or in the same time period. Thus, the biological responses to glucocorticoids are highly variable due to differential responsiveness of individual genes to the hormone (Chrousos et al 1988) and least sensitive genes will therefore require a higher duration of treatment to become fully induced. Because of this differential responsiveness, it is possible that with chronic glucocorticoid use adverse effects may continue to be effected despite tachyphylaxis to anti-inflammatory actions. If tachyphylaxis to the beneficial effects of glucocorticoids ensues then it would be appropriate to withdraw glucocorticoid therapy periodically at a rate governed by clinical status (Dixon and Christy 1980). A clinical or biochemical marker of tachyphylaxis to the anti-inflammatory actions of glucocorticoids would be of immense value in guiding the clinician. Skin blanching response to glucocorticoids correlates closely with anti-inflammatory action (Cornell and Stoughton 1985).

Blanching might therefore serve this function. It might prove useful to screen, sequentially, patients who are embarking on chronic glucocorticoid therapy to determine whether tachyphylaxis to the blanching response (anti-inflammatory) occurs and when this occurs to reduce glucocorticoid exposure. Indeed using the skin vasoconstrictor assay Linder, (Linder et al 1993) found evidence for diminution of skin vasoconstrictor responses following prolonged systemic glucocorticoid therapy. When this is observed it might be appropriate to suggest "drug holidays" from systemic glucocorticoid therapy thereby reducing risks inherent with such therapy. This approach has previously been suggested for optimizing therapy with topically applied glucocorticoids (Du Vivier 1976).

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GLOSSARY OF TERMS AND ABBREVIATIONS USED:

ACTH Adrenocorticotrophic hormone

11ß-HSD The enzyme 11ß-hydroxysteroid dehydrogenase

B Corticosterone

BDP Beclomethasone dipropionate

BLANCHING: The change in colour of the human skin in

response to topically applied glucocorticoids. The colour change presumably occurs on the basis of

vasoconstriction in the dermal vascular bed.

Ca C1₂. 2H₂0 Calcium chloride dihydrate

Classical glucocorticoid These are steroid receptors which have high (Type

II) receptors affinity for the synthetic glucocorticoid

dexamethasone compared with the naturally

occurring glucocorticoids, cortisol and corticosterone. The affinity of steroids for these

receptors parallels their anti-inflammatory

potency. These receptors have low affinity for

aldosterone.

CO₂ Carbon dioxide

DNA Deoxyribonucleic acid

DPX A plastic resin

FEV,% Forced expiratory volume in 1 second as a

percentage of forced vital capacity

FHBA 5-(4-fluorobenzoyl) 2-hydroxybenzene acetic acid

GE

Glycyrrhetinic acid

HC

Hydrocortisone

HC1

Hydrochloric acid

H,O

Water (deionized and glass distilled)

KBQ

Kilo Becquerel

KC1

Potassium chloride

KH2PO4

Monopotassium phosphate

M

Molar concentration

MSH

Melanocyte stimulating hormone

mg

Milligram

Mineralocorticoid (type I glucocorticoid) receptors

These are steroid receptors which exhibit much lower affinity for dexamethasone and very high affinity for corticosterone. These receptors have similar affinity for corticosterone and aldosterone. *In vivo*, although circulating corticosterone levels are higher than aldosterone, these receptors selectively bind to aldosterone. Recent evidence have shown that this occurs because corticosterone is denied access to these receptors, *in vivo*, by the enzyme 11ß-hydroxysteroid dehydrogenase which inactivates corticosterone (but not aldosterone).

ml

Millilitre

NaC1

Sodium chloride

Na H CO3

Sodium bicarbonate

NAD

Oxidized nicotinamide adenine dinucleotide

NADP

Oxidized nicotinamide adenine dinucleotide

phosphate

0,

Oxygen

Osteocalcin

The most abundant non-collagenous protein of bone. It is synthesized by osteoblasts and has been found to be a specific and sensitive marker of bone turnover in patients with various metabolic

bone diseases.

PGDH

15-hydroxy prostaglandin dehydrogenase

%

Percentage

RNA

Ribonucleic acid

Short Metyrapone Test

This tested the hypothalamic-pituitary-adrenal the 11-deoxycortisol response by axis metyrapone blockage of cortisol synthesis

Tris (hydroxymethly) methylamine

 μg

microgram

less than

weight per volume

THE WORLD MEDICAL ASSOCIATION, INC.

13 Chemin du Levant 01210 Ferney-Voltaire, France

DECLARATION OF HELSINKI

Recommendations guiding medical doctors in biomedical research involving human subjects.

Adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964 and as revised by the 29th World Medical Assembly, Tokyo, Japan, 1975 and by the 35th World Medical Assembly, Venice, 1983.

INTRODUCTION

It is the mission of the medical doctor to safeguard the health of the people. His or her knowledge and conscience are dedicated to the fulfilment of this mission.

The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration", and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient'.

The purpose of biomedical research involving human subjects must be to improve diagnostic, therapeutic and prophylactic procedures and the understanding of the aetiology and pathogenesis of disease.

In current medical practice most diagnostic, therapeutic or prophylactic procedures involve hazards. This applies especially to biomedical research.

Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects.

In the field of biomedical research, a fundamental distinction must be recognised between medical research in which the aim is essentially diagnostic or therapeutic for a patient, and medical research, the essential object of which is purely scientific and without implying direct diagnostic or therapeutic value to the person subjected to the research.

Special caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.

Because it is essential that the results of laboratory experiments be applied to human beings to further scientific knowledge and to help suffering humanity, the World Medical Association has prepared the following

liberty to abstain from participation in the study and that he or she is free to withdraw his or her consent to participation at any time. The physician should then obtain the subject's freely-given informed consent, preferably in writing.

- 10. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship to him or her or may consent under duress. In that case the informed consent should be obtained by a physician who is not engaged in the investigation and who is completely independent of this official relationship.
- 11. In case of legal incompetence, informed consent should be obtained from the legal guardian in accordance with national legislation. Where physical or mental incapacity makes it impossible to obtain informed consent, or when the subject is a minor, permission from the responsible relative replaces that of the subject in accordance with national legislation.

Whenever the minor child is in fact able to give a consent, the minor's consent must be obtained in addition to the consent of the minor's legal quardian.

12. The research protocol should always contain a statement of the ethical considerations involved and should indicate that the principles enunciated in the present Declaration are complied with.

II. Medical Research Combined with Professional Care (Clinical Research)

- In the treatment of the sick person, the physician must be free to use a new diagnostic and therapeutic measure, if in his or her judgement it offers hope of saving life, re-establishing health or alleviating suffering.
- 2. The potential benefits, hazards and discomfort of a new method should be weighed against the advantages of the best current diagnostic and therapeutic methods.
- 3. In any medical study, every patient including those of a control group, if any should be assured of the best proven diagnostic and therapeutic method.
- 4. The refusal of the patient to participate in a study must never interfere with the physician-patient relationship.
- 5. If the physician considers it essential not to obtain informed consent, the specific reasons for this proposal should be stated in the experimental protocol for transmission to the independent committee (1.2).
- 6. The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that medical research is justified by its potential diagnostic or therapeutic value for the patient.

ndations as a guide to every physician in biomedical research ng human subjects. They should be kept under review in the future. be stressed that the standards as drafted are only a guide to ans all over the world. Physicians are not relieved from criminal, and ethical responsibilities under the laws of their own countries.

Basic Principles

- Biomedical research involving human subjects must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation and on a thorough knowledge of the scientific literature.
- The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol which should be transmitted to a specially appointed independent committee for consideration, comment and guidance.
- 3. Biomedical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given his or her consent.
- 4. Biomedical research involving human subjects cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.
- 5. Every biomedical research project involving human subjects should be preceded by careful assessment of predictable risks in comparison with foreseeable benefits to the subject or to others. Concern for the interests of the subject must always prevail over the interests of science and society.
- 6. The right of the research subject to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the subject and to minimise the impact of the study on the subject's physical and mental integrity and on the personality of the subject.
- 7. Physicians should abstain from engaging in research projects involving human subjects unless they are satisfied that the hazards involved are believed to be predictable. Physicians should cease any investigation if the hazards are found to outweigh the potential benefits.
- 8. In publication of the results of his or her research, the physician is obliged to preserve the accuracy of the results. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.
- 9. In any research on human beings, each potential subject must be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. He or she should be informed that he or she is at

Non-Therapeutic Biomedical Research Involving Human Subjects (Non-Clinical Biomedical Research)

- 1. In the purely scientific application for medical research carried out on a human being, it is the duty of the physician to remain the protector of the life and health of that person on whom biomedical research is being carried out.
- The subjects should be volunteers either healthy persons or patients for whom the experimental design is not related to the patient's illness.
- 3. The investigator or the investigating team should discontinue the research if in his/her or their judgement it may, if continued, be harmful to the individual.
- In research on man, the interest of science and society should never take precedence over considerations related to the well-being of the subject.

tober 1983

THE UNIVERSITY OF THE WEST INDIES

FACULTY OF MEDICAL SCIENCES - ST. AUGUSTINE

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DEPARTMENT OF MEDICINE

July 19, 1994

Dr. Robin Fox
Editor
The Lancet
46 Bedford Square
London
WC1B 3SL
England.

29 JUL 1994

Dear Dr. Fox,

Ishall be grateful if you would kindly grant me permission to reproduce the following articles, which I have had published in the Lancet within recent times for inclusion as appendices in my Phd. Thesis (University of Edinburgh):-

- (1) Teelucksingh S, et al. Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. Lancet 1990; 335; 1060-1063;
- (2) Edwards, C.R.W. and Teelucksingh, S. Glycyrrhetinic acid and potentiation of hydrocortisone activity in skin. Lancet 1990; 336; 322-323 (letter);
- (3) Teelucksingh, S. and Edwards, C.R.W. Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. Lancet 1990; 2; 876 (letter);
- (4) Brown, P.H. et al. Cutaneous vasoconstrictor response to glucocorticoids in asthma. Lancet 1991; 337; 576-580;

2/.

- (5) Teelucksingh S. et al. Inhaled corticosteroids, bone formation and osteocalcin. Lancet 1991, 338; 60-61 (letter);
- (6) Teelucksingh S. et al. Liquorice. Lancet 1991; 337; 1549, (letter)
- (7) Padfield, P.L. et al. Beclomethasone and osteocalcin. Lancet 1992; 339; 370-371 (letter).

I hope to defend my thesis entitled: "Glucocorticoid and the Skin" by September, 1994.

I shall require to make five (5) photocopies of each article, with your kind permission.

With kind regards,

- 1 AUG 1994

S. Teelucksingh

LECTURER IN MEDICINE.

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ST:bd

Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid

S. TEELUCKSINGH A. D. R. MACKIE D. BURT M. A. MCINTYRE L. BRETT C. R. W. EDWARDS

The enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD), which catalyses the conversion of cortisol to the inactive steroid cortisone in man (and corticosterone to 11-dehydrocorticosterone in rodents), was demonstrated by immunohistochemistry in skin biopsy samples from healthy volunteers and from patients with psoriasis and eczema. In-vitro studies confirmed the presence of the enzyme in skin from nude mice and showed that it is inhibited by glycyrrhetinic acid, the major active component of liquorice. By means of the skin vasoconstrictor assay, glycyrrhetinic acid was shown to potentiate the action of hydrocortisone. This work suggests a novel means of targeting glucocorticoid therapy.

Lancet 1990; 335: 1060-63.

Introduction

Corticosteroids are important in the treatment of many skin disorders. Modifications of the steroid structure have produced a wide variety of clinically effective agents; however, many of the more potent agents carry a risk of skin thinning and systemic toxicity.1 Even agents with a high ratio of local to systemic activity used in the lung? (eg, beclomethasone dipropionate) for the treatment of asthma have systemic effects within recommended dose regimens.3-5 Theoretically, targeting the glucocorticoid receptor by achieving high local levels of an intrinsically weak steroid need not compromise local efficacy but may reduce the risks of systemic toxic effects. Local inhibition of metabolism of a weak steroid such as hydrocortisone offers this prospect. The skin has the capacity to metabolise cortisol to cortisone6.7 by means of 11B-hydroxysteroid dehydrogenase (11B-OHSD). We have previously shown that glycyrrhetinic acid, a constituent of liquorice, is a potent inhibitor of this enzyme system. 8.9 The aim of this study was to assess how effectively glucocorticoid receptors in human skin could be targeted by inhibition of metabolism of cortisol. The in-vitro part of the study on skin metabolism of glucocorticoid and its inhibition by glycyrrhetinic acid used skin from the nude mouse, which has previously been used as a model system for investigation of the effects of topical glucocorticoid therapy.10

Subjects and methods

We studied 23 healthy volunteers with no previous exposure to exogenous corticosteroids—12 women and 11 men of mean age 29 years (range 21–50 years). The Stoughton-McKenzie vasoconstrictor assay¹¹ as modified by Place¹² was used to determine the potency of test substances. 2 subjects were tested twice with an interval of 2 months, so a total of 25 tests were done on groups of 4–6 subjects at a time. The test solutions were hydrocortisone acctate (at concentrations of 0·1, 0·3, 1·0, 3·0, and 10 mg/ml), becomethasone dipropionate (0·1, 0·3, 1·0, 3·0, and 10 mg/ml), glycyrrhetinic acid

(20 mg/ml), and a mixture of glycyrrhetinic acid (20 mg/ml) and hydrocortisone acetate at the above concentrations. All solutions were freshly made up in 95% ethanol in coded vials and were used within 24 h of preparation. 10 μ l volumes were applied in random order to 7 × 7 mm sites outlined by silicone grease on the flexor aspect of the forearm. After it had been allowed to dry, the test area was covered with polyester film ('Melinex' 12 μ m: ICI, UK) for 16–18 h. The degree of blanching was assessed 1, 2, 3, and 6 h after removal of the occlusive dressing and scored independently by two observers on a scale of 0 = none, 1 = mild, 2 = definite, and 3 = intense blanching. All solutions were applied and test sites read blind. Time-effect curves were plotted for each test substance and the area under the curve was determined by the trapezoidal rule.

For the in-vitro studies dorsal skin from freshly killed female nude mice (nu/nu, mixed genetic background) was homogenised ('Ystral' homogeniser: Scottish Scientific Instruments Centre Ltd) in 10 ml Krebs-Ringer buffer surrounded by ice, with 20 s bursts of homogenisation and 30 s cooling intervals. Homogenates were spun at 750 g, 4°C for 5 min, and the supernatants were assayed for protein concentration ('Bio-Rad' protein assay kit). homogenates were diluted with Krebs-Ringer buffer to yield the same protein content per wet weight of skin. 400 µl volumes were incubated with shaking at 37°C for 60 min with 12 nmol/l tritiated corticosterone of specific activity 82 Ci/mmol (Amersham International) in 600 µl Krebs-Ringer with 0.2% glucose and 0.2% bovine serum albumin. Control samples containing either no homogenate or 10 µl ethanol were compared with incubations containing glycyrrhetinic acid, freshly made up in absolute ethanol, in final concentrations of 100 µmol/l, 1 µmol/l, and 0.01 µmol/l. After centrifugation, corticosteroids were extracted from the supernatant with ethyl acetate. H-corticosterone was separated from 3H-11-dehydrocorticosterone by thin-layer chromatography in a system of chloroform/95% ethanol (92/8). The percentage conversion of corticosterone to 11-dehydrocorticosterone was determined

Immunohistochemical studies used the IgG fraction of a rabbit antiserum (56-125) against purified rat liver 11B-OHSD.13 As negative controls preimmune rabbit serum and inappropriate primary antibody (to al-antitrypsin) were included. Normal human skin was obtained at laparotomy from the anterior abdominal wall and punch biopsy samples were taken from lesions of patients with psoriasis and eczema. The tissue samples were placed immediately in Bouin's fixative for 24-48 h, then subjected to routine paraffin embedding. Sections were cut at 4 µm, deparaffinised, and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by 10 min treatment with 3% hydrogen peroxide, and to minimise background staining slides were preincubated with 20% donkey serum. They were then incubated in primary antibody, preimmune serum, or inappropriate antibody at dilutions of 1 to 100 or 200 for 30 min at room temperature. This step was followed by labelled avidin-biotin immunodetection14 with biotinylated donkey antibody to rabbit immunoglobulin and peroxidase-conjugated streptavidin. The peroxidase substrate was 3,3'-diaminobenzidine tetrachloride. The

ADDRESSES Departments of Medicine (S. Teelucksingh, MRCP, A.D. R. Mackie, MRCP, D. Burt, CBiol, Prof. C. R. W. Edwards, FRCP); and Pathology (M. A. McIniyre, FRCPath, L. Brett, FIMLS), Western General Hospital, Edinburgh EH4 2XU, UK. Correspondence to Prof. C. R. W. Edwards

slides were counterstained in Mayer's haematoxylin and mounted in DPX.

The groups were compared by analysis of variance (ANOVA), followed by a paired or unpaired Student's t test as appropriate. Significance was taken as p < 0.05, and results are expressed as mean and SEM. Linear regression analysis was used to plot the dose-response relation.

Results

The dose-response relations of test substances on the skin vasoconstrictor response in normal volunteers are shown in fig 1. Hydrocortisone acetate alone produced a flat response at the lower limit of detection of the assay. The addition of glycyrrhetinic acid, however, produced significant potentiation of the cutaneous vasoconstrictor effect at hydrocortisone acetate concentrations of 10 mg/ml (p < 0.01), 3 mg/ml (p < 0.001), and 1 mg/ml (p < 0.001). Glycyrrhetinic acid alone and ethanol alone had no effect.

The high 11β -OHSD activity in skin from nude mice (mean [n=5] percentage conversion of 3H -corticosterone to 3H -11-dehydrocorticosterone $49\cdot0$ [SEM $1\cdot3]\%$) was slightly reduced by ethanol alone ($39\cdot0$ [0·7]%; $p<0\cdot05$) but was greatly inhibited in a dose-dependent way by all concentrations of glycyrrhetinic acid ($100 \mu mol/l=0$ [0]%, $p<0\cdot0001$; $1 \mu mol/l=4\cdot1$ [0·5]%, $p<0\cdot0001$; $0\cdot01 \mu mol/l=31\cdot8$ [1·5]%, $p<0\cdot05$).

Sections from normal human skin showed strong positive cytoplasmic staining for 11β -OHSD in the epidermis, with the exception of the basal cell layer (fig 2a). The luminal border of the sweat ducts stained moderately and there was some staining in the cytoplasm of sweat glands. There was no significant staining with either preimmune rabbit serum (fig 2b) or the inappropriate primary antibody (not shown). The distribution of the enzyme was similar in psoriatic and eczematous skin, although the enzyme seemed to be present in greater amounts (fig 2c and d). Mouse skin stained less intensely but all layers of the epidermis were positive (fig 3a) in comparison with normal human skin. The preimmune serum control was negative (fig 3b).

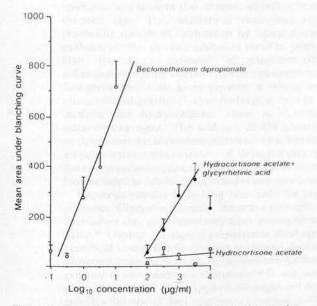


Fig 1—Relation of human vasoconstrictor response (area under blanching curve in arbitrary units × h) and dose of beclomethasone dipropionate, hydrocortisone plus glycyrrhetinic acid, and hydrocortisone alone.

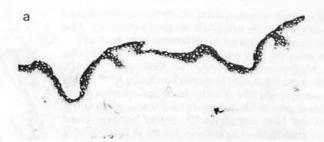








Fig 2-Localisation of 11β-OHSD.

a = normal human skin stained for 11 β -OHSD, b = normal skin with preimmune serum; c = psoriatic skin; d = eczematous skin.

Discussion

The anti-inflammatory activity of glycyrrhetinic acid has long been suspected. $^{15.16}$ Its chemical structure is similar to that of hydrocortisone but the mechanism of its anti-inflammatory action was not clear. 17 We found that glycyrrhetinic acid is a potent inhibitor of 11β -OHSD in kidney and liver $^{8.18}$ and postulated that its anti-inflammatory activity is not a direct effect but is the expression of reduced conversion of cortisol to its biologically inactive product cortisone.

The skin's capacity to metabolise cortisol was shown some time ago. ^{6,7} The fact that cortisone is the major metabolite shows that 11β-OHSD is active in skin. It is difficult to study human skin in vitro because of problems with its homogenisation. ¹⁹ The skin of nude mice is comparable to human skin as a physicochemical barrier to many drugs, including corticosteroids, ¹⁰ but unlike human skin it yields a consistent homogenate. With this tissuue we



Fig 3—Localisation of 11β-OHSD in skin of nude mouse.

a = stained for 11β-OHSD; b = preimmune serum control

have confirmed the presence of 11β -OHSD in skin and have shown that, as in kidney, glycyrrhetinic acid is a potent inhibitor of the enzyme.

Our immunohistochemical data localise the enzyme to the epidermis and confirm that enzyme activity is retained in diseased skin. This anatomical disposition makes it potentially suitable to modulation by topical therapy. To evaluate whether in-vitro inhibition could be produced in vivo, the classic bioassay¹¹ of glucocorticoid anti-inflammatory activity was used. Despite its limitations, ²⁰ the Stoughton-McKenzie assay provides a reliable index of glucocorticoid potency. ²¹ Our findings in human beings confirm that hydrocortisone alone is a weak anti-inflammatory agent. The addition of 2% glycyrrhetinic acid, however, greatly potentiated its activity. The fall-off at a hydrocortisone concentration of 10 mg/ml may relate to lower bioavailability, since at this concentration the mixture has exceeded its solubility limit and is in suspension.

Liquorice extracts have long been used for medicinal purposes. Glycyrrhetinic acid, the active principle, and its derivatives (eg, carbenoxolone) have proven therapeutic value.²² Used as 2% topical preparations these agents are beneficial in several inflammatory cutaneous disorders.^{23, 27} However, carbenoxolone has no intrinsic anti-inflammatory potency in adrenalectomised animals.¹⁵ In our volunteers glycyrrhetinic acid alone applied overnight had no effect, possibly because of low endogenous cortisol levels. The combination of hydrocortisone with glycyrrhetinic acid had a substantial effect. These findings support our hypothesis that the anti-inflammatory action of glycyrrhetinic acid is

mediated by cortisol which, in the presence of glycyrrhetinic acid, is protected from metabolism to inactive cortisone.

The long-term application of topical corticosteroids by patients with skin disease, especially if an extensive area is affected, may lead to systemic glucocorticoid toxic effects. ^{28,29} A possible advantage of applying hydrocortisone, an intrinsically weak glucocorticoid, with glycyrrhetinic acid is that the enhanced local glucocorticoid activity could have lesser systemic effects. Glycyrrhetinic acid has limited solubility and its inhibitory effect on cortisol metabolism is likely to be confined to its site of application.

Our previous studies have shown that 11β-OHSD has an important role in the protection of the non-specific mineralocorticoid receptor in tissues such as the kidney and salivary glands. Our latest findings suggest that 11β-OHSD may also modulate the access of glucocorticoids to the glucocorticoid receptor. The principle of local enzyme inhibition producing enhanced glucocorticoid activity may well be applicable to other sites where 11β-OHSD is present—eg, the lung.

We thank our colleagues who acted as volunteers; Pauline Burnet and Mary Norman for their help with the bioassay; Dr Fouad Habib, Dr Martin Waterfall, and Ms Susan Stubbs for technical advice; Ms Louise Anderson, Ms Alison Ritchie, and Mr Donald Hay of the Animal Unit for their help; Ms Susan Rae and Mrs Barbara Beattie for preparing the typescript; and Dr Carl Monder (Population Council, New York, USA) for antiserum to 11β-OHSD. S. T. was a Trinidad and Tobago Commonwealth Scholar under the auspices of the Association of Commonwealth Universities.

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BOOKSHELF

Principles and Practice of Pediatrics

Edited by F. A. Oski, C. D. DeAngelis, R. D. Feigin, and J. B. Warshaw. Philadelphia: Lippincott. 1990. Pp 2000. \$95. ISBN 0-397507070.

In size alone Dr Oski and his co-editors have produced an impressive book. It weighs over 3 kg, runs to 2000 pages, and boasts 261 contributors. Assuming that each of the contributors was given a free copy, one might initially wonder whether there are any paediatricians left to buy it, but in chapter 2 we are told that in 1985 there were 35 617 paediatricians in the United States (there are about 750 in Britain). It is written by Americans for Americans: one chapter is entitled Getting Started in the Real World: How to Set up Your Private Practice.

Lippincott's glossy advertising brochure boasts that they "give you 5 books in one", but some paediatricians may prefer to acquire their books as and when they need them rather than bulk-buying a volume that puts considerable strain on the bookshelf as well as the back. The five sections include General Pediatrics, The Fetus and Newborn, Ambulatory Pediatrics, the Sick or Hospitalized Patient, and a section curiously titled Important Things You Forget to Remember. To evaluate it as a reference book for an experienced paediatrician I looked up some recent cases where I needed to get more information. For Menke's disease, Blount's disease, CHARGE syndrome, and mitochondrial cytopathy the book gave concise and useful information. Unfortunately, Joubert's syndrome was noted only in passing. Eight marks out of ten. How did it perform as a text for junior paediatricians working for their examinations? I was impressed with the extensive background information on history taking, examination skills, paediatric pharmacology, molecular genetics, and the special needs of children with chronic illnesses. Ten out of ten. In the preface Dr Oski suggests that the book is designed to meet the needs of the medical student, but I cannot recommend it for British undergraduate use-two out of ten. Is it a good practical guide to diagnosis and treatment for the busy intern or registrar? There are several excellent sections including lists of differential diagnoses of common symptoms, normal laboratory values for different age-groups, and a well illustrated section on paediatric procedures. Nine out of ten.

This good and comprehensive text is excellent value for money and I strongly recommend it. For the second edition I would advise the publishers to put the colour plates in the text rather than at the front (to save the reader leafing backwards and forwards) and to divide it into two volumes, since its spine will not survive four years if the book is used as much as it should be.

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MALCOLM LEVENE

Modern Coronary Care

Edited by G. S. Francis and J. S. Alpert. Boston: Little, Brown/Edinburgh: Churchill Livingstone. 1990. Pp 797. £70.00. ISBN 0-316291595.

Eighty years ago Dr James Herrick was asked to see a patient with severe chest pain. The doctor thought the diagnosis was myocardial infarction, but then, in a response familiar to all of us, he spent a restless night wondering whether he had made the right decision. The patient died, necropsy confirmed the diagnosis, and the syndrome of myocardial infarction began its modern career as a recognisable disease. Eighty years on we are still discovering fundamental aspects of this fascinating condition—it was only ten years ago that the central role of coronary thrombosis, as postulated by Herrick, was finally proven, opening the way to a decade of thrombolysis. Francis and Alpert have attempted to pull together the major themes that have dominated coronary care and to present an up-to-date summary of current thoughts and management. The book is a series of reviews

SERUM HBV DNA* DURING AND AFTER THE TREATMENT

-	TNE	F dose (µg/	Post TNF		
Patient	0	10-15	100	2 wk	8-12 wk
I, severe CAH	-14			100000	1
cirrhosis	4	0	22	0	0
2, mild CAH	307	241	574	1062	213
3, mild CAH	228	157	124	606	412
4, mild CAH cirrhosis	17	4	12	11	0
5, moderate CAH	0	0	190	396	0
6, CPH/CLH	1323	961	1000	1504	934

*pg per 40 µt.

CAH = chronic active hepatitis, CPH = chronic persistent hepatitis, CLH = chronic lobular hepatitis.

received no specific antiviral therapy for at least twelve months. All were HBsAg, HBeAg, and HBV DNA positive. Patient 5 (table) proved to be HBV DNA negative on day 0 of the trial, before receiving rTNF. With one exception treatment began with daily 30 min infusion of rTNF in 100 ml of 4% albumin for 5 days. Later rTNF was given in a bolus over 10 min on a day-case basis, thrice weekly. In patient 1 (table) therapy began as 10 μg m² intramuscularly but was discontinued after three doses because of reactions; 40 days later rTNF was given intravenously. Two patients began on a dose of 10 μg/m², and four on 15 μg/m², for 2 weeks, and if no important toxicity was seen the dose was increased. The dose levels were 10, 15, 30, 60, 80, and 100 μg/m². Paracetamol 500 mg was given before each infusion.

Five of the six patients completed 9 or more weeks of treatment. An initial fall in serum HBV DNA was seen in the five who were HBV DNA positive at the start of the trial (median values after 14 days fell to 68% of pretreatment values [range 0-78%], table). In patient 6 rTNF was discontinued after 3 days, with no further treatment, but his HBV DNA returned to previous values within 28 days after an initial fall to 71% of pretreatment levels. In patient 1 serum HBV DNA fell to zero after the intramuscular injections, was low on three occasions during or shortly after intravenous therapy, and was undetectable over the subsequent 6 months. The remaining four patients completed treatment to the maximum dose of 100 µg/m2. Patients 2 and 5, however, had recurrent rigors at doses over 30 µg/m². They were given indomethacin 25 mg 30 mun before each injection, and symptoms disappeared. In three of these patients, after an initial fall in HBV DNA, an upward trend was noted throughout the trial; this trend was most dramatic in patient 5 whose serum HBV DNA became strongly positive at doses over 30 μg/m². After cessation of treatment HBV DNA rose strikingly in three patients, falling again towards pretreatment values after 2-3 months. HBsAg titres broadly paralleled changes in HBV DNA. No change in HBeAg/Ab status was seen. After 5-12 months of follow-up HBV DNA had returned to pretreatment values in patients 2, 3, and 6. HBV DNA remained negative in patients 1 and 5, and in patient 4 it was detectable intermittently (range 0-7 pg per 40 µl). Unfortunately, however, the progression of liver disease was not halted in patient 1 and he had successful liver transplantation 6 months after completing TNF treatment. All patients had a dose related increase in serum gamma glutamyltransferase (GGT) (median 58-128 IU) during treatment. Serum aspartate transaminase (AST) rose in three of the four who received higher doses of rTNF. AST doubled in patients 2 and 5 with rTNF over 30 µg/m2 in parallel with the rise in HBV DNA; values fell when treatment was discontinued. No serious clinical toxicity was seen.

With 10–15 µg/m² doses of rTNF a reduction in serum HBV DNA was seen, suggesting that rTNF was initially exerting an antiviral action against HBV. rTNF doses of over 30 µg/m² (or longer treatment with rTNF) were associated in two patients with enhanced viral replication and raised HBV DNA and HBsAg. Higher doses of TNF may be immunosuppressive—TNF is known to induce cortisone release. Alternatively, TNF may directly enhance viral replication. Treatment of HUT-78 cells with TNF reduces the subsequent replication of HIV,5 but when HIV is cultured in MOLT-4 cells TNF enhances viral replication. The induction of other cytokines may also modulate the actions of TNF; for example IL-1 has been shown to inhibit the antiviral action of

TNF against encephalomyocarditis virus cultured in fibroblasis and to inhibit the release of interferon beta from these cells. The concomitant rise in HBV DNA and AST while on treatment suggests that the increase in viral replication may have been accompanied by increased lysis of hepatocytes.

This study has shown that long-term outpatient treatment with rTNF is possible. There was little evidence of systemic toxicity despite the presence of very high peak plasma TNF. This finding is noteworthy in view of the postulated toxicity of TNF in septic shock, which is associated with much lower serum TNF.

None of our patients seroconverted to HBeAb despite the initial reduction in HBV DNA. A synergistic antiviral effect has been shown between TNF and interferon alpha in vitro studies." The addition of IFN- α as a more potent direct antiviral agent or a longer duration of therapy might result in complete viral clearance. By simulating natural patterns of cytokine production the effectiveness of therapy for chronic HBV infection might be enhanced.

We thank Dr R. Bown, Dr B. McDougall, Dr A. Forbes, and Dr J. I. Thirkettle for their help. N.S. is supported by the Wellcome Trust and J Y.N.L. by the Croucher Foundation.

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Glycyrrhetinic acid and potentiation of hydrocortisone activity in skin

SIR,-We were somewhat surprised by Dr MacKenzie and colleagues' (June 23, p 1534) comments on our report (May 31, p 1060) demonstrating potentiation of hydrocortisone activity in skin by glycyrrhetinic acid (GE), the active component of liquidice They say that similar potentiation was reported in the 1950s by Borst and colleagues' and that we failed to mention this. They also refer to a paper by Elmadjian et al' showing synergistic effects of hydrocortisone and liquorice extracts in two adrenalectomised patients. We were aware of both these papers and had cited them in our previous Lancet report demonstrating the inhibition of 11ß-hydroxysteroid dehydrogenase (11ß-OHSD) by liquorice. This report and those by Borst, Elmadjian, and their colleagues related to the mineralocorticoid-like actions of liquorice—the so-called deoxycortone-like effects-and demonstrated sodium retention and potassium loss when liquorice was given either with cortisone or hydrocortisone or when the adrenals were intact. It was not possible to draw any conclusions about the effect of liquorice on glucocorticoid action. Indeed Borst et al suggested that it did not have such an effect, as judged by the failure of liquorice to reduce the eosinophil count in healthy subjects or to improve the joint symptoms in rheumatic patients. The aim of our recent report was to examine the liquorice/glucocorticoid interaction in a well ralidated glucocorticoid bioassay". —namely, the skin rasoconstrictor assay. Our results showed that GE could indeed enhance glucocorticoid activity in addition to its well known mineralocorticoid-like effect, and thus explain Kumagai and 60-workers to clinical observations. They showed that in two patients, one with rheumatic fever and the other with lupus eyhematosus, there was potentiation and prolongation of the effect of ACTH and hydrocortisone by glycyrrhizine.

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Pre-eclampsia and HLA-DR4

Sir. 1)r Hoff and colleagues (March 17, p 661) report results from a population study that seem to indicate a negative association between HLA-DR4 and pre-eclampsia/eclampsia, mild precdampsia, and pregnancy-induced hypertension. This is in striking contrast to an earlier report of a positive association between pre-eclampsia and DR4. Hoff et al cannot explain the discrepancy. They pooled two populations, on the grounds that the direction of the association between disease state and DR4 was the same in both. However, the two populations have very different gene frequencies of DR4 (0.057 blacks, 0.158 whites)1 and they also differ in types of pregnancy hypertension. The criterion for validly combining two populations is that the marker gene frequencies are not different. In this case they are very different. Since a larger proportion of blacks than of whites are affected by the disease, the proportion of each race in the disease and control groups is different, and the frequency of DR5 would be expected to be lower in the disease group. The control group should have the same proportions of blacks and whites as the disease group

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"This letter has been shown to Dr Hoff, whose reply follows.

-En L.:

Sir.— Dr Wilton and Dr Cooper are correct to note that pooling two different racial populations which have different distributions of study variables can lead to misleading results on statistical analysis. Race can be a confounding factor but our statistical findings cannot be so easily dismissed. They come from a large historical cohort instead of a case-control study. The lower frequency of DR4 among women with the hypertensive disorders of pregnancy is observed consistently in both races. We did analyse our pooled data by the stratified chi-square method with stratification by race. Stratification climinates biased comparisons that result from confounders such as race. The significance levels were similar to those derived from the pooled analyses.

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Rectal gluten challenge and diagnosis of coeliac disease

SIR,—Dr Loft and colleagues (June 2, p 1293) report the possibility of diagnosis of coeliac disease (CD) by direct gluten challenge of rectal mucosa.

Dobbins and Rubin' were the first to describe the appearance of mild alterations in the rectal mucosa of coeliac patients when they were locally challenged with gluten. These alterations, which were greatest at 8 h post-challenge, consisted of polymorphonuclear infiltration of lamina propria and surface epithelium and depletion of goblet cells. These findings did not receive much attention until lately. Austin and Dobbins2 and I and my colleagues3 in 1988, as well as Loft and colleagues' elegant studies, (and June 2) provide new and interesting data. Unfortunately, these groups of workers used different techniques for gluten challenge and timing for post-challenge biopsy; these differences might have caused the conflicting results about the number of post-challenge (versus pre-challenge) intraepithelial lymphocytes (IEL) and the degree of cellular infiltration of the lamina propria. Some results in these studies were, however, in accord—such as the absence of an increase in post-challenge mitotic crypt cell activity.3 and the significant increase in post-challenge mucosal thickness, " which proved to be due, in part, to extravascular deposition o fibrinogen. The results of these studies strongly suggest that the rectal mucosa of coeliac patients is sensitive to gluten, in contrast with previous assumptions

How useful are these findings for the individual in need of a definite diagnosis of CD? Loft et al, who used computerised image analysis for IEL counts, regard rectal gluten challenge as a reliable test for the diagnosis of CD. We,3 however, with the longst and probably the most aggressive rectal gluten challenge, found no difference between pre-challenge and post-challenge IEL counts, by the simple and widely used technique of counting 500 successive surface epithelial cells and expression of the results as percentages of epithelial cells.

Thus, although there is evidence for sensitivity of rectal mucosa of coeliac patients to gluten, the practical usefulness of this finding remains unclear.

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Possible locus for polycystic kidney disease on chromosome 2

SIR. Autosomal dominant polycystic kidney disease (ADPKD) is one of the commoner mendelian disorders. Linkage analysis has mapped the disease gene (PKDI) to the short arm of chromosome 16,1 but several laboratories have identified families in which the disease gene segregates independently of PKD1 markers.1 - As part of a European Community programme on the prevention of inherited polycystic kidney disease (project leader Dr M. H. Breuning, Leiden) an effort is being made to map the gene (PKD2) in a large Danish family with this "unlinked" ADPKD ' Family members, whose gene carrier status has been established clinically and or by ultrasonography (over the age of 35), have been typed with the highly polymorphic DNA markers MS1, MS31, MS43a, and YNH24. The MS probes were purchased from Cellmark, UK, and YNH24 from Promega, USA. After radioactive labelling they were hybridised consecutively to 'Hybond N' filters Amersham International) containing DNA digested by Huffl Bochringer. The data were analysed with the TANKAGE accumulation of labelled liposomes was seen in inflamed scar tissue in 1 patient with suspected recurrence of malignant melanoma (groin), and in a benign thyroid cyst of a patient with an propharyngeal carcinoma. (Thin needle aspiration of cyst fluid and measurement of activity in a probe scintillation counter 2 weeks after injection of liposomes showed over twenty-fold greater activity in the cyst fluid than in a simultaneously drawn blood sample.)

In a patient with suspected recurrence of malignant melanoma the liposome scan was negative, in agreement with other imaging methods and the clinical course ("true negative"). Images of tumours were best seen 24 h and more after injection of liposomes; early images showed very low or no uptake of labelled liposomes. The distribution of liposomes (uptake in liver, spleen, bone marrow, and blood) showed considerable interindividual variation, which did not seem to correlate with radiolabelling efficiency.

The overall specificity of liposome scanning in our study was 59%, specificity 25% (which is due to the selection of patients), and diagnostic accuracy 58%. When confined to patients with oropharyngeal carcinomas, the sensitivity of liposome scanning was 67%, without a definable value for specificity. These values are low compared with the results of others,34 which may result from the relatively low number of patients and their selection. As was observed before,45 late images (24 h, 48 h, and later) seem to give the best diagnostic information. We saw a high number of "false negatives" in patients who were treated by chemotherapy at the time of liposome scanning (8/10 tumour lesions). The effect of chemotherapy and other forms of antitumour therapy on the uptake of liposomes in tumour tissues will be the subject of further investigations. The therapeutic use of liposomes (loaded with chemotherapeutic agents) will depend on the results of these studies.

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Potentiation of hydrocortisone activity in skin by glycerrhetinic acid

SIR,—Dr Teelucksingh and colleagues (May 5, p 1060) describe a response to topical hydrocortisone in skin, in which cutaneous blanching due to this steroid is apparently enhanced by coapplication of glycerrhetinic acid, a compound closely related to carbenoxolone. This enhancement is attributed to inhibition in the skin of metabolism of cortisol by 11β-hydroxysteroid dehydrogenase. Teelucksingh et al suggest that this is a novel means of targeting glucocorticoid therapy.

The potential merit of this approach depends upon the assumption that suppression of inactivation of hydrocortisone and consequent increase in tissue hydrocortisone is not accompanied by increased systemic absorption, exposing the patient in some circumstances to real risk of steroid toxicity. However, this remains an unsupported assumption because systemic absorption of hydrocortisone in the presence of glycerrhetinic acid was not measured, and without this information Teelucksingh et al cannot claim to have dissociated increased local efficacy from increased

systemic toxicity. The problem of increased local steroid toxicity is also not addressed.

Single or repeated co-application of glycerrhetinic acid with hydrocortisone might have increased percutaneous absorption of hydrocortisone. The increased blanching may thus have been wholly independent of any action on enzymatic metabolism, which was not measured in their study on human skin. These difficulties highlight the crucial importance of evaluating systemic hydrocortisone absorption in a study of this type.

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- *This letter has been shown to Dr Teelucksingh and Professor Edwards, whose reply follows.—Etc. L.

Sir,-In our paper we showed that combining hydrocortisone, an intrinsically weak glucocorticoid, with an inhibitor of its enzymatic metabolism enhances the action of hydrocortisone in producing skin blanching, the classic bioassay of glucocorticoid efficacy. Professor Greaves is concerned about the possible risks of hydrocortisone toxicity due to increased systemic absorption. Systemic side-effects from topical glucocorticoid therapy are well recognised in the setting of chronic or relapsing disease when more potent, highly bioavailable topical agents are used.' Such effects have not been described with topical hydrocortisone' except in the very young with severe disease.3 On the whole the systemic absorption of hydrocortisone from human skin is very low. The increased biological effect of hydrocortisone when co-applied with glycyrrhetinic acid implies increased access to the dennal vasculatures and perhaps the systemic circulation-but in view of the weak intrinsic action of hydrocortisone and its short half-life we proposed that this particular combination might prove to be associated with less risk than currently available potent agents.

Greaves also suggests that glycyrrhetinic acid might increase percutaneous absorption of hydrocortisone independent of inhibition of enzymatic catabolism. Our study did not address this point. We demonstrated 11B-hydroxysteroid dehydrogenase in human epidermis and showed that glycyrrhetinic acid is a potent inhibitor of this in vitro. We postulated that by inhibiting the metabolism of hydrocortisone in the epidermis, glycyrrhetinic acid was allowing greater access of hydrocortisone to glucocorticoid receptors in the dermis and hence the augmented biological action. The findings of Fuller and Verity are in keeping with our hypothesis. They showed that in the colon, also a site of 11β-hydroxysteroid dehydrogenase activity, corticosteroid responsive gene expression was enhanced by carbenoxolone (the hemisuccinate derivative of glycyrrhetinic acid). These findings therefore support our view that this enzyme protects not only the mineralocorticoid receptor7 but also the glucocorticoid receptor.

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and meningitis can be excluded. An osmotic diuretic should be available if LP is to be done.

We thank the Medical Officer of Health, Kilifi District and our colleagues on the staff of Kilifi Hospital. We are especially grateful to the KEMRI nurses and technical staff and to Dr C. Nevill of the African Medical Research Foundation for their help, and to Dr R. Snow for statistical advice. We thank Dr J. B. O. Were, Director, Clinical Research Centre, and Dr W. M. Watkins, Director, Wellcome Trust, Nairobi, for their support and encouragement, and Dr C. Newbold, Dr G. Brown, Dr N. White, Prof E. R. Moxon, and Prof B. G. R. Neville for stimulating discussions. We thank the Director of KEMRI, Dr D. Koech, for permission to publish these results. This work was funded by the Wellcome Trust, UK, and formed part of a collaborative study of the pathophysiology of malaria in children.

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Cutaneous vasoconstrictor response to glucocorticoids in asthma

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The aim of the study was to find out whether asthma patients whose airways obstruction is sensitive (CS) or resistant (CR) to corticosteroid cutaneous in their treatment also differ vasoconstrictor response to a potent topical glucocorticoid. Corticosteroid resistance was defined by failure of forced expiratory volume in 1 s (FEV,) and peak expiratory flow rate to improve by at least 15% after a 2-week trial of corticosteroids (prednisolone 20 mg daily for 1 week, then 40 mg daily for 1 week) despite more than 15% improvement with inhaled beta Beclomethasone dipropionate in concentrations of 3 μg/ml, 10 μg/ml, 30 μg/ml, and 100 μg/ml was applied to forearm skin; the site was occluded under plastic and the degree of blanching assessed after 18 h. CS asthmatic subjects (n = 31), asthma patients with mild airways obstruction (n = 26). asthma patients taking long term prednisolone (n = 13), and healthy volunteers showed similar vasoconstrictor responses. In CR asthmatic subjects (n = 15), the response (expressed in terms of either blanching intensity or the proportion of patients showing a positive response) was significantly lower than that in the CS group at concentrations of 3 μ g/ml (p < 0·01), 10 μ g/ml (p < 0·01), and 30 μ g/ml (p < 0·05), but not at 100 μ g/ml. This resistance to glucocorticoids in the skin, together with reported evidence of glucocorticoid resistance in peripheral blood leucocytes, suggests a general defect in the ability of tissues to respond to glucocorticoids in CR asthma

Lancet 1991, 337, 576, 80

Introduction

Most patients with chronic asthma respond well to inhaled corticosteroid therapy, and acute exacerbations of

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their disease respond to treatment with systemic corticosteroids. A minority of asthmatic patients, although they derive substantial benefit from bronchodilators, show little response to corticosteroids. Corticosteroid-resistant (CR) patients, in comparison with those who are corticosteroid sensitive (CS), are more likely to have had asthma for longer than 5 years, to have a family history of asthma, and to experience symptoms at night.

A series of in-vitro studies of peripheral blood mononuclear cells2-4 suggested that corticosteroid resistance was associated with a defect in monocyte responsiveness to glucocorticoids. Monocytes from asthmatic patients release a polypeptide which primes neutrophils and eosinophils for enhanced leukotriene generation when subsequently stimulated by ionophore.56 In CS asthmatic patients release of this cytokine is blocked by low concentrations of hydrocortisone (10-8 mol/l), whereas in CR patients much higher concentrations (up to 104 mol/l) do not suppress release.56 Studies of peripheral blood lymphocytes have also shown differences between CS and CR asthmatic patients. The degree of activation of T lymphocytes (as assessed by increased HLA DR and interleukin-2 receptor expression) is greater and higher concentrations of dexamethasone are required to suppress in-vitro proliferative responses to phytohaemagglutinin and to suppress interleukin-2 and interferon-gamma release in CR than in CS asthmatic patients (unpublished data).

The evidence of reduced responsiveness of airways obstruction and peripheral blood cell function to corticosteroids in CR asthmatic patients suggests that they may have a general defect in the ability of their cells to respond to glucocorticoids. It might therefore be possible to show a reduced response to corticosteroids in other tissue sites. Glucocorticoids such as beclomethasone dipropionate and budesonide benefit most patients with asthma because of their high topical potency but low systemic effect. The topical potency of a glucocorticoid can be determined by the degree of blanching produced when it is applied to the skin. This cutaneous vasoconstrictor response has been used to compare topical potencies of different glucocorticoids for many years.7 However, healthy volunteers are normally used in such comparisons, and cutaneous responses of patients with systemic diseases for which glucocorticoids are used therapeutically have not been reported. We have studied the vasoconstrictor response to beclomethasone dipropionate of patients with asthma and compared CS and CR patients.

Patients and methods

The vasoconstrictor response was studied in a group of healthy volunteers and five groups of asthmatic patients (table). Healthy subjects were recruited from hospital staff, none of whom were taking any form of corticosteroid therapy. All patients with asthma had a history of variable dyspnoca and wheeze, more than 20% diurnal variation in peak expiratory flow (PEF), and more than 15% improvement in PEF or FEV, (minimum improvement 200 ml) in response to inhaled beta-2-agonists. Atopic status, smoking history, family history of asthma, number of years with symptoms, and highest achieved FEV, in the preceding 2 years were recorded. Atopy was defined, by means of skinprick tests to a range of allergens including housedust mite, grass pollens, animal danders, and Aspergillus funigatus, as two or more reactions with a weal diameter more than 2 mm greater than the control test.

Patients with mild asthma had few symptoms and normal lung function. One group of such patients (n = 12) were taking intermittent inhaled beta agonists as sole therapy, patients (n = 14) in the other group were also taking regular inhaled corticosteroids.

(up to 2 mg beclomethasone dipropionate or budesonide daily). All of 46 patients with asthma, whether sensitive (CS; n=31) or resistant (CR; n=15) to corticosteroids, were taking inhaled corticosteroids. 8 CR patients were also taking long-term prednisolone (median 7·5 [range 5–10] mg daily; median duration of therapy 8 years), compared with only 4 CS patients. To control for possible influences of long-term systemic corticosteroid therapy, another group of asthmatic patients taking long-term prednisolone was studied. These patients either had mild airways obstruction (most were taking prednisolone for allergic bronchopulmonary aspergillosis) or their response to prednisolone had not been formally assessed. Their median dose of prednisolone (7 mg daily) and median duration of treatment (10 years) were similar to those of the CR patients taking prednisolone.

Patients with airways obstruction (FEV, below 70% predicted) in the outpatient clinic underwent a 2-week trial of oral prednisolone to determine the corticosteroid responsiveness of their asthma. Before treatment started, PEF was monitored three times daily for a week. In addition to their usual treatment, patients then took 20 mg prednisolone daily for 1 week, followed by 40 mg daily for a second week. FEV, was measured before treatment started and at the end of each treatment week; PEF monitoring continued throughout the trial. Mean PEF during the last 2 days of the baseline week was compared with that during the last 2 days of each of the treatment weeks. Corticosteroid resistance was defined as failure of initial FEV, and mean peak flow to improve by at least 15% after 2 weeks of treatment. This design allowed CS patients to be subdivided into those who were more responsive (more than 15% improvement in lung function after 1 week) or less responsive (more than 15% improvement seen only after longer duration or higher dose) to prednisolone.

Beclomethasone dipropionate was dissolved in 95% ethanol to concentrations between 1 µg/ml and 100 µg/ml. Solutions were stored at 4°C and used within 2 weeks of preparation. Preliminary tests established that there was no change in topical potency over this time. The vasoconstrictor assay was a modification of the method of McKenzie and Stoughton.¹ The subject's forearm was shaved if necessary and test sites were outlined by application of double-sided adhesive tape in which holes, 2 cm in diameter, had been cut. Test concentrations of beclomethasone dipropionate were applied to the skin. 10 µl to each site. After evaporation of the diluent, the sites were covered with plastic film ('Saran' wrap or polythene) to enhance percutaneous absorption of glucocorticoid.

Preliminary experiments in healthy volunteers confirmed that blanching was greatest 16–18 h after application of glucocorticoid. Solutions were therefore applied in the afternoon, the sites were occluded overnight, and the adhesive tape and plastic film removed the next morning. The degree of blanching was assessed 2 h later; this step allowed any tape-related erythema to resolve. Assessment was made under standard fluorescent lighting conditions by a trained observer who was unaware of the steroid responsiveness of the patient's asthma. Blanching was graded according to a 5 point scale: 0 = normal skin, 1 = faint blanching, 2 = obvious blanching, 3 = intense blanching, and 4 = intense blanching extending beyond the area to which solution had been applied. In patients who underwent a corticosteroid trial, skin testing was done before or several weeks or months after the trial.

Initially, the vasoconstrictor response was assessed in young, healthy volunteers, with concentrations of 0 μ g ml, 1 μ g/ml, 3 μ g/ml, and 10 μ g/ml. Asthmatic patients were then tested with these concentrations. Subjects in whom no blanching was detectable were retested with concentrations of 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 100 μ g/ml. Latterly, concentrations of 3 μ g/ml, 10 μ g/ml, 30 μ g/ml, and 100 μ g/ml were used.

All subjects gave informed consent and the study was approved by the Lothian Region Ethics Committee.

The numbers of patients in the study groups who showed detectable blanching at each concentration were compared by means of chi-square tests with Yates' correction. This test was also used to compare groups for atopy, smoking, and family history. Lung function data were expressed as percent predicted for age, height, and sex. Two-sample *t* tests were used for comparisons of

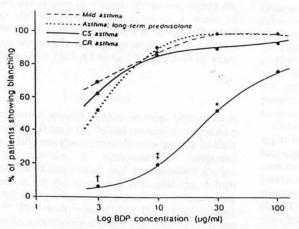


Fig 1—Proportion of asthmatic adults showing any degree of cutaneous vasoconstrictor response to beclomethasone dipropionate (BDP).

 χ^2 tests for CR vs CS: *p<0.02; tp<0.01; tp<0.001.

lung function and duration of asthma and Mann-Whitney U tests for intensity of blanching in response to a given concentration of beclomethasone dipropionate.

Results

A concentration of 3 µg/ml beclomethasone dipropionate produced blanching in most healthy volunteers. 10 µg/ml produced detectable blanching in 52 (90%) of 58 volunteers. Absence of blanching was reproducible at this concentration in the remaining 6 when they were retested with 10 µg/ml, 20 µg/ml, 30 µg/ml, and 100 µg/ml. 3 of these subjects repeatedly showed no blanching with any concentration.

16 healthy subjects who showed a positive response underwent repeat tests each month for 6 months. 15 (94%) showed consistent responses to beclomethasone dipropionate concentrations of 10 µg/ml or higher; 1 subject had five positive responses and one negative response to 10 µg/ml. At 3 µg/ml consistently positive responses were obtained in 14 (88%) subjects.

The vasoconstrictor response was similar in the patients with mild asthma whether they were taking intermittent bronchodilators or inhaled corticosteroids. These groups were therefore combined for expression of results. In the CS asthma group, there was no difference in blanching between patients whose airways obstruction improved after 1 week of prednisolone and those who responded after 2 weeks. The proportion of patients who showed any degree of blanching at each concentration of beclomethasone dipropionate was similar in the mild asthma, CS, and long-term prednisolone groups and in healthy subjects. The proportion of CR patients who showed blanching was significantly lower at all concentrations except 100 µg/ml (fig 1).

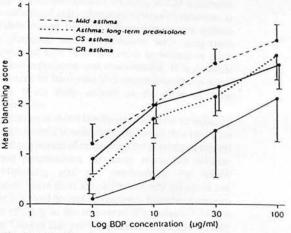


Fig 2—Intensity of cutaneous vasoconstrictor response to beclomethasone dipropionate (BDP) in asthmatic adults.

Error bars indicate 95% confidence limits.

Virtually all subjects showed dose-related vasoconstrictor response to beclomethasone dipropionate (fig 2). There was no significant difference at any concentration between the CS group and the mild asthma or long-term prednisolone group. In comparison with the mild and CS asthmatic groups, median blanching intensity was significantly lower in CR patients at 3 µg/ml (p < 0.001), 10 $\mu g/ml (p < 0.001)$, 30 $\mu g/ml (p < 0.05)$, but not at 100 $\mu g/ml$. In comparison with the prednisolone group, blanching in the CR group was significantly lower (p < 0.01) only at 10 $\mu g/ml$, though the differences at 3 $\mu g/ml$ (p = 0.08) and 30 $\mu g/ml (p = 0.13)$ approached significance. The prednisolone and mild asthma groups differed significantly only at a concentration of 30 µg/ml (p < 0.01).

Mean FEV₁ (% predicted) was slightly low in CS asthmatic subjects (82% [95% CI 77–87%]) and in those taking long-term prednisolone (77%[58–97%]). Airways obstruction was severe in the CR asthmatic group (54%, range 20–80%); the difference between the CS and CR groups was highly significant (p < 0.001).

At the start of the trial of corticosteroids, mean FEV,% predicted was 50% (95% CI 44-56%) in the CS group and 41% (31-51%) in CR patients. After the trial, FEV,% predicted improved to 73% (65-80%) in the CS group but it was unchanged (39% [29-49%]) in the CR group.

There were no significant differences among the patient groups as regards atopy, smoking, or family history of asthma (table). The duration of symptoms in the mild asthma group was shorter (mean 12·3 [95% CI 7·6-17 0] years) than in the other groups (p < 0·01). There were no significant differences among these groups in the duration of asthma, though the difference between CR and CS patients

CLINICAL CHARACTERISTICS OF STUDY PATIENTS

	n	Mean age (range) in yr	M/F	Mean FEV, (95% CI) as % predicted	No (%) current smokers	No (%) atopic	No (%) with family history	Mean time with asthma (range) in ye
Mild asthma				200 (4)	81 565	1 100 March 1		
Inhaled B, agonist alone	12	43-0 (21-63)	7/5	94 (87–101)	1 (8%)	8 167%	5 (42%)	11 7 (1-50)
Inhaled stemid	14	44 1 (19-72)	4:10	91 (82-101)	117%1	10 /71%	8 157%1	13 2 (2-30)
Asthma							was a	100 mm canage m
Long-term prednisokne	13	547(29-71)	5/8	77 (58-97)	3 (23%)	9 169%	4/3/%/	29 8 (8-55)
CS	31	49 1 (18-69)	15 16	82 (77-87)	8 1 26% 1	25 181%	14 145%1	23 2 (1-54)
CR	15	45 6 (27-66)	8,7	54 (44-64)	4 . 27%	11 .77% -	7 147%	32 9 (3-57
Normal subjects	58	35 2 (16-50)	13:25		THE SECTION OF THE SE			

approached significance (p = 0.063). However, within the CS group the 10 patients who required 2 weeks of prednisolone to show an improvement in airway calibre had had asthma longer than the 21 who responded to 1 week's prednisolone (33-3 [18-5-47-5] $v_{\rm S}$ 18-5 [12-2-24-8] years; p < 0-05).

Discussion

Glucocorticoids applied to skin undergo percutaneous absorption and influence the dermal vasculature to produce blanching. Different glucocorticoids vary greatly in their ability to provoke a vasoconstrictor response; beclomethasone dipropionate⁹ and budesonide¹⁰ are the most potent of the glucocorticoids in clinical use. A high degree of blanching reflects high topical anti-inflammatory potency. This study shows that the vasoconstrictor response to beclomethasone dipropionate is lower in asthma patients whose airways obstruction is resistant to a short course of oral corticosteroid than in CS patients.

Bioassay of topical corticosteroid formulations by the vasoconstrictor response is sensitive, precise, and reproducible.^{8,11} Variation in the degree of blanching occurs among subjects,^{12,13} but it has been assumed to be due to differences in absorption through the skin. Responses in an individual do, however, seem to be consistent from assay to assay.¹² Blanching may be detectable a few hours after application of glucocorticoid and some investigators assess the response at 6–7 h.^{8,14} The maximum response usually occurs around 18 h.⁸ Although response can be assessed more precisely by means of several assessments at different times,^{8,10} a single reading at around 18 h is satisfactory.^{11,15}

A trained eye is required to detect minor degrees of blanching, and the arm must be carefully inspected under standard lighting conditions. Although interobserver agreement on blanching scores seems good, the use of a subjective method of assessment has been criticised.¹³ An objective method might be preferable, but such techniques as reflectance spectrophotometry, doppler laser, or thermography have not been proven superior.¹³

In our study, long-term treatment with inhaled beclomethasone dipropionate or budesonide (in either low or high doses) did not affect the vasoconstrictor response. In patients taking long-term prednisolone, there was a tendency for the blanching dose-response curve to be shifted to the right but there was no difference in the proportion of patients who showed no blanching between this group and the mild asthma and CS groups. The CR asthma group tended to have more severe symptoms than patients in the other groups. However, their reduced skin response is unlikely to be due to greater use of beta agonist drugs, which have relaxant effects on vascular smooth muscle. We have not observed reduced skin blanching in patients receiving regular nebulised salbutamol in high doses for exacerbations of asthma. Treatment with other drugs, such as xanthine derivatives, did not differ between CS and CR patients. It is possible that the lifetime dose of prednisolone was higher in CR patients, but it is impossible to estimate this exposure accurately

The CR asthma patients showed evidence of a degree of fixed airways obstruction, the best recorded FEV₁ in the 2 years before the study was only 54% predicted (range 20–80%) Nevertheless, variation in FEV₁ within a patient was great in many cases, and all of the patients showed a response to beta agonists. Our observation that the blanching response is preserved in patients with acute

asthma suggest that the reduced response in CR asthmatic subjects is not related to the degree of airways obstruction at the time of skin testing. In some CR patients, severe asthma symptoms persisted despite treatment with long-term prednisolone, and as a result attempts to withdraw such treatment were infrequent and unsuccessful. It is possible that any element of corticosteroid responsiveness in these patients was being fully utilised at the time of their corticosteroid trials.

The mechanism of skin blanching in response to topically applied glucocorticoid is unknown. Although this response has been used as a means of comparing the potency of topical corticosteroid preparations for many years, the relation between blanching and the mechanisms by which glucocorticoids exert their anti-inflammatory effects is not clear. There is a good correlation between blanching activity and clinical efficacy in the treatment of psoriasis.15 Marks and Sawyer16 found that prior application of glucocorticoid to skin reduced the erythema resulting from subsequent application of arachidonic acid and histamine and postulated that inhibition of phospholipase A, activity might be involved in the mechanism of blanching. Glucocorticoids induce lipocortin 1, which inhibits the activity of phospholipase A₂.^{17,18} Concentrations of antibody to lipocortin do not differ between CS and CR asthma patients,19 though Busse20 suggested the possibility of a type 2 error in that study. Glucocorticoids also increase the sensitivity of vascular smooth muscle to the vasoconstrictor effects of noradrenaline, an effect reversed by infusions of arachidonic acid and prostacyclin.21 Although this effect could explain the blanching response, there are many other mediators of vascular tone which may themselves be affected by glucocorticoids.22

Although the mechanism of blanching is unknown, the response seems to be mediated through the glucocorticoid receptor, since blanching can be abolished by oral administration of the glucocorticoid receptor antagonist RU 486.²³ We have observed that prior application of this compound to the skin prevents the vasoconstrictor response to beclomethasone dipropionate. Budesonide has very high affinity for the glucocorticoid receptor²³ and for a given concentration produces the greatest degree of blanching ⁹

Glucocorticoids are used in many inflammatory disorders, and individual patients show differing responses to treatment. Relative corticosteroid resistance is not confined to asthma and, although variation in response may be due to differences in severity of the underlying disease process, it could also be explained by congenital or acquired defects in the ability of cells to respond to endogenous or exogenous glucocorticoids. Further investigation of skin resistance to glucocorticoid action may help explain why some patients with asthma, or other disorders treated with glucocorticoids, show a poor response to such therapy.

P. H. B. thanks Allen and Hanburys Ltd and Astra Pharmaceuticals for financial support. S. T. was Trinidad and Tobago Commonwealth medical scholar under the auspices of the Association of Commonwealth Universities. We thank Dr. R. A. Elton (Department of Medical Statistics, University of Edinburgh) for his helpful advice.

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Surfactant apoprotein-A concentration in sputum for diagnosis of pulmonary alveolar proteinosis

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Pulmonary alveolar proteinosis (PAP), a disease characterised by accumulation of surfactant in alveoli, is diagnosed on the basis of invasive biopsy procedures. We have measured apoprotein A (SP-A) concentrations in sputum to see if this is useful for the diagnosis of PAP. Sputum samples from three patients with PAP and twenty patients with other pulmonary disease were assayed using antibodies SP-A. monoclonal to concentrations were 400 times higher in patients with PAP than in the controls, suggestions that this measurement is useful for the diagnosis of PAP, especially where lung biopsy is contraindicated.

Lancet 1991; 337: 580-82

Introduction

Pulmonary alveolar proteinosis (PAP) is a rare disease of unknown actiology that is characterised by accumulation of surfactant materials in alveoli. Impaired alveolar clearance of surfactant due to abnormal function of alveolar macrophages has been implicated. The definitive diagnosis rests on histological findings after open-chest or transbronchial lung biopsy. Diagnosis by microscopic or chemical analysis of fluid from bronchoalveolar lavage^{3.5} (which is also an invasive procedure) has yet to be established. Surfactant-associated 35 kD protein (surfactant apoprotein A or SP-A) is the predominant phospholipid-associated glycoprotein in pulmonary surfactant and is specific for lung surfactant. Accumulated surfactant in alveoli in PAP contains abundant SP-A. Monoclonal antibodies to SP-A⁷ have been used to measure surfactant

concentration in amniotic fluid for the diagnosis of respiratory-distress syndrome.* ¹¹ We have used them to assay SP-A concentrations in sputum from patients with PAP.

Material and methods

Samples

Over the past 3 years we have seen three patients with PAP diagnosed by transbronchial biopsy (table 1). They had exertional dyspnoea and hypoxaemia but had no history indicating secondary alveolar proteinosis.12 They all expectorated sputum, especially in the early morning, before treatment with lung lavage. After lavage, symptoms and laboratory and chest X-ray findings improved and they expectorated little or no sputum. Ten sputum samples (0.5-2 ml) were collected on different mornings before transbronchial biopsy or lung lavage; care was taken to avoid mixing saliva with sputum. As control material single mucoid and mucopurulent sputum samples from twenty patients with other pulmonary diseases (eleven males, mean age 59 [SE 7] years) were collected The control patients had chronic bronchitis, bronchial asthma. chronic pulmonary emphysema, idiopathic pulmonary fibrosis, pneumonia, or lung carcinoma. These thirty sputum samples plus five saliva samples from the PAP patients and a bronchoalveolar lavage fluid sample from patient 2 in table (were assayed for SP-A concentration.

0.5 ml of sample and 2 ml of 10 mmol.1 "tris" HCl buffer (pH 7.4) containing 1 mmol.1 EDTA was homogenised for 1 min in an ice-cold bath (ultradisperser model-LK-21, Yamato, Tokyo). The homogenate was diluted by 0.6% sodium dodecyl sulphate. SDS)

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transferrable elements that do not resemble conventional plasmids⁵ have been isolated from these TriR/MuR S aureus transcipients, and attempts to clone the resistance are underway. There is no cross-resistance to propamidine, benzalkonium chloride, cetrimide, hexachlorophane, chlorhexidine acetate, chlorhexidine gluconate, or 9-aminoacridine. Our initial experiments indicate that the presence of TriR does not influence the rate of kill after 30 s to 2 min of exposure to triclosan. Similar experiments have suggested that the same is true for MRSA with low-level chlorhexidine resistance.

However, the possibility of a rise in MIC to triclosan should be considered if eradication of MRSA is difficult with this agent, and we would be grateful if such strains were sent to the division of hospital infection for further study.

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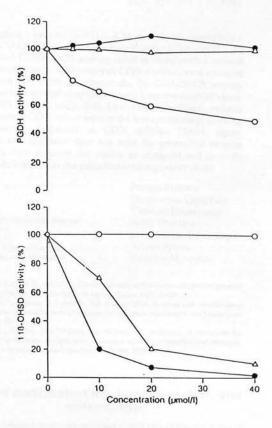
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Liquorice

SIR,-Dr Baker and Dr Fanestil (Feb 16, p 428) point out the structural homology between the prostaglandin metabolising enzyme, 15-hydroxyprostaglandin dehydrogenase (PGDH) and 11 β-hydroxysteroid dehydrogenase (11β-OHSD), which converts cortisol to its inactive product cortisone. We have previously shown that liquorice derivatives are potent inhibitors of 11B-OHSD. Baker and Fanestil speculate that by inhibiting PGDH, liquorice might, in part, exert its biological effects through prostaglandins. Their suggestion is based on data from Peskar et al' who showed inhibition of gastric prostaglandin metabolism by high concentrations of

Liquorice and its derivatives possess both mineralocorticoid and glucocorticoid properties. The mechanism of these actions was poorly understood, although the presence of an intact adrenal gland or cortisol replacement in patients with adrenal insufficiency was known to be essential.23 We found that glycyrrhetinic acid, the active component of liquorice, is a potent inhibitor of 11B-OHSD in vitro; in-vivo studies showed that this inhibition could account for both the mineralocorticoid and glucocorticoid effects of liquorice.45 To assess whether altered prostaglandin metabolism, as suggested by Baker and Fanestil, might contribute to these biological actions, we examined the effects of glycyrrhetinic acid and carbenoxolone on PGDH and 11B-OHSD activity in vitro. Human placental tissue was chosen because it is a rich source of both 11β-OHSD and PGDH.

Chorionic homogenate (protein content 1.5 mg) was incubated with 500 ng PGE, and 1 mmol/l NAD at 37°C for 15 min in a phosphate buffer containing 20% glycerol (pH 8.4). PGDH activity was measured by radioimmunoassay of the quantity of 15-keto-PGE, produced after transformation to its methyl oxime. The experiment was completed with glycyrrhetinic acid, carbenoxolone, and 5-(4-fluorobenzoyl)2-hydroxybenzene acetic acid (FHBA), a known PGDH inhibitor. Substances were tested at concentrations up to 40 µmol/l. The effects of similar concentrations of these substances on human placental 11B-OHSD were evaluated as previously described. Briefly, 0.3 mg protein from fresh placental homogenate was incubated with 12 mmol/l 3H-cortisol and 200 µmol/I NAD in Krebs-Ringer buffer at 37°C for 1 h. Steroids were extracted with ethyl acetate and separated by thin-layer chromatography. Activity of 11ß-OHSD was expressed as a



Effects of liquorice on enzymes of prostaglandin and steroid metabolism.

Upper, effects of glycyrrhetinic acid, carbenoxolone, and FHBA on 15-hydroxyprostaglandin dehydrogenase activity expressed as percentage of control; lower, effects of the same substances on 11B-hydroxysteroid dehydrogenase. . , glycyrrhetinic acid; . , carbenoxolone; (), FHBA.

percentage conversion of 'H-cortisol to 'H-cortisone. The results show that glycyrrhetinic acid and carbenoxolone had no effect on PGDH activity, in contrast to the inhibition produced by FHBA (figure, upper). FHBA had no effect on 11B-OHSD activity whereas carbenoxolone and glycyrrhetinic acid were inhibitory (figure, lower).

Liquorice ingestion has been shown to produce mineralocorticoid effects even when plasma glycyrrhetinic acid concentrations are below 2 µmol/l, the lower limit of detection of the assay.5 This evidence, together with the current findings, makes it unlikely that altered prostaglandin metabolism contributes to the salt-retaining or anti-inflammatory actions of liquorice and its derivatives.

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comparing them with cyclosporin both for efficacy and side-effects (notably nephrotoxicity, which is not exclusive to cyclosporin) are needed before we can say whether their therapeutic indices are indeed better than that for cyclosporin. In vitro minimum active concentrations do not by themselves define the therapeutic index. Furthermore new agents may still carry the risk of overimmunosuppression and will not solve a major problem encountered with all these agents in most patients-namely, disease relapse after cessation of treatment. The answer may come from new approaches fully discussed at the Paris meeting-namely, monoclonal antibodies, autoantigen peptide therapy, T cell vaccination, and so on. Immunointervention is not just a matter of selecting the best immunosuppressive drug but searching for the best strategy that allows us to slow down or stop the autoimmune process in a non-toxic, disease-specific, and long-term fashion.

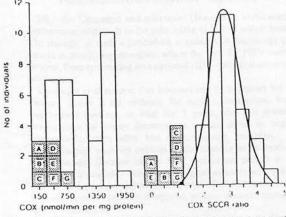
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Assessment of the mitochondrial respiratory chain

SIR,-Trounce et al' have suggested mutochondrial respiratory failure as a possible cause of ageing in man. However, the frequent scarter of control data and their overlap with those of patients limits the ability of clinicians to reach a reliable diagnosis of respiratory chain deficiencies (figure, left).23 However, we have recently observed that a constant ratio of respiratory enzyme activities is a consistent feature of oxidative phosphorylauon, irrespective of the ussue tested. This finding may be because a balanced proportion of each enzyme complex is required for optumum function of the respiratory chain. Our observation prompted us to present enzyme activities as ratios rather than absolute values



Mitochondrial enzyme activities as both absolute values and ratios

11 = patients | i = controls

We studied 7 patients (A-G) and 34 controls for cytochrome c oxidase (COX) activity, succinate-cytochrome c reductase (SCCR) activity, and COX/SCCR activity ratios in mitochondria isolated from skeletal muscle. While normal COX activities were scattered over almost two orders of magnitude, the COX/SCCR activity ratios showed a normal distribution with a narrow range of values (mean [SD] 2.9 [0.6]; range 2-4). Under these conditions, patients whose absolute COX values were in the low normal range could be unambiguously diagnosed as COX deficient (figure, right). Although this approach does not hold for generalised enzyme defects, it does improve the ability to recognise and quantify localised deficiencies in the mitochondrial respiratory chain.

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Inhaled corticosteroids, bone formation, and osteocalcin

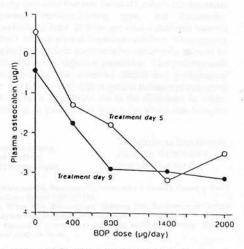
SIR,-Inhaled corticosteroids are now a part of the treatment of asthma. Low dosages of beclomethasone dipropionate (BDP), devoid of effects upon the hypothalamic-pituitary-adrenal axis, can control asthma.12 However, there is concern now that higher dosages are in use. A finding of lowered serum osteocalcin in individuals inhaling BDP 2000 µg daily suggests that high-dose therapy might be associated with adverse effects upon bone. As part of a larger study evaluating the systemic effects of inhaled BDP we measured osteocalcin across treatments using doses of BDP within the currently recommended therapeutic range (400-2000 µg daily).

We studied 16 healthy adult volunteers, using a randomised double-blind, placebo-controlled crossover design to evaluate the systemic effects of 100, 200, 350, and 500 µg BDP administered by standard metered-dose inhaler every 6 h for 10 days. Wash-out intervals of 18 days separated each treatment period. Plasma for osteocalcin measurement was collected on a fasting specimen taker: within 30 min of 0900 h 4 days before starting treatment and repeated at the beginning of the fifth and ninth days of each treatment period. Plasma octeocalcin was measured by an in-house radioimmunoassay and each individual's samples were assayed in the same batch. The difference from pre-treatment concentration at both days of testing during treatment was analysed by a three-way analysis of variance with factors subject, dose, and phase. The hypothesis of no-treatment effect was tested by a two sided i-test at each dose

During dosing with placebo plasma osteocalcin remained constant. During treatment with BDP there was a significant fall relative to placebo even at the lowest dose of 400 µg daily on treatment day 5. This effect was dose-dependent up to 1400 µg with a plateau thereafter (figure). With more prolonged treatment the plateau occurred at an even lower dose so that at day 9 of treatment plasma osteocalcin was lowered to the same extent by 800, 1400, and

2000 µg dosages (figure).

Osteoporosis is a sequel to chronic systemic glucocorticoid therapy. This occurs primarily as a result of reduced bone formation which is reflected by reduced osteocalcin values in patients on such therapy.* The finding of reduced osteocalcin in adults inhaling BDP 2000 µg daily is not too surprising because at such high dose systemic glucocorticoid effects can be expected. We have now shown that BDP even at a dose of 400 jig per day is also associated with reduced osteocalcin values. This raises the possibility that even



Plasma osteocalcin values, expressed as change from baseline.

low dosages of this inhaled corticosteroid might reduce bone formation.

Whether inhaled corticosteroids do adversely affect growth in asthmatic children remains a contentious issue. It has been assumed that paediatric dosages of BDP are unlikely to have such an effect. Whereas the clinical relevance of our current findings are yet to be established, it is important to note that we have demonstrated reduced bone formation in adults at what may be regarded a standard paediatric dose of BDP 400 µg daily.

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Revaccination against hepatitis B

SIR,—Dr Coursaget and colleagues (May 18, p 1180) suggest a cumbersome approach to the scheduling of HBV vaccine boosters. The thought of such a procedure is enough to discourage policy makers in developing countries, where the need for HBV vaccine is greatest, from embarking on a national HBV vaccine immunisation programme.

Coursaget et al assume that boosters will be necessary for HBV vaccine. There is no evidence for such a conclusion. Indeed, long-term follow-up, at least for 7 years, did not reveal any development of severe hepatitis or carrier states in high-risk homosexuals, even after loss of circulating anti-HBs. Thus immunological memory persists in the absence of antibody after immunisation. Because of the long incubation period in HBV infection it is probable that immunological memory will result in a prompt and strong anamnestic response after exposure, and that the will provide protection against serious disease, though not against reinfection.²

Certainly continued surveillance is needed to evaluate this hypothesis, particularly in populations immunised at birth or in

infancy. If such surveillance eventually reveals a need for boosters it would be more practical, in most parts of the world, to administer these at a fixed age—eg, at entry to school—than to individualise the time of boosting based on quantification of individual immune responses.

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Hadler SC, Francis DP, Maynard JE, et al. Long-term immunogenicity and efficacy
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SIR,—Dr Coursaget and colleagues' mathematical model for antibody kinetics after hepatitis B vaccination is based on anti-HBs titres measured at the time of booster vaccination, and does not require anti-HBs measurement 1 month later. This model could provide an estimate of the duration of protection in vaccination campaigns in which post-booster antibody measurements were not possible or were not planned for. In industrialised countries, however, post-booster anti-HBs titres are often available, and a less complex formula is desirable to more easily estimate the duration of protection in individuals.

TABLE I—ANTI-HBs CONCENTRATIONS (POST-BOOSTER TO AT-BOOSTER RATIO) WITH RESPECT TO AT-BOOSTER TITRES (MONTH 0)

Anti-HBs at booster (mo 0)				
IU/I	No	GMT (IU/I)	Anti-HBs ratio (SD) (mo 1/mo 0)	
10-99	28	40	197 (23-1700)	
100-999	94	256	111 (20-614)	
1000-9999	17	2180	20 (8-50)	

GMT = geometric mean titre

Our data^{1,2} obtained from anti-HBs measurements at booster vaccination 1 month later (n = 139) (table 1) and up to 4 years later (n = 53, 4 anti-HBs measurements in each) (table 11) indicate that the anti-HBs increase from at-booster to post-booster titres and the decline later on can be described by two formulas:

- 1: titre at 1 month post booster = 1700 × titre at booster 44
- 2: titre of sample = titre at 1 month post booster × time since booster (in months) -09.

Our formulae differ from that of Coursaget et al in that the exponent to titre at booster (formula 1) is slightly lower and the correction factor in formula 1 is five times higher than theirs $(1700 \times 30 \text{ days/month} = 51 000 \text{ us} 10 000)$. These variables will be determined by the vaccination scheme and dosage, age, immune status, or various random factors and would have to be established for each immunisation protocol.

However, reliance on a constant decline of titre after the 1-month post-booster anti-HBs measurement would eliminate the uncertainties associated with the effects of booster vaccination. We

TABLE II—ANTI-HBs CONCENTRATIONS AT BOOSTER AND UP TO 4 YEARS AFTERWARDS

Anti-HBs (IU/I)		Anti-HBs	Anti-HBs GMTs (IU/I) after booster vaccination (mo)					
at booster (mo 0)	No	GMT (IU/I) at booster	1	17	25	37	49	
10-99	12	45	10 839	861	571	522	292	
100-999	34	233	33 266	2972	1746	1330	925	
1000-9999	7	2748	79 068	4316	3428	2799	2051	

Antibody decline in sample taken later than 1 month post booster is independent of titre. I month post booster but is inversely proportional to time (months) since booster vaccination. Only vaccinees for whom all 5 samples were available for anti-HBs tritation were included.

preliminary study indicated that two forms of Crohn's disease exist: lipocortin-depleted, non-perforating type, and lipocortin-maintained, perforating type. If these are indeed different clinical forms of Crohn's disease several implications follow. Therapeutic trials for prophylaxis against postoperative recurrence should be targeted specifically to the high-risk population. The pathogenetic pathway accounting for the observed clinical and pathological differences should be analysed with respect to the natural history of this disease. Olaison et al indicate one of the directions in which dinicians should go to gain further knowledge about this complex disease.

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Efficacy of quinine-tetracycline for acute uncomplicated falciparum malaria in Thailand

SIR,—Several studies in Thailand during 1972–73 showed that the efficacy of short courses of quinine could be improved when tetracycline was also used. ¹² Quinine was given for 1–3 days and tetracycline for 3–10 days. Although treatment with both drugs for 3 days provided satisfactory cure rates until about 1980, since that time combined treatment with quinine and tetracycline for 7 days has become standard hospital treatment for adults in Thailand. ³ At the Bangkok Hospital for Tropical Diseases the cure rate of quinine plus tetracycline was 99.5% in 1981–844 and 100% in 1987, ⁵ since when there have been no reports of these cure rates in Thailand. During 1990–91, the response to treatment with quinine and tetracycline was evaluated in 76 adults with acute uncomplicated falciparum malaria at that hospital.

All patients had symptoms and the geometric mean asexual parasite count before treatment was 16 268/µl (range 1020–179 760). Each patient was treated orally with quinine sulphate 600 mg given every 8 h and tetracycline 250 mg every 6 h for 7 days. 70 patients were followed in hospital for 28 days. 6 patients dropped out for reasons not related to drug administration. Within 48 h after treatment 85% of patients showed clinical improvement and malaria symptoms had subsided. The mean fever dearance time was 68 h.

Parasite counts were done every 12 h during parasitaemia and then once daily for 28 days. After starting treatment, 24 patients had at least one parasite count higher than the initial count. The parasite dearance time was 89 h. 63 of 70 (90%) patients followed for 28 days in hospital were cured. 6 patients had recrudescences of their infections between 17 and 28 days after treatment (RI responses). These patients were successfully treated with either mefloquine alone or sequential treatment with sodium artesunate (a derivative of artemisinin) and mefloquine. I patient remained parasitaemic after 7 days and was thought to have an RII response to treatment. This patient had parasitaemia of 24 900/µl before treatment, 470/µl on day 7, and was negative on day 8 without further treatment. However, he had a recrudescence of falciparum infection 46 days after treatment even though after discharge he remained in Bangkok where there is no malaria transmission. He was successfully treated with a sequential course of artesunate and mefloquine.

45 patients (59%) had nausea and 30 (39%) had vomiting (1-6 h after dosing) during the first 48 h of treatment; these symptoms usually coincided with peak in fever. All patients had symptoms of cinchonism that started on the second day and persisted throughout treatment. Parasitological response was compared between the 6 patients who had RI responses and the 63 patients who were cured. The initial parasite counts before treatment and fever clearance times between the two groups was similar, but parasite clearance times of the patients whose infections subsequently recrudesced were significantly longer than those who were cured (120 h vs 86 h;

p = 0·01). Parasites from 8 patients were tested in vitro for quinine susceptibility with a microdilution radioisotope method. The mean 50% inhibitory concentration (IC_{50}) for pretreatment isolates successfully treated with quinine-tetracycline was 183·8 ng/ml (n = 5); although the mean IC_{50} from RI failures was higher (233·2 ng/ml, n = 3), the difference was not significant.

These results indicate that a 7-day regimen of quinine and tetracycline is still satisfactory for multidrug-resistant falciparum malaria in Thailand. However, a gradual increasing recrudescence rate and in vitro findings suggest that quinine-tetracycline is under increasing pressure and the failure rate may soon rise beyond 10%. New drugs or drug combinations are urgently needed to combat the rising parasite resistance in Thailand and other areas of the world where resistance is increasing.

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Beclomethasone and osteocalcin

SIR,—We have described previously (July 6, p 60) a substantial decrease in plasma osteocalcin (a marker of bone formation) in healthy volunteers after inhalation of beclomethasone 400 µg daily for 5 days. Peretz and Bourdoux (Nov 23, p 1340) subsequently reported their studies in which no change in osteocalcin was seen in 10 volunteers given inhaled beclomethasone for a week (1000 µg daily). These researchers described our results as questionable.

Peretz and Bourdoux used a commercial radioimmunoassay for the measurement of serum osteocalcin ('INCSTAR', Stillwater, Minnesota, USA). We measured plasma osteocalcin by an in-house radioimmunoassay with an antibody raised in rabbits against purified bovine osteocalcin (reference range 2-12 µg/l; interassay coefficient of variation 12%). Osteocalcin was purified and the radioimmunosassay set up as Price and Nishimoto described.1 Plasma osteocalcin concentrations can vary greatly depending upon the antibody used in the assay,2 so it is not appropriate to compare changes in osteocalcin concentrations measured by different assays, as Peretz and Bourdoux have done. The more valid comparison is between beclomethasone treatment and placebo treatment with the same assay for the measurement of osteocalcin. Our study design was randomised, placebo-controlled, double-blind cross-over (more rigorous than that of Peretz and Bourdoux), and all samples from a specific subject were run in the same batch. We were able to

EFFECTS OF INCREASING DOSES OF INHALED BECLAMETHASONE DIPROPRIONATE ON PLASMA CONCENTRATIONS OF OSTEOCALCIN

Daily dose (µg)	Baseline	Day 5	Day 9	
Placebo	8-9 (2-7)	8-9 (3-4)	8-0 (3-4)	
400	8-9 (3-1)	7.8 (2.9)*	7-2 (3-1)	
800	9.4 (5)	7-4 (3-4)*	6-6 (2-9)*	
1400	9-6 (3-5)	6-2 (2-6)1	6-2 (2-9)*	
2000	9-1 (3-5)	6-4 (2-7)1	5-0 (1-8)*	

Statistical comparison is against baseline value for each dose. Values = mean (SD)

*=p<0.05 t=p<0.001 t=p<0.005

show a dose-dependent effect of beclomethasone on plasma osteocalcin from 400-2000 µg daily with no changes on the various placebo washout periods between. Support for our data comes from Meeran and colleagues¹² observations, presented at the 1991 American Thoracic Association meeting, which showed that 1000 µg of beclomethasone reduced circulating osteocalcin concentrations.

It is always difficult to give all information in a short letter, but Peretz and Bourdoux seem to have missed our details of sampling conditions (fasting samples taken within 30 min of 0900 h). The table shows the absolute values for osteocalcin concentrations. In the figure in our previous publication osteocalcin at 0 dose of beclomethasone (placebo) was expressed as a change in osteocalcin between days 5 or 9 and the pretreatment (or day 0) osteocalcin concentration—this explains the different starting points.

Controversy is valuable in scientific research, but we agree with Peretz and Bourdoux about the need for long-term clinical studies.

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Bone-density measurement

SIR,—The first issue of *Effective Health Care*, a bulletin sponsored by the UK Department of Health, is on population-based bone screening (Jan 18, p 174). This publication raises several issues of concern to clinicians. The report, from the School of Public Health in Leeds, looked for published evidence in support of population screening to prevent fractures in elderly women. The conclusion that screening is not justified at present seems non-controversial—indeed the consensus is such that we wonder why and by whom the study was commissioned. The Leeds group claims that UK health authorities are under pressure to introduce population-based screening, but we know of no group, lay or professional, that is trying to do this. Unfortunately, coverage of this report in the lay and medical press has suggested that bone-density measurement itself is not worthwhile but the report did not purport to examine the non-screening, clinical use of bone densitometry.

Some of the controversy raised by Effective Health Care is due to inappropriate terminology. "Population-based screening" is clear but the use of "screening" when referring to opportunistic measurement of bone mineral density (BMD) is more difficult. National Health Service hospitals that have BMD equipment are in a position to respond to doctors who ask for this investigation for their patients. The clinical indications for this have been published in the USA," and an equivalent document for the UK would be welcome. The report concludes that there have been no scientific trials to assess population screening, but does not mention trials in Hull and in Aberdeen that began in 1990. Had the authors of the report contacted these two centres they would not have stated that the uptake of screening will be low, since in Hull and in Aberdeen uptake is 75% or more.

The report relies heavily on "grey literature" not published in referred journals, and it is disturbing that the final (and correct) conclusion that population screening is presently unwarranted should have been misinterpreted by some commentators as calling into question the value of BMD measurement. Four large prospective, epidemiological studies show that a low BMD predicts fracture risk? 3—indeed it is a stronger risk factor for future fracture than is hypertension for fatal stroke in the elderly% or cholesterol for ischaemic heart disease. 3 BMD is not the only risk factor for fractures associated with osteoporosis, but it is a very important factor and one that can be measured rapidly and precisely. BMD

merits further examination in prospective trials to establish its place in the screening for, and prevention of, osteoporosis.

"Effective health care" means sound clinical practice backed-up by sound clinical trials. As a publication Effective Health Care ought therefore to provide a comprehensive, non-selective review of published work and research in progress. Health authorities, in their purchasing role, could do with guidance on the value of BMD measurement in clinical practice, as opposed to screening, but the first issue of Effective Health Care does not provide it.

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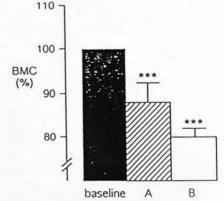
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STR,—We would like to express our concerns, and those of the British Menopause Society Council, about Effective Health Care's report on screening for osteoporosis. This bulletin, commissioned by the UK Department of Health, has been distributed to purchasing managers within the National Health Service, who will no doubt regard it as official. Many will conclude that bone densitometry is of little or no value in the management of osteoporosis. This is incorrect, and the view that screening is of little value can also be challenged.

The report makes the same mistake as Law, Wald, and Meade did, in a paper on which it draws heavily²—namely, the overlap in bone density between women with and without fracture is relatively small, and thus measurement is of no use. A woman with low bone mineral density (BMD) who is fracture-free at the time of assessment represents a false positive. Such a woman who has escaped a fracture over, for example, a 5-year period but will have a fracture during the next 5 years cannot realistically be considered as a false positive. Similar overlaps exist for blood pressure in those who have or have not had a stroke. Does that mean we should abandon blood-pressure measurement? The conclusion that the specificity and sensitivity of BMD measurement are low is flawed because their figures refer to hip fracture and not to other



Bone mineral content in distal radius (BMC) expressed as % initial value

N = 242 healthy postmenopausal women at baseline, followed for 12 years. Group A (n = 68) received a mean 5.4 years HRT followed by no treatment. Group B (n = 177) received no treatment. Values as mean (SE) ***p < 0.001