

THE CELLULAR MECHANISM OF CORTICOSTEROID RESISTANCE IN ASTHMA

by

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We are always at the brink of the known,
we always feel forward for what is to be hoped.

Jacob Bronowski (1973)
"Knowledge or Certainty"

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ABSTRACT

Chronic asthmatics normally respond to oral corticosteroid therapy with an increase in their ventilatory function. However, Grant and his co-workers identified a small proportion of severe chronic asthmatics who did not improve their ventilatory function when given oral corticosteroid. These patients were termed "corticosteroid resistant". This thesis describes the in vitro and in vivo effects of the corticosteroid, Methylprednisolone (MPS), on monocytes and T-cells from Corticosteroid Sensitive (CS) and Corticosteroid Resistant (CR) asthmatics.

In vitro colony formation by mononuclear cells (MNC) from CS asthmatics was found to be significantly more inhibited by 10^{-8} M MPS than was colony formation by MNC from CR asthmatics. This in vitro assay of corticosteroid sensitivity was subsequently used diagnostically to discriminate between CS and CR asthmatics. It was demonstrated that the unresponsiveness of MNC from CR asthmatics to MPS in vitro was attributable to the monocytes from these patients. Defects in the corticosteroid sensitivity of T-cells, eosinophils and polymorphs were also observed in vivo and in vitro ; their relationship to the monocyte defect was not clarified.

Monocytes are thought to affect the Type 1 hypersensitivity reaction seen in asthma by the production of many factors. Two of these factors are of particular relevance to this thesis: lipomodulin, which inhibits prostanoid synthesis and is induced by corticosteroid and Interleukin 1, which supports T-lymphocyte proliferation and is inhibited by corticosteroid. There is therefore reason to believe that the defect in corticosteroid responsiveness demonstrated in monocytes from CR asthmatics in vitro could reduce the therapeutic efficacy of these drugs in patients with this condition.

If this is so, these findings emphasise the importance of the actions of corticosteroid on monocyte function in the commoner CS asthma. Further investigation of this phenomenon may have wider implications for other developmental and pathological processes.

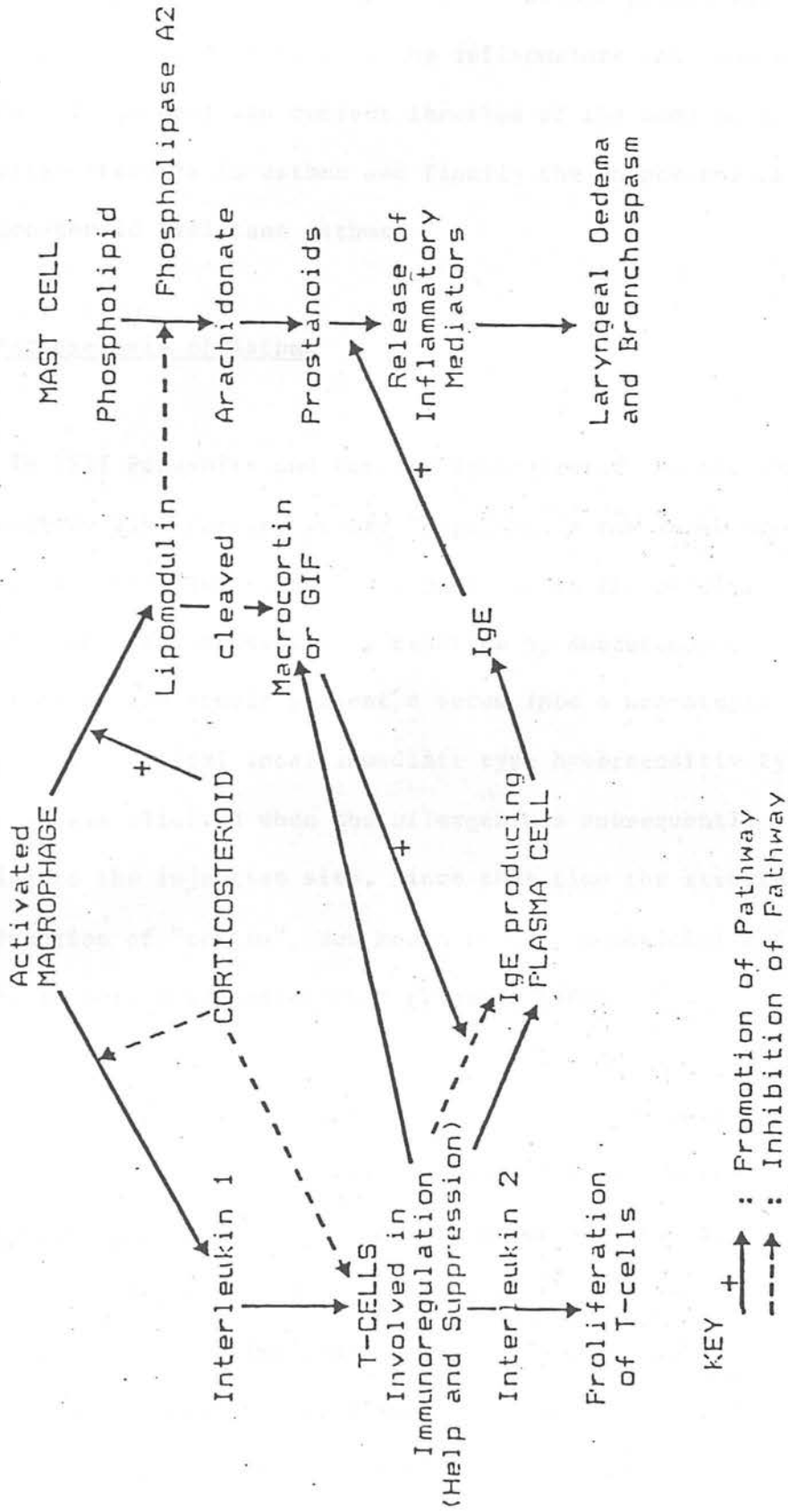
CORTICOSTEROID RESISTANCE IN CHRONIC ASTHMA

INTRODUCTION

Corticosteroids are of considerable value in the treatment of acute and chronic asthma [Crompton 1982]. However, the mechanisms by which corticosteroids act in asthma are not fully understood. There are several possibilities which include the action of corticosteroid on the production of mediators by mast cells, on IgE production by plasma cells, on the regulation of these by monocytes and T-cells and on other leucocytes including neutrophils and eosinophils [Cupps and Fauci 1982].

This thesis describes the effects that the corticosteroid, methylprednisolone (MPS), have on monocyte and T-cell function in vivo and in vitro. It exploits the recent identification of chronic asthmatics who are resistant to the therapeutic effects of corticosteroids [Carmichael et al. 1981]. By demonstrating that corticosteroid resistance is a property of the monocyte of such patients, this study emphasises the effects of corticosteroid on monocytes in the commoner corticosteroid sensitive asthmatics. This is of particular interest in view of much recent evidence that monocytes play a central role in the regulation of immunoreactivity and in the inflammatory response. Furthermore, it has been shown recently that these monocyte functions are influenced by corticosteroids (See Diagram).

CELLULAR MECHANISMS OF ACTION OF CORTICOSTEROID IN ASTHMA



Accordingly this introduction will discuss the following topics: the pathogenesis of asthma, the atopic production of IgE, corticosteroid effects on the inflammatory and immune response in general and current theories of the mode of action of corticosteroids in asthma and finally the phenomenon of corticosteroid resistant asthma.

The Pathogenesis of Asthma

In 1921 Prausnitz and Kustner demonstrated the presence of an antibody like factor, termed "reagin", in the serum of atopic subjects. They went on to demonstrate the passive transfer of a hypersensitivity reaction by subcutaneous injection of the atopic patient's serum into a non-atopic recipient. A typical local immediate type hypersensitivity reaction was elicited when the allergen was subsequently applied to the injection site. Since that time the structure and function of "reagin", now known to be Immunoglobulin E (IgE), is more fully understood [Taussig 1979].

IgE is normally produced in small amounts in response to parasite infestation and particle bound antigens. However the major significance of IgE is as a mediator of immediate type 1 hypersensitivity reactions, including those seen in allergic disorders. In atopic individuals IgE is produced in response to ubiquitous antigens otherwise known as allergens. The IgE is bound via its Fc portion to specific receptors on basophils in

the peripheral blood and more significantly to mast cells in tissues such as skin and various mucosae. Subsequent combination of the allergen with the cell bound IgE results in basophil or mast cell degranulation. The mast cell releases histamine, platelet activating factor, leukotrienes, eosinophil chemotactic factor, prostaglandins and other mediators of the inflammatory reaction characteristic of immediate type hypersensitivity. This release of inflammatory mediators may also be stimulated by non-immunological stimuli such as stress and temperature change. The result of mast cell degranulation is bronchospasm and laryngeal oedema. The hypersensitivity reaction may in turn be moderated by substances released from eosinophils.

IgE Production in the Atopic Individual

One major cause of asthma is a disorder in regulation of IgE production. It has been noted that atopic individuals do produce high levels of IgE in response to the allergen to which they are sensitised [Orgel 1975; Rachelefsky 1976].

There is a clear genetic predisposition to the formation of IgE in response to allergens [Taussig 1979]. Family studies demonstrated the association of atopic production of IgE with the HLA-A7 haplotype. Statistical analysis of such data indicates that the control of IgE responsiveness to a specific allergen is itself determined by a single Ir gene. The Ir gene

is intimately associated with genes controlling the expression of the HLA-A7 antigen.

The cellular requirements for normal IgE production are specific IgE producing plasma cells, B-Memory Lymphocytes , regulator T-Lymphocytes and monocytes [Ishizaka 1976]. The interaction between these subpopulations of lymphocytes and monocytes results in the controlled production of IgE in response to specific stimuli.

Regulation of IgE production by T-cells (including T-inducer and T-cytotoxic/suppressor cells) is mediated by soluble factors known as IgE binding factors [Ishizaka 1982]. Ishizaka and his coworkers isolated a potentiating factor which specifically promoted IgE production in their test in vitro system [Suemura et al. 1980]. They have also isolated a suppressor factor which reduces IgE production [Hirashima 1980]. Both these factors have a high affinity for IgE and differ only in their carbohydrate moiety. The control of the production of IgE potentiating and suppressor factors is currently being investigated.

Uede et al. have identified a protein capable of modulating the biologic activities of the IgE binding factors [Uede et al. 1983]. The factor, termed glycosylation-inhibiting factor (GIF), is a lymphokine derived from OX8+ cytotoxic/suppressor T-cells. GIF is capable of promoting the selective formation of IgE suppressive factors by T-

lymphocytes. GIF is currently thought to be a fragment of phosphorylated lipomodulin. This is of particular relevance to this thesis as lipomodulin synthesis, at least by monocytes and neutrophils, is known to be induced by corticosteroid as will be discussed later.

There is some direct evidence that soluble factors from T-cells may be involved in the abnormal production of IgE. For example it has been shown that T-cells isolated from patients with hyper IgE states (hyperIgEaemia, atopic states and acute graft versus host disease) release a factor which induces synthesis of IgE by plasma cells [Saryan et al. 1983]. This factor was not produced by T-cells from controls.

The notion that atopic states result from disordered immunoregulation is supported by much indirect evidence. For example cell mediated immunity is reduced in asthmatics which results in their propensity to develop viral and fungal infections [Gupta 1980]. Strannegard (1978), Brasher (1977), Tomita et al. (1982), Hsieh (1980), Gupta and Good (1981) all noted abnormalities in T- cell composition and function in atopic individuals, in particular in asthmatics, as compared with non-atopic individuals. These defects included an overall decrease in T-cell numbers, T-cell responsiveness to mitogens, active E-rosette formation and non- specific T-suppressor activity. A decrease in T-cells with receptors for IgG has also been noted in chronic asthmatics. In addition to these defects in T-cell function, Geha demonstrated that patients with

hyperIgEaemia and atopic states had reduced numbers of circulating T-cytotoxic/suppressor cells in their peripheral blood [Geha et al. 1981 ; O'Driscoll and Kay 1982].

Thus it is possible that atopy arises as a result of abnormal T- cell function [Jarret 1977; Stannegard 1979]. Jarret goes on to propose that a T-suppressor cell/T-helper cell imbalance may result in the excess stimulation of B-cell production of IgE that is seen in asthma.

Corticosteroid Mediated Immunoregulation in Man

Corticosteroids have widespread effects on the immune and inflammatory responses in man and these are extensively exploited therapeutically [Cupps and Fauci 1982]. These effects involve the depression of the number and function of circulating eosinophils, basophils and monocytes [Dunsky 1979]. The peak effect occurs 8 hours after the oral administration of corticosteroid and recovery occurs during the following 72 hours. The effects of corticosteroids on the control of the immune response are more complex and involve profound actions on monocyte and T- lymphocytes.

1. Effects on Monocytes.

Monocytes play an important role in the induction and

regulation of immune reactivity. Monocytes also contribute to and regulate the release of inflammatory mediators.

Low concentrations of corticosteroids (10^{-8} M to 10^{-9} M MPS) inhibit Interleukin 1 (IL 1) production by monocytes [Smith and Ruscetti 1981; Snyder and Unanue 1982]. Corticosteroids may also inhibit T-lymphocyte responsiveness to IL 1 [Palacios 1982]. Corticosteroids therefore inhibit the subsequent activation of T- cells by IL 1 and the production of Interleukin 2 (IL 2) by these activated T-cells. The inhibition of IL 1 production may account for the inhibitory effects that low concentrations of corticosteroids are seen to have on Phytohaemagglutinin (PHA) induced T-cell blastogenesis and colony formation in vitro [Krajewski and Wyllie 1981].

In contrast corticosteroids promote the production of the protein lipomodulin by monocytes and also neutrophils [Flower and Blackwell 1979]. The majority of lipomodulin is phosphorylated and then cleaved to form smaller molecules. One such fragment of phosphorylated lipomodulin termed "macrocortin" has anti- phospholipase A2 activity and consequently modifies prostanoid synthesis [Hirata 1982 ;Flower and Blackwell 1979]. Macrocortin is also capable of modulating the biologic activities of IgE binding factors through its postulated action as Glycosylation Inhibiting factor (GIF). GIF promotes the selective biosynthesis of IgE suppressor factor by specific regulator T-cells. Corticosteroids therefore promote lipomodulin synthesis by monocytes and neutrophils and thereby

modify the synthesis of the inflammatory mediators and selectively suppress the production of IgE.

Pharmacologic doses of corticosteroids have a variety of other less specific effects on monocyte function, the mechanisms of which are as yet unknown. These include inhibition of chemotaxis, suppression of the bactericidal and fungicidal actions and inhibition of Ia expression by monocytes [Rinehart et al. 1974; Baugren and Anderson 1977 ;Snyder and Unanue 1982]. The function of the monocyte in mixed leucocyte reactions is particularly sensitive to pharmacologic doses of corticosteroids in vivo and in vitro [Katz and Fauci 1979].

2. Effects on T-Lymphocytes.

Corticosteroids have profound effects on T-Lymphocytes in vivo and in vitro. These effects include an alteration in T-cell subpopulations and their function [Cupps and Fauci 1982].

Pharmacologic doses of corticosteroids cause a decrease in overall T-cell numbers. The maximal effect occurs six hours after the administration of a single dose of the drug with return to normality within 24 hours [Cupps and Fauci 1982]. Haynes and Fauci noted a preferential depletion of T-cells bearing Fc receptors for IgM (T_m cells) with a subsequent rise in the proportion of T-cells with receptors for IgG (T_g cells)

during the six hours following the corticosteroid dose [Haynes and Fauci 1978]. The Tg/Tm ratio returns to normal within 24 hours. It has been suggested that Tm cells have a predominantly helper cell role to play in immune regulation whereas Tg cells suppress the immune response [Moretta et al. 1976]. Recent work that utilised more accurate markers of T-cell subsets demonstrated that a single 100mg. dose of prednisolone caused preferential depletion of T-inducer cells as compared to T-cytotoxic/suppressor cells within 6 hours of the administration of the drug [Dupont et al. 1983].

Corticosteroids at low doses inhibit lectin induced T-cell blastogenesis in vitro. This is thought to be in part due to inhibition of IL 1 production by monocytes as described above. However, Cupps and Fauci suggest that corticosteroid may also directly suppress T-cell responsiveness to IL 1 and thereby inhibit IL 2 production by activated T-cells [Krajewski and Wyllie 1981]. Corticosteroids have a variety of other effects on T-cell function in vitro including inhibition of the allogenic and autologous mixed leucocyte reactions and inhibition of T-cell mediated cytotoxicity and immunosuppression [Katz and Fauci 1979, Onsrud and Thornsby 1981, Parillo and Fauci 1978, Clarke et al. 1977, Haynes and Fauci 1979, Galamud et al. 1981].

Recently Uede demonstrated that OX8+ suppressor/cytotoxic splenic T-lymphocytes treated with Freund's adjuvant release a lymphokine which is identical in its action to the GIF that is

released by monocytes and neutrophils in the presence of corticosteroid as described above [Uede et al. 1983]. Corticosteroid may soon be shown to promote the production of GIF by T-cells.

It is clear from this discussion that corticosteroids have significant effects on monocyte and T-cell function which may be relevant to the mechanism of their therapeutic action in asthma: these effects include suppression of IgE production by plasma cells and the inhibition of the production of the prostanoids. Both of these effects are mediated by the monocyte products lipomodulin and macrocortin. Of particular relevance to this thesis is corticosteroid mediated inhibition of IL 1 production by monocytes and the consequent inhibition of monocyte dependent T-cell activation and proliferation in vitro.

Corticosteroid Resistance in Asthma

Corticosteroids are amongst the most important drugs used in the treatment of acute and chronic asthma. Inhalant corticosteroids are a major component of maintenance therapy for chronic asthma whereas systemic corticosteroid therapy is administered in acute asthma [Crompton 1982].

Severe chronic asthmatics normally respond to oral corticosteroid with an increase in ventilatory function. A 7-day course of prednisolone, (20 mg. daily), normally results in a 30% increase in the patient's FEV₁ [Carmichael et al. 1981]. However, in 1981 Carmichael et al. noted the presence of a subpopulation of severe chronic asthmatics who did not improve their ventilatory function when given very high doses of oral corticosteroids (up to 60 mg. of prednisolone daily)[Carmichael et al. 1981]. These patients were termed "corticosteroid resistant" (CR). The management of such patients poses a serious clinical problem since CR asthmatics may be disabled for long periods of time and may develop episodes of severe acute asthma which do not respond to conventional oral corticosteroid therapy.

CR asthmatics show only three differences from Corticosteroid Sensitive (CS) asthmatics with respect to the clinical presentation of their disease. Firstly that CR asthma was of a longer duration than age and sex matched CS counterparts. The second difference was that a family history was significantly more common in the CR asthmatic patients. CR were also significantly more responsive to methacholine therapy than were CS asthmatics. A wide range of laboratory investigations was undertaken in an attempt to identify factors that might have been relevant to the response of asthma to corticosteroids, but with one exception these tests failed to discriminate between CR and CS asthma. The one exception was

the measurement of monocyte complement receptors (MCR) and of their enhancement (CRE) by a monocyte chemotactic factor (casein). This investigation showed that while the normal response to corticosteroids in CS asthmatics was a decrease in MCR and the degree of CRE, these changes were not observed in CR asthmatics, whose complement receptors were apparently uninfluenced by corticosteroid therapy [Kay et al. 1981]. No other studies have been performed in order to determine the markers and mechanism of corticosteroid resistance in asthma.

The further study of corticosteroid resistant asthma is of importance in two respects. Firstly, CR asthmatics represent a serious clinical problem since they may be crippled by acute episodes of their disease and unnecessarily exposed to the hazardous side effects of systemic corticosteroid therapy. Recognition of CR asthma may therefore prevent the unnecessary exposure of these patients to these drugs. Examination of CR asthma also offers the opportunity of elucidating the mechanism of action of corticosteroids in the commoner CS asthma.

This thesis describes the in vitro and in vivo effects of the corticosteroid, MPS, on leucocytes from CS asthmatics, CR asthmatics and controls. An assay of corticosteroid inhibition of colony formation by Mononuclear Cells (MNC) was used as an indicator of the in vitro sensitivity of monocyte and T-cell function to corticosteroid. Colony formation was seen to be a

sensitive indicator of both monocyte and T-cell function and was known to be responsive to inhibition by very low concentrations of corticosteroid [Krajewski and Wyllie 1981 ;Gordon et al. 1980]. The proportions of T-cell subsets, monocytes and Ia positive cells in the peripheral blood samples were determined using specific monoclonal antibodies as this was thought to effect colony formation [Filipovich 1982]. The colony cells were analysed likewise in order to determine whether low concentrations of MPS effect the proportions of cells of a given phenotype in the colonies. We also attempted to determine whether corticosteroid resistance was a property of monocytes alone or whether it was also present in T- cells. Finally, the haematological responses of CR and CS asthmatics to a single dose of prednisolone was also studied.

MATERIALS AND METHODS

Selection of Patients and Controls:

Four groups, each of 15 subjects were studied: Group 1 were known Corticosteroid Resistant (CR) Asthmatics; Group 2 were known Corticosteroid Sensitive (CS) Asthmatics; Group 3 were new asthmatics of unknown corticosteroid sensitivity, but with asthma of sufficient severity to warrant a 7-day course of systemic corticosteroid therapy; Group 4 were non-atopic controls. Essential preconditions to inclusion into the asthmatic groups were that the lowest recorded forced expiratory volume in one second (FEV1) was less than 60% of the predicted normal value and that there was a record of an increase in FEV1 of at least 30% following the use of a bronchodilator aerosol. No asthmatic included in this study had recieved systemic corticosteroid therapy during the month prior to admission.

Corticosteroid resistance was defined as an increase FEV1 of less than 15% following a seven day course of oral prednisolone (20 mg. or more daily). A patient was considered to be corticosteroid sensitive if their FEV1 had at least once increased by 30% or more during or at the end of a 7 day course of prednisolone [Carmichael et al. 1981].

Subjects were excluded from the study if there was

evidence of coexisting malignant disease, treatment with cytotoxic or immunosuppressive drugs, primary pituitary or adrenal gland dysfunction and intercurrent illness e.g. influenza and oral candidiasis.

Preliminary Documentation and Clinical Tests:

Age, sex, drug history and intercurrent illnesses were recorded for subjects in all groups. A total white blood cell count and differential white blood cell count was determined for all subjects. The best recorded FEV1 of the year was noted in patients with CR and CS asthma. The FEV1 and Peak Expiratory Flow Rate and endogenous plasma cortisol were measured in all asthmatic patients studied. A 50ml. sample of peripheral blood was withdrawn from each subject at 9.00 a.m.. A second peripheral blood sample was taken from new asthmatics at 9.00 a.m. on the day following the administration of their last dose of corticosteroid.

Preparation of Mononuclear cells from Peripheral Blood:

Peripheral venous blood was withdrawn from each subject into a sterile bottle containing 2ml. of 2% EDTA in phosphate buffered saline. Mononuclear cells (MNC) were obtained from the peripheral blood by centrifugation over Ficoll-Hypaque and washed twice in Hank's medium. [Boyum 1968].

Identification of Monocytes and T-Lymphocyte Sub-Populations
in Peripheral Blood MNC.

Subpopulations of T-lymphocytes, monocytes and Ia positive cells were quantified by means of monoclonal antibodies that were claimed by their manufacturers to define the following subpopulations:

OKT3 (Ortho-mune): greater than 95% peripheral blood T-cells (PBT cells).

OKT4 (Ortho-mune): inducer T-cell subset (65% of PBT T-cells).

OKT8 (Ortho-mune): cytotoxic/suppressor T-cell subset (35% of PBT cells).

OKM1 (Ortho-mune): peripheral blood monocytes (78% of adherent MNC and 18% of nonadherent MNC).

Ia 231: MNC bearing Ia antigens (90% of B-Lymphocytes and monocytes; 20% of null cells and activated T-lymphocytes).

Cells positive for OKT3, OKT4, OKT8, OKM1 or Ia231 were detected by indirect immunofluorescence with each of these monoclonal antibodies using fluorescein- isothiocyanate labelled Goat anti-mouse gamma immunoglobulin (GAM IgG) as the second antibody. 200 cells were counted with a fluorescent microscope under phase contact and fluorescence and the percentage of cells with membrane fluorescence was calculated. A control was included in each case that contained cells to

which only GAM IgG had been added.

Colony Formation by Peripheral Blood MNC

MNC from each subject were cultured in the absence of knowledge of the clinical data on the subject's corticosteroid sensitivity. The cells were suspended in RPMI 1640 supplemented with 10% heterologous serum with agar added to 0.3%. Forty microlitres of this suspension (2×10^5 cells) were plated onto an underlayer of 0.2 ml. of 0.5% agar, 10% heterologous AB serum (the same source of serum was used throughout) and 20ug of PHA (Difco). Methylprednisolone sodium succinate (Upjohn) was added to underlayers to give a final concentration of between 10^{-5} M to 10^{-9} M. Control cultures were set up to which only PHA was added. All cultures were in 17mm diameter wells in Multiwell dishes (Linbro 76-033-05). Cultures were incubated at 37 °C in a fully humidified atmosphere of 5% CO₂ in air [Goube de la Forest 1979; Krajewski and Wyllie 1981].

Clusters (defined as aggregates of between 10 and 40 cells) and colonies (defined as aggregates of more than about 40 cells) were scored after five days of incubation. The number of colonies and clusters were determined for one central field per well using a phase contrast microscope at a magnification of x100. Nine wells were scored in this way for each concentration of MPS. Clusters and colonies were also counted and sized using a Tektronic (4050 series) Graphic Information

system.

Cell Identification of Harvested Clusters and Colonies.

After scoring, 300 μ l of PBS was added to each well in the Linbro plate. The plate was then gently agitated for 3 minutes at 4^o C. Phase contrast microscopy indicated that this procedure detached clusters and colonies from the agar only, single cells remained embedded. The supernatant containing colonies and clusters was removed from each well and refluxed to form suspensions of cells for immunophenotyping using the monoclonal antibodies OKT3, OKT4 and OKT8 as described above.

In some cases cytopins and suspensions of intact clusters and colonies were also obtained for Non Specific Esterase (NSE) histochemistry [Pearse 1960] and staining with Giemsa. Intact colonies and clusters were also fixed in glutaraldehyde for electron microscopy. Immuno-phenotyping with monoclonal antibodies was also performed as described below.

Single Fluorescent Monoclonal Antibody Staining was performed on harvested intact colonies and clusters using the monoclonal antibodies OKT3, OKT4, OKT8, Ia 231 and OKM1 as described above. Care was taken to maintain the integrity of the clusters and colonies during processing. Harvested cells stained by indirect immunofluorescence were examined with a fluorescent microscope under phase and green fluorescence.

Double immunofluorescence was also performed on intact colonies and clusters using fluorescein conjugated OKT4 and rhodamine conjugated OKT8 and vice versa. A suspension of clusters and colonies was first incubated with OKT4 and rhodamine conjugated GAM IgG as above. The suspension of clusters and colonies was then washed and the direct fluorescein conjugate of OKT8 (Ortho-mune) applied. Clusters and colonies were also treated with OKT8 and rhodamine conjugated GAM IgG followed by incubation with the direct fluorescein conjugate of OKT4 (Ortho-mune).

Colonies and clusters stained by this technique were examined with a fluorescent microscope under phase contrast, green fluorescence and red fluorescence.

Colony Formation by Combinations of MNC from Pairs of CS and CR Asthmatics

MNC were obtained from pairs of CS and CR asthmatics as before. The clinical corticosteroid sensitivities of the members of each pair were not made known to the experimenter. Monocyte enriched MNC suspensions were prepared by lysing the T-cells with the monoclonal antibody OKT3 and complement. T-cell enriched suspensions were similarly prepared by lysis of the monocyte component of the MNC fraction with OKM1 and complement.

6×10^6 MNC were resuspended in $100 \mu\text{l}$ of RPMI 1640. $30 \mu\text{l}$ of the monoclonal antibody OKT3 or OKM1 was added and the suspension was incubated at 20°C for one hour. $150 \mu\text{l}$ of human AB serum (source of complement) and $25 \mu\text{l}$ of RPMI 1640 was then added and the cell suspension was placed in a shaking waterbath at 37°C . for one hour. The cells were then washed in 5ml . of medium and resuspended at 10×10^6 cells/ml.

The purity of cell suspensions was determined with the monoclonal antibodies OKT3 and OKM1 in conjunction with fluorescein labelled GAM IgG as described above.

The culture procedure as described above was then applied to the following MNC suspensions (10^6 cells/ml.).

- (a) MNC from the CS patient only.
- (b) MNC from the CR patient only.
- (c) Monocyte enriched MNC from the CS asthmatic (CSM).
- (d) Monocyte enriched MNC from the CR asthmatic (CRM).
- (e) T-Cell enriched MNC from the CS asthmatic (CST).
- (f) T-Cell enriched MNC from the CR asthmatic (CRT).
- (g) 40% CSM and 60% CST.
- (h) 40% CRM and 60% CRT
- (i) 40% CSM and 60% CRT
- (j) 40% CRM and 60% CST

All were cultured in the presence and absence of 10^{-8}M

MPS. In addition cells from (j) were cultured in the absence of PHA. As before cultures were incubated for five days. Clusters and colonies were sized and counted using the Tektronic Information system.

Haematological Effects of Corticosteroids In Vivo.

4 Controls, 10 CS and 10 CR asthmatics were admitted to this study . 30ml. of peripheral blood were withdrawn from each subject at 9.00 a.m.. A single 20 mg. dose of prednisolone (Upjohn) was then administered orally. Second and third peripheral blood sample were taken 6 and 24 hours thereafter.

A total white blood cell count and differential white blood cell count were performed on each blood sample. MNC suspensions were prepared as before. Subpopulations of T-cells, monocytes and Ia positive cells were quantified by indirect immuno-fluorescence with monoclonal antibodies.

RESULTS

Clinical Observations:

There was no significant difference between controls, CS and CR asthmatics with respect to age and sex ratio. There was also no significant difference between the CS and CR asthmatics in respect of the severity of their asthma on admission (as measured by their FEV1) and their plasma cortisol levels (Table 1). CS and CR asthmatics did not differ significantly in the number of patients with intercurrent illnesses. However, CS and CR asthmatics did differ significantly with respect to the number in each group taking drugs in addition to those administered as part of maintenance therapy for their chronic asthma (Table 2).

Phenotype of Leucocytes in the Peripheral Blood of Controls and Asthmatic Patients:

Analysis of the peripheral blood revealed that there were few differences between the two asthmatic groups and between the asthmatic patients and controls. As expected the asthmatic patients had an eosinophilia not found in controls (Table 3). The CS asthmatics differed in several respects from the control group. CS asthmatics had fewer monocytes and T-inducer cells than controls. A higher proportion of T-cells of the CS asthmatics were of the suppressor/cytotoxic type and consequently the ratio of T- inducer cells to T-

Table 1 Clinical Findings in Controls, Corticosteroid Sensitive and Corticosteroid Resistant Asthmatics.

	CONTROLS	STEROID SENSITIVE ASTHMATICS	STEROID RESISTANT ASTHMATICS
NUMBER	15	15	15
AGE (years) mean + s.d. (range)	44.5 + 16.9@ (24-74)	44.5 + 19.1# (19-76)	55.6 + 17.1#@ (21-78)
SEX RATIO (m:f)	9:6	10:5	9:6
FEV 1 (%) *	-	93.0 + 12.5 (62-100)	89.0 + 17.1 (59-120)
ENDOGENOUS GLUCOCORTICOID nmol/l	538 + 115 (315-1535)	504 + 148 (330-884)	503 + 137 (247-746)

@ : p = 0.04

: p = 0.05

* : Percentage of best recorded FEV 1 of year.

Table 2 : Clinical Findings in Patients with CS and CR Asthma.

PATIENT	DRUGS	INTERCURRENT ILLNESSES
STEROID SENSITIVE ASTHMATICS		
1. D.Mu	-	-
2. S.Mo	-	-
3. J.Mc	-	Diverticular disease
4. A.Tu	-	-
5. R.Pa	-	-
6. S.Vi	-	-
7. J.Ga	-	-
8. G.Cu	-	-
9. G.Mc	Tagamet	Dyspepsia
10. G.Ni	-	Diabetic
11. S.Wh	-	-
12. R.Di	-	-
13. S.Mu	-	-
14. W.Cu	-	-
15. W.Ba	-	-
STEROID RESISTANT ASTHMATICS		
1. A.Br	-	-
2. I.Ca	Belergal	Diverticular disease
3. D.Ho	-	-
4. C.Mc	Zantac, ranitidine Lasix/slowK	-
5. R.Da	Piriton	-
6. G.Go	-	-
7. A.Wa	Ampicillin	Sore throat
8. D.Ne	Slow Trasicor Aspirin	-
9. J.Co	Ampicillin/Ponstan	-
10. A.Gr	Allopurinol/Lasix Slow K / Euhypnos	-
11. A.We	Neonaclex/Septrin	-
12. J.Th	Tagamet	Mild angina
13. J.Ma	-	-
14. J.Mo	Frusemide/slow K Nifedipine/GTN	-
15. N.Hu	-	-

"DRUGS" includes medications other than aerosol bronchodilators and inhalant corticosteroids. Significantly more drugs had been prescribed for patients with CR as compared with patients with CS asthma.
(p = less than 0.02; Chi-squared test)

"INTERCURRENT ILLNESSES" are those illnesses other than asthma that patient was suffering from around the time of admission to the study.

Table 3: Number of Leucocytes ($\times 10^9/l$) of a Given Phenotype in the Peripheral Blood of Controls and Asthmatic Patients.

No. of cells ($\times 10^9/l$) of given phenotype	CONTROLS Mean + s.d. (range)	STEROID SENSITIVE ASTHMATICS	STEROID RESISTANT ASTHMATICS
OKT3	1.04 + 0.35 (0.44-1.50)	0.95 + 0.23 (0.64-1.34)	1.05 + 0.41 (0.44-2.02)
OKT4	0.79 + 0.39 (0.34-1.78)*	0.53 + 0.17 (0.32-0.92)*x	0.79 + 0.34 (0.44-1.37)x
OKT8	0.37 + 0.14 (0.12-0.55)	0.38 + 0.11 (0.23-0.60)	0.39 + 0.13 (0.19-0.65)
Ia 231	0.30 + 0.10 (0.13-0.51)	0.28 + 0.14 (0.10-0.53)	0.29 + 0.11 (0.13-0.49)
OKM1	0.73 + 0.22 (0.41-1.14)^	0.52 + 0.15 (0.24-0.72)^	0.71 + 0.29 (0.30-1.27)
Polymorphs	3.44 + 0.99 (2.35-5.58)	3.57 + 0.99 (1.74-5.44)	3.56 + 0.89 (1.99-5.38)
Eosinophils	0.15 + 0.05 (0.03-0.34)@	0.32 + 0.24 (0.00-0.70)@, #	0.30 + 0.20 (0.05-0.69)#

* : p = 0.019 (Two Sample t Test)
 x : p = 0.013 (" " ")
 ^ : p = 0.010 (" " ")
 @ : p = 0.008 (" " ")
 # : p = 0.006 (" " ")

There are no other significant differences between the groups.

suppressor/cytotoxic cells was reduced (Figure 1). The CR asthmatics resembled the controls in these respects (Tables 3 and 4).

MNC from CS asthmatics differed significantly in only one respect from MNC cells from CR asthmatics. CS asthmatics were shown to have a significantly reduced number, but not proportion, of T-inducer cells in their peripheral blood in comparison with CR asthmatics (tables 3 and 4).

Colony Formation by MNC from Controls and Asthmatic Patients.

In the presence of PHA, MNC from controls and asthmatics generated both colonies and clusters of cells in soft agar after 3 to 5 days incubation. Similar numbers of colonies were obtained from cultures of MNC from controls and asthmatics in the absence of MPS, representing a plating efficiency of approximately 0.05% (Table 5). The numbers of clusters formed was also similar for the three groups (data not shown).

The number of colonies formed by MNC from Controls and CS asthmatics was shown to be significantly more sensitive to inhibition by 10^{-8} M MPS than was colony formation by MNC from CR asthmatics (Table 5). The number of colonies that developed in the absence of MPS was denoted as 100% in order to minimise the purely technical variation in the plating efficiency between experiments (Figure 2). The concentration of

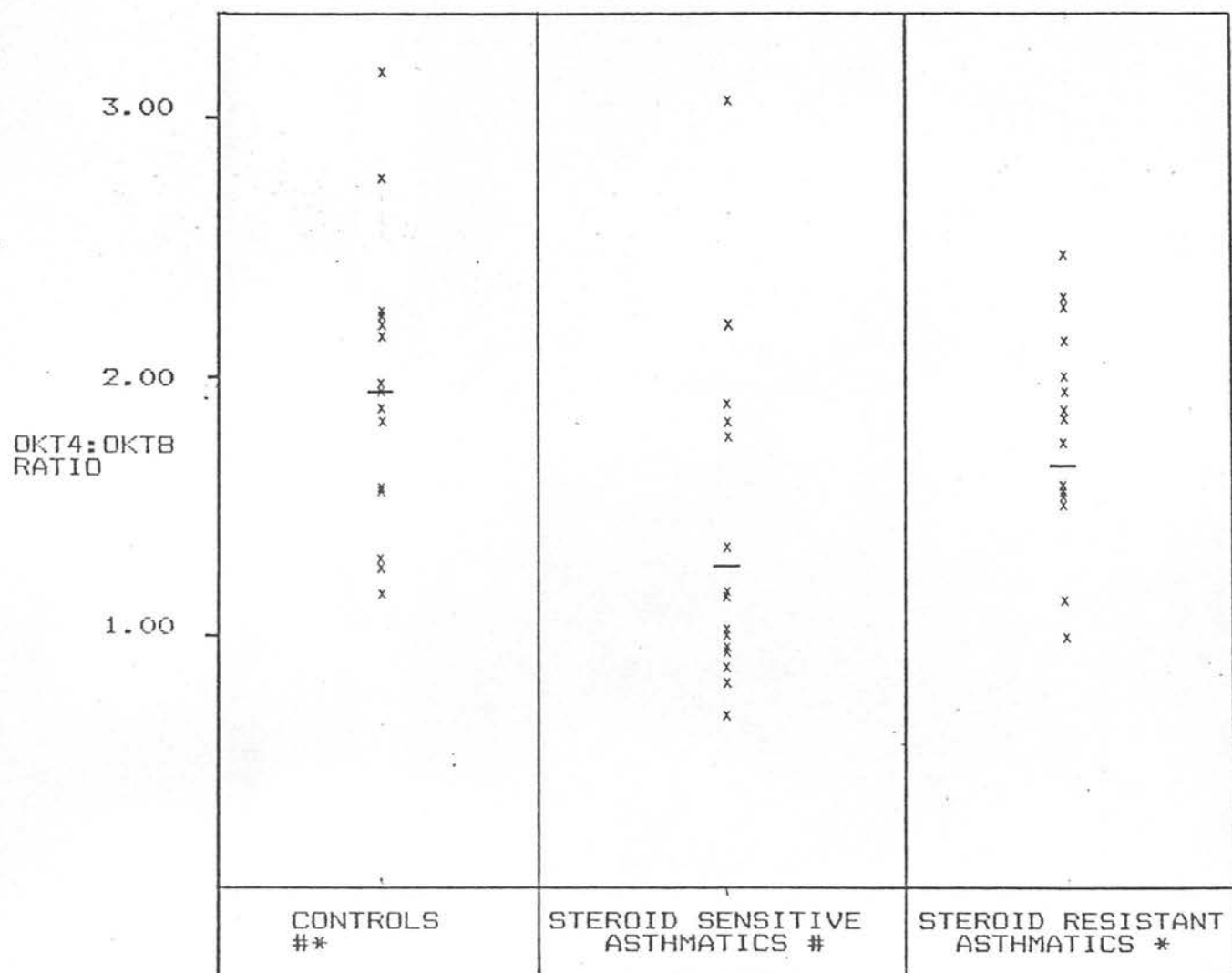
Table 4 : Proportion of Mononuclear Cells of Given Phenotype in the Peripheral Blood of Controls and Asthmatic Patients.

Percentage of mononuclear cells of specific phenotype	CONTROLS Mean + s.d. (range)	STEROID SENSITIVE ASTHMATICS	STEROID RESISTANT ASTHMATICS
OKT3 (%)	47.2 + 11.6 (22-66)	46.2 + 7.0 (30-58)	45.1 + 10.9 (32-66)
OKT4 (%)	30.9 + 9.1 (12-46)	26.0 + 7.1 (16-39)	27.2 + 7.9 (18-42)
OKT8 (%)	16.3 + 4.7* (10-27)	21.6 + 6.3* (12-35)	17.8 + 5.5 (11-28)
Ia 231 (%)	12.9 + 6.9 (7-39)	13.5 + 4.9 (4-23)	13.8 + 6.2 (5-28)
OKM1 (%)	29.3 + 9.4 (14-47)	26.7 + 7.4 (15-44)	29.0 + 10.0 (12-47)
OKT4:OKT8 ratio	1.97 + 0.64 #x (1.11-3.16) #x	1.35 + 0.68 # (0.74-3.00) #	1.59 + 0.43 (0.92-2.36) x

* : p= less than 0.01 (Two Sample t Test)
 # : p= less than 0.01 (" ")
 x : p= less than 0.05 (" ")

Figure 1

RATIO OF OKT4 +VE. (INDUCER) T-CELLS TO OKT8 +VE (SUPPRESSOR/CYTOTOXIC) T-CELLS IN THE PERIPHERAL BLOOD OF CONTROLS AND ASTHMATIC PATIENTS.



: p = less than 0.01
 * : p = less than 0.05

Table 5 : Numbers of Colonies Grown in the Presence and Absence of 10⁻⁸M Methylprednisolone.

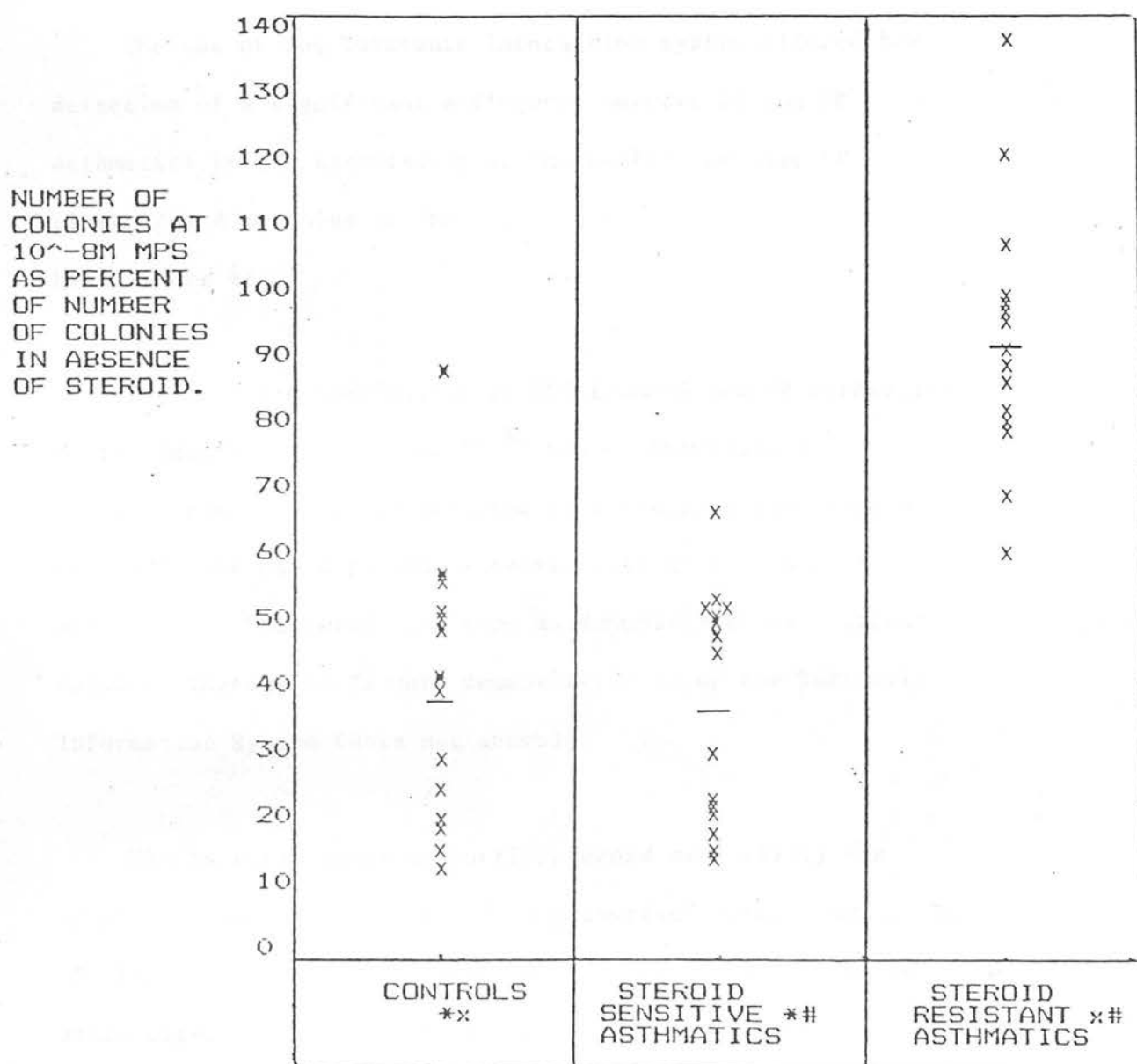
	CONTROLS Mean + s.d. (range)	STEROID SENSITIVE ASTHMATICS Mean + s.d. (range)	STEROID RESISTANT ASTHMATICS Mean + s.d. (range)
Number of Colonies No Steroid	37.7 + 5.2 (29-46)	38.3 + 6.6 (28-52)	35.3 + 9.5 (20-55)
Number of Colonies + 10 ⁻⁸ M MPS	14.7 + 8.8* (5-35)	13.9 + 7.3*# (4-26)	31.8 + 12.8# (12-66)

"Number of Colonies" = number of aggregates of cells containing more than forty cells counted in nine central fields at x100 magnification.

* : p = not significant (Two sample t test)
: p = less than 0.0001 (" ")

Figure 2

COLONY FORMATION BY MNC FROM CONTROLS
CS AND CR ASTHMATICS IN THE PRESENCE
OF 10^{-8} M MPS.



* : p = not significant (Two Sample t Test)
 x : p = less than 0.0001 (" " " ")
 # : p = less than 0.0001 (" " " ")

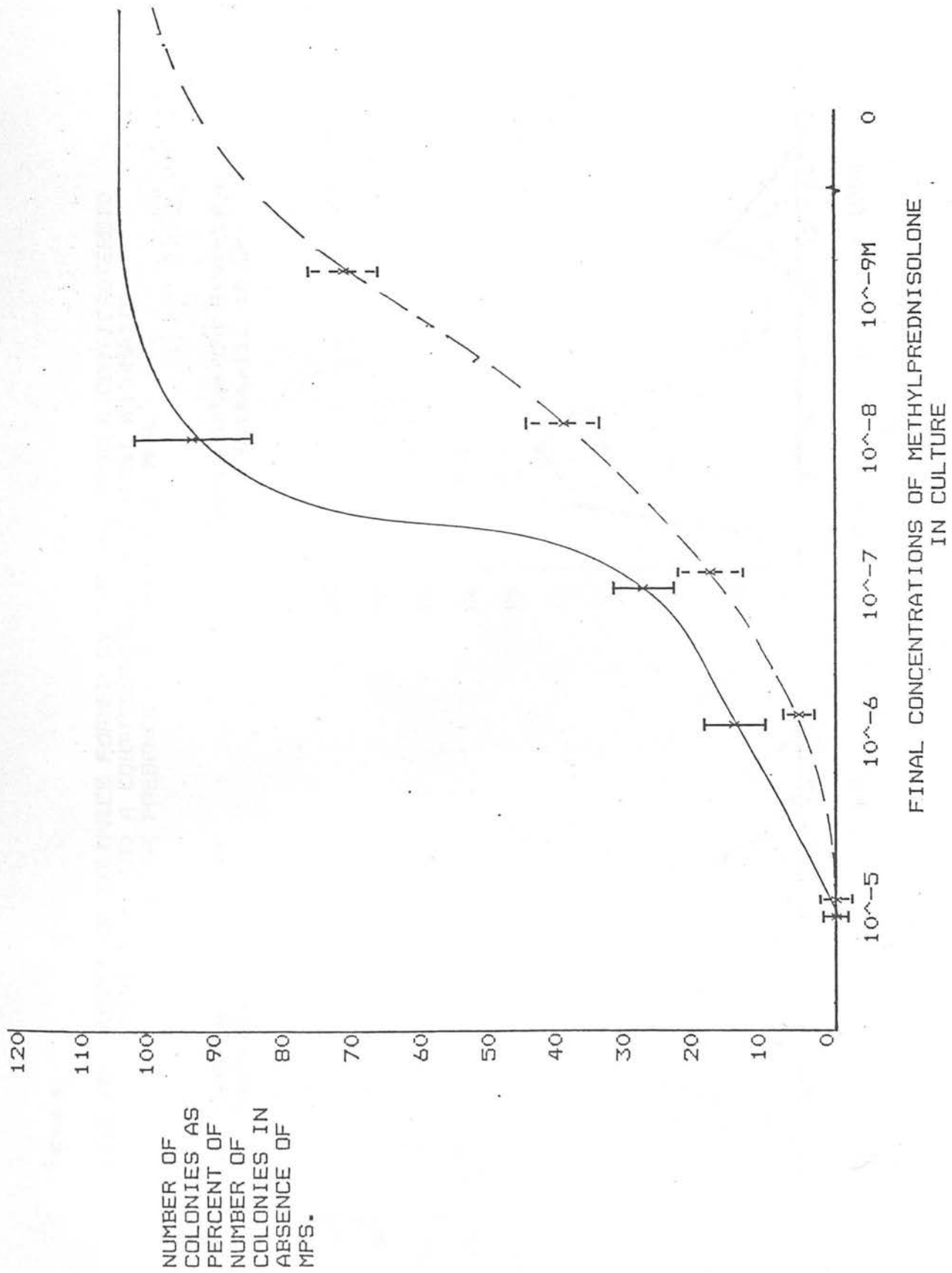
MPS causing a 50% decrease in the number of colonies formed was found to be 1.03×10^{-9} M for MNC from CS asthmatics as compared with 1.4×10^{-8} M for MNC from CR asthmatics (Figure 3).

The use of the Tektronic Information system allowed the detection of a significant difference between CS and CR asthmatics in the sensitivity of the number and size of clusters and colonies to the inhibitory effect of 10^{-8} M MPS (Figure 4).

The in vitro sensitivity of MNC from CS and CR asthmatics to the inhibitory effect of 10^{-8} M MPS on PHA-induced colony formation was not affected by a 7-day in vivo course of oral MPS. Nor was a patient's sensitivity to the in vitro effects of MPS altered over time as demonstrated by 3 repeat samples. These effects were demonstrated using the Tektronic Information System (data not shown).

The in vitro assay of corticosteroid sensitivity was of predictive value in the 6 "new asthmatic" cases studied. The MNC from 4 new asthmatics were found to be sensitive to the in vitro effect of MPS. These asthmatics subsequently responded well to a 7-day course of systemic prednisolone with an improvement in their FEV1 of greater than 30%. The MNC from 2 asthmatics were found to be resistant to the inhibitory effect of MPS in vitro and it was correspondingly found that their FEV1 had not improved by more than 5% following a 7-day course

THE EFFECT OF MPS ON COLONY FORMATION
BY MNC FROM CS AND CR ASTHMATICS.

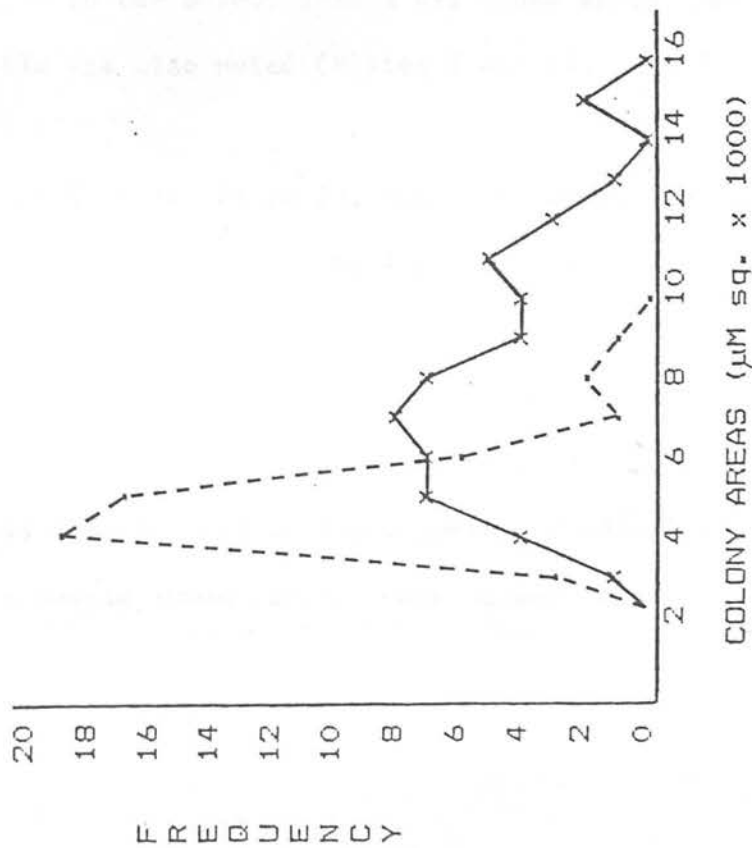


— : Steroid Sensitive Asthmatic
- - - : Steroid Resistant Asthmatic

Figure 4

SIZE AND NUMBER OF COLONIES FORMED BY THE MNC FROM A CORTICOSTEROID SENSITIVE AND A CORTICOSTEROID RESISTANT ASTHMATIC IN THE PRESENCE AND ABSENCE OF MPS.

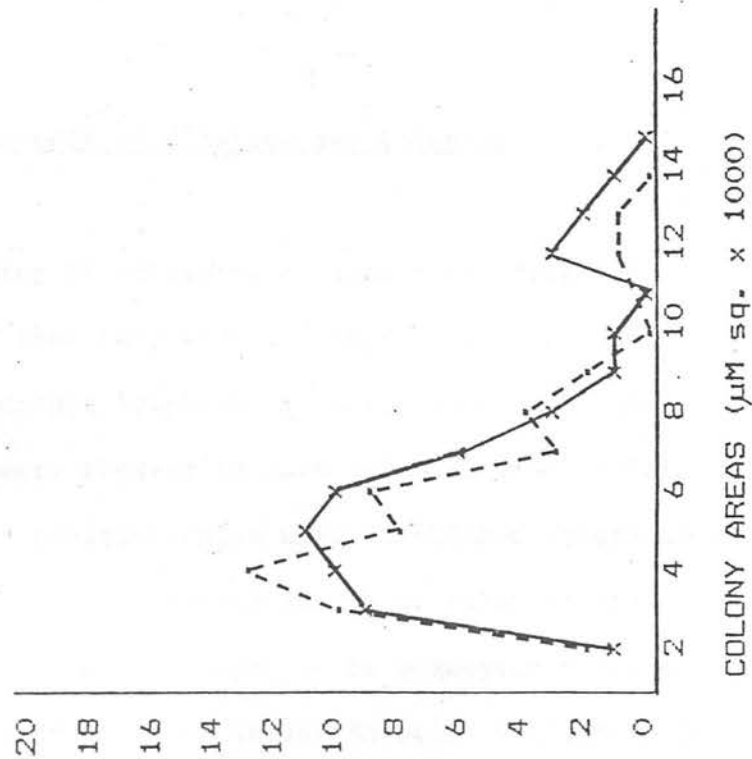
Corticosteroid Sensitive Asthmatic (A.Mc)



x = No steroid
 --- = 10⁻⁸M MPS

p = less than 0.00001 (Mann-Whitney)

Corticosteroid Resistant Asthmatic (A.Gr)



x = No steroid
 --- = 10⁻⁸M MPS

p = not significant (Mann-Whitney)

of prednisolone therapy. We were therefore able to discriminate between CS and CR asthmatics on the basis of this assay of corticosteroid inhibition of colony formation by MNC (Table 6).

The Cellular Components of Clusters and Colonies:

Giemsa staining of cytopins of intact clusters and colonies revealed that they were composed predominantly of lymphoblasts and mature lymphocytes. Mitotic figures and degenerate cells were present in each colony as well (Plate 1). One or two NSE positive cells with vacuolated cytoplasm and large cleaved nuclei were present in 95% of colonies and clusters examined and were thought to be monocytes (Plates 2 and 3). Electron microscopy of intact colonies confirmed the light microscopic findings of lymphoblasts, mature lymphocytes and degenerate cells in the colony (Plate 4). Close apposition between colony cells was also noted (Plates 5 and 6).

The presence of T-cells (Plate 7), monocytes (Plate 8), Ia positive cells (Plate 9), T-inducer cells (Plates 10a and 10b) and T-cytotoxic/suppressor cells (Plates 11a and 11b) in colonies were demonstrated using indirect immunofluorescence. The presence of T-inducer and T-cytotoxic/suppressor cells in the same colony was demonstrated in the majority of colonies examined using the double immunofluorescence method (Plates 12a and 12b).

Table 6 : Colony Formation by MNC from New Asthmatics in the Presence of 10^{-8} M MPS Before and After a 7-day Course of Oral Prednisolone .

New Asthmatic	Pre-steroid Colonies (%)*	Post-steroid Colonies (%)	Clinically Defined CS or CR Asthma
I. Ca	66	87	CR
A. Mc	48	26	CS
L. Do	40	29	CS
A. Si	25	19	CS
W. Cu	98	81	CR
S. Mo	35	48	CS

* : Colonies (%) = Number of colonies formed in the presence of 10^{-8} M MPS as a percentage of the number of colonies formed in the absence of corticosteroid.

@ : See definitions of New Asthmatic, CS and CR asthma (Materials and Methods)

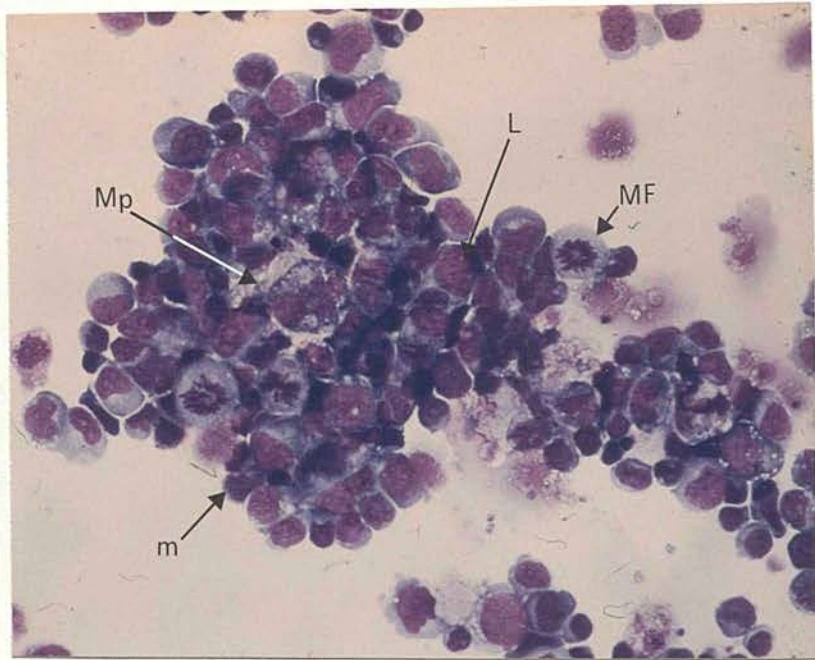
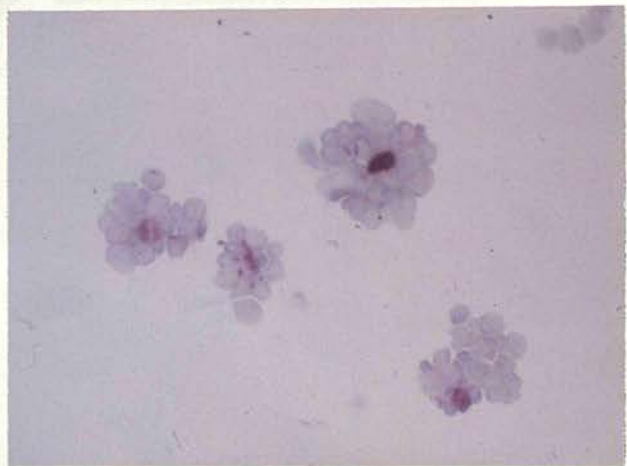
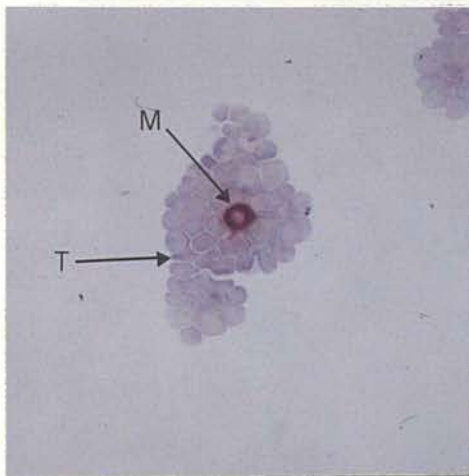


PLATE 1: Cytospins of Colonies Stained with Giemsa. Mature Lymphocytes (m), Lymphoblasts (L), Mitotic Figures (MF) and Macrophages (Mp) are present in the colony. Magnification: x200



PLATES 2 and 3: Cytospins of Colonies Stained for Non-Specific Esterase (NSE). A single NSE positive cell, thought to be a macrophage, is present at the centre of each colony (M). The central macrophage is surrounded by NSE negative T-cells (T). Magnification: x200

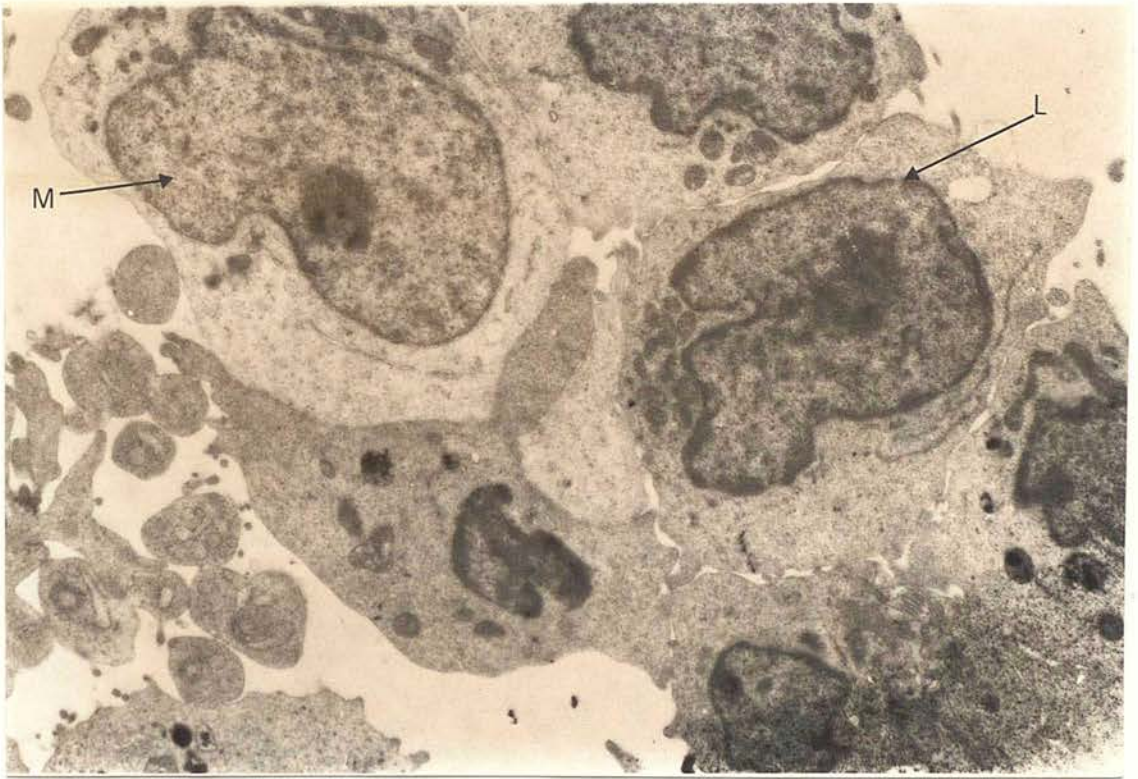


PLATE 4: Electron Micrograph of an Intact Colony. Cells showing the ultrastructural characteristics of lymphoblasts (L) and a monocyte (M) are present in the colony. Magnification: x12,000



PLATE 5: Electron Micrograph Showing Apposition between Colony Cells. Magnification: x20,000

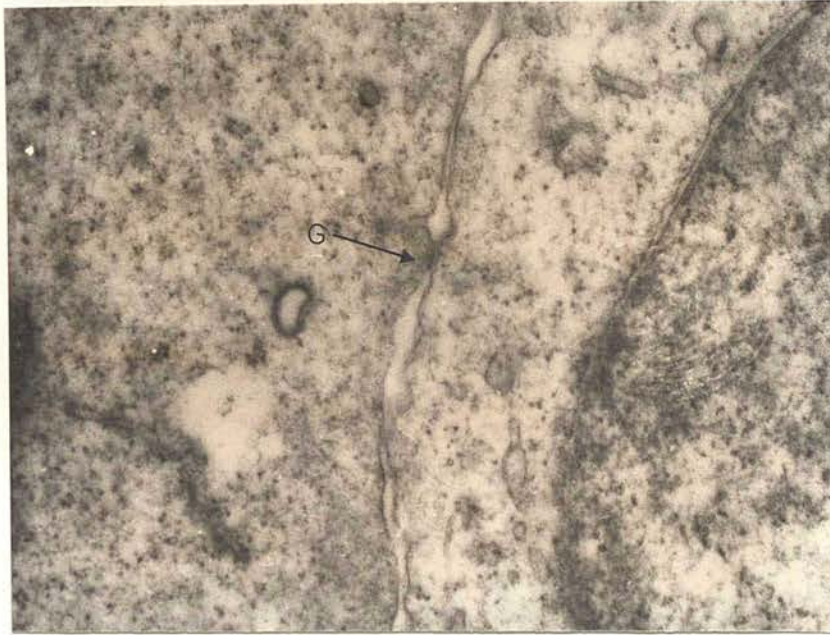


PLATE 6: Electron Micrograph Showing Gap Junction-like Structure
Between Colony Cells (G)
Magnification: x28,000

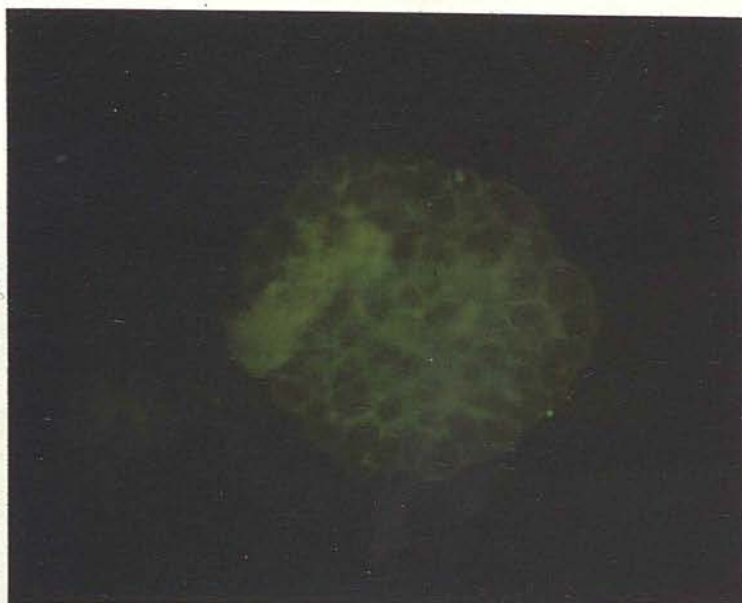


PLATE 7: Cytospin of Colony Stained with Fluorescein Labelled OKT3 (a pan T-cell marker). The majority of the cells in the colony show membrane fluorescence.
Magnification: x300



PLATE 8: Cytospin of Colony Stained with Fluorescein Labelled OKM1 (a monocyte marker). The cell at the centre of the colony shows membrane fluorescence.
Magnification: x600



PLATE 9: Cytospin of Cluster of Cells Stained with Fluorescein
Labelled Ia 231. The cells at the centre of the
cluster show membrane fluorescence.
Magnification: x600

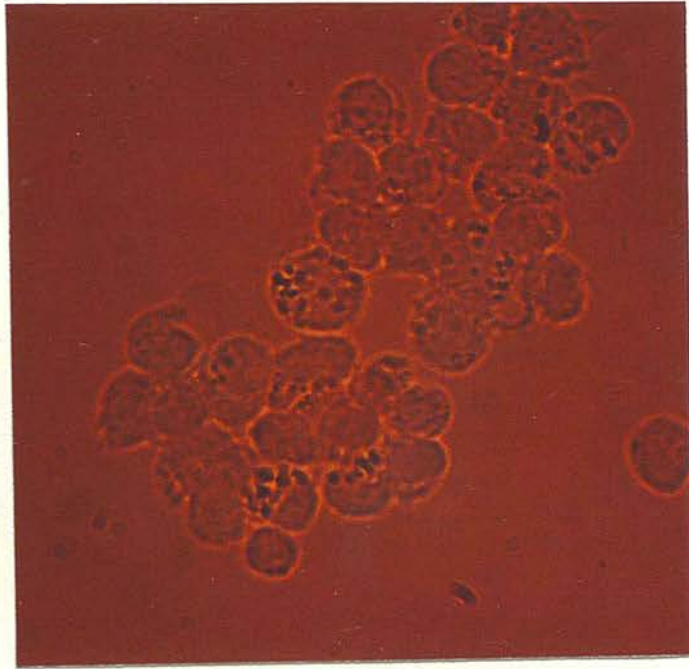


PLATE 10a: Colonies as viewed under the Phase Contrast Microscope.
Magnification: x300

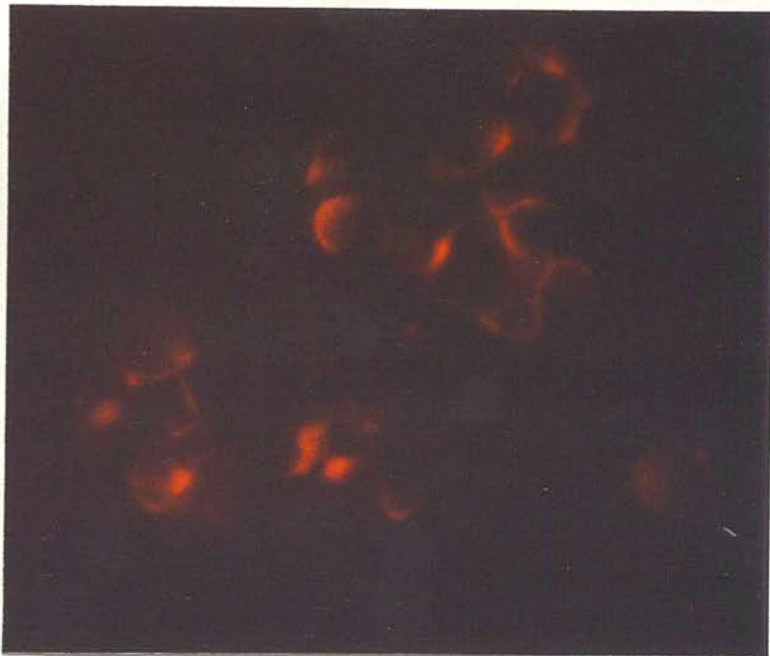


PLATE 10b: The Same Colonies Stained with Fluorescein Labelled
OKT₄ (a T-inducer cell marker). Numerous cells show
membrane fluorescence.
Magnification: x300

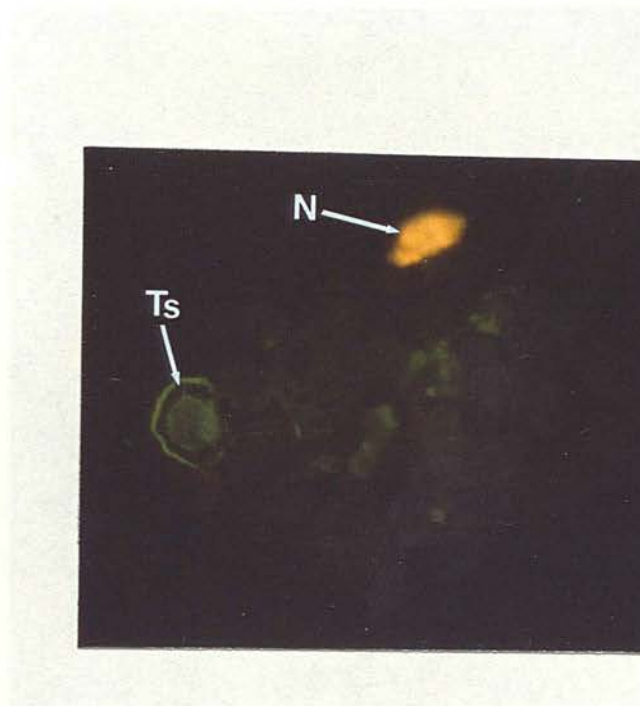


PLATE 11a: Cytospin of Colony Stained with Fluorescein Labelled OKT8 (a T-cytotoxic/suppressor cell marker). A single cell in the colony shows membrane fluorescence (Ts). There is also an area of non-specific fluorescence (N). Magnification: x300

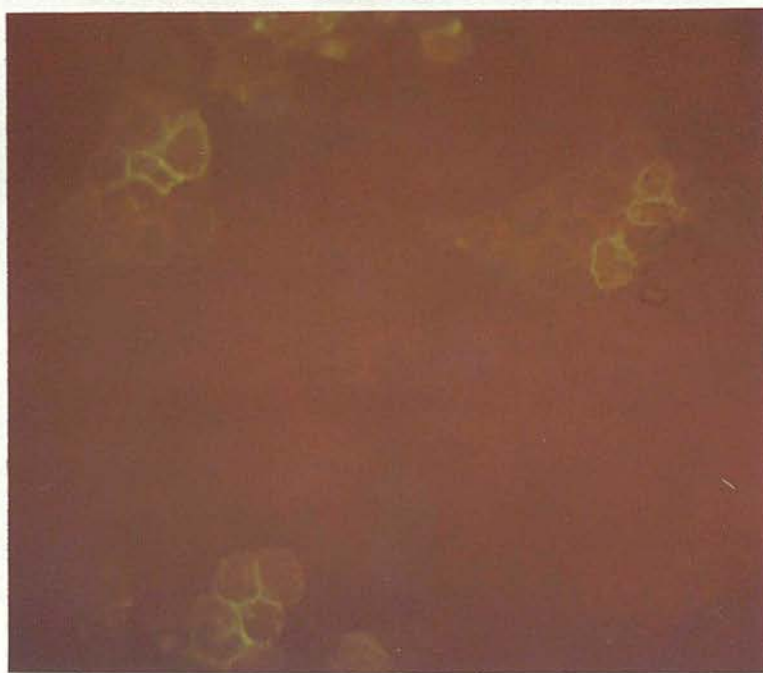


PLATE 11b: Cytospin of Clusters Stained as in Plate 11a. A minority of cells in each cluster show membrane fluorescence. Magnification: x300

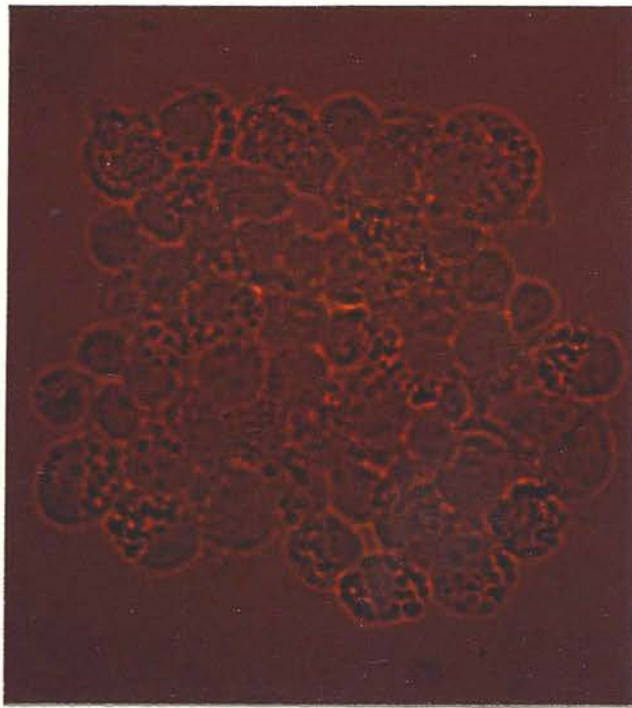


PLATE 12a: Large Colony as viewed under the Phase Contrast Microscope.
Magnification: x300

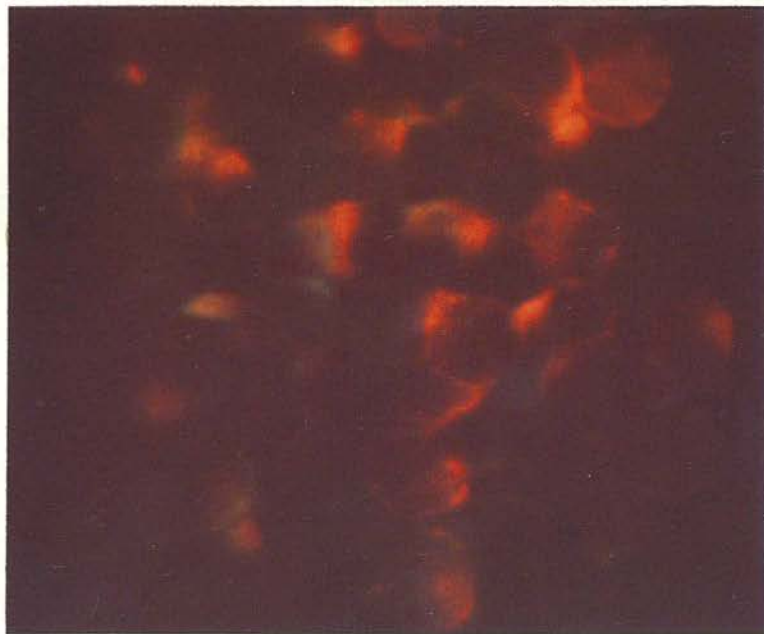


PLATE 12b: The Same Colony Stained with Rhodamine Labelled OKT₄ and Fluorescein Labelled OKT₈. The majority of cells in the colony are OKT₄ positive (Ti). A small minority of cells are OKT₈ positive (Ts).
Magnification: x300

Phenotype of T-cells Recovered From Harvested

Clusters and Colonies:

The phenotype of T-cells in suspensions of cells recovered from harvested colonies and clusters grown in the presence and absence of 10^{-8} M MPS was determined. The proportion of T-cells, as defined by OKT3, was the same for recovered cells from cultures of MNC from CS and CR asthmatics in the presence and absence of MPS. The phenotype of T-cells (as defined by OKT4 or OKT8) which were obtained from colonies and clusters grown in the absence of MPS resembled that of the input cells (Tables 4 and 7).

The most striking differences were obtained from clusters and colonies grown in the presence of 10^{-8} M MPS. Corticosteroid treated colonies and clusters from CS asthmatics differed significantly from those colonies and clusters that were generated in the absence of corticosteroid. It was shown that the proportion of T-inducer cells in clusters and colonies was significantly reduced by exposure to MPS.

In contrast, the proportions of T-inducer and T-cytotoxic/suppressor cells in the colonies and clusters from CR asthmatics were not altered by the presence of MPS in the culture medium (Table 7).

Table 7 : Phenotype of Cells Recovered from Harvested Clusters and Colonies.

		Percentage of Total Cells of Given Phenotype mean + s.d. (range)					
		10 ⁻⁸ M MPS	OKT3	OKT4	OKT8	OKT4:OKT8 ratio	
CS Asthmatics (8)	-		74.7+11.8 (55-86)	39.4+8.1 (29-53)	35.3+11.6 (19-55)@	1.24+0.47 (0.53-1.89)x	
	+		74.6+12.9 (60-86)	32.7+8.1 (22-44)	49.1+22.7 (28-67)@	0.79+0.28 (0.37-1.14)x	
CR Asthmatics (4)	-		69.6+ 2.8 (68-73)	44.0+5.3 (38-48)	25.7+ 8.1 (20-35)*	1.85+0.67 (1.08-2.30)#	
	+		73.6+ 5.8 (67-78)	45.0+10.0 (36-56)	24.3+ 6.6 (20-32)*	1.99+0.79 (1.66-2.08)#	

@ : p = less than 0.02 (Paired t Test)
 x : p = less than 0.002 (" ")
 * : p = not significant (" ")
 # : p = not significant (" ")

Colony Formation by Combinations of Mononuclear

Cells from Pairs of CS Asthmatics and CR asthmatics:

Treatment of MNC suspensions from CS and CR asthmatics with the appropriate monoclonal antibodies (OKT3 or OKM1) and complement removed about 75% of T-cells and 50% of monocytes (Table 8). In this way it was possible to prepare suspensions of MNC that were substantially enriched with T-cells (CST and CRT) or with monocytes (CSM and CRM).

CST, CSM, CRT and CRM all showed very poor colony formation in response to PHA when cultured alone. Furthermore no colony growth occurred in these cultures in the presence of 10^{-8} M MPS. When CST was recombined with CSM normal PHA-induced colony formation was seen. The CSM/CST recombination was highly sensitive to inhibition of colony formation by 10^{-8} M MPS. The recombination of CRT and CRM resulted in normal PHA induced colony formation that was not inhibited by 10^{-8} M MPS (Table 9).

When CSM was cultured with CRT, colony formation was highly sensitive to inhibition by 10^{-8} M MPS. In contrast when CRM was co-cultured with CST colony formation was highly resistant to inhibition by the same dose of corticosteroid (Table 9). This effect was demonstrated using the Tektronic Information system (Figure 5). No colony formation or "mixed leucocyte" type reaction occurred in these cultures in the

Table 8 : Preparation of Monocyte and T-cell depleted Cell Suspensions from MNC Derived from Pairs of CS and CR Asthmatics.

PATIENT	% of cells of phenotype shown			
	OKT3 before lysis	OKT3 after lysis	OKM1 before lysis	OKM1 after lysis
CS 1 CR 1	49.5% 34.0%	8.0% 12.0%	22.0% 29.0%	14.0% 22.0%
CS 2 CR 2	46.0% 20.0%	5.0% 3.0%	21.0% 36.0%	10.0% 12.0%
CS 3 CR 3	42.0% 36.0%	7.0% 7.0%	39.0% 41.0%	19.0% 17.5%

The results of three experiments are shown in the table.

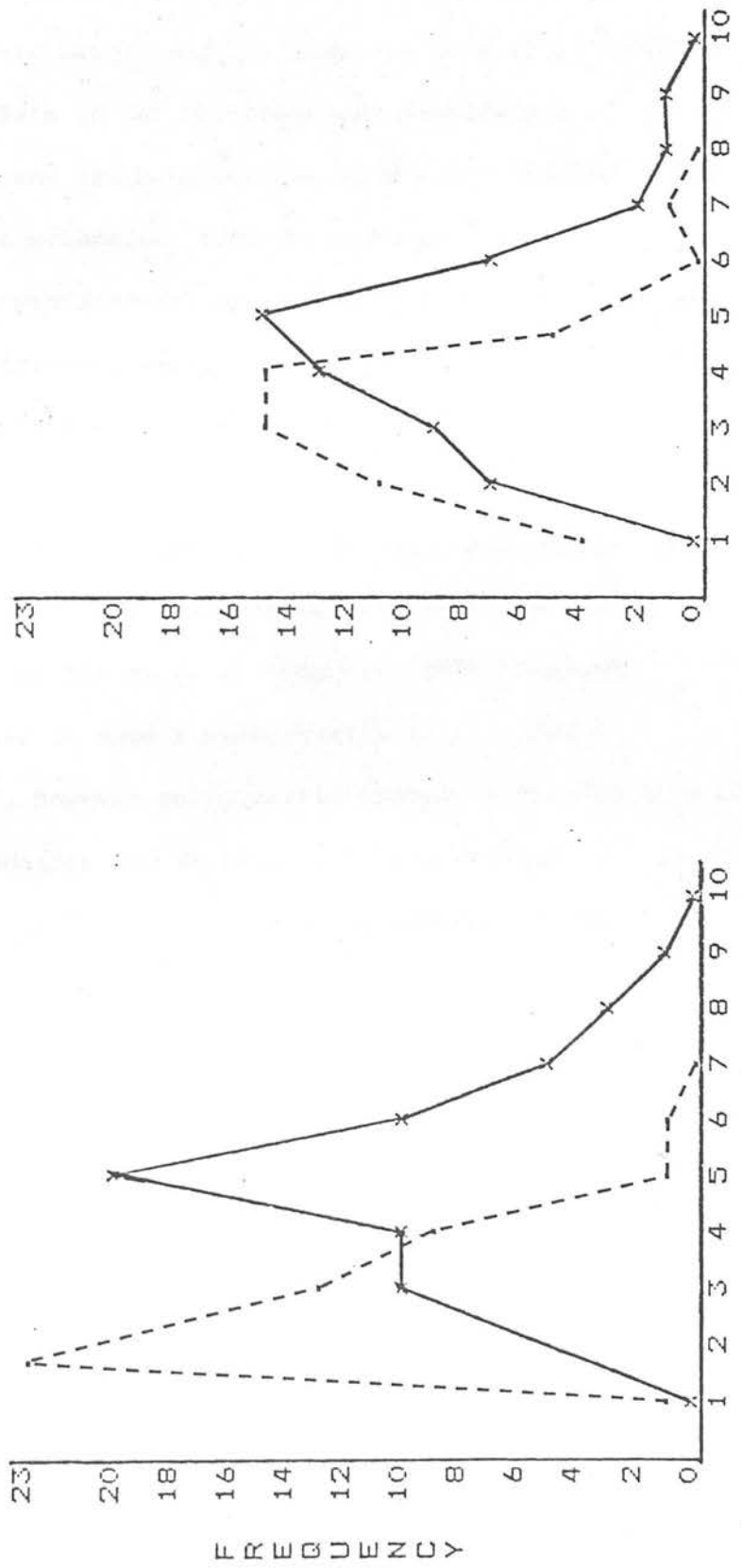
Table 9 : NUMBER OF COLONIES FORMED BY COMBINATIONS OF
MNC FROM A CS ASTHMATIC AND A CR ASTHMATIC
IN THE PRESENCE AND ABSENCE OF STEROID.

	No Steroid	+ 10 ⁻⁸ M MPS
CS monocytes (CSM) CR monocytes (CRM)	2 2	0 0
CS T-cells (CST) CR T-cells (CRT)	0 4	2 0
CSM + CST CRM + CRT	20 30	0 24
CSM + CRT CRM + CST	18 32	0 22
CRM + CST without PHA	0	0

CS : Corticosteroid sensitive
CR : Corticosteroid resistant

Figure 5 AREA AND NUMBER OF COLONIES FORMED IN THE PRESENCE AND ABSENCE OF MPS
 BY COMBINATIONS OF MNC FROM A CORTICOSTEROID RESISTANT AND
 A CORTICOSTEROID SENSITIVE ASTHMATIC.

Corticosteroid Sensitive Monocytes + Corticosteroid Resistant Monocytes
 Corticosteroid Resistant T-Cells + Corticosteroid Sensitive T-Cells



COLONY AREAS (uM sq. x 1000)

x = No Steroid
 — = 10⁻⁸M MPS

p = less than 0.00003 (Mann-Whitney) p = not significant (Mann-Whitney)

absence of PHA (Table 9).

Haematological Responses to Corticosteroids In Vivo

There were few differences between Controls and asthmatic patients in their haematological response to a single dose of prednisolone. Both CS and CR asthmatics developed a monocytopenia and lymphopenia within 6 hours (Figures 6 and 7). In addition asthmatics from both groups and controls demonstrated a preferential depletion of T-inducer cells as compared to T-cytotoxic/suppressor cells within 6 hours of the administration of the drug (Figure 8).

All but 1 of 10 CS asthmatics and all controls developed a marked neutrophilia and eosinopenia within 6 hours of the administration of the drug, as expected. This single CS asthmatic failed to show a neutrophilia in response to corticosteroid. However eosinopenia failed to develop in 4 of the 10 CR asthmatics and no neutrophilia developed in 5 CR asthmatics within 6 hours of the administration of the corticosteroid (Figures 9 and 10).

Figure 6 : EFFECT OF PREDNISOLONE ON MONOCYTES IN VIVO.

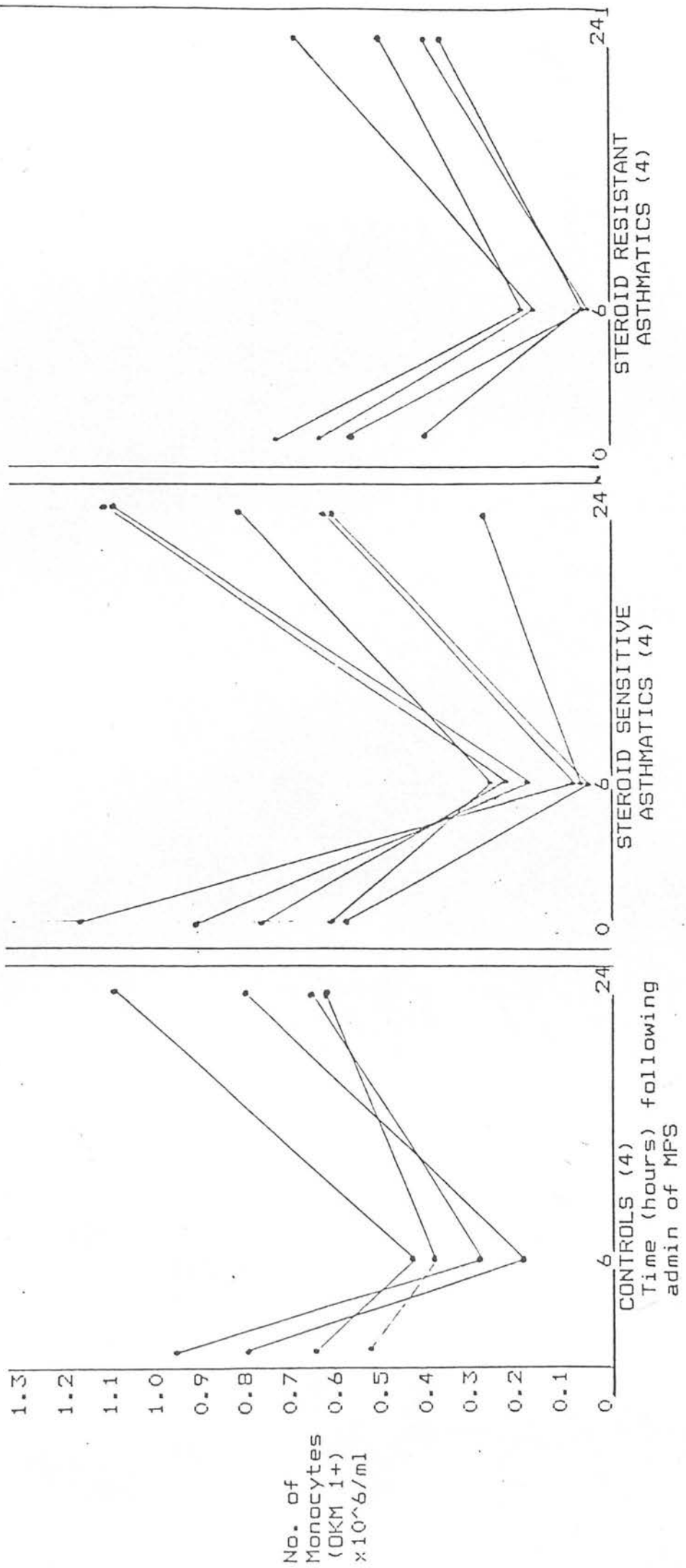


Figure 7 : EFFECT OF PREDNISOLONE ON T-CELLS IN VIVO.

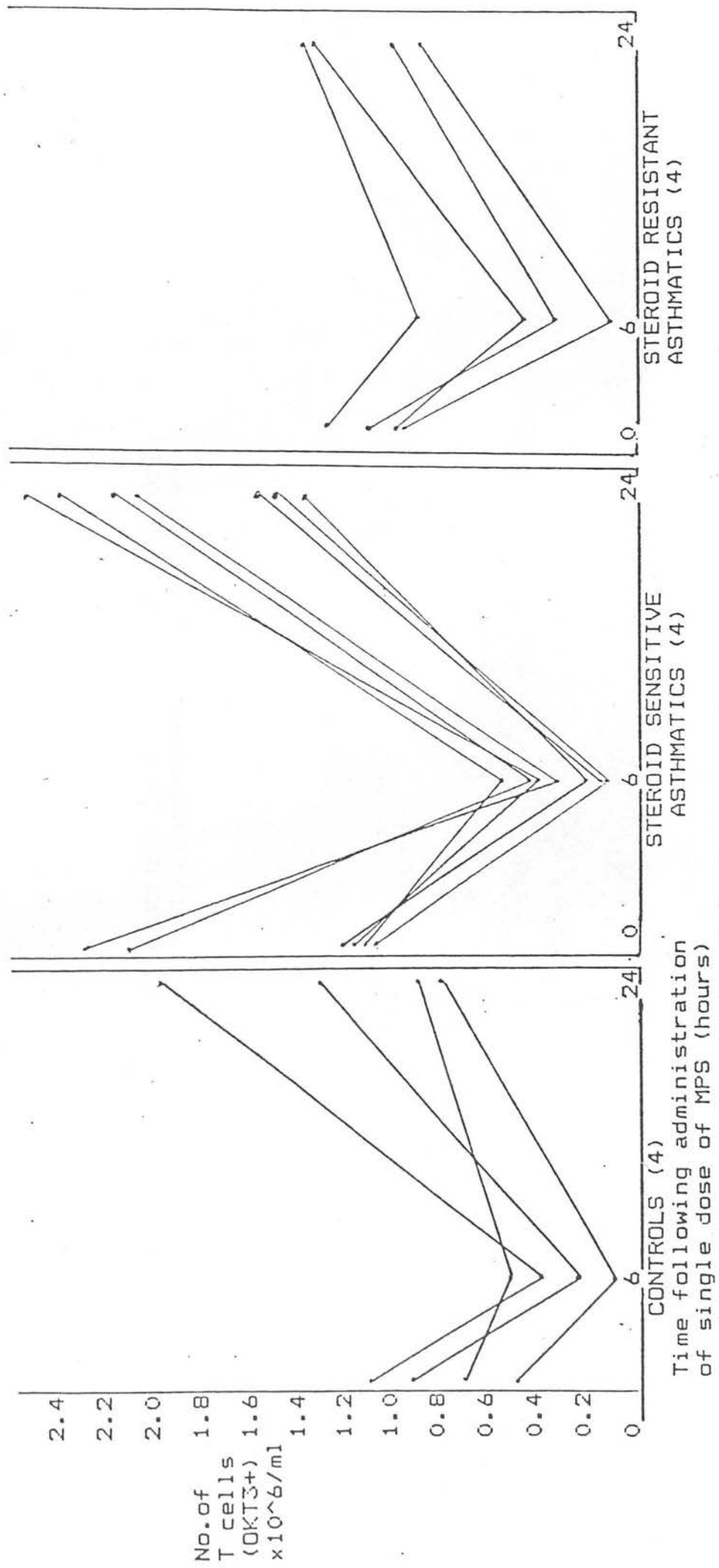


Figure 8 : EFFECT OF PREDNISOLONE ON THE RATIO OF T-INDUCER TO T-CYTOTOXIC/SUPPRESSOR CELLS IN VIVO.

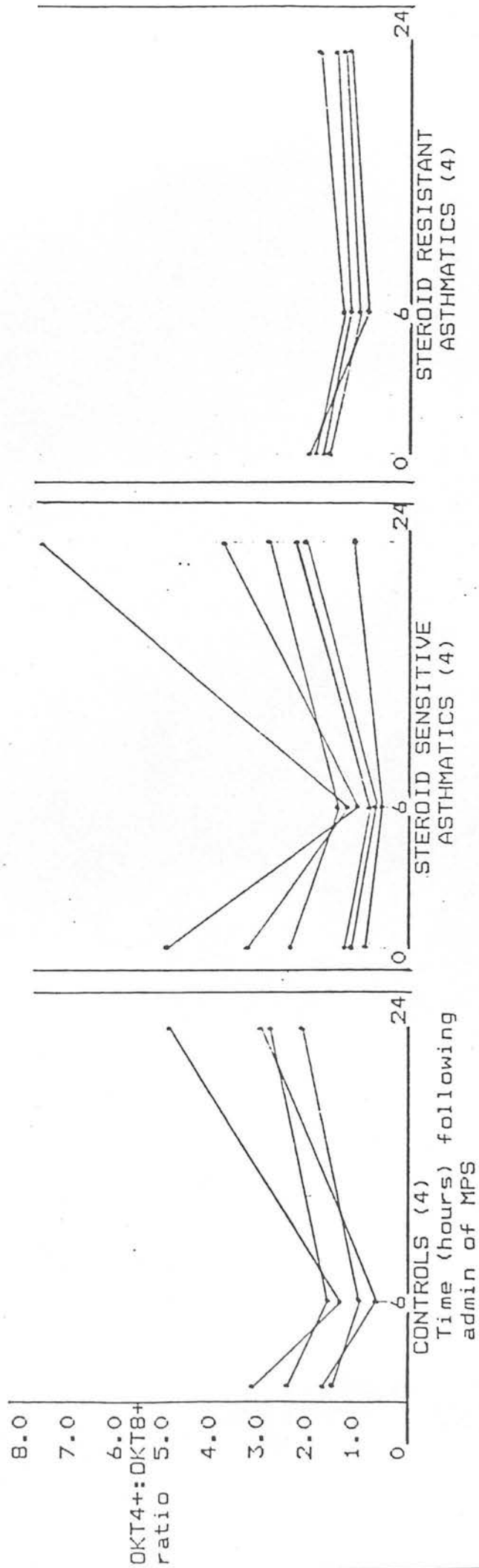


Figure 9 : EFFECT OF PREDNISOLONE ON POLYMORPHS IN VIVO.

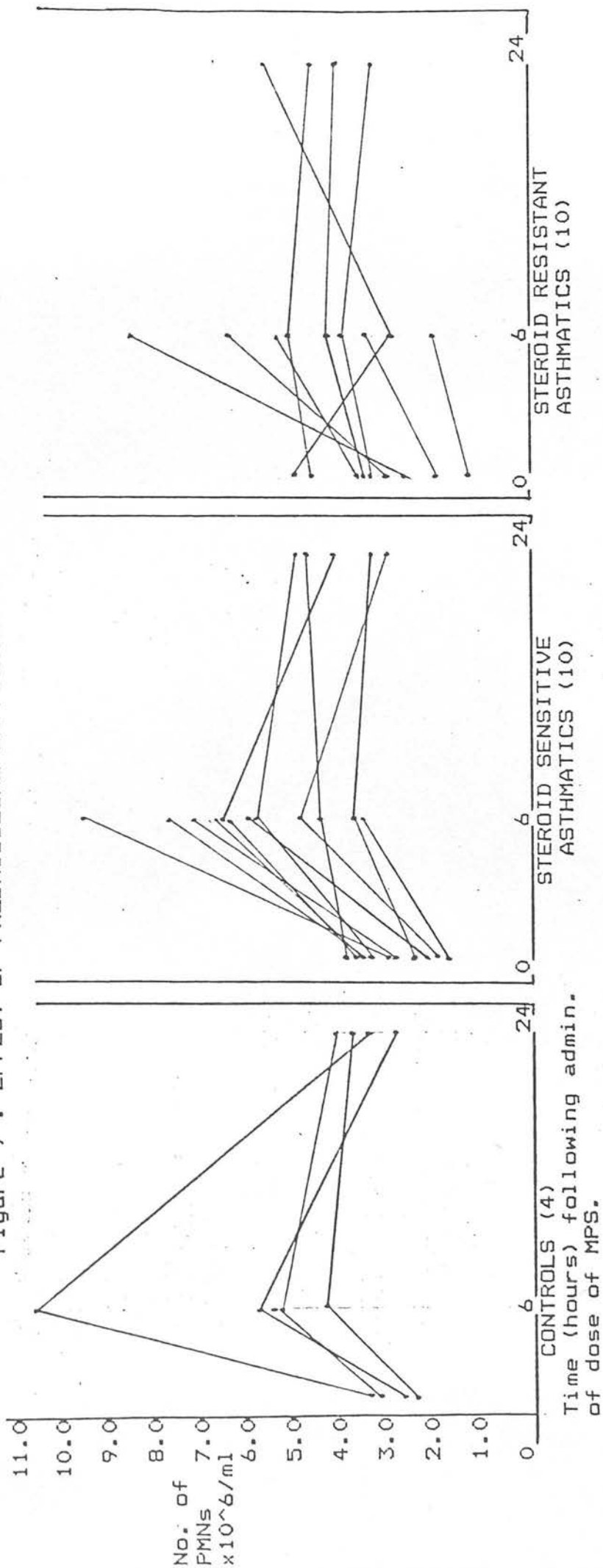
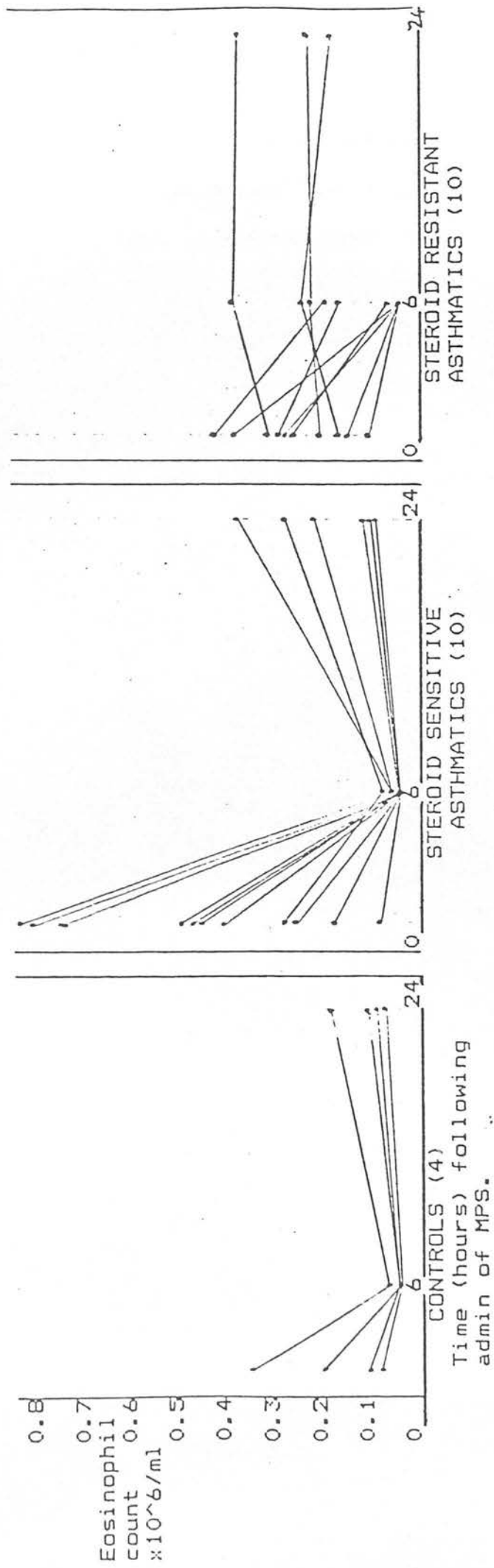


Figure 10 : EFFECT OF PREDNISOLONE ON EOSINOPHILS IN VIVO.



DISCUSSION

Corticosteroid resistant asthma became a clinically recognised entity in 1981 when Grant and his coworkers noted that a small proportion of chronic asthmatics showed no response to systemic treatment with corticosteroids [Carmichael et al. 1981]. Subsequent study of the clinical characteristics of CR asthma revealed few differences between these asthmatics and the majority of CS chronic asthmatics. All but three of a wide range of relevant investigations failed to discriminate between CS and CR asthmatics. Most importantly CR asthmatics failed to improve their ventilatory function following a 7-day course of systemic corticosteroid therapy. CR asthmatics were found to be more responsive to methacholine than were CS asthmatics. In addition monocyte complement expression (MCR) in CR asthmatics was less sensitive to inhibition by in vivo corticosteroid than was MCR expression by monocytes from CS asthmatics.

In the experiments reported in this thesis it was found that CS asthmatics were significantly more sensitive than were CR asthmatics to the inhibitory effect of corticosteroid on PHA-induced colony formation in vitro. This difference in sensitivity to corticosteroid was constant over time and was unaffected by a 7-day course of systemic corticosteroid therapy. The in vitro assay of corticosteroid sensitivity was also found to be of predictive value in six "new asthmatic" cases.

The difference in the corticosteroid sensitivities of MNC from CS and CR asthmatics in vitro was not absolute. CR asthmatics differed from CS asthmatics with respect to the range of concentrations of corticosteroid that caused inhibition of colony formation. Colony formation by MNC from both groups of asthmatics was always totally inhibited by 10^{-5} M MPS. The maximal difference in corticosteroid sensitivity occurred at 10^{-8} M MPS which represents a concentration of corticosteroid which would saturate the high affinity cytoplasmic glucocorticoid receptors described in many cell types. This concentration is also above the normal physiological range of cortisol. In addition, the non-parallel dose response curves of CS and CR asthmatics indicated that the difference between the MNC of these two groups of asthmatics was not simply a manifestation of a difference in the number of receptors for corticosteroid per responsive cell.

The CS asthmatics studied were matched to CR asthmatics with respect to age range, sex ratio, endogenous plasma cortisol and the severity of their asthma on admission to the study. Therefore the difference between the in vitro sensitivities of MNC from CR and CS asthmatics could not be explained on the basis of a difference in these clinical characteristics of the two groups of asthmatics. The proportions of monocytes, T-inducer cells and T-suppressor cells in the MNC fraction have been reported to have effects on the outcome of mitogen induced T-cell proliferation in vitro

[Gordon et al. 1980; Filipovich 1982]. As there was no significant difference between the MNC fractions from CS and CR asthmatics in these respects, however, the difference in steroid responsiveness of colony growth in vitro could not be attributed to such effects.

There is much evidence that monocytes are responsible for the initiation PHA-induced T-cell colony formation and that this function of monocytes is corticosteroid sensitive. It was demonstrated that the majority of colonies were centred on a single monocyte. It is also known that monocytes release IL 1 on stimulation by PHA and thereby initiate the proliferation of T-cells as described above. Monocyte production of IL 1 is inhibited by low concentrations of corticosteroid and inhibition of colony formation by these drugs is thought to be a manifestation of this [Krajewski and Wyllie 1981 ; Snyder and Unanue 1982]. The presence of CR monocytes was demonstrated by culturing combinations of MNC from pairs of CS and CR asthmatics. The combination of monocytes from a CR asthmatic with T-cells from a CS asthmatic resulted in colony growth which was resistant to 10^{-8} M MPS, whereas the combination of monocytes from a CS asthmatic with T-cells from a CR asthmatic resulted in corticosteroid inhibited colony growth. It therefore propose that corticosteroids fail to inhibit IL 1 production by monocytes from CR asthmatics.

Defects in corticosteroid responsiveness may also be present in leucocytes other than monocytes from CR asthmatics.

There is evidence that eosinophils, polymorphs and T-lymphocytes of CR asthmatics differ from those of CS asthmatics in response to corticosteroid. The investigation of the haematological effects of a single dose of prednisolone demonstrated that all but one of the 4 controls and 10 CS asthmatics developed an eosinopaenia and a neutrophilia within 6 hours of the administration of the drug, whereas 6 of the 10 CR asthmatics did not show any change in the numbers of circulating eosinophils and/or neutrophils in response to the corticosteroid.

Study of the clinical records showed that the CS and CR asthmatics studied in this thesis had received a similar quantity of systemic corticosteroid during the year prior to admission to this study (Dr. Ian Grant, personal communication). Although as stated earlier patients from neither group had received such therapy during the month prior to their admission to the project. It has been shown that patients who receive chronic systemic corticosteroid therapy for asthma and graft versus host disease have significantly reduced numbers of T-inducer cells in their peripheral blood as compared with untreated age and sex matched untreated controls [Moll et al. 1982 and Haynes and Fauci 1978]. The CS asthmatics studied in this thesis were also shown to have significantly reduced numbers of T-inducer cells in their peripheral blood as compared with controls. T-inducer cell numbers were not reduced in CR asthmatics hence it is possible that CR asthmatics do not show this presumptive effect of chronic systemic corticosteroid

therapy.

It was shown that the lymphocytes recovered from colonies and clusters which did develop from the MNC of CS asthmatics in the presence of 10^{-8} M MPS were substantially depleted in T-inducer cells as compared to those clusters and colonies that were not exposed to MPS. In contrast the proportion of T-inducer cells in colonies and clusters generated by MNC from CR asthmatics was unaffected by the presence of MPS in the culture medium at the same concentration.

On the basis of this evidence it seems possible that corticosteroid resistance may be expressed by cells other than monocytes from patients with CR asthma. It is conceivable that the responses of granulocytes and T-lymphocytes are themselves dependent on monocyte function. This question could be answered by direct experiments on the corticosteroid sensitivity of these granulocytes and T-cells. Thus neutrophil sensitivity to corticosteroid could be tested by an assay of induced lipomodulin production; eosinophil complement receptor enhancement following chemotactic stimuli could be compared in the presence and absence of corticosteroid ;and production of IL 2 by purified T-cells, stimulated in the absence of monocytes by IL 1 containing supernatants could be used as a test of the corticosteroid sensitivity of these cells.

The simplest interpretation of the data, however, is that the monocyte defect demonstrated in vitro in cells from CR asthmatics, is responsible for the corticosteroid resistance of their disease in vivo. This would therefore imply that the monocyte is the principle target of the therapeutic action of corticosteroids in CS asthma in vivo.

There are several ways in which the therapeutic action of corticosteroid in CS asthma might be mediated by monocytes and these fall into two main categories: a direct action involving effects of monocyte products on the synthesis of inflammatory mediators by mast cells and the indirect action of monocyte products on immunoregulatory cells causing the suppression of IgE production by plasma cells [Hirata 1982; Uede et al. 1983]. The assay used in this thesis has stressed the indirect action of corticosteroids. We would infer from the results that IL 1 production by CR monocytes is not inhibited by low concentrations of corticosteroid. This might result in the continued promotion of production of IgE in vivo. However pharmacologic doses of corticosteroid reduce the serum level of IgG but causes an increase in total serum IgE and specific IgE in vivo [Posey et al. 1978].

It therefore seems more likely that the specific defect that we have detected in monocytes from CR asthmatics in vitro is a manifestation of a general defect in the corticosteroid responsiveness of monocytes from these patients. This is supported by the data of Kay et al. on monocyte complement

receptor enhancement, which represents a different endpoint of monocyte function and which was found to be insensitive to modulation by corticosteroids in CR asthmatics [Kay et al. 1981]. Further study of corticosteroid sensitive functions of monocytes which are of greater relevance to the direct action of these drugs in asthma in vivo are required. This would undoubtedly include an assay of lipomodulin production by monocytes from CS and CR asthmatics.

In this thesis it has been demonstrated that monocyte function in CR asthmatics is insensitive to corticosteroid. The monocyte defect in CR asthma may be genetic or acquired. Interestingly there is a higher association of CR asthma with a family history of atopy than with CS asthma. Consequently a genetic basis for corticosteroid resistance was considered and HLA studies of patients with the condition have been initiated [Carmichael et al. 1981].

Recent work on the effects of corticosteroids on T-cell lines indicate that the origin of corticosteroid resistance may be a genetically determined defect in the corticosteroid receptor pathway or an epigenetic defect arising during a process such as differentiation [Gasson and Bourgeois 1983]. In addition Goodwin has shown that the MNC from elderly healthy subjects (over 65 years of age) are more resistant to the effects of corticosteroid in vitro than are MNC from young

healthy controls [Goodwin 1982]. The relevance of these findings to CR asthma may be determined in the future.

Corticosteroid resistance has been demonstrated in a small proportion of chronic asthmatics in Edinburgh. However the implications of this condition are much wider. There is no reason to suppose that corticosteroid resistance does not occur in the general population. In this case corticosteroid resistance would be of importance when the response to these hormones was essential for the success of medical therapy, as in Asthma, Sarcoidosis, Graft versus Host disease, Systemic Lupus Erythematosus and Rheumatoid Arthritis. Furthermore it is interesting to inquire whether a monocyte defect of the type described here would affect the responses of the immune system to modulation by physiological concentrations of corticosteroid and perhaps influence the course of chronic inflammatory disorders. Further investigation of the biology of corticosteroid resistance in asthma may therefore have implications for other developmental and pathological processes.

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REFERENCES

- Brasher, J.W. (1977)
IgE suppressor T-cells and atopy.
Lancet (ii) 927
- Blackwell, G.J. et al. (1980)
Macro cortin: a polypeptide causing the anti-phospholipase
effect of glucocorticoids.
Nature 287 pp. 147-149
- Boyum, A. (1968)
Isolation of mononuclear cells and granulocytes from human
blood.
Scand. J. Lab. Clin. Invest. 21 (supplement 97) pp. 77-89
- Blomgren, H. and Andersson, B. (1976)
Steroid sensitivity of PHA and PWM responses of fractionated
human lymphocytes in vitro.
Exp. Cell. Res. 97 pp. 233-241
- Carmichael, J. et al. (1981)
Corticosteroid resistance in asthma
Br. Med. J. 282 pp. 1419-1482
- Clarke, J.R. et al. (1977)
The effect of prednisolone on leucocyte function in
man.
Clin. Exp. Imm. 28 pp. 292-301
- Cline, M.J. et al. (1978)
Monocytes and macrophages: functions and disease.
Ann. Intern. Med. 88 pp. 78-88
- Crompton, G.K. (1982)
Steroids in respiratory disease.
Br. J. Hosp. Med. pp. 340-348
- Cupps, T.R. and Fauci, A.S. (1982)
Corticosteroid mediated immunoregulation in man
Imm. Rev. 65 pp. 133-155
- Dunsky, E.H. (1979)
Early effects of corticosteroids on Basophils, leucocyte
histamine and tissue histamine.
J. All. & Clin. Imm. 64 pp. 426-432
- Dupont, E. et al. (1983)
Depletion of lymphocytes with membrane markers
of helper phenotype : a feature of acute and chronic drug indu
immunosuppression.
Clin Exp. Imm. 51 pp. 345-350
- Farrar, J.J. et al. (1982)

The biochemistry, biology and role of Interleukin 2:
in the induction of the cytotoxic T-cell and antibody
forming B-cell responses.
Imm. Rev. 63 pp. 129-166

Fauci, A.S. and Dale, D.C. (1974)

The effect of in vivo hydrocortisone on subpopulations
of human lymphocytes.
J. Clin. Invest. 53 pp. 240-246

Filipovich, A.H. (1982)

The correlation between Inducer/suppressor Ratios,
generation of Con A activated suppressor cells, and mitogen
stimulated proliferation of peripheral blood lymphocytes
in patients with Alopecia Areata and normal controls.
Clin. Imm. and Immunopath. 25 pp- 21-31

Flower, R.J. and Blackwell, G.J. (1979)

Anti-inflammatory steroids induce biosynthesis of a
phospholipase inhibitor which prevents prostaglandin
generation.
Nature 278 pp. 456-457

Galanaud, P. et al. (1981)

Hydrocortisone sensitivity of in vitro antibody response:
different sensitivity of specific and non-specific B-cell
responses induced by the same agent.
Clin. Imm. & Immunopath. 18 pp. 68-75

Gasson, J.C. and Bourgeois, S. (1983)

A new determinant of corticosteroid
sensitivity in lymphoid cell lines.
J. Cell Biol. 96 pp. 409-415

Geha, R.S. et al. (1981)

Deficiency of T-suppressor cells in hyper IgE syndrome.
J.Clin Invest 68 pp.783-791

Gillis, S. et al. (1979)

Corticosteroid induced inhibition of T cell growth
factor production. I.The effect on mitogen induced
lymphocyte proliferation
J. Imm. 123 pp. 1624-1629

Gillis, S. et al. (1982)

Molecular characterisation of Interleukin 2.
Imm. Rev. 63 pp. 167-209

Goodwin, J.S. (1982)

Changes in lymphocyte sensitivity to Prostaglandin E, Histamin
Hydrocortisone, and X-irradiation with age: Studies in healthy
elderly population.
Clin. Imm. & Immunopath. 25 pp. 243-251

Gordon, M.Y. et al. (1980)

The use of a T-lymphocyte colony assay to measure mononuclear phagocyte function
Clin. Exp. Immunol. 42 pp. 156-161

- Goube de la Forest, P. et al. (1979)
Growth characteristics of PHA induced colonies in primary and secondary agar culture.
Imm. 35 pp. 917-922
- Gupta, S. (1980)
Subpopulations of human T-lymphocytes in bronchial asthma.
Int. Archs. Appl. Imm. 61 pp. 293-298
- Gupta, S. and Good, R.A. (1981)
Human T-lymphocyte subpopulations and Asthma.
Imm. Rev. 56 pp. 89-114
- Haynes, B.F. and Fauci, A.S. (1978)
The differential effect of in vivo hydrocortisone on kinetics of subpopulations of human blood T-lymphocytes.
J. Clin. Invest. 61 pp. 703-707
- Haynes, B.F. and Fauci, A.S. (1979)
Mechanisms of action of corticosteroids on lymphocyte subpopulations: V. Effects of in vivo hydrocortisone on naturally occurring and mitogen induced suppressor cells.
Cell. Imm. 44 pp. 169-178
- Hirashima M. (1980)
Regulatory role of IgE binding factors from rat T-Lymphocytes III. IgE suppressive factor with IgE binding activity.
J. Imm. 125 pp.1442-1449
- Hirata, F. (1982)
A possible mediator of glucocorticoid action. Advances in prostaglandin, thromboxane and leukotriene research.
Edited by R.Samuelson et al. (New York press)
- Hsieh, T. (1980)
Increased T-suppressor cell activity in Allergic children
Ann. Allergy 47 pp. 186-189
- Ishizaka, K. (1982)
Regulation of IgE response.
Immunology 80: Fourth International Congress of Immunology.
Edited by M. Fougereau and J. Dausset
Academic Press Inc. (London) Ltd.
- Jarret, E.E.E. (1977)
Activation of IgE regulatory mechanisms by transmucosal absorption of antigen.
Lancet (ii) pp. 223-225

- Katz, P. and Fauci, A.S. (1979a)
Autologous and allogenic cellular interactions: modulation of adherent cells, irradiation and in vitro and in vivo corticosteroids.
J. Imm. 123 pp. 2270-2277
- Katz, P. and Fauci, A.S. (1979b)
The effects of corticosteroid on immunoregulation in sarcoidosis.
Cell. Imm. 42 pp. 308-318
- Kay, A.B. et al. (1981)
Corticosteroid resistant chronic Asthma and monocyte complement receptors.
Clin. Exp. Imm. 44 pp. 576-580
- Krajewski, A.S. and Wyllie, A.H. (1981)
Inhibition of human T-lymphocyte colony formation by methylprednisolone.
Clin. Exp. Imm. 46 pp. 206-213
- Marrach, P. et al. (1982)
Non-specific factors in B-cell responses.
Imm. Rev. 63 pp. 33-50
- Mazaheri, G. et al. (1982)
Lymphocyte subsets in the allograft recipient: correlation of the helper:suppressor ratio with clinical events.
Transpl. Proc. 14 pp. 676-678
- Mizel, S.B. (1982)
Interleukin 1 and T-cell activation.
Imm. Rev. 63 pp. 51-72
- Moll, B. et al. (1982)
Inverted ratio of inducer to suppressor T-lymphocyte subsets in drug abusers with opportunistic infections.
Clin. Imm. and Immunopath. 25 pp. 417-423
- Morretta, L. et al. (1976)
Functional analysis of two human T-cell subpopulations: Help and suppression of of B-cell responses by T-cells bearing receptors for IgM or IgG.
J.Exp.Med. 146 pp. 184-200
- O'Driscoll, R. and Kay, A.B. (1982)
Leukotrienes and lung disease
Thorax 37 pp. 241- 245
- Onsrud, M and Thornsby, E. (1981)
Influence of in vivo hydrocortisone on some human blood lymphocyte subpopulations.
Scand. J. Imm. 13 pp. 573-579

- Orgel, H.J. et al. (1975)
 Development of IgE and allergy in infancy.
 J. Imm. & All. 56 pp. 296-307
- Palacios, R. (1982)
 Mechanism of T-cell activation: Role of functional relationship and HLA-DR antigens and Interleukins.
 Imm. Rev. 63 pp. 73-110
- Parillo, J.E. and Fauci, A.S. (1978)
 Mechanisms of corticosteroid action on lymphocyte subpopulations.
 J. Clin. Exp. Imm. 31 pp. 116-129
- Pearse, A.G.E. (1960)
 Histochemistry (2nd edition)
 J. & A. Churchill Ltd. (London)
- Posey, W.C. et al. (1978)
 The effects of acute corticosteroid therapy for asthma on serum Immunoglobulin levels.
 J. Allergy & Clin. Imm. 62 pp. 340
- Rachelefsky, G.F. (1976)
 Defective T-cell function in atopic children.
 J. Imm. & All. 57 pp. 569-576
- Rinehart, J.J. et al. (1974)
 Effects of corticosteroid on human monocyte function.
 J. Clin. Invest. 54 pp. 1337-1343
- Reinherz, E.L. et al. (1980)
 Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage.
 Proc. Nat. Acad. Sci. U.S.A. 77 pp. 1588-1592
- Saryan, J.A. et al. (1983)
 Induction of human IgE synthesis by a factor derived from T cells of patients with hyperIgE states.
 J. Imm. 130 pp. 242-247
- Shreiber, A.D. et al. (1975)
 Effects of corticosteroid on the human monocyte IgG and complement receptors.
 J. Clin. Invest. 56 pp. 1189-1197
- Smith, K.A. and Ruscetti, F.W. (1981)
 T-cell growth factor and the culture of cloned functional T-cells.
 Adv. Imm. 31 pp. 137-175
- Snyder, D.S. and Unanue, E.R. (1982)
 Corticosteroid inhibition of murine macrophage Ia expression and Interleukin 1 production.

J. Imm. 129 pp. 1803-1805

Suemura, M. et al. (1980)

Regulatory role of IgE binding factors. I. Mechanisms of enhancement by IgE response by IgE potentiating factor. J. Imm. 125 pp. 148-152

Strannegard, I-L. et al. (1976)

T-lymphocytes from Atopic children. Int. Arch. All. & Appl. Imm. 50 pp. 684-692

Strannegard, O. and Strannegard, I-L (1978)

T-lymphocytes in allergy. Imm. Rev. 41 pp. 149-170

Taussig, M.J. (1979)

Processes in Pathology
Blackwell (London)

Tomita, K. et al. (1982)

Active E-rosettes forming cells in children with asthma. Ann. Allergy 48 pp. 103-118

Uede, T. et al. (1983)

Modulation of the biologic activities of IgE binding factors. I: Identification of glycosylation inhibiting factor as a fragment of lipomodulin. J. Imm 130 pp. 878-884

RESISTANCE TO METHYLPREDNISOLONE BY CULTURES OF BLOOD
MONONUCLEAR CELLS FROM GLUCOCORTICOID RESISTANT
ASTHMATIC PATIENTS

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Key Words: asthma T-cell colony glucocorticoid
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 methylprednisolone

Abbreviations: MNC, mononuclear cells
 MP, methylprednisolone sodium succinate

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SUMMARY

1. In order to investigate the cellular mechanism of glucocorticoid resistance in chronic asthma, peripheral blood mononuclear cells from asthmatic patients were cultured in soft agar.

2. Cells from patients known to be clinically sensitive to glucocorticoid therapy did not differ significantly from those from clinically resistant patients in terms of their immunophenotype or the number of colonies generated by culture in the presence of phytohaemagglutinin.

3. The glucocorticoid methylprednisolone at low concentration (10 nmol/l) inhibited colony growth from cells of glucocorticoid sensitive patients, whereas there was much less inhibition of colony growth from resistant patients' cells.

4. In a small prospective study inhibition of colony growth by methylprednisolone in vitro correlated with the subsequently determined sensitivity of the patients' asthma to glucocorticoid therapy.

5. Assessment in vitro of glucocorticoid sensitivity may help to predict which patients may be spared ineffectual glucocorticoid medication. The results raise the possibility that peripheral blood mononuclear cells may respond to glucocorticoid in a similar manner to cells involved in the pathogenesis of asthma.

INTRODUCTION

Some patients with chronic asthma are resistant to systemic treatment with glucocorticoids, even in high dosage. Measurements of forced expiratory volume in one second (FEV₁) show striking differences between the response of resistant and sensitive patients to the short-term administration of systemic glucocorticoids, in sharp contrast to the considerable increases recorded in both groups after use of a bronchodilator aerosol [1]. Detailed study of a large group of glucocorticoid resistant asthmatic patients in Edinburgh has already identified some differences in the aetiology and clinical patterns of asthma between the two groups. It was considered improbable, however, that these differences were central to the phenomenon of glucocorticoid resistance. Only one of a wide range of laboratory investigations discriminated between resistant and sensitive asthmatic patients - the measurement of monocyte complement receptors and their enhancement by casein, a monocyte chemotactic factor [2]. In this paper we show that peripheral blood mononuclear cells (MNC) from glucocorticoid resistant asthmatic patients are relatively unresponsive to glucocorticoid in vitro and we suggest that this may be used to predict glucocorticoid resistance in asthmatic subjects whose clinical response to these drugs has not yet been assessed.

PATIENTS AND METHODS

The laboratory studies reported in this paper were performed on venous blood samples obtained from 41 patients with chronic asthma and 15 normal subjects (Table 1).

All 41 asthmatic patients had been shown to respond to the inhalation of an aqueous β_2 -agonist aerosol (salbutamol, 5 mg) by an increase in the FEV₁ of at least 30% from the base-line figure of less than 60% of the predicted normal value. This evidence of reversible airflow limitation was regarded as consistent with the diagnosis of asthma. The patients were, however, selected in such a way as to form three separate groups.

Group 1 consisted of 15 asthmatic patients who were 'glucocorticoid resistant' in that their FEV₁ did not increase by more than 15% after a short course of prednisolone by mouth, even when the dose was increased progressively to at least 60 (and in some cases to 1000) mg/day. Although 9 were on regular treatment with a glucocorticoid aerosol, the mean FEV₁ for the whole group was no higher on the day of blood sampling than on earlier clinic visits.

Group 2 consisted of 15 asthmatic patients who were 'glucocorticoid sensitive', as shown by an increase in FEV₁ of more than 30% after a 7-day course of prednisolone in a dose of only 20 mg/day. Nine of these patients had recently started regular treatment with a glucocorticoid aerosol and in all 15 the disease was well controlled at the time of blood sampling. This

may explain why the mean FEV₁ was significantly higher at that time than at their initial clinic visit.

Group 3 consisted of 11 'new' asthmatic patients whose response to prednisolone, in terms of FEV₁, had not previously been assessed.

As observed in the original study [1], the glucocorticoid resistant patients were on average older, had a longer history of asthma, and were more prone to nocturnal wheeze and 'morning dipping' than the sensitive patients. There was no difference between the two groups in respect of atopic status or smoking habits. The more frequent family history of asthma in the resistant patients noted in the original study [1] did not emerge in the smaller numbers included in the present series (Table 1).

The criteria for inclusion of patients in groups 1 and 2 eliminated all asthmatic patients whose FEV₁ response to glucocorticoid was in the 'grey area' between 15 and 30%, with the result that only patients who were unequivocally either 'glucocorticoid resistant' or 'glucocorticoid sensitive' were studied. Although this facilitated correlation of clinical and laboratory findings, it left unexplored the possibility that 'glucocorticoid resistance' and 'glucocorticoid sensitivity' might represent the ends of a continuous spectrum. It was hoped that the inclusion of group 3 would elucidate this point. The 11 patients in that group were asthmatics who had presented with moderately severe airflow obstruction for which a course of oral prednisolone was indicated. Their FEV₁ was measured before, and

immediately after, a 7-day course of prednisolone (20 mg/day). Laboratory studies were undertaken in all 11 cases before treatment commenced and again in 9 on its completion.

None of the asthmatic patients or the controls had co-existing malignant disease, was being treated with cytotoxic or immunosuppressive drugs, or was suffering from any form of inter-current infection, including oropharyngeal candidiasis. About half of the patients in groups 1 and 2 had previously been treated with oral prednisolone but there was no significant difference between the groups in terms of total dosage. For at least 4 weeks prior to this study no subject had received glucocorticoid therapy other than beclomethasone dipropionate by inhalation. The dose did not exceed 400 micrograms daily, which is known not to suppress pituitary-adrenal function [3]. On the morning of blood sampling the mean plasma cortisol was normal in all groups (Table 1).

Analysis of Blood Samples:

Peripheral venous blood (50 ml) was withdrawn at 09.00 hours from each subject into a sterile bottle containing 2ml of 2% EDTA in phosphate buffered sodium chloride solution (154 nmol/l). All the laboratory studies were carried out without knowledge of the clinical status of the subjects. Total leucocyte count, differential leucocyte cell count and plasma cortisol were determined on all samples.

Mononuclear cells (MNC) were obtained from the peripheral blood by centrifugation over Ficoll-Hypaque [4] and were then

washed twice in Hank's balanced salt solution. Subpopulations of T-lymphocytes, monocytes and Ia positive cells were quantified by means of monoclonal antibodies with the following specificities: OKT3 (peripheral blood T-cells); OKT4 (inducer T-cell subset); OKT8 (cytotoxic/suppressor T-cell subset); OKM1 (peripheral blood monocytes); and Ia231 (MNC bearing Ia antigens, including 90% of B-lymphocytes and monocytes, 20% of null cells, and activated T-lymphocytes). Antibodies in the OK series were obtained from Ortho Diagnostics Ltd.

Cells positive for OKT3, OKT4, OKT8, OKM1 or Ia231 were detected by indirect immunofluorescence using fluorescein-isothiocyanate labelled goat anti-mouse immunoglobulin (Meloy Ltd) as the second antibody. The percentage of cells with membrane fluorescence was calculated from study of 200 cells. For each case a negative control was included: cells to which only second antibody had been added.

Colony formation from peripheral blood mononuclear cells

MNC from each subject were cultured by using previously described methods [5,6]. For each culture, 2×10^5 cells were suspended in 40 μ l of RPMI 1640 supplemented with 10% heterologous serum and 0.3% agar, and plated on to an underlayer of 0.2ml of 0.5% agar containing 20 μ g of phytohaemagglutinin (Difco) and 10% heterologous AB serum (the same source of serum was used throughout). Methylprednisolone sodium succinate (MP; Upjohn) was added to underlayers to give a final concentration of 10 nmol/l. Control cultures received no MP. All cultures were

in 17mm diameter wells in Multiwell dishes (Linbro) and were incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air. After 5 days of incubation colonies (defined as aggregates of more than 40 cells) were counted by phase contrast microscopy at x 100 magnification in three central fields in each of three replicate wells. This relatively simple scoring method has been verified by detailed computer-assisted analysis of the size distribution of all cell clusters in the cultures. Detailed immunophenotyping of these colony cells showed them to consist mainly of OKT4 and OKT8 lymphocytes, but most colonies also included a central cell bearing monocyte markers (M.C. Poznansky, A.C.H. Gordon, I.W.B. Grant and A.H. Wyllie, unpublished work).

RESULTS

Analysis of the peripheral blood leucocytes revealed that there were few differences between the known 'glucocorticoid resistant' asthmatic patients (group 1) and the 'glucocorticoid sensitive' asthmatic patients (group 2) or between the asthmatic patients and normal control subjects. As expected, the asthmatic patients had an eosinophilia not found in controls. The resistant and sensitive patients did not differ significantly from each other or from control subjects in terms of the proportion of T-cells, T-inducer cells, Ia positive cells or cells reacting with the OKM1 monocyte monoclonal antibody. There was a slightly higher proportion of T-suppressor/cytotoxic cells in the blood of sensitive asthmatic patients as compared with controls. The mean proportion of T-suppressor/cytotoxic cells in resistant asthmatic patients was less than in sensitive asthmatics, but this difference failed to attain significance at the $p < 0.05$ level (Table 2).

In the absence of MP, MNC from both controls and asthmatic patients in groups 1 and 2 generated similar numbers of colonies in soft agar after 3-5 days incubation, representing a plating efficiency of approximately 0.05% (Table 3). Incubation during growth with MP at 10 nmol/l significantly inhibited colony numbers developing from the MNC of control subjects and sensitive asthmatics. In contrast this concentration of MP had little effect on colony development from the MNC of resistant asthmatics (Table 3).

To minimize purely technical variations in the plating efficiency and in order to permit comparison between patients, the number of colonies developing from each patient's MNC in the presence of MP at 10 nmol/l was expressed as a percentage of the number developing in its absence (Fig. 1). It was clear that the responses of individual patients, expressed in this way, fell with very few exceptions into two categories. Colony growth from MNC of glucocorticoid sensitive patients was strongly inhibited by MP in vitro, whereas growth from MNC of resistant patients was affected little by MP. In all but one of the control subjects colony growth was inhibited by MP to the same extent as that from glucocorticoid sensitive asthmatic patients.

The clinical sensitivity of the 11 asthmatic patients in group 3 to glucocorticoid was unknown at the outset of the study. In these patients, however, the response to glucocorticoid, as determined by colony inhibition in vitro, was found to correlate closely with the extent to which FEV₁ increased during subsequent treatment with oral prednisolone (Fig. 2). This is shown in Fig. 2, where percentage changes in colony number, induced in vitro by MP, are plotted against the percentage change in FEV₁, resulting from a 7-day course of oral prednisolone. An identical relationship was evident when the in vitro responses were plotted against the absolute change in FEV₁ (data not shown).

The percentage change in colony number, induced by MP in vitro, appeared to be characteristic for each subject. Thus, on retesting 6 subjects between 3 weeks and 4 months later, the divergence from the initial value was in every case less than 20%

(Fig. 3A). In particular, colony numbers from glucocorticoid sensitive patients and control subjects were strongly inhibited by MP on both occasions (i.e. to less than 60% of the untreated value). In glucocorticoid resistant patients colony numbers remained in excess of 60% of the untreated value.

Perhaps surprisingly, glucocorticoid medication did not appear to affect the extent to which colony growth was inhibited by MP in vitro. Thus in the 10 subjects tested before and immediately after the 7-day course of oral prednisolone, colony growth remained strongly inhibited in 8, and little affected in 2 (Fig. 3B).

DISCUSSION

Previous studies of the characteristics of overtly glucocorticoid resistant asthmatic patients have revealed few differences from the majority of patients with chronic asthma, who respond well to glucocorticoids [1,2]. In this paper we show that MNC from such glucocorticoid resistant patients were significantly more resistant to the effects of MP in vitro than the MNC from clinically sensitive asthmatic patients or normal controls. The criterion adopted was the ability of PHA-stimulated mononuclear cells to grow as colonies in soft agar. This phenomenon is known to depend upon interactions of monocytes and lymphocytes [6,7], but as the proportions of monocytes and T cells in the peripheral blood of the two groups of patients were similar, the observed difference in glucocorticoid responsiveness in vitro cannot be attributed merely to differences in the relative numbers of cell types under study.

MNC from individual patients with clearcut glucocorticoid resistant or sensitive asthma (i.e. groups 1 and 2) responded to MP in vitro in a consistent manner on repeated testing. The response bore no relationship to the endogenous plasma cortisol at the time of blood sampling (see Table 1). Further, the response in vitro did not change after a 7-day course of systemic glucocorticoid therapy. Hence it is probable that the mononuclear cells of each individual have a characteristic response to glucocorticoid in vitro, uninfluenced by endogenous cortisol or by the administration of exogenous glucocorticoid.

The close correlation between the response to glucocorticoid in vitro and in vivo suggests that the cell types involved in generating colonies in vitro (i.e. monocytes and T lymphocytes) may be similar to those contributing to the pathogenesis of asthma in these patients. A substantial body of evidence implicates cells of the monocyte lineage in the pathogenesis of asthma [8-10]. There are several ways in which improvement in the clinical condition of asthmatic patients treated with glucocorticoids may depend upon the effects of these drugs on such cells. Of particular relevance to the interpretation of the present results is the well documented inhibition of production of interleukin 1 (IL 1) by low concentrations of glucocorticoids [11-13]. IL 1 is a monocyte product which mediates mitogen-induced T-cell proliferation in vitro [14], and is known to support T-cell colony formation in soft agar [7]. It is also a pyrogen and promotes the synthesis of inflammatory mediators from arachidonic acid [15,16]. Other effects of glucocorticoids that may be implicated in their therapeutic action in asthma are modulation of complement receptor expression by monocytes [2] and induction in leucocytes of the phospholipase A₂ inhibitor lipomodulin [17], which also suppresses reactions mediated by immunoglobulin E [18]. It thus may be postulated that cells of monocyte lineage within the lungs of glucocorticoid resistant asthmatic patients might continue to secrete inflammatory mediators, support lymphocyte proliferation, and exist in a hyperreactive state with enhanced complement receptor expression, despite the presence of inhibitory concentrations of glucocorticoids.

We embarked on this study on the premise that glucocorticoid resistance in chronic asthma was an all or none phenomenon, which is what prompted us to restrict our investigations in vitro initially to patients whose asthma was readily recognizable on clinical criteria as sensitive or resistant. It would seem, however, from the observations on previously untreated, and thus unselected, asthmatic patients that sensitivity and resistance may form a continuous spectrum. At one end would be patients whose asthma is overtly resistant to systemic glucocorticoids, even in high dosage. It is important to recognize these patients since they may otherwise be needlessly exposed to hazardous side effects. This study indicates one way in which such patients may be identified.

Although the cellular basis of glucocorticoid resistance remains obscure, our findings suggest that a defect in mononuclear cells, and perhaps monocytes in particular, may be responsible. We are currently defining the nature of this cellular defect more precisely. We are also exploring the potential of simpler tests in vitro to identify resistant patients before they are treated with glucocorticoids either for asthma or for other diseases normally responsive to these therapeutic agents.

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TABLE 1
 DETAILS OF ASTHMATIC PATIENTS AND CONTROLS

Figures are means (\pm SD) or numbers of patients/total studied unless otherwise indicated

	GROUP 1 Glucocorticoid resistant asthmatic patients	GROUP 2 Glucocorticoid sensitive asthmatic patients	GROUP 3 New Asthmatics	CONTROLS
AGE (years)	55.7 (\pm 17.0)	44.5 (\pm 19.6)	58.2 (\pm 12.9)	49.1 (\pm 15.3)
DURATION OF ASTHMA (years)	19.3 (\pm 5.3)	11.5 (\pm 5.3)	14.0 (\pm 11.5)	-
ATOPIC	7/15	9/15	5/11	-
NOCTURNAL WHEEZE/ MORNING DIP	10/15	6/15	6/11	-
FAMILY HISTORY OF ASTHMA	3/15	3/15	2/11	-
SMOKERS	3/15	3/15	2/11	-
TOTAL DOSE OF PREDNISOLONE IN PREVIOUS 12 MONTHS (mg)	205 (range 0-1200)	169 (range 0-700)	-	-
ON REGULAR TREATMENT WITH STEROID AEROSOL	9/15	9/15	-	-
FEV ₁ (litres) (Initial clinic attendance)	1.24 (\pm 0.54)	1.59 (\pm 0.71)	1.33 (\pm 0.29)	-
FEV ₁ (litres) (Day of study)	* 1.21 (\pm 0.58)	* 2.48 (\pm 0.79)	1.33 (\pm 0.29)	-
PLASMA CORTISOL (nmol l ⁻¹) (Day of study)	506 (\pm 140)	547 (\pm 290)	535 (\pm 150)	510 (\pm 210)

* P < 0.02 (Mann-Whitney U Test)

TABLE 2

IMMUNOPHENOTYPE OF PERIPHERAL BLOOD MONONUCLEAR CELLS OF
 ASTHMATIC PATIENTS AND CONTROLS

Mean results \pm SD are shown

Phenotype	Mean percentage of mononuclear cells		
	Group 1 (resistant asthmatic patients)	Group 2 (sensitive asthmatic patients)	Controls
OKT3	45.1 (± 10.9)	46.2 (± 7.0)	47.2 (± 11.6)
OKT4	27.2 (± 7.1)	26.0 (± 7.9)	30.9 (± 9.1)
OKT8	17.8 (± 5.5)	21.6 [*] (± 6.3)	16.3 [*] (± 4.7)
Ia231	13.8 (± 4.9)	13.5 (± 6.2)	12.9 (± 6.9)
OKM1	29.0 (± 7.4)	26.7 (± 10.0)	29.3 (± 9.4)

* P < 0.01 (t-test)

TABLE 3

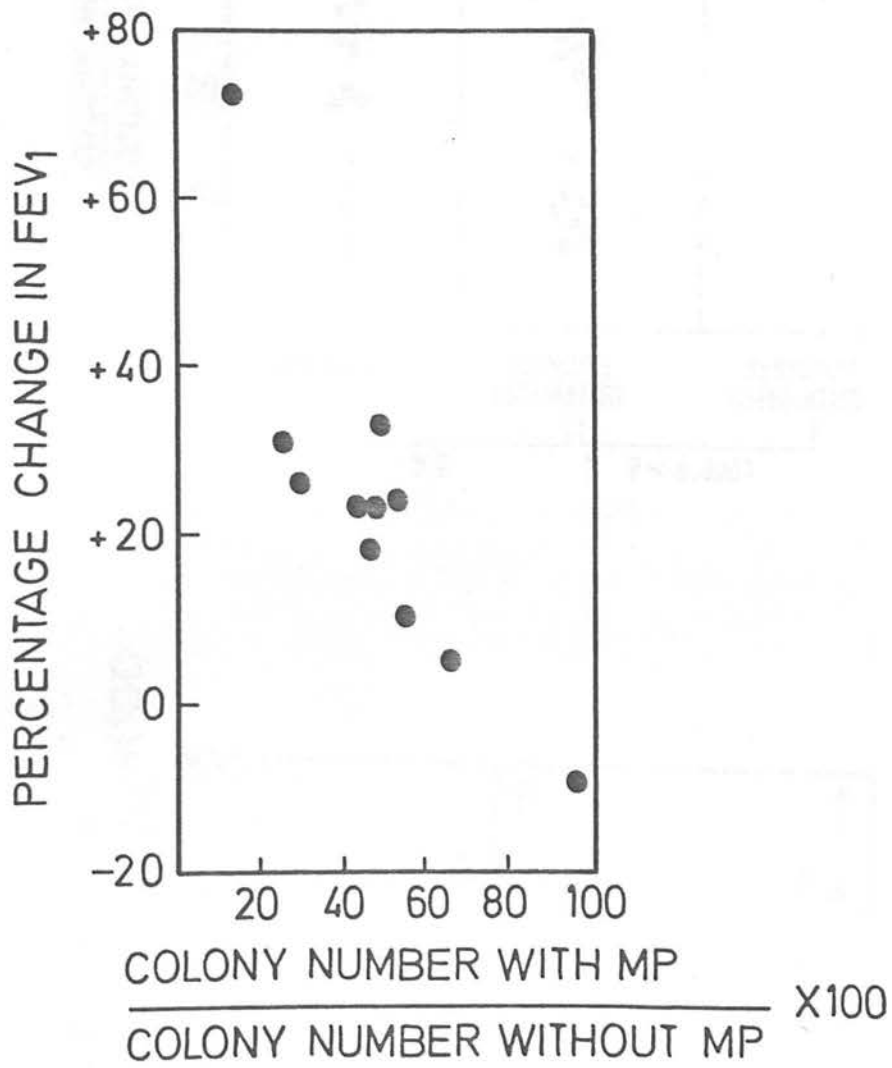
EFFECT OF GLUCOCORTICOID ON COLONY FORMATION BY PERIPHERAL BLOOD
MONONUCLEAR CELLS FROM ASTHMATIC PATIENTS AND CONTROLS

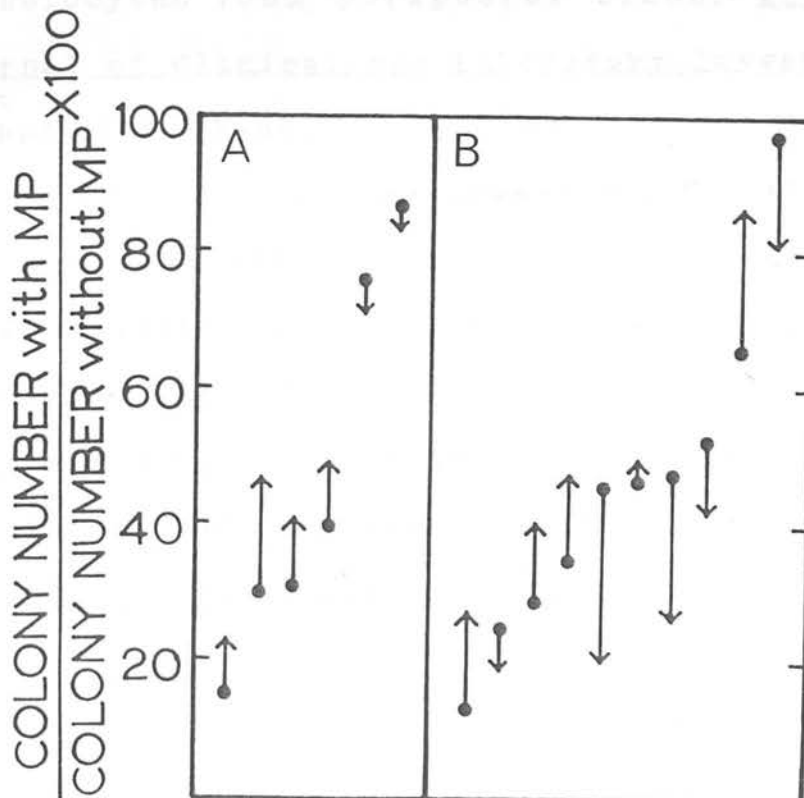
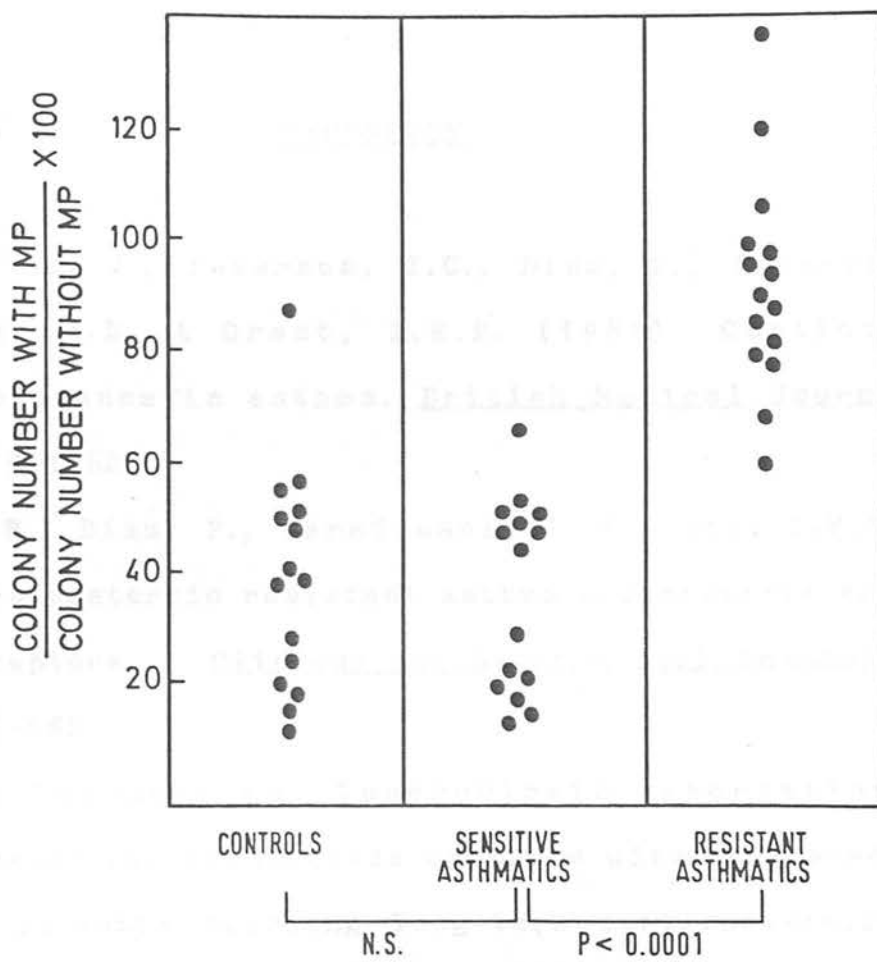
The reduction in colony number with methylprednisolone is significant for controls and sensitive asthmatic patients ($P < 0.0001$) but not for resistant asthmatic patients. Mean results \pm SD are shown.

Culture conditions	Colony Number		
	Group 1 (resistant asthmatic patients)	Group 2 (sensitive asthmatic patients)	Controls
No glucocorticoid	35.3 (\pm 9.5)	38.3 (\pm 6.6)	37.7 (\pm 5.2)
Methylprednisolone	31.8 (\pm 12.8)	13.8 (\pm 7.3)	14.7 (\pm 8.8)

LEGENDS TO FIGURES

- Fig. 1. Effect of methylprednisolone on colony formation from mononuclear cells of resistant and sensitive asthmatic patients (Groups 1 and 2) and of controls. The number of colonies developing in the presence of MP at 10 nmol/l is expressed as a percentage of the number which developed in the absence of MP, from cells of the same subject. Each point derives from a different subject.
- Fig. 2. Correlation between inhibition of colony growth by MP in vitro and relief of bronchospasm by a course of prednisolone in vivo, in the 11 unselected patients in group 3. The correlation is significant ($P < 0.005$).
- Fig. 3. Repeated estimations of inhibition of colony growth by MP in vitro. In 3A the initial values (●) and values obtained on retesting under identical conditions (point of arrow) are shown for 6 subjects (2 of them glucocorticoid resistant). In 3B initial values (●) and values obtained immediately after a 7-day course of oral prednisolone (point of arrow) are shown for 10 subjects, 9 of which came from group 3 (new asthmatics).





REFERENCES

1. Carmichael, J., Paterson, I.C., Diaz, P., Crompton, G.K., Kay, A.B. & Grant, I.W.B. (1981) Corticosteroid resistance in asthma. British Medical Journal, 282, 1419-1482.
2. Kay, A.B., Diaz, P., Carmichael, J. & Grant, I.W.B. (1981) Corticosteroid resistant asthma and monocyte complement receptors. Clinical and Experimental Immunology, 44, 576-580.
3. British Thoracic and Tuberculosis Association (1975) Inhaled corticosteroids compared with oral prednisolone in patients starting long-term corticosteroid therapy for asthma. Lancet, ii, 469-473.
4. Boyum, A. (1976) Isolation of mononuclear cells and granulocytes from peripheral blood. Scandinavian Journal of Clinical and Laboratory Investigation, 21 (Suppl. 97), 77-89.
5. Goube de la Forest, P., Lasmayous-Rion, N., Alcalay, D. & Tansor, J. (1979) Growth characteristics of PHA induced colonies in primary and secondary agar culture. Immunology, 35, 917-922.
6. Krajewski, A.S. & Wyllie, A.H. (1981) Inhibition of human T-lymphocyte colony formation by methylprednisolone. Clinical and Experimental Immunology, 46, 206-213.

7. Rosenszajn, L.A., Goldman, I., Kalachman, Y., Michlin, H., Sredni, B., Zeevi, A. & Shohaur, D. (1981) T lymphocyte colony growth in vitro: factors mediating clonal expansion. Immunological Review, 54, 157-186.
8. Joseph, M., Tonnel, A., Torpier, G., Capron, A., Arnoux, B. & Benveniste, J. (1983) Involvement of immunoglobulin E in the secretory processes of alveolar macrophages from asthmatic patients. Journal of Clinical Investigation, 71, 221-230.
9. Rankin, J.A., Hitchcock, M., Merrill, W.W., Bach, M.K., Brashar, J.R. & Askenase, P.W. (1982) IgE-dependent release of leukotriene C4 from alveolar macrophages. Nature (London), 297, 329-331.
10. Arnoux, K., Jouvin-Marche, E., Arnoux, A. & Beneveniste, J. (1982) Release of PAF-acether from human blood monocytes. Agents and Actions, 12, 713-716.
11. Smith, K.A., Lachman, L.B., Oppenheim, J.J. & Favata, M.F. (1980) The functional relationships of the interleukins. Journal of Experimental Medicine, 151, 1551-1556.
12. Smith, K.A. (1980) T-cell growth factor. Immunological Review, 51, 337-357.
13. Snyder, D.S. & Unanue, E.R. (1982) Corticosteroids inhibit murine macrophage Ia expression and Interleukin 1 production. Journal of Immunology, 129, 1803-1805.
14. Mizel, J.E. (1982) Interleukin 1 and T-cell activation. Immunological Review, 63, 51-72.

15. Mizel, S.B., Dayer, J.-M., Krane, S.M. & Mergenhagen, S.E. (1981) Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte activating factor (interleukin 1). Proceedings of the National Academy of Sciences U.S.A., 78, 2474-2477.
16. Dinarello, C.A., Marnoy, S.O. & Rosenwasser, L.J. (1983) Role of arachidonate metabolism in the immunoregulatory function of human leucocyte pyrogen/lymphocyte activating factor/interleukin 1. Journal of Immunology, 130, 890-895.
17. Flowers, R.J. & Blackwell, G.J. (1979) Anti-inflammatory steroids induce biosynthesis of a phospholipase inhibitor which inhibits prostaglandin synthesis. Nature (London), 278, 456-457.
18. Uede, T., Hirata, F., Hirashima, M. & Ishizaka, K. (1983) Modulation of the biologic activities of IgE-binding factors. 1. Identification of glycosylation-inhibiting factor as fragment of lipomodulin. Journal of Immunology, 130, 878-884.