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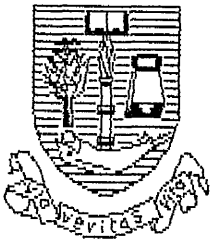

Immunological studies of natural killer cell activity in patients with atopic dermatitis.

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DECLARATION

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LIST OF ABBREVIATIONS.

The following abbreviations have been used in this thesis:-

AD	Atopic dermatitis.
ADCC	Antibody dependent cell mediated cytotoxicity.
ATP	Adenosine triphosphate.
cAMP	Cyclic adenosine monophosphate.
cGMP	Cyclic guanosine monophosphate.
E:T	Effector to target cell ratio.
FITC	Fluorescein isothiocyanate.
FCS	Foetal calf serum.
Leu	Commercial monoclonal antibodies to human leukocyte antigens manufactured by Beckton/Dickenson.
LGL	Large granular lymphocyte.
NK	Natural killer.
OKT	Commercial monoclonal antibodies to human T-cell antigens manufactured by Ortho diagnostics.
PBL	Peripheral blood lymphocyte.
PBM	Peripheral blood monocyte.
PBMC	Peripheral blood mononuclear cell.
PBS	Phosphate buffered saline.
SR	Spontaneous release.
TA	Total activity.

SUMMARY

Some patients with atopic dermatitis (AD) react abnormally to a number of cutaneous viral infections. Herpes simplex, for example can become widely disseminated with severe systemic symptoms. This susceptibility of AD patients may be due, at least in part, to deficient natural killer cell function.

This study investigated the NK cell activity of AD patients using a standard four hour chromium release assay with the erythroleukaemic cell line K562 as the target cell. The NK cell activity of patients with AD was significantly reduced as compared with normal age and sex matched controls.

The effect of the length of incubation time of the assay, removal of phagocytic/adherent cells and the use of target cells other than K562 on the standard four hour assay were examined. Increasing the incubation time from 4 to 18 hours and removing phagocytic/adherent cells resulted in an increase in NK cell activity, though the difference in response between patients and controls was maintained. In contrast the overall NK cell activity was reduced when target cells other then K562 were used, although the NK cell activity of the controls remained consistently higher than the patients.

Atopic dermatitis is a chronic relapsing disorder and to determine whether or not NK cell activity fluctuated with disease

severity NK cell activity was examined sequentially over a 12 month period in a group of patients. An inverse correlation was found between disease severity and NK cell activity, ie. the more severe the disease the lower the NK cell activity. A strong correlation was also confirmed between clinical activity of the disease and IgE level.

The reduction in NK cell activity in AD could result from either a qualitative abnormality, ie. normal numbers of effector cells with abnormal function, or a quantitative abnormality, ie. reduced numbers of effector cells. Effector cell numbers were counted using two monoclonal antibodies, HNK-1 (Leu-7) and Leu-11. Numbers were then correlated with function, as measured by chromium release. Using HNK-1 it appeared there was a reduction in the numbers of effector cells and this correlated with the reduced activity. HNK-1 does not however, stain all NK effector cells. Using Leu-11b, which is a more specific reagent, showed normal numbers of effector cells are present in the peripheral circulation of patients as compared with controls. The reduced NK cell activity in atopic dermatitis was therefore thought to be due to a functional abnormality in the effector cell's lytic cycle.

The reduction of NK cell activity could be caused by inhibitors in the serum of patients with AD. This was

investigated by incubating effector cells from both AD patients and normal controls in medium supplemented with either serum from patients with AD, normal controls or foetal calf serum (FCS). The NK cell activity was estimated following either a normal four hour assay, an extended 18 hour assay or a standard four hour assay following an 18 hour preincubation in the supplemented media. Using these three protocols no significant increase was observed in the patients' effector cell activity following incubation in medium supplemented with control serum nor was there a significant decrease in control effector cell activity following incubation in medium supplemented with patients' serum. A significant increase in NK cell activity however, was observed when either patients' or control effector cells were preincubated in medium supplemented with FCS prior to being assayed and was most likely due to the mitogenic properties of FCS.

The inverse correlation of disease severity with NK cell activity could be due to either the disorder itself or to its treatment. The mainstay of treatment in AD is topical steroids. The effect of the application of topical steroids on NK cell activity was therefore studied in ten normal volunteers. Following the application of betamethasone valerate for seven nights the NK cell activity of the volunteers was found to be reduced. This reduction was first observed on day 3 and reached its lowest level on day 8 and then gradually returned to normal

about day 18 to 22. Although the fall in NK cell activity seen in the normal volunteers was less then that observed in AD this may be a dose effect as smaller amounts of steroid would be expected to be absorbed through the intact skin of a normal control than through the skin of a patient with AD.

Stress is well recognised as one factor which may cause a flare in AD. As stress is mediated physiologically by adrenaline & noradrenaline the pharmacological effects of these agents on the activity of NK cells from normal controls was therefore studied. Betamethesone and histamine were also studied, the former because of the results of the topical steroid experiment and the latter because of its role in allergic disorders.

Following a one hour preincubation adrenaline, noradrenaline and betamethasone all inhibited NK cell activity in a dose dependent manner, while histamine slightly increased NK cell activity in a negative dose dependent manner. If added to the assay at the highest concentration without prior preincubation all of the agents caused an increased reduction in NK cell activity. A longer 4 hour preincubation with the highest concentrations of these agents resulted in a reduction in the amount of inhibition compared to that found with the other two protocols. Histamine was again found to enhance the NK cell response. After a four hour incubation, the level of enhancement observed was as great as that found following a one hour preincubation with histamine at the lowest concentration.

In conclusion, the results of this study suggest that reduced NK cell activity in AD may be related to the treatment of the disease rather than the disease itself.

CHAPTER ONE

INTRODUCTION

One of the more exciting discoveries in immunology in recent years has been that of natural cellular cytotoxicity. Natural killer, or NK, cells were first encountered during investigations into the function of T-cells in tumour control. In vivo resistance of a host to tumour growth had been mainly attributed to T cells specifically sensitised to tumour associated antigens. This was shown by adoptive transfer experiments where following transfer of cells from a sensitised donor to an unsensitised recipient, the recipient became resistant to subsequent challenge with the tumour used to sensitise the donor (Glaser, et al. 1976). Because of the recognised importance of immune or activated T-cells in the control of tumour growth it was anticipated that animals deficient of T-cells, eg. nude mice, would lack resistance to both spontaneous and induced tumours and the incidence of rapidly progressive malignancies in these animals would be much higher than in animals with competent T-cell immunity. However, no such excess was found in nude mice. Also, patients with immuno-deficiency disorders were found not to have a wide spectrum of malignant disease, as might have been expected. These findings coupled with the observation that lymphoid cells from some normal mice, rats and human donors, which had not been immunised with tumour cells or other sources of alloantigen, had significant levels of cytotoxic reactivity against certain syngeneic or allogeneic tumours (Herberman, et al. 1973: Nunn, et al. 1976: Rosenberg, et al. 1974), led to the discovery of natural cell

mediated cytotoxicity. Up to this time cell mediated cytotoxicity against tumour cells had been thought to be mediated by three types of effector cell, these were:- (i) specifically immune T-cells, (ii) antibody dependent cytotoxic cells, which depend upon IgG bound to the target cell (also known as K cells), and (iii) activated macrophages or macrophages armed with specific antibodies (Cerottini and Brunner, 1974).

Definition of natural cytotoxicity.

Spontaneous cytolysis by a discrete lymphoid subpopulation was first recognised in 1975 during studies designed to identify immune cytotoxic T-cells in humans or experimental animals with tumours (Pross and Jondal. 1975: Herberman, et al. 1975). Normal individuals were found to possess lymphocytes with the ability to recognise and kill rapidly (within hours) a variety of tumour and virus infected cells in vitro. These effector cells have become known as natural killer, or NK cells.

Natural cytotoxicity or killing may therefore be described as cytotoxicity, observed in vitro, excerted against a variety of tumour or virus infected cells by a discrete lymphoid subpopulation of mononuclear cells with the characteristics of LGL, from apparently normal unimmunised donors without the addition of external antibodies or deliberate in vitro activation of the effector cells.

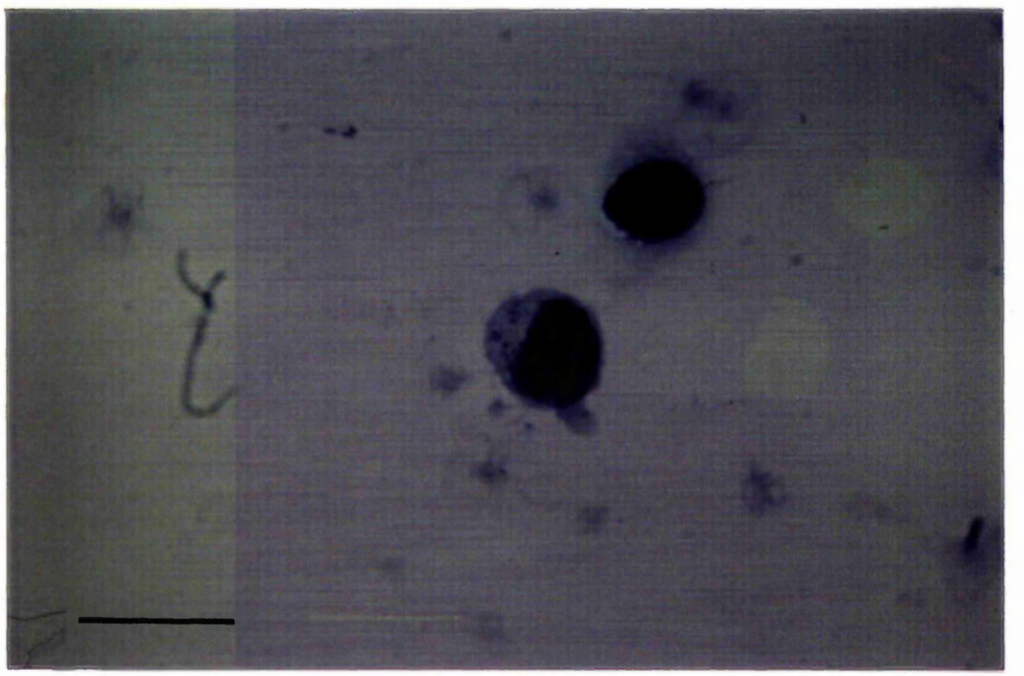
Morphological characteristics of NK cells.

In humans it has been shown that NK cell activity lies within a discrete subpopulation of non-B, non-T, non-adherent and non-phagocytic lymphocytes which lack demonstrable surface immunoglobulin and with a different morphology from typical lymphocytes (Timonen, et al. 1979: Peter, et al. 1975: Santoli, et al. 1978). NK cells have been described as large granular lymphocytes (LGL) because of their unique morphology. LGL have a pale cytoplasm, slightly eccentric reniform nucleus, a high cytoplasmic: nuclear ratio azurophilic granules in the cytoplasm and are larger than normal lymphocytes (see plate one). LGL have been shown to be the effector cells for NK activity as it has proved impossible to separate LGL from cytolytic function by either adherence to target cells or sedimentation percoll density gradients (Timonen, et al. 1981: Timonen and Saksela, 1980). In addition, lymphocyte populations enriched for LGL, show a greater level of natural cytotoxicity than do normal cell populations which in turn have a greater level of cytotoxicity than do lymphocyte populations depleted of LGL.

Origin of NK cells.

NK cells are derived from bone marrow stem cells. This has been shown by repopulating radiation chimeras with small numbers of bone marrow cells (Haller, et al. 1977). Further evidence which supports a bone marrow origin for the stem cell has come

Plate one.



Large granular lymphocyte, stained with May-Grunwald Giemsa, magnification x1,000. Bar represents 20µm.

from the observation that NK function is markedly suppressed in osteopetrotic mice, or mice treated with the bone seeking isotope ^{89}Sr. (Seaman, et al. 1979: Haller and Wigzell 1977).

Size of population and distribution.

NK cells or LGL represent a small subpopulation of lymphocytes in the blood or spleen. About 5% of circulating mononuclear cells have the characteristics of LGL, and up to 75% of these have been shown to be able to function as NK cells (Timonen, et al. 1981).

NK cells can be found in the foetal liver of man and are functionally active around the ninth week of gestation (Timonen, et al. 1981: Uksila, et al. 1983). At birth they are present in significant numbers in cord blood (Forbes, et al. 1981) though they are not as active as those found in the peripheral blood of adults. NK cell activity can be found through the body to various degrees; the highest levels being found in the peripheral blood and spleen with low levels in bone marrow, lymph node and thoracic duct and no detectable activity in the thymus (Antonelli, et al. 1981).

Mechanism of action of NK cells.

The mechanisms by which NK cells recognise, interact and

finally destroy susceptible target cells remain controversial, however, four separate steps can be identified during the killing of target cells. These are:-

i) recognition and binding of effector cells to the target cells.

ii) Triggering and activation of the lytic cycle.

iii) Release of the lytic agent.

iv) Target cell death and recycling of the effector cell.

i) Target cell recognition and binding.

The chemical nature of the structures on the target cell recognised by receptors on the NK cell is unknown, although a number of theories exist.

Both glycoproteins and glycolipids have been suggested (Roder, et al. 1979: Roder, et al. 1979: Young et al. 1981). The capacity of some simple sugars to inhibit NK cell cytotoxicity has been cited as evidence for a lectin-ligand like interaction in effector cell/target cell binding (Stutman, et al. 1980: MacDermott et al. 1981). It has been demonstrated recently however, that such sugars may exert their inhibitory influence at a post-recognition stage in the lytic cycle (Vose, et al. 1983). Effector cell recognition may also be influencedby the degree of

target cell sialylation, ie. the more sialic acid there is present on the target cell membrane, the less efficient the effector cell binding and therefore the more resistant the target cell will be to NK cell mediated lysis (Yogeeswaran, et al. 1981: Werkmeister, et al. 1983).

More recently the transferrin receptor has come under scrutiny. One study suggested that NK cell interaction with K562 target cells is mediated through the recognition by effector-borne transferrin of transferrin receptors expressed by the target cell (Vodinelich, et al. 1983: Baines, et al. 1983). Another study however, found contradictory evidence showing that neither apo-transferrin nor iron saturated transferrin caused a significant inhibition of lysis (Werkmeister, et al. 1983).

From the results of "cold competition" analysis, in which the capacity of unlabelled cells to compete with chromium 51 labelled cells for lysis by NK cells is measured, it would appear that both common and non crossreactive recognition structures exist which are expressed by different target cell populations (Ortaldo, et al. 1977: Callewaert, et al. 1979). NK cells reactive with recognition structures expressed by a target cell may be specifically removed from the effector population by absorption onto target cell monolayers (Jensen and Koven, 1979). Depletion of an effector population by this method does not prevent lysis of a different target cell type (Phillips et al. 1980)

This implies that target receptors of different specificities are distributed among NK cell populations. This theory is supported by preliminary evidence from the results of clonal analysis of both human and murine NK cell populations. There exists therefore, at least limited clonal distribution of target cell selectivity (Hercend et al. 1983: Kedar, et al. 1982).

The effector cell/target cell binding is most likely to involve a receptor-ligand interaction between a structure on the surface of the target cell and a receptor on the surface of the NK cell. The exact nature of the receptor is not known but in the mouse pretreatment of the effector cells with trypsin, papain or pronase prevents target cell binding and subsequent cytolysis (Roder, et al. 1978). Both these functions regenerate over a four hour time course. Preincubation for 18 hours with inhibitors of protein synthesis also decreases both target cell binding and cytolysis. These observations suggest that the receptor on the surface of the NK cell is a de-novo synthesised protein with a turnover time similar to that of other membrane proteins.

Although effector cell recognition of, and binding, to the target cell is a prerequisite for lysis it is obvious that this

alone is not enough. Normal cells may be bound by NK cells but are not lysed, (Brooks, et al. 1981), and variants of K562, which exhibit increased resistance to natural cytotoxicity, have identical effector cell binding to their more susceptible counterparts (Kimber, et al. 1983). It is likely therefore, that the sensitivity of a cell to NK lysis is influenced by inherent resistance mechanisms which operate at a post recognition stage of the lytic cycle. The exact nature of such a mechanism is unclear though evidence for both protein synthesis dependent (Hudig, et al. 1981: Kunkel and Welsh, 1981), and independent mechanisms (Kimber, et al. 1983) exists.

ii) Triggering and activation of the lytic cycle.

Triggering and activation of the lytic cycle follows target cell binding. The cellular events which result in this activation and subsequent target cell death are not yet fully understood.

A stimulus-secretion model has been proposed for NK cell mediated killing. Following contact with an appropriate target cell, the NK cell is triggered to secrete a factor, or factors, which then result in the lysis of the target cell (Roder, et al. 1980). This part of the lytic cycle would appear to be controlled by cyclic nucleotides. It has been shown that the induction of cyclic AMP by prostaglandins inhibits cytotoxicity (Hall et al. 1983) whereas cyclic GMP, or its inducers, including interferon,

cause augmentation of cytotoxicity (Katz, et al. 1982).

iii) Release of the lytic agent.

One of the first measurable events after target cell binding is a rapid burst of superoxide anion, O_2^-, generation, which can be measured in a chemiluminometer (Roder, et al. 1982: Helfand, et al. 1982). If O_2^- production is blocked by superoxide dismutase an inhibition of NK cell activity results, however, O_2^- is not thought to be the sole lytic agent. Patients with Chediak-Higashi syndrome have been found to produce normal amounts of O_2^- but are unable to lyse NK targets (Roder, et al. 1983). It has been postulated that O_2^- may act as a cofactor or activator for a later step in the chain of events leading to lysis of the target cell.

Another early event in the lytic cycle is the methylation of phospholipids within the NK cell, along with an increase in phospholipase A_2 activity (Hoffman, et al. 1981). The role of enzymes, or their products, however, remains to be established.

It is also possible that the azurophilic granules in the cytoplasm of NK cells have an active role in target cell lysis. Exposure to Sr^{2+} causes degranulation of LGL, with a subsequent reduction in cytotoxicity (Neighbour and Huberman, 1982). Further evidence that the granule contents may be involved in target cell death has come from the observation that purified

granules from rat LGL are directly cytotoxic for a variety of cell types (Henkart, et al. 1984).

Treatment of effector cells with monensin, a carboxylic ionophore which compromises cellular secretions, also inhibits NK cell mediated lysis (Carpen, et al. 1981). Observations that a variety of protease inhibitors can compromise NK cell activity has indicated a possible role for lysosomal proteases in the lethal hit (Hudig, et al. 1981). The exact nature of the event and in particular the role of cytotoxic factors awaits further clarification.

iv) Recycling of effector cells.

After administering the lethal hit to the bound target cell, the effector cell disengages and is then free to recycle and bind to another target cell. This capacity of effector cells to recycle allows a small population to be more effective. If NK cells killed on a one to one basis the effector population would have to be much larger than it is. The rate of recycling has been shown to vary between individuals although it is fairly constant within an individual (Ullberg and Jondal, 1981).

Surface markers.

There have been many studies aimed at the identification and classification of the effector cell by surface markers. A

large proportion of human peripheral blood NK cells have been shown to bear receptors for gamma globulin although evidence exists for cytotoxicity mediated by FcγR negative cells as well (West, et al. 1977: Kay, et al. 1979). The observation that non-rosetting lymphocytes, as well as those forming both high and low avidity rosettes with sheep erythrocytes, can exhibit natural cell-mediated cytotoxicity suggests that NK cells are not necessarily divorced from the T-cell lineage (West, et al. 1977: Potter and Moore, 1979). In addition studies with heterologous anti-T cell antisera have provided indirect evidence for the possession of some T-lymphocyte markers by human NK cells (Kaplan and Callewaert, 1978). More recent evidence suggests that immature NK cells display the OKT3 marker during development in the bone marrow and spleen, and that the T3 marker is lost when the NK cells reach a more mature stage in the peripheral blood (Abo et al. 1983). Other monoclonal antibodies reactive with T-cells have also been shown to recognise a proportion of human LGL: eg. both the OKT8, cytotoxic/suppressor marker and the OKT10 marker, which reacts with early thymocytes, bind to approximately 20% and 60% of human LGL respectively (Ortaldo, et al. 1981). In addition both OKM1, which identifies polymorphonuclear leukocytes, and Mac 1, which reacts with macrophages, have been observed to react with NK cells (Ortaldo, et al. 1981).

The first monoclonal antibody, specific for NK cells, to be

described was HNK-1, now known as Leu-7 (Abo and Balch, 1981), Leu-7 has been shown to be reactive with most, but not all, NK cells (Lanier, et al. 1983). Leu-7 also reacts with cells expressing the T-cell associated antigens Leu-1, Leu-2 and Leu-4 (Lanier, et al. 1983). Leu-7 has now been superceded by Leu-11 which is a more specific monoclonal antibody and unlike Leu-7, Leu-11 does not react with Leu-1, Leu-3 or Leu-4 positive cells. However, cells positive for the Leu-2a antigen have been found that also express the Leu-11 marker but the density of the 2a marker on Leu-11 positive cells is much lower than that on Leu-7 positive cells (Lanier, et al. 1983). Another potentially useful monoclonal antibody to be described recently is B73.1 which apparently recognises an epitope expressed on the Fc R of both human granulocytes and LGL, but is operationally specific for NK cells in lymphocyte populations (Perussia, et al. 1983).

As the technique of producing and selecting monoclonal antibodies is improved it is likely that a monoclonal antibody which is non-crossreactive will be developed. This may resolve the question of heterogeneity among human NK cells, ie. is heterogeneity due to the existence of a variety of discrete, but related, cell types or do these differences simply reflect variation in the level of differentiation within the NK cell population.

Variations in NK cell activity due to age, sex, and disease.

Age.

In mice, ageing has been shown to have an effect on NK cell activity (Herberman, et al. 1975). Mice under three weeks of age lack detectable cytotoxic activity, which appears between four to five weeks of age, reaches a peak between five to eight weeks, and then gradually declines to low levels of NK cell activity between six to twelve months of age.

In contrast to the age dependence of NK cell activity in mice, age has not been found to have any major effect on human NK cell activity (Fernandes and Gupta, 1981: Penschow and MacKay 1980). NK cell activity can be detected in mononuclear cells isolated from cord blood at birth (Forbes, et al. 1981: Antonelli, et al. 1981). After birth natural cytotoxic levels increase gradually to high levels in adulthood but, unlike mice, no dramatic decrease in activity is seen late in life.

Sex.

In human blood it has been found that males have a slightly higher level of NK cell activity than females, this difference has also been shown in cord blood (Pross and Baines, 1982: Penschow and MacKay, 1980: Antonelli, et al. 1981). Within an individual levels of NK cell activity remain fairly stable; even when tested over many years. Individuals may therefore, be categorised as having either high of low levels of natural cytotoxicity (Pross and

Baines, 1982).

Disease.

Low NK cell activity has been described in many diseases including malignant disorders such as breast cancer (Cunningham Rundles, et al. 1981), melanoma (Hersey, et al. 1978), Leukaemia (Linvat, et al. 1980), and other diseases such as SLE (Karsh, et al. 1981), Hashimoto's thyroiditis (Seybold, et al. 1981) and multiple sclerosis (Benczur, et al. 1980). No correlation however, has been found between low NK cell function and susceptibilty to disease in any of these disorders.

Mechanisms by which disease may depress NK cell activity may envolve one or more component(s) of the NK cell system and modulating factors such as interferon and prostaglandins (Koren, et al. 1981: Targen, et al. 1981: Bankhurst, 1982).

NK cell activity can be enhanced by interferon of all types (Ortaldo, et al. 1983). In mice inducers of interferon such as polyinosinicpolycytidilic acid (poly I:C) can also cause an increase in NK cell activity (Djeu, et al. 1979). The mechanism of action of interferon is unclear although it probably involves the induction of NK cell precursors and an augmentation of the potential of existing mature effector cells.

The lymphokine interleukin 2 (IL-2) has recently been shown to potentiate NK cell activity in man (Hersey, et al. 1981). IL-2 is also known to be an inducer of interferon (Kasahara, et al. 1983), therefore, it is not clear whether IL-2 alone is enough to augment lysis or whether it acts indirectly through the production of interferon (Weigent, et al. 1983).

A number of mediators including the prostaglandins E1, E2 and D2 can cause a depression of NK cell function (Droller, et al. 1978) possibly via an increase in intracellular cAMP (Goto et al. 1983). There is also evidence for cellular suppression of NK cell activity by macrophages, thymocytes and granulocytes (Jondel, et al. 1983: Nair, et al. 1981: Kay and Smith, 1983).

The role of NK cells in vivo.

The original studies which led to the discovery of NK cells suggested that they were involved in immunological surveillance. Animal studies have shown a correlation between the NK cell activity of different strains of mice and the ability of the strain to reject NK sensitive lymphoma cells (Kiessling, et al. 1975).

Clinical Observations.

The immunological surveillance role of NK cells is further supported by various clinical observations. Patients with Chediak-Higashi syndrome, which is characterised by impaired NK

cell function, have an increased incidence of lymphoid malignancy (Roder, et al. 1980: Dent, et al. 1966).

Role in tumour surveillance.

Following a renal transplant the recipient receives immunosuppresive therapy to prevent graft rejection, this results in a depression of NK cell function and an increased risk of developing both reticuloendothelial tumours and carcinomas (Lipinski, et al. 1980: Penn, 1977). These results are suggestive that NK cells form at least part of an immune surveillance system. Other conflicting evidence does not support this view: i) the vast majority of carcinoma patients have normal levels of NK cell activity in the early stages of the disease (Cross and Baines, 1976). ii) freshly isolated tumours are relatively insensitive to NK mediated lysis (Vose and Moore, 1980), iii) lymphocytes isolated from tumour biopsies lack NK cell activity (Moore and Vose, 1981) and iv) interferon is relatively ineffective at inducing NK cell activity against autologous biopsy derived target cells (Vansky, et al. 1980). However, established tumours that have evaded the surveillance system are likely to be resistant in vitro. They are therefore inappropriate models for the immunological processes involved in in vivo. prevention and surveillance. The case for an NK cell mediated surveillance system therefore remains unproven.

Role in viral infections.

The observation that NK cells undergo activation during the early stages of viral infection has led to speculation that they may also play a role in the host's defences against viruses (Welsh and Zinkernagel, 1977). NK cell activation during the early stages of a viral infection may be due to virally induced interferon production. However, inactivated virus may also cause an elevation of NK cell function in the absence of detectable interferon production (Kirchner, et al. 1982). Evidence supporting NK cell recognition of virus infected cells has come from studies in which it has been shown that virus-infected targets are more susceptible to lysis than their uninfected counterparts (Welsh and Hallenbeck, 1980). Susceptibility of a host to viral infection may be influenced by the genetically-determined level of natural cytotoxicity, and depletion of NK cell function in vivo. should result in reduced host resistance. Ten out of eleven mouse strains showed a correlation between resistance to murine cytomegalovirus and the level of virus-induced NK cell activity (Bancroft, et al. 1981). Beige mice, which are naturally NK deficient, showed an increased susceptibility to infection with the same virus (Shellam, et al. 1981).

Role in haemopoiesis.

NK cells have also been shown to have a role in the survival

of haemopoietic or lymphoid grafts. The success of human bone marrow grafts has also been shown to be related to the NK cell activity of the recipient (Dokhelar, et al. 1981). Haemopoietic or lymphoid grafts fail to survive in lethally irradiated F1 hybrids of certain murine strains (Cudkowicz and Bennett, 1971). This phenomenon, known as hybrid resistance, was subsequently found to be effected by NK cells (Lotzova, et al. 1983).

NK cells have the ability to destroy bone marrow cells in vitro. (Hansson, et al. 1981). This observation together with their role in hybrid resistance has raised the possibility of a primary physiological role for NK cells in the regulation of the differentiation of normal haemopoietic tissue. If true, the anti-tumour and anti-viral activity they also possess may be a fortuitous by-product of their capacity to interact with membrane determinants and to lyse those cells carrying inappropriate markers.

Supporting evidence has come from studies which demonstrated that NK cells can inhibit granulocyte macrophage colony forming cells (Hansson, et al. 1982) and that NK like cells may regulate haemopoiesis in aplastic anaemia (Bacigalupo, et al. 1980). An NK lymphocytosis associated with a neutropenia has also been described (Pross et al. 1981).

NK cells may also have a regulatory influence on B-cells. It has been observed that they can suppress the response of B-cells to pokeweed mitogen (Arai, et al. 1983.). Cells expressing the HNK-1 (Leu-7) marker have been observed in the follicular areas of human lymphoid tissues (Ritchie et al. 1983), where, it has been suggested, they may play a role in B-cell differentiation.

In addition to cytolysis NK cells may also be able to perform an immunoregulatory role through the production and release of soluble factors. Studies have revealed that lectin stimulation of highly purified populations of LGL results in the production of a number of lymphokines: eg. IL-2, interferon and colony stimulating factor (Kasahara, et al. 1983). It would not be unreasonable therefore to suggest that under appropriate conditions NK cells may, through the action of lymphokines, be able to influence the immunological microenvironment.

NK cells are therefore not the laboratory artifacts they were first thought to be, they are in fact a subpopulation of mononuclear cells with the characteristics of LGL and the ability to lyse , spontaneously, a variety of target cells, and are able to produce various lymphokines. It has been suggested that they may be involved in immunosurveillance, patients with Chediak-Higashi have an increased incidence of lymphoid malignancy (Dent et al. 1966), haemopoiesis, NK cells have been shown to be able to destroy bone marrow cells <u>in vitro</u> (Hanson et al. 1981), and possibly regulation of antibody production, NK cells can suppress the B-cell response to pokeweed mitogen and have been found in the follicular areas of lymphoid tissues (Arai, et al. 1983: Ritchie, et al. 1983). As yet there have been no reports of any correlation between low

NK cell function and susceptibility to disease, although it has been shown that virus infected cells are more susceptible to NK lysis than their uninfected counterparts (Welsh and Hallenbeck, 1980) and that the ability of mice to resist viral infection correlates well with NK cell activity (Bancroft, et al. 1981).

Quite how NK cells recognise their targets is, as yet, unknown. It has been suggested that effector cell recognition of target borne transferrin receptors may be the mechanism as a direct correlation has been found between transferrin receptor expression and susceptibility to NK cell lysis, however it was not possible to inhibit lysis with either excess iron, saturated transferrin receptors or affinity purified transferrin receptors (Borysiewicz, et al. 1986). Recent reports have suggested that the T-cell receptor for antigen is involved, however, Young and his coworkers (1986) have shown that a functional T-cell receptor β chain is absent in fresh LGL making it extremely unlikely that the T-cell receptor is envolved in target cell recognition.

Atopic Dermatitis.

One such disease which may allow insights into the mode of action of NK cells is atopic dermatitis (AD). Patients with AD are recognised as having an increased incidence of certain cutaneous viral infections such as warts and molluscum contagiosum (Currie, et al. 1971: Soloman and Talner, 1966). They also handle certain other viral infections abnormally. Vaccinia virus is now almost of historical importance but both this and the much more common herpes simplex type I can result in Kaposi's varicelliform eruption, a serious and potentially fatal condition in a patient with AD (Baer, 1959: Rajka, 1975). As NK cells have the ability to lyse certain tumour and virally infected cells (Roder and Pross, 1982) it was of interest to determine whether NK cell function was altered in patients with AD and if possible to elucidate the nature of the defect.

Atopic dermatitis is a pruritic cutaneous condition with characteristic clinical features and a chronic relapsing course. Classically it is seen in patients who also have a personal or family history of "atopic disease" that is, asthma, allergic rhinitis and urticaria. AD usually presents at a very early age, one study (Rajka, 1975) has shown that 60% of patients had onset of AD in the first year of life and that 85% of patients with AD were under five years old. Some patients become free of AD during childhood and studies of patients from 15 to 20 years old

have shown that about 40% show a clearing of the disease (Musgrove and Morgan, 1976). Adult onset of AD does occur, but it is unusual.

Immunological features of Atopic Dermatitis.

Humoral.

Serum IgE levels are elevated in 80% of patients with AD (Juhlin, et al. 1969). The evidence for the existence of an antigen-IgE-mast cell mechanism as the primary cause of AD is at best tenuous for several reasons. Firstly, raised IgE levels are seen in patients whose conditions have no historical or clinical evidence for AD or allergic respiratory disease, eg. patients with parasitic infections. Secondly, serum IgE levels remain high during remissions although, during prolonged disease free periods, levels gradually fall towards normal (Johansson and Tuhlin, 1970). Thirdly, AD occurs in patients with low or undetectable levels of serum IgE, including those with agammaglobulinaemia (Peterson, et al. 1962). It would therefore, appear that the raised serum IgE levels seen in AD may be due to a fault in the control mechanism of IgE production rather than the cause of the disorder.

Cell mediated.

Cell mediated immunity in patients with atopic dermatitis may also be abnormal. As early as 1895 patients with AD were observed to be unusually susceptible to severe cutaneous

infections with herpes simplex and vaccina (Kaposi, 1895). These patients also have an increased incidence of warts and Molluscum contagiosum (Currie, et al. 1971: Solomon and Telner. 1966). Increased incidence of delayed type hypersensitivity (DTH) reactions in patients with AD was thought to be a result of the atopic disorder (Epstein and Mohigern, 1964), however, in retrospect these findings are now thought to represent the high frequency of exposure to sensitising agents, ie. topical medications such as neomycin, among patients with AD. In fact, it is now thought that there is a decrease in DTH in patients with AD, this was suggested as long ago as 1937 when it was observed that there was a low incidence of contact sensitivity in patients with AD (Rostenberg, 1937). Since then it has also been shown that there is a low incidence of sensitisation to poison oak and dinitrochlorobenzene in AD (Jones, et al. 1973: Palacios, et al. 1966). Depressed DTH responses to tuberculin, streptokinase-streptodornase (SKSD) and candida have also been noted, however, the ability of lymphocytes from patients with AD to transform *in vitro* in response to these allergens has been shown to be relatively normal, despite cutaneous anergy (Elliott and Hanifan, 1979 a).

Depressed *in vitro* cellular immune responses have been reported. Two patients with severe AD both showed total cutaneous anergy and their lymphocytes failed to respond when cultured

with the mitogen phytohaemagglutinin (PHA) during periods of disease activity. However, during remission the lymphocyte transformation improved (Lobitz, et al. 1972). Elliott and Hanifan (1979 b) found that the abnormal responses to PHA were reversible *in vitro* if the cells were kept in culture for two to four days. One explanation to have been put forward for these results suggests that there are immature circulating lymphocytes that are deficient in mitogen responsiveness. (Elliott and Hanifan, 1979 a).

It has been observed that the chemotactic response of neutrophils and monocytes from patients with severe AD is depressed and that this depression of chemotaxis is reversible with clinical improvement (Rogge and Hanifan, 1976). Evidence has been put forward suggesting that a serum derived inhibitory factor is responsible for the depression of chemotaxis in these patients (Snyderman, et al. 1977).

A more recent *in vitro* observation has been the report of reduced levels of suppressor T-cells or suppressor T-cell activity in patients with AD (Canonica, et al. 1979: Faure, et al. 1982: Rola-Pleszcynski and Blanchard, 1981). This area of investigation is confused by poor definition of the patients studied with few details of presence or absence of respiratory and cutaneous symptoms and the severity of the atopic dermatitis.

The methods used to investigate the T-cell subsets have also varied in these studies, with some groups using the T_μ and T_γ method of quantitation (Cooper, et al. 1983) and others using monoclonal antibodies (Leung, et al. 1981). The T_μ, T_γ method of quantitation relies on the observation that T cells that carry receptors for IgM function as helper cells while T cells that carry receptors for IgG function as suppressor cells. This method however is not as accurate as that using monoclonal antibodies. It has been suggested that the low numbers of, and/or defective suppressor T-cells may be responsible for the high serum IgE levels in patients with AD. However, as the maturation of suppressor/cytotoxic T- cells is dependent on the helper/inducer subset as well there may also be a defect in the helper/inducer population. A defect in this population would explain the observations of decreased antigen and mitogen responsiveness of peripheral blood lymphocytes (PBL) from patients with AD. (Elliott and Hanifan, 1979a: Elliott and Hanifan, 1979b).

Scope of present thesis.

Natural killer cell activity in atopic dermatitis.

Studies of PBL NK cell activity in patients with AD have given conflicting results. Some groups report that NK cell activity against a tumour cell line target is reduced (Kusiami and Trentin, 1982). Others have found normal or raised levels of NK cell activity against tumour cell line targets (Viander, et al. 1982: Strannegard and Strannegard, 1980). While others have reported that against autologous and allogenic skin fibroblasts it is raised (Leung, et al. 1982).

In summary, individuals with AD are recognised as having abnormal humoral immunity and altered cellular reactivity, as well as a susceptibilty to viral infection. Defective NK cell activity may explain the link between susceptibility to viral infections and AD as it is well known that NK cells will lyse virally infected cells (Welsh and Hallenbeck, 1980). The possibilty exists that defective NK cell activity is an integral part of the disease or it may be an epiphenomenon related to AD, therefore to try and resolve this question this thesis examines:-

1) NK cell activity in AD in comparision with age and sex matched controls using the assay described below.

2) The methodology of the cytotoxic assay to determine whether variations in the method (eg. incubation length, removal of phagocytic/adherent cells or the use of target cells other than K562) caused the variations reported in NK cell activity in AD.

3) Sequential NK cell activity in a group of patients

with AD to establish if a correlation exists between clinical activity and NK cell activity.

4) Effector cell numbers in AD patients and normal controls using the monoclonal antibodies HNK-1 (Leu-7) and Leu-11b.

5) The possible presence of serum inhibitors specific for NK cells.

6) The effect of topically applied steroids (the mainstay of treatment of AD) on the NK cell activity of normal volunteers.

7) The effect of betamethasone *in vitro*. and because of the recognised effect of stress in causing the disorder to flare and the role histamine has in allergic disorders, the effect of adrenaline, noradrenaline and histamine were also studied *in-vitro*.

CHAPTER TWO

MATERIALS AND METHODS

Materials and Methods.

Resume of methods available for the study of NK cells.

Introduction.

There are several methods available for the estimation of natural killer cells both function and numbers. These are:-

1. Cytolysis.
2. Chemiluminescence.
3. Target cell/effector cell conjugates.
4. Monoclonal antibody techniques.
5. Histological stains.

1. Cytolysis.

Cytolysis estimates the natural killer cell's ability to lyse certain target cells, usually a tumour cell line, such as K562. There are a large number of cell lines available for use in cytolytic assays, ranging from naturally occuring tumours to virally transformed cell lines including T- and B-cells, and some non-T non-B lines such as the myeloid cell line K562.

The most common cytolytic assay is one based on isotope release. Tumour cells are labelled with a radioactive isotope, usually chromium 51 in the form of chromate ($^{51}CrO_4^{-2}$).

Chromium binds to large molecular weight intracellular proteins and is released when the plasma membrane is damaged. Chromium released from damaged cells is not reutilised, therefore the amount of chromium released is nearly proportional to the number of cells damaged.

Parameters affecting the outcome of cytolytic assays.

There are a number of methodological factors which can affect the outcome of the chromium release assay:-

a) The size and geometry of the wells in the microtitre plate.

Three types of microtitre plate are available, flat, round and V bottomed. As cytolysis depends upon effector: target cell contact, the geometry of the well will therefore influence the outcome of a cytctoxicity assay and as contact is facilitated in plates with round or V bottoms they will have a greater influence on the result of an assay.

The size of well may also be an important consideration. In a smaller vessel the chance of effector cell/target cell contact is increased. However, it has been shown (Miller and Dunkley. 1974) that in plates with concave bottoms volume does not matter as the cells tend to pellet during the assay. It has also been shown, however, that if the culture is kept in suspension the amount of chromium released is inversely dependent on the volume.

b) Presence of macrophages.

NK cell activity can be suppressed in vitro. by the presence of macrophages or monocytes in the effector cell population (Jondal et al. 1982). Conversely removal of macrophages or monocytes by either (a) incubation with carbonyl iron followed by passage over a strong magnet or (b) incubation on glass or plastic for one hour at 37°C results in an increase in natural killer cell activity.

The mechanism by which macrophages suppress natural killer cell activity is uncertain. There are three possibilities. (1) Macrophages may bind target cells thereby masking them from the effector cells. (2) Macrophages and monocytes may produce prostaglandins which inhibit NK cell activity (Droller et al. 1978). Or (3), it may be a combination of both these factors.

c) Choice of target cell.

While natural killer cells show a specific ability to lyse tumour and virus infected cells, their ability to kill is not uniform, i.e. effector cells from the same source will lyse different target cells to varying degrees (Allavena and Ortaldo (1984). Certain target cells are also more resistant to lysis by NK cells than others (Jondal et al. 1978).

d) Incubation length.

There are two controls included in the cytolytic assay. The

first, spontaneous release (SR), measures the amount of isotope that leaches out of the target cells during the course of the assay. The second, total activity (TA), measures the amount of isotope that would be released if all the target cells were lysed. Incubation length is important from the point of the level of spontaneous release; the longer the incubation the more likely it is that isotope will leach out of the target cells and give rise to a high spontaneous release. Also NK cells are well documented as being multi-hit, therefore, the longer the incubation period, the higher the percentage isotope release (Ullberg and Jondal 1981). It is possible therefore, that during longer incubations, differences in NK cell activity could be masked because of this. Four hour incubations are therefore, more preferable than eighteen hours for these reasons.

e) Use of "filler cells".

The two controls for cytolytic assays, SR and TA, are both important as they are the standards against which the activity of the effector cells is measured. Spontaneous release of chromium, if too high may mask effector cell activity. The choice of target cell and the length of assay are, therefore, important. Spontaneous release however, can be reduced by the addition of what are termed "filler cells" (Dunkley et al. 1974). "Filler cells" may be fixed white blood cells or populations of cells with no active NK cell function such as mouse thymocytes.

2. Chemiluminescence.

Chemiluminescence has been recently suggested as an alternative method of measuring NK cell activity. Following phagocytosis of bacteria by polymorphonuclear leukocytes there is a burst of superoxide production which is involved in the killing of the ingested bacteria. NK cells have also been shown to generate superoxide when mixed with target cells (Helfand et. al. 1982). It is unknown whether or not this is involved in the killing step. This burst of superoxide generation can be measured using a chemiluminometer.

The disadvantage of this method is that it is indirect, ie. it does not show if any actual killing takes place. Patients with Chediak-Higashi syndrome have defective NK cell activity as measured by chromium release (Roder et al. 1980), although using chemiluminescence it has been shown that superoxide generation does occur (Roder et al. 1983). Recent evidence suggests that the cytolytic mechanism is not oxygen dependent. Patients with chronic granulomatous disease, a disorder where phagocytic cells fail to produce toxic oxygen derivatives, have normal natural killer cell function (El-hag and Clark. 1984). It has also been shown that the addition of catalase or superoxide dismutase to NK cell assays has no effect on natural killer cell-mediated cytotoxicity (Abrams and Brahmi 1984).

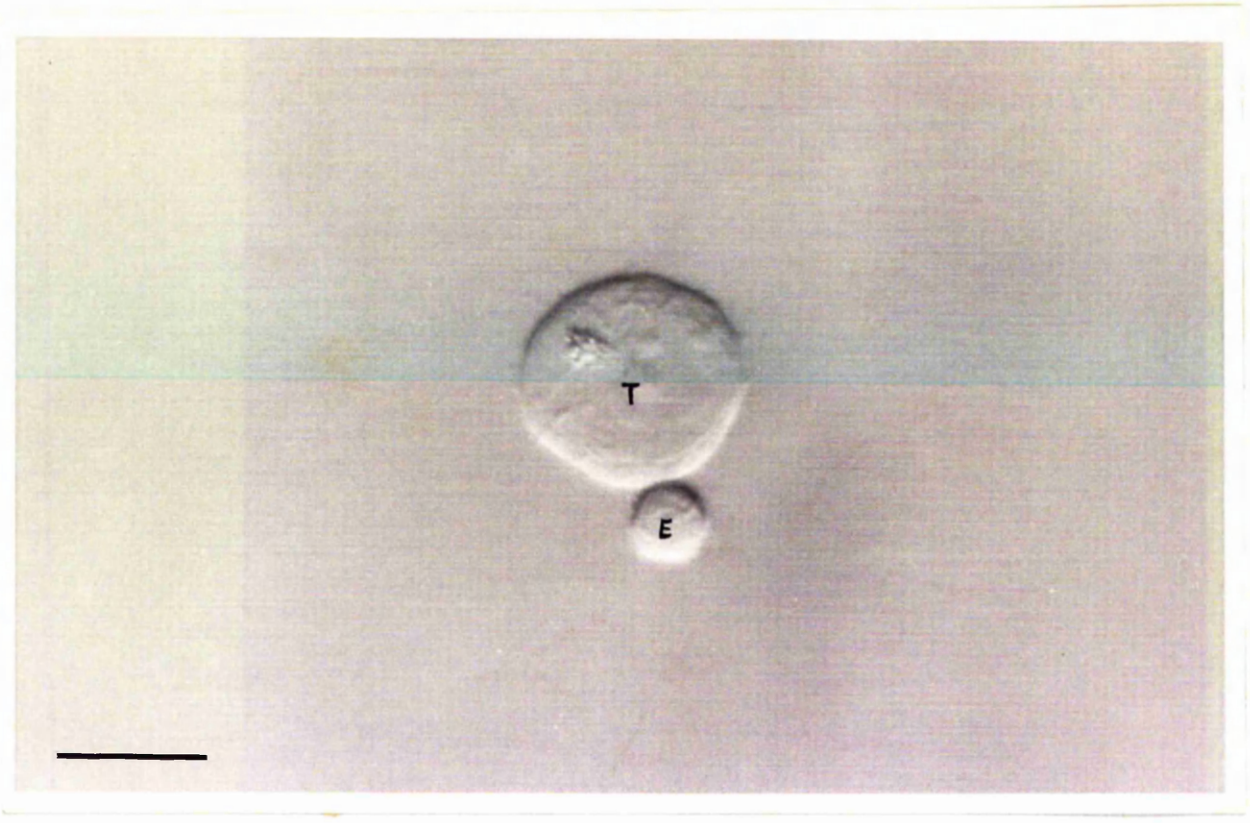
3. Target cell/effector cell conjugates.

Conjugation assays examine events at the single cell level. They are dependent on the fact that NK cells bind to their targets prior to lysis. Equal numbers of effector cells and target cells are incubated together for thirty minutes at 37°C. The resulting cell mixture is examined microscopically under phase contrast and the percentage of lymphocytes that have formed conjugates with the much larger tumour cells can be estimated (Grimm and Bonavida 1977). See plate two.

This assay provides a useful estimation of the number of cells capable of binding to the target cells. It gives, however, no measure of their ability to kill the target cells. Lymphocytes from patients with Chediak-Higashi syndrome bind to the target cells normally but cannot then lyse the target (Roder et al. 1983).

A modified version of this assay overcomes this disadvantage and allows the visualisation of cytolysis (Grimm and Bonavida 1979). Target cells and effector cells are allowed to interact together in agarose rather than medium. The agarose with the effector cell/target cell conjugates is then plated out and incubated for various times at 37°C. The plates are then stained with trypan blue and fixed with formaldehyde. Only the killed target cells are stained with the trypan blue, and as the agarose prevents the effector cell from moving away from its target both the number of effector cells and active effector cells may be counted.

Plate two.



Effector cell/target cell conjugate, photographed using Nomarski differential interference contrast optics.

Magnification x1.000. bar represents 20µm.

T= target cell

E= effector cell

4. Monoclonal antibody techniques.

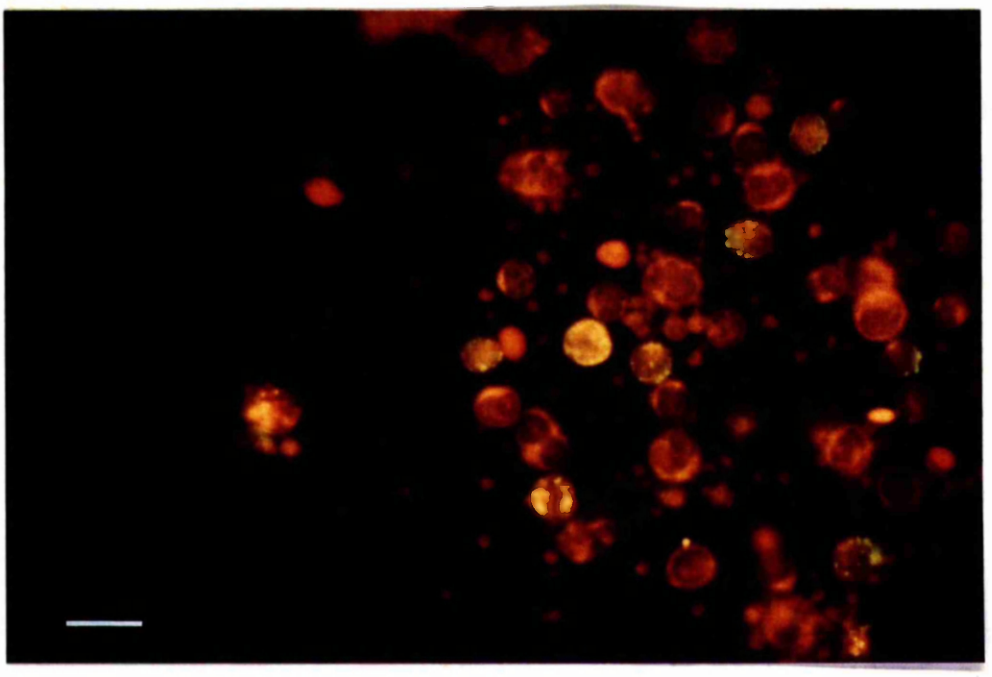
There are now several monoclonal antibodies available that are specific for cell surface markers on natural killer cells, eg. HNK-1, Leu-11a and Leu-11b (Abo and Balch 1981., Lanier et at. 1983). These can be used in fluorescent or peroxidase techniques to measure the percentage of positive cells within a population. See plate three.

An advantage of the fluorescent antibody method is that after labelling with the fluorescent conjugate, the cells can be separated into two populations on a fluorescent activated cell sorter (FACS): NK marker positive and NK marker negative. Functional studies can then be carried out on the enriched and depleted populations of cells (Loken and Herzenberg 1975: Ortaldo et al. 1981).

5. Histological stains.

Finally natural killer cells can be studied stained with histological stains, such as giemsa. Human NK cells have been shown to have the unique morphology of large granular lymphocytes (LGL) (Timonen et al. 1979). See plate one. In practice this stain may be used on cytocentrifuge preparations although it is a purely quantitative method and gives no estimation of the functional ability of the counted cells. However, as only about 5% of peripheral blood lymphocytes (PBL) have the morphology of LGL and as activated cytotoxic T-cells also have this morphology it is difficult to gain a true insight as to the actual numbers present in the circulation.

Plate three.



Fluorescent micrograph of PBMC stained with Leu-11b and FITC conjugated goat anti-mouse IgM. Magnification x500, bar represents 20μm.

Micrograph shows both Leu-11b positive cells, with associated fluorescence and red counter stain, and negative cells red counter stain only.

Materials and Methods used in this study.

Cell culture medium.

The medium used throughout was R.P.M.I. 1640 (GIBCO, Paisley Scotland) obtained as a times ten concentrate and made up to the working dilution as follows:-

Twenty ml. of the concentrate was added to 180 ml. of a solution made up of 2.65 g/l. Hepes (Flow laboratories) and 0.85 g/l. of sodium hydrogen carbonate (BDH Chemicals, Poole, England). The pH of the medium was then adjusted to 7.5 by the addition of 5N sodium hydroxide.

Separating mixture for isolation of mononuclear cells from venous blood.

The separating mixture used for the isolation of peripheral blood mononuclear cells was ficoll/isopaque. This was made up of a 9% solution of ficoll (Pharmacia Fine Chemicals, Milton Keynes) and a % solution of isopaque (Nyegaard & Co. AS. Oslo) which was made up by adding 48 ml. of distilled water to 40 ml. of isopaque 440.

83 ml. of isopaque were added to 2000 ml. of 9% ficoll to give 283 ml. of mixture with specific gravity between 1.076 and 1.078. This was then sterilised by filtration and stored in the dark at 4^{o}C.

White cell counting fluid.

White cell counting fluid was made up of 1% solution of

acetic acid in distilled water plus two or three drops of a 2% trypan blue solution.

Preparation of effector cells.

Venous blood was taken from both patients and, where possible, age and sex matched controls, and added to sterile bottles containing preservative free heparin (Evans, Speke, Liverpool). The mononuclear cell layer was isolated by layering two volumes of whole blood over three volumes of Ficoll/Isopaque and centrifuging at 200g for 30 minutes in an MSE multex centrifuge. The mononuclear cell layer was removed and the cells washed twice in RPMI containing 100 units of preservative free heparin/ml. (Weddel Pharmaceuticals Ltd., London) to prevent aggregation of cells. The cells were then given a final wash in RPMI containing 5% heat inactivated foetal calf serum (GIBCO) L-glutamine 2mmol/ml. (GIBCO) and 2 U/mL. of penicillin/steptomycin (GIBCO). For overnight incubations 8µl of gentamicin was also added (Nicholas Laboratories).

Purity was assessed visually using white cell counting fluid and was never less than 95% mononuclear cells. Viability was assessed using trypan blue exclusion and was also never less than 95%. The final concentration of cells was adjusted to $2x10^6$/ml. two dilutions of this suspension were made to achieve concentrations of $1x10^6$ and $5x10^5$ cells/ml.

Removal of phagocytic and adherent cells.

Two methods were employed for the removal of phagocytic and adherent cells.

1) Carbonyl iron ingestion.

Isolated mononuclear cells were incubated with 5 mg/10^7 cells carbonyl iron (GAF Manchester) for 20 minutes at 37°C. followed by passage over a strong magnet which removed the phagocytic cells.

2) Adherence to glass or plastic.

Adherent cells were removed by incubating the isolated mononuclear cells in glass or plastic universals for one hour at 37°C. In each case the recovered cells were washed twice in medium containing supplements before being resuspended and counted.

Target cells.

K562 were used as the target cells in all the standard cytotoxicity assays. Other cell lines used in this study were BJAB, CCRF-CEM, MOLT-4 and Namalva. See table one for characteristics of each cell type. All cell lines were maintained in continuous culture in RPMI 1640 medium containing 10% heat inactivated foetal calf serum 200 units of penicillin/streptomycin and 2 mmol/ml. of L-glutamine. The cells were kept at a temperature of 37°C. in an atmosphere of 5% CO_2 95% air, and were subbed routinely every two days or 24 hours before being used in an assay.

Table 1.

Target cell line characteristics.

			Immunoglobulin			
Cell Line	Type	EBG	H	L	SIG	SEC
BJAB[1]	BLCL	-	M	K	+	+
CCRF-CEM[2]	TLCL	-	-	-	-	-
K562[3.4]	CML	-	-	-	-	-
MOLT-4[5]	T-AL	-	-	-	-	-
Namalva[6]	BL	+	M	L	+	+

Key:

BLCL	B-Lymphoblastoid cell line
TLCL	T-Lymphoblastoid cell line
CML	Chronic myeloid leukaemia
T-AL	T-acute Lymphatic Leukaemia
BL	Burkitt's Lymphoma
EBG	Epstein Barr Genome
SIG	Surface Immunoglobulin
SEC	Secreted Immunoglobulin

1. Menezes et al. (1973)
2. Foley et al. (1965)
3. Lozzio and Lozzio (1975)
4. Andersson et al. (1979)
5. Minowada et al. (1972)
6. Nyormoi et al. (1973)

Labelling of target cells.

An aliquot of the cell culture medium containing $3x10^6$ cells was incubated with 0.2 ml.of ^{51}Cr. (Na_2CrO_4, radiochemical Centre, Amersham) containing 20 megabequerals per ml. at 37°C. for one hour. The cells were then washed six times with RPMI plus supplements. After the final wash the cells were resuspended in 10ml. of medium and counted. The final concentration was adjusted to $2x10^4$/ml.

Standard cytotoxic assay.

The cytotoxic assay was carried out in V bottomed microtiter plates (Flow). 0.1 ml. of effector cells at the various dilutions, $2x10^6$/ml., $1x10^6$/ml. and $5x10^5$/ml. , were incubated with 0.1 ml. of labelled target cells, at $2x10^4$/ml. for 4 hours at 37°C. in an atmosphere of 5% CO_2 95% air. This achieved effector to target cell ratios of 100:1, 50:1 and 25:1. Two controls were included, the first estimated the degree of spontaneous release (SR) of radioisotope from the target cells which were incubated with 0.1 ml. of RPMI alone. The second estimated the total activity (TA) by completely lysing the target cells with the addition of 5N NaOH to wells containing 0.1 ml. of target cells at a concentration of $2x10^4$/ml. 100% release of bound isotope was regularly achieved. All tests and controls were carried out in quadruplicate.

Following incubation 0.1 ml. of cell free supernatant was removed from each well and counted for three minutes in a gammacounter (LKB 1282 compugamma) to assess the radioisotope release.

Modification of cytotoxic assay: to test In Vitro. effects of various pharmacological agents.

The microtiter plates were set up as stated for the standard cytotoxicity assay with the following modifications:
0.09 ml. of effector cells at the various concentrations were aliquoted along with 0.01 ml. of the following four pharmacological agents. I) adrenaline, original concentration of 1 mg./ml. (Antigen Ltd., Roscrea, Ireland). II) Noradrenaline in the form of noradrenaline acid tartrate, original concentration of 1 mg./ml. (Winthrop Laboratories, Surbiton-upon-Thames, Surrey). III) Histamine, in the form of histamine acid phosphate, original concentration 1 mg./ml. (BDH Chemicals, Poole, England). This was made up in phosphate buffered saline pH 7.4 and filter sterilised before use, and IV) betnesol, in the form of betamethasone sodium phosphate, original concentration of 4 mg/ml. (Glaxo Laboratories Ltd., Greenford, England). The pharmacological agents were added to each well so as to effect final concentrations of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the original. The plates were then incubated at $37^{o}C$ for one hour before the addition of 0.09 ml. of labelled target cells and 0.01 ml. of medium to each well.

The control wells, to estimate the degree of SR and TA, each received 0.02 ml. of medium. Also included in the controls for this experiment were wells containing 0.09 ml. of effector cells at the various concentrations plus 0.09 ml. of target cells plus 0.02 ml. of medium, to act as a control for the wells with the

various pharmacological agents added to them. The four pharmacological agents themselves had no effect on SR or on effector cell viability.

Further variations of this assay were:-

1) microtitre plates were set up as outlined above, with the following modifications. 0.02ml. of the various pharmacological agents used were added at the same time as the 0.09 ml. of target cells, and only one concentration of each agent was used namely 10^{-2} final concentration of the original.

2) the effector cells were incubated for four hours with the various drugs at a final concentration of 10^{-2} of the original at $37^{o}C$. Followed by three washes in RPMI plus supplements before being counted and plated out in a standard cytotoxicity assay.

Modification of cytotoxicity assay to test effects of incubation of effector cells in medium supplemented with 10% homologous autologous or foetal calf serum.

In the standard assay the medium is supplemented with 5% FCS, in the following protocol the medium was modified and supplemented with either (a) 10% patient's serum, (b) 10% control serum or (c) 10% FCS. The standard cytotoxicity assay was then carried out with patients effector cells in medium containing either patient's serum, control serum or FCS and control effector cells in medium containing patient's serum, control serum or FCS.

Two further modifications of this assay were used.

1) Plates were set up as outlined above and incubated with 0.1 ml. labelled target cells overnight at 37°C. "Filler cells" were used in the spontaneous release wells of overnight assays to reduce the level of spontaneous release. The "filler cells" used were mouse thymocytes isolated on the day of the assay and added to the SR wells at a ratio of 100 thymocytes to 1 target cell.

2) Effector cells were incubated overnight in the various supplemented media at 37°C. The labelled target cells were added the following day and a standard cytotoxicity assay carried out.

Regime for the testing of the effect of topically applied steroids on the NK cell activity of normal subjects.

Ten normal healthy volunteers with no past history, personal or family, of atopic disease were asked to apply 15 grams of betamethasone valerate (Glaxo laboratories) to their trunk immediately following a hot bath for seven nights. (The full details of the study group are given in table 15). Venous blood samples for the estimation of NK cell activity were taken at regular intervals, days 0, 3, 7, 11, 15, 18 and 22. Blood samples were always taken between 9.00 and 10.00 am.

The above protocol was repeated with a further four volunteers using the base of the ointment (i.e. containing no active steroid). Regular blood samples were again taken on days 0, 3, 8, 15, 18, and 22 for the measurement of NK cell activity. Again the samples were taken between 9.00 and 10.00 am.

Calculation of results.

Results were expressed as percentage lysis according to the following formula where CPM is counts per minute, SR is spontaneous release and TA total activity.

$$\%\text{lysis} = \frac{\text{Mean CPM of sample - Mean CPM of SR}}{\text{Mean CPM of TA - Mean CPM of SR}} \times 100$$

IgE methodology.

The total serum IgE level was measured by the paper radio-immunosorbent test (PRIST) technique using commercially avaible kits (Pharmacia diagnostics AB, Uppsala, Sweden).

Briefly, 50 µl of patient's serum which had been diluted 1:10 with an IgE free diluent (horse serum) was layered onto a paper disk coated with anti-human IgE antibody. The disk and the patient's serum were then incubated together for three hours at room temperature. Following incubation the disk was then washed three times prior to 50 µl of ^{125}I-labelled anti-human IgE being layered onto the disk, which was then incubated overnight at room temperature. The following day the disk was again washed three times in PBS prior to being counted in an LKB minigamma gamma counter.

A standard curve was set up at the same time using samples with a known amount of IgE and the level of IgE in the patient's sample was then estimated from this standard cruve. In the event of the level of the patient's IgE being higher than the top standard further dilutions of the serum were made and the assay repeated.

The normal range for IgE in serum using the Pharmacia PRIST method is 0 - 110 IU/ml.

Fluorescent antibody technique.

The indirect method of staining cells was used. Briefly, $4x10^5$ cells were incubated with either 5 µl of HNK-1 (a gift from Dr. J.C. Roder.) or 5 µl of Leu-11b (Beckton/Dickinson) for 45 minutes at room temperature, both monoclonal antibodies had 200 ug of protein per ml. Following incubation the cells were washed twice in cold PBS before being incubated with 50 µl of a 1:50 dilution of an FITC conjugated goat anti-mouse IgM antibody (Melloy Labs diagnostic division, Springfield, Virginia, USA.) for one hour at $4^{\circ}C$. The conjugate contained $\geqslant 0.4$ mg of specific antibody per ml. Following the second incubation the cells were washed a further two times in cold PBS prior to cytocentrifuge preparations being made. These preparations were then fixed in methanol for five minutes and then counterstained with a 0.001% solution of thyazine red (Raymond A. Lamb, London.) for one hour at $4^{\circ}C$. The preparations were then examined under UV light using a Leitz Ortholux fluorescent microscope. 200 cells were counted and the number giving positive staining was expressed as a percentage of the total.

Clinical details of patients.

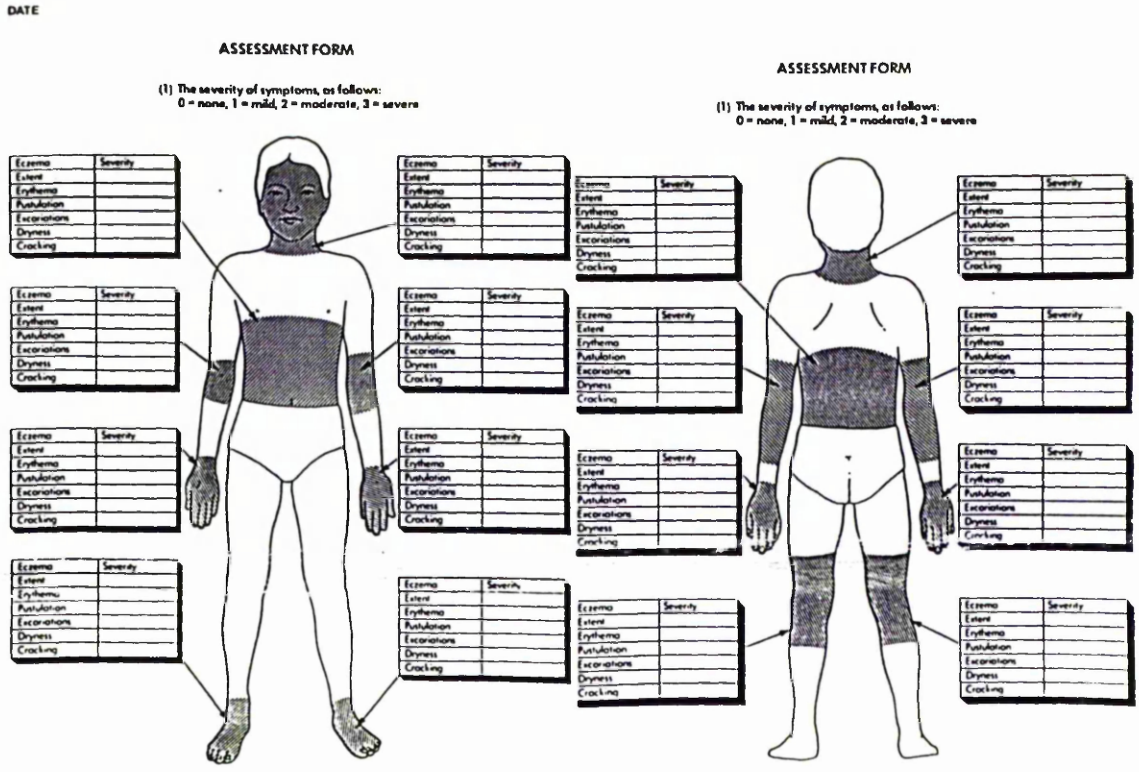
All patients had long standing atopic dermatitis, diagnosed on the basis of the following criteria:-
Age of onset in early childhood, previous or current flexural involvement, previous or current lichenification, a positive past and/or family history of other atopic diseases and a raised serum IgE level, 200 I.U./l.

Clinical scoring of patients.

Sixteen body sites were examined on each visit to the clinic and scored for the following features: extent of involvement, erythema, pustulation, excoriations, dryness and cracking on a 0 to 3 scale. See figure one. The values are then added and the total is the patients clinical score. The higher the total therefore, the worse clinically the patient.

As this method of scoring patients is subjective, patients were therefore assessed by the same clinician on each occasion they visited the out-patients department of the hospital.

Figure 1.



Assessment form showing the body sites and the symptoms used to establish the clinical condition of patients with AD.

Statistical analysis of results.

The statistical significance of the results obtained in this study were determined by either the paired or unpaired Student's t-test, where the data was normally distributed, or by the Mann-Whitney test when it was not.

Correlation coefficients were obtained using Pearson's product moment correlation test.

All statistical analysis was carried out on a North Star advantage microcomputer.

CHAPTER THREE

NATURAL KILLER CELL ACTIVITY OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH ATOPIC DERMATITIS.

The aim of this experiment was to ascertain whether or not NK cell activity was reduced in patients with atopic dermatitis.

Clinical Details of Patients.

Twenty patients with atopic dermatitis were studied, 14 female, 6 male, with an age range of 14 to 54 years (mean age 26 yrs). All of the patients had long standing atopic dermatitis, i.e. a chronic relapsing pruritic skin condition with the typical features of AD. Additional diagnostic criteria included age of onset in early childhood, previous or current lichenification, a positive past personal and/or family history of other atopic diseases, a raised serum IgE level >200 IU/ml. All six criteria were present in eleven of the patients, five were positive in another five patients, of the remaining four patients, two were positive for four features and two for three. Seventeen patients had a past history of other atopic disease and seventeen had a positive family history of atopy. No patient was on systemic corticosteroid therapy at the time of the study, though one patient had received intermittent short courses of prednisolone for asthma. These details are summarised in table two.

Control Subjects.

Nine non-atopic controls were used on thirteen occasions, all controls were age matched within ten years of the patients' age, the controls were also sex matched in six out of nine occasions.

TABLE 2

Number of patients	20	
Female:Male	14:6	
Flexural involvement	19 (95%)	All but No. 19.
Lichenification	19 (95%)	All but No 19.
Age of onset (years)		
< 2	14	All but Nos. 5, 6, 11, 12, 17 & 19.
2 - 5	3	Nos. 5, 6, & 17
> 15	3	Nos. 11, 12 & 19
Past history		
Other atopic disease	17 (85%)	All but Nos 1, 12 & 13.
Family history		
Other atopic disease	17 (85%)	All but Nos. 5, 13 & 17.
Raised IgE		
> 200 IU/ml	19 (95%)	All but No. 17.

Clinical details of patients with atopic dermatitis.

Results.

The results of this experiment showed that NK cell activity was very much reduced in patients with atopic dermatitis as measured by ^{51}Cr. release cytotoxicity assay. Figure 2. At each effector to target cell ratio the mean value for the patient group was significantly lower than that of the corresponding control group. $p < 0.004$. by Mann-Whitney test.

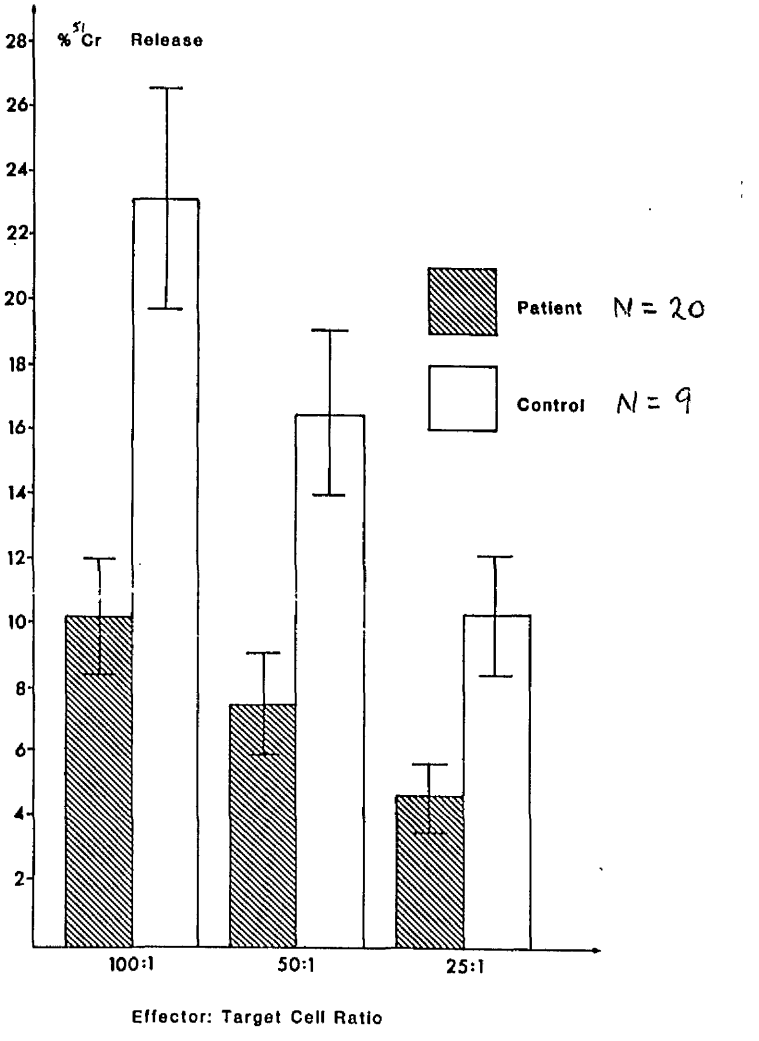
From the individual results, displayed in table 3, it can be seen that the mean NK cell activity of the patients was 44.1% at 100:1, 45.4% at 50:1 and 44.6% at 25:1 of the control values.

Plotting the results individually, as in figure three, it may be observed that there is some overlap between patients and controls, although the level of NK cell activity for the majority of the patients lies below the mean value for the control group.

Serum IgE levels and NK cell activity in AD.

Serum IgE levels were also examined in this group of patients. It has been suggested that serum IgE may be connected with disease activity although this is unlikely. The contradiction to this theory being the 20% or so of patients with normal levels of serum IgE (Juhlin, et al. 1969). It was, however, thought to be a worthwhile exercise to ascertain whether or not there was a correlation between NK cell activity and serum IgE levels.

FIGURE 2



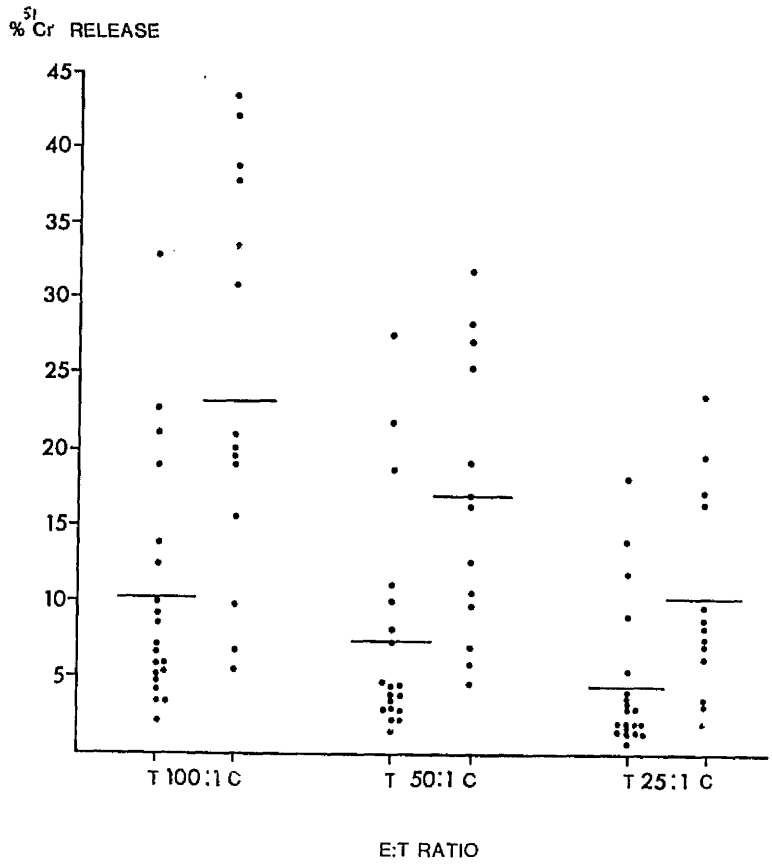
Mean and SE of chromium release following a four hour cytotoxicity assay for patients with AD and normal healthy age and sex matched controls.

TABLE 3

	Atopics					Controls				
	Age (yrs)	Sex	100:1	50:1	25:1	Age (yrs)	Sex	100:1	50:1	25:1
1	19	F	3.5	3.0	1.7	20	M	19.6	16.4	8.7
2	22	F	10	8.1	2.1	"	"	"	"	"
3	30	F	2.3	2.3	0.7	"	"	"	"	"
4	21	M	3.4	1.6	1.5	"	"	"	"	"
5	27	M	5.9	4.5	2.0	"	"	"	"	"
6	20	F	4.3	4.5	2.0	-	F	20.1	17.0	9.6
7	16	F	5.3	3.9	3.0	"	"	"	"	"
8	23	F	13.6	6.7	4.1	28	F	30.8	19.1	6.3
9	21	F	6.6	2.9	3.1	27	F	15.6	12.6	7.1
10	45	F	8.6	11.0	9.0	47	F	6.8	5.9	7.5
11	25	M	9.2	7.5	5.5	30	M	5.6	4.6	3.3
12	54	M	4.8	4.6	2.0	56	M	37.7	28.3	16.5
13	42	F	7.2	3.9	3.3	37	F	21	10.5	8.4
14	21	F	32.7	27.6	18.3	22	F	38.7	25.4	19.6
15	25	F	5.8	2.3	1.5	24	F	9.8	7.0	3.5
16	23	F	5.4	3.6	1.5	28	F	43.3	27.4	17.7
17	30	F	21.1	9.9	3.5	24	F	19.0	9.8	2.1
18	22	F	12.6	3.0	1.4	"	"	"	"	"
19	21	M	22.7	21.8	11.9	20	M	33.5	31.7	23.6
20	14	M	19.1	18.8	14.0	"	"	"	"	"
	Mean		10.2	7.5	4.6			23.1	16.5	10.3
	SE		1.7	1.6	1.0			3.4	2.5	1.8

Results for atopic Vs. normals for NK cell activity

FIGURE 3



Percentage chromium release for individual patients (T) and controls (C) at the various E.T. ratios. Mean values for each group are indicated by the bars.

Raised IgE levels were found in nineteen of the twenty patients, only one was within the normal range, i.e. 50 IU/ml. In fifteen of the twenty patients, the IgE level was significantly higher than normal, (table 4) with the range being from 1,013 to 35,000 IU/ml. (mean 8,812 IU/ml). Regression analysis was carried out on these figures. No correlation could be found between percentage ^{51}Cr. release and IgE level or percentage ^{51}Cr. release and log IgE level. (Normal range for IgE is 0-110 IU/ml.).

Conclusions.

Results reported from different groups have been at variance. Kusiami and Trentin, in 1982 reported that NK cell activity is reduced in these patients. Others have reported normal or raised levels of activity (Viander, et al. 1982; Strannegard and Strannegard. 1980).

The results of the above experiment have shown that the NK cell activity of patients with atopic dermatitis is greatly reduced compared with that of normal controls, as measured by the method used in this laboratory. It would also appear that the level of serum IgE has no effect on the level of NK cell activity of PBMC from patients with atopic dermatitis.

It is possible that variations in assay methodology could explain the normal or raised levels of NK cell activity found in some laboratories. It was decided therefore, to investigate this possibility further.

TABLE 4

Age	Sex	IgE IU/ml.	Log IgE
19	F	3,975	3.599
22	F	1,013	3.005
30	F	3,508	3.545
21	M	35,000	4.544
27	M	773	2.888
20	F	15,125	4.179
16	F	8,375	3.922
23	F	2,400	3.380
21	F	7,250	3.860
45	F	1,230	3.089
25	M	13,625	4.134
54	M	8,000	3.903
42	F	375	2.574
21	F	6,650	3.822
25	F	208	2.318
23	F	5,431	3.734
30	F	50	1.698
22	F	255	2.406
21	M	2,100	3.322
14	M	18,500	4.267

Serum IgE and Log serum IgE levels for patients with AD.

CHAPTER FOUR

THE EFFECT OF VARIATIONS IN ASSAY METHODOLOGY ON THE NK CELL ACTIVITY OF PATIENTS WITH AD AND NORMAL CONTROLS

Introduction.

There are differences in assay methodology between the groups of workers that reported normal or raised levels of NK cell activity in patients with AD and the assay methodology used in this study. The aim of the following experiments was therefore to ascertain whether or not differences in assay technique could account for the differences in results between the different groups of workers.

The major differences in assay methodology between this study and those of Viander et. al. (1982) and Strannegard and Strannegard. (1980) were:

1. Length of time of incubation of effector cells with targets.
2. Removal of phagocytic/adherent cells.
3. Use of target cells other than K562.

Each of these variables was checked in turn to assess what difference, if any, they made to the amount of NK cell activity of patients with AD and controls.

1. Effect of increasing the incubation time on the level of NK cell activity of PBMC from patients with AD and normal controls.

The reduced levels of NK cell activity in AD seen following a four hour assay could be due to abnormal activation of effector

cells ie. NK cells from patients with AD may require longer to reach control levels of cytolytic activity after the addition of the target cells and given a longer incubation time it might be possible for effector cells from patients with AD to "catch up" with those from normal controls.

NK cell activity was studied in nine patients (5 female, 4 male) with AD and nine age and sex matched healthy controls in a) a four hour assay and b) an 18 hour assay.

Results.

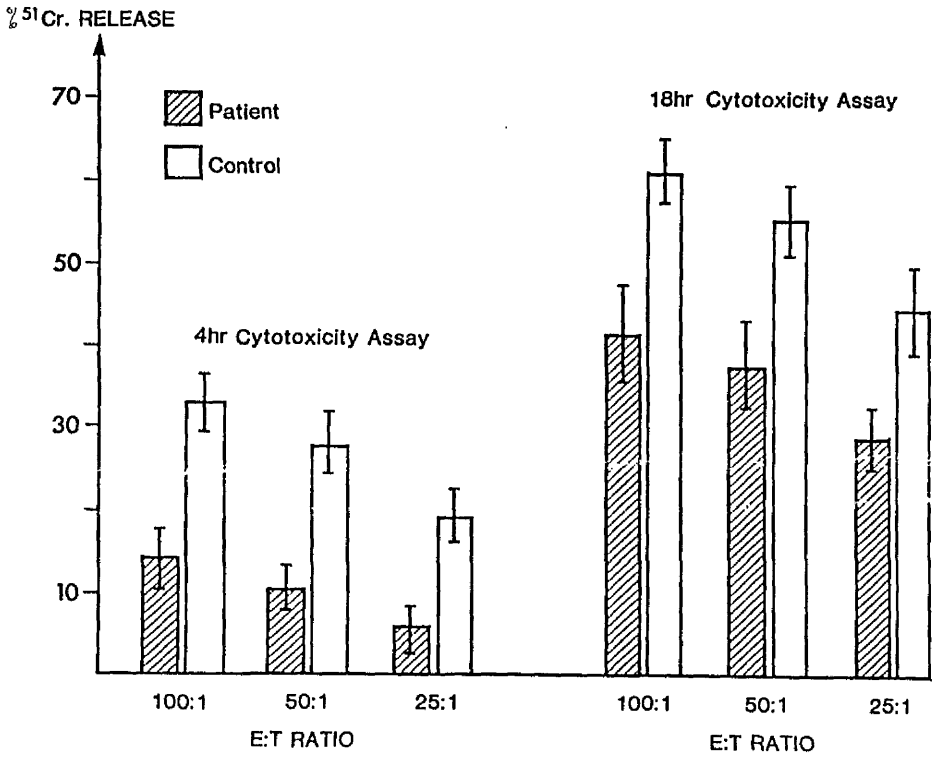
Following an 18 hour cytotoxicity assay the NK cell activity of PBMC from the AD patients increased significantly compared with that found following a 4 hour cytotoxic assay, 14.4 vs 41.9, at an E:T ratio of 100:1, $p < 0.0005$ using Mann-Whitney test (table 5). The NK cell activity of the control subjects had also increased with the longer assay time (33.3 vs 61.4) with the same significant degree of difference. However, the level of NK cell activity in the control group was still higher (61.4 vs 41.9) than that of the patients. It can be seen that the difference between the two groups following an 18 hour incubation was not as great as after a 4 hour incubation and did not reach the same level of significance $p<0.02$ (100:1) following 18 hours vs $p<0.004$ (100:1) following 4 hours. (Figure 4).

Table 5.

	% of ^{51}Cr. Release					
	Patient			Control		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1
4 hours	14.4	10.9	5.9	33.3	28.1	19.2
18 hours	41.9	37.8	29	61.4	55.5	44.6

Mean ^{51}Cr. release during a four hour and eighteen hour cytotoxicity assay for patients with AD and age and sex matched controls

Figure 4.



Mean ±SE of chromium release following either a standard four hour cytotoxicity assay or an 18 hour cytotoxicity assay for patients with AD and age and sex matched healthy controls.

2. Removal of phagocytic and adherent cells.

The presence of macrophages or monocytes in the effector cell population during a cytotoxic assay is known to cause suppression of NK cell activity (Jondal, et al. 1982). It is possible that the suppression caused by the presence of phagocytic or adherent cells in the effector cell population is greater in patients with AD than in normal healthy controls. The phagocytic/adherent cells were, therefore, removed from the effector population prior to the cytotoxic assay, by

1. Carbonyl iron ingestion followed by passage over a strong magnet.

or

2. Adherence to glass or plastic.

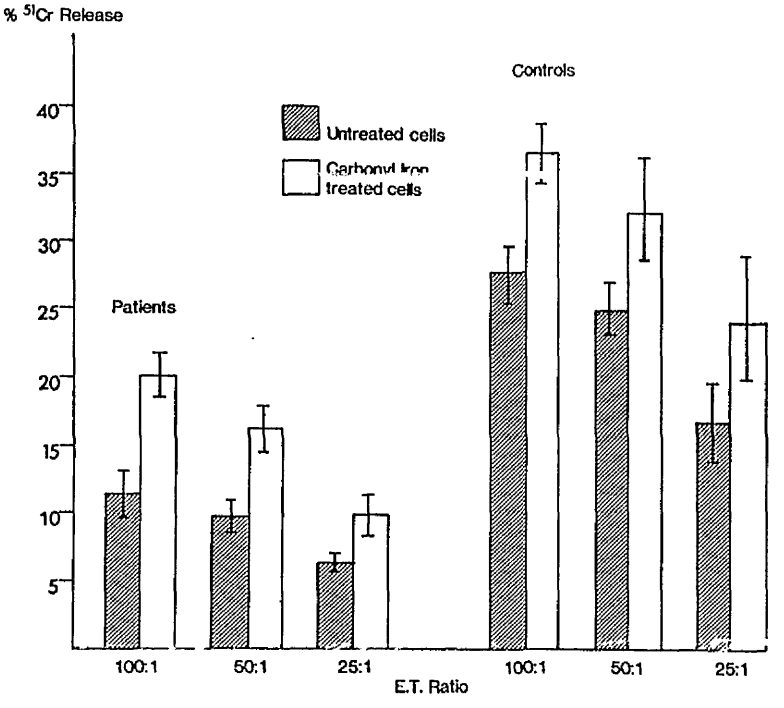
Carbonyl iron ingestion.

PBMC from eight patients with AD (5 female, 3 male) and five age and sex matched healthy controls (3 female, 2 male) were treated as described above prior to the standard four hour ^{51}Cr. release assay. The levels of NK cell activity found after removal of carbonyl iron reactive cells were compared to those found in untreated intact PBMC populations from the same patients and controls.

Results.

From figure 5, it can be seen that carbonyl iron treatment

Figure 5.



Mean ±SE of chromium release before and after the removal of phagocytic cells by carbonyl iron ingestion for patients with AD and age and sex matched healthy controls.

of the effector cell population resulted in an increase in NK cell activity of PBMC from both the patients with AD and the control subjects. The difference between the patients and controls following carbonyl iron treatment had the same degree of significance as that found before treatment. $P < 0.005$. These results are summarised in table 6.

Glass adherence.

PBMC from 9 patients with AD (5 females, 4 males) and 4 age and sex matched controls (2 females, 2 males) were treated as described above prior to the standard four hour ^{51}Cr. release assay for NK cell activity. The levels of NK cell activity found after removal of adherent cells was compared with that found in untreated intact PBMC populations from the same patients and controls.

Results.

From figure 6 it may be seen that the effect of the removal of adherent cells from the population of effector cells is to increase the level of NK cell activity. The observed increase was not as great as that found following carbonyl iron treatment nor is it statistically significant. $p < 0.03$ at the 100:1 E:T ratio for the patients group and $p < 0.5$ for the control group at the same E:T ratio. these results are summarised in table 7.

3. Natural killer cell activity against various target cells.

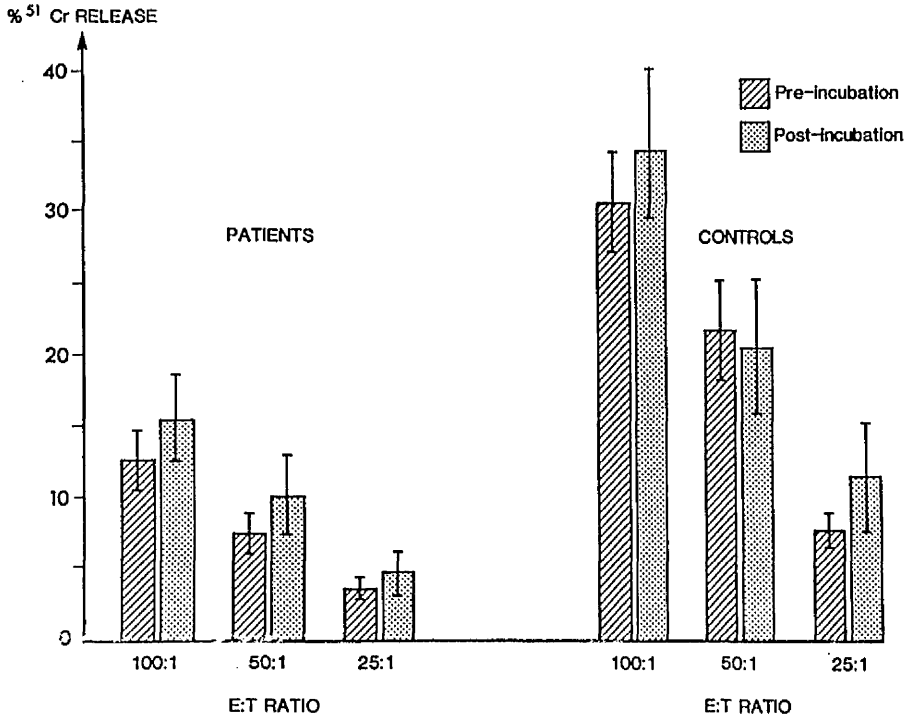
It has been shown that peripheral blood NK cells from an

Table 6.

	% of ^{51}Cr Release					
	Patient			Control		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1
Untreated	11.5	9.9	6.5	27.7	24.9	16.8
Carbonyl iron treated	20.2	16.3	10	36.7	32.3	24.2

Mean ^{51}Cr release during a four hour cytotoxicity assay before and after removal of phagocytic cells for patients with AD and age and sex matched controls.

Figure 6.



Mean ±SE of chromium release before and after removal of adherent cells by incubation in glass or plastic for patients with AD and age and sex matched controls.

Table 7.

	% ^{51}Cr Release					
	Patient			Control		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1
Untreated	12.7	7.6	3.7	30.8	21.8	7.6
Plastic/Glass Incubated	15.7	10.2	4.8	34.6	20.6	11.4

Mean ^{51}Cr. release during a four hour cytotoxicity assay, before and after removal of adherent cells, for patients with AD and age and sex matched controls.

individual show varying degrees of activity against different target cells (Santoli, et al. 1976). It was decided therefore, to investigate whether the difference in NK cell activity observed between patients with AD and normal controls was due to differences in reactivity against the target cell used, namely K562, rather than any differences in the lytic ability of the effector cells.

Four different target cells were used: BJAB, CCRF-CEM, Molt-4 and Namalva. CCRF-CEM and Molt-4 are recognised target cells (Phillips et al. 1980: Callewart, et al. 1979). BJAB and Namalva were included due to their availability within this department. Full details of these cells were given in table one. Peripheral blood NK cell activity was measured in ten patients against each of the target cells in a four hour cytotoxic assay. As with previous experiments cells from age and sex matched healthy controls were also measured. The ratio of males to females, patients and controls, used in each experiment was:-

		M F		M F
a) BJAB	Patients	4: 6	Controls	3: 5
b) CCRF-CEM	Patients	3: 7	Controls	2: 4
c) Molt-4	Patients	5: 5	Controls	2: 2
d) Namalva	Patients	3: 7	Controls	1: 5

Results.

The results of this experiment showed that the overall level of NK cell activity, for both patients and controls, was reduced against all four target cells compared with that found

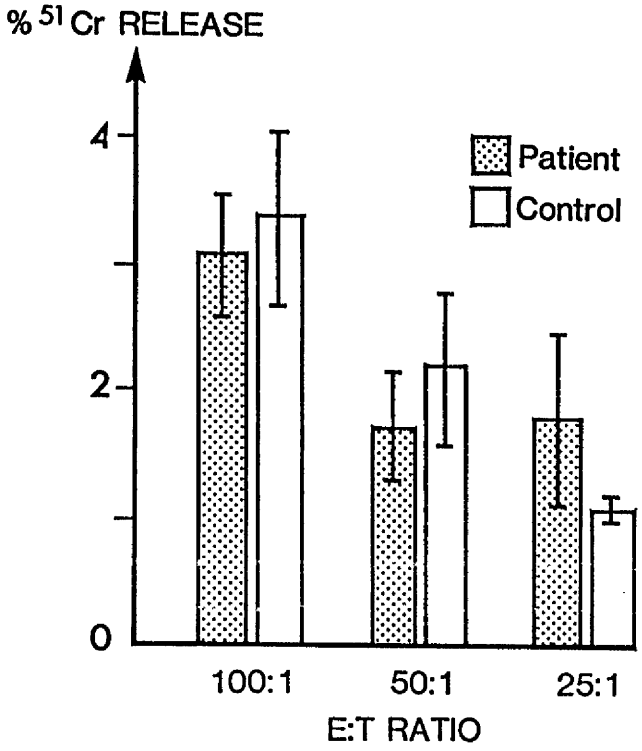
when K562 was the target. See figures 7 a, b, c, and d. The level of NK cell activity of the control subjects remained higher than that of the patients, although with BJAB and Namalva, the difference was very small, indeed the level of activity was so low for both patients and controls that these two target cells might be referred to as NK resistant and not suitable as targets. A significant difference between the patients and controls was only demonstrated with CCRF-CEM ($p < 0.05$ at 100:1) this was less significant than that found with K562 ($p < 0.004$ at 100:1).

The sensitivity of the various target cells to lysis by NK cells was CCRF-CEM > Molt-4 > BJAB > Namalva. and all of these were less sensitive than K562. These results are summarised in table 8.

Conclusions.

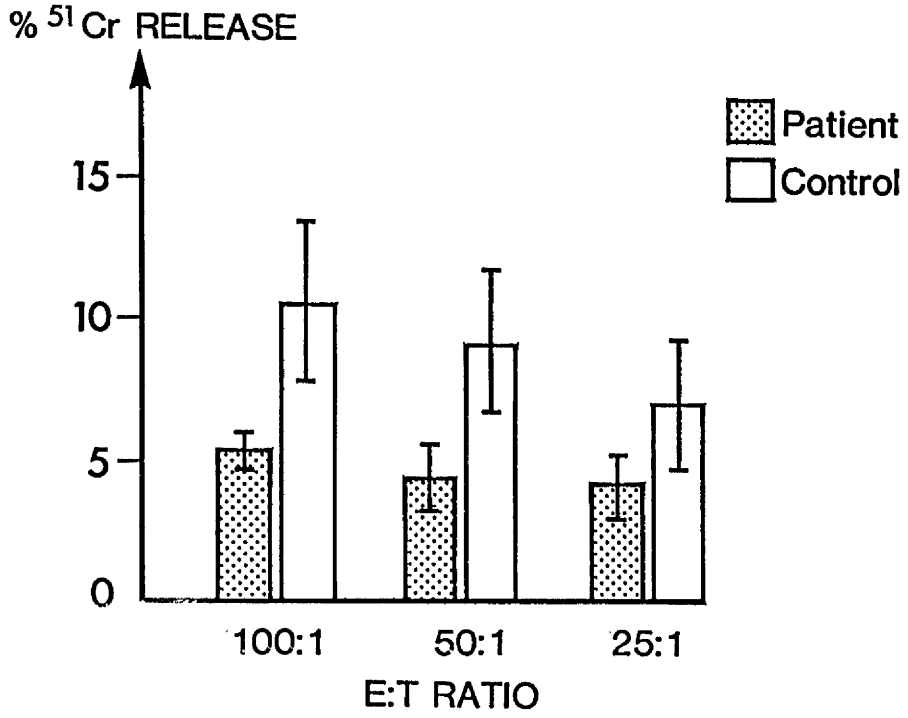
The above results demonstrate that the methodology used in the assay does not account for the varying results obtained by other investigators. The extended 18 hour incubation time caused an increase in NK cell activity in the patient's effector cells. However, there was also a similar, though lesser, increase in the level of activity seen in the control group. The difference between the two groups was therefore less following an 18 hour incubation as compared with that after four hours but it remained statistically significant. One may therefore assume that abnormal activation of effector cells is not the cause of the reduced levels of NK cell activity in patients with AD as even with an 18 hour incubation the patient's effector cells still do not reach the level

Figure 7a.



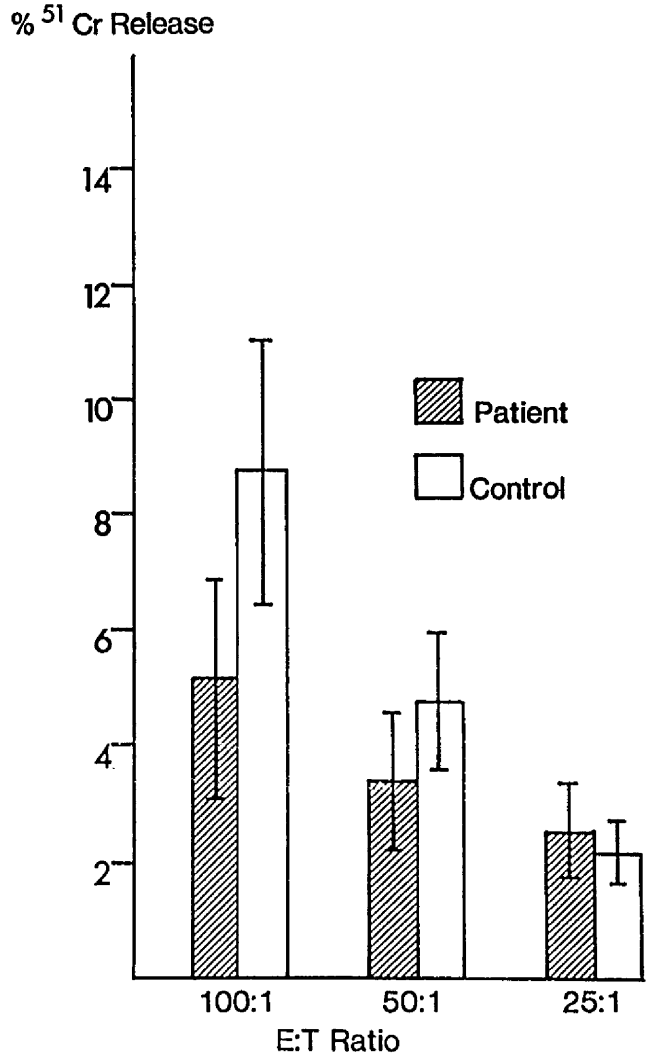
Mean ± SE of chromium release for patients with AD and age and sex matched controls using BJAB as the target.

Figure 7b.



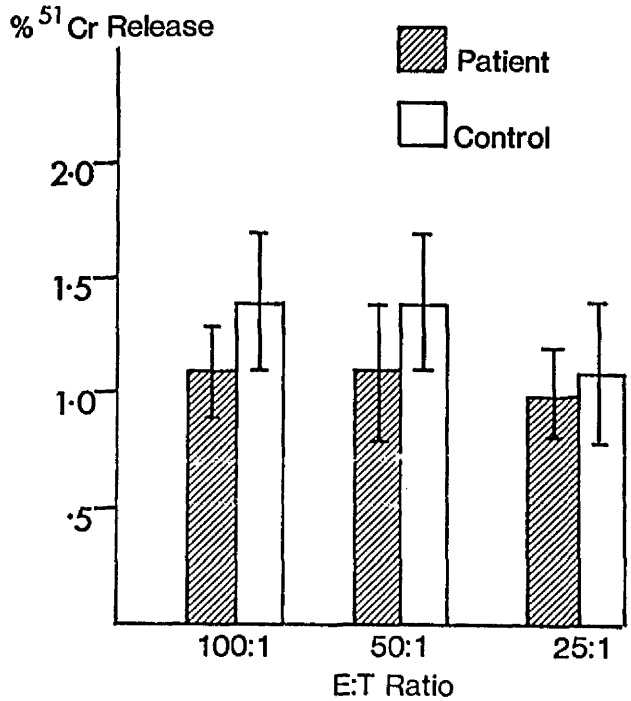
Mean ±SE of chromium release for patients with AD and age and sex matched controls using CCRF-CEM as the target.

Figure 7c.



Mean ±SE of chromium release for patients with AD and age and sex matched controls using MOLT-4 as the target.

Figure 7d.



Mean ±SE of chromium release for patients with AD and age and sex matched controls using Namalva as the target.

Table 8.

	% ^{51}Cr Release					
	Patients			Controls		
E:T Ratio / Target Cell	100:1	50:1	25:1	100:1	50:1	25:1
BJAB	3.1	1.7	1.8	3.4	2.2	1.1
CCRF-CEM	5.5	4.5	4.2	10.8	9.3	7.1
Namalva	1.1	1.1	1.0	1.4	1.4	1.1
Molt-4	5.2	3.4	2.6	8.8	4.8	2.2

Mean ^{51}Cr Release during a four hour cytotoxicity assay, with target cells other than K562, for patients with AD and age and sex matched controls.

of activity of the control subjects.

Removal of phagocytic/adherent cells from the effector cell population also caused an increase in the level of NK cell activity in both patients and controls. Removal by carbonyl iron treatment of the effector population caused a greater increase in activity than that by adherence to glass or plastic. Neither of these methods closed the gap between the two groups. If there is inhibition of NK cell activity in AD then it is unlikely to be due solely to the presence of phagocytic/adherent cells.

The third experiment showed that the choice of target cell did affect the level of NK cell activity, which, depending on the target cell, could result in a highly significant difference, a slightly significant difference or no difference at all between the patients and controls. However, the level of activity in the controls was always greater than in the patients, to a varying degree, with BJAB and Namalva proving to be NK resistant targets. This suggests that the variation is due to the inherent ability of the target cells to resist lysis rather than differences within the effector populations. If the difference in NK cell activity was due to different specificities within the effector cell population then it would be expected that some target cells might be more easily lysed by effector cells from individuals with AD than from controls. This was not demonstrated.

Of the parameters investigated none could be said to account for the varying results between investigators. The level of NK cell activity in patients with AD was found to be consistently lower than that of the controls throughout this group of experiments. The cause of this reduction is unclear but there are several possibilities. The reduced levels of effector cell function may be due to the disorder itself, reduced numbers of effector cells, reduced function in the effector cell population, the presence of an inhibitory factor or even the treatment of the disease.

CHAPTER FIVE

Sequential study of NK cell activity in patients with AD.

Introduction.

NK cell activity was monitored in a group of patients with AD sequentially over a twelve month period. NK cell activity of normal individuals remains relatively constant over extended periods of time (Pross and Baines 1982). As AD is a chronic relapsing disorder it was of interest to establish whether or not NK cell activity correlated with the clinical severity of the disorder or whether patients with AD retained a relatively stable, albeit reduced NK cell function.

Seven young adults (3 males and 4 females with ages ranging from 19 to 30 years, mean age 24) were selected for close follow up of their atopic dermatitis. The patients were examined on a total of 52 occasions: three patients on six occasions, and one patient on seven, eight, nine and ten occasions. At each the disease activity was assessed by clinical examination of sixteen body sites, (figure 1) for the six clinical features described in materials and methods. The patients were examined by the same clinican on each occasion in order that subjectivity be kept to a minimum. A peripheral blood sample was taken on each occasion for NK cell activity and serum IgE levels. A sample from an age and sex matched healthy control was also included.

Results.

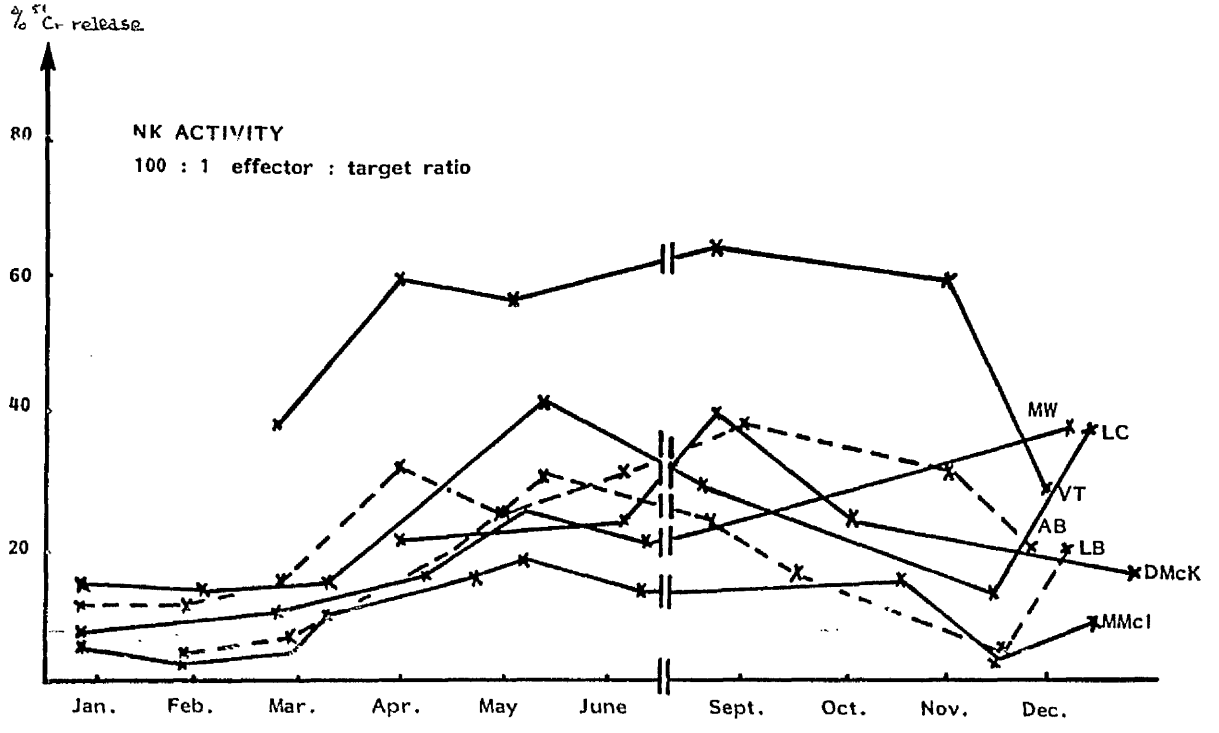
The level of NK cell activity of these patients was again observed to be very much reduced in respect to the control subjects.

subjects, 19.3 vs 32.3 at 100:1, 14.7 vs 27.6 at 50.1, 9.5 vs 19.9 at 25:1 (mean NK levels for the duration of the experiment). A seasonal variation in the NK cell activity of these patients was observed with the lowest values for NK cell activity recorded in the period January to March, see figure 8. Over the next three months NK cell activity rose towards normal levels. During this time the patients also had a relative remission of their dermatitis. The pattern in the autumn was less clear though there was some rise in the disease activity and the NK cell activity was observed to fall. The patient with the highest disease activity also had the lowest NK cell activity. The patient with the lowest disease activity (MW) went into virtual clinical remission over the course of the study and her NK cell activity was also found to have risen to normal levels. Figure 9 displays the disease activity of each of the patients throughout the study.

NK cell activity was found to correlate with clinical score throughout the period of study, at all three effector to target cell ratios, in an inverse manner, i.e. the more active the disease clinically (the higher the score) the lower the NK cell activity, $r = -0.400$, $p < 0.01$.

The group as a whole also showed the recognised relationship between clinical activity and both serum IgE and log IgE

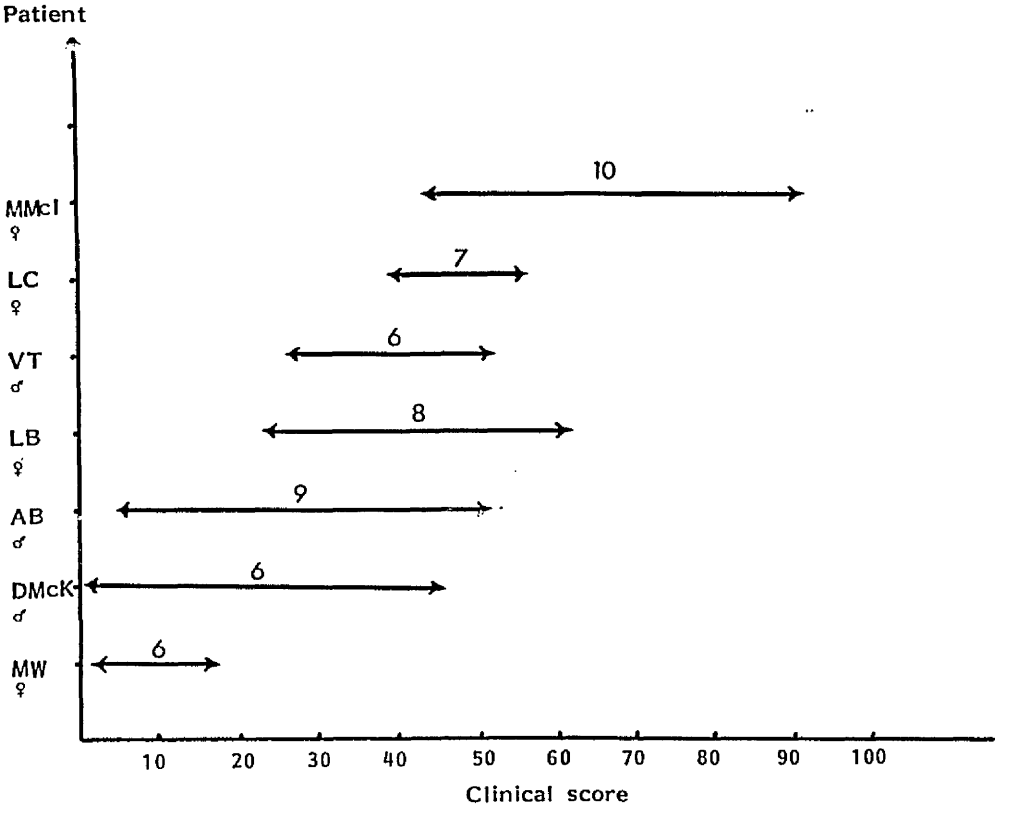
Figure 8.



NK cell activity of patients throughout sequential study.

As measured by chromium release following a four hour cytotoxicity assay.

Figure 9.



Clinical score ranges of patients throughout sequential study. The number on top of the bar refers to the number of occasions each patient was examined.

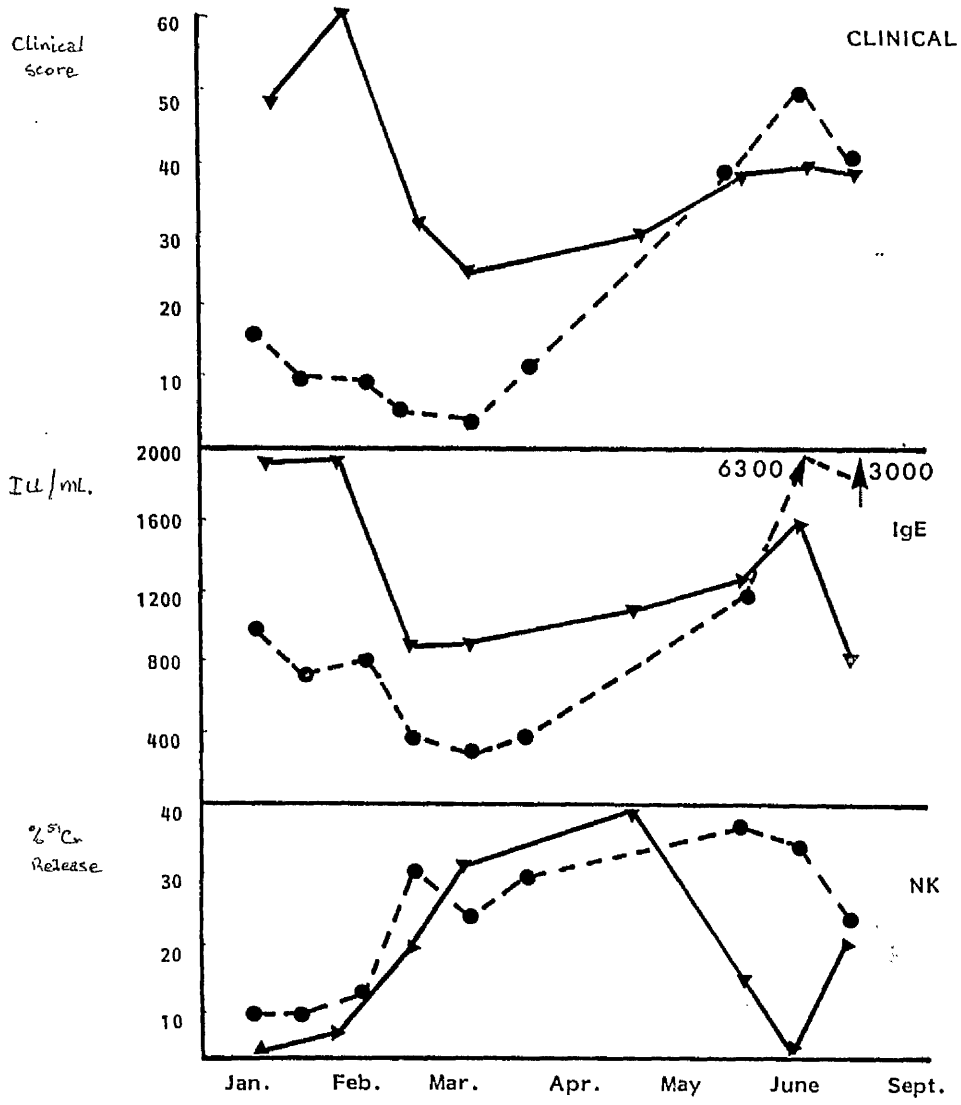
level. Two of the patients who showed this relationship most clearly are displayed in figure 10. However, IgE and log IgE did not show a significant relationship to NK cell activity. These results are summarised in table 9.

One of the patients, a young man, was discovered to have a serious alcohol problem during the course of the study. Interpretation of his results (his NK cell activity was consistently high/normal) in the light of his co-existent liver disease was impossible, therefore, his results were excluded from subsequent analysis.

Conclusions.

This experiment confirmed that reduced amounts of NK cell activity are found in individuals with AD. An inverse relationship between NK cell activity and disease activity was demonstrated ie, the more active the disease the lower the NK cell activity. Seasonal variation in both the level of NK cell activity and the disease activity were also shown. As expected no relationship between either serum IgE levels or log serum IgE levels and NK cell activity was found.

Figure 10.



Relationship of clinical activity, serum IgE level and NK cell activity for two of the patients throughout the sequential study.

——▲—— L B

- - - -● D M K

Table 9.

	NK cell activity		
	100:1	50:1	25:1
Clinical Score	-0.400+++	-0.411+++	-0.405+++
IgE	0.286	0.263	0.220
Log IgE	0.270	0.238	0.163

+++ $p < 0.01$

Correlation coefficients for NK cell activity with clinical score, IgE level and Log IgE level for patients with AD.

CHAPTER SIX

FLUORESCENT MARKER STUDIES

Introduction.

The reduced NK cell activity in patients with AD could be due to either a quantitative defect of NK cells ie. a reduction in the absolute number of effector cells present, or a qualitative defect ie. normal numbers of effector cells but with reduced activity. The following experiment was designed to investigate these possibilities further. The percentage of effector cells present in the peripheral blood of patients with AD and normal controls was estimated using a fluorescent sandwich technique. Two monoclonal antibodies were used, HNK-1 (Leu-7) and Leu-11b. The percentage of positively staining cells was then correlated with functional activity as measured by the standard chromium release assay.

Results.

HNK-1 (Leu-7).

The experimental group consisted of 22 patients with AD (16 female, 6 male) and 24 control subjects (12 female, 12 male). The difference in NK cell activity, as measured by chromium release, between patients and controls was found to be highly significant at each of the E:T ratios examined (table 10), $p < 0.005$ by Mann Whitney test. There was a highly significant difference in the numbers of HNK-1 (Leu-7) positive cells for the patients as compared with the normal controls (11.1 vs 17.4) $p < 0.005$ by Mann-Whitney test (table 11.). The results of regression analysis showed that the line of percentage ^{51}Cr. release

Table 10.

	% Cr. Release					
	Patient			Control		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1
Expt. 1	20.1±2.4	14.9±2	9.5±1.7	35±2.5	30.8±2.7	22.9±2.5
Expt. 2	12.7±1.6	8.2±1.2	3.8±0.6	26.7±2.2	20.7±2.1	13.1±1.6

Mean ±SE of chromium release following a standard four hour cytotoxicity assay for patients with AD and age and sex matched normal controls, (Experiment 1 HNK-1, Experiment 2 Leu-11b).

Table 11.

	% Positive staining cells	
	HNK-1	Leu-11b
Patient	11±1.1	12.8±1.0
Control	17.4±1.3	14.1±0.8

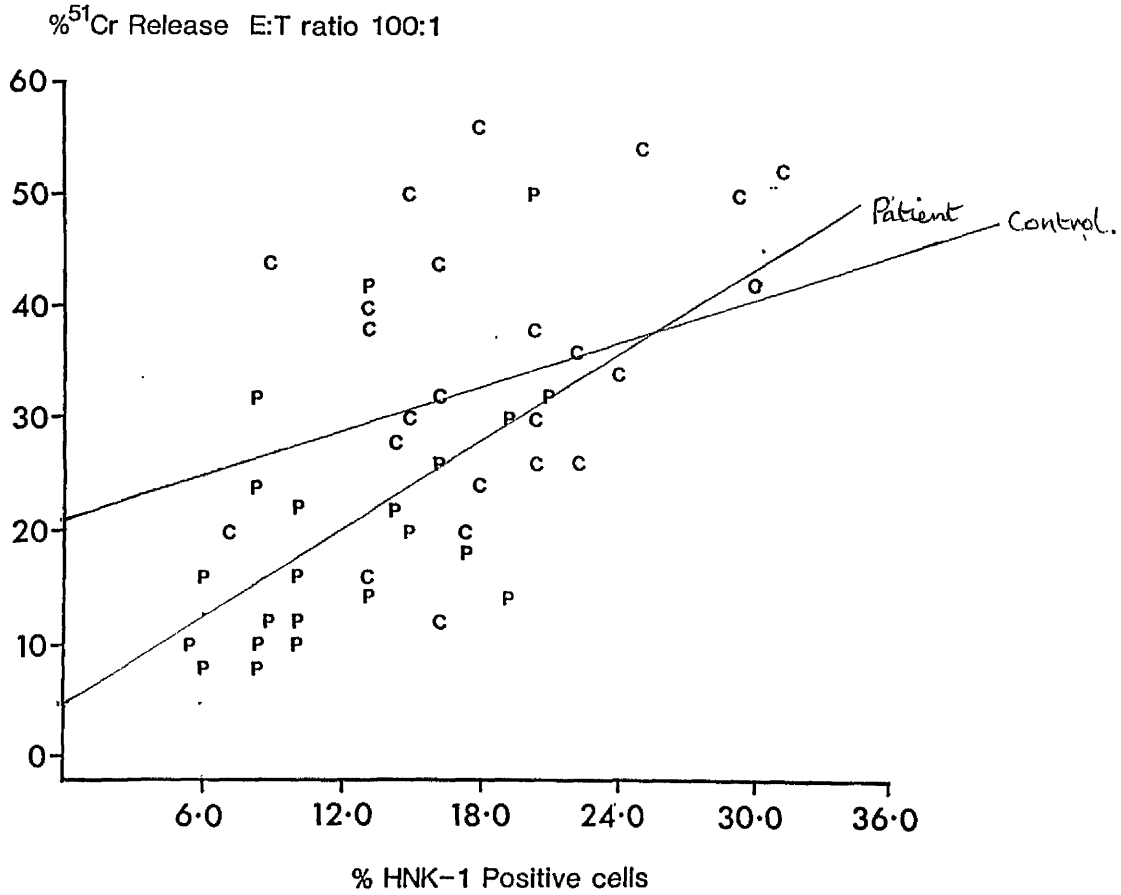
Mean ±SE of percentage positive staining cells using either HNK-1(Leu-7) or Leu-11b for patients with AD and age and sex matched normal controls.

against percentage HNK-1 positive cells for the patients had a large gradient, 1.39, and a small intercept, 4.6. While a similar line for the control subjects had a small gradient, 0.72, and a large intercept, 20.8. This suggests that there are two separate lines for patients and controls. From figure 11 it can be seen that the patients are clustered round the bottom part of the graph whereas the controls are to be found further up. This suggests that there are more effector cells present in the control population giving rise to greater chromium release which supports the hypothesis that reduced NK cell activity is due to reduced numbers of effector cells as stained by HNK-1. A significant correlation was found between the percentage of NNK-1 staining cells and chromium release for both the patients and controls. For the patient group the correlation was found to be most significant at the 100:1 E:T ratio, $r= 0.572$, $p< 0.01$, than at the 50:1, $r=0.507$, $p< 0.02$, or the 25:1, $r= 0.473$, $p< 0.05$. The correlation for the control subjects was not significant at the 100:1 E:T ratio but was found to be significant at the 50:1, $r= 0.497$. $p< 0.02$ and at the 25:1, $r= 0.486$, $p<0.05$.

Leu-11b.

As HNK-1 has been shown to be reactive with most but not all NK cells (Lanier, et al. 1983) the above experiment was repeated using Leu-11b which is said to be more specific than HNK-1. The experimental group consisted of 30 patients with AD (22 female, 8

Figure 11.

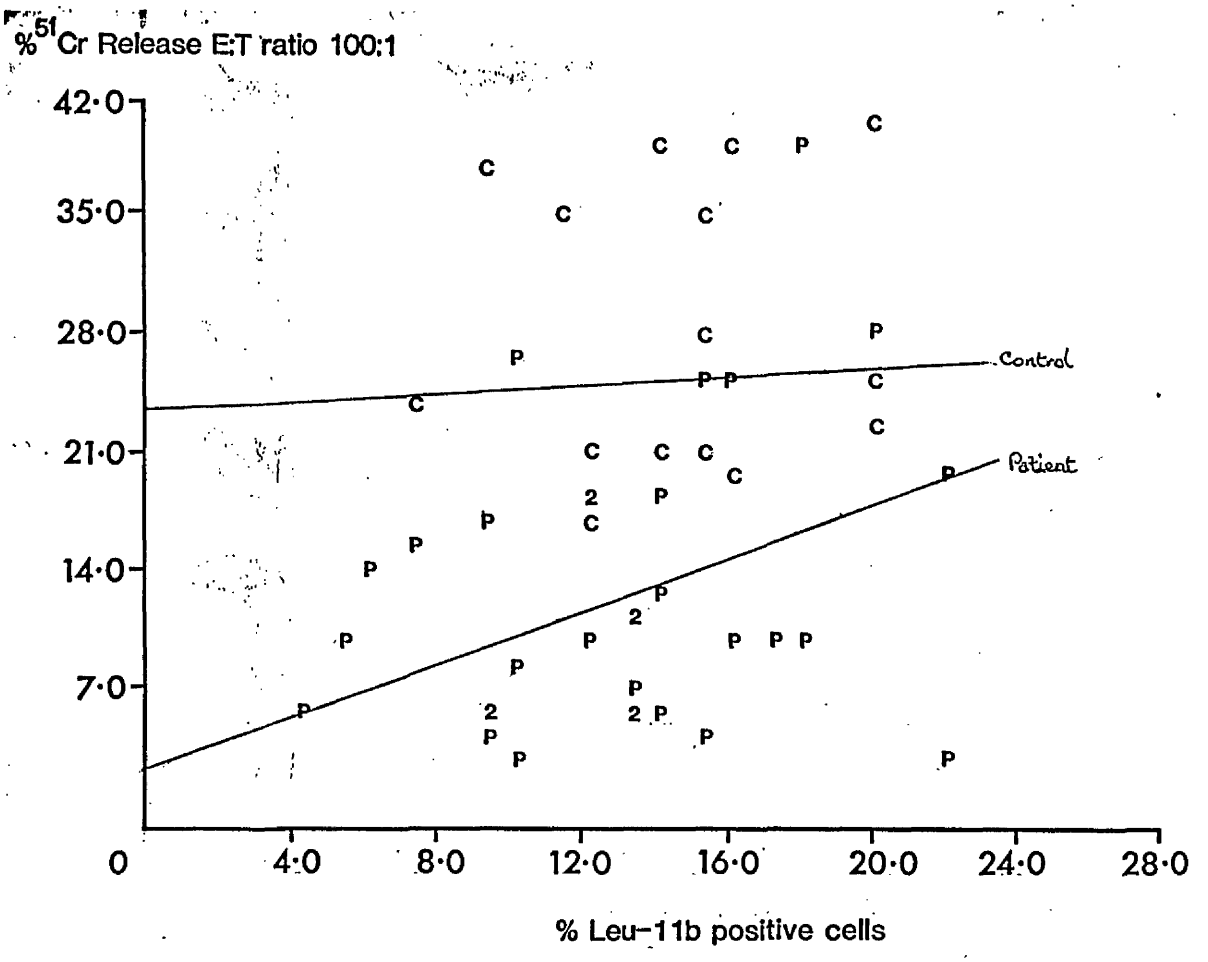


Percent ^{51}Cr. release Vs percentage HNK-1 positive cells for patients with AD (P) and normal controls (C).

male) and 17 controls (7 female , 10 male). The difference in NK cell activity between patients and controls was again found to be highly significant, $p < 0.005$ by Mann-Whitney test, at each of the E:T ratios examined (table 10). The number of Leu-11b positive cells was not significantly different in the patients with AD compared to the controls, (12.8 vs 14.1) $p < 0.3$ by Mann-Whitney test (table 11). The results of regression analysis again showed that the line of percentage ^{51}Cr. release against percentage Leu-11b positive cells for the patients had a large gradient, 0.6, and a small intercept, 3.2. While a similar line for the controls had a small gradient, 0.16, and a large intercept, 23.1. These results also suggest that there are two separate lines for patients and controls. When the results are displayed graphically,as in figure 12, it can be seen that there is not as much clustering as was found with HNK-1. The different values obtained for gradient and intercept for patients and controls coupled with the observations of the lack of clustering and the similar numbers of Leu-11b positive staining cells suggests that there is no difference in effector cell numbers, that would account for the differences in activity of patient and control effector cells, rather there are two sets of kinetics involved.

Unlike HNK-1 no correlation was found between the percentage of Leu-11b positive staining cells and activity at any of the E:T ratios for both patients and controls.

Figure 12.



Percent ^{51}chromium release Vs. percentage Leu-11b positive cells for patients with AD (P) and normal controls (C).

The figure "2" indicates that there are two points on the graph that are too close together to be separated.

Conclusion.

The results of this experiment have shown that there is a correlation between the number of HNK-1^+ cells and percentage chromium release but not between Leu-$11b^+$ cells and percentage chromium release. A significant difference in the number of HNK-1^+ but not Leu-$11b^+$ cells was found between patients and controls. The results of regression analysis for both monoclonals suggests that the kinetics of the patients' response are different from those of the normal controls. As Leu-11b is said to be a more specific monoclonal antibody than HNK-1 these results suggest that the reduction in NK cell activity in patients with AD is more likely to be due to a functional difference in the effector cells between patients and controls. The defect in the patients' effector cells may be an inability to bind the target cells or ineffective killing or a poor recycling capability. There are several possible underlying causes of the reduced function. The effector cells could be inhibited by the presence of a factor in the circulation of these patients caused by the underlying condition itself, or the treatment of the disease by topical steroids could effect the function of the NK cells.

CHAPTER SEVEN.

Investigation of serum inhibitory factors.

Introduction.

The presence of inhibitory factors in the circulation of patients with AD could account for the low levels of NK cell activity despite the presence of normal numbers of Leu-11b positive staining cells. Nair and Schwartz (1982) have reported the existence of soluble factors, produced by unstimulated lymphocytes, that are able to inhibit natural killing. It has also been reported by Barrett et al. (1982) that preincubation or assaying of effector cells in medium containing maternal or neonatal serum results in inhibition of cytotoxicity. In 1983 Sibbitt et al. showed that serum from patients with SLE could cause up to 30% inhibition of cytotoxicity. It has been observed that the chemotactic responses of neutrophils from patients with AD are reduced and that this reduction is due to the presence of a serum derived inhibitory factor (Rogge and Hanifin. 1976 : Snyderman, et al. 1977). The degree of impairment of chemotaxis was also found to correlate with the severity of the disease and improved with clinical remission (Hanifin, et al. 1980). It is therefore possible that a similar factor may be the cause of the reduced levels of NK cell activity seen in atopic dermatitis.

The following study was designed to examine the effects on NK cell activity of incubating effector cells from patients with AD and age and sex matched controls in medium supplemented with

10% autologous serum, 10% homologous serum or 10% foetal calf serum (FCS).

NK cell activity was assessed in ten patients with atopic dermatitis and ten age and sex matched healthy controls. Three different protocols were used in this experiment, these were:-

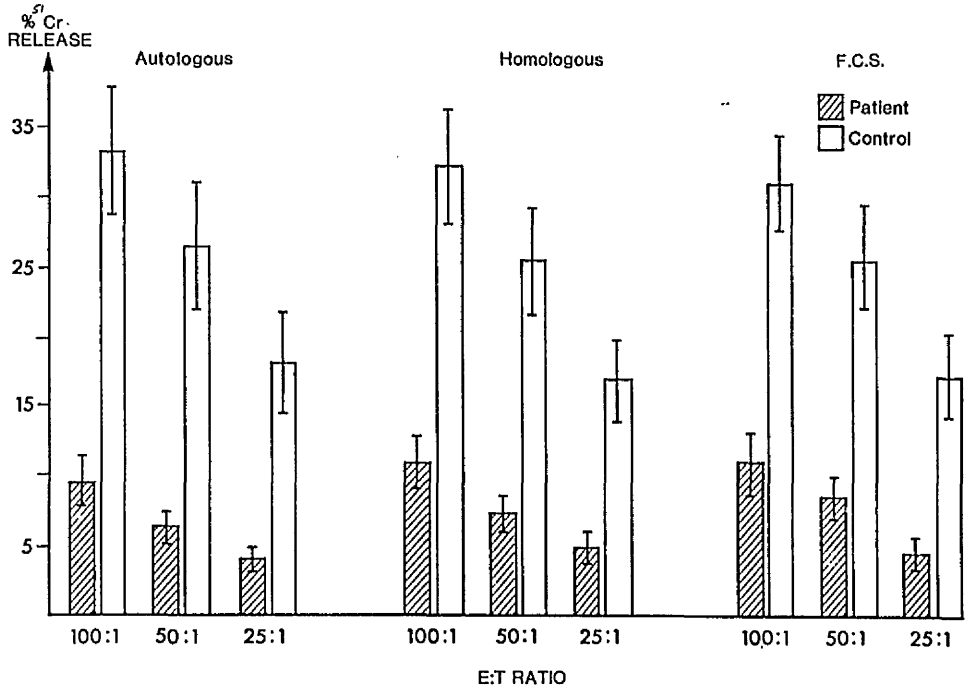
1. Standard four hour cytotoxicity assay in supplemented medium.
2. Eighteen hour cytotoxicity assay in supplemented medium.
3. Overnight incubation of effector cells in supplemented medium followed by a standard four hour cytotoxicity assay, also in supplemented medium.

Results.

Four hour cytotoxic assay in supplemented medium.

From figure 13 it can be seen that the NK cell activity of the controls is significantly higher than that of the patients in all three groups $p < 0.005$. The incubation of patients effector cells in medium containing 10% control serum resulted in a slight and insignificant increase in NK cell activity compared with the amount of activity found following incubation of cells in medium supplemented with 10% autologous serum. The converse was also found to be true, ie. incubation of control effector cells in

Figure 13.



Mean ±SE of chromium release following a standard four hour cytotoxicity assay in supplemented medium for patients with AD and age and sex matched controls.

medium containing 10% patient serum resulted in a slight, and statistically insignificant, decrease in NK cell activity compared with control effector cells incubated in medium containing 10% autologous serum. Incubation of effector cells in medium supplemented with 10% FCS resulted in a slight enhancement of NK cell activity for both the patients and the controls. There was however, no significant difference between the three groups ie. those incubated in medium supplemented with 10% autologous, 10% homologous or 10% FCS. These results are summarised in table 12.

As no significant differences were seen in effector cell activity following a four hour assay it was decided to extend the incubation time.

Effect of an 18 hour cytotoxic assay in supplemented medium.

The results of this experiment were very similar to those found after a four hour incubation although the percentage $^{51}Cr.$ release was much higher, see figure 14, following an 18 hour incubation. The percentage difference, approximately 23% between the patients and controls remained the same (statistical significance, $P < 0.005$). These results are summarised in table 13.

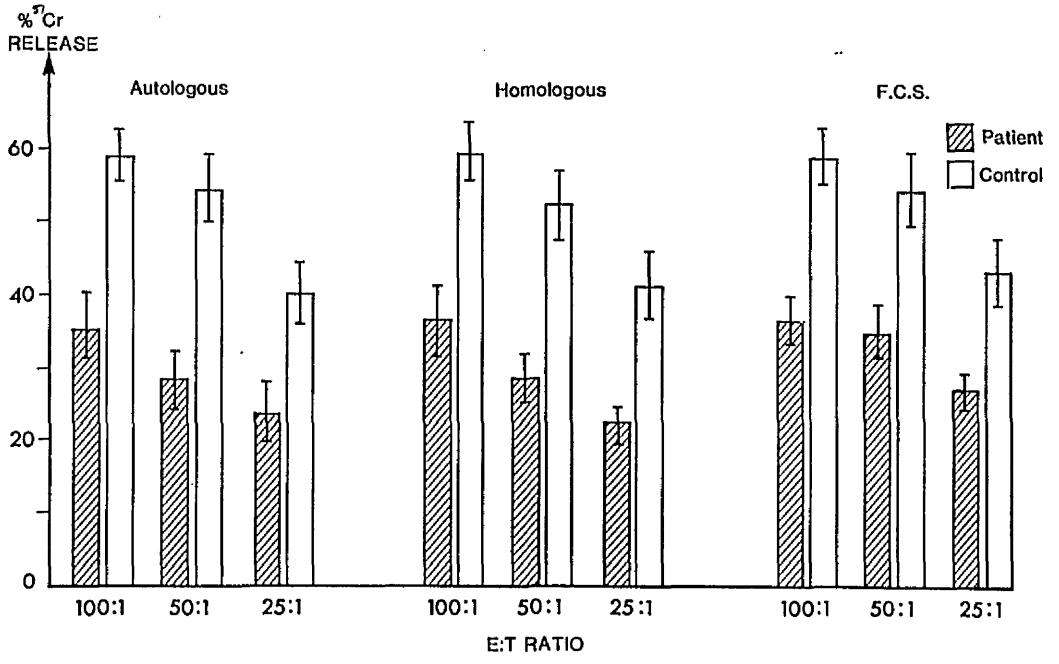
As an 18 hour incubation in the presence of labelled target cells showed an increase in the activity of both patient and

Table 12.

	% of ^{51}Cr Release								
	Autologous			Homologous			FCS		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1	100:1	50:1	25:1
Patient	9.6	6.4	4.1	11.0	7.4	4.9	11.1	8.6	4.5
Control	33.4	26.6	18.3	32.4	25.6	16.9	31.2	25.6	17.3

Mean ^{51}Cr. release for patients with AD and normal controls during a standard four hour cytotoxicity assay in supplemented medium.

Figure 14.



Mean ±SE of chromium release following an 18 hour cytotoxicity assay in supplemented medium for patients with AD and age and sex matched controls.

Table 13

	% ^{51}Cr Release								
	Autologous			Homologous			FCS		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1	100:1	50:1	25:1
Patient	35.6	28.3	23.8	36.5	28.6	22.2	36.4	34.7	26.9
Control	58.7	54.4	40.0	59.5	52.4	41.1	58.9	53.9	42.9

Mean ^{51}Cr. release for patients with AD and normal controls during an 18 hour cytotoxicity assay in supplemented medium.

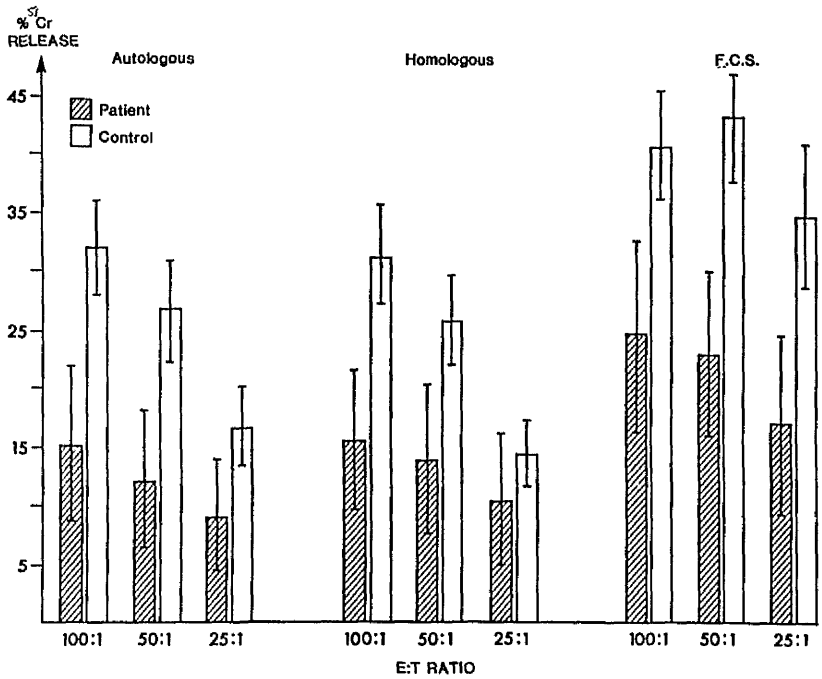
control effector cells. It was then decided to examine the effect of preincubation of the effector cells overnight in supplemented medium prior to a standard four hour cytotoxicity assay.

Effect of prior 18 hour incubation in supplemented medium on the results of a standard four hour cytotoxic assay.

Prior incubation of effector cells in supplemented medium had no significant effect on the NK cell activity of either the patients or the controls, as seen in figure 15. As in the previous experiment the level of NK cell activity of the control subjects remained higher than that of the patients. However, it was observed that effector cells (patients and controls) incubated in medium supplemented with 10% FCS had a very much higher level of NK cell activity than those incubated in medium containing either 10% autologous or homologous serum. Indeed the amount of ^{51}Cr. release for patient's effector cells, preincubated in medium containing 10% FCS, reached levels of activity similar to those from control subjects whose effector cells had been incubated in medium supplemented with either 10% autologous or 10% homologous serum. However, similar increases in NK cell activity were also seen in the controls following 18 hour preincubation in medium supplemented with 10% FCS.

These results are summarised in table 14.

Figure 15.



The effect of overnight incubation of effector cells from patients with AD and age and sex matched controls in supplemented medium on the mean ±SE of chromium release following a standard four hour cytotoxicity assay.

Table 14.

	% ^{51}Cr. Release								
	Autologous			Homologous			FCS		
E:T Ratio	100:1	50:1	25:1	100:1	50:1	25:1	100:1	50:1	25:1
Patients	15.3	12.2	9.1	15.6	14	10.5	24.8	23.0	17.2
Controls	32.1	26.8	16.8	31.2	25.8	14.5	40.7	43.2	34.6

Mean ^{51}Cr. release for patients with AD and normal controls during a standard four hour cytotoxicity assay following 18 hour incubation of patient and control effector cells in supplemented medium.

Conclusions.

The findings of these experiments failed to show the presence of a serum inhibitory factor in AD that could be responsible for the reduction in NK cell activity. They do, however, confirm both the reduction seen in the NK cell activity of these patients and the effect of an 18 hour cytotoxicity assay on the level of activity observed (compare figure 4 with figure 14).

CHAPTER EIGHT

The effect of topically applied steroids on the NK cell activity of normal subjects.

Introduction.

Topical steroids form the main course of treatment in AD, systemic steroids are used but only very rarely. Systemic steroid therapy has been shown to depress peripheral blood NK cell activity (Parrillo and Fauci. 1978). It is possible that systemic absorption of topically applied steroids may be the cause of the reduction seen in NK cell activity. The following experiment was designed to investigate the effect of topical steroid application on the peripheral blood NK cell activity of normal volunteers.

Ten normal healthy volunteers were used in this study, five female and five male, their ages ranged from 19 years to 31 years. All of the volunteers had no past personal or family history of atopic disease. Within the group of female volunteers one had a history of cold urticaria and one had contact sensitivity to jewellery. Within the male group one had acne and one had a past history of urticaria. These details are summarised in tables 15 and 16.

Results.

Peripheral blood NK cell activity was found to have fallen within the group as a whole as early as day 3, see figure 16. This decrease reached a statistically significant level by day 7: $p<0.05$ at the 100:1 ratio using the paired t-test. The level of NK cell activity then rose slowly until base line levels were again achieved by day 22.

Table 15.

Study Group.

10 Normal Volunteers.

	Male	Female
Number	5	5
Age (Mean)	26-31 (27.6)	19-31 (23.0)
Past History Family History Atopy	No	No

Details of Study Group.

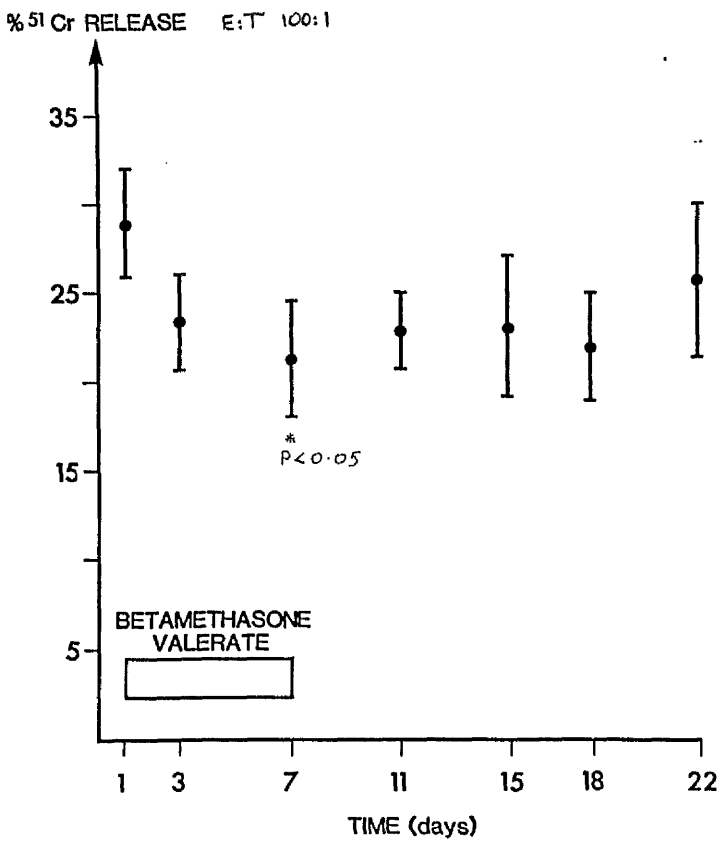
Table 16.

Study Group.

	Past Medical History
Male	1 acne
	1 Urticaria (10 years ago)
Female	1 cold urticuria
	1 contact dermatitis (earrings)

Past medical history of study group.

Figure 16.



The effect of topical steroid application on the NK cell activity of normal volunteers as shown by the mean ±SE of chromium release following a four hour cytotoxicity assay.

If the two groups are analysed separately, ie, males and females (figure 17), it may be seen that the NK cell activity of each group behaves slightly differently. In the female group NK cell activity is seen to drop through the period of application of the steroid ointment. It then rises slightly and drops again at day fifteen.

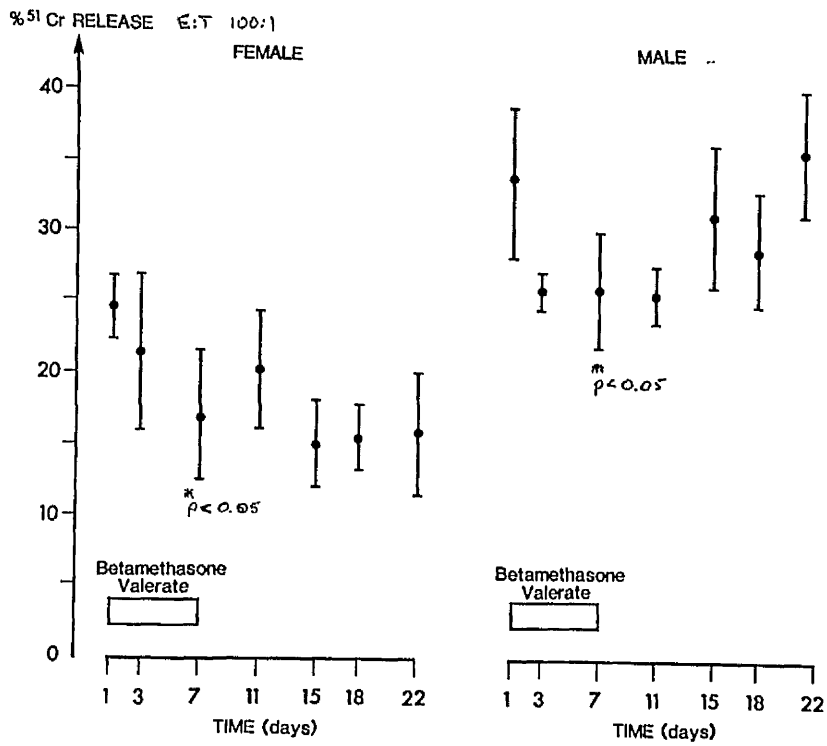
In the male group, however, the NK cell activity reaches its lowest level by day 3 and stays at this lower level during the period of application after which it rises to base line levels. There is also a slight dip around day 18, similar to the female group, but it is not as pronounced.

Effect of base alone.

To confirm that the observed reduction was due to the effect of the topically applied steroid and not to some other factor or component of the ointment, the experiment was repeated using the base for the steroid. Four of the original group of volunteers were recruited to repeat the study. The NK cell activity of these four subjects was then compared following the application of the base alone.

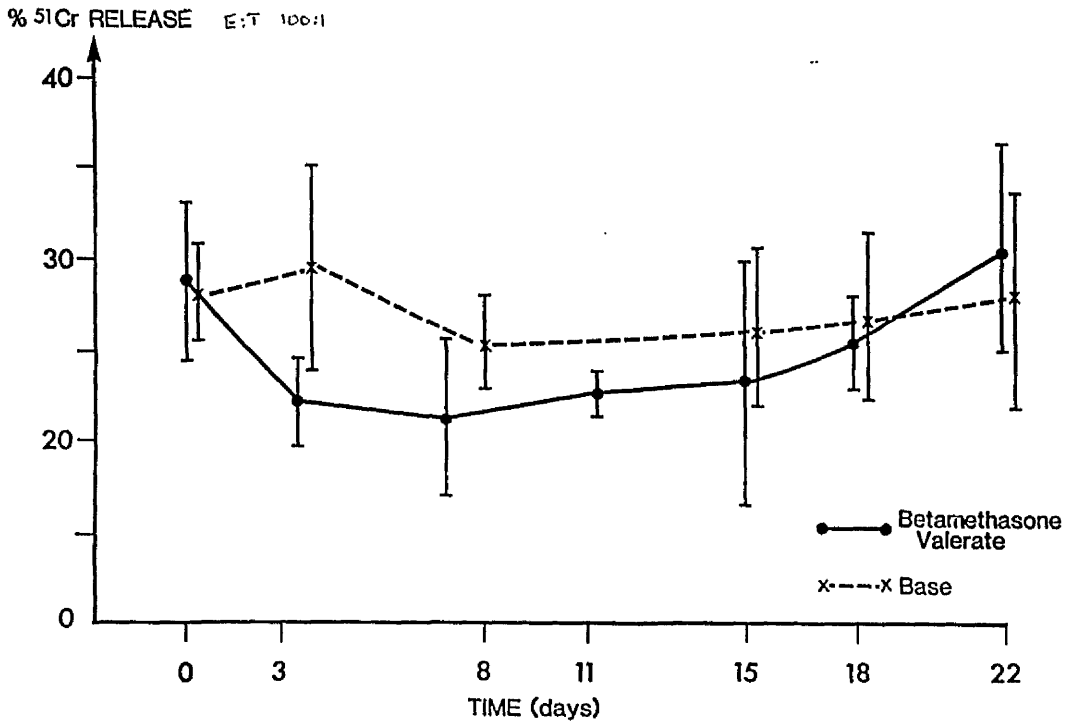
After an initial slight rise the activity was observed to fall (figure 18). The drop in activity was, however, not as large as that found when the steroid was used. It was also found that the difference between these two groups was not statistically significant. The difference between the two groups can be more

Figure 17.



The effect of topical steroid application on the NK cell activity of the male and female volunteers as shown by mean ±SE of chromium release following a four hour cytotoxicity assay.

Figure 18.



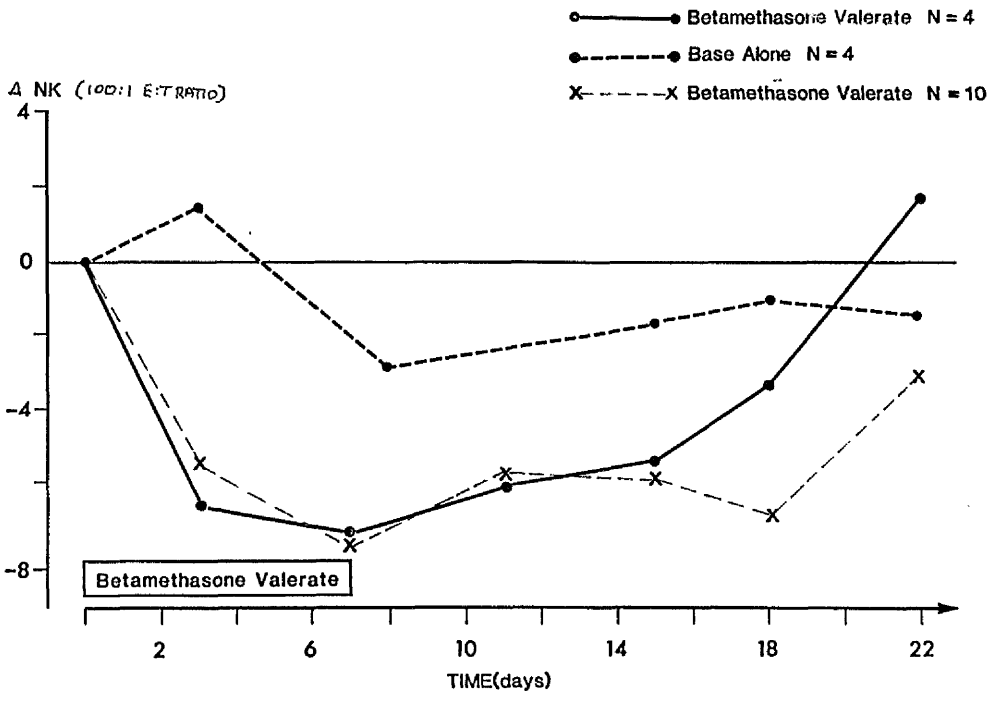
The effect of topical steroid ointment base alone on the NK cell activity of normal volunteers as shown by mean ±SE of chromium release following a four hour cytotoxicity assay.

clearly seen in figure 19. This graph was made by subtracting the baseline NK cell activity, before the subjects applied any base/ointment, from that found on sampling days and plotting this difference against time.

Conclusion.

The results of this experiment would suggest that NK cell activity of normal healthy individuals may be reduced following the application of topical steroids. However, they did not cause the NK cell activity of these individuals to fall to levels seen in patients with AD. Therefore, it would appear that topical steroids are not the only cause of the reduced levels of NK cell activity.

Figure 19.



Change in NK cell activity of normal volunteers following the application of a topical steroid or base alone as shown by mean of chromium release following a four hour cytotoxicity assay.

Chapter Nine

The in vitro. effect of adrenaline, noradrenaline, betamethasone and histamine acid phosphate on the NK cell activity of normal controls.

Introduction.

Stress is known to be a factor in causing patients with AD to flare i.e. for the disease to become active. As the physiological effects of stress are mediated by the products of the adrenal glands it was decided to investigate the effect of adrenaline and noradrenaline on NK cell activity *in vitro*. Histamine was also investigated, because of its primary role in allergic reactions. Betamethasone was included to ascertain whether or not a greater reduction in NK cell activity could be achieved by steroid treatment *in vitro* compared with that induced *in vivo* by the application of topical steroids (chapter eight). The effect of these four agents on NK cell activity was assessed using PBMC from six normal subjects, two female and four male in each group of experiments.

The results are expressed as percentage inhibition. This was achieved as follows. The controls (without the agent) were taken as 100% chromium release, and the percentage isotope release from the groups that had either of the four agents present was subtracted from 100%, the balance was therefore the percentage inhibition.

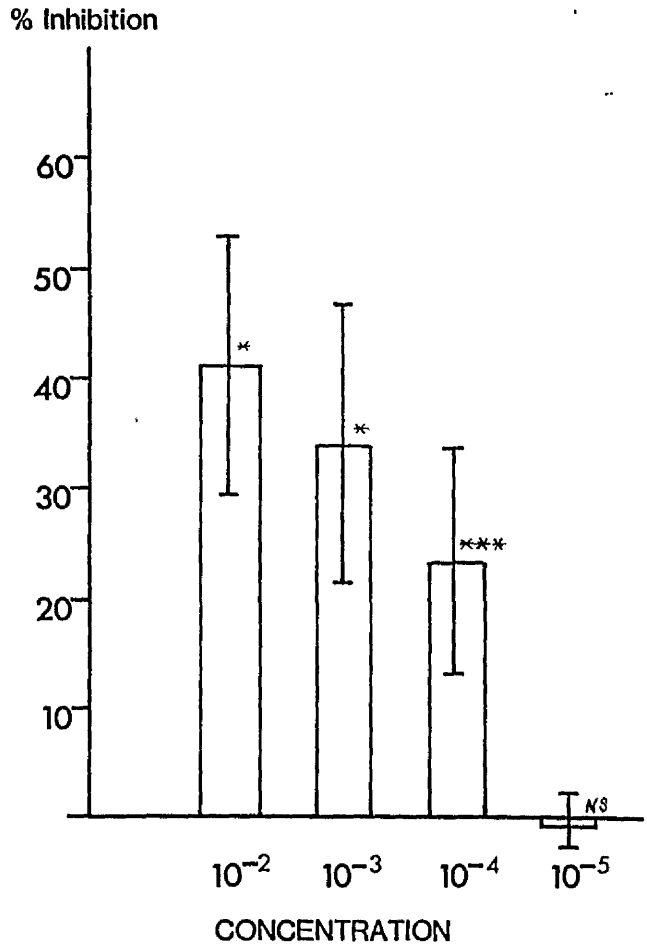
Results.

The effect of preincubation of effector cells for one hour with various concentrations of adrenaline, noradrenaline, betamethasone and Histamine acid phosphate on the NK cell activity of normal subjects.

Adrenaline, noradrenaline and betamethasone were found to inhibit NK cell activity at each of the E:T ratios in a dose dependent manner following a one hour preincubation. Figure 20 a, b, and c. (These figures show only the 100:1 ratio).

Histamine on the other hand, was found to have slight stimulatory effect on the NK cell activity of the normal subjects in a

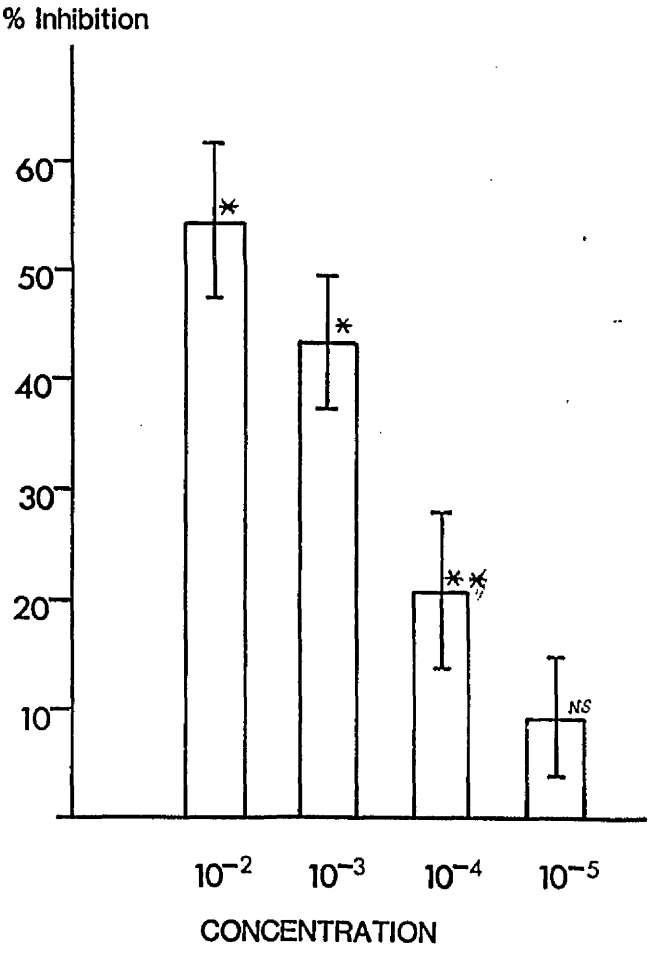
Figure 20 a.



Mean ±SE inhibition of NK cell activity following preincubation for one hour of effector cells with adrenaline at concentrations of 0.05 mM (10^{-2}) to 0.00005 mM (10^{-5}) E:T ratio 100:1

* $p \leq 0.005$, *** $p \leq 0.05$, 10^{-5} N.S.

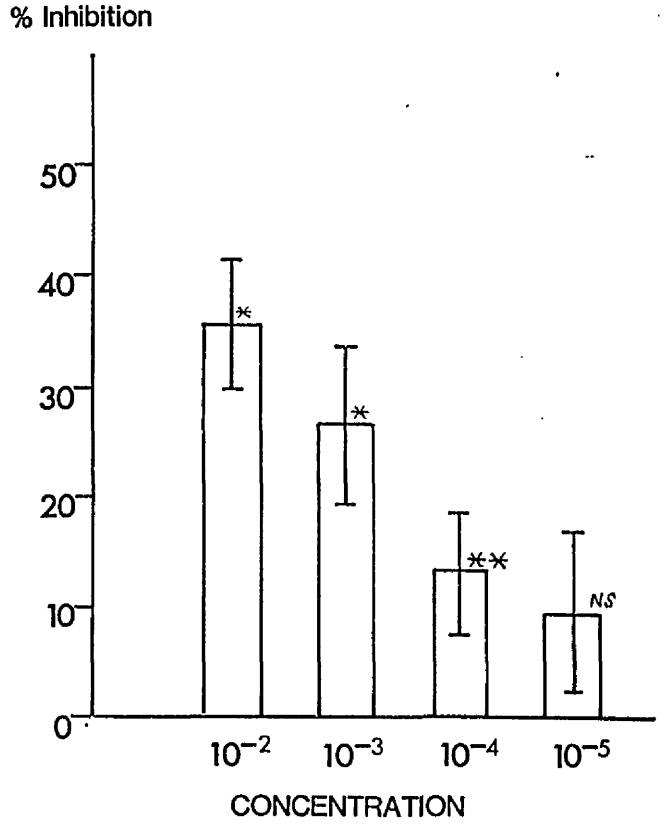
Figure 20 b.



Mean ±SE inhibition of NK cell activity following preincubation for one hour of effector cells with noradrenaline at concentrations of 0.02 mM (10^{-2}) to 0.00002 mM (10^{-5}) E:T ratio 100:1

*$p < 0.005$, ** $p < 0.02$, 10^{-5} NS.

Figure 20 c.



Mean ±SE inhibition of NK cell activity following preincubation for one hour of effector cells with betamethasone at concentrations of 0.07 mM (10^{-2}) to 0.00007mM (10^{-5}) E:T ratio 100:1

* $p<0.005$, ** $p<0.05$, 10^{-5} NS.

negative dose dependent manner i.e. the lower the concentration the higher the NK cell activity. See figure 20 d.

Addition of adrenaline in ten fold dilutions ranging from 0.05mM to 0.0005mM resulted in 41% inhibition (at 0.05 mM) to about 1% enhancement (at 0.0005mM). Ten fold dilutions of noradrenaline ranging from 0.02mM to 0.0002mM resulted in 54 (at 0.02mM) to 10 percent inhibition (at 0.00002mM), addition of betamethasone in concentrations from 0.07mM to 0.00007mM resulted in 35 (at 0.07mM) to 10 percent (at 0.00007mM) inhibition. The percentage increase in NK cell activity following preincubation with histamine ranged from about 1% at the 0.03 mM concentration to about 8% at the 0.00003mM concentration. The levels of inhibition for adranaline, noradrenaline and betamethesone were found to be statistically significant up to the 10^{-4} concentrations ie. $p \leq 0.005$ at the 100:1 ratio for the highest concentration to $p < 0.05$ at the 10^{-4} concentration, although at the lowest concentration the level of inhibition was not significant. The percentage increases in NK cell activity following preincubation with histamine acid phosphate however, were not found to be significant. The results of this experiment are summarised in table 17.

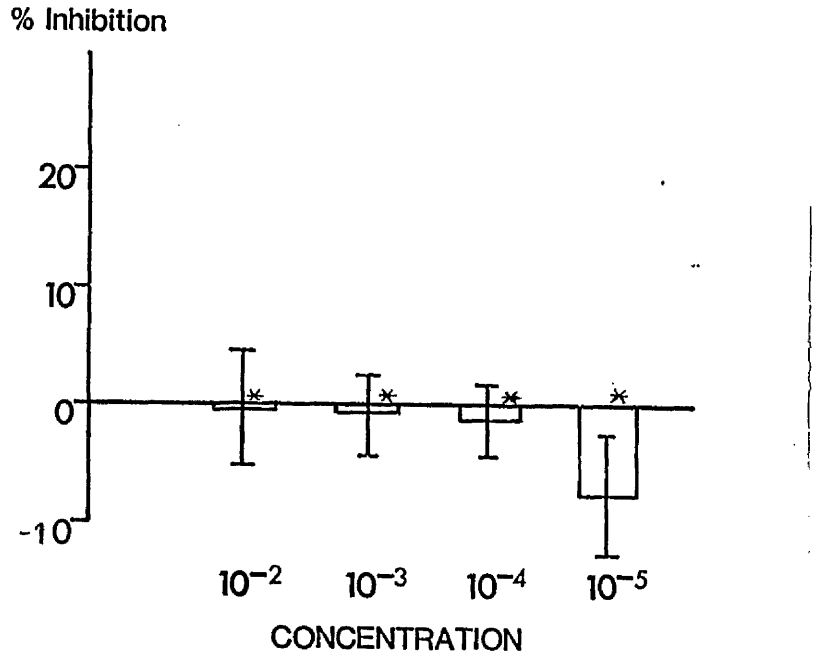
<u>NK cell activity of normal subjects following the addition of adrenaline, noradrenaline, betamethasone and histamine acid phosphate at the start of a standard four hour cytotoxicity assay.</u>

Table 17.

		% Inhibition			
	Concentration	10^{-2}	10^{-3}	10^{-4}	10^{-5}
D	Adrenaline	41.3	34.1	23.6	-0.7
R	Noradrenaline	54.2	43.3	20.8	9.0
U	Betamethasone	35.8	26.7	13.4	9.6
G	Histamine Acid Phosphate	-0.35	-0.93	-1.5	-7.7

Percentage inhibition of NK cell activity at the 100:1 E:T ratio following one hours preincubation with adrenaline, noradrenaline, betamethasone and histamine acid phosphate at various concentrations prior to a standard four hour cytotoxicity assay.

Figure 20 d.



Mean ±SE inhibition of NK cell activity following preincubation for one hour of effector cells with histamine acid phosphate at concentrations of 0.03 mM (10^{-2}) to 0.00003mM (10^{-5})

E:T ratio 100:1

* N.S.

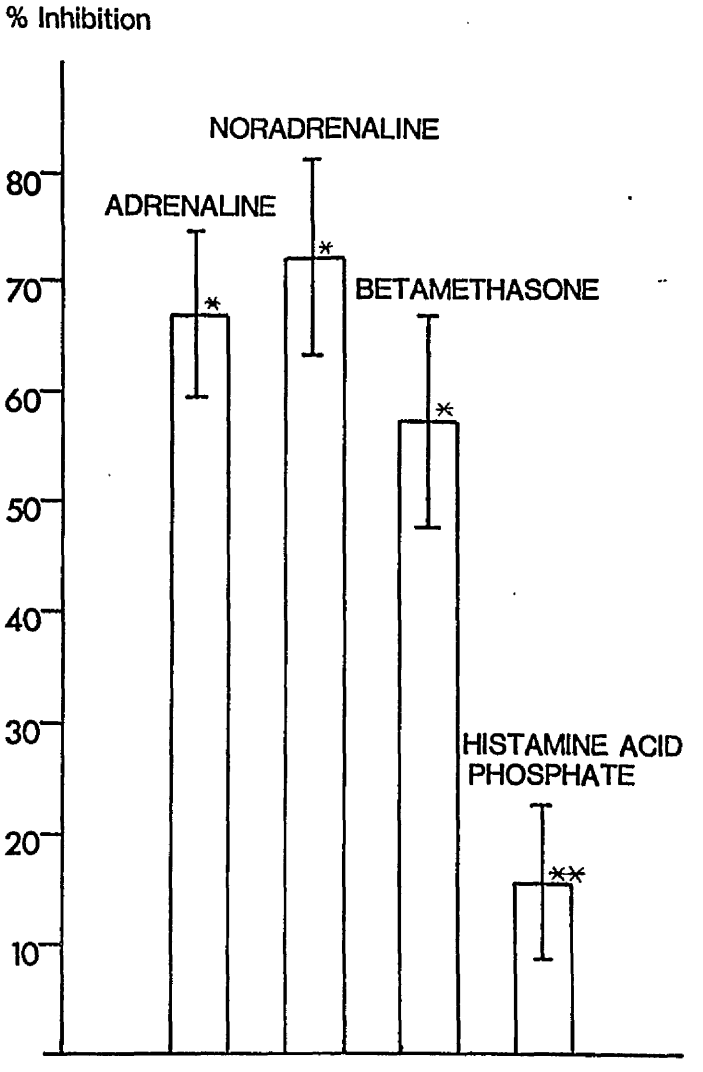
The above experiment was repeated with two alterations to the method. Firstly, the different agents were added at the beginning of the assay with the target cells and secondly they were added at their highest concentrations. When the agents were added with the target cells a much greater inhibition resulted than that which was found following one hours pre-incubation. See figure 21. The percentage inhibition increased by about 25% for adrenaline ($p < 0.005$), 15% for noradrenaline ($p < 0.005$), 15% for betamethasone ($p < 0.005$) and 16% for histamine ($p < 0.05$). These results are summarised in table 18.

<u>The effect of a four hour preincubation of effector cells with adrenaline, noradrenaline, betamethasone and histamine acid phosphate on the NK cell activity of normal subjects.</u>

From the results of the previous experiments it was decided to investigate the effect of a longer preincubation of effector cells with these agents at their highest concentrations prior to setting up the cytotoxic assay.

Longer preincubation with adrenaline, noradrenaline or betamethasone resulted in lower levels of inhibition of NK cell activity than those seen following either one hours preincubation or addition of these agents with the target cells. See figure 22. It was also observed again that histamine caused a slight increase in the level of NK cell activity. The level of enhancement

Figure 21



Mean ±SE inhibition of NK cell activity following the addition of adrenaline 0.05 mM, noradrenaline 0.02 mM, betamethasone 0.07 mM and histamine acid phosphate 0.03 mM at the start of a standard four hour cytotoxicity assay.

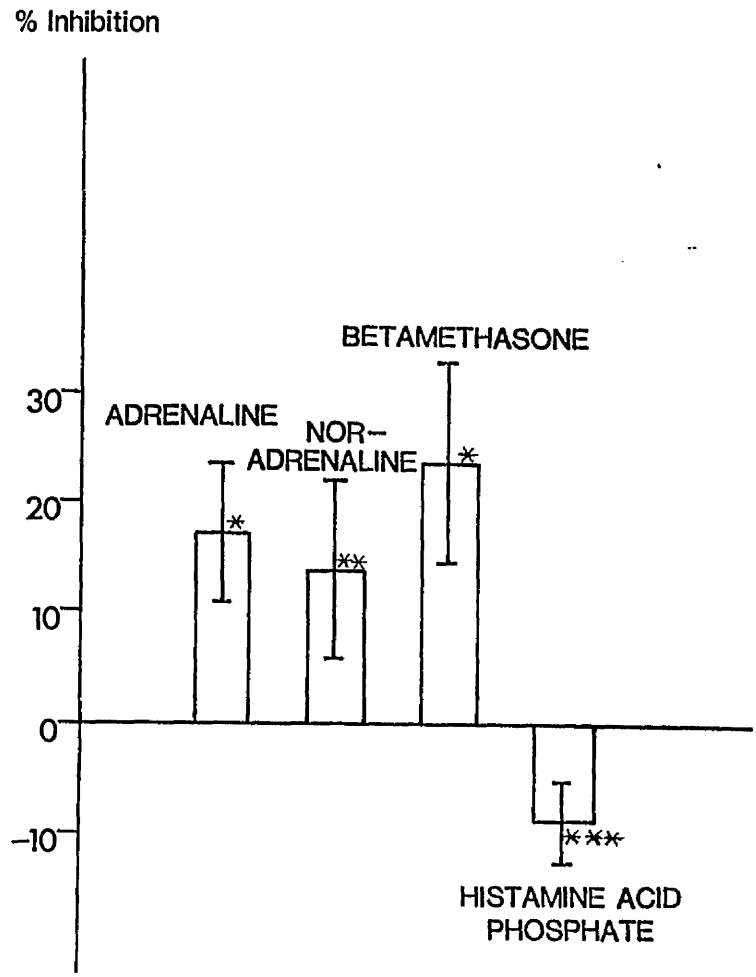
E:T ratio 100:1 * $p < 0.0005$ ** $p < 0.05$

Table 18

Drug	% Inhibition
Adrenaline	67.0
Noradrenaline	72.1
Betamethasone	57.4
Histamine Acid Phosphate	15.4

Percentage inhibition of NK cell activity at the 100:1 E:T ratio following the addition of adrenaline, noradrenaline, betamethasone and histamine acid phosphate at a concentration of 10^{-2} at the start of a standard four hour cytotoxicity assay.

Figure 22.



Mean ±SE inhibition of NK cell activity following a four hour pre-incubation of effector cells with adrenaline at 0.05 mM, noradrenaline 0.02 mM, betamethasone 0.07 mM and histamine acid phosphate 0.03 mM prior to a four hour cytotoxicity assay.

E:T ratio 100:1 * $p<0.03$ ** $p<0.2$ *** $p<0.05$

following a four hour preincubation was as great as that found following one hours preincubation with histamine at the lowest concentration. The inhibition caused by adrenaline, and betamethasone was not as significant as was found previously $p < 0.05$, and that caused by noradrenaline was not significant. The enhancement seen following preincubation with histamine was only slightly significant, $p < 0.05$. These results are summarised in table 19.

Conclusions.

From the results of this experiment it may be seen that adrenaline, noradrenaline, betamethasone and histamine have a dose dependent effect on NK cell activity in vitro. Adrenaline, noradrenaline and betamethasone cause inhibition of the response whether they are added with the target cells or are preincubated with the effector cells for either one or four hours prior to setting up the assay. It was observed that the longer the preincubation was the smaller the resulting inhibition. Histamine, on the other hand, caused a slight enhancement following a one hour preincubation with the effector cells, but in a negative dose dependent manner, i.e. the lower the concentration the greater the enhancement. A four hour preincubation with histamine, at the highest concentration resulted in enhancement as great as that found following the one hour preincubation at the lowest concentration. Histamine was

Table 19

Drug	% Inhibition
Adrenaline	17.1
Noradrenaline	13.9
Betamethasone	23.5
Histamine Acid Phosphate	-9.0

Percentage inhibition of NK cell activity at the 100:1 E:T ratio following a four hour preincubation with adrenaline, noradrenaline, betamethasone and histamine acid phosphate at a concentration of 10^{-2} prior to a standard four hour cytotoxicity assay.

found to cause inhibition of the NK cell response only when added at the same time as the target cells.

It would, therefore, appear that the effect of these agents is only transient and that following addition to the effector cell population they are metabolised, hence the reduced effect following the preincubations. Histamine on the other hand, appears to have its enhancing effect once it has been metabolised.

These results indicate that at the concentrations used these agents have a very significant effect on NK cell activity *in vitro*. However, whether or not they have the same effect *in vivo* cannot be determined from these results. Adrenaline and noradrenaline were both used at pharmacological rather than physiological doses and the dose responsiveness of these two drugs suggest that at physiological concentrations they would have no inhibitory effect on NK cell activity.

The results also show that very low concentrations of betamethesone can inhibit NK cell activity *in vitro* therefore, one may assume that if sufficient amounts of topical steroid could penetrate the skin then it could cause a quite marked reduction in NK cell activity *in vivo*.

CHAPTER TEN

DISCUSSION

Abnormal immunological functions, both humoral and cellular, have been reported in patients with atopic dermatitis. Reports of altered natural killer cell function have been conflicting. Some groups report that NK cell activity, in vitro, against tumour cell targets is reduced (Kusiami and Trentin. 1982) while others have found normal or raised levels of activity (Viander et al. 1982: Strannegard and Strannegard 1980). In addition raised levels of activity against autologous and allogeneic skin fibroblasts have been reported (Leung et al. 1982).

The NK cell activity of patients with AD, as measured in this laboratory, was found to be very significantly reduced compared to those of age and sex matched healthy controls. This finding raises two major points of discussion. Firstly, other workers have found normal/high NK cell activity against tumour cell targets (Viander et al. 1982: Strannegard and Strannegard 1980). This discrepancy could be due to variation in the patients selected or differences in the methods used to assay for NK cell activity, and I propose to discuss these first. Secondly, possible causes for the reduced NK cell activity in AD are investigated in a series of experiments and these are considered in the second part of the discussion.

Viander et al. (1982) found that the NK cell activity of a group of 12 male patients with AD was slightly higher than that

of the control group. The same incubation time (4 hrs), and the same target cell (K562) were used in their study as were used in experiments in this thesis. On close examination of the data on the patients they assessed it can be seen that one had bronchial asthma as well as AD. The NK cell activity of this individual was 833.3 lytic units, this was approximately seven times higher than that of the next highest patient and 104 times that of the lowest. It has recently been reported that the NK cell activity of individuals with asthma is significantly higher than that of normal healthy controls (Timonen and Stenius-Aarniala. 1985). It would appear that the overall level of NK cell activity for the group of patients was pushed up because of this one individual.

Strannegard and Strannegard (1980) also found increased NK cell activity in a group of atopic children compared with normal age matched controls. All of the subjects were atopic having asthma, allergic rhinitis and/or atopic dermatitis but the number of each is not stated. This study was looking at atopy in a general sense and it is possible therefore, that the level of NK cell activity of the subjects with asthma was causing the overall group level to be elevated as in the study by Viander et al. It was also found that within the patients examined in this study those with active asthma as well as AD had a higher NK cell activity than those with a past history of asthma which had been inactive for some time.

Another patient variable is alcohol consumption. Saxena et al. (1980) found increased levels of NK cell activity in a group of 32 alcoholics. One patient in this study was found to have an alcohol problem and his NK cell activity was consistently high/normal in spite of his atopic dermatitis. Patient selection could therefore affect the outcome of NK cell assays, however, differences in the laboratory methods might also affect the result of NK cell assays.

There were several differences in the assay methodology as used in this study and those of Viander et al. and Strannegard and Strannegard, namely :- the length of incubation, removal of macrophages and the target cell used. Each of these parameters was examined in order to exclude the possibility that the observed differences in NK cell activity between patients and controls was due to differences in assay technique.

Increasing the length of incubation of effector cells with target cells to 18 hours, instead of four, resulted in an increase in NK cell activity for the patients. However, the activity of the effector cells from the controls also increased during the longer incubation and the difference between the patients and controls was maintained. With increased incubation time one would expect that, if defective activation of the patient's effector cells was the cause of the reduced NK cell activity, the patients would achieve the same level of activity

as the controls. The fact that the activity of the patients did not attain the same level as the controls, or indeed, reduce the difference, suggested that the reduced NK cell activity was not due to differences in activation of effector cells between patients and controls. The observation that the chromium release increases during this longer incubation suggests that the patient's effector cells are able to kill their targets but that the low levels seen in atopic dermatitis are due to a cause other than a defect or deficiency in effector cell activation, eg. reduced numbers or reduced functional ability of effector cells. The results of studies of effector cell numbers with function would suggest that there is in fact a qualitative abnormality in patient's effector cells. Strannegard and Strannegard (1980) found that NK cell activity was increased in atopic patients following an 18 hour incubation, however, they used a different target cell, P3HR1, and the difference only reached significance in a subgroup of older children. Also it should be pointed out that 18 hour assays are unreliable due to the variability in spontaneous release over long incubation times.

Macrophages and monocytes in the effector population have been shown to inhibit the NK cell response (Jondal, et al. 1982). Melewicz et al. (1981 a and b). have reported both increased numbers of Fc-IgE receptor bearing monocytes and enhanced monocyte-IgE dependent killing in atopic disorders. Jensen et al. (1984) have also shown that the addition of autologous monocytes to the cytotoxic assay reduces NK cell activity. It is therefore conceivable that there is an <u>in vivo</u> activation of monocytes during acute episodes of AD and that effector cell-monocyte

interaction may cause the reduction in NK cell activity in patients with AD. As Viander et al. (1982) found almost normal NK cell activity in their study it was of interest to examine the effect that removal of macrophages and monocytes, by the two methods available, glass/plastic adherence in the case of Viander et al. and carbonyl iron ingestion in the case of Lever et al. (1984), might have on the outcome of an NK assay.

Removal of macrophages and monocytes increased NK cell activity although the NK cell activity of the patients remained lower than the controls. The levels of ^{51}Cr. release for the patient's effector cells almost doubled while that for the controls went up by about 30% following carbonyl iron treatment. Depletion by adherence to glass or plastic also resulted in an increase in NK cell activity for both groups; this increase, however, was not as great as that observed following carbonyl iron treatment. The control NK cell activity remained significantly greater than that of the patients, it is therefore unlikely that reduced NK cell activity in AD is due to effector cell/monocyte interaction following activation of the monocyte population as suggested by Jensen et al. (1984) although it may be a contributory factor.

The third difference in methodology to be investigated was the use of alternative target cells to K562 in the standard four hour assay. Phillips et al. (1980) published results which

suggested that NK cells may be a heterologous population with subpopulations of cells which recognise different antigenic specificities on target cells. It is therefore possible that in AD there is a deficiency in one or more subpopulation(s) of NK effector cells which could account for the different levels of activity that have been reported in studies where different target cells have been used. Strannegard and Strannegard (1980) found raised NK cell activity in a group of atopic children using a Burkitt's lymphoma, P3HR1, as the target.

Four target cells were tested on both patients and controls, the overall NK cell activity was reduced against all four of the targets compared to that found when K562 was the target. The range of activities observed was, CCRF-CEM> Molt-4> Namalva> BJAB. Only CCRF-CEM gave a significant difference between the patients with AD and the controls by Student's t-test. Although the other three cell lines showed no significant difference in activity between the patients and the controls the activity of the control effector cells was still greater than that of the patients. It is possible that had larger groups of patients and controls been used greater differences would have been found. The cell lines which proved to be the least sensitive as targets were the two B-cell lines, both of which have immunoglobulin, IgM, on their surface. It is possible that this surface immunoglobulin prevents the NK cell from binding and so protects the cell from lysis. It is also interesting to note the low level of activity

against Namalva, a Burkitt's lymphoma derived cell. This compares with the level of activity found by Strannegard and Strannegard (1980), they used another Burkitt's lymphoma, P3HR1, as the target. They found that NK cell activity was higher in the atopic group than in the control group, 10% as against 7%, however this difference only reached significance in a subgroup of older children.

The results obtained with the four alternative target cells, CCRF-CEM, Molt-4, Namalva and BJAB, may be explained in the light of findings by Hudig et al. (1981) who found that it was possible to increase the sensitivity of a "slow" NK target cell, ie, one that has a long lag phase before lysis occurs by preincubating it with an inhibitor of protein synthesis. Some target cells may actively resist NK lysis through the use of self repair mechanisms which would keep the membrane intact. Support for this theory has come from the results of a study by Kunkel and Welsh (1981) who showed that the treatment of NK resistant target cells with metabolic inhibitors renders them sensitive to lysis. If tumour cells do have a self repair mechanism then it is possible that what is thought to be differences in specificity on the part of the effector cell may in fact be differences in the self repair ability on the part of the target cell. Roder et al. (1979) showed that although NK cells carried unique specificities there were widespread crossreactions between target structure complexes suggesting that NK cells are to some extent polyspecific while having heterogeneity in the recognition structure. These results supported the earlier

work of Ortaldo et al. (1977) who showed that thirteen out of nineteen cell types, including fresh tumour cells (unrelated to the target cell), cryopreserved cells, normal lymphocytes and human fibroblasts could inhibit NK cell activity when added to a cytotoxic assay. These two observations suggest that the differences observed in sensitivity to lysis by target cells may indeed be due to the active resistance of the target cell rather than the hetrogeneity of the effector cell. A further method of self protection of the target cell might be its ability to release prostaglandins. Droller et al. (1978) showed that if prostaglandin production by the target cell was inhibited then there was an increase in the effector cells activity.

Active resistance, along these lines, would explain the results obtained with CCRF-CEM, Molt-4, Namalva and BJAB. The NK cell activity of patients with AD is significantly reduced against K562, if this reduction is due to a defect or inhibition of effector cell function then one would expect that no matter what target cell type was used the activity of the patients effector cells would be lower than that of the controls. This is in fact the case although in some instances that difference was only very slight. It is clearly important to use the most sensitive target cell possible otherwise differences in effector cell activity in different populations may be overlooked. The results also indicate that Namalva and BJAB are unsuitable as NK targets.

The main points of this first part of the discussion are:-

1. Patient selection may influence the outcome of NK cell assays because of the variability of NK cell activity in other atopic disorders, such as asthma and the influence of other factors such as alcohol.

2. Differences in NK cell activity between patients with AD and controls are not due to differences in assay methodology.

The above results suggest that the difference in NK cell activity between patients with AD and normal controls is a genuine one and not due to differences in assay methodology. It was therefore necessary to evaluate other possible causes for this reduction. The first possibility to be investigated was that the disease itself was the cause of the reduced NK cell activity in patients with AD.

A sequential study of the NK cell activity of a group of patients with AD revealed a correlation between disease severity and NK cell activity. That is, the more active the disease the lower the NK cell activity. This is in agreement with the observations of Hanifin et al. (1980) who reported that the degree of impairment of other cellular functions, the chemotactic response of neutrophils, of patients with AD correlated with the

clinical severity of their disorder. A seasonal variation in the NK cell activity of these patients was also observed with the lowest values being recorded in the period January to March. Over the next three months NK cell activity was found to rise towards normal levels and during this time the patients also had a relative remission of their dermatitis. There was a rise in disease activity in the autumn and the NK cell activity was observed to fall. One patient went into virtual clinical remission over the course of the study and also showed a gradual improvement in her NK cell activity. This improvement took from June to December. This may reflect the time taken for the pool of existing inhibited or defective effector cells to be replaced with normal effector cells.

The observation that NK cell activity varied with clinical severity suggested that the disease itself caused the reduction in NK cell activity. This could be a quantitative or a qualitiative defect, ie, it is possible that there are reduced numbers of effector cells present in the circulation of patients with AD, alternatively, normal numbers of cells could have reduced function. This was investigated in the next group of experiments.

Two different monoclonal antibodies were used to estimate the numbers of effector cells, HNK-1 (Leu-7) and Leu-11b. When numbers were correlated with function, as measured by chromium release, conflicting results were obtained.

The results obtained with HNK-1 (Leu-7) showed a correlation between activity and the numbers of HNK-1^+ cells for both patients

and controls. This suggested that the reduction in the activity was due to fewer effector cells in the circulation of the patients. On the otherhand, Leu-11b showed no correlation between numbers of effector cells and function which suggested that the defect was due to a difference in the functional ability of the effector cells. Regression analysis of the curve of percentage positive cells Vs. chromium release gave similar results for both monoclonals, ie. the patients curve had a large gradient and a small intercept while the curve for the controls had a small gradient and a large intercept. This suggested that there were either two sets of different kinetics, for patients and controls, or that they were on the same curve with the patients falling on the lower half of the curve and the controls higher up. On further examination of the curve produced with the results of using HNK-1 it may be seen that there is a clustering of patients and controls with the patients falling in the lower quarter of the graph and the controls in the higher quarter. Whereas with Leu-11b there is no clustering of patients or controls. Therefore the results using HNK-1 suggest a quantitative defect while those obtained with Leu-11 suggest a qualitative abnormality. The question then arises; which is correct?

It has been shown that HNK-1 does not stain all NK cells (Lanier et al. 1983) and is therefore less specific that leu-11b. One may therefore assume that the results obtained using Leu-11 are most likely to be more accurate than those obtained using HNK-1. This coupled with the results of the regression analysis

would suggest that there is no difference in the numbers of effector cells present in both patients with AD and controls but that there is a difference in the number of functional effector cells. Jensen (1985) showed that the target cell binding and lysis kinetics of effector cells from patients with AD were normal, using a single cell assay, however the number of active effector cells was found to be reduced, ie. NK cells bound normally to their targets and those that went on to lyse the target did so normally, however there was a reduction in the number that did go on to the lytic step. Jensens results therefore support the hypothesis that the defect in the effector cells is qualitative.

An alternative explanation of these results exists. That is, as a difference was found in the numbers of HNK-1^+ cells and not Leu-$11b^+$ between patients and controls it is possible that the effector cells are required to carry both markers in order to be fully functional, ie. to be HNK-1^+ and Leu-$11b^+$. This would suggest that the effector cells may be HNK-1^- Leu-$11b^+$ in patients with AD and HNK-1^+ Leu-$11b^+$ in the controls. This explanation conflicts with the findings of Lanier et al. (1983) who found that effector cells with the Leu-11^+ 7- (HNK-1^-) phenotype were more active than those with the Leu-11^+ 7^+ phenotype. However, Lanier et al. (1983) used only three individuals, and without the benefit of double labelling

although a larger population was studied, it is not possible to make a definite statment. From the available data, assuming Leu- 11b to be more specific than HNK-1, it is possible that NK effector cells from patients with AD express the Leu-11 marker without being able to perform the function due to either inhibition of the cellular response or immaturity of the effector cells. It would therefore appear that there are normal numbers of effector cells present, as defined by the Leu-11b monoclonal marker, but there is a reduction in the number of functional cells. Another possibilty for the observation of reduced NK activity in AD is that the effector cells have migrated to the skin Leung et al. (1982) have shown increased NK cell activity to autologous skin fibroblasts. It has been shown, however that the infiltrating cells are predominatly T helper cells and that NK cells are seen only occasionally (Leung et al. 1981: R.S. Lever, personal communication).

A possible cause of this NK effector cell dysfunction could be the presence of an inhibitory factor. Rogge and Hanifin (1976) observed that in severe cases of AD there was impaired lymphocyte transformation and neutrophil chemotactic responses. Hanifin et al. (1980) showed that this depression correlated with the disease state and suggested that it was due to a factor in the patient's plasma. Suppression of NK cell activity of normal controls has been observed following incubation of effector cells in media supplemented with sera from patients with systemic lupus erythematosus (Sibbitt, et al. 1983), or maternal, ie. newly delivered mothers, or neonatal sera (Barrett, et al. 1982). These observations suggest that there may be an inhibitory substance present in the circulation of patients with AD that is responsible for the reduced NK cell activity. If an inhibitory factor is present in the serum of patients with AD it might be expected to

reduce the NK cell activity of effector cells from control subjects conversely it may be possible to increase the activity of "atopic" cells by incubation in medium supplemented with normal serum. However, from the results it would appear that there is no inhibitory factor specific for NK cells as there appears to be for chemotaxis, although a very slight increase in the NK cell activity of patient's effector cells incubated in medium supplemented with control serum was observed. Likewise, a similar very slight decrease in control NK cell activity was observed when the assay was carried out in medium supplemented with patient's serum. However, neither of these proved to be significant.

Longer incubation, 18 hours instead of 4, failed to reveal the presence of an inhibitory substance in the serum of patients. Also there was no enhancement of the slight increase seen in the NK cell activity of patients effector cells following a four hour assay in medium containing 10% control serum. Likewise there was no further decrease in control effector cell activity when assayed for 18 hours in medium containing 10% patient's serum. The longer incubation did, however, reinforce the earlier findings on the effect of 4 and 18 hour incubations on NK cell activity, ie. NK cell activity increases with longer incubations, both the patients and the controls, but the difference between them remains the same. The most interesting results were found with a standard 4 hour assay following an 18 hour preincubation of effector cells in the various supplemented media. The major difference observed was the marked increase in the NK cell activity of the cells that had been incubated in medium containing

10% FCS seen in both patients and controls. This increase was most likely due to the recognised stimulatory effects of the FCS (Zielske and Golub 1976). The activity of patient's effector cells that had been incubated in 10% FCS rose to a level similar to that of control cells that had been incubated in 10% autologous serum. This finding suggests that the effector cells from patients with AD have the potential to reach the same level of activity as unstimulated control cells. Hanifin et al. (1980) observed that the decrease in neutrophil chemotaxis correlated with the severity of the disease, this would suggest that an inhibitory factor for chemotaxis is either absent during remission, or present only in very small amounts, and increases in concentration during episodes of flare. Although all the patients investigated had moderate to severe episodes of AD when studied, no inhibitory factor was observed with the three protocols employed, ie. the four hour assay, 18 hour assay or the 18 hour preincubation followed by a four hour assay. This suggests that the low levels of NK cell activity in AD are not due to the presence of a specific inhibitor of NK cell function such as that postulated by Hanifin et al. (1980) for chemotaxis.

If the defect in NK cell function of patients with AD is not due to an inhibitory factor and not due to reduced numbers of effector cells it is possible that the treatment of the disease is the cause. The mainstay of treatment of AD is with topical steroids. It has been shown that systemic steroids can cause

depression of NK cell activity (Parillo and Fauci, 1978). The possibilty exists therefore that systemic absorption of topically applied steroids may cause the reduced levels of NK cell activity. This hypothesis would explain the relationship of NK cell activity to clinical activity, as the disorder worsens so the patient will use more steroids. However, it does not explain the persistently low levels of NK cell activity that are found in a few patients with inactive disease and little or no steroid use.

The effect of the application of topical steroid was investigated in a group of ten normal volunteers. NK cell activity was observed to falland reached statistical significance on day seven. A smaller reduction in NK cell activity was observed when the base alone was used, this reduction was not statistically different from the original base line values. In neither case, ie ointment or base, was the fall in NK cell activity as great as that seen in AD.

There are three possible reasons as to why the results are inconclusive. Firstly, although the steroid preparation used was strong, the period of application, seven nights, may not have been long enough. The NK cell activity of the group fell during the period of steroid application and then started to recover. Statistical significance was achieved on day seven, the last day of application of the steroid. It is possible that if the period of application was longer an even greater reduction in NK cell

activity would have been seen. However, seven days was thought to be long enough, due to the side effects of steroid treatment. Secondly, it is likely that patients with active dermatitis absorb greater amounts of steroids than volunteers with an intact skin. Lastly, if the group that applied the base was larger, it is possible that the difference between that group and the group that used the steroid would have been larger and therefore significant.

The observation that NK cell activity correlated with the severity of the disorder and the observation of Hanifin et al. (1980) that the chemotactic response also correlated with the disease state may be explained by the use and effect of topical steroids. Patients with active disease use greater amounts of stronger steroids than patients in remission which would cause greater inhibition of cellular function, with clinical improvement both the quantity and strength of steroid used decreases which would reduce the amount of suppression.

A further possibility for the reduced NK cell activity is the presence of pharmacological mediators. Stress is well recognised to be a trigger in AD, and some of the pharmacological mediators of stress were found to cause inhibition of the NK cell response of normal effector cells at pharmacological doses *in vitro*. Adrenaline and noradrenaline caused a dose dependent inhibition of NK cell activity which was statistically significant at a concentration of 0.0005 mM for -adrenaline and 0.0002mM for

noradrenaline. These mediators act via the adenyl cyclase receptor which catalyses the conversion of ATP to cAMP (Nimmo, 1984). It is well documented that cAMP and its inducers such as prostaglandins, histamine, theophylline etc. can cause inhibition of NK cell activity (Roder and Klein 1979: Katz et al. 1982: Goto et al. 1983: Hall et al. 1983). It would therefore appear that adrenaline and noradrenaline could mediate their effect via cAMP. The greatest levels of inhibition were found when these two agents were added at the start of the four hour assay. Conversely, the lowest levels of inhibition were found following a four hour preincubation of effector cells with these agents. This suggests that the effect is short lived and that adrenaline, noradrenaline and the cAMP they induce are metabolised and that once removed the effector cells then start to return to normal. Katz et al. (1982) also noted that, following the addition of cAMP inducers, NK cell function was reduced but that this reduction only lasted two hours before returning to normal baseline levels. Although these results have shown that adrenaline and noradrelanine have a profound effect on NK cell activity in vitro at pharmacological doses, it does not necessarily follow that similar effects would occur under physiological conditions in vivo.

As topical betamethesone appeared to reduce NK cell activity in vivo. its effect in vitro was therefore examined. A very significant reduction in NK cell activity was observed when betamethesone was added to the assay. The reduction was shown

to be dose dependent and was statistically significant at a concentration of 0.0007mM. Betamethesone is thought to have its effect through binding to membrane phospholipids (Nimmo 1984). It is therefore possible that a defect in target cell binding occurs, although the killing step is probably still intact as it has been shown that steroids do not inhibit ADCC (Parillo and Fauci 1978). Again, as with adrenaline and noradrenaline, the degree of inhibition is time dependent ie. the longer the betamethesone is in contact with the effector cells the less the resulting inhibition. Therefore betamethesone must be either metabolised or lost through membrane turnover. This *in vitro* finding also strengthens the hypothesis that topically applied steroids may cause a reduction in NK cell activity.

The effects of incubating effector cells with histamine was also studied as histamine release is well recognised to be of importance in related allergic conditions such as urticaria. It was observed, following one hours preincubation, that there was an increase in NK cell activity that was negatively dose dependent ie. the lower the dose the greater the NK cell activity. This conflicts with histamines reported ability to suppress NK cell activity. It has been reported that histamine causes inhibition of NK cell activity via the induction of cAMP (Katz et al. 1982: Hall et al. 1983) This apparent contradiction may be explained by the findings of Safko et al. (1981) who showed that while the addition of histamine at concentrations of 10^{-3}M

causes a 200% rise in the level of cAMP that PBMC can produce, pretreatment of the cells with very low doses of histamine, 10^{-6}M., caused a reduction in the amount of cAMP produced. The concentrations of histamine used in this study were of the order of 0.3×10^{-4}M. and lower, it is therefore likely that these lower concentrations of histamine were causing a reduction in the amount of cAMP being produced and therefore increasing the NK cell activity. However, the addition of the highest concentration of histamine, 0.03 mM, at the start of the assay caused inhibition of the NK cell response. This suggests that the concentration used was enough to cause inhibition. In contrast, a four hour preincubation of effector cells with this concentration of histamine caused an increase in NK cell activity which was of a similar magnitude to that seen following one hours preincubation with histamine at the lowest concentration, 0.00003mM. This again suggests that the effect of histamine is short lived due to its being metabolised or to the loss of bound histamine through membrane turnover. As histamine concentration falls so the cAMP production falls with a resultant increase in NK cell activity. The normal level of histamine in the blood is very low, approximately 1ng/ml., which is approximately 10^{3} times lower than the lowest concentration used in this experiment. It is tempting to suggest that at even lower concentrations histamine would have an even greater effect on NK cell function. However, in the light of the very different effects histamine has at higher concentrations ie 10^{-3}M. compared to those at lower concentrations eg. $10^{-3}$to 10^{-6}M.,

it is impossible to predict its effect *in vivo*. The action of histamine is generally a local one and is short lived it is therefore unlikely that at physiological doses histamine would have any measurable effect om circulating NK cells.

CHAPTER ELEVEN

CONCLUSIONS

The results of this study have shown that the natural killer cell activity of PBMC from patients with atopic dermatitis is greatly reduced.

A sequential study of NK cell activity in AD revealed a correlation between disease severity and NK cell activity, suggesting that the reduction might be an integral part of the disease process or an epiphenomenon related to the disease. i.e. Does the disease itself cause the reduction in NK cell activity or is there some other cause such as the treatment?.

If the disease is causing the reduction in NK cell activity then it may be through a reduction in effector cell numbers or by inhibition of effector cell function.

Fluorescent marker studies with two monoclonal antibodies (HNK-1 and Leu-11b) against NK cell surface markers gave different results, for reasons given elsewhere it was assumed that the results obtained with Leu-11b were the more correct. These suggested that the kinetics of the effector cell response were different for patients and controls, indeed it has been shown, using a single cell assay, that there are normal numbers of effector cells present in the circulation of patients with AD but that only a percentage of them are fully functional (Jensen, 1985).

The possibilty that the reduction in NK cell activity was due to the presence of an inhibitory factor was therefore investigated. No inhibitory factor, specific for NK cell function, was detected. This inferes that the cause of the reduction is due to something other than the disease.

The last point to be investigated therefore was whether the treatment of the disorder was responsible for the reduction in NK cell activity.

Topical steroids, which form the mainstay of treatment in AD, were found to cause a reduction in the NK cell activity of normal subjects. The observed decrease in NK cell activity was less than that seen in patients with AD. Because of the effect topical betamethasone had on NK cell activity *in vivo* the *in vitro* effect was also examined. A much greater reduction in NK cell activity was observed *in vitro* than that found *in vivo*, these results support the hypothesis that steroids applied topically may cause a reduction in NK cell activity.

Pharmacological doses of adrenaline and noradrenaline were also found to cause a significant reduction in NK cell activity *in vitro*. However, the results suggested that, at physiological concentrations, there would be no detectable effect on NK cell activity.

Preincubation of effector cells with histamine at various concentrations was found to cause a slight enhancement of NK cell activity. In contrast, if histamine was added at the start of an assay an inhibition of the NK cell response was observed. Histamine is only present in very small concentrations, less than 1ng/ml. in the blood of normal subjects, although elevated levels have been found in individuals with AD, up to 3ng/ml. (Ring, et al. 1979). higher concentrations of histamine have been reported in the skin of patients with AD, 16ug/g of skin compared with 9.2ug/g of skin for controls. (Johnson, et al. 1960). The levels of histamine *in vivo* are lower than those studied *in vitro*. In addition as the main action of histamine is local and probably short lived, it is unlikely that histamine is envolved in the reduced NK cell activity in AD.

The results of this study show that NK cell activity is indeed reduced in AD. They do not show if there is a pathological mechanism

in AD which causes the primary reduction in NK cell activity but rather they suggest that the reduction is an epiphenomenon which may be related to the treatment of the disease in the following hypothetical way.

When a patient with AD relapses, via an antigenic trigger or through stress or a combination of both there is an increase in inflammation, monocytes may aggregate at the site of inflammation and release products such as prostaglandins which could inhibit NK cell activity. The patient will then increase the amount and strength of topical steroids to control the flare, these may be absorbed in high enough concentrations to cause further inhibition of NK cell activity. With improvement less steroids are used topically which may result in less inhibition of NK cell activity. The consistently low levels of activity that are seen may be due to the chronic relapsing nature of the disorder. ie. The effector cells do not have enough time to fully recover their function between episodes of flare. Mice treated with steroids have been found to take from six to eight weeks to regain their baseline levels of NK cell activity. It has also been found in humans that it takes up to 96 hours for NK cell activity to return to normal following a single dose of 8mg of hydrocortisone taken orally (Seaman, et al. 1978: Parillo and Fauci, 1978). The results of patient MW would appear to support this contention.

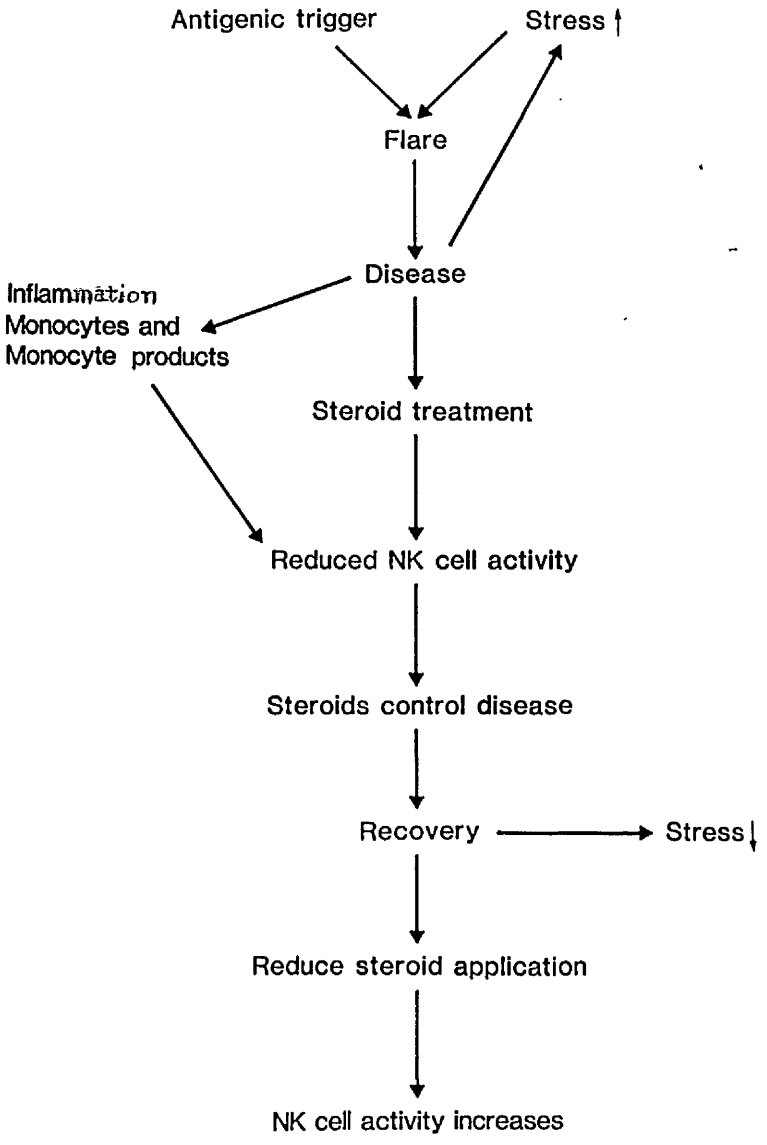
The disease, treatment, recovery cycle may also explain the observation of normal numbers of effector cells but with reduced function. As AD is a chronic disorder so the treatment of the disorder is also long term. It may be postulated that the continued use of quite mild steroids over a long period of time

may cause chronic inhibition of NK cell function (it has been shown in this study that even very low concentrations of steroids may inhibit NK cell function in vitro). The dose of steroid may not be enough to cause lymphopenia but may be enough to maintain the inhibition of the NK response. It is possible that once the level of NK cell activity is reduced it may only require very small doses to maintain the reduced level of activity. Figure 23 illustrates the disease, treatment recovery cycle.

The role of steroids in AD therefore needs more closer examination. This would require access to patients before they ever use steroids, finding such a group of patients would be very difficult as patients usually have been prescribed steroids by their general practitioner before they see a dermatologist. One solution would be to test the NK cell activity of mononuclear cells from cord blood from infants whose mothers or fathers have AD and compare this with cord blood from infants born to non atopic parents. Then follow the progress of these children for one year (AD usually develops in the first year of life). If the child develops AD it would then be possible to retest the NK cell activity before starting treatment. This would answer several questions, 1) is NK cell activity already reduced in these individuals ?, 2)does development of the disease lead to reduced levels of NK?, 3) or is the treatment the cause of the reduction in NK cell activity.

In retrospect it may have been useful to look further at the role of macrophages in AD. Removal of phagocytic cells by carbonyl iron ingestion resulted in a significant increase in the NK cell activity of patients with AD. This treatment, while being effective

Figure 23.



Possible mechanisms involved in reduced NK cell activity in patients with AD.

in removing phagocytic cells, is not 100% effective. It would be of interest therefore to examine what effect indomethacin treatment of effector cells would have on NK cell activity. It is possible that a greater increase in NK cell activity could be achieved if macrophage function was completely abrogated, rather than trying to reduce the numbers present. This would reveal just how much of a role macrophages had in AD, if any?.

A further study that may have proved useful would have been to examine ADCC in these patients. It is known that NK cells can also function in ADCC and that ADCC is unaffected by steroids, therefore if ADCC were normal in these patients then it may be said with increased confidence that topical steroid treatment was the cause of the reduced NK cell activity in AD, although the role of steroids would require further investigation as suggested above.

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