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A STUDY OF NATURAL KILLER CELLS

Marian Jean Herman

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This is to certify that the thesis entitled:
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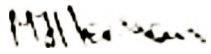

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1) Supernatant derived from a human hepatocellular carcinoma cell line (PLC/PRF/5) depresses natural killer (NK) cell activity.
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3) Prostaglandin E_2 depresses natural cytotoxicity by inhibiting interleukin-1 production by large granular lymphocytes.
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ABBREVIATIONS

AA	aplastic anemia
ADCC	antibody-dependant-cellular-cytotoxicity
Ag	antigen
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHS	Chediak-Higashi syndrome
con A	concanavilin A
cpm	counts per minute
^{51}Cr	^{51}chromium
c-RPMI	complete RPMI 1640
CTL	cytotoxic T lymphocyte
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	decays per minute
E.coli	escherichia coli
E:T	effector:target
FCS	foetal calf serum
g	gravity
GVH	graft versus host
HCC	hepatocellular carcinoma
HLA	human leukocyte antigen
hr	hour
Ia	I associated
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin-1
IL-2	interleukin-2
Ir	immune response

LGL	large granular lymphocyte
LPS	lipopolysaccharide
M	molar
μCi	microcurie
MEM	minimal essential medium
mg	milligram
μg	microgram
μg/mℓ	microgram per millilitre
MHC	major histocompatibility complex
min	minute
mℓ	millilitre
μℓ	microlitre
mM	millimolar
MNC	mononuclear cell
NA	non-adherent
NH_4Cl	ammonium chloride
NK cell	natural killer cell
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PG	prostaglandin
PGE_2	prostaglandin E_2
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SEM	standard error of mean
s/n	supernatant
SRBC	sheep red blood cell
TCGF	T cell growth factor

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ABSTRACT

These studies involved the isolation of populations of peripheral blood leukocytes enriched for large granular lymphocytes (LGL), cells thought to be responsible for natural killer activity.

It was found that the degree of cytotoxicity of LGLs could be modulated by various substances, namely, PLC/PRF/5 cell supernatants, interferon, certain monosaccharides and prostaglandin E_2. This modulation appears to be due, at least in part, to the regulation of interleukin-1 (IL-) production by LGLs.

LGLs are able to produce IL-1 in response, not only to LPS and Staphlococcus aureus but also to a variety of NK sensitive target cells. The degree of sensitivity of these cells, to NK lysis, correlates with their ability to stimulate IL-1 production by LGLs.

The observed decrease in cytotoxic activity of LGLs from patients with advanced malignant disease can be ascribed to a defect in IL-1 production by these LGLs, an effect which can be partially corrected by in vitro interferon treatment.

Treatment of target cells with IL-1 increased cytotoxicity of cancer patients LGLs to normal levels. This effect

appears to be a result of increased binding of LGLs to the target cells.

It is postulated, therefore, that LGLs, coming into contact with K562 cells, produce IL-1 which acts on the target cells enhancing their ability to bind further LGLs and thereby increasing the cytotoxicity of the latter.

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CHAPTER 1

1. LITERATURE REVIEW AND GENERAL INTRODUCTION

1.1 The nature and occurrence of neoplastic disease

Cancer is a neoplastic disease process which may affect multicellular organisms and which is characterized by seemingly uncontrolled multiplication and spread within organisms of abnormal forms of their own cells.

The development of manifested malignant tumours is an extremely complicated process, commonly divided into two main phases: initiation and promotion. Generally initiation involves an interaction between carcinogen and genome, whereas promotion is the summation of factors allowing initiated cells to become manifest as a tumour. One among many factors operating at the promotional step could be the host's immune competence. The concept of immune surveillance and its importance for carcinogenesis has been an area of great controversy in recent years. (Moller and Moller, 1979).

In the last analysis it is probably true that each example of neoplastic disease is the outcome of a unique set of circumstances including the genetically determined uniqueness of the individual affected, the virsuses carried by that person or animal and the uniqueness of the pattern of his exposure to relevant enviornmental factors.

1.1.1 The surface properties of tumour cells

The first clear demonstration that changes in cell surface properties might be of importance in malignancy was made by Coman, when in 1944, he carried out micromanipulation studies of squamous cell carcinoma of the skin and normal epidermis. He found that tumour cells were more readily separated from each other than normal epidermal cells, suggesting that the surfaces of the tumour cells were less adhesive.

Studies of cell surface and cell membranes have expanded greatly in recent years. It has become clear that the major factors concerned with biological control in metoza either directly involve the cell surface or such factors must enter the cell through the membrane. Disturbance of such control mechanisms are likely to be of fundamental importance in the development of neoplasia. The following are the major areas of investigation into cell surfaces in cancer:

a) Surface adhesive factors
b) Cell surface shape and movements
c) Biochemical constitution of the surface
d) Immunology of cell surfaces
e) Cell functions and intercellular communications.

1.2 The concept of immune surveillance

The most obvious function of the immune system is to recognise and respond to foreign materials introduced into the

the body, and to eliminate them by the production of specifically reactive antibody molecules and sensitized effector cells.

The idea that immune responses are the principal defence mechanism against neoplasia has deeply influenced cancer research during the past decades. This postulate, although put forward by Paul Ehrlich in 1909, lay dormant until it was reformulated in 1959 and crystallized as a general theory of "immunologic surveillance" by Burnet (1970a,b, 1971). Although the immune surveillance concept is commonly accepted as established fact, a small chorus of critics has developed during recent years (Stutman, 1974, 1977; Kripe and Boros, 1974).

The main contention of the theory of immunologic surveillance is that immune functions have evolved in part, as a mechanism to prevent the emergence of cancer cells that arise through somatic mutation (Burnet, 1970a,b 1971). The purported role of immune surveillance is not to mediate the regression of established tumours, but rather to seek and destroy clinically unrecognised in situ tumours (Burnet, 1970a,b, 1971). In a way, the established tumour would represent a failure of the immune surveillance mechanism.

1.2.1 General aspects of immune surveillance

In a review on cancer biology Burnet stated "It is by no means inconceivable that small accumulations of tumour cells

may develop and because of their possession of new antigenic potentialities provcke an effective immune reaction resulting in regression of the tumour and no clinical hint of its existance" (Stutman, 1977). This "forshadowing" of the theory has remained almost unchanged in essence. For example:- "The thesis is that when aberrant cells, with proliferative potential, arise in the body, they will carry new antigenic determinants. When a significant quantity of new antigens has developed, a thymus-dependant immunologic response will be initiated which eventually eliminates the aberrant cells in essentially the same way as a homograft is destroyed" (Burnet, 1970a).

Thomas (1975) extended the immune surveillance concept further by postulating that the mechanisms for homograft rejection evolved primarily as a natural defence against neoplasia. This theory of immune surveillance has generated many experimental studies, discussion and controversy. One of the reasons for the controversy is that the concept is rather complex and leads to a series of predictions some of which are as follows:

a) Tumour cells have antigens which could be recognised by the immune system. The resulting reactivity of the host, against the tumour, would lead to its eventual elimination.

b) The part of the immune system that is involved in the resistance against tumour growth is the same as that involved in rejection of transplants of normal tissues.

c) Immune depression is necessary for the development of

detectable tumours.

d) Immunosupression would be expected to cause increased susceptibility to tumour development, and

e) One requisite action of carcinogenesis and/or tumour promoters might be to suppress host defences.

Contrary to the predictions of the immunosurveillance theory, immunologically priviliged sites, such as the anterior chamber of the eye, possibly the brain, and the hamster cheek pouch do not have an increased incidence of neoplastic disease. Similarly, the incidence of malignant tumours in diseases associated with a state of anergy, such as leprosy and sarcoidosis is reportedly low (Olenich, 1969; Brinker and Wilbeck, 1974). Finally, if the genetics of tumour evolution in the absence of an intact immune system is considered, a polyclonal tumour cell proliferation would be favoured over monoclonal neoplasms. However, evidence based on studies of genetic markers does not support this premise. The majority of tumours investigated have thus far been classified as unicellular proliferative expansions. The occurrence of multiple tumours as well as neoplasms of various histological types in immunosuppressed hosts is relatively uncommon (Fialkow, 1974; Friedman and Fialkow, 1976).

The main support for the immune surveillance hypothesis has come from evidence related to prediction (d), since naturally occurring or induced immunodepression has been associated with a greater incidence of some types of tumours. In experimental animals this has been most clearly

demonstrated with tumours induced by oncogenic virsuses. Polyoma virus is widespread in nature, yet wild mice do not develop polyoma tumours (Huebner, 1963). However, administration of the virus to newborn mice of some inbred strains, when their immune system is not fully developed, results in neoplastic growth. Suppression of immunity, particularly by procedures that affect thymus-dependant immune responses, has been shown to greatly increase the incidence of polyoma tumours (Law, 1965, 1966; Law and Ting, 1965; Levy and Wheelock, 1974; Levy et al., 1979; Liotta et al,. 1977).

Similarly, neonatal thymectomy of chickens results in an increased frequency of tumours in strains that are genetically resistant to the malignant effects of Marek disease virus (Payna, 1972). There is also considerable evidence that immunodepression is associated with an increased incidence of immune deficiency diseases, lymphomas and leukemia (Doll and Kinlen, 1970; Fraumeni, 1969). Gatti and Good (1971) estimated that the incidence of tumours in these patients was about 10 000 times that seen in the general age matched population. Allograft recipients receiving immunosuppressive agents, mainly prednizone and azathioprine, have also been found to have an increased incidence of tumours (Greene et al., 1981; Penn and Starzl, 1972). Again lymphomas were prominant but the majority of the tumours were of epithelial origion.

Patients with Hodgkins disease, other types of cancer, or patients with arthritis or other benign disease who

received chemotherapeutic (mainly alkylating) agents have been subsequently found to develop primary malignancies, mainly leukemias and lymphomas, with relatively high frequency (Cancalloe et al., 1974; Hyman, 1969; Puri and Campbell, 1977; Reimer et al., 1977; Roberts and Bell, 1976; Tchernia et al., 1976).

The effects of immunosupression on tumour induction in experimental animals by chemical carcinogens has been even less clear cut (Stutman, 1975). There has also been a general failure to observe more rapid tumour growth in nude mice (stutman, 1979). A likely explanation for many of the discrepancies in results regarding the possible association of immune depression and tumour development is that various effector mechansims might be involved in host resistance. Results might vary with (1) the type of effector mechanism that plays a predominant role in each form of cancer and (2) the nature and extent of immune deficiency.

Such a likelihood of diversity of effector mechanisms involved in immune surveillance may be offered as a response to most of the criticisms that have been raised against the hypothesis. When information about thymus-dependant immunity became known, and particularly when T cells were found to play a controlling role in allograft rejection, the immune surveillance hypothesis was modified to stress the key role of this effector mechanism in anti-tumour resistance (Burnet, 1970). Related to this has been the emphasis placed on expression, in tumours, of tumour-associated

transplantation antigens. Since structures would have to be recognised by T cells in order to get effective T cell mediated resistance to tumour growth. It has been possible to obtain strong evidence for a major in vivo role of T cells in virus-induced tumours (Berenson et al., 1975; Collavo et al., 1974; Glaser et al., 1976; Gorezynski and Norbury, 1974) however, little comparable evidence exists for spontaneous tumours or even for transplantable chemical carcinogen induced tumours.

These observations have led to the suggestion (Klein and Klein, 1977) that immune surveillance may be operative only against tumours induced by oncogenic virsuses which have strong transplantation antigens and, for which immune T cells have been shown to be important in resistance. T cell mediated immunity should be viewed however as only one of a series of possible host defence mechanisms. Target cell structures, other than tumour-associated antigens, might be involved in recognition by other types of effector cells; and in T cell deficient individuals the other effector mechanism might still be functional and capable of resisting tumour growth.

1.2.1.1 Mechanisms of subversion of the host immune response

Since the occurrence of malignant disease in individuals with seemingly normal immune systems would, in essence, imply a failure of immunological surveillance, protagonists of

the immunosurveillance concept have proposed several mechanisms whereby tumours may evade the host immune responses:

1. Lack of antigen expression:

   Successfully evolving tumours may lack immunogenicity and, as such, preclude recognition by immune effector cells. Although certain virus and chemically induced tumours do lack detectable antigenicity, the vast majority of all tumours tested elicit some, albeit often weak, immune reactivity. Hence it is unlikely that this rationale is applicable to tumours.

   The surveillance system may exert a selection pressure on evolving neoplasms resulting in an outgrowth of non-immunogeneic clones. This postulate is challanged by evidence that non-immunogenic tumours grown in vitro, in the absence of an immune system, do not become immunogenic (Prehn, 1970).

2. Antigenic modulation and surface redistribution:

   Neoplastic cells may undergo antigenic modulation in response to anti-tumour immunity by diminishing or enveloping exposed cell surface target antigen. The genetic instability of neoplastic cells could also result in a continuous production of new tumour associated antigens, independant of immunological pressures (Nowell, 1976).

3. Enhancement and self-enhancement:

   The possibility that an anti-tumour immune response

may even stimulate tumour growth has been put forward by Prehn (1976). This phenomenon is termed countersurveillance or immune-stimulation and has been observed in vitro and in vivo.

4. The production of blocking factors (Figure 1.1):
The developing tumour may resist the immune response by inducing suppression of either the afferent or efferent arc of cell mediated or humoral immune mechanisms. For example, the blocking of immune anti-tumour cytotoxic cells by soluble tumour antigen or antigen-antibody complexes and the coating of tumour target cells with "blocking factors", preventing adequate effector target cell interaction (Currie and Alexander, 1974; Hellstrom and Hellstrom, 1974).

5. The lack of appropriate Ir genes:
The absence of appropriate immune response (Ir) genes that regulate susceptibility to oncogenesis would adversely compromise host resistance and lead to tumour development. An example of Ir gene influence on oncogenesis is provided in the marmoset primate model. Marmosets are susceptible to lymphoma development following infection with Herpes Saimiri virus, whereas the same lymphotropic virus colonizes its natural host, the squirrel monkey, in an innocuous fashoin. This resistance to oncogenesis is probably related to a more efficient antiviral humoral immune response in the squirrel monkey (Deinhart et al., 1974).

What then are the likely alternatives to T cell mediated immunity in anti-tumour resistance and immune surveillance There are a variety of possibilities including macrophages, natural killer (NK) cells together with other natural effector cells and antibody-dependant-cellular-cytotoxicity (ADCC) The evidence in support of a role for each will be discussed.

1.2.2 The possible role of macrophages in immune surveillance

Cells of the mononuclear phagocyte lineage originate in the bone marrow and emigrate from the blood to function in tissues (van Furth, 1975, 1980). Differentiation, growth and survival of mononuclear phagocytes appears to be dependant on a lineage-specific growth factor which is selectively destroyed by the differentiated cells (Tushinski et al., 1982). Mononuclear phagocytes, recognised for a century as being involved in phagocytosis, intracellular killing and digestion are now viewed as important secretory cells, elaborating an extensive variety of products of diverse structure and biological activities (Nathan et al., 1980). The production of these products is, essentially, independant of the functional activity of the macrophage, whereas the majority require induction by agents as diverse as lymphokines, lipopolysaccharide (LPS), immune complexes and tumour promoters to name a few. Distinctive surface structures enable macrophages to recognize "foreign" material and to participate in complex cellular and humoral interactions, notably inflammation and immunity, anti-microbial and anti-tumour

defences and the remodelling of tissue and wound healing (van Furth, 1975, 1980; Nelson, 1976; Nathan et al., 1980; Unanue and Rosental, 1980).

The mechanisms by which these cells recognise "foreign" as opposed to "self" components are still poorly defined. Functional activity of macrophages and their ability to resume proliferation in tissues remote from the bone marrow is presumed to be under the control of the microenviornment. It is not clear whether the extraordinary versatility of the cells and their mulciplicity of functions can be fulfilled by a single cell type or whether this reflects the operation of macrophage subpopulations with different properties and functions, or, whether, this is a reflection of different stages of maturation (Walker, 1976; Forster and Landy, 1981).

There is extensive literature attesting to the capactiy of mononuclear phagocytes to either promote or impair proliferation and viability of tumour cells in vitro (Nelson, 1976; James et al., 1977; Keller, 1980b, 1981). The extent to which the various activities of tumour-suppressive macrophages become evident depends on the metabolic and functional state of effector cells (Forster and Landy, 1981), the susceptibility and repair capacity of the target cell, its ability to subvert host defence and the ratio of effector

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Killing of tumour cells by macrophages can take place

either in the absence of or with the help of immunospecific structures. In particular via arming with antibody (ADCC) (Levy and Wheelock, 1974; Nathan and Cohn, 1980; Chow et al., 1981) or with T cell derived structures (specific macrophage arming factor) (Levy and Wheelock, 1974; Alexander, 1976; den Otter, 1981).

Activated macrophages manifest "spontaneous" killing of tumour cells with some selectivity. That is, they are able to distinguish between the "normal" and the "transformed" state (Hibbs et al., 1972; Keller, 1981). The altered topography of tumour cells is somehow registered as "non-self" or "activated-self". Activated macrophages have an impressive potential to destroy tumour cells under a variety of circumstances and probably via different mechanisms. Exposure of activated macrophages to pharmacological stimuli such as tumour-promoting phorbol esters, triggers a rapid cytolytic process (4 - 6 hours) which has been attributed to hydrogen peroxide secreted by macrophages (Nathan et al., 1979). The manifestation of another type of spontaneous cytotoxicity, mediated by activated macrophages against a wide variety of syngeneic, allogeneic and xenogeneic tumour cells, required prolonged interaction (24 - 36 hours) and close contact between effector and target cells (Keller, 1980b).

Affirmation of the tumourcidal efficacy of activated macrophages in vivo has been provided by reports of the growth of various transplanted tumours being inhibited by inoculation of activated syngeneic or allogeneic macrophages

(Alexander et al., 1973; Fidler, 1974; Keller, 1976, 1977; den Otter, 1981). Inhibition of tumour growth was particularly efficient when macrophages were injected into the site of the tumour inoculum. Keller (1983) presented findings showing that (1) various "anti-macrophage" agents reduced natural anti-tumour resistance when administered locally briefly before tumour cell challange. (2) Local pretreatment with immunostimulants or local adoptive transfer of immunostimulant induced macrophage-type cells markedly augmented local resistance without significantly affecting resistance in other compartments and (3) immunostimulant-enhanced local resistance is readily nullified by local administration of the anti-macrophage agents previously mentioned (Keller, 1980b,c 1982a,b).

It seems that both activation and inactivation of macrophage tumouricidal capacity are largely local phenomena (Freedman et al., 1980; Keller, 1980c). It has been suggested that it is at the foci of infection where lymphocytes must recruit and activate macrophages for anti-bacterial immunity to be expressed (MacKaness, 1969). This may also be valid for macrophages in tumours (Holden et al., 1969; Nelson et al., 1981). Various workers have found that macrophages from regressing tumours are capable of killing neoplastic cells whereas macrophages from progressing tumours kill inefficiently, if at all (Russel and McIntosh, 1977; Russel et al., 1977). Earlier work has suggested that tumours which do not metastasise are immunogenic and are characterized by extensive macrophage infiltration, whereas metastatic

tumours evoke little host response(Birbeck and Carter, 1972; Eccles, 1978). More recent studies, however, do not support the existance of such a close relationship between the number of macrophages in a tumour and its metastatic potential or its ability to mount an immune response(Evans and Lawler, 1980; Evans and Eidlen, 1981; Talmadge et al., 1981; Keller, 1982b).

Quite apart from their effects on the proliferation and viability of tumour cells, macrophages influence vascularization of the tumour, a process known to critically affect the host-tumour interaction (Brem et al., 1976; Polverini et al., 1977). Further variabilities which may decisively influence the outcome of the host tumour interaction derive from the tumour itself. Certain tumours, at least at some stages of growth have been shown to stimulate cells of the mononuclear phagocyte system by increasing monopoiesis by the bone marrow, (Baum and Fisher, 1972; Otu et al., 1977; Nelson et al., 1981) by inducing monocytosis (Golde, 1975; Eccles et al., 1976; Wood et al., 1979; Joshua et al., 1980; Nelson et al., 1981), or by enhancing macrophage functional activities (Old et al., 1961; Levy and Wheelock, 1974; North and Kirsten, 1977; Otu et al., 1977; Hooper et al., 1979; Nelson et al., 1981).Conversely tumour-bearing animals often manifest diminished capacity to mobilise mononuclear phagocytes into inflammatory exudates , and exhibit depressed macrophage responsiveness to chemotactic stimuli in vitro (Eccles and Alexander, 1974; Normann and Sorkin, 1976; Snyderman et al., 1976; Woods and Papadimitriou, 1977),

depressed macrophage mediated resistance to infection with Listeria monocytogenes (Spitalny and North, 1977) and decreased phagocytic capacity (Levy and Wheelock, 1974).

Tumours may subvert host defences by inducing the activation of suppressor cells (Mikulski and Muggi, 1978; Kennard and Zolia-Pasnev, 1980). Apart from suppressor T cells and from blocking antigen-antibody complexes (Keller and Jones, 1971; Hellstrom and Hellstrom, 1974) monocytes and macrophages, and their secretory products such as prostaglandin E_2 (PGE_2), may also be implicated in the depression of cell-mediated immunity (Kirchner et al., 1974; Nelson et al., 1976; Goodwin et al., 1977; Krakauer et al., 1977; Schultz et al., 1979; Joshua et al., 1980). From findings such as these it appears that the type of tumour cell and the actual stage of tumour growth are important in deceiding whether the interaction will result in enhancement or suppression of host defences. (Fig. 1.1).

There is considerable evidence of host surveillance mechanisms against neoplasia. Prominent amongst these are activated macrophages, which have the capacity to destroy neoplastic cells in vitro with considerable selectivity, and which appear to be operative in vivo against primary tumours and metastases. Macrophage mediated tumour cell lysis appears to circumvent the problem of cellular resistance to killing often encountered with cytotoxic drugs. It seems likely that the major area of macrophage action is the recognition and effective elimination of transformed cells in the early

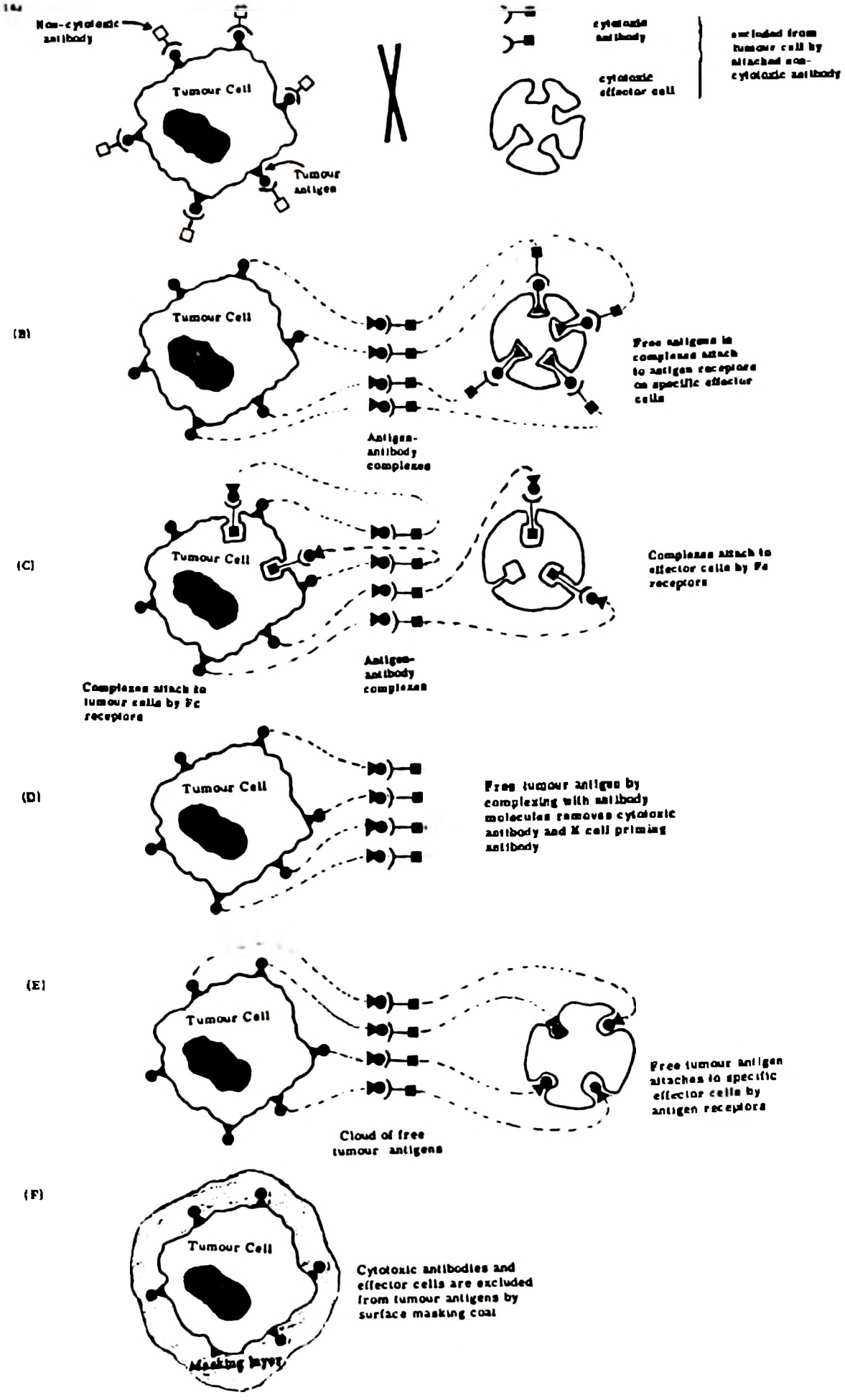

FIGURE 1.1 A DIAGRAM OF POSTULATED MECHANISMS WHEREBY TUMOUR CELLS SUBVERT THE HOST IMMUNE RESPONSE.

phase of primary tumour growth and/or its secondary spread, rather than destruction of a large tumour burden.

1.2.3 The possible role of antibody dependant cellular cytotoxicity in immune surveillance

In addition to any direct cytotoxic effects on tumour cells, macrophages (Haskill and Fett, 1976; Key and Haskill, 1982), polymorphonuclear cells (PMN's) and natural killer(NK) cells (Landazuri *et al.*, 1979; Ojo and Wigzell, 1978; Timonen *et al.*, 1982), may also act in co-operation with antibodies to produce ADCC. ADCC has been detected against a variety of experimental (Blair *et al.*, 1976; Harada *et al.*, 1975; Haskill and Fett, 1976; Lamon *et al.*, 1975; Landazuri *et al.*, 1979; Pollack *et al.*, 1972) and human tumours (Hakala *et al.*, 1974; Jondal and Gunven, 1977). Phagocytosis of tumour cells by macrophages or PMN's may also occur and may be dependant on the presence of anti-tumour antibodies (Key and Haskill, 1982); but there is very little evidence for a significant role of ADCC in *in vivo* anti-tumour resistance. This is probably due, in part, to difficulties in distinguishing between ADCC and the direct anti-tumour effects of antibody on various effector cells.

In a variety of experimental tumour systems (Herlyn *et al.*, 1980; Ting and Herberman, 1976) *in vivo* administration of antibody has resulted in some protection against progressive tumour growth, and it has often been suggested that ADCC was a major mechanism for the observed effects.

However, in only a few studies has evidence been provided to support this possibility. Furthermore, most of the supportive data are indirect and not conclusive. For example, tumour cells isolated from antibody-producing mice have been shown to be coated with antibody, and are thereby susceptible to lysis by macrophages (Haskill and Fett, 1976). Phagocytosed tumour cells within macrophages have also been noted at the site of tumour growth (Key and Haskill, 1982). Shin and his associates (1975, 1976, 1978) used a somewhat more direct approach to support an in vivo role for alloantibodies in ADCC. Syngeneic IgG antibodies were shown to have limited effects on mice with large tumour burdens. This appeared to be related to a shortage of macrophages at the tumour site since activity could be restored by local addition of macrophages. Herberman (1983) has put forward the following approach to explain the possible importance of this mechanism. In a tumour system in which in vivo administration of antibody could be shown to have protective effects, normal recipients of antibodies and tumour cells could be compared with animals selectively depleted of one or another type of potential effector cells. Loss of activity of antibody, for example in animals with selective deficits in NK cells and macrophages, would point toward an important collaboration between these components of the immune system. Further evidence for mediation of anti-tumour effects by ADCC would be provided by restoration of antibody activity by adoptive transfer of a purified effector cell population into deficient recipients.

Clearly, since there is a paucity of evidence for an in vivo role of ADCC with tumour cell lines, the question of possible involvement of this mechanism in immune surveillance becomes highly speculative. This effector mechansim should, however, be kept in mind and appropriate investigative studies performed. In addition to the possible role for antibody specifically induced by tumour-associated antigens, natural anti-tumour antibodies have been demonstrated (Choe et al., 1979, 1981; Houghton et al., 1980; Martin and Martin, 1975; Menard et al., 1977), which might interact with effector cells and mediate ADCC. It should be noted that virtually all the evidence presented for a possible role of macrophages or NK cells in immune surveillance would also be compatible with the mediation of their effects by collaboration with such natural antibodies.

1.3 Natural killer cells

1.3.1 Introduction

NK cells were discovered some ten years ago in the course of experiments assigned to reveal specific cell mediated anti-tumour immunity in cancer patients and tumour bearing animals (Takasugi et al., 1973; Kiessling et al., 1975; Oldham et al., 1975). It became clear that a particular subpopulation of normal peripheral blood lymphocytes found in a wide range of mammalian and avian species are capable of spontaneously lysing a wide variety of tumour cells, tumour-derived cell lines and certain virus infected cells. The derivation of

NK cells remains unclear and controversial, but it seems likely that these cells are either derived from a pathway of development within the T cell lineage, or from bone marrow precursor cells and merely share the same characteristics with cells of other better known lineages (Herberman, 1983).

In the past few years, NK cells have attracted increasing attention, particularly because of the possibility of their playing an important role in the defence against cancer. NK cells seem to be important in immune surveillance and play a role in microbial infections as well as having other functions. There is, in fact, much evidence to suggest a wide range of in vivo functions for NK cells namely:

a) NK cells mediate resistance against NK-sensitive transplantable tumours
b) they play a major role in resistance against metastatic spread of tumour cells
c) they have been implicated in immune surveillance against certain types of tumours
d) they secrete interferon, interleukin-1 and interleukin-2 and therefore appear to have a regulatory role
e) they mediate natural resistance against infection by some microbial agents
f) they play a central role in natural resistance against some bone marrow transplants and
g) they possibly also contribute to the development of certain diseases including graft versus host disease, atopic dermatitis, asthma and some cases of aplastic anemia (Herberman, 1983). (Table 1.1).

TABLE 1.1 POSSIBLE FUNCTIONS OF NK CELLS

1) Natural killer activity and destruction of tumour cells.

2) Natural killer activity against microbial agents (virus infected targets, parasites, fungi and bacteria.

3) Destruction and/or control of growth and development of some normal haematopoietic and thymus cells.

4) Contribution to graft versus host disease or autoimmunity (eg. aplastic anemia).

5) Secretion of interferon, IL-1, IL-2 and consequent immunoregulatory activity.

1.3.2 Characteristics of NK cells

At present the general consensus is that NK cells in both man and experimental animals are non-phagocytic, non-surface adherent, partly sheep-erythrocyte rosette forming (in man), or partly Thy-1 positive (in mouse) lymphocytes with a receptor for the Fc part of IgG on the plasma membrane (Kiessling et al., 1975; Herberman et al., 1977; West et al., 1977). Most functional NK cells are found in peripheral blood and spleen, with some minor activity in bone marrow, lymph nodes, gut and lungs (Reynolds et al., 1981). It is noteworthy that lymph and thymus are devoid of NK activity indicating compartilization of these cells. It is also obvious on the basis of this distribution that NK activity is not due to a small number of cytolytic T lymphocytes (CTL), an opinion entertained by some authors only a few years ago. The differentiation of NK cells is bone marrow dependant (Haller et al., 1977). In mice, the response of NK cells in the spleen is age dependant, with an apparent low cytolytic activity in young and old animals, but a strong killing potential at the age of five to eight weeks (Kiessling et al., 1978). In man there is no clear age dependancy. Active NK cells can be detected in foetal liver during the ninth week of gestation (Toivaven et al., 1981). Cord blood contains NK cells with a weaker reactivity than adult blood and some reports describe a low reactivity in the circulation of adult females (Timonen, 1983).

NK cells in man and rats and, to a certain extent in

mice, have a characteristic morphology in Giemsa-stained cytocentrifuged smears. They are relatively large lymphocytes with a high cytoplasmic to nuclear ratio and a pale cytoplasm with azurophilic granules often in the area close to the notch formed by the kidney-shaped nucleus (Timonen et al., 1979). This cell type has been termed large granular lymphocytes (LGL). Approximately 5-10% of peripheral blood cells in man are LGLs (Timonen et al., 1981). The distribution of LGLs in various organs parallels NK cell activity. NK activity in vitro is demonstrable in percoll gradient cell fractions containing LGLs (Reynolds et al., 1981; Timonen et al., 1981). An enriched population of LGLs can be obtained by centrifugation of non-adherent peripheral blood lymphocytes (PBL) on discontinuous percoll gradients. Further enrichment can be attained by depletion of conventional T cells by sheep-erythrocyte rosette formation or by depletion using appropriate monoclonal antibodies and complement or a fluoresence-activated cell sorter (Timonen et al., 1981; Hercend et al., 1983). Purified populations of LGLs have, during the past two years, been a subject of extensive investigation, including studies of their cytochemistry, the biochemical events involved in the lytic phenomenon, surface marker analysis, the mechanism of killing at single cell level and the analysis of cell cultures grown in the presence of T cell growth factor (TCGF).

1.3.3 Cytochemistry of NK cells

Like T lymphocytes and monocytes, but unlike B cells, LGLs

are anti-naphthyl-acetate esterase (ANAE) positive (Timonen et al., 1979; Grossi et al., 1982). The cytoplasmic activity is diffuse and in this respect resembles the monocytic enzyme distribution. It has not been established whether the ANAE reactivity of LGLs can be inhibited by sodium fluoride, a property which would also point towards the similarity of monocytes and LGLs. The cytochemistry of LGLs also resembles that of T cells: they are lysosome and peroxidase negative, and positive for β-glucuronidase and tartrate-sensitive acid phosphatase (Timonen et al., 1981). As a whole cytochemical methods have not offered possibilities to distinguish LGLs from other lymphoid cells or monocytes.

1.3.4 Surface markers on NK cells

With the availability of monoclonal antibodies against purified LGLs and subpopulations of lymphocytes, some additional information on the lineage of NK cells has been obtained. As shown in Figure 1.2, LGLs express antigenic determinants characteristic for both T lymphocytes and myelomonocytic cells (Ortaldo et al., 1981). Abo et al (1983) have put forward the hypothesis that NK cells move through three stages of differentiation before reaching maturity; with each of these stages they have associated a certain phenotype. According to their theory immature NK cells found in the very young foetus express the HNK-1 antigen. As the foetus matures and the lytic potential of the NK cells increase they aquire the OKT3 antigen, a pan T cell marker. NK cells of this phenotype are thought to have low cytolytic activity

and can be found in the bone marrow of adults. However mature active NK cells present in peripheral blood and spleen are thought to have lost the OKT3 antigen and gained the OKM1 antigen (a monocyte marker), while still retaining the HNK-1 antigen. These stages of "differentiation" of NK cells are said to be paralled by not only an increase in cytolytic activity but also an alteration in morphology until they resemble the peripheral blood large granular lymphocytes. Data in Fig.1.2 shows the various antigens found exclusively on LGLs in addition to those shared with either T cells or cells of the myelomonocytic lineage. However LGLs are thought to form a heterogenous population and it is speculated that the lineage of LGLs could differ, some being similar to macrophages while others resemble T cells.

1.3.5 Specificity of NK cells

NK cells have been found to react with a wide variety of target cells. In vitro passaged leukemia cell lines are particularly susceptible to lysis but some carcinoma and sarcoma lines as well as some in vivo transplanted tumours are also susceptible to rapid lysis. It has recently become clear that a limited assortment of normal cells may also be lysed by NK cells. These are mainly subpopulations of thymocytes, bone marrow cells, macrophages and cultured foetal fibroblasts. Despite the broad range of susceptible targets, selectivity is seen mainly with tumour cells and normal cells are usually quite resistant to lysis.

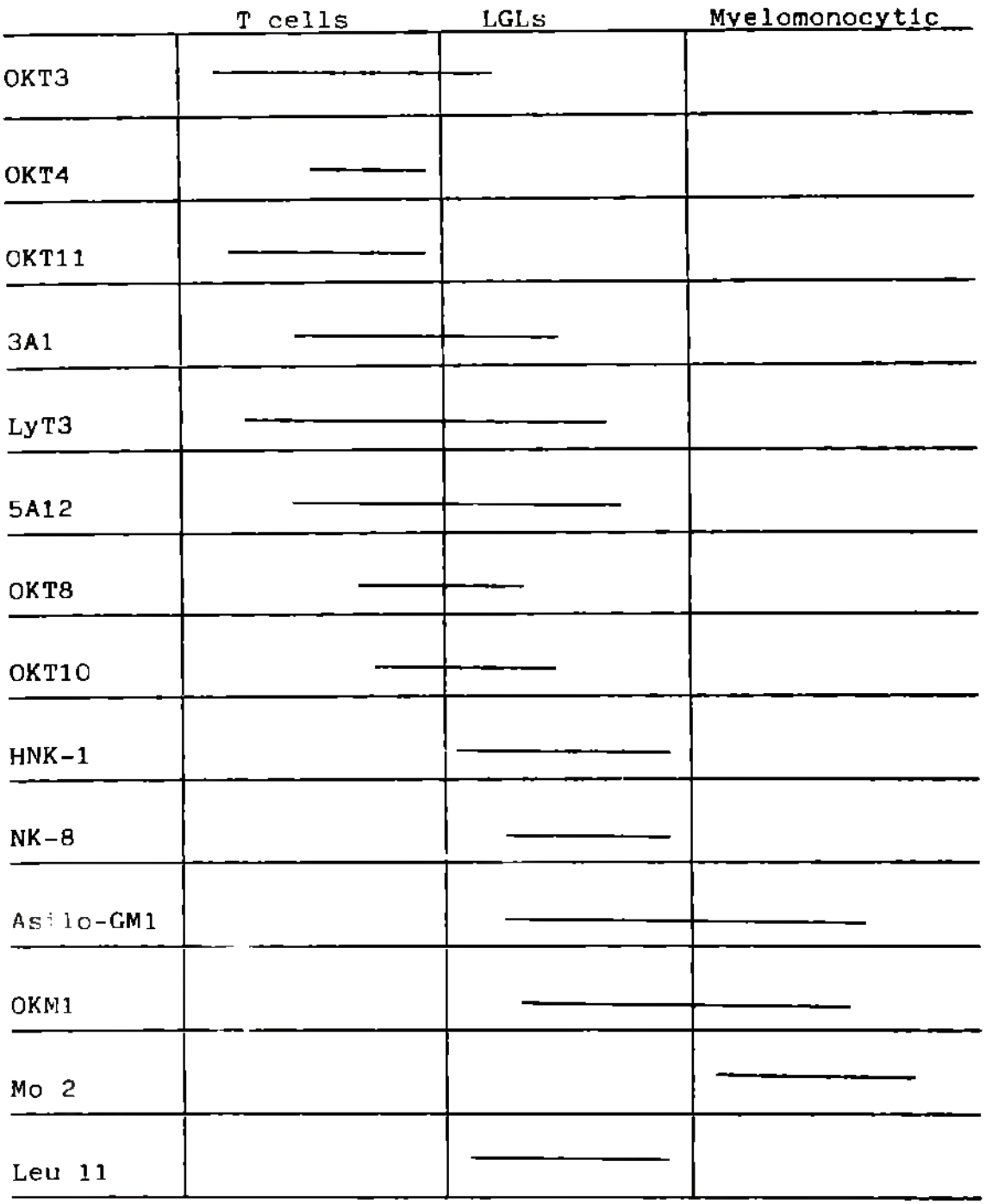

FIGURE 1.2 REACTIVITY OF T CELLS, LGL AND MYELOMONOCYTIC CELLS IN HUMAN PERIPHERAL BLOOD WITH VARIOUS MONOCLONAL ANTIBODIES

When NK cells stimulated with interferon are tested, lysis may be seen against some targets that usually resist the action of spontaneous NK cells. The available evidence favour the possibility that the effector cells are simply more active and are therefore able to lyse usually more resistant targets.

Prolonged incubation of the cytotoxicity assay from the usual four hours to eighteen hours also broadens the range of target cells susceptible to NK lysis. This phenomenon is probably due to augmentation of cytolysis by interferon secreted by the NK cells on prolonged incubation with the target cell.

An important issue is whether primary tumour cells are susceptible to NK lysis. Limited information is available but it is becoming clear that at least some primary tumour cells are susceptible.

Initially most investigators beleived that NK cells would recognize one common structure on target cells and although this view is still held by some, evidence for recognition of multiple specificities has accumulated. With such data have also come indications for subsets of NK cells, each recognizing a limited number of target cells. For example normal human donors have been found to vary in their patterns of reactivity against various target cells (Herberman and Ortaldo, 1980).

NK cells are thought to bind to their targets using one of two alternative modes. The first involves a receptor-ligand interaction between a target structure on the plasma membrane of the target cell and a recognition receptor on the surface of the NK cell. In the second the target cell is coated with antibody specific for a target-cell antigen and the NK cell then binds the Fc portion of the antibody to complete the necessary bridge.

The nature of the recognition receptor is not known, but in the mouse pretreatment of NK cells with protelytic enzymes such as trypsin, papain and pronase eliminated both target cell binding and cytotoxicity (Roder et al., 1978). These two functions regenerated in parallel over a four hour time course and could be blocked by cycloheximide. Treatment of non-trypsinized cells with minimum concentrations of protein synthesis inhibitors such as puromycin or cycloheximide also decreased both target-cell binding and lysis. It is likely therefore that the NK recognition receptor is a de novo synthesised protein with a turnover time similar to that of other membrane proteins. The ability of simple sugars to inhibit target-effector binding in the mouse (Stutman et al., 1980) suggests that lectin like receptors are involved.

The target structure has been more extensively analysed (Roder et al., 1979a,b,c). Preincubation of human PBL with solubilized glycoproteins from NK sensitive, but not NK resistant, target cells, inhibited binding to the homologous

intact target cells by approximately 50% (Roder et al., 1979a). However these molecules were not related to any known surface proteins including viral and major histocompatibility complex (MHC) products.

In some target cells the target structure may represent early antigens that are lost upon differentiation or maturation. Hence immature thymocytes from neonates, but not mature thymocytes and T cells, were sensitive to NK-mediated cytolysis in the mouse (Hansson et al., 1979a,b). Human thymocytes were also NK sensitive (Ono et al., 1977) and there was an inverse correlation between the sensitivity of foetal fibroblasts and the age of the donor foetus (Saksela et al., 1979). In the human there is good correlation between controlled differentiation in K562, U937 of GM86 tumour cell lines and increased resistance to NK mediated cytolysis (Gidlund et al., 1981). It could be hypothesized that NK cells recognise "foetal like" antigens which are lost upon differentiation. An intriguing alternative hypothesis has been developed by Karre (1981) who suggests that NK cells are triggered by a lack of self-MHC antigens of the class 1 type. It is known that undifferentiated lines express less MHC antigens. The changes that occur in the target structure during differentiation are not yet known. However, studies in the mouse (Gronberg et al., 1981; Durdik et al., 1980; Yogeeswaran et al., 1981) have revealed a negative correlation between NK sensitivity and the amount of cell surface sialic acid (Yogeeswaran et al., 1981) whereas a positive correlation between asilo-GM_2 and NK sensitivity was found

in two independant studies (Yogeeswaran et al., 1981; Young et al., 1981). These results suggest that sialic acid may mask the target structure but further work is needed to implicate GM_2 or other gangliosides. It has recently been proposed that the mechanism of effector-target interaction involves the binding of effector borne transferrin to a target borne transferrin receptor (Baines et al., 1983). One could predict a correlation between the rate of proliferation in a target and its sensitivity to NK lysis since the expression of transferrin receptor is directly related to the proliferation rate (Towbridge and Omary, 1981; Sutherland et al., 1981). Alternatively it has been suggested that the transferrin receptor itself may be the target structure (Vodinelich et al., 1983).

1.3.6 Mechanism of NK mediated lysis

The development of a single cell assay for dissociating NK mediated binding and lysis in the mouse has advanced knowledge in this field (Roder and Kiessling, 1978; Roder et al., 1978). The model has some similarities to, but also distinct differences from earlier work performed in the cytolytic T cell field (Granger and Kolb, 1968; Henney, 1977). These results have been confirmed and extended in the human (Carpen et al., 1981).

The lytic cycle can be divided into four discrete stages:

1) recognition and binding
2) triggering and activation of the lytic mechanism

3) release of a lytic substance from the NK cell

4) target cell death.

(shown schmatically in Fig. 1.3).

Binding of the effector cell to the target cell is the first necessary prerequisite for lysis. This step is temperature independant, unlike T cell binding, and Ca^{2+} and Mg^{2+} dependant. Membrane and cytoskeletal integrity may also be important in this step. Dimethylsulfoxide (DMSO) , a dipolar solvent, inhibits NK mediated binding (Roder et al., 1981). Although the mechanism of action of the drug is unclear it is thought that the plasma membrane is destabilized leading to disorganisation of integral membrane proteins including the recognition receptors.

The triggering mechanism in NK cells subsequent to target-effector binding is unknown but indirect evidence implicates cyclic nucleotides. In both the mouse (Roder and Klein, 1979) and the human (Katz et al., 1982) the addition of cyclic adenoside monophosphate (cAMP), or inducers thereof, to highly enriched NK cells caused inhibition of cytolysis, whereas cyclic guanosine monophosphate (cGMP) and interferon (Tovey et al., 1979) caused augmentation. Although specific antagonists blocked these effects, levels of intracellular cAMP and cGMP in the NK cell before or after target-effector contact, have not been measured. It is possible therefore that cAMP, which is known for its ability to inhibit cell locomotion, could simply inhibit NK cell recycling.

FIGURE 1.3 A SCHEMATIC MODEL OF NK CYTOLYSIS

Step 1 involves a divalent cation-dependant and specific contact between the target and effector which is inhibited by EDTA

Step 2 involves an interaction between the NK receptor and target structure which triggers an energy-dependant intrinsic arming of the NK cell which is inhibited by dinitrophenol or sodium nitrate

Step 3 the lytic moiety is activated and brought into close opposition with the target cell membrane by a dynamic process which is inhibited by colchicine. The actual killing event may involve a lysosome derived serine esterase (NK lymphotoxin) which is inhibited by phenylmehtyl sulfonyl fluoride and di-isopropyl flurophosphate. These lytic molecules bind to an acceptor site on the target cell membrane.

Step 4 target cell death follows and is thought to result from colloid osmotic lysis.

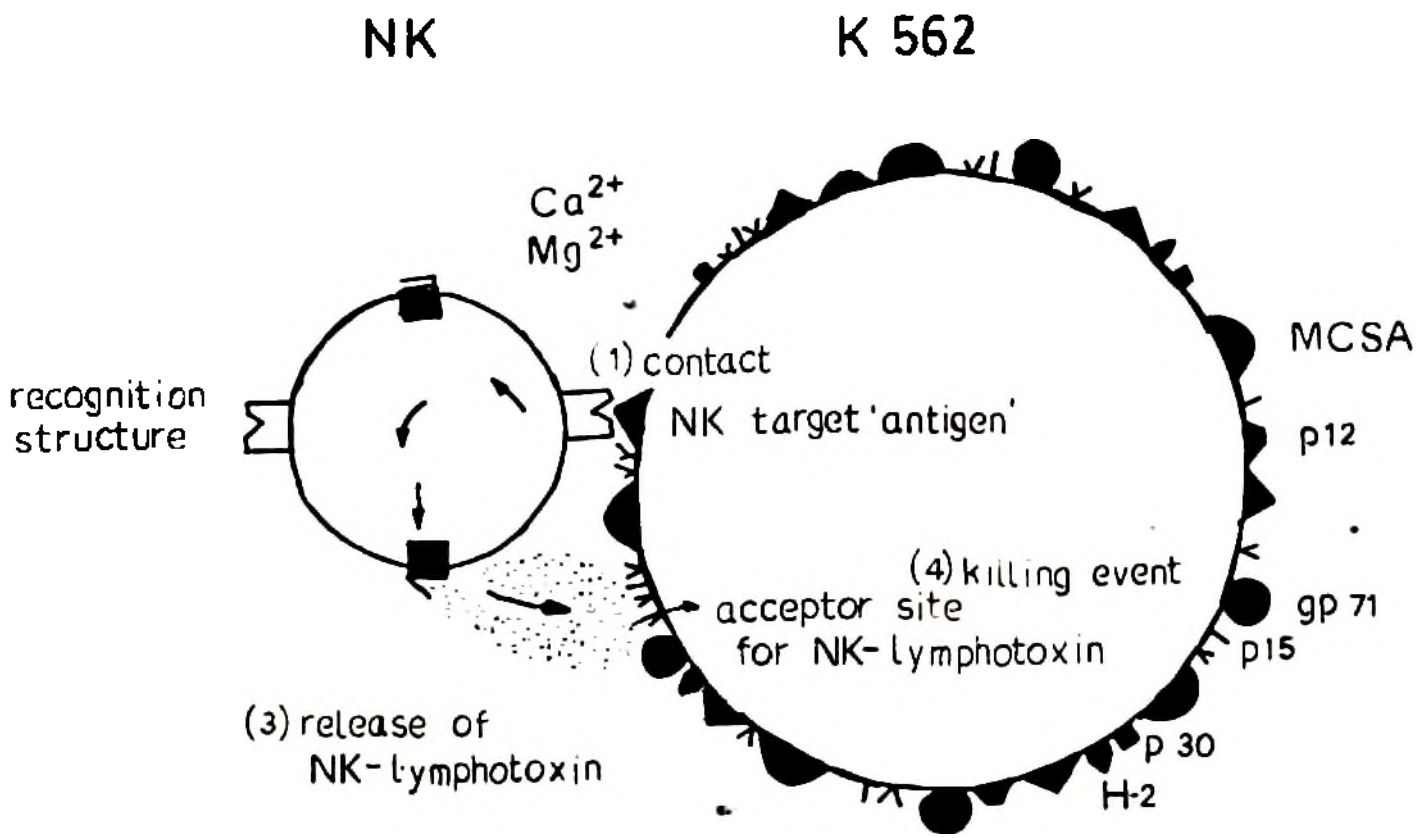

FIGURE 1.3 A SCHEMATIC MODEL OF NK CYTOLYSIS.

Binding of NK cells to their target cells does not necessarily lead to activation of the lytic machinery. This seems to require other stimuli, the nature of which have not been clarified. The activation is energy and calcium dependant involving various enzymatic activities such as chymotrypsin-like proteases (Rudig et al., 1981; Goldfarb et al., 1981) and phospholipids (Hoffman et al., 1981). Neither DNA nor protein synthesis is needed for endogenous NK activity. Morphologically, the activation of the lytic machinery is revealed by the orientation of the golgi apparatus in the LGL towards the target cell, and a subsequent secretory process (Carpen et al., 1981).

After the secretion phase the NK cell detatches from its target cell, which then proceeds to a killer cell independant osmotic lysis. This happens 30 to 60 minutes after initial contact. The NK cell is refractory to rebinding for approximetly two hours, after which it is again capable of lytic activity, a phenomenon known as recycling (Ullberg and Jondal, 1981; Ortaldo et al., 1982).

Another hypothesis suggests that soluble toxic molecules are released by the NK cell and bind to specific acceptor sites on the target cell membrane (Roder et al., 1981). Human peripheral blood lymphocytes stimulated by lectins have been shown to release soluble factors which are toxic to NK sensitive but not NK resistant targets (Wright and Bonavide, 1981). The existance of acceptor sites for such molecules on the target cell membrane has been inferred from

data showing that NK resistant mutants of Yac cells bound normally to the NK cell but resisted lysis (Roder et al., 1981).

1.3.7 Modulation of NK activity

1.3.7.1 Enhancement

It has become apparant that NK cells produce γ-interferon (Djeu et al., 1981). The interferon producing capability of NK cells signifies that these cells play a major role in immunoregulation which goes far beyond their ability to lyse susceptible target cells. Interferon has anti-viral as well as antiproliferative properties (Epstein, 1978; Friedman, 1981; Stewart, 1981). It can inhibit virus dissemination by direct interference with viral replication and it interferes with tumour cell proliferation (Herberman et al., 1979). In addition, it has immunoregulatory activity. It regulates mitogenic responses and antibody production, and augments cytotoxic functions in NK cells (Trinchieri et al., 1978; Herberman et al., 1979), monocytes (Jett et al., 1980; Lo Bugilo et al., 1981) and T cells (Heron et al., 1976; Zarling et al., 1978). NK cells, by producing interferon, may participate in regulation of several arms of the host defence mechanism (Fig. 1.4).

Purified α and γ interferon from fibroblasts or leukocytes has recently been shown to augment NK cytolysis after in vivo and in vitro administration (Gidlund et al., 1978;

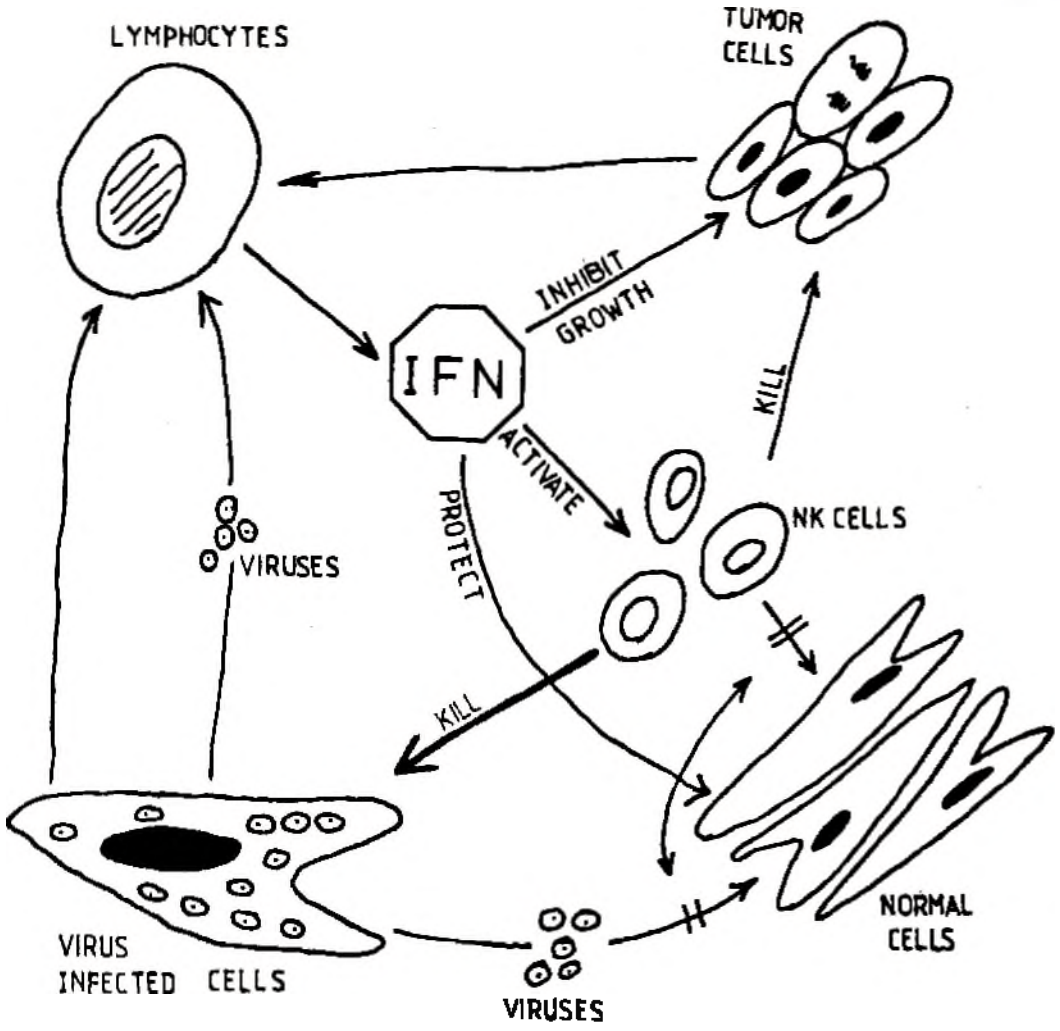

FIGURE 1.4 A MODEL FOR THE EFFECTS OF INTERFERON ON NK ACTIVITY IN VITRO.

Viruses, virsus infected cells, most tumour-derived cell lines and other IFN inducers stimulate lymphocytes to produce IFN, which inhibits the growth of rapidly dividing cells, inhibits viral replication in susceptible cells and enhances NK activity. IFN renders normal cells, and some tumour cells, resistant to lysis by NK cells whereas virus-infected cells are not protected.

Gresser et al., 1978) and anti-interferon antibodies block the NK enhancement observed in both mice (Gidlund et al., 1978) and humans (Herberman et al., 1975, 1979). Inducers of interferon such as tilorone, statalon, Newcastle disease virus (Gidlund et al., 1978); poly-iniosinic-poly-cytodylic acid (Oehler and Herberman, 1978; Oehler et al., 1978) and tumour derived or virus infected cells (Santoli et al., 1978; Trinchieri et al., 1978). also enhance NK mediated cytolysis in humans, rats and mice (Table 1.2).

In the mouse the enhancement of endogenous NK activity appears to reflect an increase in the intrinsic lytic potential of individual effector cells rather than recruitment or clonal expansion (Roder et al., 1978). The actual mechanism of interferon induced enhancement is still unknown but could conceivably involve an increase in the recycling rate of NK cells so that the total number of targets contacted and lysed with time is greater.

It is not known if interferon augments the triggering signal provided by the NK target or whether interferon itself is the signal. The latter possibility is supported by the observations that:

1) anti-interferon antibody inhibits NK activity in normal, non-interferon activated populations (Herberman et al., 1979; Arhlund-Richter et al., - submitted for publication)
2) certain patients with a profound depression in their ability to produce interferon have a concomitant

TABLE 1.2 MECHANISM OF AUGMENTATION OF NK ACTIVITY BY INTERFERON

A. Evidence in support of differentiation of pre-NK cells into mature NK cells

1) Differences in the phenotype of spontaneous NK cells and interferon induced NK cells.
2) Activation of cytotoxicity against K562 cells by LGLs not forming conjugates with foetal fibroblasts.
3) Reports that interferon induces increased proportions of lytic conjugate forming cells in the human single cell agarose assay.

B. Evidence for increase in activity of mature NK cells

1) There is no alteration in the percentage of lymphoid cells forming conjugates with NK susceptible target cells after interferon treatment.
2) In the human single cell agarose assay, interferon treatment resulted in an increased rate of target cell lysis.
3) Analysis of spontaneous or interferon augmented human NK activity by enzyme kinetic studies indicated only a change in the velocity of the reaction (Vmax).

depression in NK activity (Virelizier et al., 1977). Alternatively there are many targets which do not induce any detectable interferon secretion but are nevertheless lysed efficiently by NK cells which suggests that interferon is not an obligatory requirement for NK mediated cytolysis (Vose et al., 1979). It is possible that interferon simply blocks target derived inhibitory products such as prostaglandin (Droller et al., 1978a,b), by competing for the same ganglioside receptor on the NK cell. If interferon actively induced cAMP, which it does in most cells (Friedman et al., 1977), NK cytolysis would be greatly inhibited rather than enhanced since it has been shown that increasing cAMP levels suppresses NK activity (Roder and Klein, 1979). However, several hours prior to the increase in intracellular cAMP, interferon causes a transient increase in cellular cGMP (Tovey et al., 1979). Since cGMP also causes a slight enhancement of NK activity (Roder and Klein, 1979) it is possible that interferon enhances NK activity by virtue of its cGMP inducing effect.

Interferons have, however, a negative regulatory role at the target cell level: if target cells are pretreated with interferon they are partially protected from lytic attack (Trinchieri and Santoli, 1978; Welsh et al., 1981). The development of this protective effect is slower than that of the augmenting effect of interferon on effector cells. It could therefore be speculated that the point at issue concerns the down regulation of NK cells at a later stage of the cytolytic attack. Nevertheless, this protection may be of

importance in clinical trials in which interferons are injected into cancer patients.

Other physiological compounds augmenting NK activity include TCGF (Henney et al., 1981), retinoic acid (Goldfarb and Herberman, 1981), some antibodies against histocompatitiblity antigens (Saxena and Adler, 1979; Brunda et al., 1981) and IL-1 which operates at the level of the target cell (Herman et al., - submitted for publication). A more detailed mechanism of the augmentation by these substances has not yet been evualated.

1.3.7.2 Suppressor cells and NK activity

Several groups of investigators have provided evidence for a role of suppressor cells in the inhibition of NK activity (Lotzova, 1980; Santoni et al., 1980). However these cells have been difficult to detect and study in detail thus little is known of their nature and the mechanisms of suppression.

Suppressor cells for NK activity in various cancer patients have been described (Vose and Moore, 1979; Mantovani et al., 1980).Uchida and Mickshe (1981b) reported the presence of suppressor cells in carcinomatous pleural effusions from patients with lung cancer. There were populations of both nylon wool non-adherent suppressor lymphocytes and adherent monocyte suppressor cells in these effusions. These suppressor cells, which required a twenty four hour preincubation with the effector cells to mediate their effect,

did not reduce the number of effector-target conjugates. The decrease in cytotoxicity was irreversible and not abrogated by indomethacin suggesting that prostaglandins played no part in the suppression. In addition to macrophages, suppressor cells have been identified among T cells (Tarkkanen and Saksela, 1982), null cells (Cudowicz and Hochman, 1979) and granulocytes (Kay and Smith, 1983).

1.3.7.3 Inhibitors of NK activity

There are several techniques for regulating NK activity. Prostaglandins, especially of the E and A series, as well as macrophages (possibly through their capacity to produce prostaglandins) suppress NK activity (droller et al., 1978; Brunda et al., 1980; Santoni et al., 1980). Other inhibitory substances include estrogens (Seaman et al., 1978), cortisol (Parrillo and Fauci, 1978), immune complexes (Kay et al., 1979; Pape et al., 1979) and cytophilic monomeric IgG (Sulica et al., 1982).

1.3.8 The effect of disease on NK cell activity

Low NK activity has been described in association with malignant disease (Pross and Baines, 1976; Takasugi et al., 1977; Forbes et al., 1981; de Boer et al., 1982; Kadish et al., 1981; Cunningham-Rundles et al., 1981), leukemia (Linvat et al., 1980; Behelak et al., 1976; Ziegler et al., 1981; McGeorge et al., 1982; Fernandes et al., 1979), aplastic anemia (Linvat et al., 1980), chronic ulcerative herpes

simplex infection (Siegal et al., 1981), chronic renal failure (Badger et al., 1981; Lang et al., 1981), fibrocystic breast diseases (Cunningham-Rundles et al., 1981), and diseases such as rheumatoid arthritis (Karsh et al., 1981), systemic lupus erythmatosus (Karsh et al 1981; Silverman and Cathcart, 1980) and multiple sclerosis (Benczur et al., 1980; Santoli et al., 1981). In none of these disorders is there an invariable correlation between low NK function and disease, and certain of these reports are contrary to the findings of others (Santoli et al., 1981; Silver et al., 1982). The existance of NK depression has been reported in association with radiation therapy (Blomgren et al., 1980), extensive surgery (Gillou et al., 1982), cytotoxic chemotherapy (Badger et al., 1981), with prednisone therapy (Parrillo and Fauci, 1978) and diethylbestrol treatment (Kalland and Haukman, 1981). Removal of these agents results in recovery of normal NK function in most patients. The mechanism by which various diseases depress NK activity are complex, poorly understood and probably involve more then one component of the NK system as well as modulating substances such as interferon and prostaglandins (Koren et al., 1981; Targan, 1981).

A decrease in NK activity in multiple sclerosis is accompanied by a decrease in the ability to synthesise or respond to interferon (Benczur et al., 1980). A similar defect was inferred by Hersey et al. (1982) in Fanconi's syndrome.

Elevated NK activity in vivo has usually been associated

with activation by interferon. A number of inflammatory disorders such as atopic dermatitis (Leung et al., 1982), chronic hepatitis (Kakumu et al., 1978; Dienstag and Bhan, 1980), incipent renal transplant rejection (Gillou et al., 1982) and graft versus host disease (Dokelar et al., 1981; Lopez et al., 1979) have all been correlated with an increase in NK activity although it is not known if interferon plays a role in these circumstances.

1.3.9 The role of NK cells in resistance

1.3.9.1 Resistance against tumour cell lines

There is substantial evidence for an important role of NK cells in resistance against tumours in vivo, particularly those which show susceptibility to cytolysis by NK cells in vitro. A major approach has been to look for a correlation between resistance to the growth of the tumour cell lines in vivo and levels of NK activity in recipients.

Beige mice, with low NK activity (Roder and Duwe, 1979), have provided a convenient model for examining the role of NK cells in resistance of transplantable tumour lines to growth in vivo. Several NK susceptible syngeneic tumour lines have been shown to produce a greater incidence of tumours with high metastatic potential in beige mice as compared with the normal heterozygous littermates (Karre et al., 1980; Talmadge et al., 1980).

The transfer of increased anti-tumour resistance with cell populations enriched in NK cells (Kasi et al., 1979; Tam et al., 1980), and the inhibition of anti-tumour resistance by antisera reactive with NK cells (Pollack, 1982) support the contention that NK cells play a key role in the limitation of growth of transplanted NK susceptible tumour cell lines. However, there are two limitations to such evidence:

1) The administration of cells or antibodies may affect other effector mechanisms in addition to NK cell reactivity. Although the transferred populations were enriched for NK cells, they could also contain other lymphoid cells which may play an accessory role in anti-tumour resistance.

2) Induced alterations in NK reactivity are somewhat transient and could have led to secondary alterations in other effector functions that may be regulated by NK cells.

To obtain more direct evidence regarding the role of NK cells in the rapid elimination of tumour cells in vivo, ^{125}I deoxyuridine (^{125}IUdR) labelled tumour cells were intravenously injected into mice and clearence of radioactivity from the lungs and other organs monitered for two to four hours (Riccardi et al., 1979, 1980b). In mice with high NK activity there was greater clearence of radioactivity than seen in mice with low NK activity. It appears, therefore, that NK cells alone, without the need for other accessory cells, can efficiently eliminate tumour cells. However, the effectiveness of this natural resistance mechanism appears to be rather limited, even with tumour cells that are highly

susceptible to NK cell lysis and development of progressively growing tumours can occur in animals with good NK activity.

1.3.9.2 Resistance against metastasis

There are increasing indications that NK cells may play a major role in host resistance against metastasis. The ability of NK cells to rapidly eliminate intravenously innoculated tumour cells might provide a very effective control of haematogenous spread of tumours. Experimental support for this possibility first came from the finding that cells from lung metastases of transplantable tumours in mice were more resistant to NK cell attack than locally growing tumour cells (Gorelik et al., 1979). That metastatic cells might represent those cells which evaded elimination by relative resistance to NK activity was supported by the demonstration that selection of NK resistant variant tumour cells in vitro was accompanied by an increase in the metastatic capability of such cells (Gorelik et al., 1982a).

That NK cells are important in resistance against metastatic growth of transplantable tumours has further been supported by the following observation: suppression or augmentation of NK activity of mice was associated with parallel alterations in resistance to artifical metastases produced by intravenous innoculation of tumour cells (Hanna and Fidler, 1980, 1981). In addition, nude mice, which have elevated levels of NK activity, were found to have increased resistance

to lung metastases, whereas beige mice, with deficient NK cell activity, developed numerous lung metastases (Hanna, 1980; Talmadge et al., 1980; Gorelik et al., 1982b).

Since the above mentioned experiments were performed with inbred mice lacking certain cell populations it was important to evualate the role of NK cells in the develpoment of spontaneous metastases from locally growing tumours. Gorelik et al. (1982b) approached this issue by surgically excising tumours growing the footpads of mice treated repeatedly with either anti-asilo GM-1 (an anti mouse NK cell antibody) or normal rabbit serum. It was found that the number of lung metastases in animals with depressed NK activity was substantially higher than in mice with normal levels of NK activity, confirming the work performed in beige mice. Similarly Hanna and Schneider (1983) showed that oestradiol-17 (a substance which decreases NK activity) treated mice had a higher incidence of spontaneous pulmonary metastases.

The available evidence seems to suggest that NK cells play a major role in resistance against the metastatic spread of tumours. The presence of NK cells in the blood may allow them to be particularly effective in elimination of most, if not all, tumour cells which seed into the circulation. Therefore the development of agents and protocols which can induce substantial augmentation of NK activity may be expected to lead to more effective prevention and control of metastatic dissemination of tumours.

1.3.9.3 Resistance against primary tumours

There is some evidence that NK cells can play a role in the defence against the growth of primary tumours. The majority of spontaneous mammary tumours or C3H mice (Serrate and Herberman, 1982a) and lymphomas in AKR mice (Nunn *et al.*, 1977) are susceptible to lysis by NK cells. Similarly some human tumours are significantly lysed by NK cells (Rosenberg *et al.*, 1972; Zarling *et al.*, 1979; Axberg *et al.*, 1980; Vanky *et al.*, 1980; Mantovani *et al.*, 1982; Pattengale *et al.*, 1982; Serrate *et al.*, 1982b). Such lysis can be appreciably augmented when the effector cells are pretreated with interferon. Of particular importance has been evidence that NK cells from autologous or syngeneic individuals react against primary tumour cells (Serrate and Herberman, 1982a).

Another line of evidence in support of a role for NK cellsin interactions with autologous primary tumour cells *in vivo*, is the demonstration that NK cells accumulate at the site of tumour growth (Gerson, 1980). NK cells have been detected in small spontaneous mouse mammary carcinomas and in small primary mouse tumours induced by murine sarcoma virus (Becker, 1980; Gerson, 1980). In contrast NK activity has usually been undetectable in large tumours in mice or in clinical tumour specimens (Gerson, 1980). This may be due, in part, to the presence of suppressor cells which have been demonstrated in cell suspensions from several tumour specimens (Gerson, 1980; Gerson *et al.*, 1981; Vose, 1980; Eremin, 1980; Allavena *et al.*, 1981; Uchida and

Mickshe, 1982).

1.3.9.4 The role of NK cells in immune surveillance against tumours

A question of paramount importance is whether NK cells are involved in immune surveillance against the initial development of spontaneous or carcinogen induced tumours. There are several pieces of circumstantial evidence consistent with, or suggestive of, a role for NK cells:

1) Patients with the genetically determined Chediak-Higashi syndrome have a high risk of developing lymphoproliferative disorders (Dent et al 1966). In recent detailed studies on several patients with this disease (Roder et al., 1980, 1982) all patients investigated were found to have profound deficits in NK and K cell activity, whereas a variety of other immune functions, including cytotoxicity against tumour cells by T cells, monocytes and granulocytes were essentially normal.
2) Similarly, beige mice, which have an analogous genetic defect also have a substantial (Roder and Duwe, 1979; Roder et al., 1979a) but incomplete (Brunda et al., 1980) selective deficiency in NK activity. Aged beige mice have recently been shown to have a high incidence of lymphomas (Loutit et al., 1980; Haliotis et al., 1982) whereas heterozygous mice without the beige phenotype had a lower incidence (Haliotis et al., 1982).
3) Another human genetic abnormality, X-linked lymphroliferative disease (Purtilo et al., 1977) has been assoc-

lated with a defect in the ability to control proliferation of B cells infected with Epstein-Barr virus (EBV). Recently, low NK activity has been found in such individuals, and this defect was proposed to be involved in the pathogenesis of the disease (Sullivan et al., 1980; Seeley et al., 1982). In support of this possibility, cells with the characteristics of NK cells have been found to inhibit the proliferation of autologous EBV-infected B cells in vitro (Masucci et al., 1982).

4) Patients on immunosuppressive therapy after kidney allograft transplants have a high risk of developing tumours including both reticuloendothelial tumours and a variety of carcinomas (Penn and Starzl, 1972). Patients on such treatment regimens have been found to have low NK activity and this has been suggested as a contributing factor to the subsequent development of malignancy (Lipinski et al., 1980).

5) Patients with paroxysmal nocturnal haemoglobinuria also have a high risk of developing leukemia in addition to exhibiting low NK activity (Yoda et al., 1982).

Each of these lines of evidence fits one of the major predictions of the immunosurveillance theory, that tumour development would be associated with and, in fact, proceeded by, depressed immunity.

A related prediction of the immune surveillance theory is that carcinogeneic agents would cause depressed immune

function, thereby impairing the ability of the host to reject transformed cells. This postulate has been examined by many with regard to the possible role of mature T cells and humoral immunity and conflicting results have been obtained (Stutman et al., 1975). In contrast initial and still fragmentary data suggests a contribution of NK cells to resistance against the development of at least certain types of tumours. The results of studies performed in this field support the possibility that one of the requisites for tumour induction by carcinogeneic agents may be interference with host defences, including those mediated by NK cells.

Based on the available data the hypothesis that NK cells are responsible for early surveillance against malignancies of the haematopoietic system and possibly blood borne metastases from solid tumours would appear to be valid. Further studies, however, are needed to directly demonstrate a role for NK cells in immune surveillance. Demonstration of increased tumourogenesis in association with selective NK cell depression and reduced tumour formation when NK cell activity is selectively augmented would provide further evidence in support of this hypothesis. Several practical problems have limited vigorous pursuit of such experimental protocols. In addition to the long periods of time required for such studies and the difficulties in identifying the most relevant experimental carcinogen models, completely selective and sustained alterations of NK activity are not easily found or produced.

Many observations have been presented in support of a fundamental role for NK cells in anti-tumour surveillance, such a theory must however take into account several seemingly paradoxical observations:

1) the vast majority of cancer patients in the early stages of disease have normal levels of NK activity (Pross and Baines 1976; Takasugi et al., 1977; Forbes et al., 1981; Hersey et al., 1978; de Boer et al., 1982; Kadish et al., 1981; Cunningham-Rundles et al., Eremin et al., 1981; Heidenreich et al., 1977).
2) fresh tumours are often relatively insensitive to lysis by NK cells (Werkmeister et al., 1979; Vose and Moore, 1980).
3) lymphocytes isolated from tumour biopsies often lack NK activity (Eremin et al., 1981; Vose et al., 1981; Moore and Vose, 1981).
4) interferon is relatively ineffective at inducing NK activity against autologous biopsy-derived target cells (Vanky et al., 1980).
5) the level of NK activity in irradiated leukemic patients after successful transplantation of bone marrow does not correlate with the prevention of relapse (Linvat et al., 1980).

All of these observations are however compatible with the hypothesis which states that NK cells are circumvented by tumour variant formation (Roder et al., 1981; Gronberg et al., 1981; Durdik et al., 1980; Poste and Fidler, 1980). As shown in the mouse, early neoplastic cells may be highly

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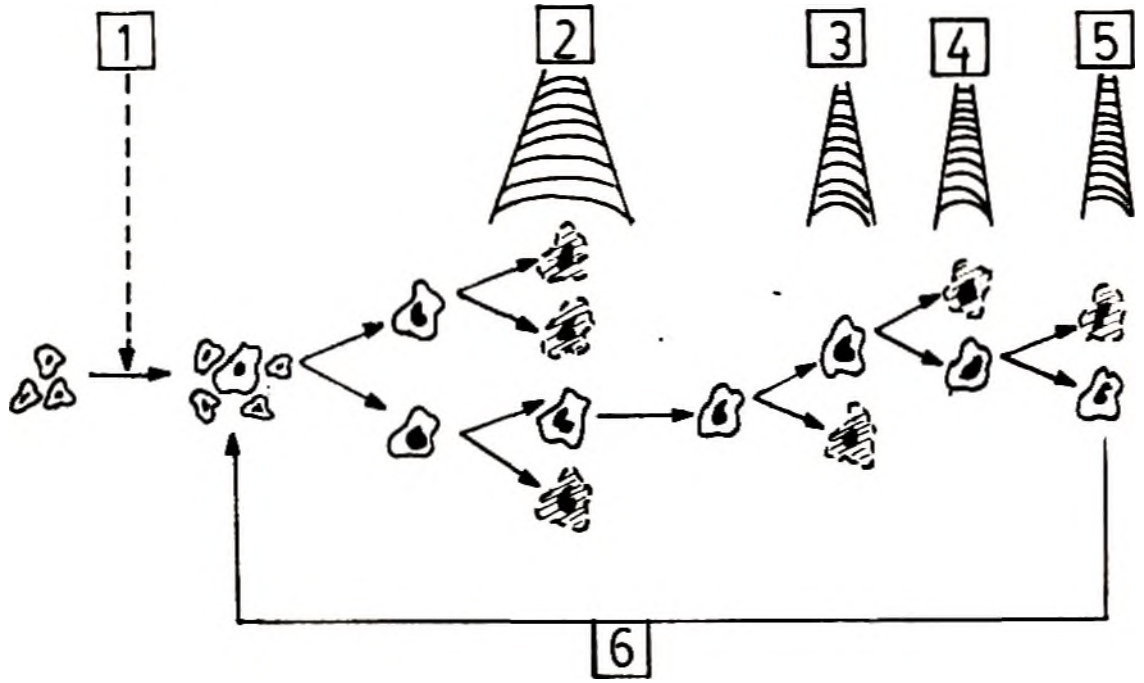
All of these observations are however compatible with the hypothesis which states that NK cells are circumvented by tumour variant formation (Roder et al., 1981; Gronberg et al., 1981; Durdik et al., 1980; Poste and Fidler, 1980). As shown in the mouse, early neoplastic cells may be highly

sensitive to natural cytotoxicity (Roder et al., 1981; Durdik et al., 1980; Gorelik et al., 1982), whereas variants can be selected which resist NK cell lysis and develop into an observable tumour. This mechanism may also be operative in metastasis formation. (Fig. 1.5).

Escape from NK cell surveillance may also result from the local suppression of NK activity by tumour products, for example prostaglandins (Bankhurst, 1982), tumour surface "antigens" (Roder et al., 1979a; Ortaldo et al., 1977) or carcinogens and tumour promoters as has been shown with urethane (Gorelik and Herberman, 1981) and phorbol esters (Keller et al., 1982).

1.3.9.5 Resistance against microbial infections

There have been an increasing number of suggestions that NK cells are important in resistance to microbial infections. Most of the studies are related to virsuses with several investigators showing that cells infected with a variety of virsuses became considerably more sensitive to lysis by NK cells (Bloom et al., 1980; Santoli et al., 1980). Persistantly virally infected tumour cells grow poorly in nude mice apparently as a result of interferon induction and subsequent reactivity of NK cells (Bloom et al., 1980). Furthermore, resistance to infection by several types of viruses in vivo has been found to correlate with NK activity. There is considerable evidence for a role of NK cells in genetic



1. A neoplastic transformation occurs via somatic mutation, viral or chemical induction.
2. Proliferating tumour cells are held in check by NK cells, which pre-exist at high numbers.
3. After a sensitizing period of several days, some of the remaining tumour cells are lysed by immune T cells assuming that the tumour is antigenic.
4. Immune T cells are triggered by tumour antigens to release macrophage-activating factors which induce macrophages to lyse remaining tumour cells.
5. Finally, small amounts of anti-tumour antibody may appear in the blood and bind to any remaining tumour cells thus rendering them susceptible to ADCC.
6. Tumour cells escaping from the primary tumour during metastasis are lysed by NK cells during transport to, or at, the metastatic foci (assuming NK variants have not arisen).

FIGURE 1.5 THE PLACEMENT OF NK CELLS IN A HYPOTHETICAL SEQUENCE OF ANTI-TUMOUR SURVEILLANCE.

resistance of mice to severe infection by herpes simplex virus type 1 (Lopez, 1980). Some of the data are not entirely supportive of this association (Kirchner et al., 1982). A correlation has been found between susceptibility to severe herpes virus infection in patients with low NK activity against virally-infected target cells (Lopez et al., 1982). It also seems likely that NK cells play an important role in natural resistance to infection by mouse cytomegalovirus (Shellam et al., 1982). There have also been some indications that natural genetic resistance to another herpes virus, Mareks disease virus, in chickens may be mediated by NK cells (Lam and Linna, 1980). Cells with the characteristics of NK cells were found to transfer resistance to susceptible newborn chicks. In contrast to these suggestions indicating an important role of NK cells in protection against infection by several virsuses, it should be noted that there is evidence against a role of NK cells in resistance to virsuses such as lymphocyte chriomenigitis virus (Welsh and Kiessling, 1980), influenza virus (Haller et al., 1980) and mouse hepatitis virus (Krichner and Schnidler, 1982).

NK cells may also be involved in resistance against certain other types of micrbial infection. A correlation between NK activity and resistance of mice to the malarial parasite Babesia microti has been documented (Ruebush and Burgess, 1982). Beige mice were found to be highly susceptible to infection whereas herteozygous normal mice were more resistant to this parasite.

There is also some suggestive evidence for a role of NK cells in infection by Trypanosoma cruzi a parasite which has been shown to be susceptible to cytotoxicity by NK cells in vitro (Hatcher and Kuhn, 1982).

NK cells are thought to function in resistance of mice to cryptococcus neoformans (Murphy and McDaniel, 1982). During the first two weeks after infection by this agent nude mice were found to be more resistant to growth of the organism than euthymic mice. Cells with the characteristics of NK cells were found to mediate inhibition of colony formation by the fungus.

1.3.10 Other functions of NK cells

NK cells may not be involved exclusively in immune surveillance and protection against foreign antigens. There is evidence that they also play a role in regulating the response to bone marrow transplants and the development of certain immature cells such as undifferentiated thymocytes.

Mice given lethal doses of radiation are no longer able to reject skin grafts, but can still reject bone marrow grafts from entirely unrelated strains. Nude, athymic mice have a similar ability . This bone marrow response cannot therefore be a thymus dependant property is a skin graft rejection. Beige mice, however, tend to accept bone marrow transplants more readily than mice with normal NK activity.

The genetics of bone marrow rejection also indicate that cells other than T lymphocytes are involved. Additionally,a number of treatments which affect NK activity seem to affect natural bone marrow resistance. It now appears very likely that NK cells are the principal effectors of natural resistance to bone marrow grafts.

NK cells may also be involved in the graft versus host (GVH) response. The mechanism involved in this process has never been clear, but there appears to be a correlation between the incidence of GVH disease and the level of NK activity in the patient prior to a marrow transplant. When patients are given immunosuppressive treatment to allow a bone graft to take, their typical T cell mediated functions do not reappear for three months or longer; NK activity, however, returns earlier, often within days and certainly within one month. Furthermore when GVH disease appears, usually about seven days after the graft, it is accompanying the return of NK activity. While these correlations suggest that NK cells may cause GVH disease, there are alternative explanations. GVH disease may, for example, be the result of elevated production of interferon which, in turn, enhances NK activity.

It has recently been shown that bone marrow stem cells are susceptible to in vitro NK cytolysis (Herberman and Ortaldo, 1981). Thus another function of NK cells may be to regulate the normal differentiation of certain cells such as haematopoietic stem cells. There may be a certain point in the differentiation pathway when stem cells are most suscep-

tible to in vitro NK cytolysis (Herberman and Ortaldo, 1981). Thus another function of NK cells may be to regulate the normal differentiation of certain cells such as haematopoietic stem cells. There may be a certain point in the differentiation pathway when stem cells are most susceptible to this control. Lysis of stem cells by NK cells may not be the only control mechanism NK cells may merely inhibit stem-cell growth. There is, however, no clearly recognised role for NK cells in stem-cell regulation.

Another population of normal cells sensitive to NK activity are undifferentiated thymocytes. NK cells may be involved in regulating the number of T cells that eventually mature. There is evidence that, of the thymocytes developing in the thymus, only one in ten thousand ever exits. The others die within a few days of maturation. This may not be an expression of the cells' limited life-span but the result of down regulation similar to that which may happen to bone marrow stem cells.

The question of whether haematological disorders involving bone marrow progenitors could be due to an "over regulation" of NK cells exists. One haematological disorder where there have been several reports indicating a possible autoimmune aetiology is aplastic anemia (AA) (Kagan et al., 1976; Hoffman et al., 1977). Several different groups have described lymphocytes, mainly of T cell nature, from AA patients which can suppress erythropoiesis (Torok-Storb et al., 1978) or myelopoiesis, in vitro, (Bacigalupo

et al., 1980). These latter workers found that the patients myeloid colony formation could be enhanced by removing T_γ cells from the marrow. These suppressor T_γ cells could also suppress normal allogenic colony formation (Bacigalupo et al., 1980). The exact relationship between T_γ suppressor cells (T cells with Fc receptors for gamma globulin) described here and NK cells which are also FcR^+, is however, unclear and further investigations of a possible relationship between this suppressor cell and NK cells would be intresting.

A considerable proportion (30-50%) of AA patients have been found to have deficient NK and ADCC activity (Linvat et al., 1980). If AA is caused by NK cell mediated autogolous marrow destruction high NK cell activity levels in these patients would be expected. There are, however, several difficulties in interpreting the absence of such a correlation. Most probably the absence of NK cells in the circulation of some of these patients could be secondary to a general stem cell deficiency and may be caused by an effect of the stem cell common to NK cell and other cell types. The low NK activity among some of the AA patients could also be explained by the migration to the bone marrow of NK cells.

Very little information regarding correlations between NK cells and other types of anemia exists. One report describes a 19 year old man with pure red cell aplasia. He showed an unusually high proportion of lymphocytes with the morphology of LGL in both the blood and bone marrow. He also had leukocytes displaying strongly elevated NK activity

against the erythroblastoid leukemic line, K562, in vitro. In addition his non-T blood lymphocytes strongly inhibited autologous and allogeneic erythroid colony formation in vitro but not granulocyte-monocyte growth (Hansson and Kiessling, 1983). Another report of a young man with AA has shown that LGLs, isolated from the blood of this patient, could inhibit the growth of allogenic and autologous bone marrow in vitro. In this instance the numbers of LGLs were not significantly increased although in vivo levels of interferon were raised (Goss et al., - submitted for publication). Although these case reports are only suggestive of NK cells as being at least partially responsible for this type of anemia, they point towards an important area of clinical researech.

NK cells are defined by their lytic capicity although a postulated regulatory mechanism is likely to be important. They are thought to secrete interferon as well as IL-1 and IL-2. The fact that these cells are capable of secreting such soluble mediators makes a regulatory function seem probable. The type of NK cells "policing" an organism as a first line of defence against malignant clones could be extended to their playing a role against the uncontrolled growth of normal and transformed cells.

1.3.11 Characteristics of cultured NK cells

Purified LGL populations have been used as starting cell populations in attempts to culture NK cells in vitro. The

only growth factor detected which maintains the proliferation of cytolytically active NK cells is TCGF. Initially the cells are induced to divide by phytohaemagglutinin (PHA). Subsequent culturing is then possible with the aid of lectin free growth factor (Timonen et al., 1982, 1983; Hercend et al., 1983). Cultured NK cells are morphologically LGLs but their surface phenotype is closer to that of conventional T cells. They partially express antigen detected by the T associated OKT3 monoclonal antibody and almost all of them have high affinity receptors for sheep erythrocytes, although the initial purified LGL population has been depleted of such cells. Quite recently, LGLs have been cloned in the presence of conditioned medium derived from the supernatant of lymphocyte cultures stimulated with phorbol myristate acetate (PMA) or PHA (Hercend et al., 1983). The surface phenotypic and functional analysis of the clones revealed clear clonality of NK cells. These clones have been cytotoxic only to K562 cells although occasionally activity against some solid tumour-derived target cells has also been observed in some clones.

The expression of antigenic determinants detected by the monoclonal antibodies OKM1, OKT3, OKT8 and OKT4 have also been variable in individual clones. It remains to be established how common this heterogeneity is in NK cells in vivo since, only one in seventy, and at most one in five, LGLs are initially proliferative in cultures (Timonen et al., 1983). In mice, NK cell clones have been reported to be homogenous in their target cell selectivity (Dennert

et al., 1981). It is not clear whether this apparent discrepancy in the results from human and animal studies is due to technical differences in the procedures to initiate the cultures or to a real biological phenomen.

1.4 Soluble mediators and carcinogenesis

Increasing evidence suggests that cytokines, the hormones of the immune system that maintain the stability or homeostasis of this system, have the potential to greatly influence the development of cancer (Evans, 1982). Prevention of carcinogenesis (Gresser, 1977; Evans and DiPaolo, 1981; Ransom et al., 1982), destruction of preneoplastic cells (Ransom and EVans, 1982), suppression, as well as eradication of fully tumourgenic cells due to the presence of various cytokines have been reported (Fidler et al., 1981; Khan et al., 1982; Papermaster et al., 1980; Cheever et al., 1982; Klein et al., 1981; Valentine et al., 1982). These are important developments in the quest for the prevention and eradication of cancer, because cytokines are both important molecular probes in the search to understand neoplasia and potentially powerful new agents for the prevention, control and elimination of malignancies. To determine the role of cytokines in cancer it will be necessary to delineate their physiologic function and the way in which they influence carcinogenesis.

Lymphokines are lymphocyte glycoproteins possessing typical hormone activities namely secretion by one cell, specific interaction with another target cell and physiologic

regulation under feedback control of specific vital function (Cohen et al., 1979; Deweck et al., 1980). Some common cytokines are IL-1, IL-2, lymphotoxin and interferon to name a few. Lymphokines are often leukocytotrophic in their target cell detection and immunoregulatory in their action. Interleukocyte secretory activities were first recognised in 1966 (Bloom and Burnet, 1966; David, 1966). Interleukin-1 is mitogenic for T cells and stimulates them to secrete IL-2 (Smith et al., 1980a). It has also been found to play a role in NK activity (Herman et al., submitted for publication). IL-2 promotes the continued replication of T cells in culture (Smith et al., 1980b). Interferon, although not formally labelled as an interleukin, stimulates NK cell activity (Djeu et al., 1978) and thus has interleukin-type properties. As the actions of other cytokines continues to be defined, many and possibly all, will be shown to have interleukin-type activities.

The cytophilic behaviour of lymphokines and cytokines, although frequently lymphocytotrophic, can be quite diverse, as evidenced by the ability of IL-1 to interact with fibroblasts and synovial cells (Schmidt et al., 1982; Mizel et al., 1982), as well as the ability of interferon and lymphotoxin to affect the growth and/or function of a variety of non-lymphoid normal cells and tumour cells (Brouty-Boyle, 1980). In addition to being secreted by lymphocytes, some lymphokines are released by non-lymphoid cells. Thus although lymphokines are primarily thought of as lymphocyte-derived immunoregulatory hormones, these cytotrophic,

bioactive glycoproteins can be secreted by cells other than lymphocytes with the hormonal action of lymphokines extending beyond the immune system.

Just as lymphokine secretion can be pluricellular in origion and lymphokine target direction can be pluricytotrophic, the regulatory actions of these glycoprotein hormones are pluripotential and multifacted. Like most groups or classes of hormones, lymphokines display stimulatory, inhibitory and antagonistic actions. Lymphokine stimulation is exemplified by:

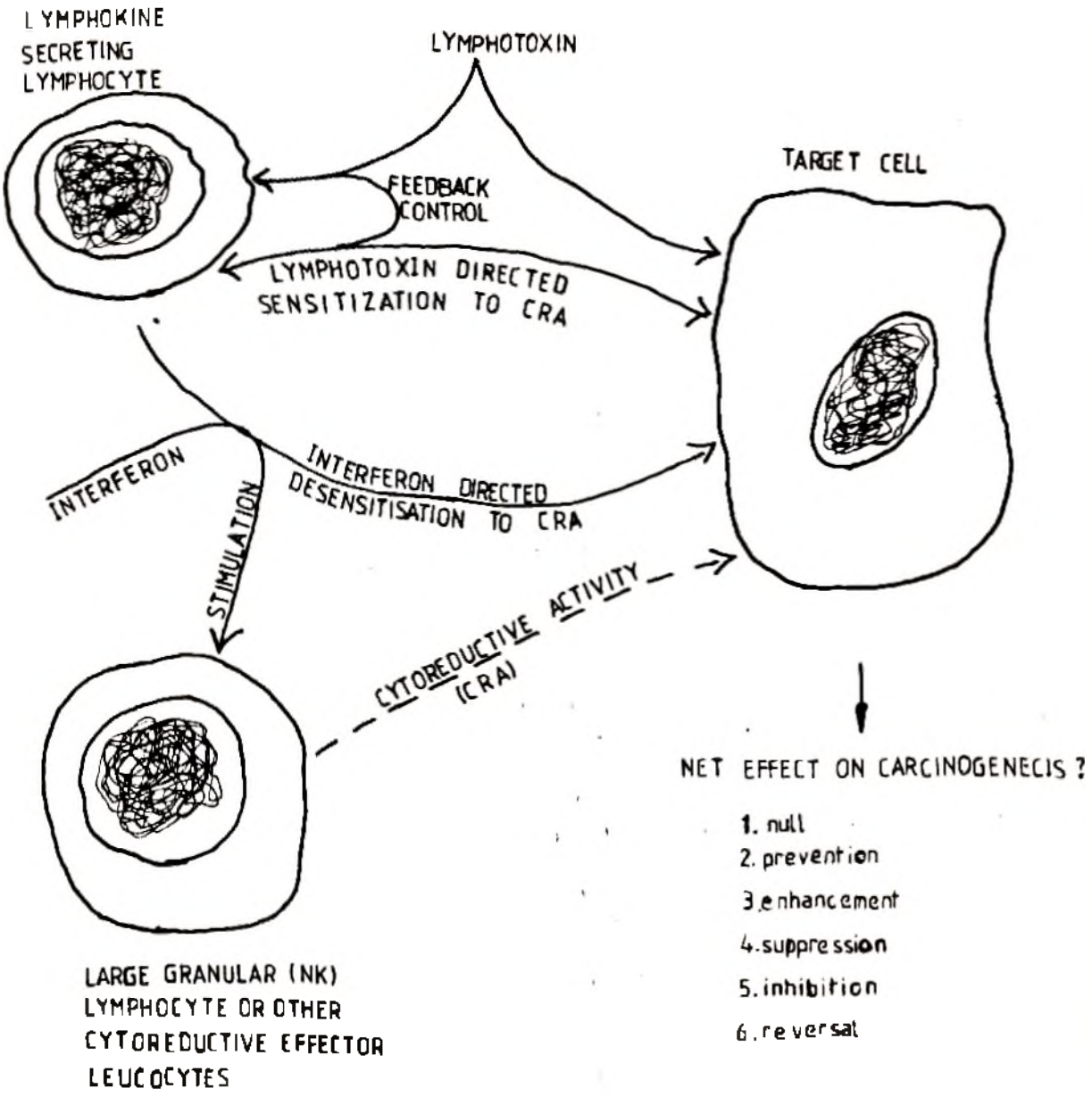
1) the induction of IL-2 secretion by IL-1
2) T cell proliferation induced by IL-2
3) by the enhancement of the sensitivity of tumour cells to NK cell destruction by IL-1 and lymphotoxin and
4) the enhancement of NK activity by interferon.

Less well appreciated but increasingly recognised and potentially of great physiologic importance are antagonistic or disparate lymphokine regulatory actions. The ability of interferon to inhibit tumour cell growth and viral carcinogenesis and yet to enhance certain types of carcinogenesis is one example (Brouty-Boyle and Little, 1977).

Homeostasis, the natural progression toward stability in normal body states, is achieved through a balance of self-regulatory mechanisms. It is the net result of pluralistic additive, synergistic, inhibitory and antagonistic mechanisms. Until recently, many of the actions of cytokines on carcinogenesis and tumour growth could be attributed

only tentatively to one or more of these soluble mediators present in the preparations understudy. The increasing availability of purified lymphokines, present in conjunction with specific antibodies and inhibitors, however, now permits precise assignment of anti-carcinogenic activities to individual lymphokines. As a result, it is becomming possible to identify both the cytokine components and their mechanisms of action in immunologic defences directed against cancer. The ability of lymphoid cells and their products to participate in a variety of homeostatic responses has been recognised for many years (Fidler, 1980). Recent observations suggest that the stimulatory and inhibitory activities of, naturally immune, lymphoid cells for carcinogenesis (Prehn, 1971) may be mediated, in part, through lymphotoxin (Evans et al., 1982). Moreover, emerging evidence suggests that the actions of several lymphokines, individually or in concert, may be the important and possibly critical interactions necessary for the physiological prevention and control of the initiation and progression of cancer.

One example where the participation of, and perhaps the balance between, the actions of several lymphokines affects the regulation of host defence against cancers is the stimulatory, yet potentially antagonistic, actions of interferon and lymphotoxin in NK cells destruction of preneoplastic, as well as fully tumourgenic, cells (Fig. 1.6). Interferon is able to increase NK cell cytolytic destruction of tumour cells directly but paradoxically increases the resistance of tumour cells to NK cell lysis (Trinchieri and



Lymphotoxin and interferon can originate intrinsically and/or extrinsically in relation to the reaction of effector and target cells.

FIGURE 1.6 PATHWAYS OF POTENTIAL LYMPHOTOXIN AND IFN STIMULATORY AND INHIBITORY ACTIONS IN NATURAL LYMPHOID CELL-MEDIATED PREVENTION, CONTROL AND ERADICATION OF CARCINOGENESIS.

Santoli, 1978). Conversely, lymphotoxin has no direct effect on the cytotoxicity mediated by NK cells, yet it increases the sensitivity of preneoplastic and tumour cells to NK cell destruction (Ransom and Evans, 1982). Lymphotoxin enhancement of tumour cell sensitivity, furthermore, is rapid, occuring within thirty minutes, whereas interferon induced resistance to NK destruction occurs later, two to three hours after exposure of target cells to interferon (Evans, 1983). A lymphotoxin-like soluble mediator has been identified in NK cell mediated cytolysis (Wright and Bonavida, 1981). It is, however, not yet known whether during NK cell cytolysis lymphotoxin participates directly in the cytolytic destruction of the tumour target cell or enhances the sensitivity of the target cell to destruction or both. Lymphotoxin secretion is also subject to feedback type regulation (Rundell and Evans, 1979), which could limit the amounts of effective lymphotoxin generated in an NK cell reaction.

Lymphokine stimulatory and inhibitory actions and feedback regulation may also contribute to the different stages of carcinogenesis (Prehn, 1977). Lymphotoxin has been shown to be at least partially responsible for the inhibition of carcinogenesis by normal spleen lymphocytes (Evans et al., 1983). Lymphotoxin, moreover, can inhibit carcinogenesis at several points or stages. Firstly, it can prevent the development of radiation carcinogenesis or chemical carcinogenesis by an, as yet, unidentified noncytotoxic mechanism (Evans and DiPaolo, 1981). After carcinogen exposure, lymphotoxin can irreversibly inhibit the further development

of initiated and phorbol diester-promoted transformation (DiPaolo et al., 1981). Subsequent stages in carcinogenesis are susceptible to the various cytotoxic or cytoreductive action of lymphotoxin. Tumour cells are also susceptible to the growth-inhibitory, and to a lesser extent, the cytolytic activities of this hormone (Evans and Heinbaugh, 1981). Susceptibility to lymphotoxin cytotoxicity, furthermore, can develop either transiently or permantly during the preneoplastic stages. It is however as a permanent genotypic alteration once expressed during the neoplastic stages of carcinogenesis (Ransom and Evans, 1982; Evans et al., 1981).

Lymphotoxin, therefore has the potential to alter the development of cancer through several diverse mechanisms. The net result can be prevention or inhibition of carcinogenesis. Alternatively, stimulation of carcinogenesis is possible should the hormone be down regulated or otherwise inhibited during modulation of natural lymphoid cell cytotoxicity (Evans et al., 1982). Down regulation may be one means by which the developing cancer cells can circumvent and escape inherent homeostatic mechanisms for the prevention and control of cancer. Elucidation of the mechanisms by which the cytokines, individually and collectively, maintain homeostasis is necessary to select the cytokine activities that warrent monitering in individuals at increased risk of carcinogenesis. Three recent important observations require further discussion. Lymphotoxin can induce or stimulate interferon secretion (Svedersky, 1982) and thereby indirectly stimulate NK cell activity. This mechanism of NK cell stimu-

lation, in conjunction with lymphotoxin-induced increased target cell sensitivity to NK cells, would amplify the net target cell destruction. This is one example of the complex synergistic, potential anti-carcinogenic activities of the lymphokines. Interactions with other hormones are also possible. Two examples are:

1) hydrocortisone has been shown to directly inhibit lymphokine secretion (Gillis et al., 1982) and
2) insulin has recently been shown to negate the growth inhibitory action of lymphotoxin in insulin-responsive tumour cells (Evans, 1983).

The potential, therefore, exists for combinations of lymphokines and non-immunologic hormones to result in either stimulation or inhibition of carcinogenesis. The extent to which lymphokines actually regulate carcinogenesis remains to be defined, because the additive and synergistic actions of lymphokines constitute a relatively new area of immunology.

Lymphokines are natural and pharmacologically augmentable immunophysiological regulators. As such, they can be powerful positive or negative forces in the intervention and management of cancer. Little is known about the normal quantities of lymphokines secreted. It is important that lymphokine secretion and function in healthy individuals, as well as those considered at increased risk for carcinogenesis be assessed. Alone, various lymphokines may be insufficient and ineffective as cancer preventive and/or therapeutic agents, In combination, however, lymphokines

offer an adjunct to preventive, diagonistic and therapeutic modalities of a powerful force in the prevention, control and eradication of cancer.

CHAPTER 2

2. ISOLATION OF LARGE GRANULAR LYMPHOCYTES FROM HUMAN PERIPHERAL BLOOD

2.1 Introduction

Natural killer (NK) cells are non-adherent, non-phagocytic mononuclear cells having spontaneous cytotoxic capacity against tumour target cells *in vitro*. It is becoming increasingly apparent that these cells have other functions in addition to the original postulate of immune surveillance. Roles for NK cells in the rejection of bone marrow transplants, as regulators of cellular development, as major producers of interferon and as effectors against microbial infections have been hypothesised (Biddison and Palmer, 1977; Blazer *et al*., 1980; Herberman, 1982).

It has recently been shown that human NK cells are morphologically large granular lymphocytes (LGL), being approximetly 50% larger than a typical lymphocyte and having azurophilic granules in their cytoplasm (Saksela *et al*., 1979a; Timonen *et al*., 1979a,b). They also have a higher proportion of cytoplasm thus a relatively lower density than other lymphoid cells which makes their isolation possible. The technique employs the use of discontinuous density gradients prepared with percoll - a non-toxic inert silica polymer (Timonen and Saksela, 1980).

As this method fractionates cells displaying NK activity efficiently, resulting in effective enrichment and good

cell yields, it was decided to employ the technique in the isolation of LGLs in order to study them further.

The purity of the LGL fraction was monitered using monoclonal antibodies and complement, immunofluorescent studies and phagocytosis of candida albicans and peroxidase staining.

It has previously been shown that treatment of lymphocytes with OKT11A, a monoclonal antibody which reacts with all human thymocytes and sheep red blood cell rosetting lymphocytes, and complement markedly decreases NK cell activity. Although approximetly 90% of the $OKT11A^+$ cells are $OKT3^+$, NK cell activity resides within the $OKT11A^+$ cell population which is $OKT3^-$, since $OKT3^+$ cell depletion fails to decrease NK activity (Zarling et al., 1981).

Due to the fact that any contaminating B cells in the LGL fraction would fluoresce with fluorescent labelled goat-anti-human immunoglobulin this technique was used to moniter B cell contamination.

Candida albicans was added to the LGLs to moniter phagocytosis by any contaminating monocytes. Myeloperoxidase staining was also utilized to moniter monocyte contamination.

The most common method for assaying NK activity, a ^{51}chromium release assay, was used to determine in which of the percoll fractions NK activity could be found. Monocyte

depleted peripheral blood mononuclear cells, further enriched for LGLs as previously described, were used as effector cells. LGLs were mixed in various ratios with ^{51}chromium labelled target cells. The target cells utilized were K562 cells, a line established by Lozzio and Lozzio (1975) from a pleural effusion of a patient with chronic myelocytic leukemia, in blast crisis. This cell line has several advantages, namely its high sensitivity to NK cell lysis in both short and long term assays, spontaneous release of ^{51}Cr is low, it is HLA-A B C negative, Epstein Barr nuclear antigen negative, is easily maintained in suspension culture and is comparatively insensitive to spontaneous monocyte mediated lysis (Pross and Baines, 1980).

2.2 Materials and methods

2.2.1 Separation of mononuclear cells from human peripheral blood

Mononuclear (MN) cells were isolated from heparanized human peripheral blood from normal, healthy donors, by centrifugation on a Ficoll-Hypaque density gradient according to the method of Boyum (1968).

The band of mononuclear cells at the plasma - Ficoll interface was collected, washed three times in physiological saline, and the cells resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2mM L-glutamine, 100 units/mℓ penicillin and 100 mg|mℓ strepto-

mycin which will be referred to as complete-RPMI (c-RPMI),

2.2.2 Depletion of adherent cells from mononuclear cell preparations

Mononuclear cells in c-RPMI were adjusted to a concentration of 2 x 10^6/mℓ. 30 x 10^6 cells were placed in a 75cm² 250mℓ Falcon tissue culture flask and incubated in a 5% CO_2 humidified enviornment at 37°C for 2 hours. The medium containing non-adherent cells was poured out and the flasks washed twice with medium. The cells were pelleted and resuspended in c-RPMI, counted and checked for contaminating macrophages.

2.2.3 Depletion of B cells on nylon wool columns

Nylon wool from leukopak leukocyte filters was washed overnight in 0.02M hydrochloric acid then rinsed repeatedly in distilled water until the pH was neutral. It was then dried, teased apart and autoclaved in aluminium foil. The nylon wool was stored in this way until used.

The required amount of nylon wool (3.5g/50mℓ syringe) was teased apart in RPMI 1640, using forceps, until no resistance was felt. 50mℓ syringes were packed by placing small pieces of the saturated nylon wool into the syringe. The "column" was washed with approximetly 100mℓ of medium. At this stage the columns could be stored for up to 7 days at 4°C.

Before use, 50mℓ RPMI 1640 supplemented with antibiotics, glutamine and 4% FCS was passed through the column to correct the pH. The column was then incubated at 37°C for 30-60 minutes prior to cell loading.

Between 500 and 1000 x 10^6 cells in RPMI 4% FCS were slowly pipetted onto the column. The column was fitted with a stop-cock. When the cells were pipetted onto the column the stop-cock was opened slightly to allow the cells to pass into the nylon wool always ensuring that the nylon wool did not run dry. The column was incubated at 37°C for 60 minutes before the cells were slowly eluted and the column washed with 200mℓ RPMI 4% FCS. The cells were then centrifuged at 1000rpm for 7 minutes and the pellet resuspended in c-RPMI and counted (Julius et al., 1973).

2.2.4 Separation of mononuclear cells depleted of monocytes and B cells using discontinuous percoll density gradients

To prepare density gradients for centrifugation percoll was mixed in various concentrations with c-RPMI. The starting solution of percoll was a mixture of 92 parts percoll and 8 parts of 10X concentrated phosphate buffered saline (PBS) (formula in grams|litre 2KCl, $2KH_2PO4$, 80NaCl, $21.6Na_2HPO_4$, $7H_2O$, pH 7.4). Seven different concentrations of percoll in c-RPMI were prepared, beginning with a concentration of 40% percoll, increasing by 2.5% each time. The osmolality of all solutions was adjusted to 285m osm/kg.

The gradient was formed by layering 2mℓ of each percoll concentration into a 15mℓ falcon centrifuge tube (the most dense solution being at the bottom). 5-7 x 10^7 non-adherent mononuclear cells in 1mℓ c-RPMI were poured onto the gradient which was centrifuged at 550g for 30 minutes at room temperature according to the method of Timonen and Saksela (1980).

Cells from each layer of the gradient were collected, using a sterile pasteur pipette, and washed once with saline. Viability studies adjugded the cells in all but the top fraction to be over 95% viable.

2.2.5 Purity of fractions from the percoll gradient

Cells from different fractions of the percoll gradient were washed and resuspended in approximetly 0.5mℓ RPMI 5% FCS, at a concentration of 6 x 10^6/mℓ. These cells were incubated with OKT3 monoclonal antibody (Ortho, Raritan, New Jersey) at a final dilution of 1:100, at room temperature for 45 minutes. After the initial incubation 200µℓ of rabbit complement was added to each tube and incubated for 60 minutes at 37°C. Control cells treated with complement alone were incubated at the same time. After the second incubation a small aliquot was removed from each tube and the cell viability determined using trypan blue exclusion dye. The control cells incubated with complement alone were used to determine the backgrcund cytotoxicity.

The degree of contamination of the LGL fraction with B cells and monocytes was judged to be less than 2% as assessed by immunofluroscence with anti-human Ig, the injestion of opsonised yeast and using myeloperoxidase staining techniques.

2.2.6 NK activity of fractions from percoll gradients as measured by a ^{51}Cr assay

A cytotoxicity (chromium release) assay measures the ability of an effector cell to kill a target cell without prior sensitization to that cell. It is carried out by incorporating radioactive chromium into the target cells (Kadish et al., 1981) so that if these labelled cells are lysed the radioactivity is released into the surronding medium. Thus by removing a sample of the supernatant and measuring its radioactivity the cytotoxic ability of the effector cells can be calculated.

The K562 cell line, used in the above mentioned assay, was maintained as a stationary suspension culture in 25cm^2 tissue culture flasks in c-RPMI. The line was subcultured three times a week and 24 hours prior to use in an assay.

Approximetly 2 x 10^6 target (K562) cells were labelled with 100µCi of sodium chromate ($Na_2\ ^{51}CrO_4$ specific activity 250 - 500 mCi/mg) for 1½ hours at 37°C. The cells were then washed three times in sterile, physiological saline and resuspended to a concentration of 1 x 10^5 cells/mℓ in

c-RPMI, after determining the cell number and percentage viability. Only cell populations with viability greater than 90% were used. Cells from each fraction of the percoll gradient were tested against ^{51}Cr labelled K562 cells. Triplicate cultures were established in flat bottomed microtitre plates. Effector cells at various concentrations (such that there were effector to target ratios of 7:1, 15:1, 30:1) in 100µℓ of c-RPMI were added to 100µℓ ^{51}Cr labelled target cells (1×10^4 target cells/well). Cultures were incubated at 37°C for 4 or 18 hours. Samples were harvested by pipetting 100µℓ of supernatant from each well into tubes which were counted in an autogamma counter - to measure the amount of radioactivity in each well. Spontaneous ^{51}Cr release was measured by collecting supernatants from triplicate culture wells containing 1×10^4 target cells and c-RPMI only. Maximal release was determined by measuring the total ^{51}Cr released from an aliquot of 1×10^4 labelled target cells and 100µℓ triton x100. The percentage cytotoxicity was always calculated relative to the spontaneous release using the equation:

$$\% \text{ cytotoxicity} = \frac{\text{test cpm} - \text{SR}}{\text{MR} - \text{SR}} \times 100$$

SR = Spontaneous release

MR = Maximal release

2.3 Results

2.3.1 Purity of percoll gradient fractions

Cells from fractions 2 and 3 of the percoll gradient were adjudged to be large granular lymphocytes after being examined by transmitted light microscopy. The percentage of viable cells from each fraction of the percoll gradient after treatment with OKT3 monoclonal antibody and complement was assessed using trypan blue dye. The results are tabulated in Table 2.1.

2.3.2 NK activity of cells from percoll gradients

The cytotoxic activity of cells from each fraction of percoll gradients was assessed using a chromium release assay. The percent cytotoxicity was calculated at three different effector to target cell ratios and on each occasion was maximum when cells from fractions 2 and 3 were employed. These results confirm those of previous workers (de Landazuri et al., 1981; Timonen and Saksela, 1980). The results of the assays employing effector to target ratios of 15:1 and 7:1 are not shown as they exhibit a similar pattern to those obtained when a ratio of 30:1 was used. (Table 2.2 Fig.2.1).

When contaminating T cells were removed from the LGL fraction by treatment with OKT3 and complement the cytolytic activity of this cell population was unchanged (results not shown) showing that any contaminating T cells did not

TABLE 2.1 TREATMENT OF PERCOLL CELL FRACTIONS WITH OKT3 MONOCLONAL ANTIBODY AND COMPLEMENT

Cell system	% viable cells (mean ± SD)
Percoll fraction 2 + complement	92.8 ± 0.3
Percoll fraction 2 + OKT3 + complement	89.2 ± 0.7
Percoll fraction 3 + complement	91.5 ± o.4
Percoll fraction 3 + OKT3 + complement	89.6 ± 1.9
Percoll fraction (4+5) + complement	92.4 ± 1.6
Percoll fraction (4+5) + OKT3 + complement	2o.8 ± 1.8
Percoll fraction (6+7) + complement	92.1 ± 1.7
Percoll fraction (6+7) + OKT3 + complement	36.7 ± 3.5

TABLE 2.2 THE CYTOTOXIC ACTIVITY OF PERCOLL FRACTION CELLS AGAINST ^{51}CR LABELLED K562 CELLS 18 HOUR ASSAY

Cell system	% lysis (mean ± SD)		
	effector : target		
	30:1	15:1	7:1
Fraction 1	-	-	-
Fraction 2	46 ± 4.4	30 ± 5.8	25 ± 6.1
Fraction 3	55 ± 2.0	44 ± 4.0	35 ± 11.3
Fraction 4	34 ± 5.9	27 ± 3.5	23 ± 3.5
Fraction 5	16 ± 1.0	10 ± 2.1	8 ± 1.0
Fraction 6	12 ± 2.5	10 ± 3.1	3 ± 1.0
Fraction 7	8 ± 3.2	4 ± 2.0	0 ± 0.6

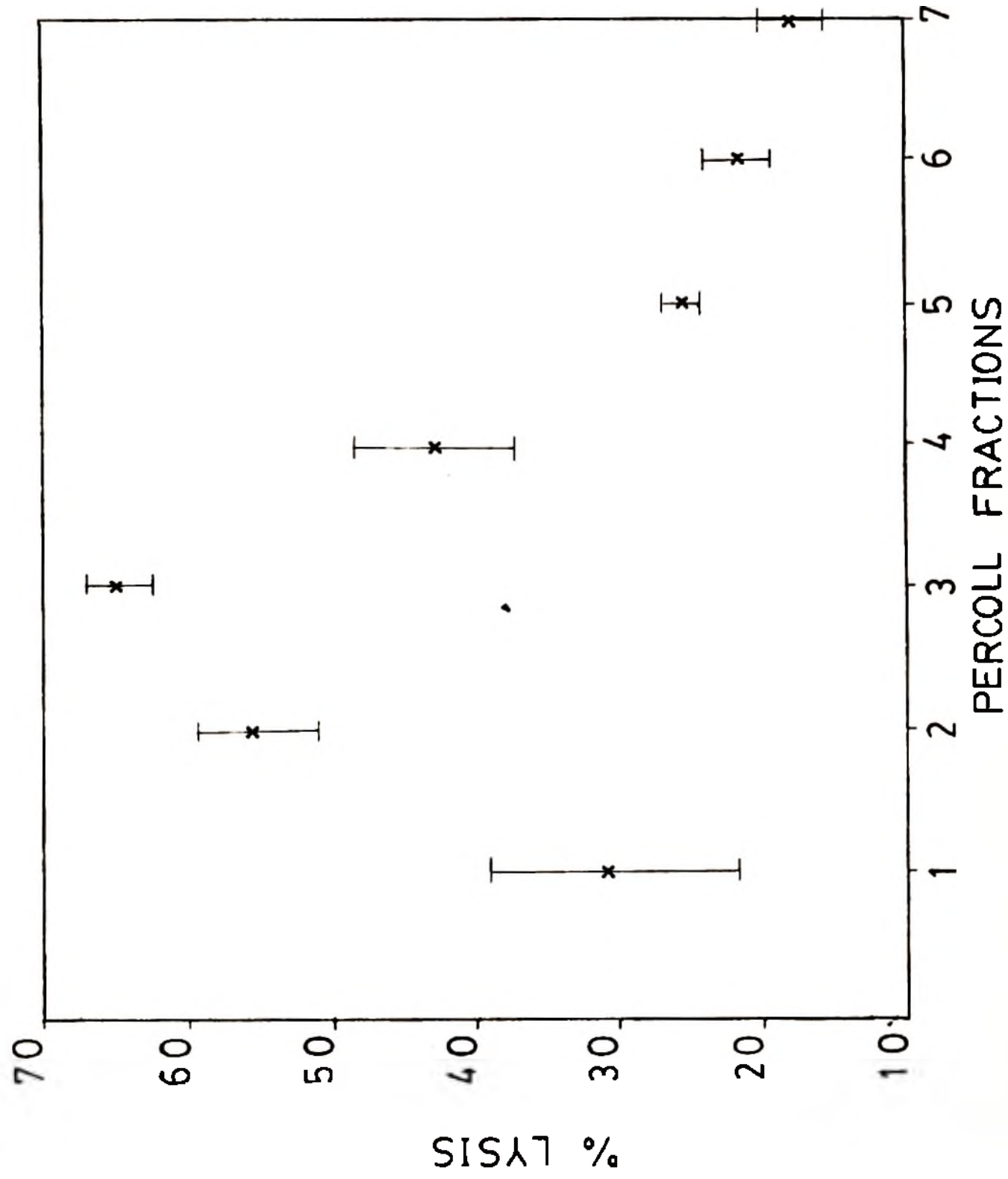


FIGURE 2.1 THE ABILITY OF CELLS FROM DIFFERENT FRACTIONS OF PERCOLL TO LYSE ^{51}CR LABELLED K562 CELLS.

affect cytotoxicity.

2.4 Discussion

These results confirm the work of Timonen and Saksela (1980) in demonstrating the enrichment of peripheral blood mononuclear cells for NK cells by density gradient centrifugation. Morphological studies of the low density cells isolated from percoll gradients revealed these cells to be LGLs, the characteristic feature being azurophilic granulation in the cytoplasm and a high cytoplasmic:nuclear ratio compared with other lymphoid cells. The present results showing a concomitant enrichment of LGL in fractions with strongest NK activity further support this conclusion.

Approximetly 60% of the LGLs were found to express the OKM1 antigen (a surface marker present on monocytes) and 70 - 90% to express the HNK-1 marker (an antigen found on most LGLs), - these results will be presented and discussed in greater detail in a subsequent chapter. The work of Zarling et al., (1981) showing that the majority of LGLs do not posess the OKT3 antigen (a pan-T cell antigen) was also confirmed.

The enrichment of human NK cells by the gradient centrifugation technique has obvious advantages compared with other methods used to fractionate NK cells. It has been clearly shown that human NK cells are heterogenous with regard to subclass specific markers (Herberman and Holden,

1978; Saksela et al., 1979). Thus fractionation based on membrane properties are only moderatly efficient in NK cell enrichment. By the present method functional NK activity peaked strongly in two fractions with good cell yields.

Adsorption and elution of effector cells from target cells has previously been shown to be an efficient method for enrichment of NK cells (Saksela et al., 1979; Timonen et al., 1979). However, irrelevant conventional lymphocytes adhere to adsorbing target cells to some degree so that pure populations of LGLs cannot be produced by this method. Furthermore, NK cells enriched by this method have passed the recognition and activation stages of regulatory mechanisms in NK activity. By enriching NK cells by the gradient centrifugation method, the purity of LGLs is substantially inproved without the activation of effector cells.

CHAPTER 3

3. THE EFFECTS OF PLC/PRF/5 CULTURE SUPERNATANTS AND INTERFERON ON THE NK ACTIVITY OF PURIFIED LGLS

3.1 Introduction

The NK activity *in vitro* and *in vivo* has been found to be diminished in tumour bearing animals (Herberman *et al.*, 1975) and in patients with malignant disease - more particularly in those with widely disseminated tumours (Pross and Baines, 1970). Similar results have been reported in patients with leukemia (Takasugi *et al.*, 1973) and in those with large solid tumours (Vose *et al.*, 1977).

Studies in our laboratory, performed on lymphocytes from patients with large mass hepatocellular carcinoma, have indicated that in general, NK activity is reduced. Whether this is due to an intrinsic malfunction of the NK cells and/or their precursors or to the presence of a suppressor factor or cell population, has until now, not been elucidated.

The work of Keong and Rabson (1983) in our laboratory has shown that the supernatant from the cell line PLC/PRF/5, derived from a patient with hepatocellular carcinoma by Alexander *et al.*, (1976), contains factors that are highly immunosuppressive to lymphocyte mitogenesis and, in addition, inhibit NK activity of normal non-adherent mononuclear cells. It is not known whether this suppression is due to the induction of a suppressor cell, or, results from a direct

action of the supernatant on NK cells. Other investigators (Zaunders et al., 1981) have described inhibitors of NK cell activity in culture supernatants of melanoma and Chang liver cells.

PGE_2, which is produced primarily by mononuclear phagocytes (Kennedy et al., 1980) and tumour cells (Droller et al., 1979), has been shown to inhibit NK activity (Droller et al., 1978). Studies by Keong and Rabson (1983) indicate that, addition of indomethacin to PLC/PRF/5 cultures, does not abrogate the observed suppression caused by the supernatant.

Bloom (1980) and Zarling et al., (1980) have found natural cytotoxicity to be stimulated by interferon and interferon inducers both in vivo and in vitro. Although the molecular mechanism of interferon action on NK cells is unknown Taragan and Dorey (1980) have indicated that interferons promote the differentiation of pre-NK cells to mature NK cells and Bloom (1980) has suggested that this mediator augments the cytolytic activity of already mature NK cells.

The purpose of this study was to examine the effect of PLC/PRF/5 culture supernatants alone and in conjunction with interferon, an the NK activity of purified LGLs isolated from human peripheral blood. The standard chromium release assay (as described in chapter 2.2.6) was used in this study.

3.2 Materials and methods

3.2.1 Cell lines and preparation of culture supernatants

The hepatoma cell line PLC/PRF/5, established and described by Alexander et al., (1979) was used in this study. It was grown as a stationary monolayer in 25cm^2 tissue culturs flasks in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2mm L-glutamine, 100 units/mℓ penicillin, 100µg/mℓ streptomycin and 50µg/mℓ gentamycin (culture medium). The cells were subcultured weekly after trypsinization with trypsin-EDTA.

Approximetly 1 x 10^6 cells in 5mℓ of culture medium was innoculated into each 25cm^2 tissue culture flask and tha cultures were allowed to grow for 3 days to attain confluency. The spent medium was discarded, each culture washed three times with MEM, then incubated with culture medium for a further 24 hours. The culture medium was then harvested, centrifuged at 3000rpm to remove cell debris, aliquoted and stored at -20°C until required. Each batch of supernatant was tested for suppressive activity in a blastogenesis assay, based on the method described by Thurman et al., (1973) - modified by Keong (Msc dissertation).

3.2.2 Isolation of LGL from peripheral blood

LGL from human peripheral blood were isolated as described

in chapter 2.2.1 - 2.2.4.

3.2.3 The effect of PLC/PRF/5 culture supernatants on NK activity

Culture supernatants were assayed for inhibition of NK cell function by comparing the levels of cytotoxicity of cells, from each of the percoll gradient fractions, against ^{51}Cr-labelled K562 cells.

In certain assays cells from the high density percoll fractions were added to purified LGLs in equal numbers in the assay, ie. these wells contain twice the number of effector cells, and either one or both of the cell populations were pulsed with the culture supernatant.

The standard NK assay as described in chapter 2 was performed. Effector cells were those isolated from percoll gradients and pulsed, for 24 hours, either with complete medium (control system) or PLC/PRF/5 supernatants. The effector cells were treated with culture supernatant for 24 hours as suppression of NK activity was found to be at a maximum at this time. Three different effector : target ratios namely, 30:1, 15:1 and 7:1 were employed in the assay system. Each assay was incubated for either 4 or 18 hours.

As an additional control the effect of the culture supernatant on spontaneous release, by labelled K562 cells was determined. This was to ensure that no cytotoxic effects

were exerted on the target cells by supernatant alone.

3.2.4 The effect of interferon on NK activity

Crude interferon (IFN) from human leukocytes incubated with blue tongue virus and standardized to 16 000 IU/mℓ against an international standard (NIH) was a gift from Professor B Schoub (National Instituts of Virology). Recombinant A interferon (Roche, Johannesburg) was also utilized in these studies.

LGL at a concentration of 4-6 x 10^6 in 2 mℓ c-RPMI were incubated with or without 1000 IU/mℓ IFN for one hour. At the end of the incubation period the cells were washed three times in RPMI 1640 and assayed for cytotoxicity. To exclude the possibility that this agent had a direct cytotoxic effect it was (in some experiments) added directly to target cells and ^{51}Cr release assayed.

3.2.5 The combined effect of PLC/PRF/5 culture supernatants and interferon on NK activity

LGL to be used in cytolytic assays, were pulsed for 24 hours with PLC/PRF/5 supernatant before being washed three times and 1000 IU/mℓ interferon added (to 4-6 x 10^6 cells in 2mℓ medium) for a further 2 hours, the reverse procedure was also carried out, ie. pulsing first with interferon followed by the culture supernatant for 2 and 4 hours respectively.

3.3 Results

3.3.1 The effect of PLC/PRF/5 supernatants on NK activity

The levels of cytotoxicity exhibited by purified LGLs (in 4 and 18 hour assays), pulsed with culture supernatant, were markedly reduced when compared to those cells pulsed with complete medium, as illustrated in Fig. 3.1 and in Tables 3.1, 3.2 and 3.3.

The suppression mediated by the supernatant on the various cell populations, harvested from percoll gradients, is tabulated in Table 3.1.

Table 3.4 shows that there appeared to be no suppressor cell population induced when the various cell fractions were pulsed with culture supernatant and mixed with LGLs in cytolytic assays.

Radiolabelled K562 cells assayed in the presence of culture supernatant alone showed similar levels of spontaneous release to those assayed in complete medium (results not shown), thus indicating that the supernatants did not exert cytotoxic effects on the target cells.

Chromium labelled K562 cells pulsed with supernatant and assayed with LGLs were lysed normally, indicating that the supernatant affects the effector rather than the target

Key to Figure 3.1

A - LGL pulsed with complete medium

B - LGL pulsed with culture supernatant for 24 hours

C - LGL pulsed with interferon for 2 hours

D - LGL pulsed with culture supernatant for 24 hours followed by interferon for 2 hours

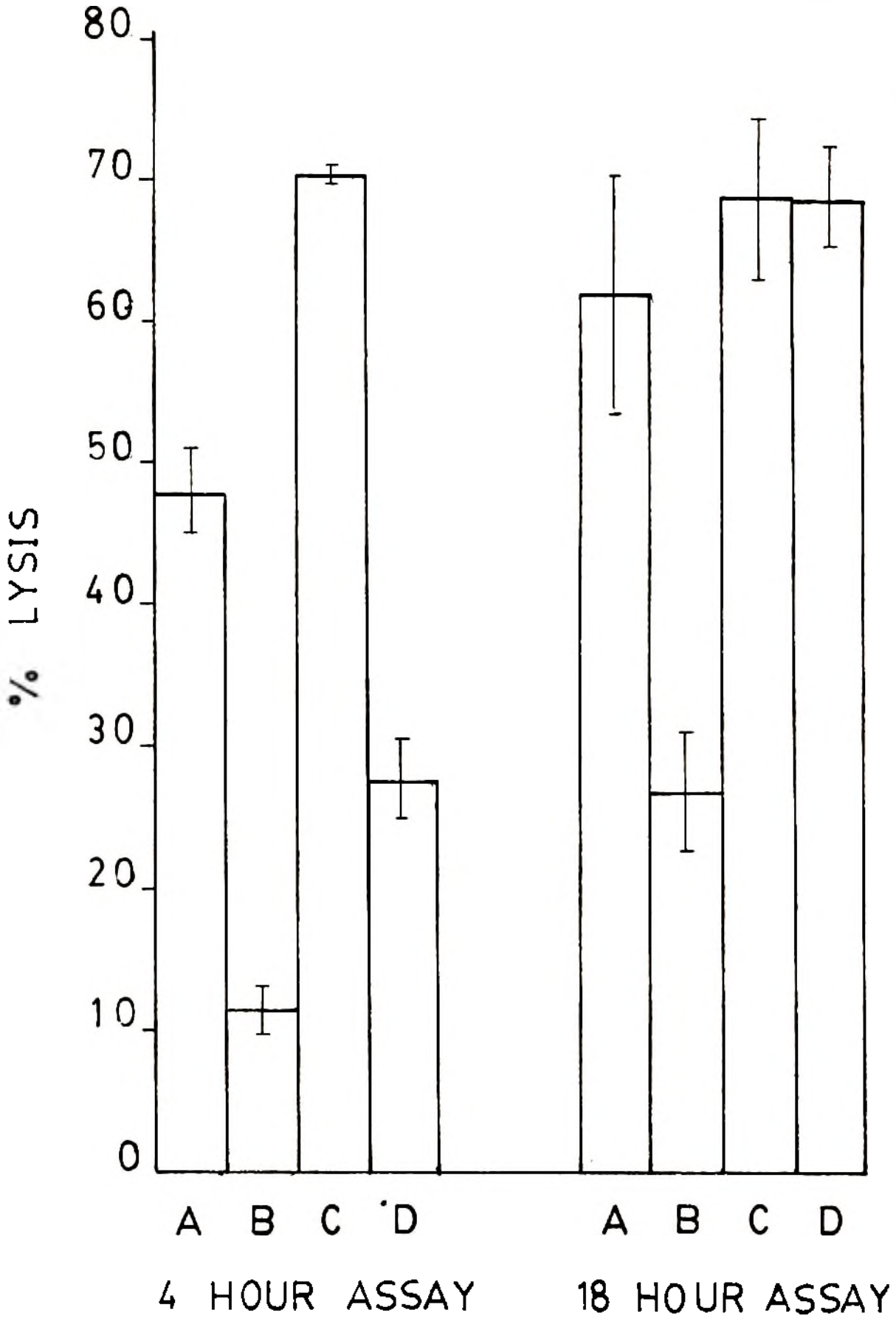


FIGURE 3.1 THE EFFECT OF PLC/PRF/5 S/N AND IFN ON THE ABILITY OF PURIFIED LGLS TO LYSE ^{51}CR LABELLED K562 CELLS.

TABLE 3.1 THE EFFECT OF PLC/PRF/5 SUPERNATANT ON THE NK ACTIVITY OF PERCOLL GRADIENT PURIFIED CELLS E:T 30:1 18 HOUR ASSAY

Cell system	% lysis (mean ± SD)	%suppression
Unfractionated nylon wool non-adherent (NA) MNC	34 ± 10.0	
Unfractionated nylon wool NA MNC + PLC/PRF/5 s/n	27 ± 5.1	21
Percoll fraction 2 cells	54 ± 5.3	
Percoll fraction 2 cells + PLC/PRF/5 s/n	23 ± 3.5	58
Percoll fraction 3 cells	55 ± 2.3	
Percoll fraction 3 cells + PLC/PRF/5 s/n	28 ± 3.1	50
Percoll fraction 4 cells	36 ± 5.1	
Percoll fraction 4 cells + PLC/PRF/5 s/n	29 ± 4.7	18
Percoll fraction 5 cells	27 ± 2.1	
Percoll fraction 5 cells + PLC/PRF/5 s/n	24 ± 1.0	10
Percoll fraction 6 cells	23 ± 2.0	
Percoll fraction 6 cells + PLC/PRF 5 s/n	16 ± 0.6	32

TABLE 3.2 THE EFFECT OF PLC/PRF/5 SUPERNATANTS, INTERFERON OR BOTH ON THE NK ACTIVITY OF LGL 18 HOUR ASSAY

Cell system	% lysis (mean ± SD)	(% suppression)
	E:T 30:1	15:1
LGL + complete medium	62 ± 8.9	46 ± 2.5
LGL + PLC/PRF/5 supernatant	27 ± 4.0 (57)	21 ± 4.8 (55)
LGL + interferon	87 ± 5.6	66 ± 4.4
LGL + PLC/PRF/5 supernatant + interferon	87 ± 3.5 (0)	69 ± 1.3 (0)

TABLE 3.3 THE EFFECT OF PLC/PRF/5 SUPERNATANTS, INTERFERON OR BOTH ON THE NK ACTIVITY OF LGL 4 HOUR ASSAY

Cell system	% lysis (mean ± SD)	% suppression
LGL + complete medium	48 ± 3.0	
LGL + PLC/PRF/5 supernatant	12 ± 1.5	76
LGL + interferon	70 ± 0.8	
LGL + PLC/PRF/5 supernatant + interferon	28 ± 2.5	42

TABLE 3.4 THE EFFECT OF PULSING CELLS FROM HIGH DENSITY PERCOLL FRACTIONS WITH PLC/PRF/5 SUPERNATANTS AND ADDING THEM TO LGL IN A ^{51}CR ASSAY 18 HOUR ASSAY

Cell system	% lysis	% suppression
LGL + complete medium	54 ± 4.2	
LGL + PLC/PRF/5 s/n	33 ± 4.0	38
Percoll fractions (4-7) + complete medium	28 ± 3.0	
Percoll fractions (4-7) + PLC/PRF/5 s/n	25 ± 2.7	11
LGL + percoll fractions (4-7) + complete medium	77 ± 5.2	
LGL + percoll fractions (4-7) each + PLC/PRF/5 s/n (each population pulsed seperatly then mixed 1:1)	56 ± 5.0	27

cells.

3.3.2 The effect of interferon on NK activity

The augmentation of cytotoxicity, by interferon, on LGLs employing radiolabelled K562 cells as targets is shown in Fig. 3.1 and Tables 3.2 and 3.3.

The results of the experiments in which different cell populations from the percoll gradients were treated with IFN and mixed are presented in Table 3.5 and Figure 3.2. From these it appears that interferon augments the cytotoxicity mediated by the LGLs only, however, both the crude IFN and the recombinant IFN gave similar results.

3.3.3 The combined effect of PLC/PRF/5 supernatants and interferon on the NK activity of LGLs

The cytolytic ability of LGLs, pulsed sequentially with supernatant and IFN, against ^{51}Cr labelled K562 cells is shown in Fig. 3.1 and Tables 3.2 and 3.3. A noticable difference in the results from the 4 and 18 hour assays can be seen, whereas the suppression of cytotoxicity mediated by the culture supernatant is apparent at both assay times. When the supernatant pulse is followed by one with IFN the cytotoxicity in the 18 hour assay is not suppressed but in the 4 hour assay it is suppressed by 42%. The reverse procedure of pulsing effector cells first with IFN followed by a pulse with the supernatant reduced the lysis of K562 cells

TABLE 3.5 THE EFFECT OF INTERFERON ON THE CYTOTOXIC ABILITY OF LOW AND HIGH DENSITY PERCOLL GRADIENTS 18 HOUR ASSAY

Cell system	% lysis (mean ± SD)	
E:T	30:1	7:1
LGL + complete medium	40 ± 5.0	25 ± 4.0
LGL + interferon	73 ± 6.9	57 ± 12.2
LGL + high density cells (1:1)	58 ± 9.3	37 ± 8.7
LGL + [high density cells (1:1) + IFN]	61 ± 4.5	37 ± 6.7
[LGL + IFN] + high density cells (1:1)	71 ± 0.6	59 ± 9.8
LGL + high density cells (1:1) both + IFN	90 ± 8.5	77 ± 16.1
High density cells + complete medium	19 ± 9.2	13 ± 7.8
High density cells + IFN	22 ± 8.2	16 ± 9.2

Key to Figure 3.2

A - Unfractionated nylon wool non-adherent (NA) MNC

B - Unfractionated nylon wool NA MNC pulsed with supernatant for 24 hours

C - Percoll fraction 2 cells

D - Percoll fraction 2 cells pulsed with supernatant for 24 hours

E - Percoll fraction 3 cells

F - Percoll fraction 3 cells pulsed with supernatant for 24 hours

G - Percoll fraction 4 cells

H - Percoll fraction 4 cells pulsed with supernatant for 24 hours

I - Percoll fraction 5 cells

- Percoll fraction 5 cells pulsed with supernatant for 24 hours

K - Percoll fraction 6 cells

L - Percoll fraction 6 cells pulsed with supernatant for 24 hours

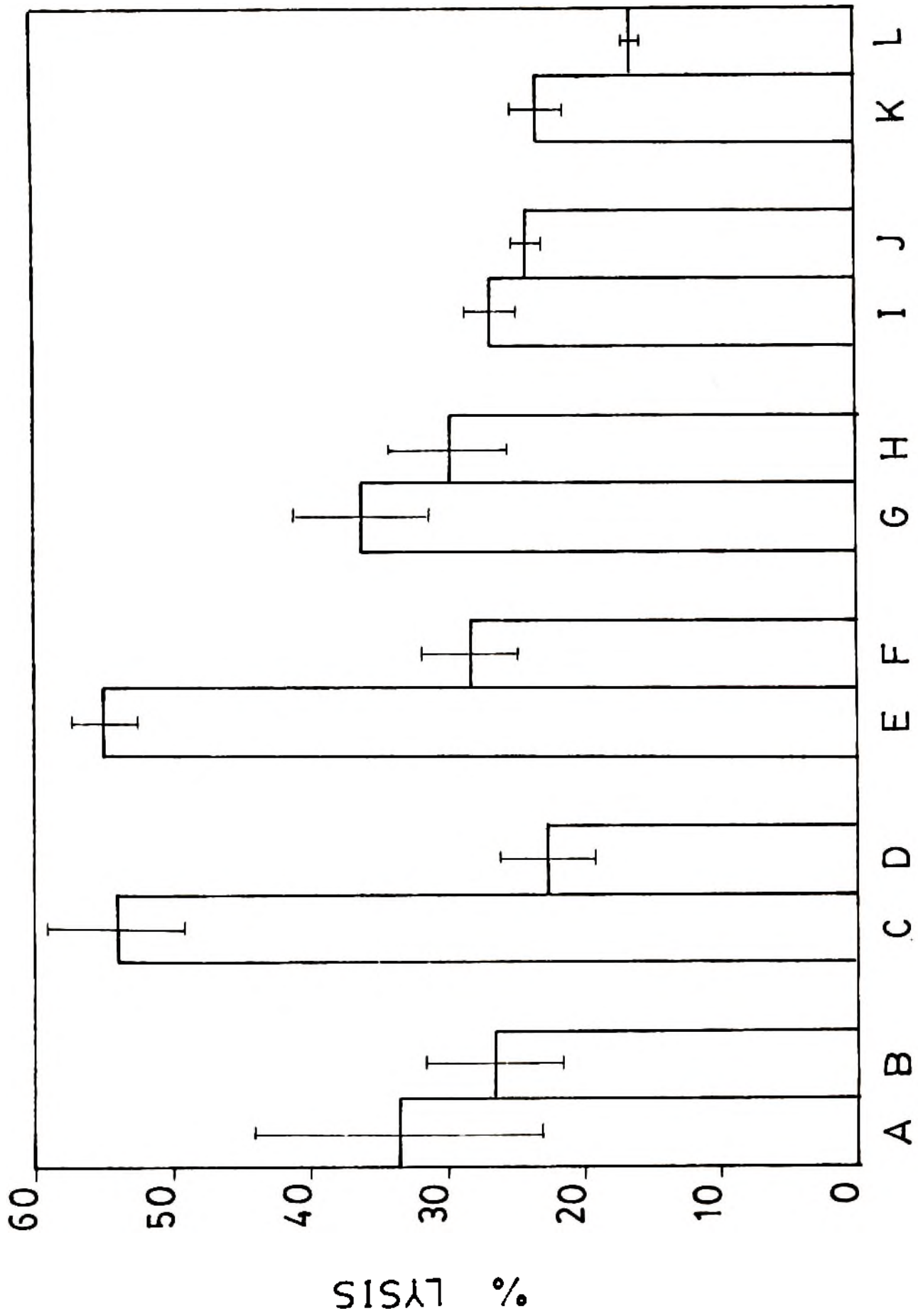


FIGURE 3.2 THE EFFECT OF PLC/PRF/5 S/N ON THE LYTIC ACTIVITY OF LYMPHOCYTES, ISOLATED ON PERCOLL GRADIENTS, AGAINST ^{51}CR LABELLED K562 CELLS

in the assay when compared to the value of LGLs pulsed with IFN only (results not shown). This however was probably due to the cells being incubated with IFN for 4 hours. Auget (1980) reports that the removal of bound IFN by a subsequent incubation of the cells in IFN free medium reduces the NK ctivity at least to the level of the control.

3.4 Discussion

In the present study the PLC/PRF/5 supernatant has been shown to contain factors capable of markedly suppressing NK activity of LGLs. This suppression can be partially corrected by subsequent treatment of effector cells with IFN, a well known augmentor of NK activity (Trinchieri et al., 1978; Santoli and Kaprowski, 1979; Herberman et al., 1979; Bloom et al., 1979; Zarling et al., 1980). The mechanism by which IFN augments NK activity is unclear and will be discussed further in a later chapter.

Previous studies on the kinetics of induction of suppression by the PLC/PRF/5 supernatant have shown that significant suppression of cytotoxicity is induced after 6 hours of pretreatment of effector cells and reaches a maximum at 24 hours. This suppression is partially reversible (Keong et al., 1983). These studies also demonstrate that suppression was mediated at the effector cell level.

We have shown that when mononuclear cells from more dense fractions of percoll gradients were pretreated with

the PLC/PRF/5 supernatant, and added to LGLs in a ^{51}Cr release assay, no inhibition of cytotoxicity was observed (results not shown), suggesting that the culture supernatant was not capable of activating a population of NK suppressor cells but was acting directly on the LGLs. However the possibility of the activation of a suppressor cell population within the LGL fraction cannot be excluded.

The PLC/PRF/5 supernatant could mediate suppression in one of several ways:

1) by adhering to the effector cell thus interfering with effector-target cell interaction.
2) by causing an inhibition of intracellular activity or by inhibition of the cytotoxic mechanism of the effector cells
3) NK cell mebabolism, glycoprotein or glycolipid synthesis could be inhibited
4) by interfering with the production of or function of certain helper lymphokines which may be required for NK cell activity.

The first four of these mechanisms is discussed further in chapter 4.

The second and third postulated mechanisms would be extremely difficult to investigate as little is known concerning the lytic event. It has been established that NK activity can be abolished by inhibition of chymotrypsin-like enzymes and inhibitors of phospholipid methylation and phospholipase A_2 (Hoffman et al., 1981; Quan et al., in press;

Hattori et al., 1983). Agents which augment cAMP and which interfere with cytoskeletal protein all negatively affect target cell lysis. It is also possible that the PLC/PRF/5 supernatant affects the secretory apparatus of the LGLs. Further purification of the supernatant and more detailed examination of its effects at the cellular level are, however, required for the mode of action to be evaluated.

The fourth possibility leads to the consideration of the role of IFN in this system. The results of this study have confirmed that pretreatment of effector cells with IFN causes an increase in NK activity (Trinchieri et al., 1978; Santoli and Kaprowski, 1979; Herberman et al., 1979; Bloom et al., 1979; Zarling et al., 1980) and the pretreatment of target cells with IFN protects them (results not shown). The mechanism by which IFN stimulates the cytotoxicity is, as yet, uncertain. Targan and Dorey (1980) have presented evidence that IFN causes a maturation of pre-NK cells to become fully active, a theory supported by Saksela et al.,(1979).

That human PBL, on exposure to certain human cell lines including K562, produce IFN, is widely accepted (Trinchieri et al., 1978; Peter et al., 1980). There also appears to be a correlation between the susceptibility of certain tumour cell lines to NK lysis and their ability to induce IFN release (Gustafsson and Lundgren, 1981). Previous studies in our laboratory have shown that co-culture of PBL with K562 cells for 18 hours results in the production of significant

levels (± 40 units) of IFN by the effector cells. However, if the PLC/PRF/5 supernatant was added to the system the levels of IFN decreased 10 fold (± 4 units). It is possible therefore that a factor is present in the supernatant which is capable of reducing the induction of IFN. Thus accounting for the reduced levels of cytotoxicity exhibited by LGLs pretreated with PLC/PRF/5 supernatant.

Although the reason for the depression in cytotoxicity produced by the PLC/PRF/5 supernatant and also the nature of the inhibiting factor is not known, it is possible that these findings may have significance in the understanding of how malignant tumours proliferate even in the presence of active NK cell activity. It is likely that the release of factors by the tumour may inhibit local NK cell activity thereby allowing the tumour to grow.

CHAPTER 4

4. THE EFFECT OF PLC/PRF/5 CULTURE SUPERNATANTS AND INTERFERON ON THE ABILITY OF LGL TO BIND K562 TARGET CELLS

4.1 Introduction

Little is known regarding the nature of the "recognition structure" on NK cells, or the target sites involved in target-effector interaction. It has, however, become apparent that there are at least five phases in the lytic cycle:

1) recognition
2) target cell binding
3) programming for the lytic event
4) degranulation
5) the phase of lysis

(Rager-Zisman and Bloom, 1982).It has been inferred that, in the initial phase, a surface determinant on the target cell is recognised by surface receptors on the NK cells (Herberman et al., 1975; Kiessling et al., 1975; Roder et al., 1979).

Target cell binding can occur in the presence of Mg^{2+} and the absence of Ca^{2+}. The addition of Ca^{2+} to the system triggers the programming for lysis. After target cell binding has occurred, but prior to lysis, NK activity can be blocked by inhibitors of chymotrypsin-like enzymes. Inhibitors of phospholipid methylation and of phospholipase A_2 appear to inhibit cytolysis (Hoffmen et al., 1981; Quan et al.,

in press; Hattori et al., 1983). Phenothiazine derivatives inhibit the calcium dependant step. Drugs that augment c-AMP and inhibitors of cytoskeletal proteins, colchicine and cytochalasin B also block lysis. Carpen et al., (1981) showed that a carboxylic ionophor, monensin, blocked NK activity and increased staining of cytoplasmic granules of LGL. It is on this basis that these workers postulated that an intact secretory apparatus is a prerequisite for NK cytolysis. Strontium has been shown to have a similar effect on NK activity (Neighbour and Huberman, 1982). Protein, RNA synthesis and cell division are not required for NK cytolysis, there is also no evidence that any accessory cell or factor is required for cytotoxicity (Herberman, 1982).

Thus when the amount of lysis of target cells in a test system differs from that of a control, one could postulate that there is less killing because:

1) a "blocking factor" is preventing the binding of the effector and target cell or
2) that there is an intrinsic defect of the effector cell such that it is unable to lyse the target to which it has bound.

An example of the latter possibility is observed in people suffering from Chediak-Higashi syndrome (CHS). These patients have recurrent infections (Blume and Wolff, 1972) and abnormal lysosomal granules (Root et al., 1972). Those who survive the infectious complications often succumb to a lymphroliferative disorder later in life (Dent et al., 1966).

They also appear to have an impairment in NK cell function (Halitosis et al., 1980; Klein et al., 1980). Katz et al., (1982) showed that this overall deficiency of NK activity in CHS patients is secondary to an inability of potentially cytotoxic lymphocytes, capable of binding to target cells, to complete the lytic sequence.

Roder et al., (1979) reported that glycoproteins, isolated from the surface of various tumour cells, selectively bind to NK cells thereby inhibiting their attachment to the intact target cells.

Stutman et al., (1980) demonstrated that, in mice, the binding of NK cells to target cells can be blocked by certain sugars (chapter 5). This is another stage in the "lytic cycle" at which killing of the target cell can be prevented.

There has been considerable work describing the effects of interferon on NK cells. This has resulted in controversy concerning the effect of IFN on the binding of target and effector cells. Although there is some disagreement on whether interferons affect the number of effector-target cell conjugates formed, there seems little doubt that the rate of target cell lysis is increased. When an NK cell lyses a target it can dissociate from that cell and, after 2 hours, bind to and lyse another target cell, this is termed recycling. It appears that if NK cells are treated with IFN they recycle more rapidly (Herberman, 1982).

The present study was performed in an attempt to determine how the PLC/PRF/5 culture supernatants prevented normal NK cell lysis. The results shown in chapter 3 indicated that the culture supernatants act directly on the NK cells rather than by activating a suppressor cell population which could in turn affect the NK cells. It was, therefore, of intrest to determine whether the culture supernatants prevent target-effector binding or inactivation of some part of the lytic machinery.

4.2 Materials and methods

The effects of PLC/PRF/5 culture supernatants and interferon on the binding of K562 target cells to LGLs and the subsequent lysis of the bound target cells, was examined. This was achieved by using a target binding assay based on the methods of de Landazuri *et al*., (1981) and Reynolds *et al*., (1981).

4.2.1 Isolation of LGL

LGL derived from peripheral blood were separated as described in chapter 2.2.1 - 2.2.4.

4.2.1 Preparation of culture supernatants

PLC/PRF/5 supernatants were prepared as described in chapter 3.2.1.

4.2.3 Treatment of effector cells with PLC/PRF/5 cuture supernatants

Percoll purified populations of LGLs were pulsed with culture supernatants for 4 or 24 hours as described in chapter 3.

4.2.4 Treatment of target cells with PLC/PRF/5 supernatants

To ensure that there was no direct effect of the culture supernatants on the target cells, these cells were pulsed with the supernatants for 4 hours before being incorporated in a target cell binding assay.

4.2.5 Interferon pretreatment of effector cells

Purified populations of LGLs were pulsed with IFN as described in chapter 3.

4.2.6 Pretreatment of purified LGLs with PLC/PRF/5 supernatants and interferon

LGLs were pulsed first with culture supernatants followed by interferon in a manner similar to that outlined in chapter 3.2.3.

4.2.7 Target binding assay

Mixtures of LGLs (5×10^6) and K562 cells (1×10^6) were

centrifuged at 200g for 8 minutes after which the supernatant was discarded and the pellet gently resuspended in 0.5mℓ medium then incubated at 37°C for varying periods of time.

The test system was prepared in a similar manner, the only difference being that effector cells which had been treated either with culture supernatant or IFN; or target cells which had been incubated with supernatant were used.

After the incubation the pellet was stained with trypan blue dye and examined microscopially on a haemocytometer. The use of trypan blue dye made it possible to determine the number of lytic and non-lytic conjugates. The proportion of effector cells bound to targets, bound target cells and the proportion of lytic and non-lytic conjugates was determined. A minimum of 400 cells was counted.

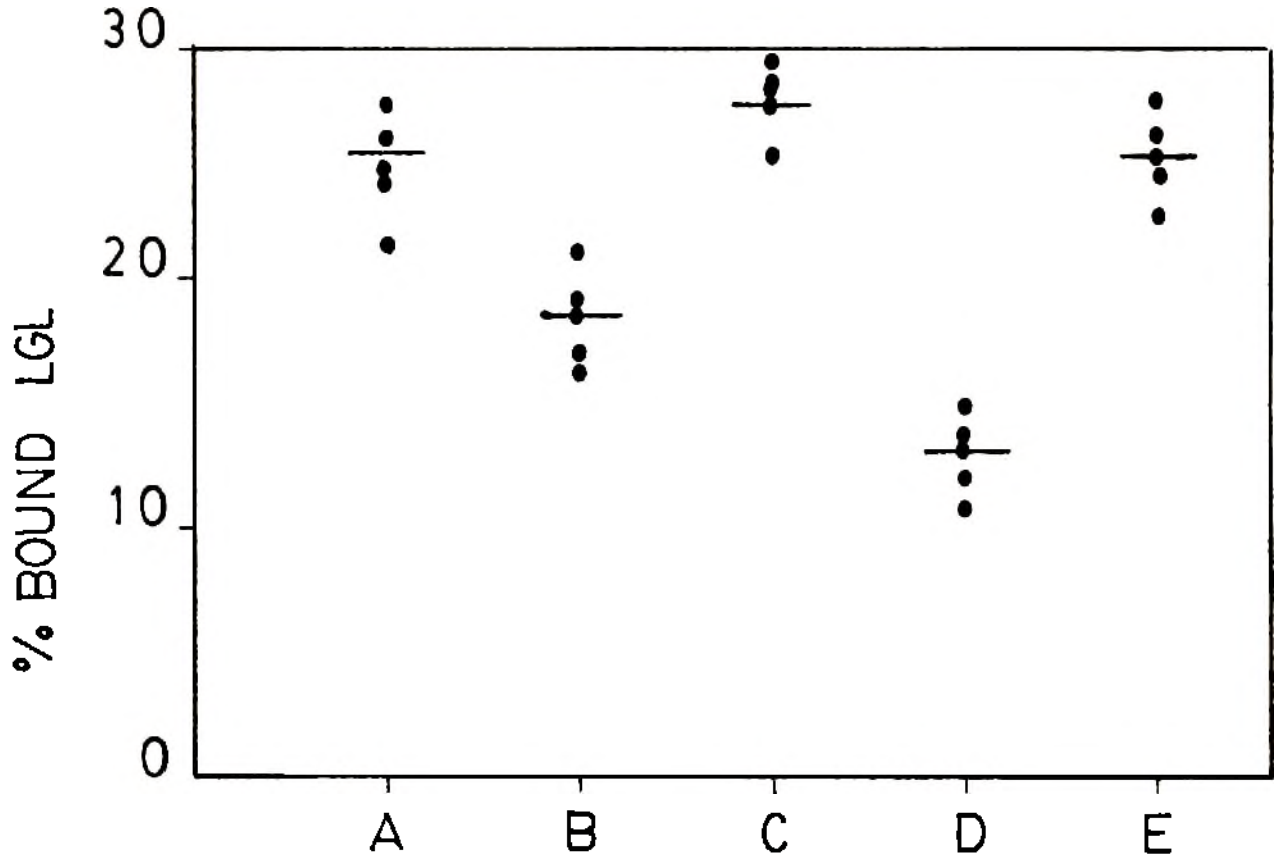
4.3 Results

4.3.1 The effect of PLC|PRF|5 supernatants on the ability of LGLs to bind and lyse target cells

The proportion of purified LGLs which had bound to target cells (target binding cells - TBC) was slightly reduced in the test system (Table 4.1 and Fig. 4.1) while the percentage of bound target cells was greatly reduced (Table 4.2 and Fig. 4.2). Although the proportion of bound target cells in the test system was lower than that of the control, the percentage of bound targets which had been lysed was only

TABLE 4.1 THE EFFECT OF PLC/PRF/5 SUPERNATANTS OF INTERFERON ON THE NUMBER OF LGLS BOUND TO K562 CELLS

Cell system	% target binding cells (mean ± SD)	
	2 hours	3 hours
LGL + complete medium	15.5 ± 3.4	25.3 ± 4.1
LGL + PLC/PRF/5 s/n	12.0 ± 2.1	17.7 ± 3.4
LGL + interferon	16.9 ± 3.2	27.2 ± 3.7
LGL + PLC/PRF/5 s/n + interferon	12.8 ± 2.7	14.3 ± 3.4
K562 cells + PLC/PRF/5 s/n	15.7 ± 2.7	26.0 ± 3.0



Key

A LGL + complete medium

B LGL + PLC/PRF/5 s/n 24 hours

C LGL + IFN 2 hours

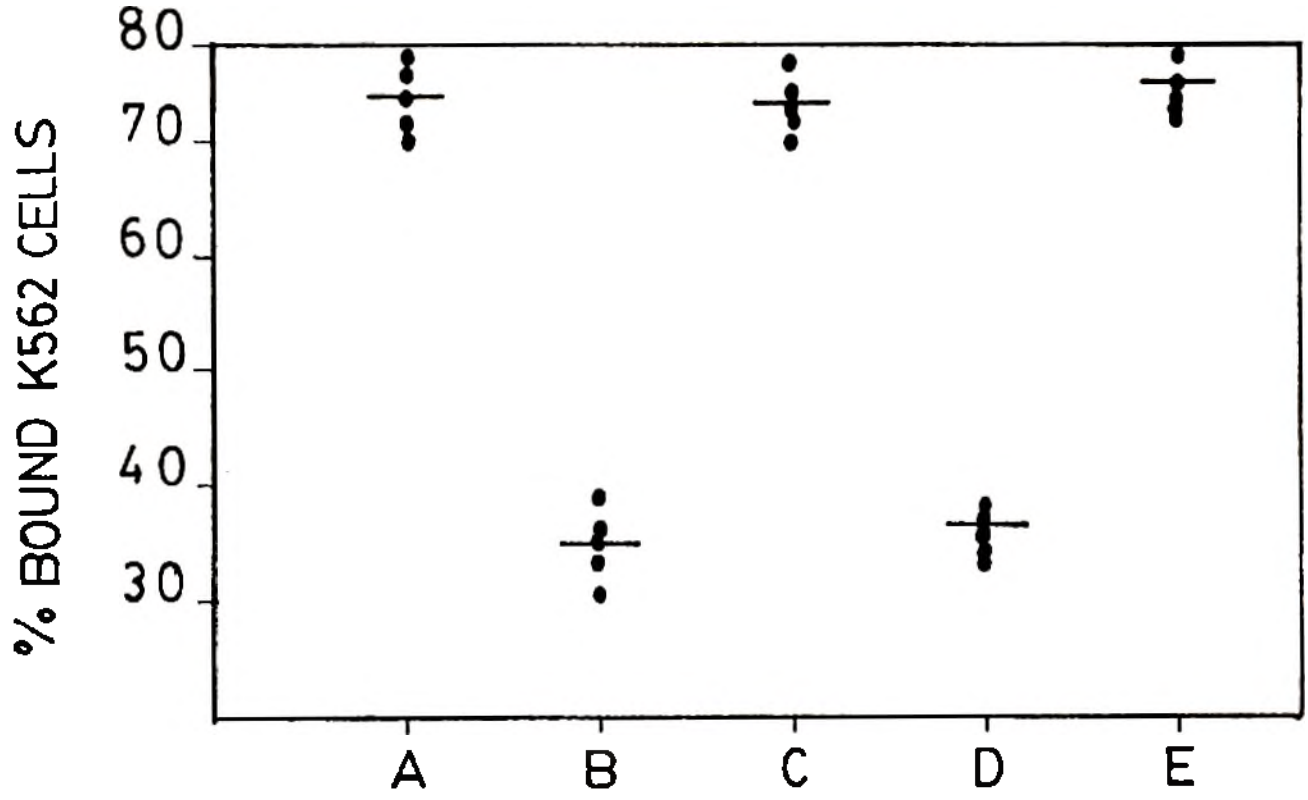
D LGL + PLC/PRF/5 s/n then IFN

E K562 cells + PLC/PRF/5 s/n 3 hours

FIGURE 4.1 TARGET CELL BINDING ASSAY USING LGL OR K562 CELLS PULSED WITH PLC/PRF/5 S/N AND/OR IFN.

TABLE 4.2 THE EFFECT OF PLC/PRF/5 SUPERNATANTS OR INTERFERON ON THE NUMBER OF K562 CELLS BOUND BY LGLS

Cell system	% bound target cells (mean ± SD)	
	2 hours	3 hours
LGL + complete medium	66.0 ± 12.6	77.4 ± 8.4
LGL + PLC/PRF/5 s/n	32.0 ± 1.7	35.0 ± 5.4
LGL + interferon	61.5 ± 9.4	76.3 ± 7.2
LGL + PLC/PRF/5 s/n + interferon	31.7 ± 2.4	36.2 ± 3.2
K562 cells + PLC/PRF/5 s/n	65.5 ± 7.7	78.8 ± 6.3



Key

A LGL + complete medium

B LGL + PLC/PRF/5 s/n 24 hours

C LGL + IFN 2 hours

D LGL + PLC/PRF/5 s/n then IFN

E K562 cells + PLC/PRF/5 3 hours

FIGURE 4.2 TARGET CELL BINDING ASSAY USING LGL OR K562 CELLS PULSED WITH PLC/PRF/5 S/N AND/OR IFN.

slightly reduced in the test system (Table 4.3 and Fig. 4.3), indicating that although fewer conjugates are formed those effector cells which do bind K562 cells are able to lyse them in a manner comparable to the control.

4.3.2 The effect of PLC/PRF/5 supernatants on target cells as the proportion of E:T conjugates formed

Pretreating the target cells with supernatants had no effect on the number of TBC, the percentage of bound target cells or the proportion of bound, dead target cells (Tables 4.1, 4.2, 4.3 and Figs. 4.1, 4.2, 4.3).

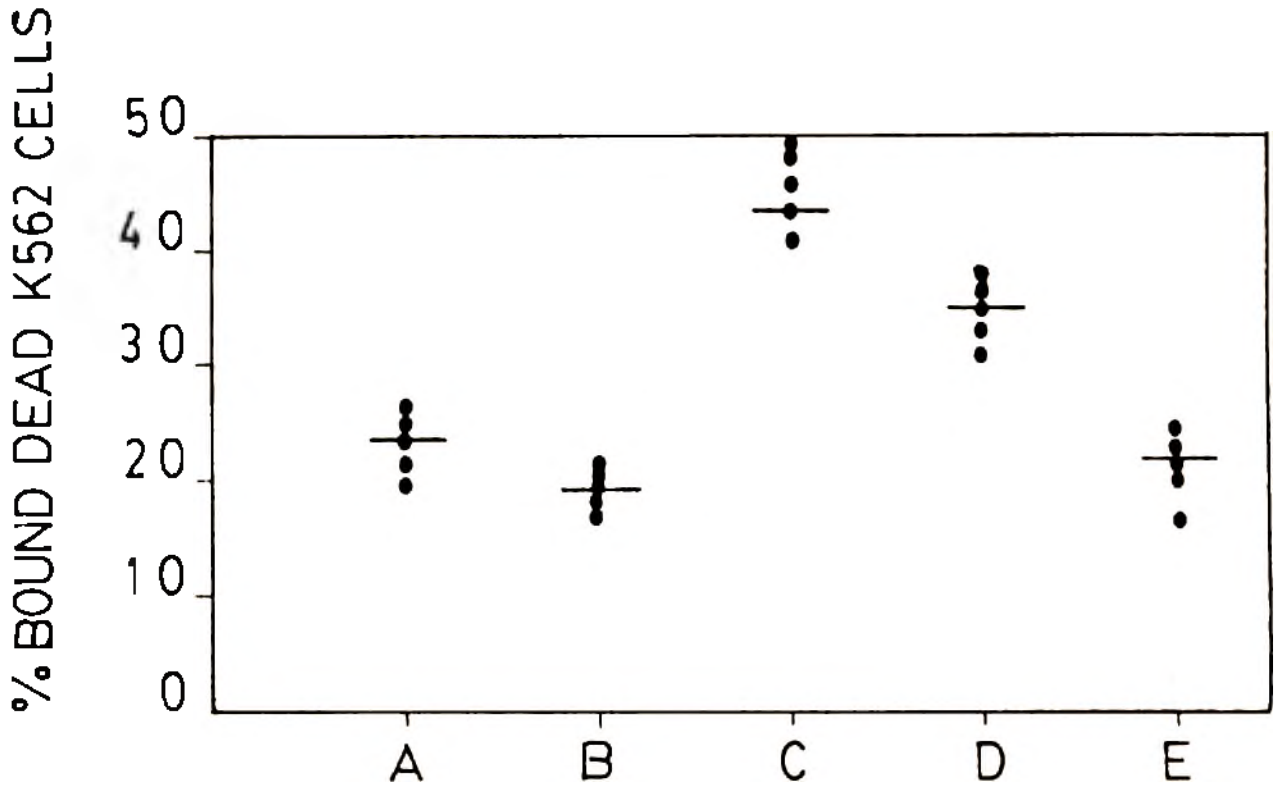
4.3.3 The effect of interferon on the ability of purified LGLs to bind and lyse target cells

Neither the percentage of TBC nor the proportion of bound target cells was raised after IFN treatment (Table 4.1, 4.2 and Figs. 4.1, 4.2), however, the proportion of bound, dead target cells was greatly increased (Table 4.3) showing that although IFN does not increase the percentage of conjugates formed it does augment the ability of conjugated effector cells to lyse the target cells.

4.3.4 The effect of treatment with PLC/PRF/5 supernatant and IFN on the ability of LGLs to bind and lyse target cells

TABLE 4.3 THE EFFECT OF PLC/PRF/5 SUPERNATANTS OR INTERFERON ON THE NUMBER OF DEAD BOUND K562 CELLS

Cell system	% dead bound target cells (mean ± SD)	
	2 hours	3 hours
LGL + complete medium	17.5 ± 5.0	24.2 ± 4.7
LGL + PLC/PRF/5 s/n	15.1 ± 1.5	19.2 ± 2.1
LGL + interferon	40.5 ± 9.8	47.0 ± 8.0
LGL + PLC/PRF/5 s/n + interferon	37.2 ± 3.2	35.9 ± 4.3
K562 cells + PLC/PRF/5 s/n	16.5 ± 3.2	23.0 ± 4.5



Key

A LGL + complete medium

B LGL + PLC/PRF/5 s/n 24 hours

C LGL + IFN 2 hours

D LGL + PLC/PRF/5 s/n then IFN

E K562 cells + PLC/PRF/5 s/n 3 hours

FIGURE 4.3 TARGET CELL BINDING ASSAY USING LGL OR K562 CELLS PULSED WITH PLC/PRF/5 S/N AND/OR IFN.

Although both the proportion of TBC and bound K562 cells in the test system were both reduced (Table 4.1 and 4.2) the percentage of bound target cells that were lysed was similar to that of the cells treated with IFN alone (Table 4.3 and Fig. 4.3). This result suggests that although the supernatant partially inhibits binding between target and effector cells, those effector cells which do form conjugates are able to kill target cells normally.

4.4 Discussion

In the previous chapter the suppression of target cell lysis by PLC/PRF/5 supernatant treated LGLs was outlined. The existance of a suppressor cell population which was activated by the culture supernatant is possible although unlikely.

It is generally accepted that the cytotoxic activity of NK cells is based on the interaction of receptors on the NK cell with "antigens" on the surface of target cells. Studies on the specificity of natural cytotoxicity have shown that NK cells exhibit different patterns of cytolysis against a range of target cells (Takasugi and Mickey, 1976) consistent with the interaction of receptors on NK cells with different antigens on the target cells. Further support for specific NK-target cell interactions come from analysis of specificity using competitive inhibition with unlabelled target cells. This work showed that cytotoxicity could be inhibited by some but not all target cells (Kiessling et al.,

1975; Hersey et al., 1975).

Extensive analysis of the results of such studies suggested that antigens detected by NK cells were, in some instances, expressed on a wide variety of cultured cells whereas others were more restricted in their distribution (Takasugi et al., 1977; Ortaldo et al., 1977). More direct evidence for binding of receptors on NK cells to antigens on target cells was obtained by visualization of these interactions in target cell binding assays by Roder and Kiessling (1978).

A prerequisite for NK mediated lysis is therefore the ability of the effector cells to bind to tumour target cells. The results obtained from target cell binding experiments indicate that the PLC/PRF/5 supernatants are capable of interfering with the binding of effector lymphocytes to tumour targets. When either enriched LGLs or K562 cells were individually treated with culture supernatant and incorporated in conjugate assays, inhibition of binding was only observed when the effector cells had been treated with the supernatant. It is therefore likely that the supernatant inhibits target cell binding by masking some surface receptor present on the NK cell rather than by coating membrane structures on the tumour target cells.

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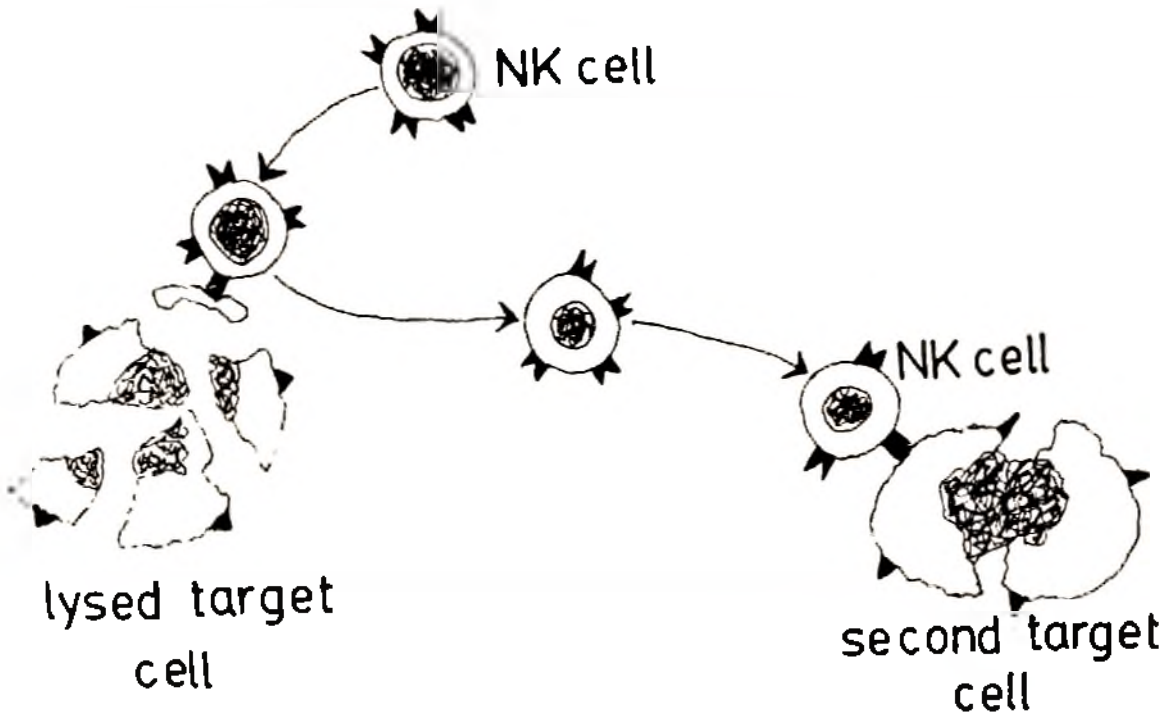
Although the reason for the decrease in cytotoxicity observed by PLC/PRF/5 supernatant treated LGLs and the nature of the inhibiting factor is not known, these observations

may have significance in the understanding of how malignant tumours proliferate even in the presence of active NK cell activity. It is likely that the release of factors by the tumour may inhibit local NK cell activity thereby allowing growth of malignant cells.

Zaunders et al., (1981) have presented data defining neutral glycoproteins found in the culture supernatant of the M M 200 and Chang cell lines. These glycoproteins were identified as cell-membrane structures recognised by NK cells which were spontaneously shed into the culture medium.

Spontaneous shedding of cell surface macromolecules in vitro is a well recognised phenomenon (Bystryn, 1977; Grimm et al., 1976; Leong et al., 1978). If similar shedding of NK antigens occurs in vivo it is conceivable that inhibition of NK cell activity in the host may occur and hence be of biological significance in aiding tumour growth.

The role of interferon in this system has long been recognised; although there remains some controversy as to the way in which NK cell lysis is enhanced by interferon. The results in this study showed that treatment of lymphocytes with interferon did not significantly increase the number of lymphocytes binding to target cells, the maximum number of target cells lysed in the effector-target cell conjugates increased however. This confirms the work of Trinchieri et al., (1981) and Taragan and Dorey (1980) who found that the rate of cytotoxicity was greatly increased when IFN



NK cells recycle. After an NK cell has bound to a target cell and lysed it, it will dissociate from the dead target and be able to bind and lyse another cell. A two hour recovery period is usually required but interferon shortens the time of recycling.

FIGURE 4.4 RECYCLING OF NK CELLS.

CHAPTER 5

5. THE EFFECTS OF VARIOUS SIMPLE SUGARS ON THE ABILITY OF LGL TO BIND TO AND LYSE K562 TARGET CELLS

5.1 Introduction

Carbohydrates are characteristic molecules on cell surfaces and are often associated with proteins and lipids. The carbohydrate moieties of glycoproteins and glycolilpids have numerous functions, one of the most important being their role in recognition (Sharon, 1980; Stutman et al., 1980).

Certain simple sugars, such as mannose-6-phosphate, are known to serve as recognition markers, and to play a role in directing the intracellular traffic of glycoprotein enzymes normally present in lysosomes. Cell surface sugars may also act as receptors for various physiological and non-physiological agents. Pronounced changes in these cell surface sugars are observed during differentiation and on transformation of normal cells to malignant cells (Sharon, 1980).

Cellular interaction, in certain systems, is mediated by the interaction of carbohydrate binding proteins on one cell with specific oligosaccharide receptors on another. Simple sugars such as galactose inhibit the aggregation of cells when added into the system (Sharon, 1980).

Cell surface saccharides act as receptors for viruses

and bacteria, for example E.coli adhere to white blood cells and epithelial cells through units of mannose on the surface of such cells. This carbohydrate specific interaction is mediated by a mannose specific lectin on the surface of the bacteria and can be inhibited by the addition of free mannose to the system (Sharon, 1980).

Thus the role of surface sugars in cellular functions is clearly very diversified. Interactions between lectin-like structures and either simple sugars or complex glycoproteins or glycolipids from the cell membrane are not only important in cell adhesion and aggregation (Balsamo and Lilien, 1975; Vicker, 1976; Gartner et al., 1978; Hausman and Moscana, 1975), cell differentiation (Balsama and Lilien, 1975; Grabel et al., 1979; Muramatsua et al., 1979) and binding of virus and bacteria to host cells (Gelb and Lerner, 1975; Gesner and Thomas, 1966; Laver, 1973; Ofek et al., 1977; Bar-Shavit et al., 1977) but also in phenomena such as platlet activation (Gartner et al., 1978), virus induced malignant transformation (Kalcker, 1965; Hatawaka, 1973, 1974) and phagocytosis (Brown et al., 1975).

Cell surface sugars of gangliosides (which are unique glycolipids that are selectively concentrated in the plasma membrane of cells) serve for the attachment of other biologically active molecules, for example, interferon. The incubation of gangliosides with IFN inhibits the antiviral activity of this mediator. Moreover, mouse cells that do not respond to treatment with IFN become responsive after the

incorporation of gangliosides into their surface membrane, indicating that gangliosides and IFN can interact at the cell surface and that these complex carbohydrates may have a function in the antiviral activity of IFN.

Roder et al., (1979) showed that glycoproteins isolated from the surface of various tumour cells selectively bound to NK cells and specifically inhibited their attachment to the intact target cell.

Stutman et al., (1980) presented evidence that natural cytotoxic cell mediated cytolysis in mice can be blocked by certain monosaccharides, namely D-mannose and D-galactose. On the basis of this and other results, they proposed that the effector cells have "lectin-like" receptors which recognise neutral sugars on the surface of a target cell (sugars that are probably part of glycoproteins or glycolipids in the cell membrane). Saturation of these binding sites by the appropriate sugars prevents effector-target interaction and thus cell lysis. They did not however, distinguish whether the effect was on binding of cells or on the lytic process; nor whether the lectin-like receptors were on the target or effector cell.

In view of the apparent blocking of NK activity by certain sugars in mice, this study was performed in an attempt to find possible similarities between the effect of the PLC/PRF/5 supernatants and various sugars on the target binding and subsequent lysis of K562 cells by purified

human peripheral blood derived LGLs.

5.2 Materials and methods

5.2.1 Purification of LGLs

LGLs from human peripheral blood were isolated as described in chapter 2.2.1 to 2.2.4.

5.2.2 The effect of various sugars on the NK acitvity of LGLs

Various sugars, both mono- and disaccharides were added in 0.05mℓ of c-RPMI, in varying concentrations directly to chromium release assays. In other experiments either the effector or target cells were pretreated with the sugar for 2 hours prior to those cells being incorporated in a 4 or 18 hour cytolytic assay as described in chapter 2.2.6. Stock solutions of all sugars used were made in c-RPMI and sterilized by filtration prior to use.

5.2.3 The effect of sugars and interferon on the NK activity of purified LGLs

In some experiments LGLs were pulsed with sugars for 2 hours and, after washing, they were incubated , for a further 2 hours, with 1000IU/mℓ IFN before being incorporated in a 4 or 18 hour ^{51}Cr release assay.

5.2.4 The effect of sugars on the target binding abiltiy of LGLs

The effect of various sugars on the target binding ability of LGLs to K562 target cells was examined in a manner similar to that described in the previous chapter.

5.3 Results

5.3.1 The effect of various sugars on the ability of LGLs to lyse K562 cells

The effects, on cytolysis, of incorporating sugars, at varying concentrations , in ^{51}Cr release assays are illustrated in Figs. 5.1 and 5.2. Fig. 5.1 shows that high concentrations of all sugars tested suppressed cytolysis, but in Fig. 5.2 it can be seen that only D-mannose and methyl-D-mannoside suppress killing when lower concentrations of sugar are used.

The results of pulsing the target cells with sugars are illustrated in Fig. 5.3. This graph shows that cytolysis was unaffected. However, when the LGLs were incubated with various sugars a marked decrease in killing, in certain instances, was visible. This would suggest that the sugar affects the effector rather than the target cell.

5.3.2 The combined effect of IFN and sugars on cytolysis

Key to Fig. 5.1

1 - LGL + medium

2 - LGL + mannose

3 - LGL + galactose

4 - LGL + glucose

5 - LGL + N-acetyl glucosamine

6 - LGL + methyl-mannoside

7 - LGL + rhamnose

8 - LGL + fucose

9 - LGL + lactose

10 - LGL + sucrose

11 - LGL + maltose

X - sugar concentration in the assay 100mM

O - sugar concentration in the assay 50mM

O - sugar concentration in the assay 25mM

The results of a typical experiment are illustrated.

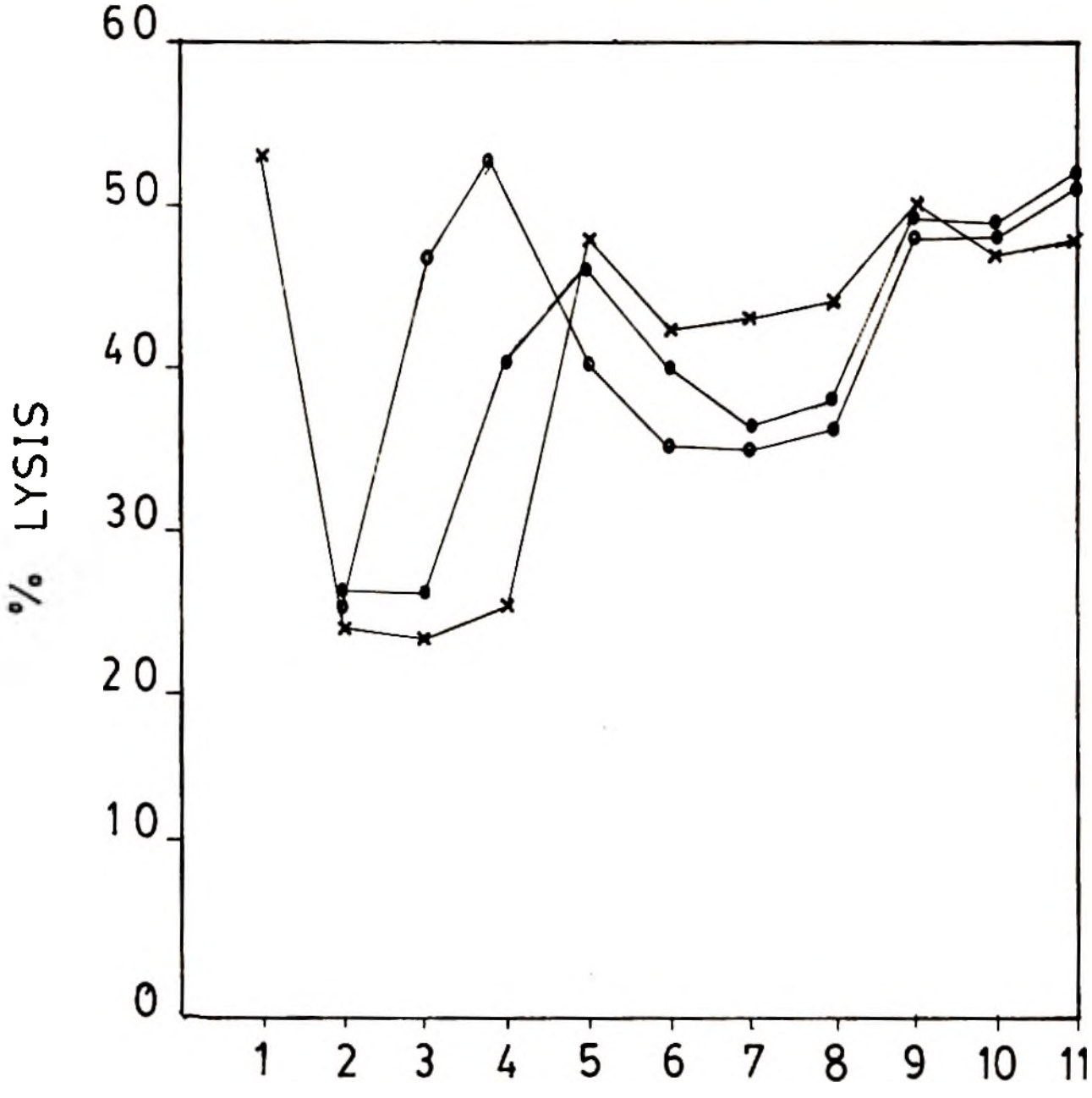


FIGURE 5.1 THE EFFECT OF SUGARS ON THE NK ACTIVITY OF LGL AGAINST K562 CELLS.

Key to Fig. 5.2

1 - LGL + medium

2 - LGL + mannose

3 - LGL + galactose

4 - LGL + glucose

5 - LGL + N-acetyl glucosamine

6 - LGL + methyl-mannoside

7 - LGL + rhamnose

8 - LGL + fucose

9 - LGL + lactose

10 - LGL + sucrose

11 - LGL + maltose

X - sugar concentration in the assay 5mM

O - sugar concentration in the assay 10mM

The results of a typical experiment are illustrated.

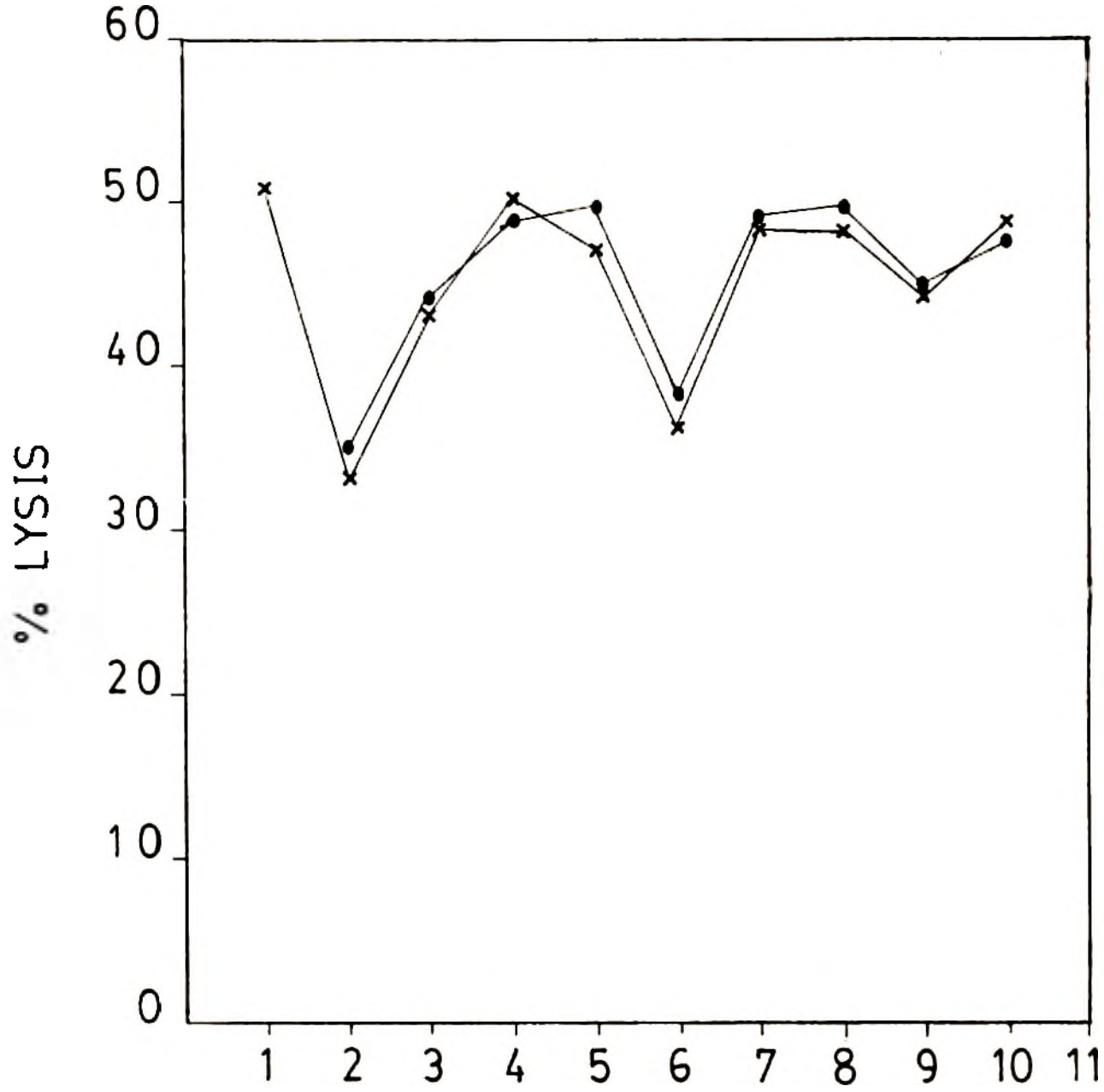


FIGURE 5.2 THE EFFECT OF SUGARS ON THE NK ACTIVITY OF LGL AGAINST K562 CELLS.

Key to Fig. 5.3

A - LGL pulsed with sugar for 2 hours

B - K562 cells pulsed with sugar for 2 hours

1 - cells + medium

2 - cells + mannose

3 - cells + galactose

4 - cells + glucose

5 - cells + N-acetly glucosamine

6 - cells + methyl-mannoside

7 - cells + rhamnose

8 - cells + fucose

9 - cells + lactose

10 - cells + sucrose

11 - cells + maltose

The results of a typical experiment are illustrated.

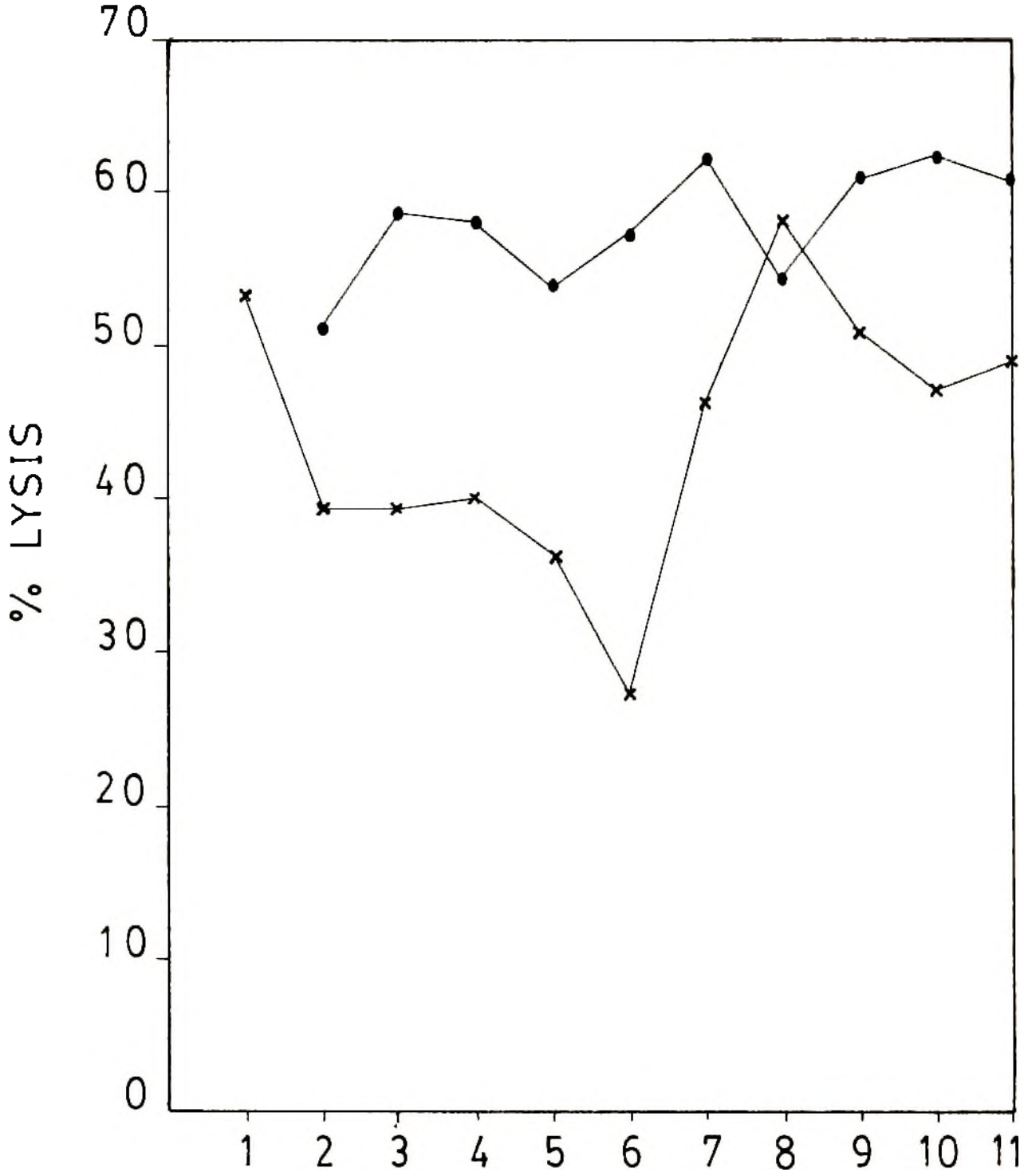


FIGURE 5.3 THE EFFECT, ON NK ACTIVITY, OF PULSING EITHER EFFECTOR OR TARGET CELLS WITH SUGARS FOR 2 HOURS.

Fig. 5.4 shows the reduction in lysis after treating LGLs with D-mannose and methyl-D-mannoside alone or followed by IFN in a 4 hour assay. In an 18 hour assay however, the level of cytotoxicity of all of the effector cells treated with IFN is markedly improved. This result is similar to that shown in Fig. 3.1 which illustrated the effect of pulsing LGLs with PLC/PRF/5 supernatants followed by IFN.

5.3.3 The effect of sugars on effector-target cell binding

Figs. 5.5, 5.6 and 5.7 show the effects of sugars on:

1) the ability of LGLs to bind target cells (Fig. 5.5)
2) the ability of target cells to be bound by LGLs (Fig.5.6)
3) the ability of LGLs which bind to target cells to lyse them (Fig. 5.7).

It can be seen that only mannose and methyl-mannoside inhibit all three of the above mentioned parameters and that even if these effector cells are subsequently treated with IFN the binding is not restored. However, IFN treated LGLs were found to lyse greater numbers of K562 cells (Fig. 5.7).

5.4 Discussion

A role for various carbohydrates has been implicated in the initial target-effector interaction of the NK cytolytic pathway (Roder et al., 1979; Zaunders et al., 1981; Stutman et al., 1980; MacDermott et al., 1981; Ades et al., 1981).

Key to Fig. 5.4

A - duration of assay - 4 hours

B - duration of assay - 18 hours

1 - LGL pulsed with medium - 2 hours

2 - LGL pulsed with IFN - 2 hours

3 - LGL pulsed with mannose - 2 hours

4 - LGL pulsed with galactose - 2 hours

5 - LGL pulsed with glucose - 2 hours

6 - LGL pulsed with methyl-mannoside - 2 hours

7 - LGL pulsed with mannose - 2 hours then IFN

8 - LGL pulsed with galactose - 2 hours then IFN

9 - LGL pulsed with glucose - 2 hours then IFN

10 - LGL pulsed with methyl-mannoside - 2 hours then IFN

The results of a typical experiment are illustrated.

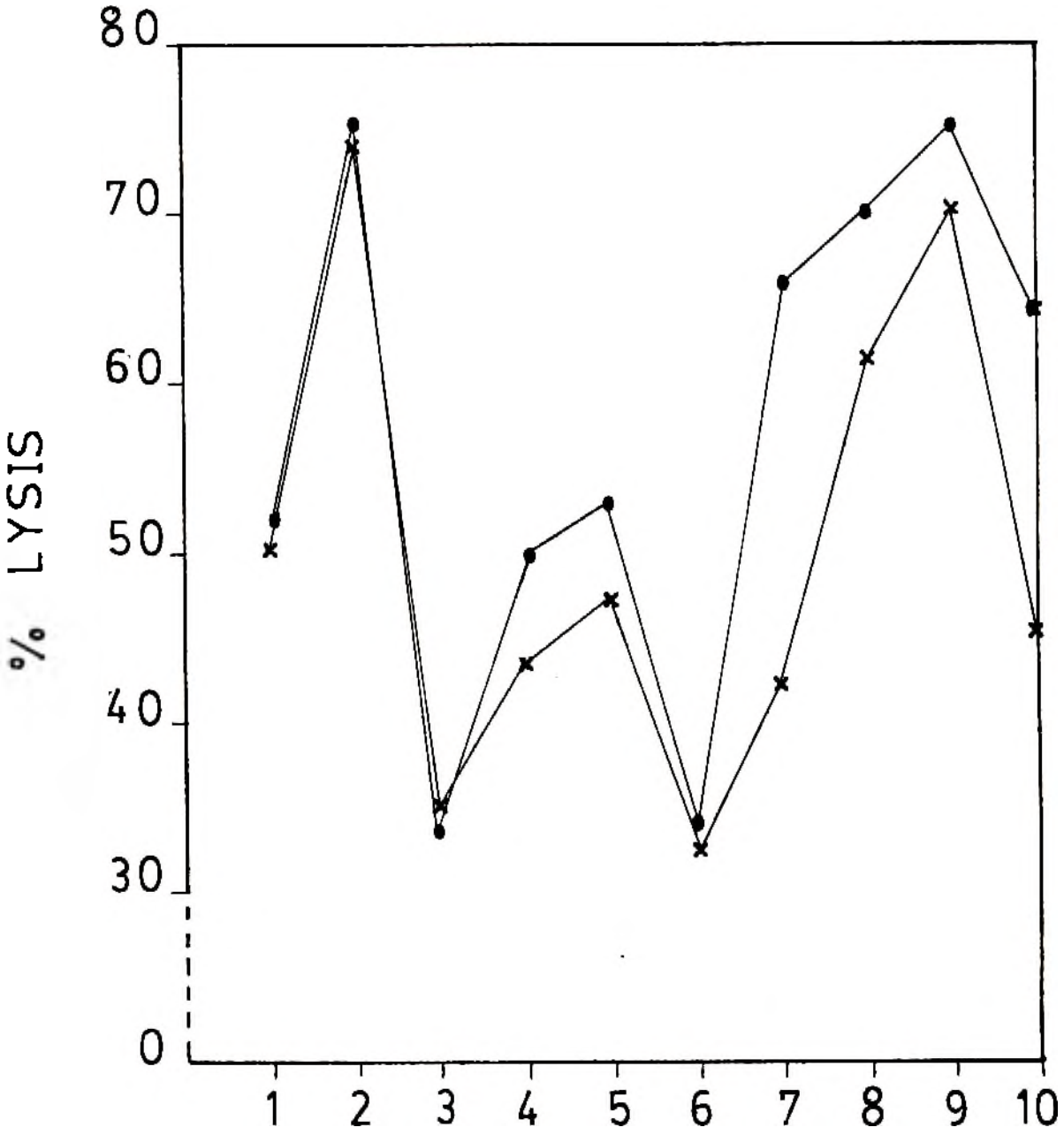


FIGURE 5.4 THE COMBINED EFFECT OF SUGARS AND INTERFERON ON THE NK ACTIVITY OF LGL AGAINST K562 CELLS.

Key to Fig. 5.5

1 - LGL pulsed with medium

2 - LGL pulsed with IFN - 2 hours

3 - LGL pulsed with mannose - 2 hours

4 - LGL pulsed with galactose - 2 hours

5 - LGL pulsed with methyl-mannoside - 2 hours

6 - LGL pulsed with mannose - 2 hours then IFN

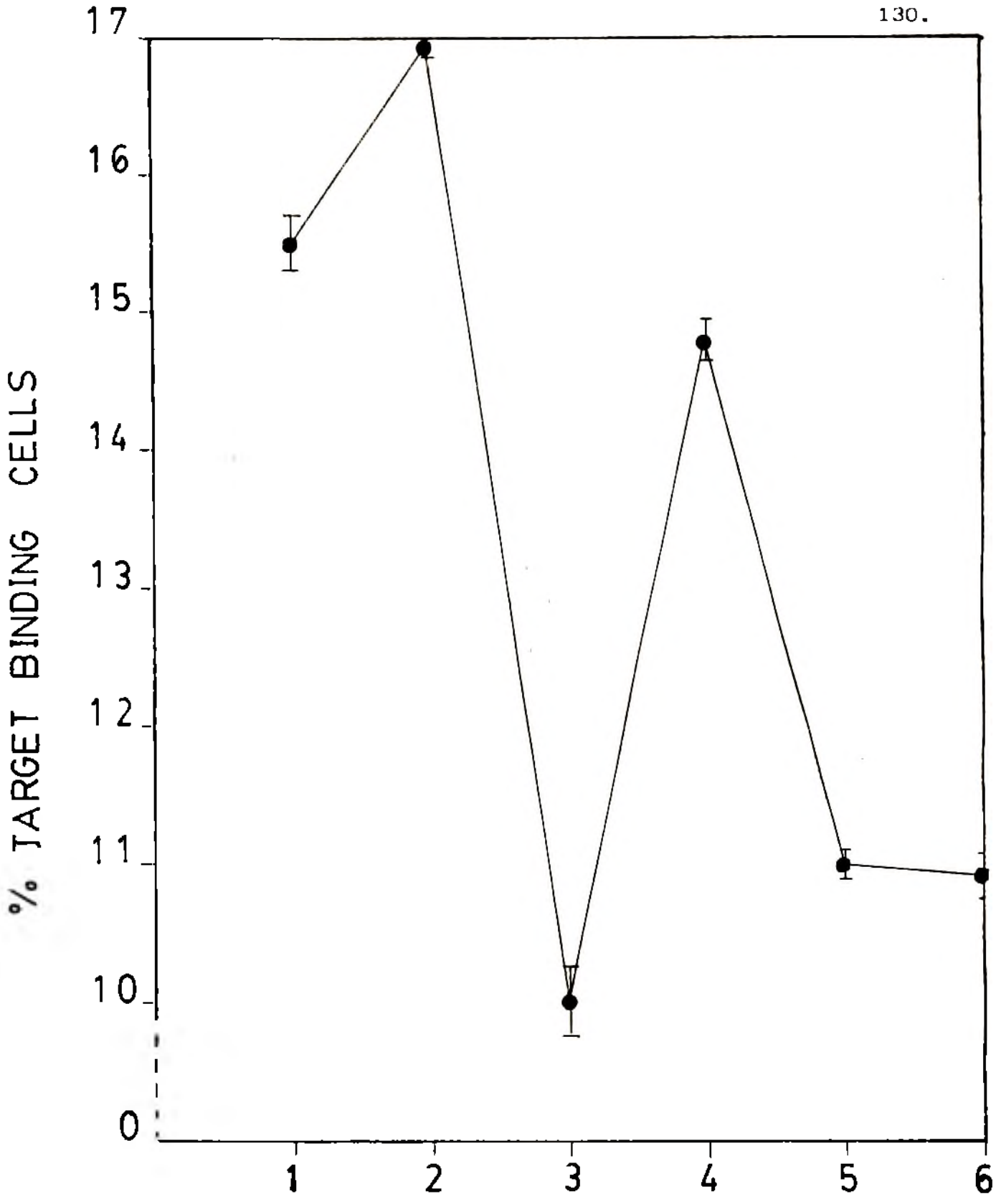


FIGURE 5.5 THE EFFECT OF SUGARS AND INTERFERON ON THE PROPORTION OF TARGET BINDING CELLS.

Key to Fig. 5.6

1 - LGL pulsed with medium

2 - LGL pulsed with IFN - 2 hours

3 - LGL pulsed with mannose - 2 hours

4 - LGL pulsed with galactose - 2 hours

5 - LGL pulsed with methyl-mannoside - 2 hours

6 - LGL pulsed with mannose - 2 hours then IFN

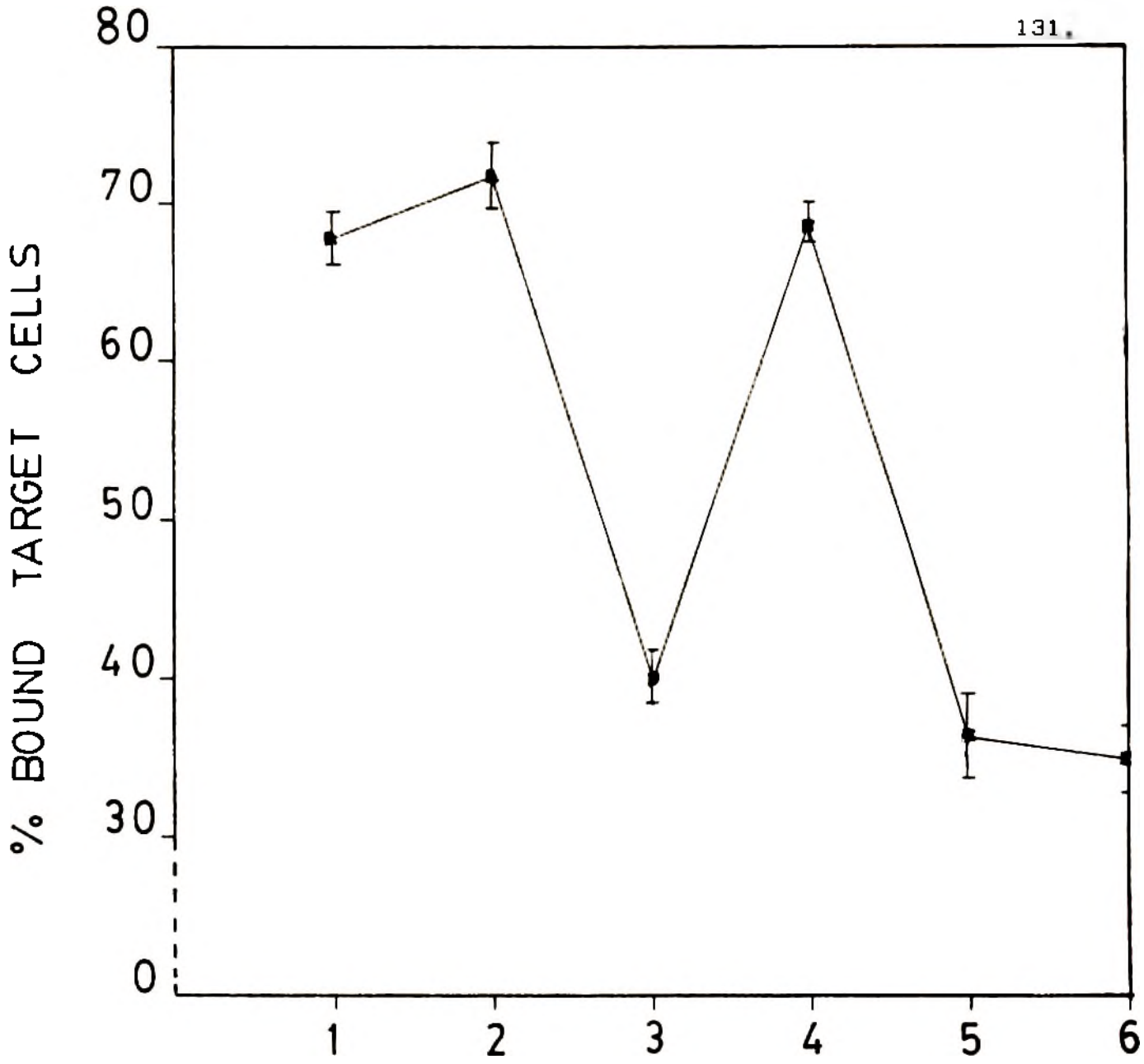


FIGURE 5.6 THE EFFECT OF SUGARS AND INTERFERON ON THE PROPORTION OF BOUND TARGET CELLS.

Key to Fig. 5.7

1 - LGL pulsed with medium

2 - LGL pulsed with IFN - 2 hours

3 - LGL pulsed with mannose - 2 hours

4 - LGL pulsed with galactose - 2 hours

5 - LGL pulsed with methyl-mannoside - 2 hours

6 - LGL pulsed with mannose - 2 hours then IFN

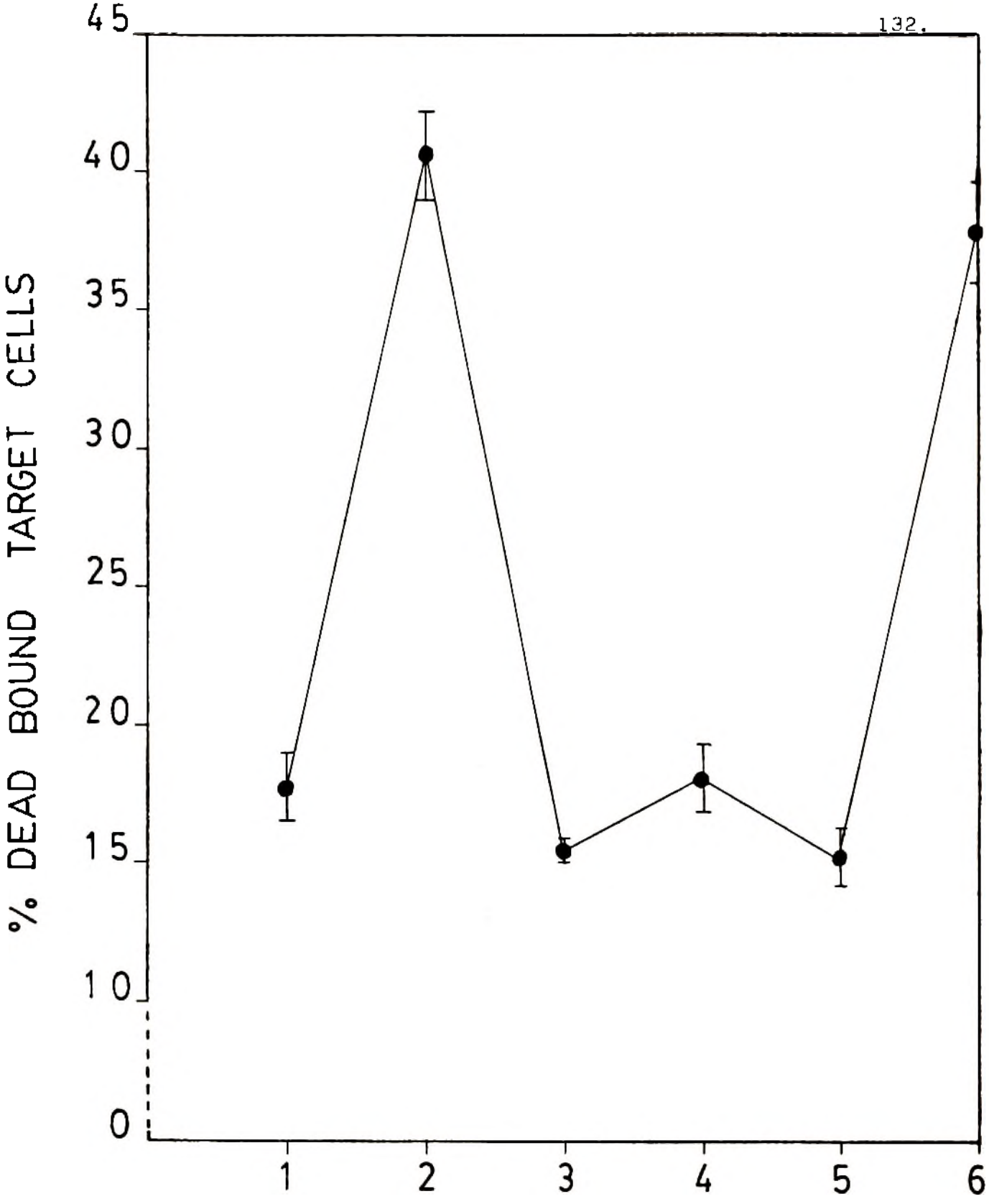


FIGURE 5.7 THE EFFECT OF SUGARS AND INTERFERON ON THE PROPORTION OF DEAD, BOUND TARGET CELLS.

This hypothesis has emerged from simple sugar inhibition studies (Stutman et al., 1980; MacDermott et al., 1981; Ades et al., 1981) to direct isolation and characterization of putative recognition structures from susceptible target cells (Roder et al., 1979; Zaunders et al., 1981). The majority of findings support the concept that NK cells are polyvalent and can recognise a wide variety of target cells of diverse origin (Herberman, 1982; Roder et al., 1981). More recently Forbes et al., (1981) have demonstrated that selective hexose phosphates particularly mannose-6-phosphate could strongly inhibit NK cytolysis.

In this study the effect of various simple sugars on the binding and lysis of K562 target cells by peripheral blood LGLs was studied, in view of the reported findings of simple sugars and the apparent similarity to the effects of the PLC/PRF/5 supernatant.

From a panel of saccharides tested, mannose and methyl-mannoside were found to have the greatest effect on NK cell function. The results of suppression with mannose confirms the work of Stutman et al., (1980) and Werkmeister et al., (1983) while contrasting with the work of others (MacDermott et al., 1981; Forbes et al., 1981). No significant inhibition was found with any of the other sugars tested.

The addition of IFN to the system, after treatmant of effector cells with sugar, did not cause greater numbers of target and effector cells to bind. It did, however, enhance

lysis of those target cells which were bound by LGLs. This effect was more apparent in an 18 hour assay than the 4 hour chromium release assay. It seems likely that IFN acts by enhancing the recycling capacity of those effector cells unaffected by the sugar thus causing them to complete each lytic cycle more rapidly thereby enabling them to lyse greater numbers of target cells in the assay period.

Present investigations indicate the possible importance of selected sugar moieties in recognition and in subsequent steps of the lytic cycle.

The studies outlined in this, and the previous chapter, namely the inhibition of NK cell activity with certain monosaccharides and the PLC/PRF/5 supernatant are consistent with the concept that NK cells may recognise the target cell by virtue of lectin-like receptors which are capable of interaction with distinct cell surface carbohydrate moieties presumably in the from of glycoproteins or glycolipids.

CHAPTER 6

6. EFFECTOR-TARGET CELL CONJUGATE FORMATION IN SOFT AGAR AFTER TREATMENT OF THE CELLS WITH PLC/PRF/5 SUPERNATANTS INTERFERON AND SIMPLE SUGARS

6.1 Introduction

Despite the fact that the mechanism by which NK cells recognise their targets is poorly understood, binding is an essential first step in the events which lead to target cell lysis. The study of NK cell - target cell interaction has employed many of the techniques initially developed for examining cytotoxic T cell interactions. Some of these include the formation and enumeration of effector-target conjugates - as described in chapter 4 (Berke and Amos, 1973) and more recently the immobilization of these conjugates in agarose to permit analysis of single lytic interactions (Grimm et al., 1979).

It is widely accepted that interferons are capable of augmenting NK activity (Trinchieri et al, 1978; Herberman et al., 1979; Taragan and Dorey, 1980; de Landazuri et al., 1981) but the mechanism of this augmentation remains unclear. Previously this increased cytotoxic ability of NK cells has been described using a ^{51}Cr release assay (Herberman et al., 1979; Zarling et al., 1979; Ault and Weiner, 1979). Due to this assays' technical limitations the question of whether the IFN induced augmentation was related to :

1) an increased number of NK cells
2) activation of individual NK cells to lyse targets more rapidly or
3) a combination of the two

could not be adequatly examined.

A similar shortfall in our laboratory was experienced when determining the effect of the PLC/PRF/5 supernatants on NK activity. Although this problem was, to some extent, overcome by using the target binding assay described in chapter 4, the possibility of an alteration in the recycling rate of effector cells still could not be examined.

The method of immobilizing effector-target conjugates in agarose has been shown to be extremely useful to date. Silva et al., (1980) have demonstrated that in vitro IFN augments normal NK function by recruiting previously non-lytic "pre-NK" cells to display their cytotoxic potential as well as by enhancing the kinetics of lysis. Taragan and Dorey, (1980) have also shown that the augmentation of natural cytotoxicity by IFN is due to both recruitment of effector cells capable of binding to, but not lysing targets, and to activation of this population of cells and already active NK cells to recycle more rapidly.

The obvious advantages of the immobilization of conjugates in agarose led to the use of this technique in the present study, in an attempt to gain further knowledge of the mechanism by which the PLC/PRF/5 supernatants were able

to reduce natural killing *in vitro*.

6.2 Materials and methods

6.2.1 Preparation of cell suspensions

LGLs isolated from human peripheral blood as described in chapter 2.2.1 to 2.2.4, were pulsed with either PLC/PRF/5 supernatant for 24 hours, interferon for 2 hours, various sugars for 1 hour or medium (the control system) as described in chapter 3.2.3.

6.2.2 Single cell assay in agarose

The assay was performed using a modification of the method of Katz *et al.*, (1982) based on the origional descriptions of Grimm and Bonavida (1979) and Bradley and Bonavida (1981).

2×10^5 effector cells and an equal number of K562 target cells were mixed in a total volume of 0.2mℓ medium in a 10mℓ roumd bottomed centrifuge tube. At higher effector to target ratios more than one lymphocyte binds to a single target cell and therefore the percentage of conjugates cannot be accuratly determined. Tubes were centrifuged at 1000rpm for 5 minutes and incubated at 37°C for 15 minutes. The cells were gently resuspended and the mixture carefully added to 0.5mℓ 0.5% agarose in RPMI 1640 which was pre-cooled at room temperature to 37°C. Cells were mixed in the agarose with a pre-warmed pasteur pipette before being pipetted

onto glass slides which had been coated with 0.5% agarose. After the cell mixture in agarose had solidified the slides were placed in sterile petri dishes and covered with medium. The dishes were incubated at 37°C, 5% CO_2 in a humidified environment for various time intervals. After the incubation the medium was removed and 0.1% trypan blue dye added for 8 minutes. Slides were washed in saline three times before being examined by transmitted light microsocpy.

Spontaneous target cell death was determined by incorporating target cells alone into the agarose and counting the number of dead cells on a slide at each timepoint. Corrections for spontaneous target death were made by applying the following formula to calculate the percentage target binding cells (TBC) with dead targets.

(% TBC with dead targets) - (% spontaneous dead targets x % TBC with dead targets).

The percentage of active killer cells for a given lymphocyte population at a certain time is calculated using the formula:
(% bound cells that are toxic at x hours) (% of lymphocytes bound to K562 cells).

Using the results from the single cell assay in agarose in conjunction with those obtained in a chromium release assay (performed as described in chapter 2.2.6) using an ettector:target (E:T) ratio of 5:1 (ie. 5 x 10^4 effector

cells:10^4 target cells) it is possible to calculate the maximum recycling capacity (MRC) which estimates the number of target cells killed by an active NK cell in the 4 hour cytotoxicity assay. Since the dose response curve from the ^{51}Cr release assays resembles Michaelis-Menton enzyme-substrate kinetics it is possible to use the Lineweaver-Burke equation in the analysis of data.

$$\frac{1}{V} = \frac{Km}{Vmax} \times \frac{1}{T} + \frac{1}{Vmax}$$

where T is the initial number of target cells and V the number of lysed target cells. Vmax is the number of target cells killed when the system is saturated with target cells. When the E:T ratio is 5:1 the formula is:

$Vmax = 1.4 \times 10^3 + 4.2 \times 10^2 \times$ (% lysis at 5:1)

The MRC is calculated by dividing Vmax by the absolute number of NK cells ie. the percentage of active NK cells multiplied with the number of effector cells in the Vmax (10^5).

6.3 Results

6.3.1 The effect of PLC/PRF/5 supernatant, IFN or sugars on binding, lysis and recycling of LGLs

The results expressed in Table 6.1 are calculated from those shown in Tables 6.2 and 6.3 that is, by combining the data from cytotoxicity assays with that from single cell assays

TABLE 6.1 THE EFFECT OF PLC/PRF/5 SUPERNATANTS, INTERFERON OR SUGARS ON NK PARAMETERS AGAINST K562 CELLS

Cell system	Vmax x 10^3	%TBC	%lytic TBC	%active NK cells	estimated MRC
LGL + medium	12.7	12	27	3.2	3.9
LGL + interferon	21.2	11	45	5.0	4.3
LGL + PLC/PRF/5	4.1	6	24	1.8	2.9
LGL + glucose	12.2	11	26	2.9	4.0
LGL + mannose	3.5	5	28	1.6	3.4

TABLE 6.2 THE EFFECT OF PLC/PRF/5 SUPERNATANTS, INTERFERON OR SUGARS ON THE BINDING AND LYSIS OF K562 CELLS IN A SINGLE CELL ASSAY IN AGAROSE 4 hours

Cell system	% TBC (mean ± SD)	% lytic TBC
LGL + medium	12 ± 1.4	27 ± 1.9
LGL + interferon	11 ± 2.7	45 ± 0.7
LGL + PLC/PRF/5	6 ± 2.0	24 ± 2.1
LGL + glucose	11 ± 1.5	26 ± 2.5
LGL + mannose	4 ± 1.0	28 ± 1.8

TABLE 6.3 THE EFFECT OF PLC/PRF/5 SUPERNATANT, INTERFERON OR SUGARS ON THE NK ACTIVITY OF LGLS E:T 5:1 4 hours

Cell system	% lysis (meam ± SD)
LGL + medium	27.0 ± 5.6
LGL + interferon	47.2 ± 3.4
LGL + PLC/PRF/5 s/n	6.4 ± 3.0
LGL + glucose	25.8 ± 4.8
LGL + mannose	5.0 ± 3.1

in agarose. Table 6.3 illustrates the marked suppression of NK activity caused by the PLC/PRF/5 supernatant and by mannose. The degree of lysis of target cells in these assays is much lower than that shown in previous chapters because a ratio of 5:1 E:T cells was employed. The data in Table 6.2 indicates that decreased lysis exhibited by PLC/PRF/5 supernatant, or mannose treated cells is due to a decrease in the numbers of conjugates fromed between effector and target cells.

The first column of Table 6.1 shows the Vmax, that is the number of target cells killed when the system is saturated with such cells, to be decreased when the effector cells were pulsed with either the culture supernatant or mannose. Glucose treatment of LGLs had no effect however. IFN treatment of LGLs resulted in an increased Vmax.

The second two columns of Table 6.1 repeat the information gained from target cell binding assays, namely that the PLC/PRF/5 supernatant and mannose decrease the binding of effector and target cells

The percentage of active NK cells (column 4) is enhanced by IFN treatment of LGLs, and decreased by treatment of the cells with culture supernatant and mannose.

The estimated MRC (column 5), that is the number of target cells killed by an active NK cell in 4 hours, is increased by interferon. This result confirms the work of

Taragan and Dorey (1980). The MRC is, however, unaffected by mannose. The decrease in the MRC caused by treatment of the LGLs with PLC/PRF/5 supernatant, like the increase resulting from IFN treatment is significant. It is likely therefore that there are substances in the culture supernatant which block a stage in the lytic cycle subsequent to binding.

6.4 Discussion

This study demonstrated that, using a single cell assay, direct measurement of the frequency of active NK cells is possible. The decrease in NK activity exhibited by PLC/PRF/5 supernatants and mannose treated LGLs was shown to be secondary to an inability of potentially cytotoxic LGLs to bind to target cells. However, those LGLs which could bind to target cells were able to lyse them in a manner comparable to that of untreated cells. However, the "recycling" of PLC/PRF/5 treated LGLs was found to be defective. Thus the defect induced by the supernatant treatment occurs both at the binding stage of the lytic cycle and at a later, as yet unknown, stage as well.

In vitro interferon enhanced the overall cytolytic capacity of a population of LGLs (Vmax). This increase is likely to be secondary to:

1) an activation of previously non-lytic NK cells. These could be LGLs which bound to target cells but which did not have the ability to lyse them
2) an increase in the percentage of "active" NK cells and

3) an increase in the recycling capacity of interferon treated LGLs.

Taragan and Dorey (1980) have demonstrated the existance of sub-populations of peripheral blood lymphocytes which can bind sensitive NK targets but which do not produce lysis. Results of this study have confirmed this work in showing that a small population of inactive target bound LGL can be rapidly programmed to kill. This can be interpreted to mean that a subset of "pre-NK" cells exists. Such cells have NK receptors and can bind targets but remain inactive until they interact with appropriate stimuli such as IFN that initiates their killer potential.

Another aspect of NK augmentation relates to alteration in the kinetics of lysis. In addition to being able to directly visualize the frequency of active NK cells, the single cell assay is a sensitive technique to assess the kinetics of lysis of cytotoxic lymphocyte populations.

Interferon appears to rapidly activate "pre-NK" cells, which have NK receptors and can bind to targets but are inactive killers, to become "activated-NK" cells. Thus there is a reserve of potential killer cells rapidly available to be activated when appropriatly stimulated. In addition IFN can stimulate already "active-NK" cells to become activated, and kill target cells more rapidly. Since both of these processes are rapidly reversible *in vitro* (Zarling *et al.*, 1979) there is a suggestion that this may be important and

rigidly controlled *in vivo*. That this system could be turned on to respond to exogenous or endogenous stimuli, such as viral infections, and then turned off once they are no longer present.

Thus using a single cell assay to measure the differences in the frequency and kinetics of lysis of IFN activated, PLC/PRF/5 supernatant or mannose treated NK cells. Augmentation in cytotoxicity caused by IFN is due to both recruitment of new effector cells and an activation of both new and already active NK cells. Mannose, however, inhibits the binding of effector and target cells, whereas the PLC/PRF/5 supernatant affects both the binding and recycling of NK cells.

The effect of the PLC/PRF/5 supernatant appears not to be simply the result of decreased binding as was the case with mannose. However, it does contain a "blocking factor", possibly a glycoprotein, which inhibits the initial interaction of target and effector cells. This supernatant also inhibits a later stage of the lytic cycle, possibly by inhibiting various intracellular substances.

CHAPTER 7

7. DEPRESSED NK ACTIVITY IN PATIENTS WITH ADVANCED CANCER

7.1 Introduction

Studies on the NK activity of lymphocytes isolated from animals with experimental tumours or humans with spontaneous tumours have yielded variable results. Low NK activity has been described in association with numerous disorders including: malignant disease (Pross and Baines, 1976; Takasugi et al., 1977; Forbes et al., 1981; Hersey et al., 1978; de Boer et al., 1982; Kadish et al., 1981; Cunningham-Rundles et al., 1981), leukemia (Linvat et al., 1980; Behelak et al., 1976; Ziegler et al 1981; McGeorge et al., 1982; Fernandes et al., 1979), aplastic anemia (Linvat et al., 1980) chronic renal failure (Badger et al., 1981; Lang et al., 1981), rheumatoid arthritis (Karsh et al., 1981) and systemic lupus erythematous (Karsh et al., 1981; Silverman and Cathcart, 1980) to name a few.

The correlation between low NK cell function and severity of disease is not absolute. Some investigators have been unable to show any NK cell abnormality in patients with multiple sclerosis (Santoli et al., 1981) or rheumatoid arthritis (Silver et al., 1982).

Conversely Pross and Baines (1980) reported depressed NK cell activity with ovarian cancer especially those with advanced disease. Gerson (1980) studied patients with a

variety of tumours and found depressed NK activity predominially in patients with neuroblastoma. Depressed NK activity appeared to be correlated with the extent of disease but not necessarily with the size of the primary tumour. Some of the patients with large localised tumours had high levels of NK cell activity. Patients with early operable breast cancer have normal NK cell activity but studies on patients with lung cancer have yielded conflicting results (Cannon et al., 1977; Eremin, 1980).

NK cell depression due to various types of therapy, such as radiation therapy (Blomgren et al., 1980), extensive surgery (Gillou et al., 1982), cytotoxic chemotherapy (McGeorge et al., 1982), prednisone (Parrillo and Fauci, 1978) and diethylbestrol (kalland and Haukaas, 1981) have been reported. Rubin and his colleagues (1982) postulated that immunosuppressive therapy may also result in reduced NK cell frequency and defective recycling capacity of the NK cells. Removal of the above mentioned agents results in recovery of normal NK cell function in most patients. Guillo et al., (1982) have also shown that NK cell function may gradually recover in spite of long term immunosuppression with steroids and azathioprine.

In other studies suppression of NK cell activity in peripheral blood lymphocytes, lymph nodes and in situ in cancer patients has been attributed to suppressor cells (de Boer et al., 1982; Uchida and Mickshe, 1981; Allavena et al., 1981; Eremin and Coombs, 1981). This conclusion is based on

the inhibitory effect of tumour associated lymphocytes on NK cells *in vitro*. The mechanisms by which various diseases bring about NK cell depression are very complex and may involve one or more components of the NK cell system as well as modulating substances, such as IFN and prostaglandins (Koren *et al*., 1981; Taragan, 1981; Bankhurst, 1982). Benczur and his colleagues (1980) reported a depressed NK cell activity in patients with multiple sclerosis which was accompanied by a decrease in the ability to make or respond to IFN. A similar defect has been inferred by Hersey and coworkers (1982) in Fanconi's syndrome and by Kadish *et al*., (1981) in a study of low NK cell activity donors with breast cancer. In most instances, however, the mechanisms underlying low NK activity have not been elucidated.

Previous work in our laboratory has shown non-adherent peripheral blood lymphocytes from patients with advanced hepatocellular carcinoma (HCC) to have depressed NK cell activity against both the K562 and an established hepatocellular carcinoma cell line (Son *et al*., 1982). In the light of the numerous reports on the NK activity of cancer patients these adults suffering from HCC provided an ideal system in which the possible NK cell defect could be investigated. A recently described IgM monoclonal antibody HNK-1 which defines a differentiation marker expressed on NK and K cells (Abo and Balch, 1981) was also utilized in this study. Cells identified by this antibody and isolated by fluoresence activated cell sorters (FACS) have been shown to display almost all of the NK cell function in standard *in*

vitro tests. Correlation between HNK-1^+ cell frequency and NK cell functional activity has been clearly demonstrated in further studies (Abo et al., 1982).

7.2 Materials and methods

7.2.1 Patients

30 patients with histologically proven HCC were studied. All patients were Black and none were receiving any form of therapy at the time of study. 12 other patients with various types of cancer and differing tumour burdens were also studied. They too were not receiving any form of therapy at the time of investigation. Normal healthy laboratory workers served as controls.

7.2.2 Preparation of LGL

LGLs from peripheral blood samples were isolated and purified as outlined in chapter 2.2.1 to 2.2.4 and the purity of the LGL fraction checked as described in chapter 2.2.5.

7.2.3 Cell lines

The K562 cell line was used as a target cell in the chromium release assay. It was maintained in culture as described in chapter 2.2.6.

7.2.4 Chromium release assay

The ^{51}Cr release assay was performed as outlined in chapter 2.2.6 and used to determine the NK cell activity of both normal and patient LGLs.

7.2.5 Pretreatment of LGLs with interferon

The effect of IFN on the ability of both patient and normal LGLs to lyse K562 cells was assessed as described in chapter 3.2.4.

7.2.6 Target cell binding assay

LGLs isolated from the normal blood and cancer patients were assessed for their ability to bind K562 target cells using the method described in chapter 4.2.7.

7.2.7 Immunofluoresence assay

Cell surface antigens defined by the HNK-1 monoclonal antibody were enumerated by direct immunofluoresence assays. The monoclonal IgM antibody fluorscein isothiocyanate (FITC) conjugated HNK-1 (Leu-7; Becton Dickinson and Co., Sunnyvale) was used at a concentration of 50 µg/mℓ.

Briefly, 2 x 10^5 cells were resuspended in 100µℓ RPMI 1640 supplemented with 5% FCS and 0.01% sodium azide. 5µℓ HNK-1 antibody was added to the cell suspension and incubated for 30 minutes at 4°C in the dark. The cells were then washed twice in the medium supplemented with FCS and sodium azide,

resuspended in approximetly 100µℓ medium and a drop was examined under high power for fluoresence.

7.2.8 Complement mediated lysis

Cell surface antigens defined by the Leu 11b, OKM1, OKT3, OKT8 and OKT4 monoclonal antibodies were enumerated by complement mediated lysis.

LGLs from HCC patients and normals were resuspended at a concentration of 1×10^6 cells in 100µℓ RPMI 1640 medium supplemented with 5% FCS and 0.01% sodium azide and incubated with 5 µℓ of the appropriate monoclonal antibody for 30 minutes at room temperature. 50 µℓ of rabbit complement was added to each cell mixture and incubated for 1 hour at 37°C. Appropriate controls, such as cells with complement and no antibody, were included in the experiment. After the second incubation period, the cell suspensions were washed and the percentage of viable cells adjuged by examination under the light microscope after the addition of trypan blue dye.

7.3 Results

7.3.1 Quantitation of LGLs

Although the total mononuclear cell population was decreased in HCC patients, the proportion of LGLs obtained by percoll density gradient centrifugation was comparable to that of controls. In both patients and controls 70-90% of the cells

isolated from the 40-45% interface of percoll gradients exhibited surface antigens (Leu-7) characteristic of LGLs (Table 7.1). In both groups less than 1% of the LGL fraction injested opsonised candida albicans or had characteristic staining properties of monocytes as adjuged by peroxidase staining (Table 7.1). Approximetly 50% of LGLs isolated from both patients and controls expressed the Leu 11b antigen, and 70% of the LGLs exhibited the OKM1 antigen. A small proportion of both populations of LGLs expressed the OKT8 antigen, although less than 10% were stained with OKT3, a pan T cell marker, likweise there were very few contaminating B cells in the LGL population (Table 7.1).

7.3.2 Cytotoxic activity of enriched LGLs and the effect of interferon

As illustrated in Fig. 7.1 considerably decreased lysis of K562 cells was exhibited by patient LGLs in comparison to normals. Cytolytic activity of both control and patient LGLs was improved by pulsing the cells with 1000IU IFN for 1 hour. IFN had no direct cytotoxic effect on the target cells (results not shown). NK cell activity was also assessed in a group of patients with other types of cancer. Table 7.2 shows that the NK activity of the LGLs of some of these patients was depressed and this decrease in cytotoxic activity correlated well with the size of the tumour.

7.3.3 Target cell binding

TABLE 7.1 CHARACTERISTICS OF LGL PREPARATIONS FROM CONTROLS AND PATIENTS WITH HCC

Percent cells reacting with:	(mean ± SD) controls	patients
Leu 7	84.4 ± 4.9	80.3 ± 6.4
Leu 11b	50.5 ± 7.8	46.0 ±12.5
OKM1	66.8 ± 3.3	68.7 ± 3.5
OKT3	10.1 ± 2.9	8.7 ± 4.9
OKT8	17.8 ± 3.2	15.0 ± 7.0
OKT4	4.4 ± 1.2	ND
Anti-human Ig	0.2 ± 0.1	1.0 ± 1.4
Peroxidase positive cells	2.7 ± 1.2	2.0 ± 1.4

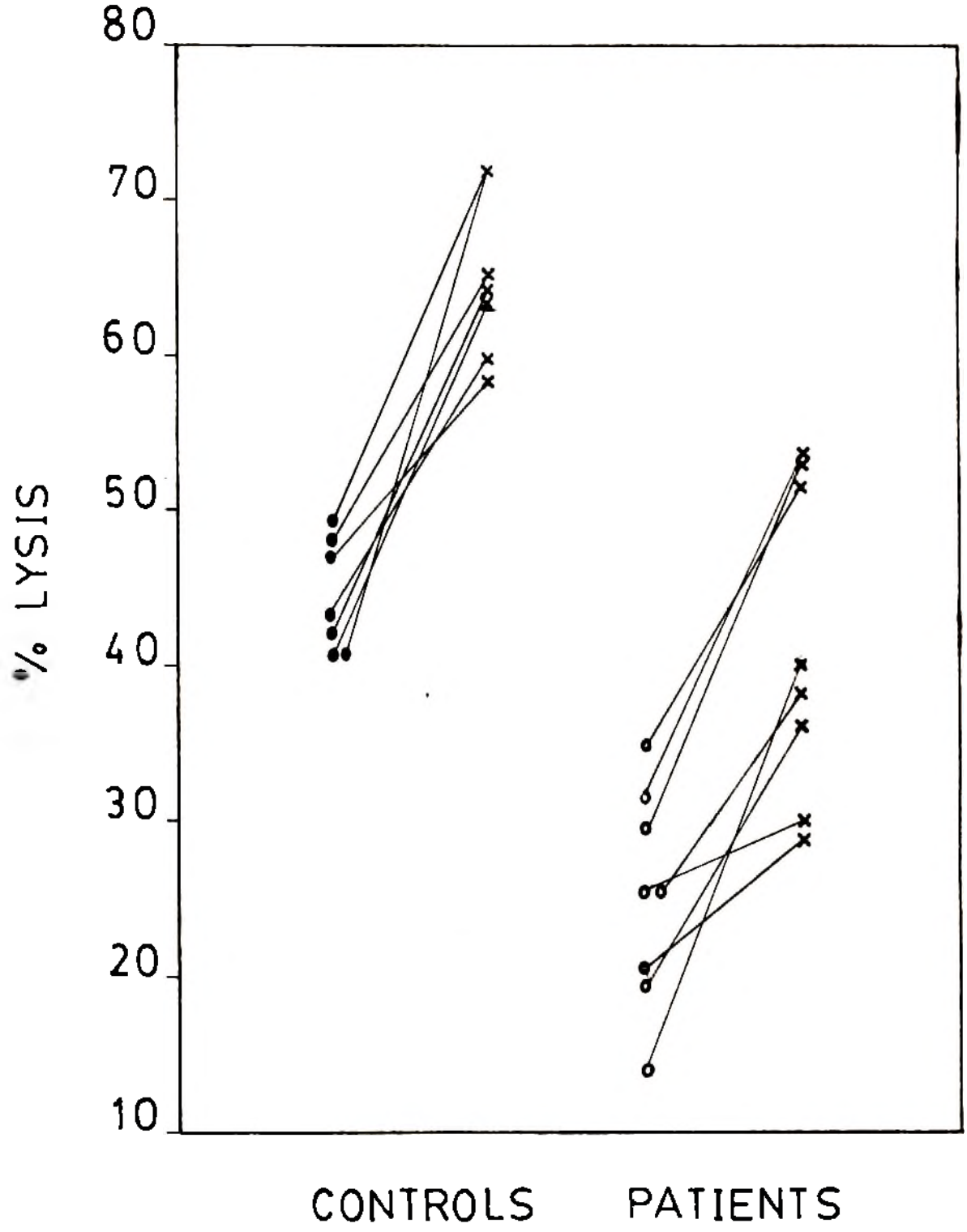


FIGURE 7.1 IMPROVED CYTOTOXICITY OF CONTROL AND PATIENT LGLS AFTER BEING PULSED WITH 1000IU/ML INTERFERON.

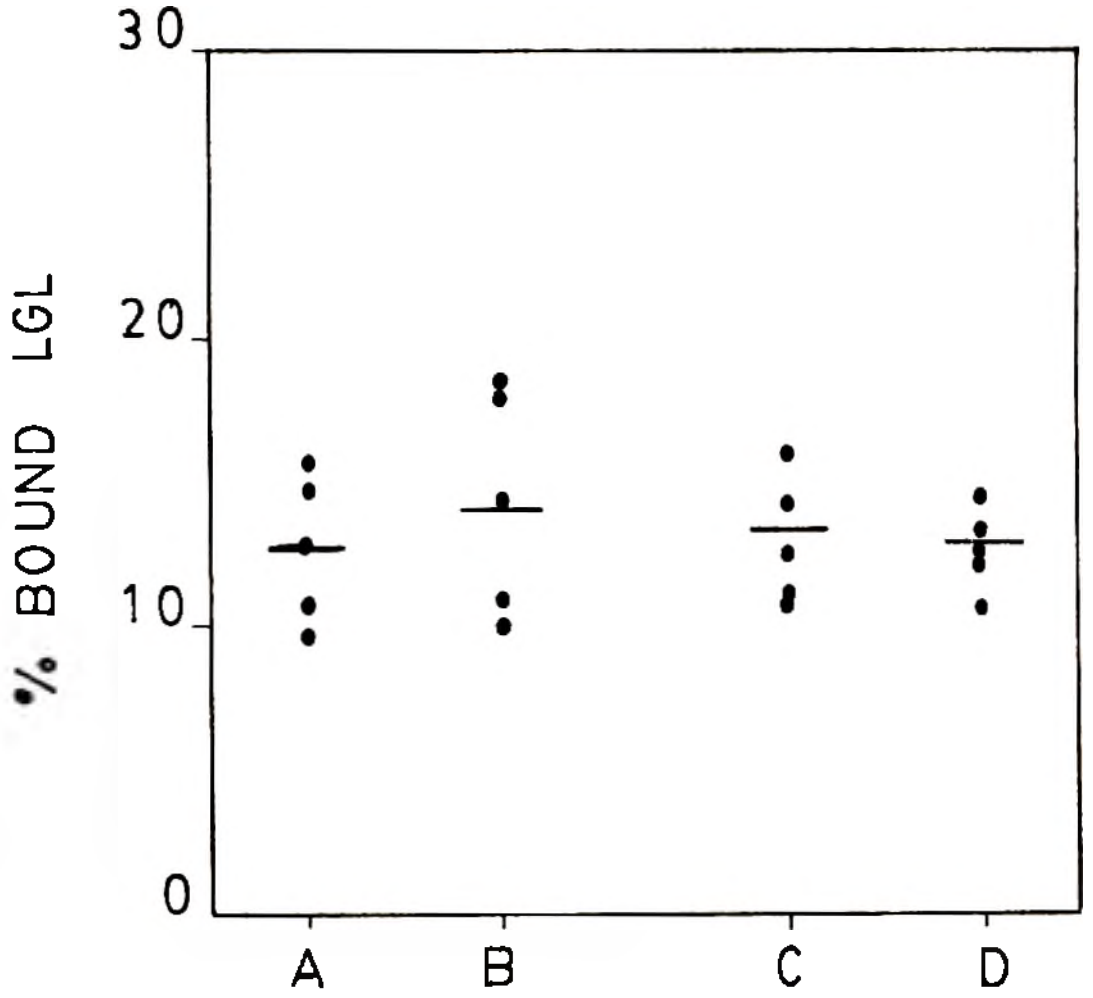
TABLE 7.2 RELATIONSHIP OF TUMOUR BURDEN WITH CYTOTOXICITY BY LGLS

Tumour	Tumour burden	% lysis
Ca ovary	large	14
Ca kidney	large	27
Soft tissue sarcoma	large	19
Ca stomach	large	35
Ca cervix IIIB	moderate	10
Ca cervix IIB	moderate	39
Ca breast	moderate	38
Ca salivary gland	moderate	37
Ca oesophagus	small	34
Ca prostate	small	38
Ca oesophagus	small	47
Ca oesophagus	small	40
Controls		41-67

LGLs from patients and normals were incubated with K562 cells for 3 hours and the degree of target cell binding was assessed microscopically. In Fig. 7.2 and 7.3 it can be seen that although similar numbers of LGLs from both groups bound to target cells, fewer K562 cells bound patient LGLs as compared to the control LGLs. This defect could not be improved by adding IFN into the binding assay. Fig. 7.4 shows that the actual killing of bound target cells was reduced when patient LGLs were used and that this defect could be corrected by the addition of IFN to the system. Addition of IFN into the control system enhanced the percentage of target cells lysed. IFN is thought to act by increasing the recycling capicity of LGLs as discussed in chapter 4.4.

7.4 Discussion

The cytolytic ability of NK cells isolated from patients with cancer is the subject of much controversy.This study clearly illustrates considerably reduced NK cell activity from LGL of untreated patients with HCC and certain other cancers. In our laboratory, it has previously been shown that non-adherent mononuclear cells from HCC patients are deficient in the ability to kill cell lines other than the one used in this study. It is therefore apparant that this defect is not confined to the killing of the K562 cell line (Son et al., 1982). Son and his colleagues showed that the effect was not due to a serum factor since normal NK cell activity could not be abrogated by incubation of NK cells with the serum of HCC patients. In the same study patients



Key

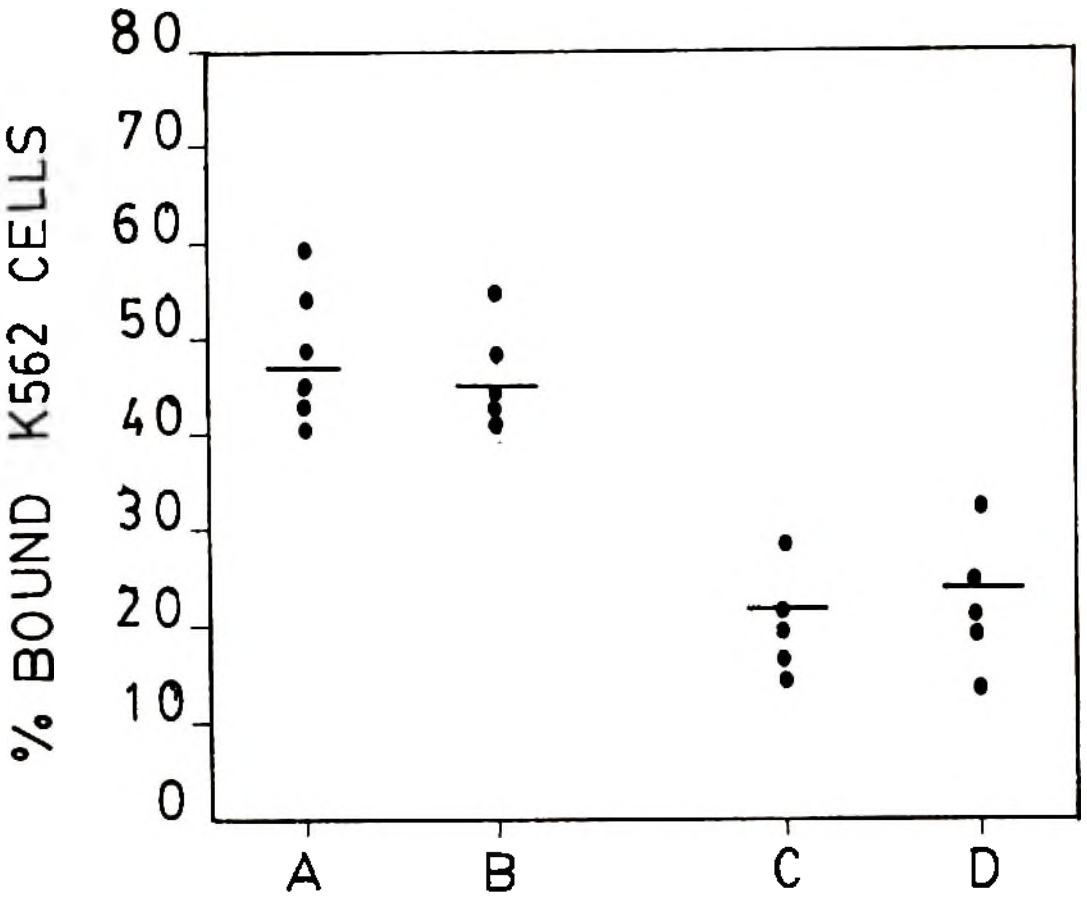
A control LGL

B control LGL + IFN

C patient LGL

D patient LGL + IFN

FIGURE 7.2 TARGET BINDING ASSAY OF CONTROL LGL AND HCC PATIENT LGL, TO K562 CELLS, AFTER INCBATION WITH 1000IU/ML INTERFERON.



Key

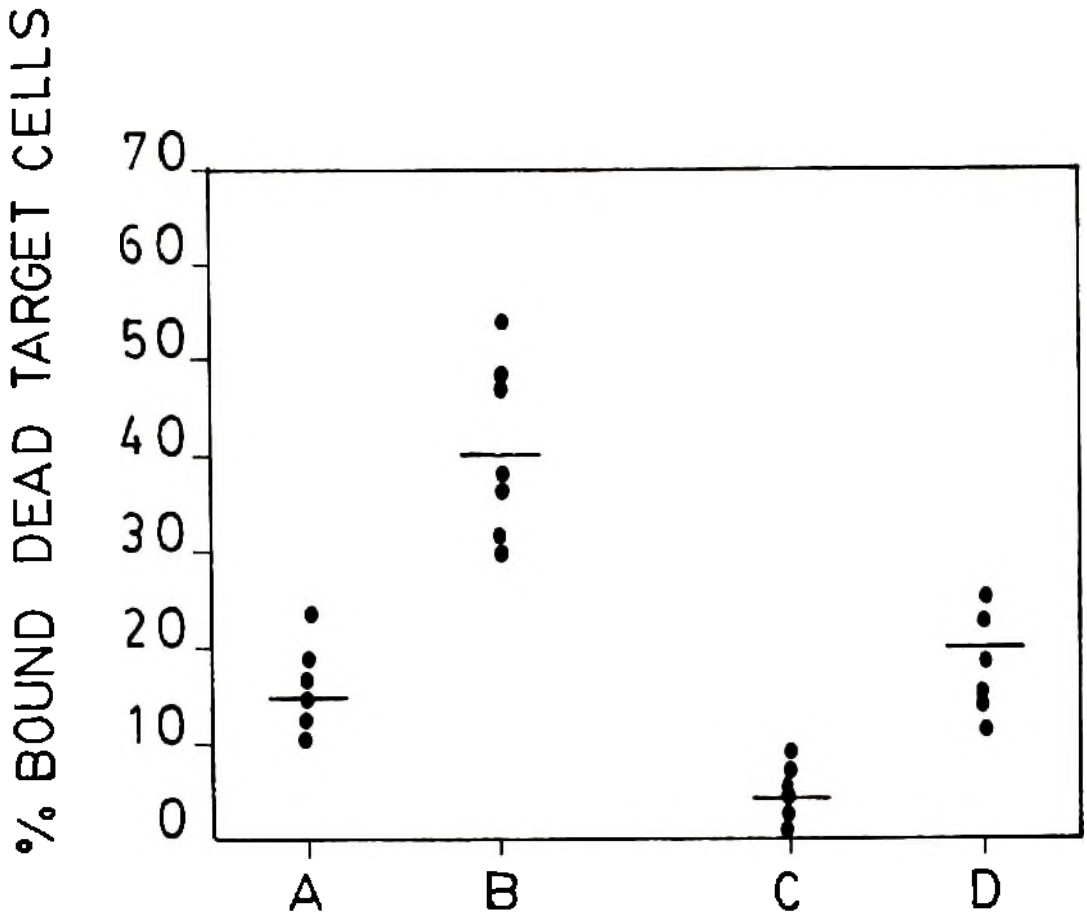
A control LGL

B control LGL + IFN

C patient LGL

D patient LGL + IFN

FIGURE 7.3 TARGET BINDING ASSAY OF CONTROL LGL AND HCC PATIENT LGL, TO K562 CELLS, AFTER INCUBATION WITH 1000IU/ML INTERFERON.



Key

A control LGL

B control LGL + IFN

C patient LGL

D patient LGL + IFN

FIGURE 7.4 TARGET BINDING ASSAY OF CONTROL LGL AND HCC PATIENT LGL, TO K562 CELLS, AFTER INCUBATION WITH 1000IU/ML INTERFERON.

with a variety of acute and chronic liver diseases were also shown to have normal levels of NK cell activity. It was concluded therefore, that the liver itself was not the cause of depressed NK cell function.

The degree of depression of NK cell activity in cancer patients has been postulated to be related to the tumour burden. Natural killing would thus be low when the tumour load was large (Forbes et al., 1980). In the group of HCC patients examined the tumour load was not formally assessed but since all patients had massive enlargement of the liver with one or more defects visualized on a liver scan it is likaly that the tumour mass was extremely large. Due to the very rapid growth of these tumours, all the patients had advanced disease at the time of study and it was not possible to test NK cell activity in any patients with early HCC. In the group of patients with other cancers the correlation between the size of the tumour burden and NK cell activity was not absolute but good. Treatment of patients' cells with trypsin, to remove any proteins which could be blocking target-effector contact, followed by a "recovery period" did not lead to increased NK cell function (results not shown). This indicated that the defect observed is unlikely to be due to a "blocking" factor on the surface of the effector cell.

None of the patients studied were recovering from any form of therapy at the time of examination and although the cause of depression of NK cell activity is unknown various

postulates have been put forward.

It is possible that the NK cell activity is depressed by factors released from the tumour or by suppressor cells such as those observed in the spleens of C.parvum treated mice (Savary and Lotzova, 1982). Alternatively NK cells could require constant priming by IFN, a process which may be disturbed by malignant disease. In this study human leukocyte IFN and recombinant IFN was shown to cause considerable enhancement of NK cell activity. This observation has previously been documented in both in vitro and in vivo models (Einhorn, 1980; Einhorn et al., 1978; Herberman et al., 1979). The mechanisms by which IFN increases the NK cell activity of LGLs is unclear, however a number of possibilities exist. One view is that IFN activates NK cells in a manner similar to that in which lymphokines activate macrophages. It has also been suggested that IFN binds to receptors on NK or pre-NK cells and triggers new messenger RNA production thus protein synthesis of new proteins involved in the cytolytic process (Ortaldo et al., 1980). Alternatively IFN could activate "pre-NK" cells causing them to mature. Saksela and his co-workers (1979) have shown that human mononuclear cells, depleted of functional NK cell activity by selective adsorption to target cells, could be induced to become cytolytic after treatment with IFN.

Senik and his colleagues (1980) inferred that IFN mediates its effects via various regulatory cells. This seems unlikely, however, as IFN is known to be effective even in

absence of T cells B cells and macrophages. There is, however, some evidence to indicate that NK cell activity is under control of regulatory lymphocytes (Cudkowitz and Hochman, 1979; Pollack and Emmons, 1979) and it is possible that IFN could mediate its effect on NK cells activity via these cells.

After an NK cell has lysed a target cell it dissociates from that cell and, after approximetly 2 hours, the LGL is able to bind and kill another target cell. This recycling capacity has been demonstrated by Timonen and co-workers (1979). If the LGLs are treated with IFN, they are thought to recycle more rapidly this may be another mechanism of NK cell augmentation by IFN.

Another reason for the decreased NK cell activity found in cancer patients is that the peripheral blood LGLs could have become depleted due to selective recruitment or entrapment at the tumour site. If this were the case NK cell activity of the tumour infiltrating lymphocytes would be expected to be high. In a number of studies the opposite has been found (Gerson, 1980; Eremin, 1980; Mantovani et al., 1980). In this study although the total number of mononuclear cells was decreased in those patients with advanced cancer, the relative number of LGLs was comparable to that of the controls. Work utilising various monoclonal antibodies revealed that similar numbers of LGLs were present in the peripheral blood of patients and normal controls.

Abo et al., (1983) indicated that there are three

distinct stages of NK cell differentiation namely:

1) cells expressing the HNK-1 antigen but neither the OKT3 nor the OKM1 antigen. These cells ($HNK^+T3^-M^-$) represent the earliest definable stage of NK cell differentiation. Such cells have been identified in 13-17 week old foetuses and are found infrequently in the bone marrow of children and adults. Morphologically, they are small agranular lymphocytes, and exhibit very low cytotoxicity.

2) $HNK^+T3^+{}_M{}^-$ cells, LGLs expressing the HNK-1 and OKT3 antigens. These cells are thought to represent an immature population of NK cells, and are found primarily in neonates and in the bone marrow of adults. They have sparse cytoplasmic granules and express relatively low levels of cytolytic activity.

3) $HNK^+T3^-M1^+$ cells , this population represents the most mature form of of NK cells and exhibit the highest levels of cytotoxic activity. These cells have the morphology of LGLs and are found predomintaly in adult blood and spleen. The distribution of these cells has been correlated precisely with NK cell functional activity.

In the light of the reported stages of NK cell differentiation the possibility that NK cells isolated from some of the HCC patients were of an immature phenotype was examined. A study was conducted in which the OKT3, OKM1 and various other antigens on LGLs from patients and normals was measured. However, there were no significant differences in the LGL populations, it therefore seems unlikely that the decrease in NK cell function could be attributed to a decased number of mature LGLs.

CHAPTER 8

8. DEFECTIVE INTERLEUKIN-1 PRODUCTION BY NATURAL KILLER CELLS FROM PATIENTS WITH ADVANCED CANCER

8.1 Introduction

Modulation of NK cell lysis by agents such as interferon (Kadish et al., 1981), IL-2 (Henney et al., 1981) and prostaglandins (Droller et al., 1978) is well documented.

IL-1, previously known as lymphocyte activating factor, is a cellular mediator produced by macrophages (Oppenheim and Gery, 1982; Farrar and Koopman, 1979). IL-1 has a wide variety of effects, it can:

1) stimulate ^{3}H thymidine incorporation by mouse and human T cells (Oppenheim et al., 1980; Gery, 1982)
2) regulate B cell differentiation (Rosenstreich and Mizel, 1978; Unane and Kiely, 1977; Mizel and Rosenstrich, 1979; Sztein et al., 1979)
3) play a role in control and growth of bone marrow cells (Mizel and Ben-Zvi, 1980)
4) affect the generation of cytolytic T cells (Oppenheim et al., 1979)
5) have numerous other non-immunological effects on various cell types in vivo.

IL-1 is known to serve as a differentiation signal (Rosenberg and Lipsky, 1981) and to bring about changes in cell membranes.

IL-1 has been postulated to affect the total response to bacterial antigens or tumours by simultaneously stimulating several lines of host defence (Oppenheim, 1982).

It has been previously demonstrated in this study, and by others, that patients with hepatocellular carcinoma (Son et al., 1982) and other patients with large tumour burdens (Pross and Baines, 1976) have diminished NK activity (Herman et al - submitted for publication). In most studies the K562 cell line was employed as a source of target cells. Although the cause of the decreased NK cell activity in cancer patients has not been clearly defined, it has been found, that these patients have functionally active NK cells which are present in normal numbers (Steinhauer et al., 1982; Herman et al., - submitted for publication) and which do not lack the ability to produce IFN on induction with virus or tumour cells (Kadish et al., 1981).

Although IL-1 has not been described as playing a role in natural cytotoxicity directly, it has a wide variety of effects on immunologic reactions. This study was therefore carried out in order to assess its role, if any, in NK cell mediated lysis, by employing LGLs obtained from the blood of healthy volunteers and patients with HCC and other malignancies.

The work outlined in this chapter indicates that LGLs, on contact with lipopolysaccharide (LPS) release an IL-1 like substance (this finding has also subsequently been reported by Scala et al., 1984). More intrestingly LGLs appear

to secrete IL-1 not only in response to LPS but also when coming into contact with K562 cells. The IL-1 released by LGLs acts on the K562 cells to stimulate their binding to LGLs thereby increasing natural cytotoxicity. The IL-1 production by LGLs from patients with extensive malignant disease was found to be grossly defective and the cytotoxic activity of these cells can be markedly improved by pretreating the target cells with IL-1 containing supernatants (or purified IL-1) prior to culturing them with LGLs. Furthermore, the augmentation of cytotoxicity by IFN is thought to be, in part, due to increased IL-1 production by treated LGLs.

8.2 Materials and methods

8.2.1 Patients

Three groups of patients were studied. The first consisted of 30 Black adults with histologically proven HCC and the second, 12 adults with a variety of malignant disease. The latter comprising carcinoma of the oesophagus, stomach, prostats, kidney, salivary gland, cervix, breast and ovary and a soft tissue sarcoma. Four of these patients had a small tumour burden, four had a moderate and four a large tumour mass. All the patients with HCC had large primary tumours. The tumour burden was based on size and extent of local and distant spread. None of the patients had received treatment at the time they were investigated. Once again, normal healthy laboratory workers served as controls. Another group of patients consisting of four with tuberculosis, two of whom

also had cirrhosis and one patient suffering from cirrhosis only (five patients in all) were also examined.

8.2.2 Preparation and identification of LGLs

LGLs were isolated from peripheral blood samples as outlined in chapter 2.2.1 - 2.2.4, and the purity of the LGL fraction checked as described in chapter 2.2.5.

8.2.3 K562 cells

The K562 cell line was maintained in culture as described in chapter 2.2.6 and utilized in ^{51}Cr assays, target binding assays and in the stimulation of IL-1 production.

8.2.4 Cytotoxicity assay

The ^{51}Cr release assay was used to determine the effect of IL-1 on the ability of LGLs to kill radiolabelled target cells as described in chapter 2.2.6.

8.2.5 Target cell binding assay

The ability of mixtures of LGLs and K562 cells to bind to each other was determined as outlined in chapter 4.2.7.

8.2.6 Production of IL-1 by LGLs

1×10^6 enriched LGLs/mℓ were cultured for 24 hours in the

presence and absence of either varying amounts of LPS (*E.coli* 0127:B8, Difco Laboratories) or varying numbers of K562 cells. Supernatants were harvested and assayed for IL-1 activity by measuring their ability to stimulate the proliferation of BALB/c mouse thymocytes. Both Concacavilin A (con A) activated thymocytes and unactivated thymocytes were used. 0.1 mℓ of 10 x 10^6/mℓ thymocytes were incubated with either con A and 0.01 mℓ of the cell culture supernatants or 0.02mℓ of the cell culture supernatant alone for 72 hours in round-bottomed microtitre plates. ^{3}H-thymidine (24 Ci/mol, Amersham, England) was added for at least 6 hours prior to culture termination. Cultures were harvested with a multiple automatic harvester and ^{3}H-thymidine incorporation assessed by liquid scintillation counting.

In certain experiments cells producing IL-1, from both patients and controls were lysed after 24 hour stimulation with LPS, to investigate the possibility that LGLs isolated from patients could synthesise but not release IL-1.

8.2.7 Effect of IL-1 on ^{51}Cr release assay and cell conjugates

1 mℓ of IL-1 containing supernatants derived either from LPS or K562 treated LGLs was added to 2 x 10^6 enriched LGLs or K562 cells and allowed to incubate for 1 hour at 37°C. The cells were washed three times in RPMI 1640 and used in either cytotoxicity assays or tested for their ability to form cell conjugates. In some experiments the IL-1 used was

derived from 24 hour cultures of adherent monocytes (1 x 10^6/mℓ) treated with LPS (20mg/mℓ).

8.2.8 The effect of interferon on IL-1 production

Varying concentrations of recombinant A IFN were incubated with LGLs for 1 hour at 37°C prior to incubation of these LGLs with either LPS or K562 cells for 24 hours to measure IL-1 production. The LGLs were washed three times after IFN treatment thus no IFN was present in the final cultures.

8.2.9 The effect of anti-IL-1 antibody on supernatants postulated to contain IL-1

Supernatants harvested from LGLs cultured either with LPS or K562 cells which had been shown to stimulate thymocyte proliferation were incubated for 2 hours at room temperature with 5µℓ of anti-IL-1 antibody, a gift from Dr. C Dinerallo, Tufts University School of Medicine, Boston. Appropriate controls of active supernatant and normal rabbit serum were included. All supernatants were then re-assayed for thymocyte stimulatory activity.

8.2.10 Comparision of the numbers of LGLs and monocytes required to produce IL-1

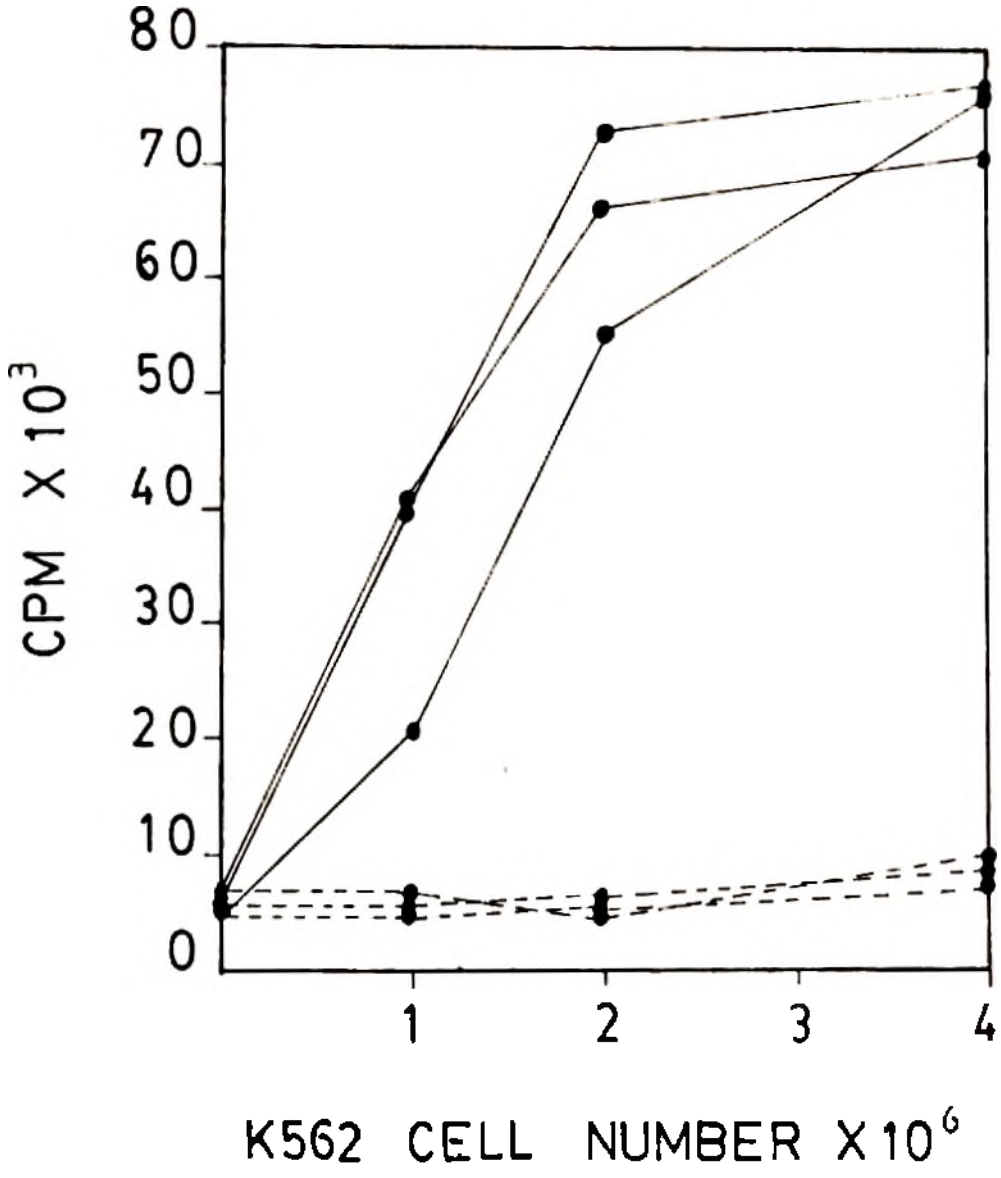
Varying numbers of LGLs and monocytes were stimulated with LPS, the supernatants harvested after 24 hours and assayed as described in 8.2.6.

8.3 Results

8.3.1 Production of IL-1 by LGLs

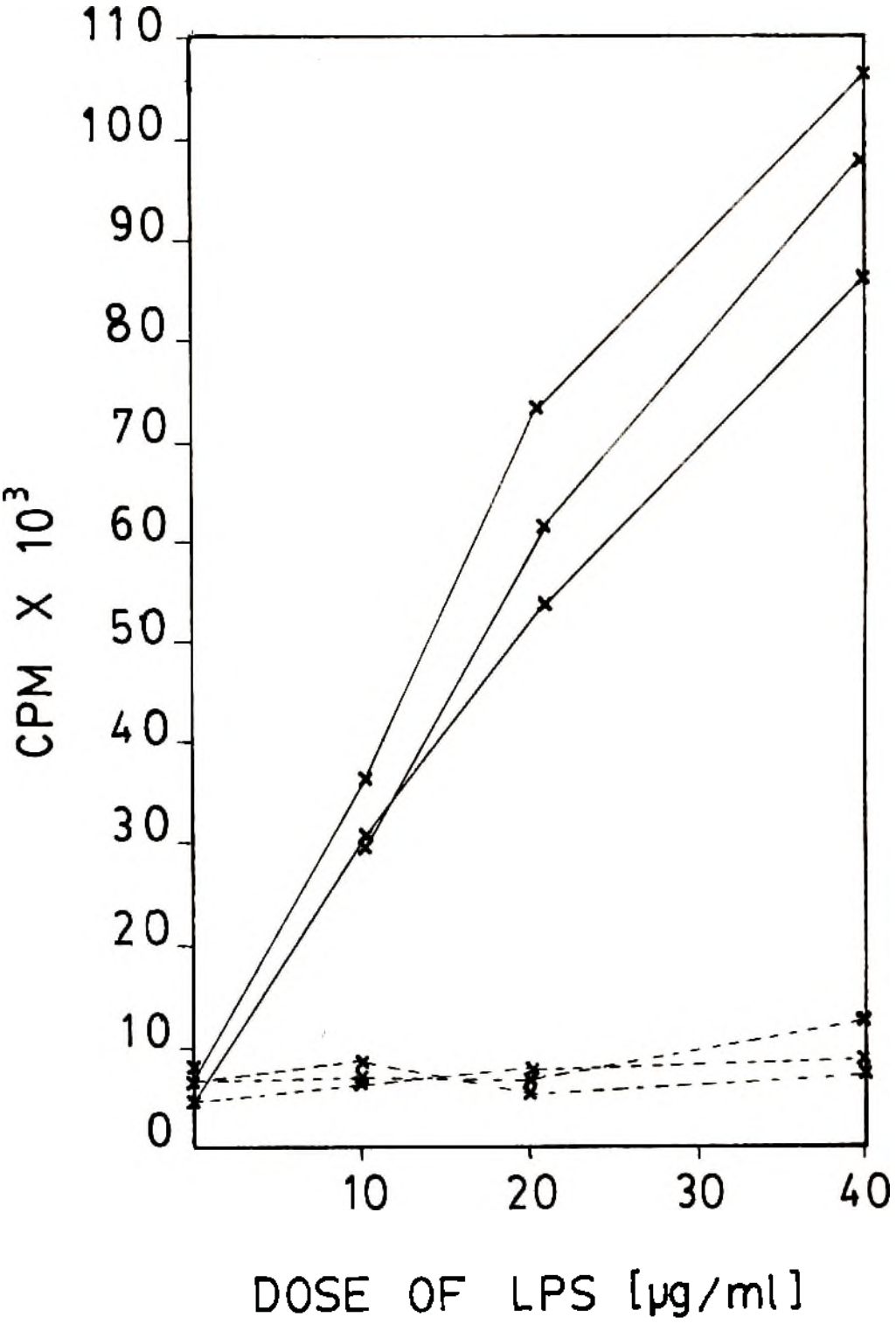
Patient or control LGLs were treated with either LPS or K562 cells for 24 hours, after which supernatants were collected and assayed for IL-1 activity by their ability to cause proliferation of con A stimulated or unstimulated mouse thymocytes. As shown in Figs. 8.1 and 8.2 markedly reduced amounts of IL-1 were produced by all the HCC patients LGLs as compared to controls when either varying doses of LPS or different numbers of K562 cells were employed to stimulate IL-1 production. Similarly, when LGLs were treated with LPS or K562 cells for varying periods of time considerably less IL-1 was produced by patient cells as compared to controls (Figs. 8.3 and 8.4). In the 12 patients with various other malignant disease IL-1 production by both LPS or K562 stimulated LGLs correlated well (though not absolutally) with the percent cytotoxicity as assessed by release of ^{51}Cr from labelled target cells (chapter 7) (Table 8.1). Both of these parameters correlated fairly well with the extent and degree of malignancy in most cases.

LGLs isolated from the 5 patients suffering from various forms of chronic illness (other than malignant disease) showed normal levels of IL-1 production when stimulated with either LPS or K562 cells (results not shown). This observation would indicate that although LGLs, isolated from blood of patients with malignant disease, show decreased IL-1



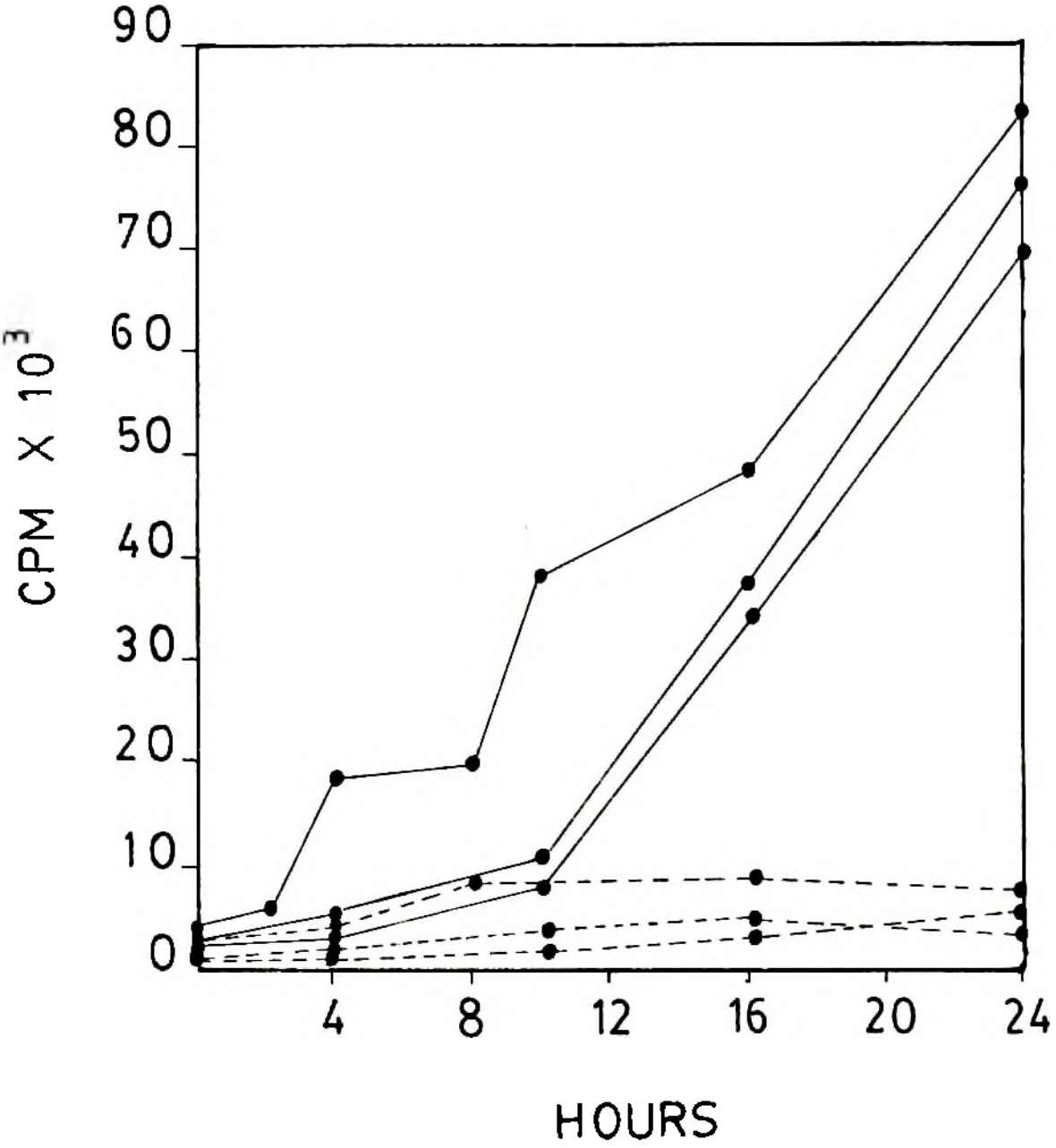
The results of three typical experiments are illustrated.

FIGURE 8.1 PRODUCTION OF IL-1 BY LGLS OF HCC PATIENTS (----) AND CONTROLS (——) STIMULATED BY INCREASING NUMBERS OF K562 CELLS.



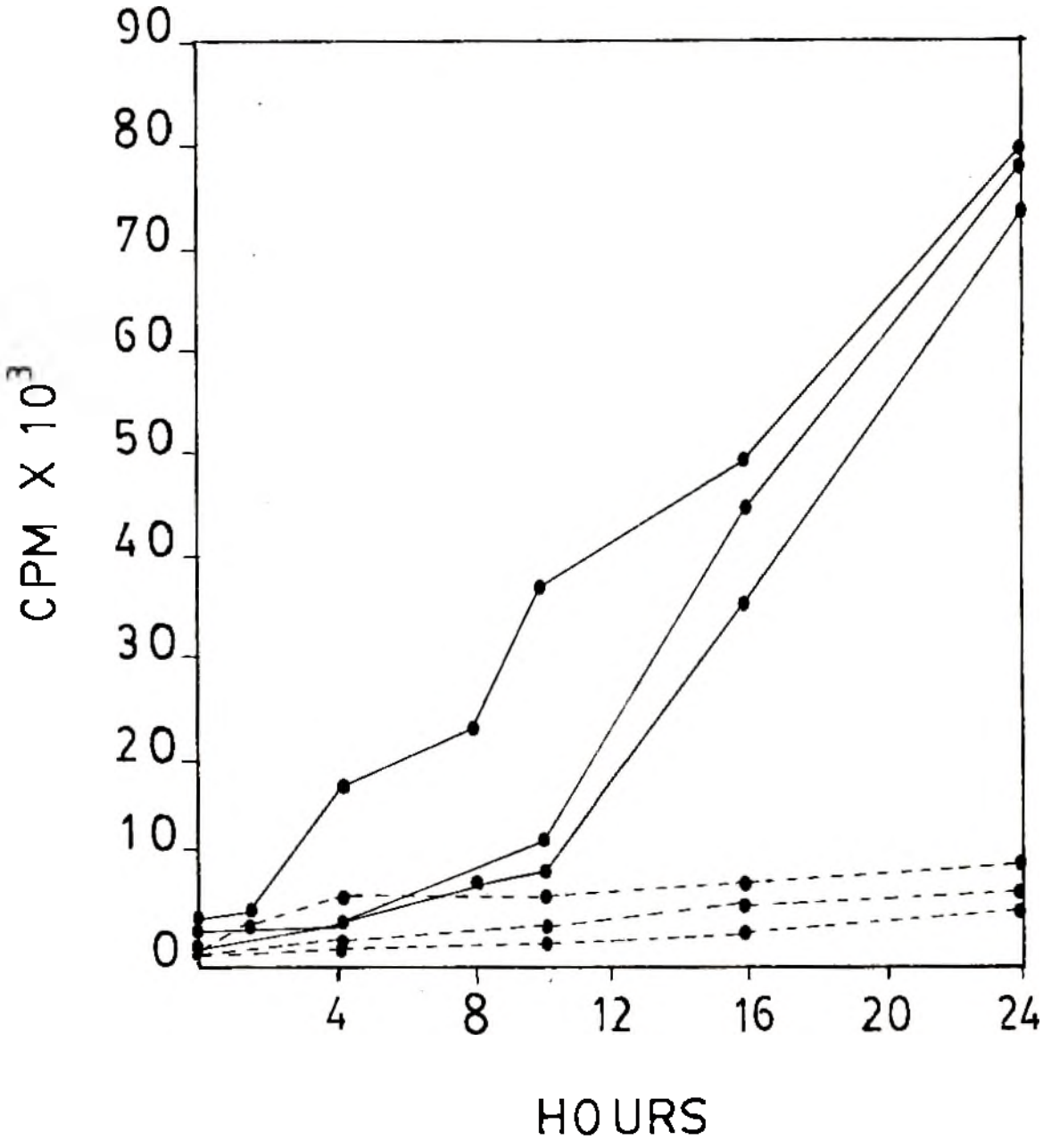
The results of three typical experiments are illustrated.

FIGURE 8.2 PRODUCTION OF IL-1 BY LGLS OF HCC PATIENTS (----) AND CONTROLS (——) STIMULATED BY INCREASING DOSES OF LPS.



The results of three typical experiments are illustrated.

FIGURE 8.3 TIME OF PRODUCTION OF IL-1 BY LGLS FROM HCC PATIENTS (----) AND CONTROLS (——) STIMULATED BY 2 x 10^6 K562 CELLS.



The results of three typical experiments are illustrated.

FIGURE 8.4 TIME OF PRODUCTION OF IL-1 BY LGLS FROM HCC PATIENTS (----) AND CONTROLS (———) STIMULATED WITH 20µg/mℓ LPS.

TABLE 8.1 RELATIONSHIP OF TUMOUR BURDEN WITH CYTOTOXICITY AND K562 OR LPS INDUCED IL-1 PRODUCTION BY LGLS

Tumour	Tumour burden	% lysis	IL-1 production*	
			K562	LPS
Ca ovary	large	14	14	24
Ca kidney	large	27	27	26
Soft tissue sarcoma	large	19	10	10
Ca stomach	large	35	7	9
Ca cervix IIIB	moderate	10	11	11
Ca cervix IIB	moderate	39	72	72
Ca breast	moderate	38	88	89
Ca salivary gland	moderate	37	93	88
Ca oesophagus	small	34	49	48
Ca prostate	small	38	104	91
Ca oesophagus	small	47	85	85
Ca oesophagus	small	40	85	86
Controls		41-67		

*IL-1 production by patient LGLs is expressed as a percentage of that produced by control LGLs

production, this is not the case with all cells isolated from all malnourished chronically ill people.

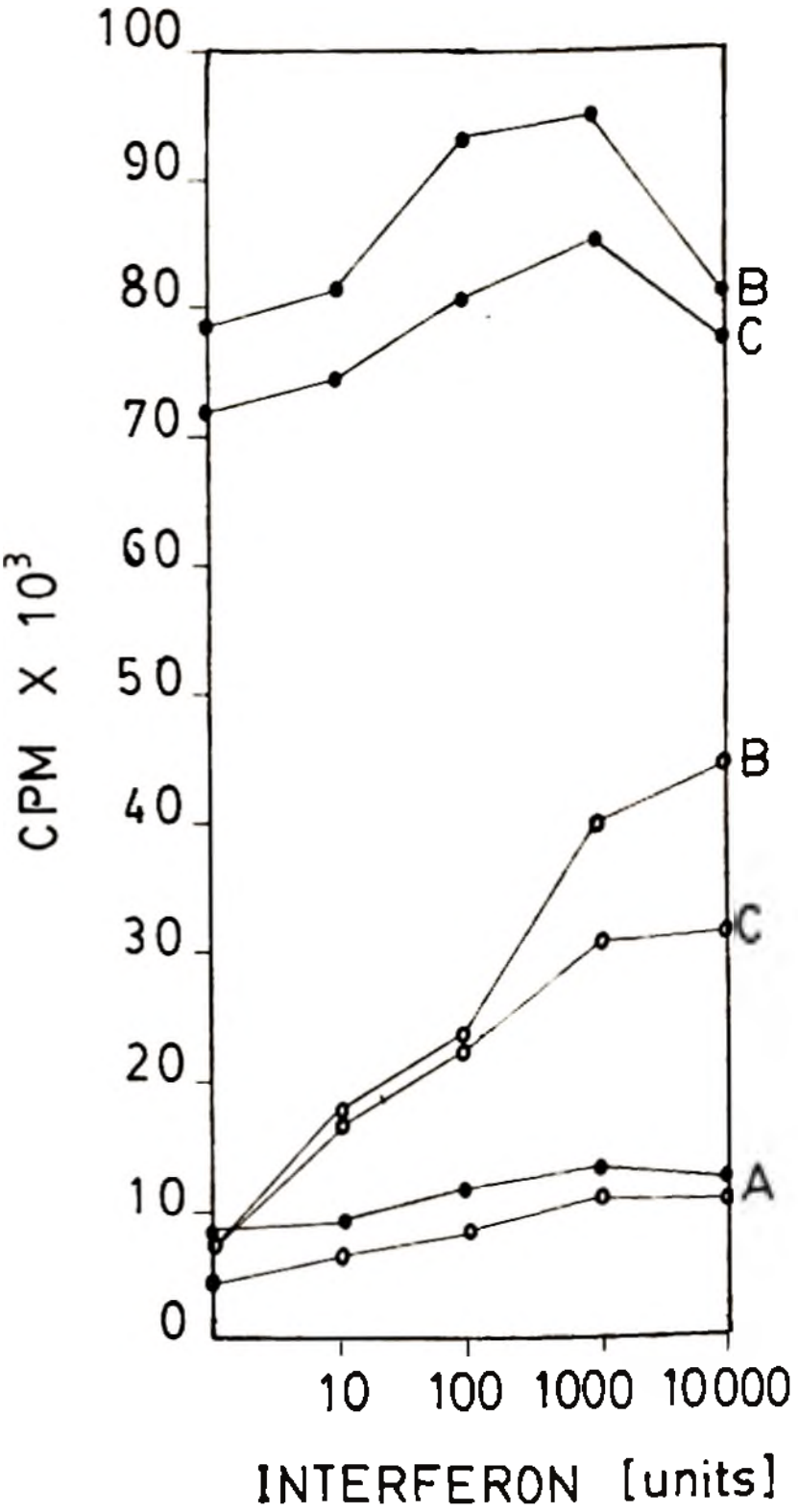
The possibility that LGL, isolated from HCC patients, could synthesise but not release IL-1 was examined. This was achieved by lysing the LGLs after LPS stimulation. Data tabulated in Table 8.2 shows that cell lysis had no effect on the amount of IL-1 in the supernatant. It is unlikely therefore that HCC patients LGLs can synthesise but not release IL-1.

8.3.2 The effect of interferon on IL-1 production

Enriched LGLs were pulsed for 1 hour with varying doses of IFN. After washing, these cells were incubated with either LPS or K562 cells for a further 24 hours. Supernatants were collected and tested for IL-1 activity. As shown in Figs. 8.6 and 8.7 IFN treatment caused some increase in IL-1 production by control LGLs but a considerable increase in the amount of IL-1 released by patients LGLs. IL-1 production by patients LGLs, however, did not reach the level of IL-1 produced by normal LGLs. IL-1 release was assessed at various times after treating LGLs with 1000 IU/mℓ of IFN and even after 3 hours significant increases could be observed (Figs. 8.5, 8.6 and 8.7). After 8 hours a very considerable boost in IL-1 production from either LPS or K562 cell stimulated LGLs was noted, and although IFN treated cells continued to produce greater amounts of IL-1 at all times tested, the increase tended to diminish after 16-24 hours.

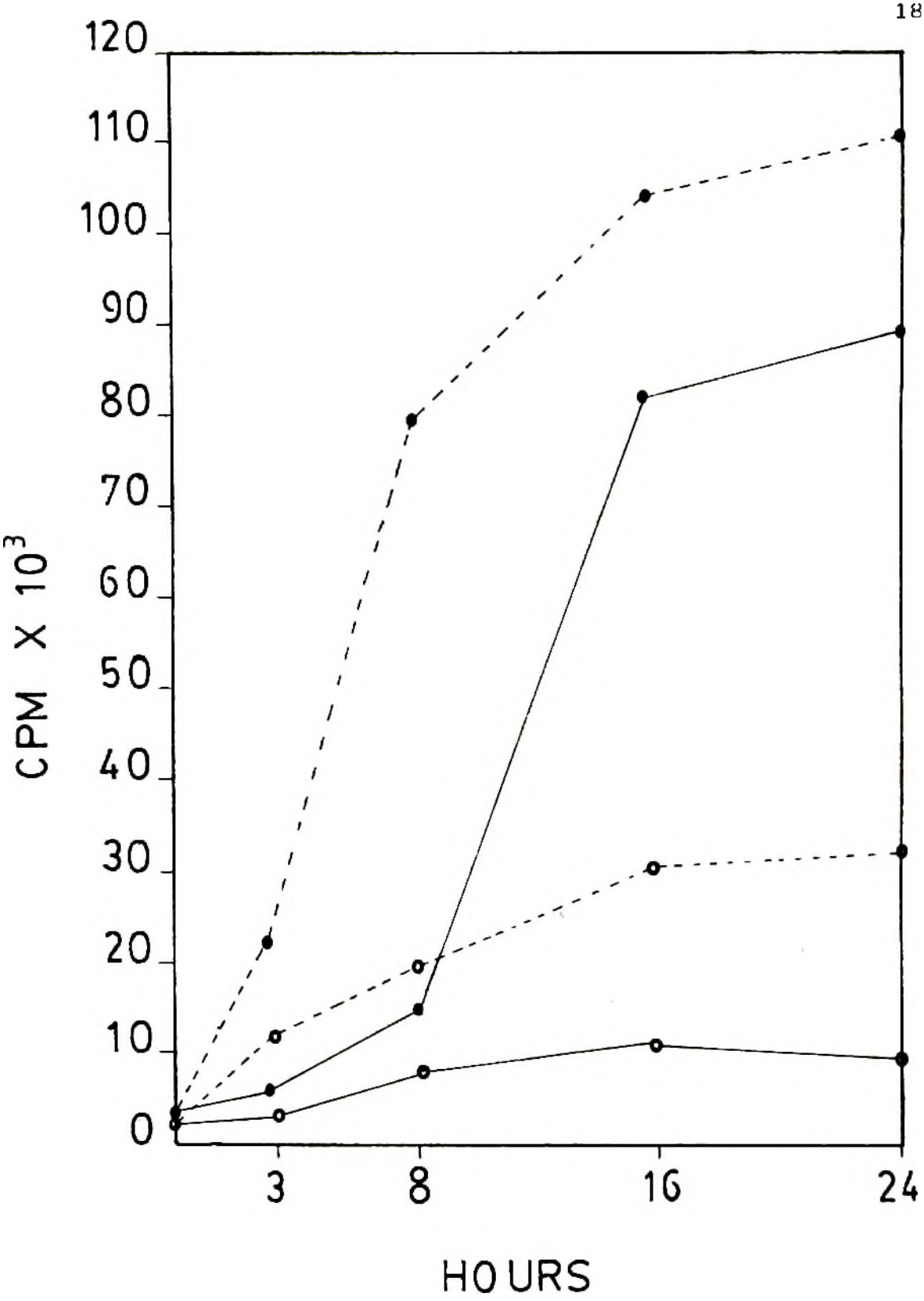
TABLE 8.2 LYSIS OF HCC PATIENTS LPS STIMULATED LGLS DOES NOT INCREASE THE AMOUNT OF IL-1 CONTAINED IN THE SUPERNATANT

Cell system	dpm (mean ± SD)
IL-1 secretion by control LGL	7465 ± 1021
IL-1 secretion by control LGL + IL-1 contained in the cells	7906 ± 978
IL-1 secretion by HCC patient LGL	2645 ± 623
IL-1 secretion by HCC patient LGL + IL-1 contained in the cells	3012 ± 984



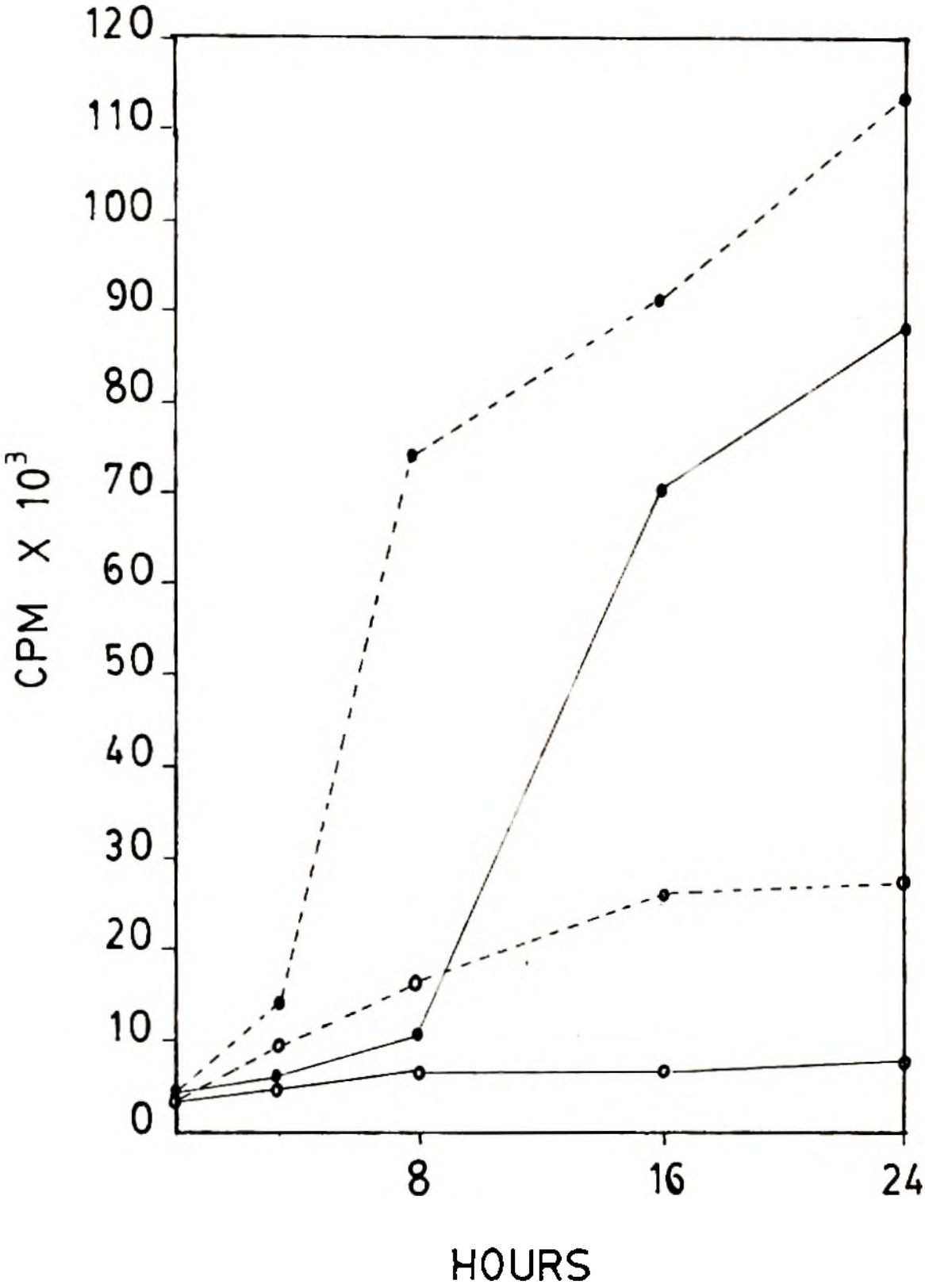
The results of a typical experiment are illustrated.

FIGURE 8.5 THE EFFECT OF VARYING DOSES OF IFN ON THE PRODUCTION OF IL-1 BY (A) UNSTIMULATED (B) LPS STIMULATED AND (C) K562 STIMULATED LGLS FROM CONTROLS (●—●) AND HCC PATIENTS (○—○)



The results of a typical experiment are illustrated.

FIGURE 8.6 THE EFFECT OF IFN ON K562 CELL INDUCED IL-1 PRODUCTION BY CONTROLS (•) AND HCC PATIENTS (O) UNTREATED (——) AND IFN TREATED (---).



The results of a typical experiment are illustrated.

FIGURE 8.7 THE EFFECT OF IFN ON LPS INDUCED IL-1 PRODUCTION BY CONTROLS (●) AND HCC PATIENTS (O) UNTREATED (——) AND IFN TREATED (---).

IL-1 production is known to be stimulated by various lymphokines (Dinirello, 1984). The possibility that contaminating T cells may stimulate IL-1 production by secreting IL-2 therefore had to be examined. It was found, however, that IL-2 was not present in the IL-1 containing supernatant and that removal of any contaminating T cells with OKT3 monoclonal antibody and complement did not affect the levels of IL-1 produced (results not shown).

The production of IL-1 by varying numbers of LPS stimulated LGLs and monocytes is illustrated in Fig. 8.8. From this data it is apparent that were 1% of the LGL enriched population monocytes, there would have been insufficient monocytes to produce the IL-1 found in the supernatant. (In chapter 2.2.5 a maximun of 1% contaminating monocytes was found in the LGL enriched cell population). The minumum number of monocytes required to produce, even small quantitise of IL-1, is 0.5 x 10^6cells. It is apparent, therefore, that neither contaminating monocytes nor T cells are responsible for IL-1 production in the LGL enriched population.

8.3.3 Cytotoxic activity of enriched LGLs and the effect of IL-1

As indicated in chapter 7 considerably less lysis of target K562 cells was exhibited by patient LGLs as compared to normals. Cytotoxic activity of both patient and normal LGLs could be increased by IFN treatment (1000 IU IFN for 1hour). When these effector cells were pulsed with IL-1 for 1 hour

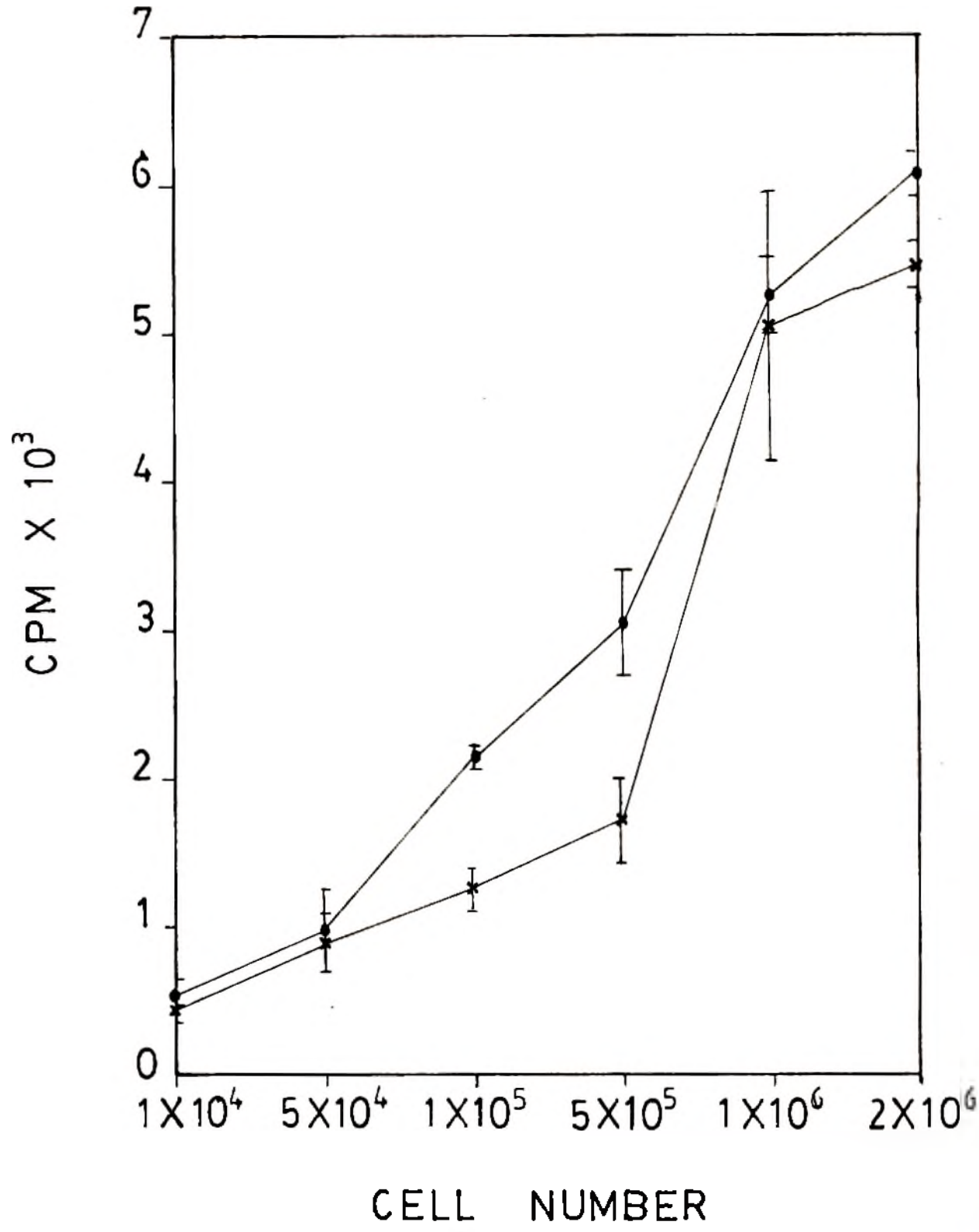


FIGURE 8.8 THE PRODUCTION OF IL-1 BY VARYING NUMBERS OF LPS STIMULATED MONOCYTES (●——●) OR LGLS (X——X).

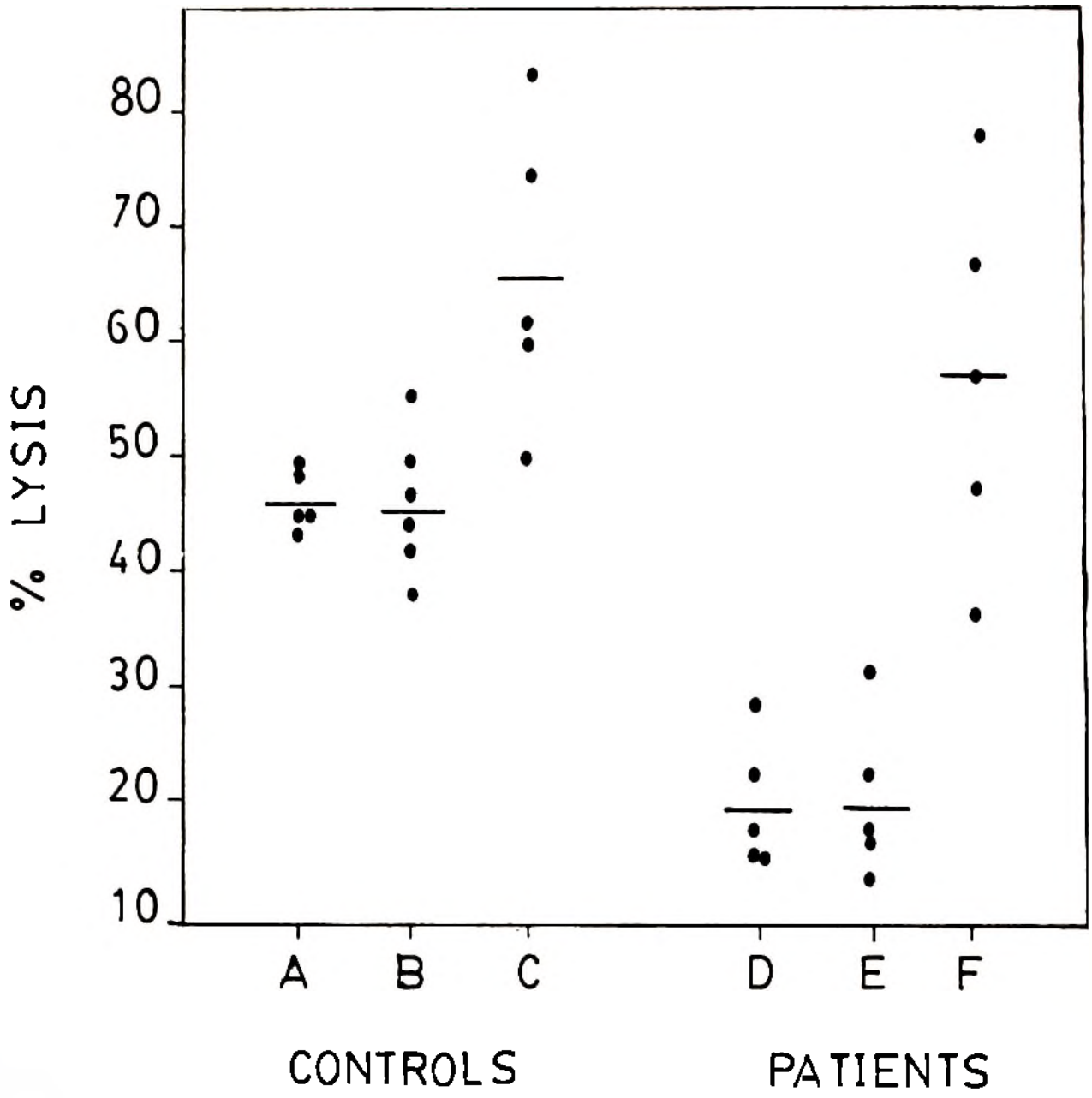
no significant change in cytotoxicity was discernable. However, when the K562 target cells were treated with IL-1, derived from LPS or K562 stimulated normal LGLs or monocytes, (or purified IL-1 - results not shown) a marked increase in lysis was observed (Fig. 8.9). LPS alone had no effect (results not shown). IL-1 added directly to ^{51}Cr release assays also had an enhancing effect (results not shown).

8.3.4 Target cell binding assays

Fewer K562 cells bound patient LGLs as compared to control LGLs (Fig. 8.10). This defect could not be improved by the addition of IFN to the binding assay (chapter 7), but was corrected by treating the target cells with IL-1 derived from any of the above mentioned sources. Treatment of the LGLs with IL-1 had no significant effect on the target cell binding (Fig. 8.10).

8.3.5 Antiserum to IL-1 abolishes thymocyte proliferation induced by IL-1 supernatants

Supernatants treated with medium, normal rabbit serum or anti-IL-1 antibody were tested for their ability to stimulate thymocytes to proliferate. As indicated in Fig. 8.11 active supernatants treated with either medium or normal rabbit serum were able to stimulate thymocyte proliferation. However, those supernatants treated with the antibody to IL-1 lost the ability to stimulate thymocyte proliferation thus showing that the thymocyte stimulatory substance in these super-



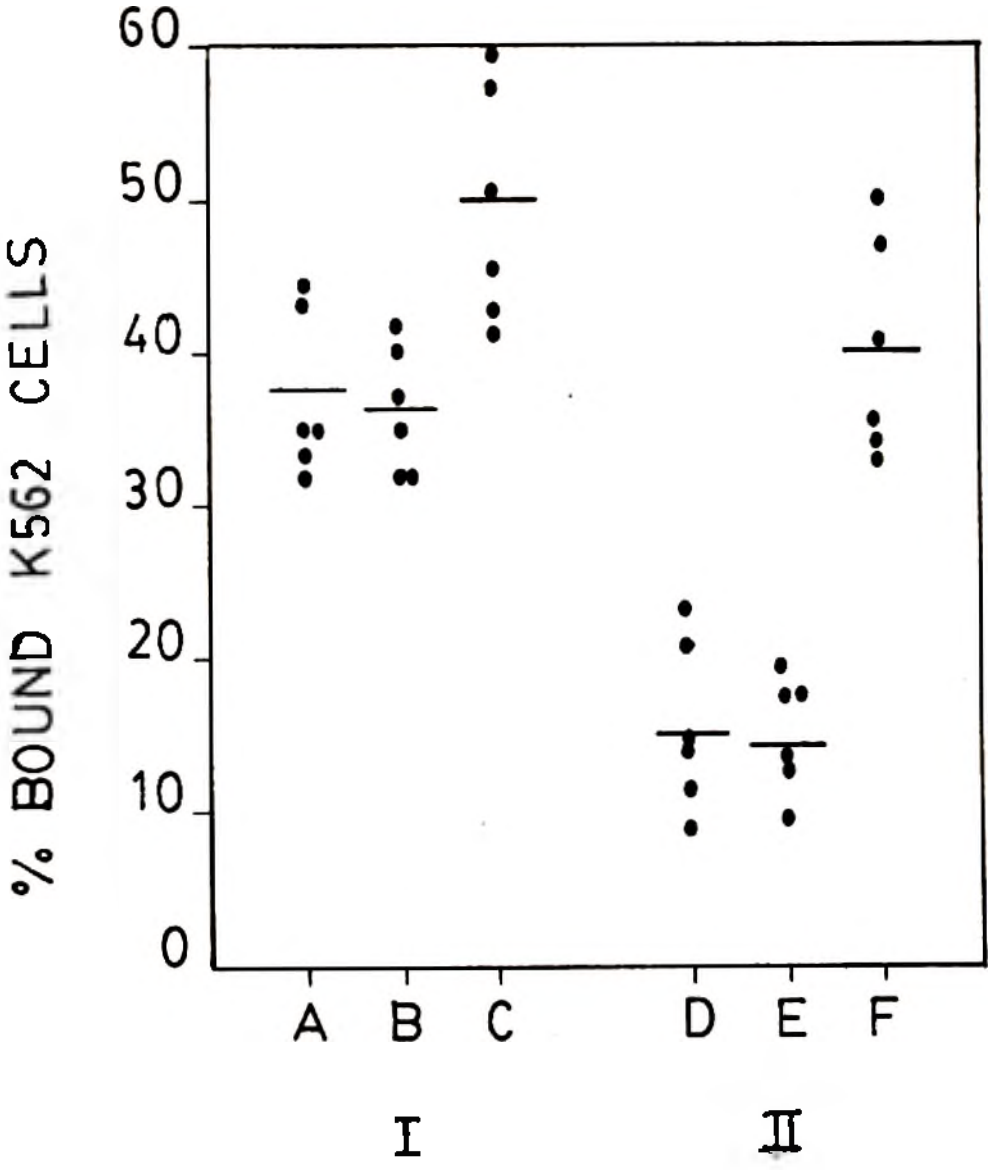
Key

A, D LGL

B, E IL-1 treated LGL

C, F IL-1 treated K562 cells

FIGURE 8.9 CHROMIUM RELEASE ASSAY OF CONTROL AND PATIENTS LGLS AFTER INCUBATION OF EFFECTOR AND TARGET CELLS WITH IL-1.



Key

A, D LGL

B, E IL-1 treated LGL

C, F IL-1 treated K562 cells

FIGURE 8.10 TARGET CELL BINDING OF CONTROL LGL (I) AND PATIENT LGL (II) AFTER INCUBATION OF EFFECTOR OR TARGET CELLS WITH IL-1.

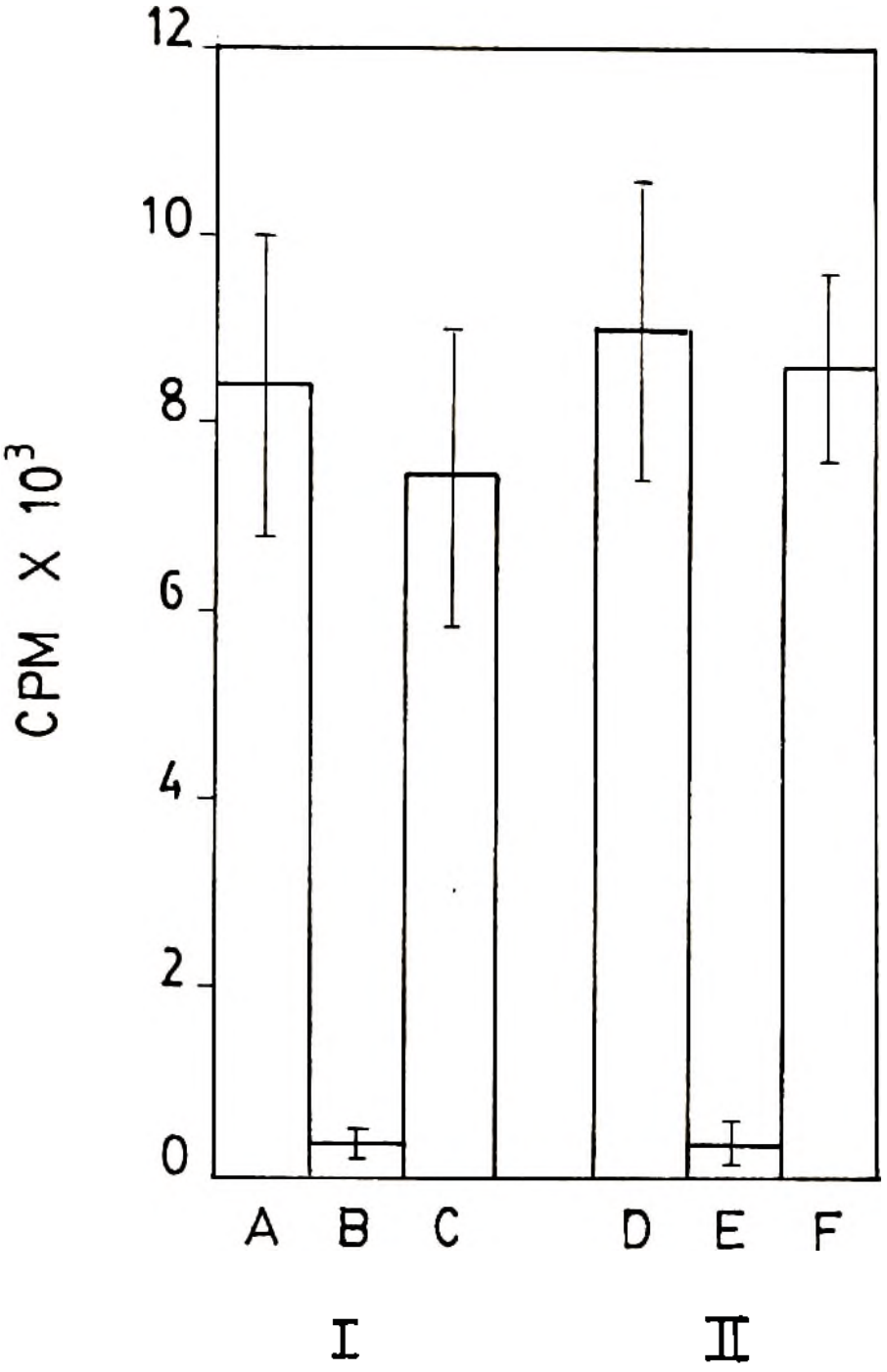


FIGURE 8.11 THE EFFECT OF ANTI-IL-1 ANTIBODY (B,E) OR CONTROL RABBIT SERUM (C,F) OR MEDIUM (A,D) ON IL-1 CONTAINING SUPERNATANTS MADE FROM:
I LGL + LPS II LGL + K562 CELLS

natants was IL-1.

8.4 Discussion

The results presented in this chapter indicate that LGLs produce IL-1 or an IL-1 like substance (this has also now been shown by Scala et al., 1984); that the production of this substance by LGLs is stimulated not only by LPS but also by K562 cells (an NK susceptible target cell); that IL-1 production by LGLs is considerably depressed in patients with large tumour burdens; that the decreased LGL-induced cytotoxicity and binding to target cells, which was described in the previous chapter, can be corrected by treating the target cells but not the effector cells with IL-1; and that IFN increases IL-1 production by normal and patient LGLs.

IL-1 has previously been described as a macrophage-derived "hormone like" factor of molecular mass 12 000 - 16 000 that has a variety of effects on immunologic and inflammatory reactions, and is assayed by measuring augmentation of thymocyte proliferation (Trinchieri et al., 1981). The finding that the supernatant of stimulated LGLs markedly increased the proliferation of murine thymocytes without stimulating proliferation of mature human T cells (results not shown) suggests that this material was IL-1. Furthermore, restoration of depressed cytotoxicity, and target effector cell conjugate formation in the cancer patients could be obtained with IL-1 containing supernatants (and subsequently with purified IL-1 - results not shown) derived from either LGLs or adherent monocytes.

Further evidence in favour of this substance being IL-1 stems from the results in Fig. 8.11. This figure illustrates the abolition of thymocyte proliferation by IL-1 containing supernatants treated with anti-IL-1 antibody. The pyrogenic effect of supernatants shown to stimulate thymocyte proliferation has also been demonstrated (results not shown).

The IL-1 like substance produced by LGLs was shown to have a molecular mass of 12 000 - 16 000 and to exhibit an elution profile similar to that of monocyte derived IL-1 when passed through a sephadex G50 column (results not shown). This finding is further evidence in favour of the substance produced by LGLs being IL-1.

LGLs are known to be a heterogenous population of cells and it is possible that those "monocytic" LGLs which label with the OKM1 antibody produce IL-1. Unstimulated LGLs produce only minimal amounts of IL-1, but production of this mediator could be stimulated by LPS, and more intrestingly, by K562 cells. Thus it is possible that, in the early stages of the cytotoxic reaction, contact between the target and effector LGLs could result in activation of IL-1 production by the latter. It is known that tumour cells induce IFN production by lymphocytes (Trinchieri et al., 1978) and it, therefore, seems likely that these IL-1 containing supernatants also contain interferon. However, it would seem unlikely that the effects noted in this study could be attributed to IFN rather than IL-1, because, although IFN does augment NK cell cytotoxicity, Trinchieri et al., (1981) have shown, and here it has been confirmed, that this mediator

has no effect on target binding. Furthermore, treatment of target cells with IFN has been shown to render them less susceptible to lysis by LGLs, a finding not consistent with the results documented above (Trinchieri et al., 1981).

That IL-1 is important in the cytotoxic reaction is illustrated by the observation that patients with malignant disease, whose LGLs have impaired cytotoxic activity, also produce considerably reduced amounts of IL-1. Although LGLs from all HCC patients tested had depressed cytotoxicity and impaired IL-1 production, some of the patients with smaller tumour burdens showed normal cytotoxicity and IL-1 production. The cytotoxic defect in HCC patients could be restored by treating the target cells with IL-1 containing supernatants and indeed the cytotoxic ability of normal LGLs could also be markedly enhanced by this treatment. These effects were observed if the IL-1 employed was derived from LGLs activated with LPS or K562 cells, or if it was classical IL-1 produced by LPS stimulated monocytes. In view of these findingd it is likely that many of the non-specific means of augmenting NK cell activity which have previously been described, are mediated through IL-1 production either by monocytes or LGLs. It is of intrest that when LGLs from either patients or controls were pulsed with IL-1 no increase in cytotoxicity was observed. Improved lysis could only be obtained by treating target cells with IL-1 suggesting that this mediator must activate the target cell to either release a factor that stimulates LGL activity or to develop a receptor which allows greater binding of effector LGLs. A similar model has been

proposed by Wright and Bonavida (1982) who showed that, following binding of the effector to the target cell, the target cell activates the NK cell to release cytotoxic factors important in lysis.

In the present study although similar numbers of LGLs were obtained from patient and control blood, fewer target cells were bound by patients LGLs as compared to controls. If however, the K562 cells were first treated with IL-1, greater numbers of these cells bound either patient or control LGLs. In fact, the number of K562 cells which bound to patient LGLs approximated the normal values. This finding again suggests that IL-1 influences the target K562 cells in such a way that greater numbers will be bound by LGLs.

It has previously been shown (Son et al., 1982), and confirmed in this study, that depressed NK cell cytotoxicity by the LGLs of patients with cancer could be improved by treating the effector cells with IFN. This effect is thought not to be due to increased binding of LGLs to target cells, but rather to increased recycling of the LGLs or to augmentation of the lytic efficiency of the NK cells (Katz et al., 1982). In experiments reported here it has been shown that IFN also stimulates IL-1 production in a dose dependant manner. More dramatic, however, was the considerable shift in the IL-1 kinetic curve induced by IFN. Whereas the majority of IL-1 was released from LPS or K562 treated LGLs in 16 hours, this was reduced to 8 hours when the cells were pulsed with IFN. It would appear that IFN aids in the release of IL-1 from stimulated cells. LGLs from both normals and

patients with cancer showed these IFN induced increases in IL-1 production, but this was not as marked for cancer LGLs as normals, indicating a basic defect in the latters' ability to produce IL-1.

In the light of these results a model to explain cytotoxicity against K562, and perhaps other tumour cells, can be postulated. The first step would be the interaction of LGLs with susceptible target cells resulting in the release of IL-1 from the effector cells. The IL-1 acting on the target cells, stimulates (1) their ability to bind large numbers of LGLs and (2) the possible production of receptors on the target cell surface thus rendering previously non-susceptible cells to become susceptible to lysis by LGLs. Alternatively, IL-1 could act as a bridge between the effector and target cell by binding to the surface thus causing increased binding of effector and target cells.

IFN, in addition to its previously described effects, will accelerate the rate of release and amounts of IL-1 produced by LGLs. The depression of LGL activity in patients with advanced cancer is not due to a decrease in the number or proportion of LGLs in the peripheral blood but can be ascribed to an inability of these LGLs to produce IL-1. This defect can be improved, to some extent, by *in vitro* IFN treatment. Although the reason for the decreased IL-1 production is not known a similar depression in IL-1 production by peripheral blood monocytes in patients with large tumour burdens has also been observed (Herman *et al.*, 1984). Studies designed to explore whether the above model

applies to only NK sensitive targets but not to NK resistant targets will be discussed in chapter 10.

Recent studies suggest that IL-1 can synergise with other lymphokines to enhance NK cell activity against certain tumour target cells (Dempsey et al., 1982). IL-2 incubated with human blood mononuclear cells causes an increase in NK cell activity. In the presence of IL-1 the effect of IL-2 on the generation of NK cell activity. When IL-1 is added to cultures containing IL-2 and IFN there is maximum natural killing of tumour targets.

The fact that IL-1 is important in natural killing has important implications for the role of fever in immunotherapy for certain tumours. In vivo IFN treatment is also associated with fever and although the various lymphokines mediate their effects at different cell levels in vitro this is not necessarily the case in vivo.

CHAPTER 9

9. DEFECTIVE INTERLEUKIN-1 PRODUCTION BY MONOCYTES FROM PATIENTS WITH MALIGNANT DISEASE

9.1 Introduction

Recent evidence indicates that macrophages play an important role in resistance against tumours. Tumour cells co-cultured with macrophages may be lysed in at least two distinct circumstances. The interaction may require antibody which gives specificity to the lysis (ADCC) or the interaction may be independant of antibody (Adams and Nathan, 1983). In the latter instance the cytolytic activity of human peripheral blood monocytes can be enhanced by treating them with lymphokines (Hammerstrom, 1979; Cameron and Churchill, 1979) and IFN (Jett et al., 1980; Stanwick et al., 1980). Although the mechanism of action of these mediators is not yet clear, it has been shown that a series of steps are required for cytotoxicity (Pace et al., 1983). The first of these does not cause cytolytic activity but instead primes macrophages to respond to a second signal which then triggers the onset of killing (Meltzer, 1981; Pace and Russel, 1982). The mediator responsible for priming has now been identified as IFN (Pace et al., 1983) which has, in addition, been shown to have a number of stimulatory effects on macrophages including their ability to bind and phagocytose antibody coated erythrocytes (Vogel and Rosenstreich, 1979; Vogel et al., 1983).

Interleukin-1 is a macrophage derived hormone like factor that has a mulciplicity of effects on immunological and inflammatory reactions (Oppenheim and Gery, 1982). Although IL-1 has not been shown to be important in macrophage tumour cytotoxicity, Meltzer and Oppenheim (1977) have previously indicated that the capacity of macrophages to kill tumour cells correlates directly with their capacity to produce IL-1. In this chapter IL-1 production by monocytes from patients with advanced malignant disease was examined and found to be impaired. Furthermore, IL-1 production by normal monocytes isolated from healthy volunteers and patients could be considerably increased by treatment of these cells with IFN (Herman et al., 1984).

9.2 Materials and methods

9.2.1 Patients

Two groups of Black adults were studied as described in chapter 8. Briefly the first consisted of 10 adults with histologically proven HCC, and the second, 11 adults with a variety of malignant diseases. The tumour burden was considered to be large in 5 of these patients and in all 10 of those with HCC; it was moderate in three patients and small in the remaining three patients. The tumour burden was, once again, assessed clinically, radiologically and isotopically and was based on the size of primary tumour and extent of local and distant spread. None of the patients has received treatment at the time they were investigated.

9.2.2 Preparation of adherent cells

Mononuclear cells were obtained from the peripheral blood of cancer patients and controls by Ficoll-Hypaque density gradient centrifugation as described in chapter 2.2.1 - 2.2.4. After washing in RPMI 1640, the mononuclear cells were resuspended in c-RPMI at a concentration of 2.5×10^6 cells/mℓ. 30mℓ of the mononuclear cell suspension was incubated in 140 x 15 mm glass petri dishes for 2 hours at 37°C in a humidified CO_2 incubator. Non-adherent cells were decanted and the remaining adherent cells washed three times. Adherent cells were removed from the glass dishes by treatment with EDTA for 15 minutes at 37°C then washed three times before being resuspended in c-RPMI.

9.2.3 Production of IL-1 by adherent peripheral blood monocytes

1×10^6 adherent cells/mℓ were cultured for 24 hours in the presence and absence of either varying amounts of LPS or varying numbers of K562 cells. This leukemic cell line was maintained as described in chapter 2.2.6. Adherent cell supernatants were harvested and assessed for IL-1 activity as outlined in chapter 8.2.6.

9.2.4 The effect of interferon on IL-1 production

The effect of recombinant A IFN on monocyte IL-1 production was assessed in a manner similar to that described in chapter 8.2.8.

9.3 Results

9.3.1 Production of IL-1 by adherent cells

Patient or control adherent cells were treated with either LPS or K562 cells for 24 hours, after which the supernatants were collected and assayed for IL-1 acitivity. As indicated in Fig. 9.1 markedly reduced amounts of IL-1 were produced by patients' cells as compared to controls when either varying numbers of K562 cells or increasing amounts of LPS were employed to stimulate IL-1 production. Similarly, when adherent cells were treated with LPS or K562 cells for varying periods of time considerably less IL-1 was produced by patients as compared to controls at all time periods (Fig. 9.2). (In some experiments the K562 cells used to stimulate IL-1 production were either heat-killed or cycloheximide treated to ensure that these cells were not producing the IL-1 measured - results not shown). IL-1 production by either LPS or K562 stimulated cells was also assessed in patients with only moderate or small tumour burdens. IL-1 production generally correlated with the extent and degree of the malignancy. It was markedly defective in patients with large tumour burdens but was normal in those with moderate or small tumour burdens (Table 9.1). Monocytes isolated from 5 patients suffering from various forms of chronic illness (other than malignant disease) showed normal levels of IL-1 production when stimulated with either LPS or K562 cells (results not shown).

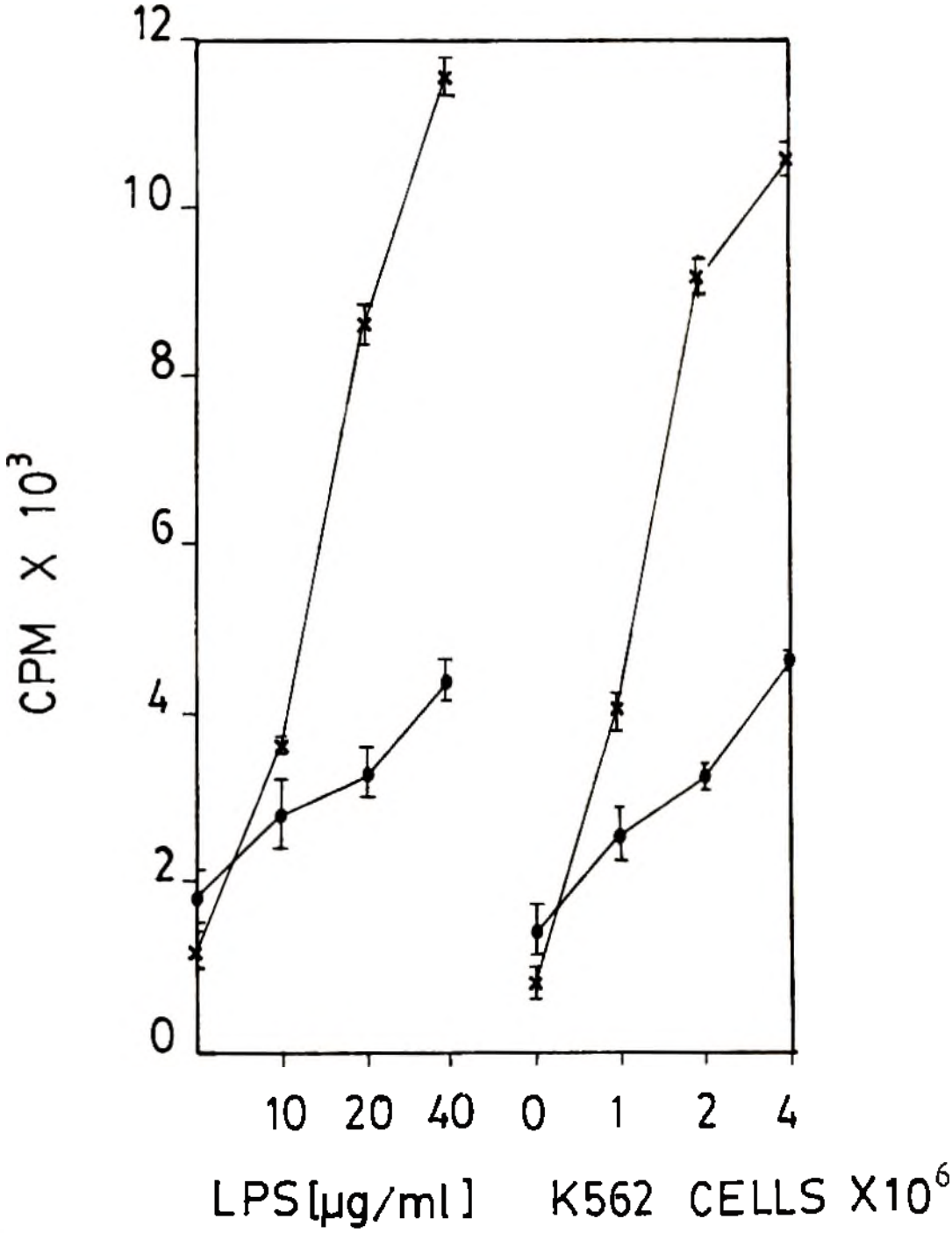


FIGURE 9.1 IL-1 PRODUCTION BY ADHERENT CELLS OF CONTROLS (X——X) AND PATIENTS WITH HCC (O——O) WHEN ACTIVATED BY VARYING DOSES OF LPS OR INCREASING NUMBERS OF K562 CELLS.

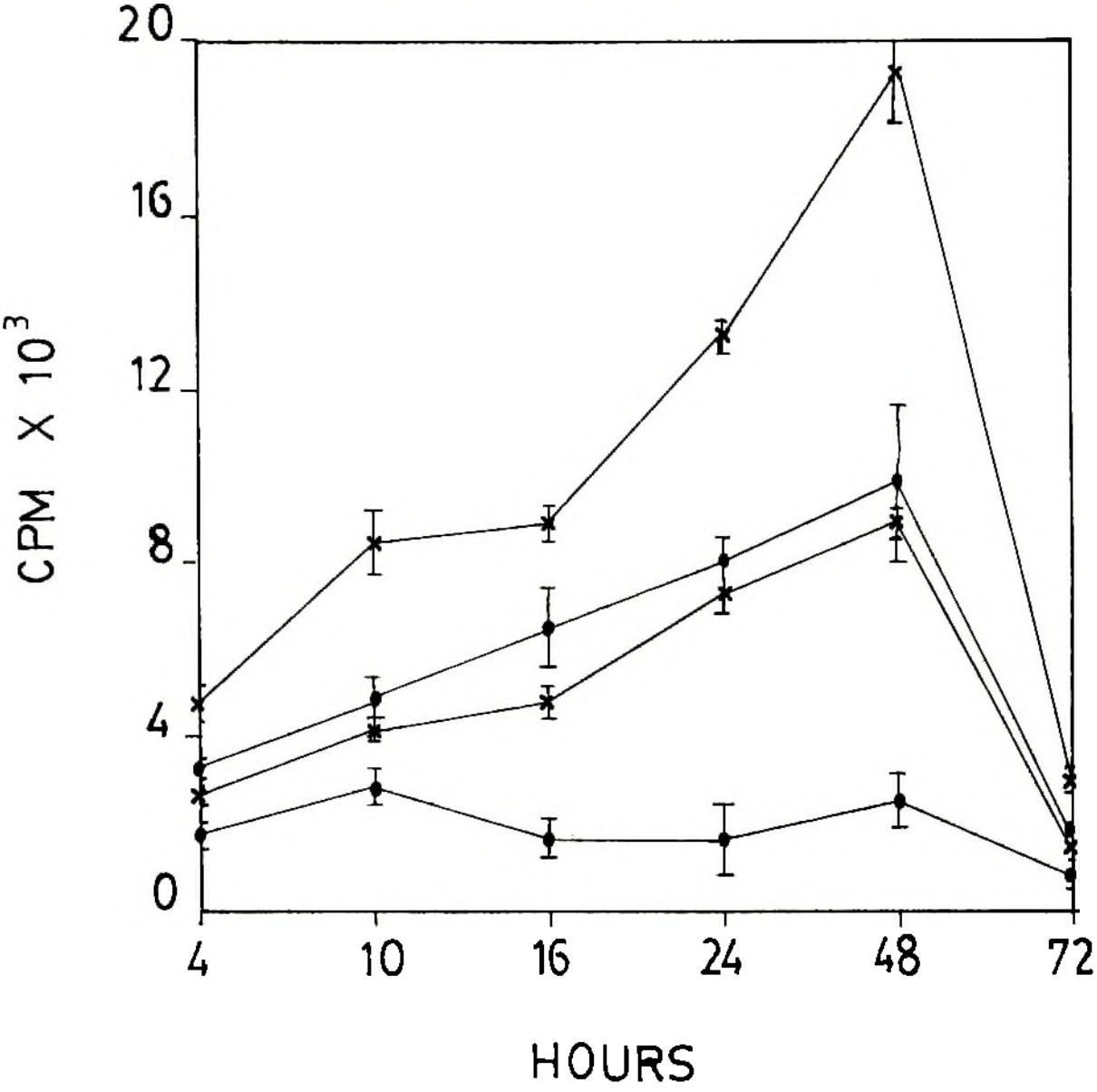


FIGURE 9.2 THE EFFECT OF IFN ON IL-1 PRODUCTION BY CONTROL (X——X) AND PATIENT (O——O) MONOCYTES ACTIVATED BY a) LPS AND b) K562 CELLS. THE UPPER TWO CURVES REPRESENT IFN TRAATED CELLS.

TABLE 9.1 IL-1 PRODUCTION BY ADHERENT CELLS FROM PATIENTS WITH MALIGNANT DISEASE. IL-1 IS EXPRESSED AS A PERCENTAGE OF THE CONTROL VALUE

Tumour	Tumour burden	IL-1 production	
		K562	LPS
Ca kidney	large	45	52
Ca ovary	large	34	36
Soft tissue sarcoma	large	10	25
Ca stomach	large	8	8
Ca ovary	large	43	46
Ca cervix IIB	moderate	80	81
Ca breast	moderate	104	102
Ca salivary gland	moderate	98	95
Ca prostate	small	98	94
Ca oesophagus	small	88	81
Ca oesophagus	small	90	92

9.3.2 The effect of interferon on IL-1 production

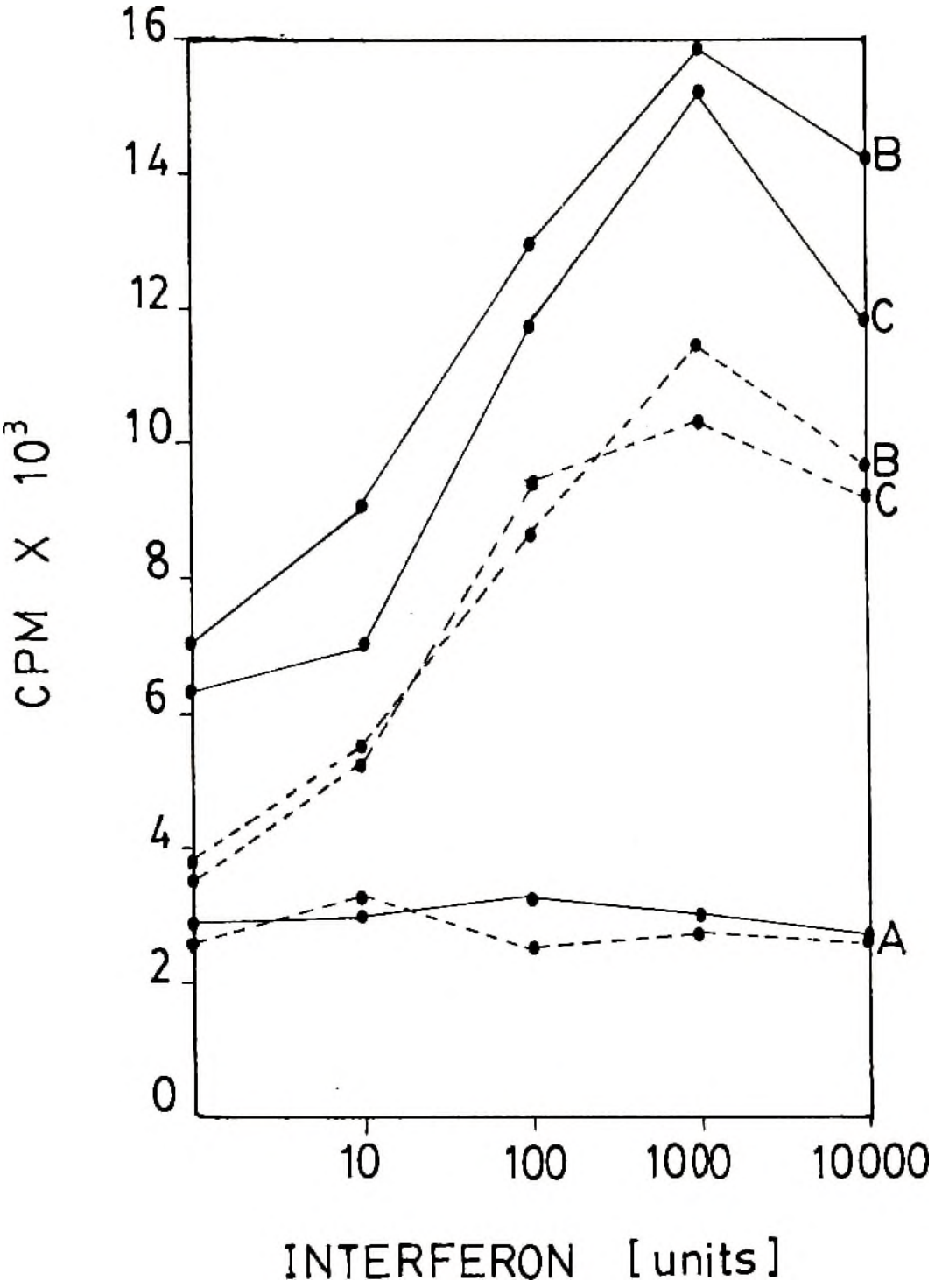
Adherent cells were pulsed for 1 hour with 1000IU of IFN and after washing were incubated for varying times with either LPS or K562 cells. Supernatants from these cultures were collected and tested for IL-1 activity. As seen in Fig. 9.2 significantly increased amounts of IL-1 were produced by IFN treated adherent cells of both controls and patients. After 48 hours IL-1 production decreased dramatically, an event which was not influenced by prior treatment with IFN.

IL-1 production by adherent cells was also assessed after treating the cells with varying doses of IFN. IFN alone did not stimulate IL-1 production, a second stimulus of either LPS or K562 cells was required. IFN, at all doses tested, caused an increase in IL-1 production by control and patient LPS or K562 treated adherent cells. Even after *in vitro* IFN, however, IL-1 production by adherent cells from the patients with advanced malignancy did not approximate those of normal cells (Fig. 9.3).

9.4 Discussion

The major findings in this chapter are:

1) that production of IL-1 by adherent cells can be stimulated by treating the cells not only with LPS but also with K562 cells
2) that IFN enhances IL-1 production by both normal and patients adherent cells and



Key

A monocytes treated with IFN

B monocytes treated with IFN and LPS

C monocytes treated with IFN and K562 cells

FIGURE 9.3 THE EFFECT OF INCREASING DOSES OF IFN ON IL-1 PRODUCTION BY CONTROL (●—●) AND PATIENT (●--●) MONOCYTES.

3) that IL-1 production by the adherent cells of patients with large mass tumours is depressed.

A number of agents are known to stimulate adherent cells to produce IL-1. These include LPS, components of mycobacteria and muramyl dipeptide (Oppenheim and Gery, 1982). In addition , antigens and mitogens, by activating lymphocytes, may stimulate monocytes to produce IL-1. This occurs either by a cell-contact dependant pathway (Unanue, 1980) or by the production of other lymphokines (Moore et al., 1980). The finding that K562 cells can activate adherent cells to release IL-1 is of interest in the context of mononuclear cell cytotoxic activity against tumour cells. The increased susceptibility of IL-1 treated tumour target cell binding and lysis by NK cells was discussed in chapter 8. The release of this cytokine is thought to be an important anti-tumour mechanism.

The finding that IFN increases IL-1 production by LPS or K562 stimulated monocytes confirms the work of Arenzanz-Seisdedos and Virelizer (1983) who indicated that the effect of both α or β IFN on IL-1 production by LPS treated monocytes could be abolished by antiserum to the relevant IFN. It is worth noting that IFN without an additional stimulant failed to significantly stimulate IL-1 production and similarly it has previously been shown that IFN, under endotoxin free conditions, does not induce macrophages to become cytolytic (Pace et al., 1983). The decrease in IL-1 production by stimulated monocytes observed after 48 hours

could not be abrogated by interferon treatment. It appears, therefore, that IFN primes the adherent cells whereas LPS or malignant K562 cells trigger them for IL-1 secretion. As IL-1 may be identical to endogenous pyrogen, this finding may explain the fever observed in patients receiving therapeutic interferon.

Malignant disease induces profound changes in monocyte function and both stimulation and inhibition of mononuclear phagocyte function has been reported. These changes include increased clearence of particles from the blood of patients with malignant disease (Sheagren, 1967), decreased movement of monocytes to the site of inflammation in cancer patients (Catalona, 1972) and decreased macrophage chemotaxis associated with increased phagocytic ability in tumour bearing mice (Meltzer and Stevenson, 1978). The considerable defect in IL-1 production by adherent monocytes described in this chapter was observed in all patients with HCC or other large tumours. Monocytes from patients with moderate or small tumour masses produced normal amounts of IL-1. The cause of the defect of mononuclear phagocyte IL-1 production in the patients with large mass tumours is unknown. It is possible that tumours themselves may produce factors which inhibit IL-1 production, or that the mononuclear phagocytes may be depleted of this cytokine due to chronic activation. This defect, however, further illustrates the complex abberations in mononuclear cell functions which exists in cancer patients.

CHAPTER 10

10. INTERLEUKIN-1 AFFECTS THE INTERACTION BETWEEN NK CELLS AND TUMOUR TARGET CELLS

10.1 Introduction

Interleukin-1 is a cytokine produced by monocytes and other cells, including LGLs, which is a key mediator of host responses to microbial invasion. It is a true hormone produced during infection and inflammation and exhibits biological activities accounting for several aspects of the acute phase reaction. Until recently, however, IL-1 has not been implicated in natural killer cell interactions. Dempsey et al., (1982) have shown that IL-1 alone has no effect on NK cell cytotoxicity at the effector cell level but synergises with both IFN and IL-2 in the enhancement of NK activity. Both IFN and IL-2 are known augmentors of NK activity on their own (Kadish et al., 1981; Henney et al., 1981).

In the light of the data presented in chapter 8 which indicates that LGLs synthesize and release IL-1, and that this IL-1 appears to influence NK-K562 interactions, this study was undertaken to establish the importance of IL-1 in the NK cell - target cell interaction.

The concept of IL-1 playing a role in natural killing, although fairly recent, ought not to be altogether foreign. It is well known that most, if not all in vitro and in vivo inducers of IFN production are also stimulators of IL-1

production or produce fever themselves in humans (Dempsey et al., 1982).

That IL-1 and endogenous pyrogen are different names for a similar if not identical substance is now widely accepted. The role of fever in host defence has been the subject of much research (Atkins, 1960). The study of the use of microbial toxins in treatment of mailgnant tumours began many years ago. These clinical experiments took place when it was noted that "spontaneous regression" occurred in certain cancer patients who concurrently developed post operative infections. It now seems likely that the induction of IL-1 release played a role in these "regressions". There is currently considerable interest in the clinical studies on the use of hypothermia as treatment or adjunt therapy for cancer.

Although little is known concerning IL-1 functionally, it seems fairly clear that it increases the lipid viscosity of certain cell membranes thereby increasing the number of antigen binding sites, and the availibity of receptors for antigens and mitogens (Oppenheim and Gery, 1982). The ability of IL-1 to induce T cell differentiation, maturation and IL-2 production is well documented (Oppenheim and Gery, 1982). The intracellular mechanism involved in promotion of cell cycling is, however, not yet elucidated. Few studies have investigated the molecular events following direct contact of IL-1 with the cell membrane although it is known that the responses are very rapid.

With the possible membrane "maturational" effects of IL-1 in mind, one of the mechanisms by which IL-1 was thought to affect target cell binding could be by increasing the number of surface "receptors" for the effector cell on the target.

IFN is yet another cellular mediator whose effects on cells are extremely diverse. This substance is probably better known for its cytolytic enhancing properties (Djeu et al., 1978; Gidlund et al., 1978). Although when NK sensitive target cells are pretreated with IFN the opposite occurs, they become resistant to lysis by an, as yet, unknown mechanism (Trinchieri et al., 1981).

The aim of this work was therefore to examine the effect of IL-1 on various target cells, and their interactions with LGLs with a view to elucidating the mechanism by which cell binding and lysis is increased. This study also investigated whether tumours which are highly susceptible to NK cell attack would stimulate LGLs to release greater amounts of IL-1 than those tumours which are more resistant to NK cell attack. In addition, this chapter examined the effect of IFN on the susceptibility of K562 target cells to NK cell lysis confirming the work of Trinchieri et al., (1981) and extending it to examine the effect of IFN pretreatment of K562 cells on their ability to stimulate IL-1 production by LGLs and on the expression of β-2 microglobulin on the surface of these cells.

10.2 Materials and methods

10.2.1 Preparation and identification of LGLs

LGLs were isolated from human peripheral blood as described in chapter 2.2.1 - 2.2.4 and the purity of the percoll enriched fraction checked as outlined in chapter 2.2.5.

10.2.2 Target cell lines

The following cell lines were used to stimulate the production of IL-1 and as target cells in cytotoxicity assays: K562, PLC/PRF/5, GM4672, Yac-1 and normal skin fibroblasts.

The human erythromyeloblastic cell line K562 was maintained in culture as described in chapter 2.2.6. PLC/PRF/5, a human hepatocellular carcinoma cell line was cultured as described in chapter 3.2.1. The human B cell lymphoma, GM4672 was grown in suspension culture, in c-RPMI and subcultured twice weekly. A mouse T cell lymphoma, Yac-1, was also maintained in suspension culture in c-RPMI and subcultured biweekly. Normal skin fibroblasts were grown in monolayers in c-RPMI.

10.2.3 Cytotoxicity assay

The standard chromium release assay as outlined in chapter 2.2.6 was used in determining the effect of IL-1 on the degree to which the previously mentioned cell lines were lysed

by LGLs. The method of IL-1 pretreatment of cells was similar to that described in chapter 8.2.7 although in some experiments the time of pretreatment of target cells with IL-1 was varied.

In some experiments K562 cells were first treated with actinomycin D (an RNA synthesis inhibitor) for 30 minutes at 37°C before IL-1 was added, in order to investigate the possibility of the target cell synthesizing a new receptor or some other substance as a result of IL-1 treatment.

In other experiments anti-IL-1 antibody was added together with the IL-1 supernatants to K562 cells to demonstrate that increased cell binding and lysis was indeed the result of IL-1 treatment.

10.2.4 Production of IL-1 by LGLs

The five above mentioned cell lines were used to stimulate the production of IL-1 from LGLs in a manner similar to that described in chapter 8.2.6. 1 x 10^6 LGLs were stimulated with an equal number of target cells for 18 - 24 hours, whereafter the supernatants were harvested and tested for IL-1 activity as described previously (chapter 8.2.6).

10.2.5 The effect of IFN pretreatment of target cells on cytotoxicity, IL-1 production and cell surface expression of β-2 microglobulin

K562 cells were pulsed with varying quantities of recombinant

A IFN for different periods of time before being incorporated as target cells in ^{51}Cr assays as described previously. IFN pretreated K562 cells were also used to stimulate IL-1 production from LGLs as described in chapter 8.2.6.

In some experiments 1 x 10^6 IFN treated and untreated K562 cells were incubated with fluorscein-labelled anti-β-2 microglobulin antibody for 30-60 minutes at 4°C in the dark. The quantities of antibody used had previously been shown to label 70% of peripheral blood lymphocytes. After the incubation period the cells were washed and viewed for fluorescence under a Reichel Zetopan fluorescent microscope using transmitted light.

10.3 Results

10.3.1 The effect of IL-1 on lysis of various target cell lines by LGLs

The data presented in Fig. 10.1 shows that IL-1 pretreatment of ^{51}Cr labelled target cells, at 37°C for 1 hour, increases their susceptibility to lysis by LGLs. Normal skin fibroblasts, Yac-1 cells and GM4672 cells were all killed poorly and although their lysis could be improved slightly by IL-1 pretreatment the degree of cytotoxicity did not match the extent to which K562 cells were killed.

In some experiments the time of pretreatment of target cells was varied. Fig. 10.2 shows that the enhancement of

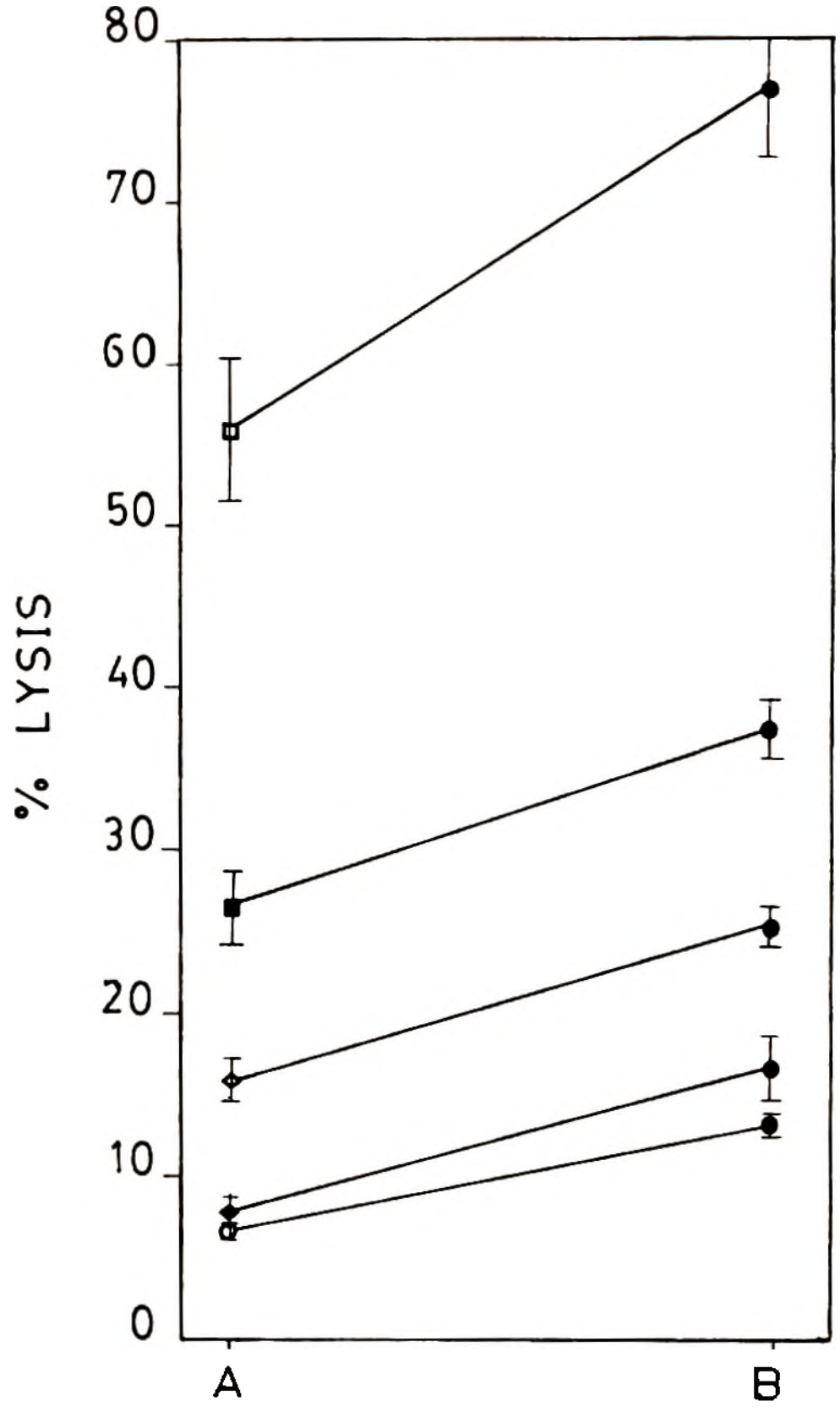
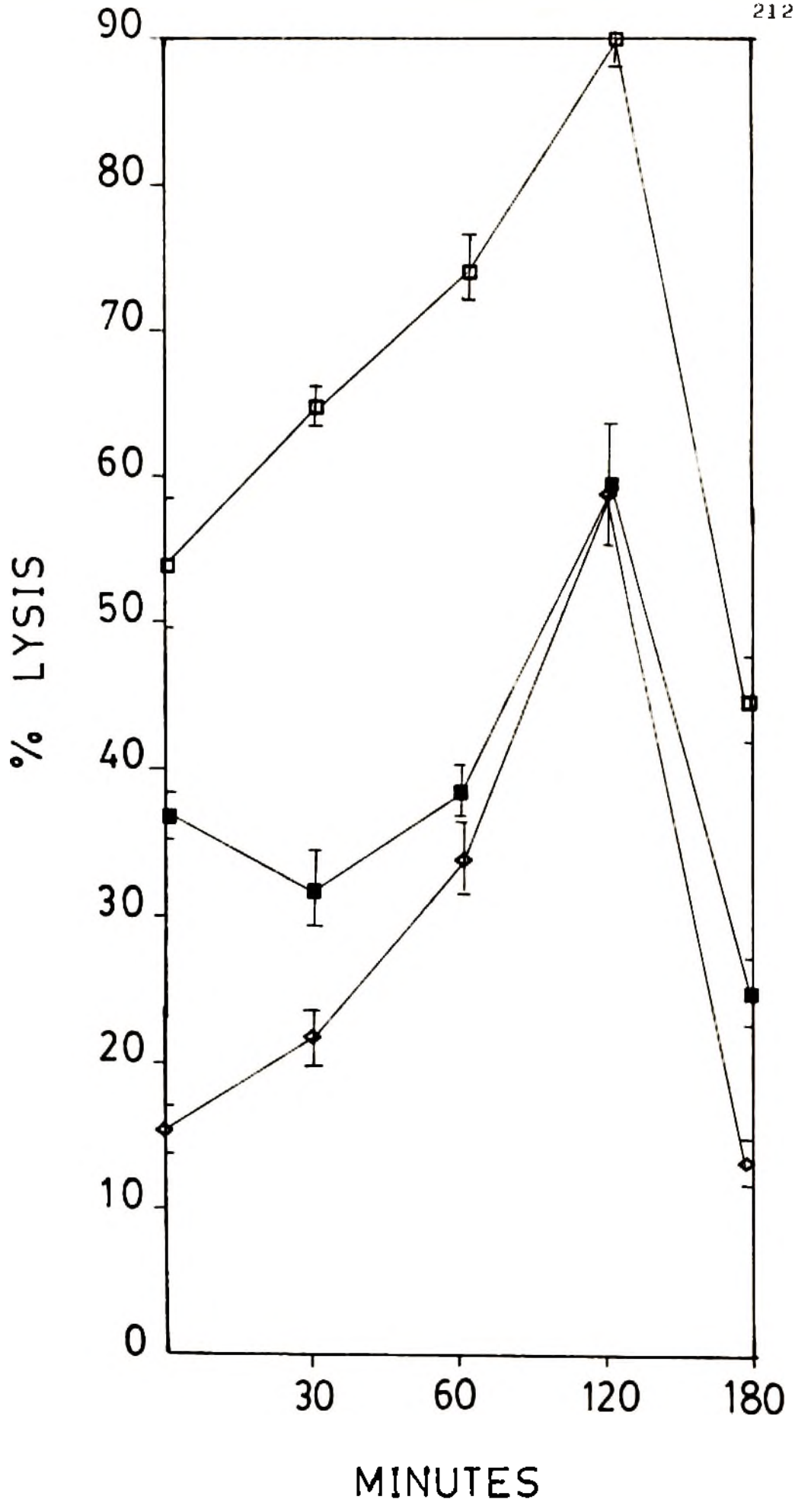


FIGURE 10.1 THE PERCENT LGL CYTOTOXICITY OF VARIOUS TARGET CELLS BEFORE (A) AND AFTER (B) A 1 HOUR PULSE WITH IL-1 CONTAINING SUPERNATANT.

FIGURE 10.2 THE EFFECT OF TREATING VARIOUS TARGET CELLS, FOR DIFFERENT TIME PERIODS, WITH IL-1 CONTAINING SUPERNATANTS.


90
80
70
60
50
40
30
20
10
0
% LYSIS
30
60
120
180
MINUTES

cytotoxicity by IL-1 peaked after 2 hours of treatment, where even the lysis of GM4672 cells was increased to over 50%. Increased lysis of K562 cells and PLC/PRF/5 cells also peaked after 2 hours of IL-1 treatment.

10.3.2 Inhibition of IL-1 enhancement of K562 cell lysis by actinomycin D

The results expressed in Fig. 10.3 illustrate the increased lysis of IL-1 treated K562 cells. This graph also shows that this increased lysis is abolished if K562 cells are treated with 1 µg|mℓ actinomycin D for 30 minutes at 37°C, thus revealing that inhibition of RNA and therefore protein-synthesis abolishes the effect of IL-1.

10.3.3 Treatment of IL-1 containing supernatant with anti-IL-1 antibody abrogates its effect on the cytotoxicity and the binding of target cells

The two graphs in Fig. 10.4 show that when K562 cells are treated with IL-1 containing supernatant they are:

1) lysed to a greater extent by LGLs - this increased binding appears the result of
2) increased binding of the target cells.

Both of these effects can be abolished when the supernatant is first treated with anti-IL-1 antibody. (A similar increase in binding and lysis of target cells was shown when purified IL-1 was utilized in such assays - results not shown).

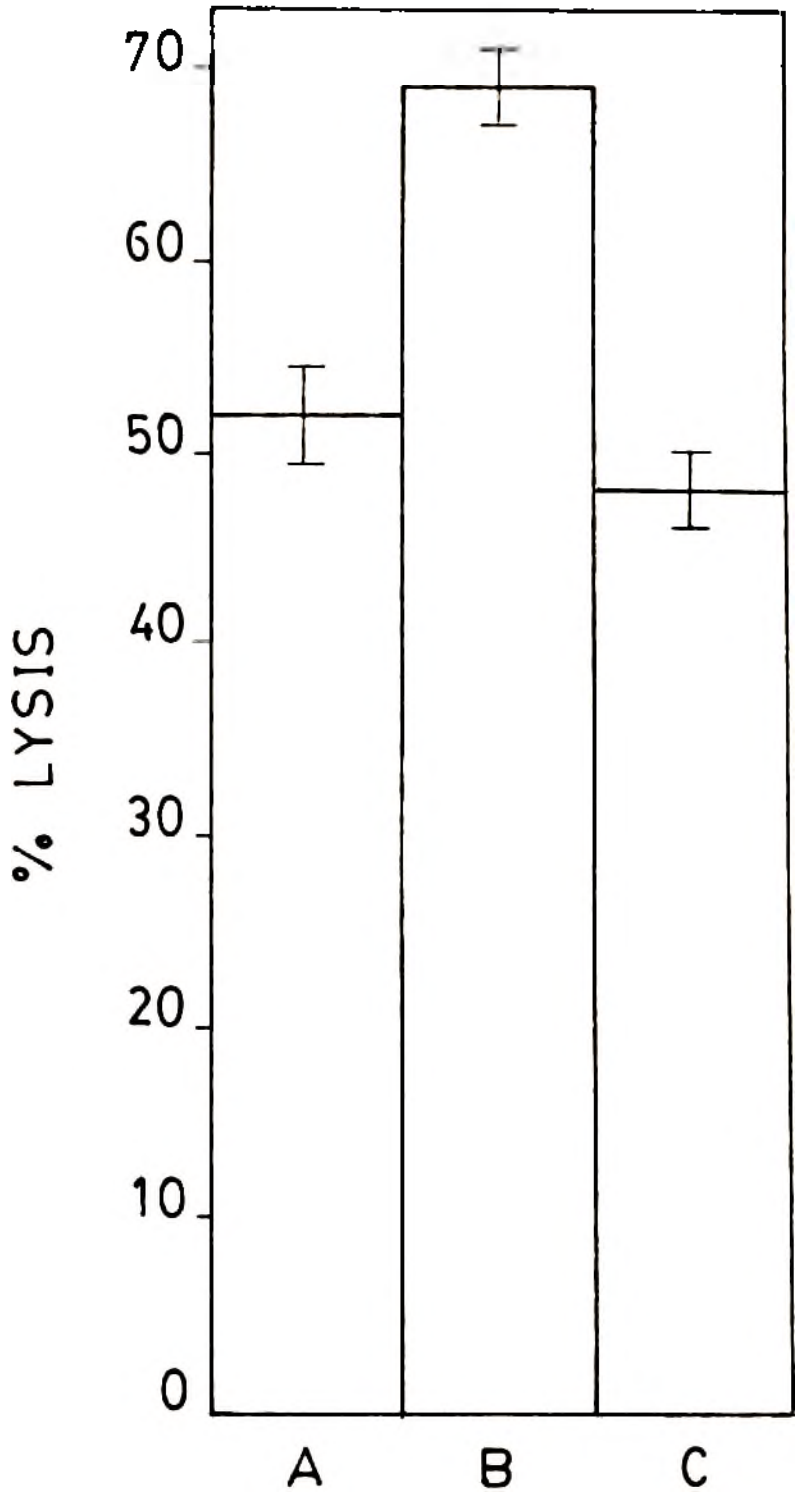


FIGURE 10.3 THE PERCENT CYTOTOXICITY OF LGLS AGAINST (A) K562 CELLS (B) K562 CELLS TREATED WITH IL-1 SUPERNATANT AND (C) K562 CELLS TREATED FIRST WITH ACTINOMYCIN D (30 MIN) THEN IL-1 SUPERNATANT.

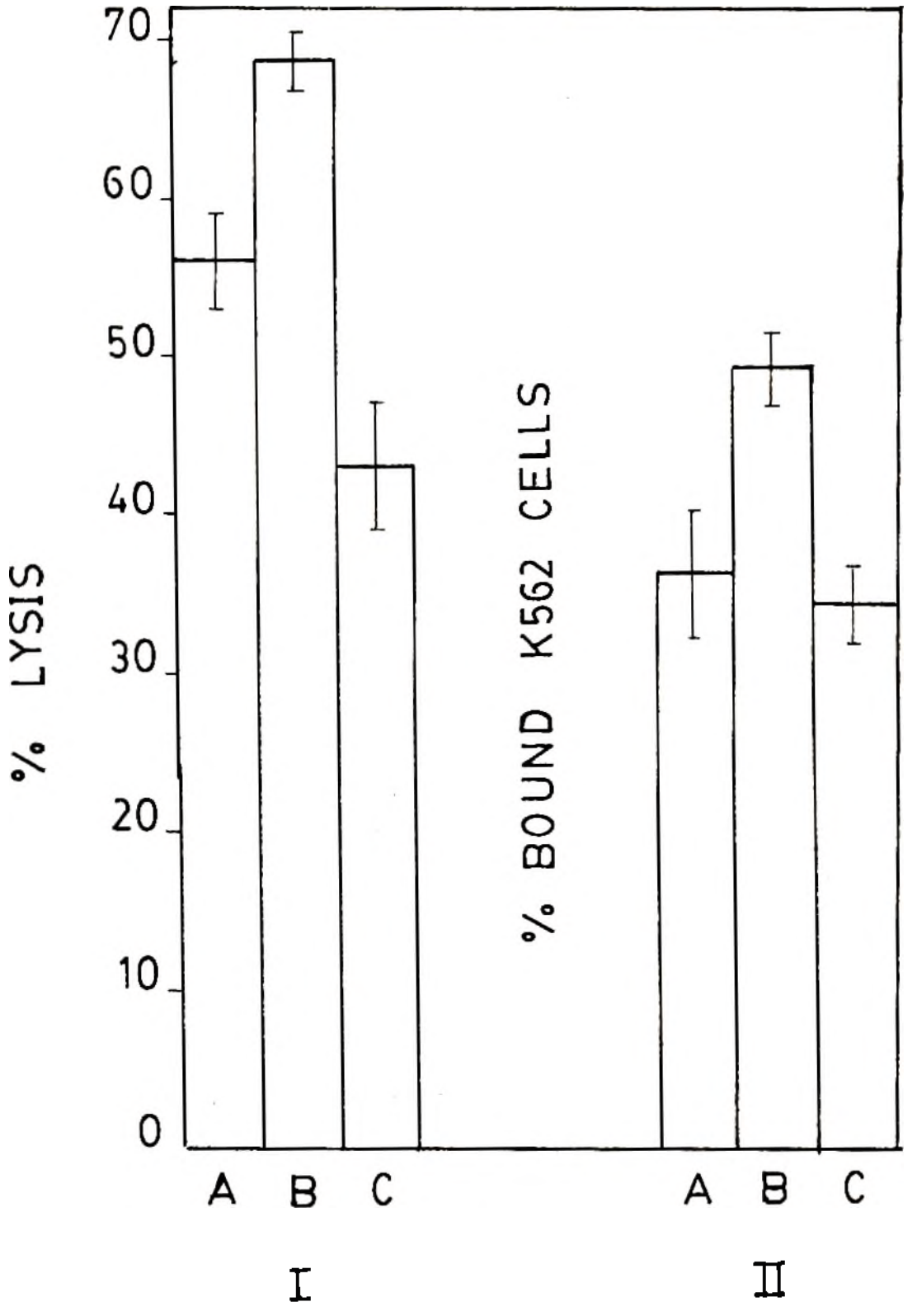


FIGURE 10.4 THE PERCENT CYTOTOXICITY (I) AND PERCENT BOUND K562 CELLS (II) OF NORMAL LGLS AGAINST (A) K562 CELLS (B) K562 CELLS TREATED WITH IL-1 AND (C) K562 CELLS TREATED WITH IL-1 AND RABBIT ANTI-HUMAN IL-1 ANTIBODY.

10.3.4 Correlation of cytotoxicity and stimulation of IL-1 production by various target cells

Fig. 10.5 relates the degree to which various cells are killed by NK cells and their ability to stimulate IL-1 production from purified LGLs. It should be noted that those target cells which are killed to the greatest extent by LGLs, such as K562 cells, are able to stimulate larger amounts of IL-1 than those which are lysed to a lesser degree such as Yac-1 and skin fibroblasts. When heat killed or cycloheximide treated cells were used to stimulate IL-1 similar results were obtained, indicating that it was indeed the LGLs producing the IL-1 and not the target cells (results not shown).

10.3.5 Pretreatment of K562 cells with IFN renders them more resistant to NK cell activity and reduces their ability to stimulate IL-1 production by LGLs

The data in Fig. 10.6 illustrates the reduction in the percentage of dead target cells after these cells have been pulsed with varying amounts of IFN for 4 to 16 hours. After 16 hours of IFN treatment, cytotoxicity was reduced at all concentrations of IFN, but particularly when 200 or 500 IU of IFN were employed. A 4 hour pulse of target cells with IFN protected only those cells which had been treated with 200-500 IU.

Fig. 10.7 illustrates that the K562 cells require an

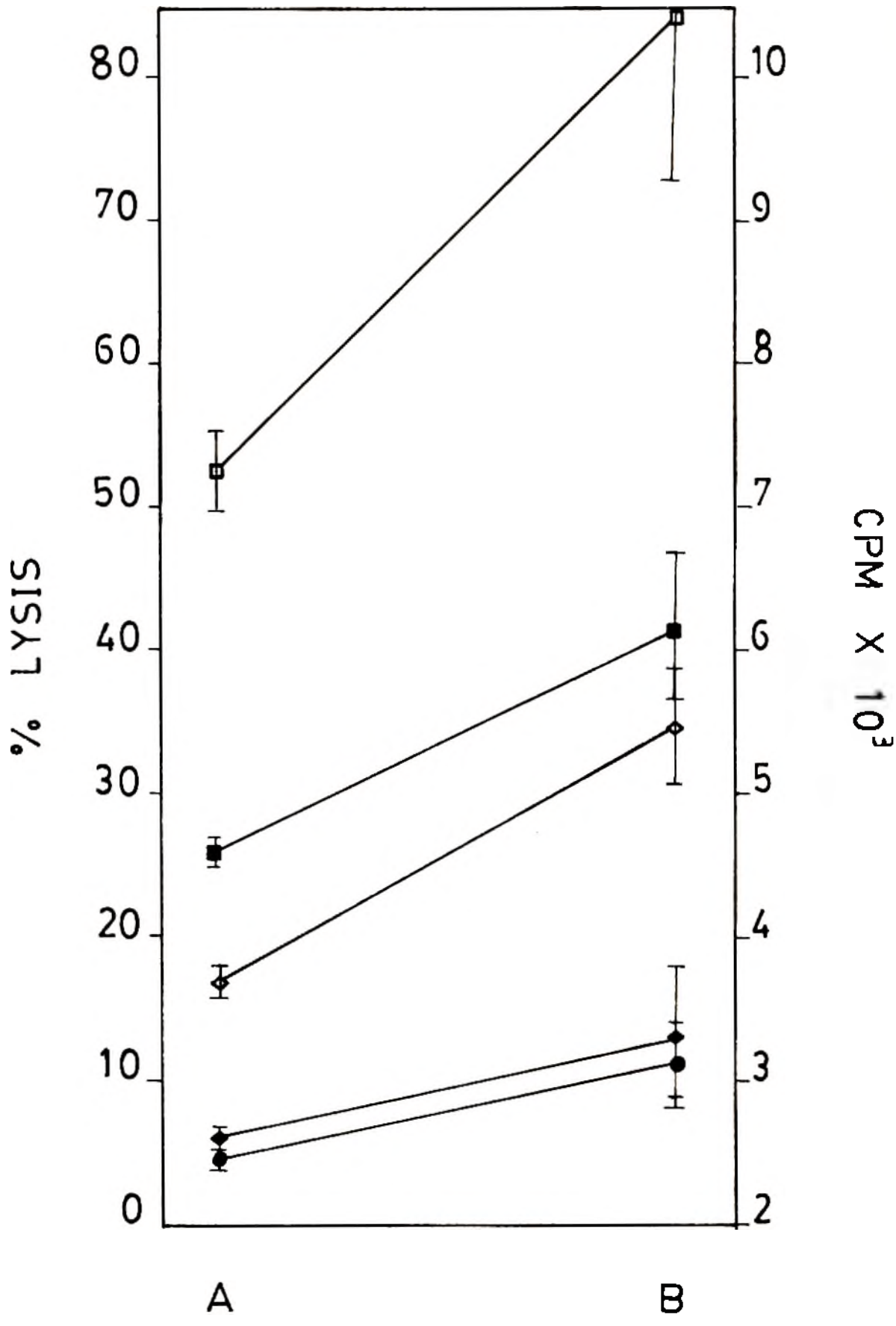


FIGURE 10.5 CORRELATION OF (A) PERCENT CYTOTOXICITY OF VARIOUS CELL LINES AND (B) THEIR ABILITY TO STIMULATE IL-1 PRODUCTION FROM LGLS.

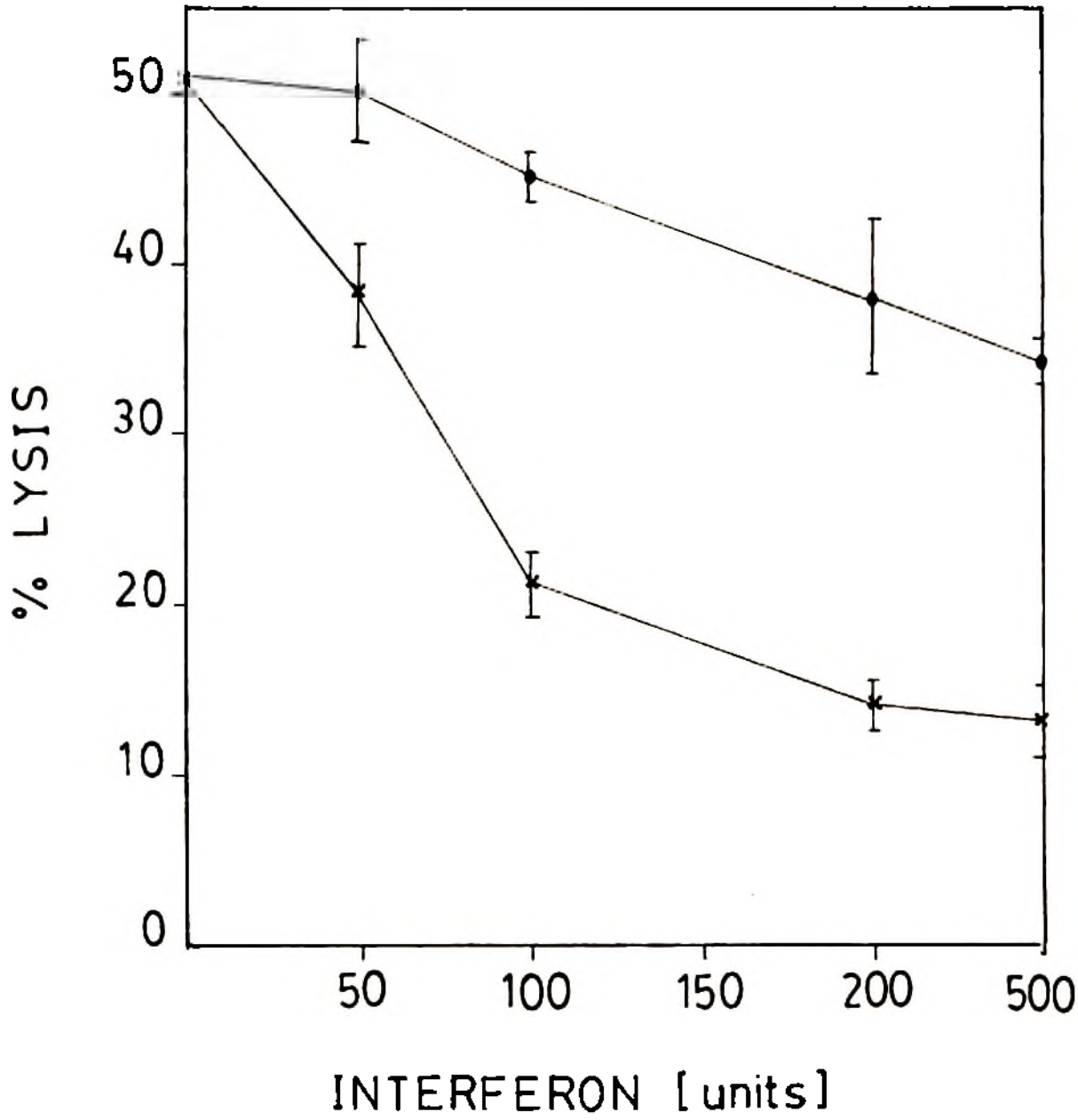


FIGURE 10.6 THE CYTOTOXIC ACTIVITY OF LGLS AGAINST K562 CELLS TREATED WITH VARYING CONCENTRATIONS OF IFN FOR 4 HOURS (●——●) OR 16 HOURS (X——X).

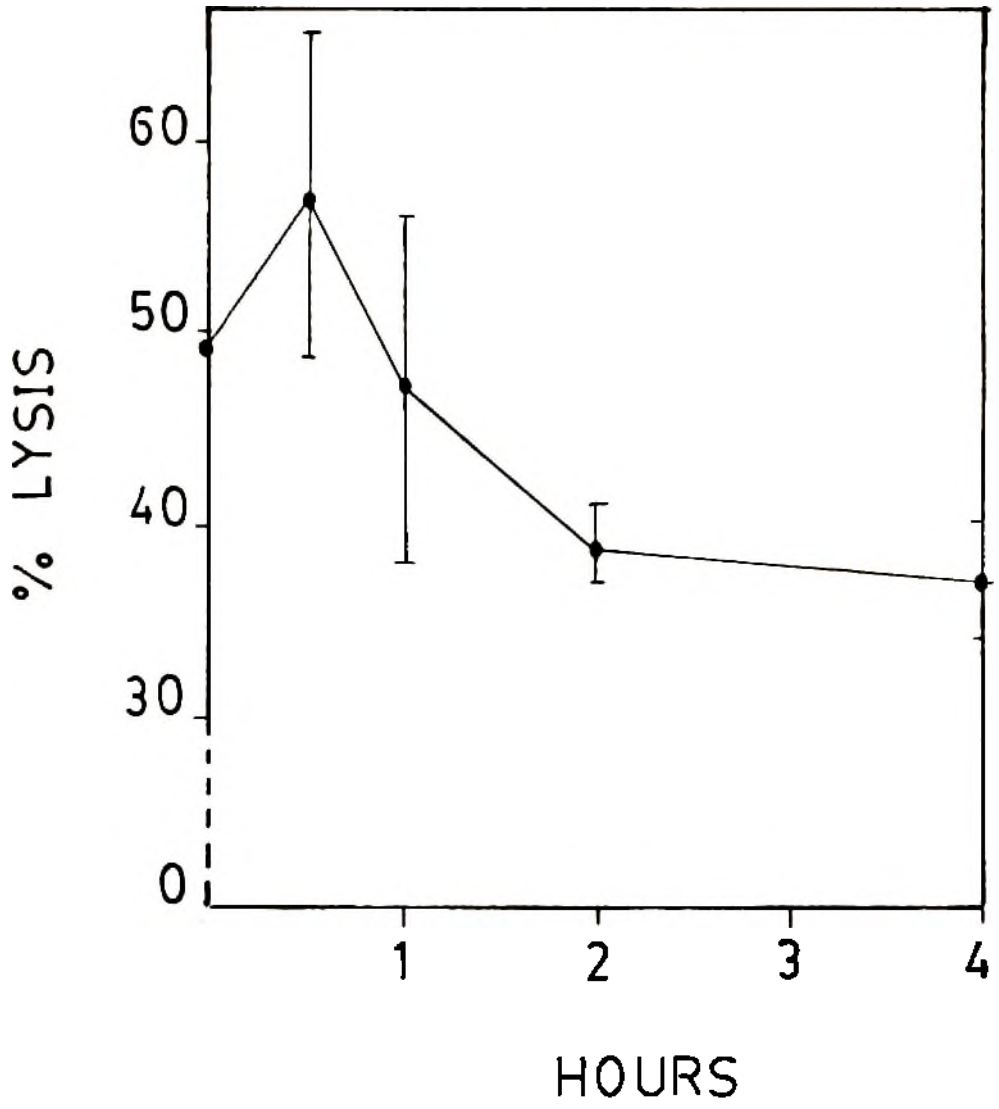


FIGURE 10.7 TREATMENT OF K562 CELLS WITH 500IU IFN/mℓ AFFECTS THE DEGREE TO WHICH THEY ARE LYSED BY LGLS.

IFN pulse of at least 2 hours in order to aquire resistance to NK cell lysis. Treatment of K562 cells with 500 IU of IFN for only 30 minutes resulted in enhancement of lysis.

In all experiments IFN treatment had no effect on the spontaneous and maximal release of chromium by K562 cells (results not shown).

The degree to which IFN pretreatment of K562 cells affected their ability to stimulate IL-1 production by LGLs was examined. The results are illustrated in Figs. 10.8 and 10.9. The results shown in Fig. 10.8 indicate that pulsing K562 cells for 16 hours with IFN renders them less able to stimulate IL-1 production, at all concentrations of IFN used. Doses of IFN over 100 IU were most effective. Treatment of K562 cells with IFN for 4 hours was only effective when the target cells were treated with 500 IU. Treatment of cells with less IFN caused only a slight decrease in the IL-1 stimulatory ability.

Fig. 10.9 shows that treatment of K562 cells with 500 IU IFN for brief periods of time - up to 1 hour - does not affect their ability to stimulate IL-1 production, this effect is apparent only after treatment for 2-4 hours.

In all of the above experiments 24 hour supernatants from 1×10^6 K562 cells were collected and tested for IL-1 activity as controls. Thymocyte proliferation was never found to be above background.

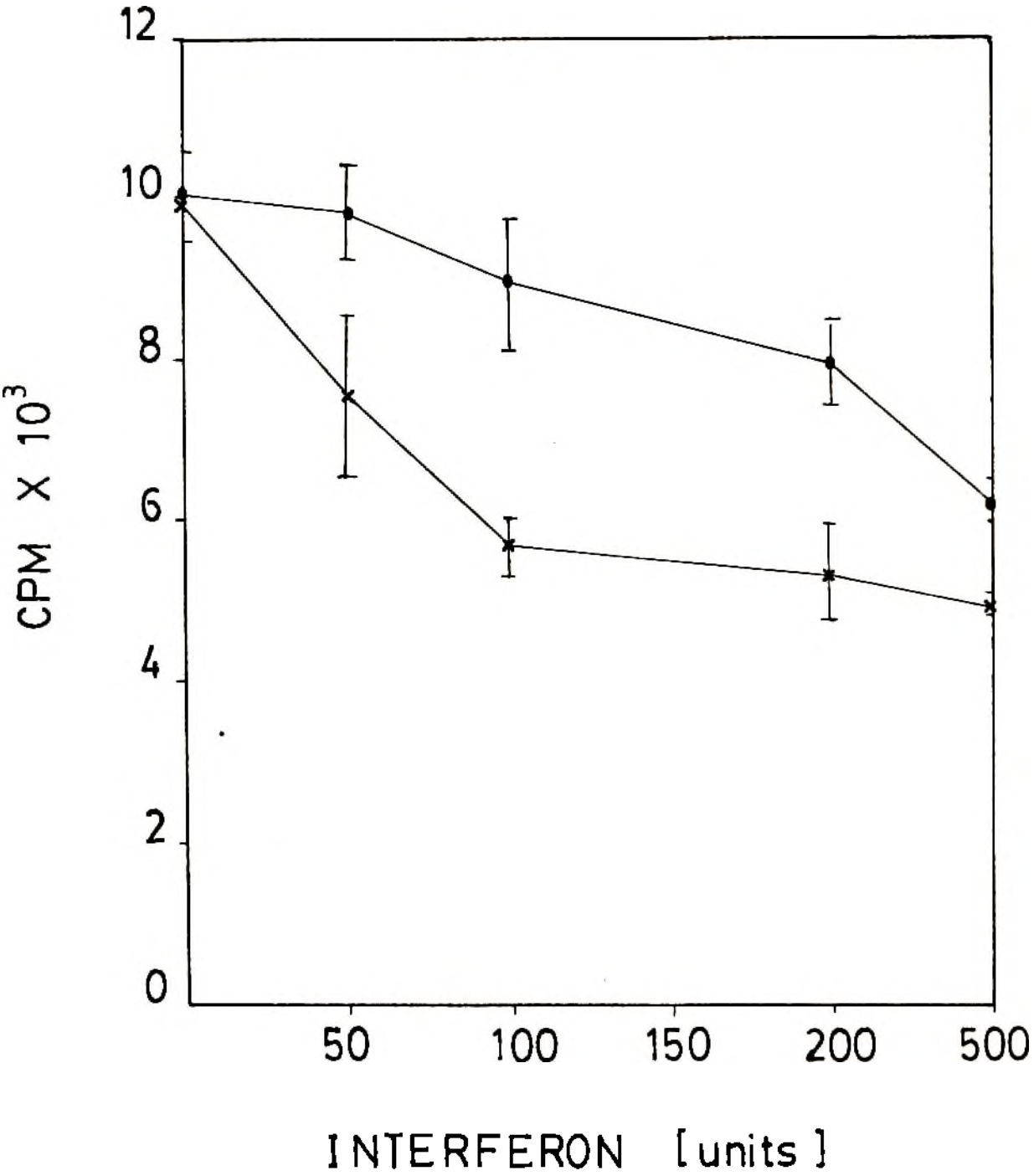


FIGURE 10.8 IL-1 PRODUCTION BY LGLS STIMULATED WITH K562 CELLS TREATED WITH VARYING CONCENTRATIONS OF IFN FOR 4 HOURS (•—•) OR 16 HOURS (X—X).

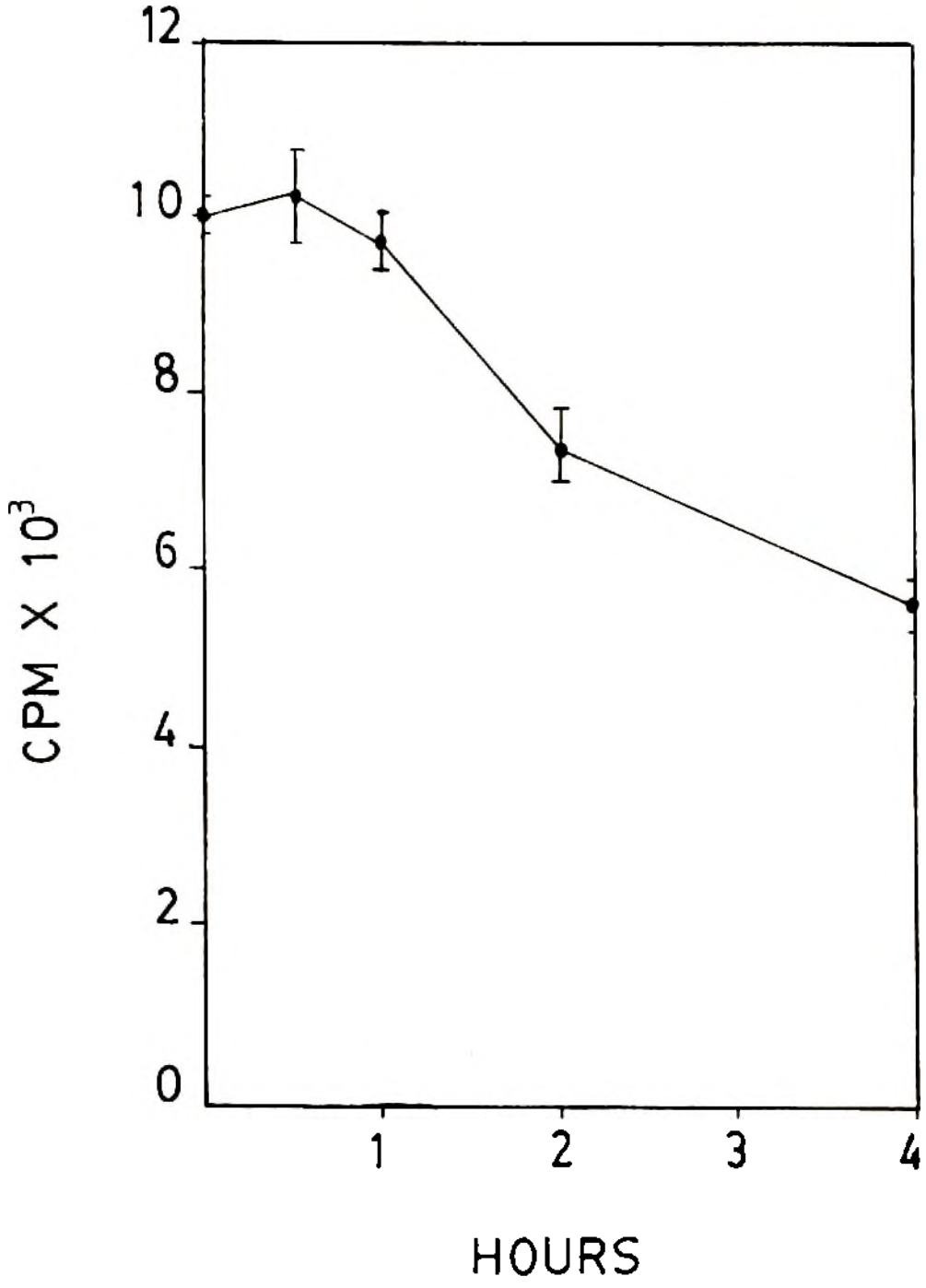


FIGURE 10.9 TREATMENT OF K562 CELLS WITH 500IU OF IFN/mℓ FOR VARYING PERIODS OF TIME AFFECTS THEIR ABILITY TO STIMULATE IL-1 PRODUCTION BY LGLS.

10.3.6 Pretreatment of K562 cells with IFN does not induce the synthesis of cell surface β-2 microglobulin

The possibility that IFN treatment of K562 cells induced the synthesis of surface HLA (a known function of IFN - Sanderson and Beverley, 1983) was examined. This was achieved by measuring the presence of cell surface β-2 microglobulin on K562 cells, before and after IFN treatment. No cell surface β-2 microglobulin was detected on K562 cells before or after IFN treatment however (results nor shown).

10.4 Discussion

The major findings of the experiments reported in this chapter are:

1) cells resistant to NK attack can be induced to become more susceptible after IL-1 treatment
2) this effect is dependant on RNA synthesis
3) susceptible target cells stimulate LGLs to produce greater quantities of IL-1 than resistant target cells
4) IFN treated K562 cells resist attack by NK cells and do not stimulate IL-1 production from LGLs.

Although IL-1 has been shown to stimulate the binding of target cells, the mechanism by which this occurs is unknown. However, this phenomenom is abrogated by RNA synthesis inhibitors such as actinomycin D and when the K562 cells and IL-1 are incubated at 4°C (results not shown).

Ransom and his co-workers (1983) have reported a similar finding with lymphotoxin. Lymphotoxin specifically increased the sensitivity of tumour cells to NK cell lysis. The IL-1 used in this study was found not to be directly cytotoxic to the target cells. It was also found to have no effect on red blood cells (results not shown) thus the IL-1 does not appear to act by simply causing target cells to become "sticky".

One mechanism for the IL-1 enhancement of target cell susceptibility to NK cell cytotoxicity may reside in the ability of IL-1 to alter the composition of the target cell plasma membrane. Certain pertubations in the plasma membrane may result in an increase of receptors for the NK cells, Ransom et al., (1983) have postulated that lymphotoxin induces reduction of membrane repair mechanisms leading to enhanced cell lysis and it is possible that IL-1 mediates its effects in a similar manner. Alternatively, IL-1 could bind to the target cell surface and aid in either the subsequent binding or lytic steps of cytolysis.

It is of interest to examine the data expressed in Fig. 10.2 more closely. This graph illustrates the stimulation of target cell lysis by IL-1. The enhancement of lysis caused by IL-1 is greatest after a 2 hour incubation of target cells and IL-1. However, the increase is no longer observed after a 3 hour incubation. Prolonged exposure of target cells to IL-1 was not found to affect the viability of the cells (results not shown). However, it is possible that after a certain period, target cells become resistant to

the effects mediated by IL-1.

The finding that the anti-IL-1 antibody abrogates only the enhancement of lysis caused by IL-1 and not all lysis, implies that IL-1 is more likely to be important in increasing the sensitivity of resistant cells rather than instrumental in the initial lysis of target cells by LGLs.

It has been postulated that transferrin is important in the recognition stage of NK cell lysis (Vodinelich et al., 1983; Baines et al., 1983). Support for this postulate is found in the work of two groups of workers. Stutman and his colleagues (1980) have shown that rapidly proliferating normal thymic and bone marrow cells, which have large amounts of cell surface transferrin receptors, express antigens capable of competing in lytic assays. Baines and his co-workers (1983) have proposed that NK cells regulate growth and differentiation of cells via a mechanism which moniters the uptake of a common but essential metabolite or co-factor, such as transferrin, which could result in a strong interaction between NK cell and target leading to elimination.

Vodinelich and his colleagues (1983) correlated target cells expression of transferrin receptors with the sensitivity of the cells to NK cell lysis. They have hypothesised that this receptor is a target structure. If this were the case, it is possible that IL-1 increases the density of transferrin receptors on the target cells, thus causing them to bind greater numbers of LGLs, thereby increasing target

cell lysis. Preliminary findings have shown that IL-1 may act by increasing either the number of K562 cells with transferrin receptors or the density of transferrin receptors on these cells - or both (results not shown).

Tumours which are highly susceptible to NK cell attack will stimulate LGLs to release greater amounts of IL-1 than those tumours which are more resistant to NK cell lysis. This finding is potentially of great importance as it could be one of the mechanisms by which neoplastic, pre-neoplastic or metastasing cells escape destruction by the immune system. It is possible that tumour cells which do not stimulate IL-1 production are less susceptible to cytotoxicity and more likely to metastasize.

It is widely accepted that many tumour cells and tumour cell lines stimulate the release of IFN by mononuclear cells (Trinchieri et al., Peter et al., 1980). Gustafsson and Ludgren (1981) reported a correlation between the susceptibility of certain tumour cell lines and their ability to stimulate IFN production. It has previously been found (Herman et al., 1984) that IFN enhances both the rate and production of IL-1. It is possible that when large quantities of IFN are released, the production of IL-1, in response to neoplastic cells, is also enhanced, resulting in elimination of such cells. However, should the immune system not recognise the neoplastic cell as foreign, or be unable to respond to the foreign cell due to various "blocking factors" less IFN would be produced. This could result in decreased IL-1

production and enhanced tumour growth. Keong (Msc dissertation 1982) reported that supernatants from the PLC/PRF/5 hepatocellular carcinoma cell line, decreased the production of IFN by peripheral blood leukocytes. Suggesting that another mechanism of evasion of the immune system could be the secretion of substances by neoplastic cells, which prevent IL-1 release.

Studies reported here have also confirmed the work of Trinchieri et al., (1981) showing that IFN treated K562 cells are less susceptible to NK cell lysis. This finding has been extended to show that IFN treated K562 cells are less able to stimulate IL-1 production by LGLs. Sanderson and Beverley (1983) reported that IFN causes a profound increase in surface HLA antigens. These cells are no larger than normal but the density of these antigens appears to increase. The possibility that more primitive or immature cells with little or no surface HLA are more susceptible to NK cell attack has been investigated by Brunda et al., (1981). They have indicated that cytotoxicity can be increased by treating cells with antibody to HLA. It is possible that when target cells are treated with IFN they synthesize HLA antigens rendering them less sensitive to NK cell attack. However, no β-2 microglobulin was detected on IFN treated K562 cells. It can be concluded therefore, that it is not the synthesis of HLA antigens which induces resistance in IFN treated K562 cells.

That IFN treated K562 cells resist attack by NK cells

and do not stimulate IL-1 production needs to be elucidated. Werkmeister et al., (1983) showed sodium-butyrate differentiated K562 cells to heve greater amounts of cell surface sialic acid which is associated with a decreased susceptibility to NK mediated cytolysis. The presence of sialic acid on the cell surface has been implicated in a variety of depressed cell mediated responses and it has been suggested that effective antigenic expression by some tumours may be masked by the presence of this substance (Werkmeister et al., 1983). It is possible that IFN treatment of K562 cells leads to an increased expression of cell surface sialic acid which could mask target recognition structures. However, biochemical comparisions of the glycoproteins and glycolipid content of both IFN treated and untreated K562 cells are needed to facilitate a greater understanding of the early recognition events in NK mediated cytolysis.

Both IFN and IL-1 are known to affect intracellular levels of cyclic nucleotides (Trinchieri et al., 1979; Oppenheim et al., 1980). It is possible, therefore, that the mechanisms by which these cytokines affect NK cell activity involves the alteration of intracellular quantities of cGMP and cAMP.

cAMP is known to influence the cytotoxic sensitivity of normal (Ebbesen and Arnung, 1973) and transformed cells (Boyle et al., 1976). Saito and co-workers (1983) demonstrated that treatment of certain cells with glucose results in an alteration of intracellular cAMP levels accompanied by

an increase in the sensitivity, of such cells, to lysis.

IFN is known to cause a transient increase in intracellular cGMP which is associated with its ability to enhance NK cytolysis. However, the level of intracellular cAMP rises after 18-24 hours (Trinchieri et al., 1979). This increase in cAMP could account for the decreased sensitivity of IFN treated K562 cells observed in this chapter. It is possible, therefore, that the increased lysis of IL-1 treated target cells is a manifestation of altered levels of intracellular cyclic nucleotides.

The seemingly paradoxical effects of IFN should be considered, particularly with regard to IFN therapy. It appears, from these results, that in vivo IFN administration could result in tumour protection rather than stimulation of the immune system.

Knowledge of the lytic cycle and various mediators of NK cell activity is extremely limited. Of importance are the findings that IL-1 plays a role in this system and also that IFN has differing effects on target and effector cells. Thus the need for clear definition of surface structures on tumour cells is becoming increasingly apparent. It seems likely that the surface antigens of tumour cells which do not stimulate IFN and IL-1 production by LGLs differ in some ways from those cells which are able to stimulate lymphokine production. Modulation of these resistant tumour cells may prove to be an important mechanism in the understanding of

LGL-target cell interactions.

This investigation identifies a potentially physiologic role for IL-1 and demonstrates that a cytokine can enhance the activity of the normal immune system by affecting the immunological target to a greater degree than the effector mechansim. These may be important mechansims in the control of cancer.

CHAPTER 11

11. PROSTAGLANDIN E_2 DEPRESSES NATURAL KILLER CELL CYTOTOXICITY BY INHIBITION OF INTERLEUKIN-1 PRODUCTION BY LARGE GRANULAR LYMPHOCYTES

11.1 Introduction

Prostaglandins (PGs), especially of the E series, have often been linked with malignancy. Prostaglandin E_2 (PGE_2) is present in high concentrations in many natural and experimentally induced tumours. Tumour bearing patients often show elevated levels of PGs in their sera and in actual tumours (Karim, 1976). High levels of PGE_2, or PGE_2 metabolites, have been found in the serum of patients with squamous-cell carcinoma of the lung (Seyberth et al., 1975) and a variety of other malignancies in man (Demers et al., 1977). Administration of indomethacin and other PG synthetase inhibitors can slow the growth of certain tumours in mice (Lynch et al., 1968a; Lynch and Salomon, 1979). Furthermore, Bennett et al., (1975) reported a correlation between PGE_2 production by human breast cancer and metastasis, in that tumours with high PGE production were more likely to have metastasised to bone. PGE_2 has been shown to inhibit PHA induced lymphocyte proliferation (Smith et al., 1971) and lymphokine production (Lomnitzer et al., 1976). More recently PGE_2 has been shown to be a potent inhibitor of NK cell activity (Bankhurst, 1982). At concentrations of PGE_2 in the physiologic range (10^{-8}M) significant suppression of NK cell activity could be observed employing different target cells and a variety of effector:target ratios. Furthermore, the

suppressive effect of PGE_2 was directed at the effector cell population but did not affect the target cell (Herman and Rabson, 1984).

We have recently observed (Herman et al., - submitted for publication) that purified LGLs, when activated by LPS or a variety of tumour cells including K562 cells, release considerable amounts of IL-1 which is important in the effector:target interaction (chapter 8). As PGE_2 is a potent inhibitor of NK cell activity, this study was undertaken to assess whether various concentrations of this PG could inhibit IL-1 production by enriched LGLs.

In the light of the results obtained regarding the inhibition of NK activity with PLC/PRF/5 supernatant it seemed appropriate too to study the effects of these supernatants on IL-1 production.

11.2 Materials and methods

11.2.1 Preparation and identification of LGLs

LGLs were isolated from peripheral blood as outlined in chapter 2.2 and the purity checked as described in the same chapter.

11.2.2 K562 target cells

The K562 cell line was maintained in suspension culture as

described in chapter 2.2.6.

11.2.3 Cytotoxicity assay

The standard ^{51}Cr release assay, as described in chapter 2.2.6, was used to assess the effect of PGE_2 on NK cell activity. Enriched LGLs were treated with various concentrations of PGE_2 for differing time periods. After washing the cells three times they were used in cytotoxicity assays.

11.2.4 Production of IL-1 by PGE_2 pulsed LGLs

1 x 10^6/mℓ enriched LGLs were cultured for 24 hours in the presence and absence of 20 µg/mℓ LPS as described in chapter 8.2.6. To assess the effect of PGE_2 on IL-1 production, LGLs were pulsed, for 4 hours, with varying concentrations of PGE_2 ranging from 10^{-3}M to 10^{-9}M. After washing, the cells were stimulated with LPS for a further 18 hours, after which supernatants were collected and assessed for IL-1 activity as described in chapter 8.2.6.

11.2.5 The effect of PLC/PRF/5 supernatant on IL-1 production by LGLs

In some experiments 1 mℓ of the supernatant was added to cultures of LGLs and LPS or, LGLs and K562 cells. To assess the effect of the culture supernatant on mouse thymocytes in the final IL-1 assay, PLC/PRF/5 supernatant was added to the thymocytes together with active IL-1, which was also

tested alone with the thymocytes. In other experiments LGLs were cultured for varying periods of time before the addition of culture supernatant to these cultures.

11.3 Results

11.3.1 The effect of PGE_2 on the cytotoxic activity of purified LGLs

The effect of various concentrations of PGE_2 on NK cell activity of enriched LGLs against labelled K562 cells is shown in Fig. 11.1. The levels of lysis exhibited by LGLs, pulsed for 4 hours with PGE_2, are markedly reduced. Although very marked suppression of lysis was observed at concentrations of 10^{-6} to 10^{-3}M, considerable inhibition of cytotoxicity could be seen at 10^{-8}M. Furthermore, when LGLs were pulsed with PGE_2 for even 1 hour 50% inhibition of lysis was seen. Maximum inhibition of lysis was seen, however, when the cells were treated with PGE_2 for a period of 3 or 4 hours (Fig. 11.2).

11.3.2 The effect of PGE_2 on IL-1 production by LGLs

As can be seen in Fig. 11.3, LGLs pulsed for 4 hours with varying concentrations of PGE_2 before being stimulated with LPS produced considerably less IL-1. Inhibition of IL-1 production was dose dependant but even at concentrations of 10^{-9} and 10^{-8}M significant suppression of IL-1 production

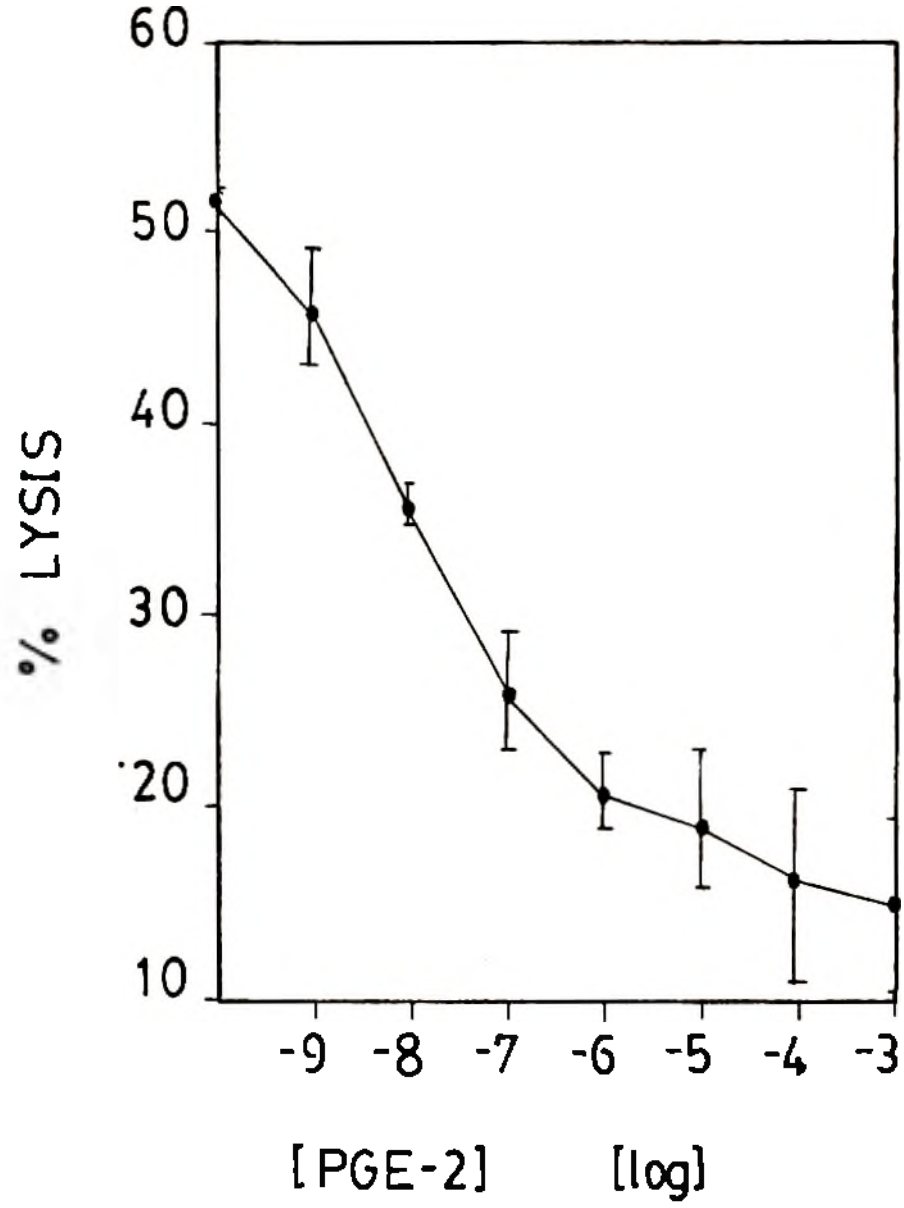


FIGURE 11.1 THE EFFECT OF VARYING CONCENTRATIONS OF PGE_2 ON THE PERCENTAGE CYTOTOXICITY OF PURIFIED LGLS AGAINST K562 TARGET CELLS.

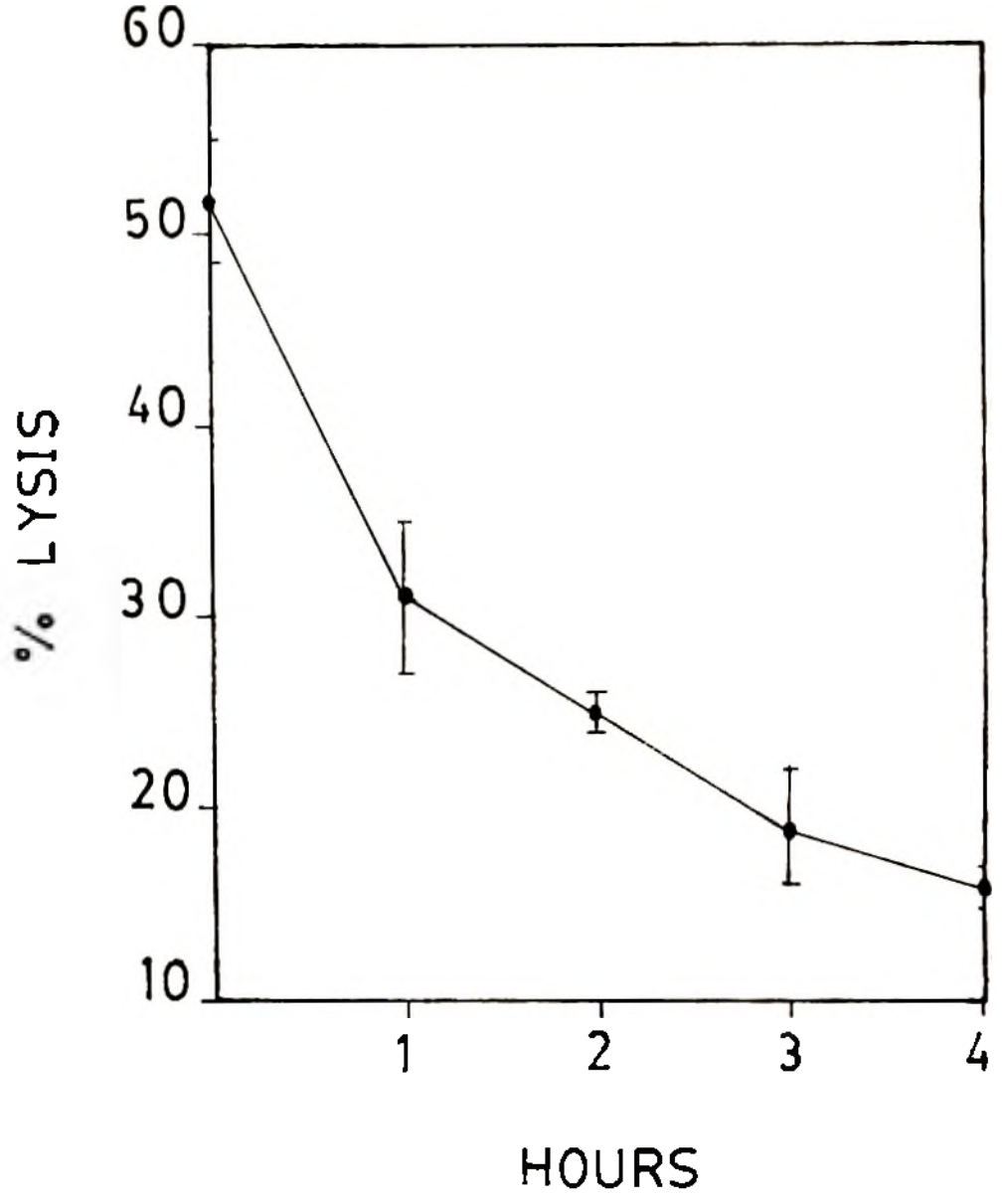


FIGURE 11.2 THE EFFECT OF PGE_2 INCUBATED WITH LGLS FOR VARYING TIME PERIODS ON THEIR CYTOTOXIC ACTIVITY.

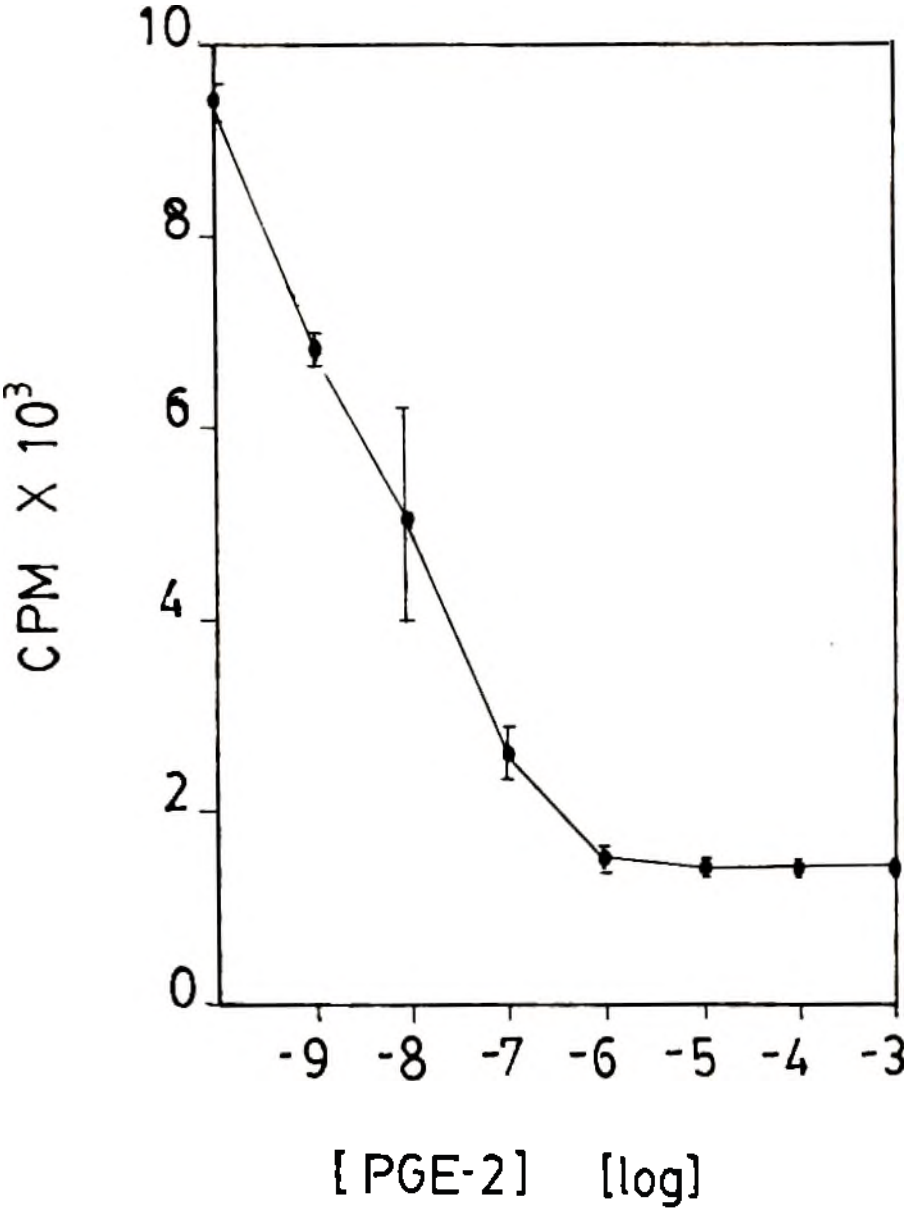


FIGURE 11.3 THE EFFECT OF VARYING CONCENTRATIONS OF PGE_2 ON THE PRODUCTION OF IL-1 BY LPS STIMULATED LGLS.

was seen. Although a 4 hour treatment with PGE_2 produced the greatest suppression of IL-1 production, when the cells were treated with 10^{-7}M PGE_2 for only 1 hour, considerable inhibition of IL-1 production could be observed (Fig. 11.4).

11.3.3 The effect of PLC/PRF/5 supernatant on IL-1 production by LGLs

The data expressed in Figs. 11.5 and 11.6 show a decreased production of IL-1 in the presence of PLC/PRF/5 supernatant. However, column F of each graph illustrates that PLC/PRF/5 supernatant added to mouse thymocytes together with IL-1 halves the proliferation of the thymocytes. It is not clear therefore, whether the culture supernatant affects the production of IL-1 by LGLs, when stimulated with LPS or K562 cells, or, whether it disturbs the mode of action of IL-1 on mouse thymocytes, or even simply inhibits the proliferation of the thymocytes. (It is known that PLC/PRF/5 supernatant inhibits lymphocyte blastogenesis (Keong and Rabson, 1983). Columns B, C, D and E of each graph show that the PLC/PRF/5 supernatant, added to stimulated LGLs up to 3 hours after stimulation inhibits IL-1 production.

Due to the apparent difficulties it is not possible to clearly deduce the effect of PLC/PRF/5 supernatant on IL-1 production.

11.4 Discussion

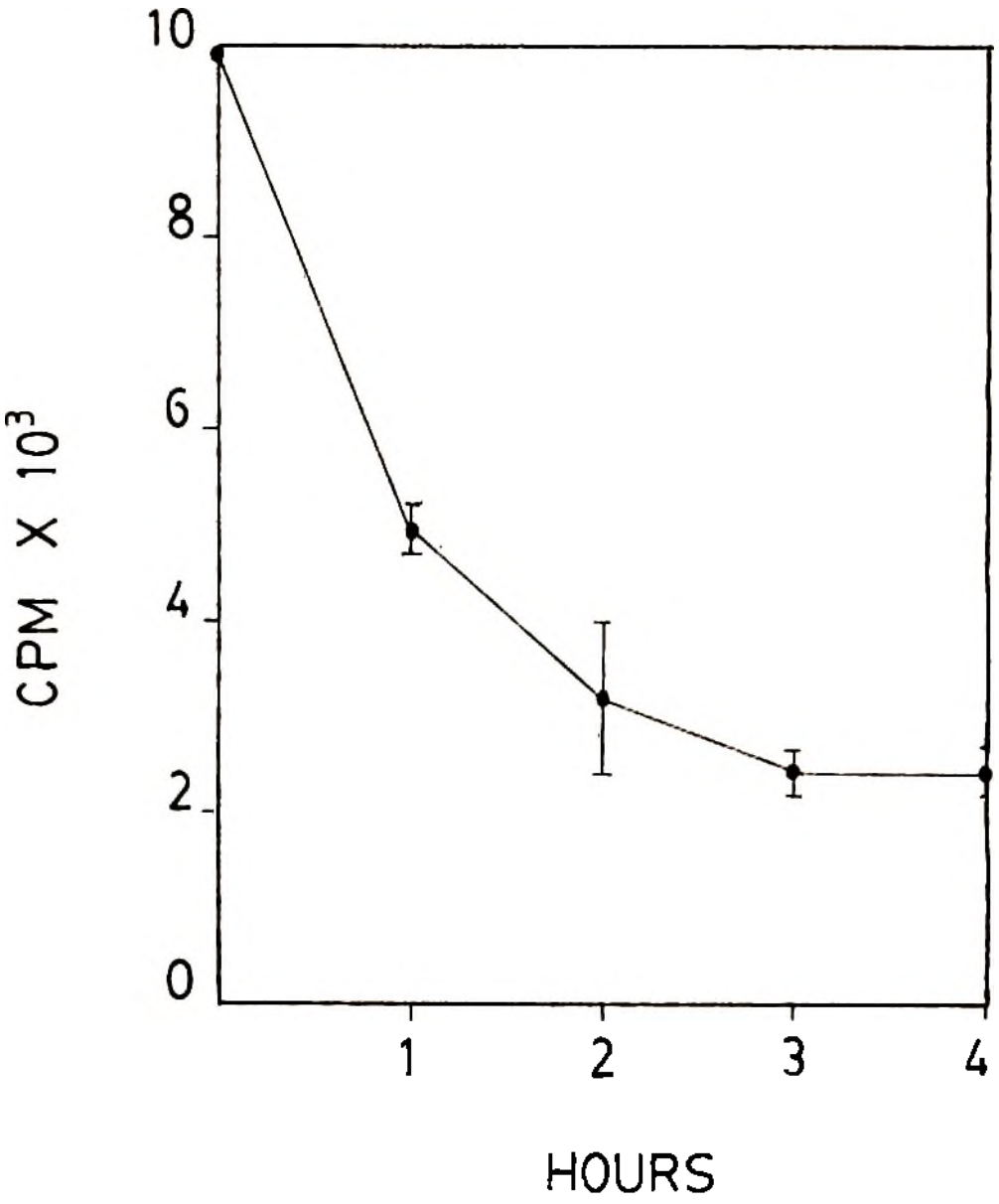


FIGURE 11.4 IL-1 PRODUCTION BY ENRICHED LGLS TREATED WITH 10^{-7}M PGE_2 FOR VARYING TIME PERIODS.

Key to Fig. 11.5

A IL-1 production by LGLs + LPS 24 hours

B IL-1 production by LGLs + LPS + PLC/PRF/5 s/n 24 hours

C IL-1 production by LGLs + LPS 1 hour then LPS + PLC/PRF/5 s/n 23 hours

D IL-1 production by LGLs + LPS 2 hours then LPS + PLC/PRF/5 s/n 22 hours

E IL-1 production by LGLs + LPS 3 hours then LPS + PLC/PRF/5 s/n 21 hours

F Addition of PLC/PRF/5 s/n + IL-1 (produced as in A) to mouse thymocytes

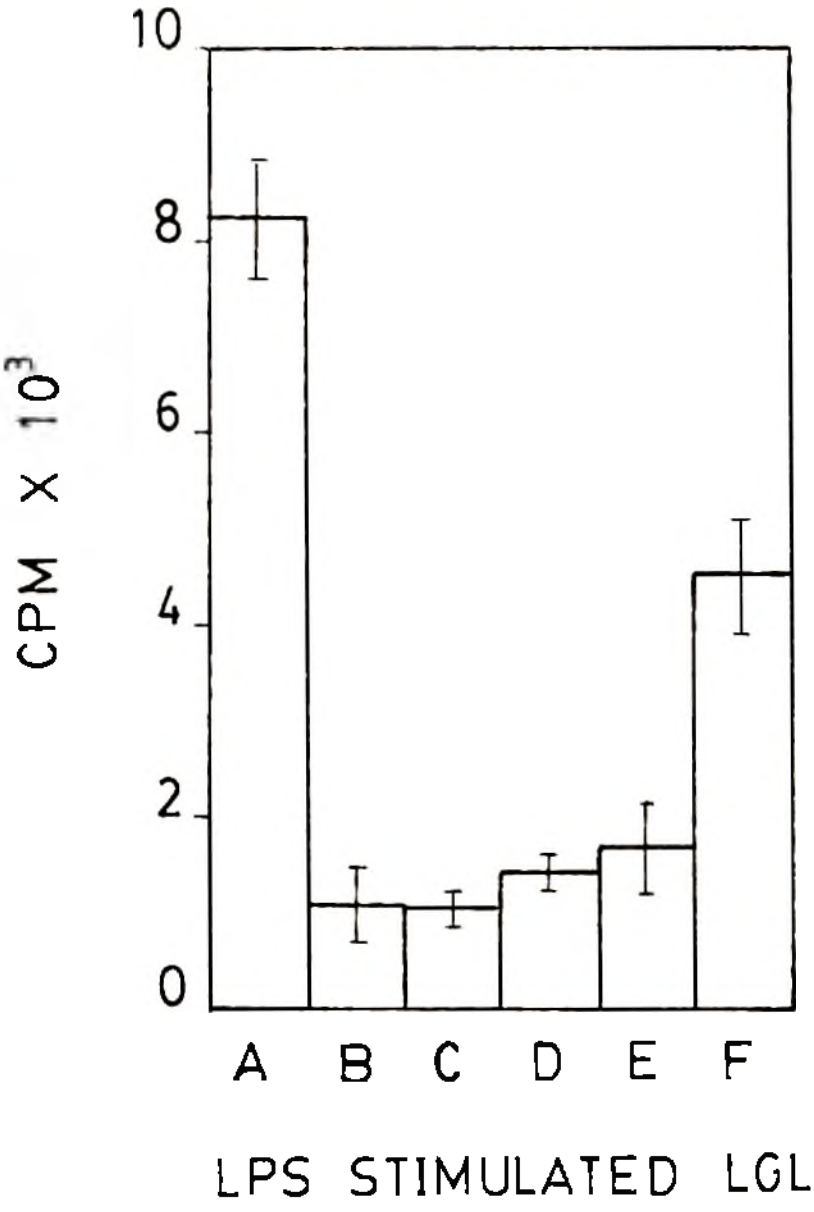


FIGURE 11.5 PLC/PRF/5 SUPERNATANTS AFFECT EITHER IL-1 PRODUCTION BY LPS STIMULATED LGLS, OR STIMULATION OF MOUSE THYMOCYTES BY IL-1.

Key to Fig. 11.6

a IL-1 production by LGLs + K562 24 hours

B IL-1 production by LGLs + K562 + PLC/PRF/5 s/n 24 hours

C IL-1 production by LGLs + K562 1 hour then K562 + PLC/PRF/5 s/n 23 hours

D IL-1 production by LGLs + K562 2 hours then K562 + PLC/PRF/5 s/n 22 hours

E IL-1 production by LGLs + K562 3 hours then K562 + PLC/PRF/5 s/n 21 hours

F Addition of PLC/PRF/5 s/n + IL-1 (produced as in A) to mouse thymocytes

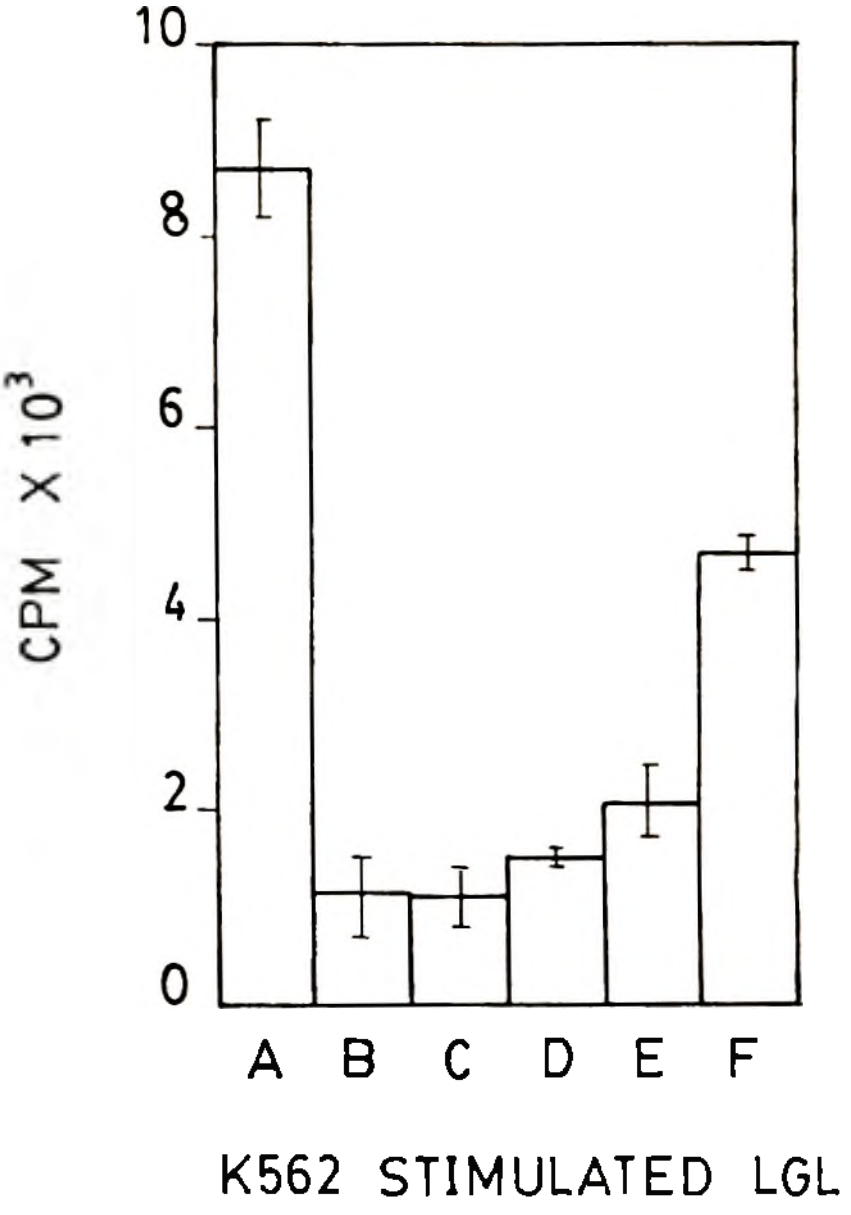


FIGURE 11.6 PLC/PRF/5 SUPERNATANTS AFFECT EITHER IL-1 PRODUCTION BY K562 STIMULATED LGLS, OR STIMULATION OF MOUSE THYMOCYTES BY IL-1.

The data presented in this chapter confirm the work of others that PGE_2 has a marked suppressive effect on NK activity (Droller et al., 1978a; Brunda et al., 1980). Of intrest, however, was the finding that the dose and time kinetics of the suppression of cytotoxicity were almost identical to the inhibition of IL-1 production shown by PGE_2 treated NK cells. These findings suggest that the inhibition of lysis was mediated via inhibition of IL-1 production. This finding is compatible with studies discussed in chapter 8, which indicate the important role of IL-1 in LGL-target cell interactions. Target cells treated with IL-1 bind considerably greater numbers of LGLs resulting in enhanced cytotoxicity. It would be expected, therefore, that agents which inhibit LGL IL-1 production will also depress NK cell activity. Although the cause of the depressed IL-1 production is not known, PGE_2 has also been shown to inhibit IL-1 production by monocytes (Oppenheim et al., 1980).

The finding that PGE_2 could decrease NK cell activity by affecting IL-1 production leads to speculation on its mode of action. It seems likely that PGE_2 could affect IL-1 production by causing a loss of surface Ia (I associated) antigen expression on the surface of monocytes or LGLs. This hypothesis is based on the work of Gilman et al., (1983), Steeg et al., (1982) and Beller and Ho, (1983) all of whom studied aspects of Ia expression on IL-1 production by monocytes.

Beller and Ho (1983) reported that mononuclear phago-

cytes become Ia negative within 24 to 48 hours and no longer function as accessory cells for antigen processing. Furthermore, they lose their ability to produce IL-1. Gillman et al., (1983) have shown that a monoclonal antibody directed against Ia, reduced IL-1 production by 60% from endotoxin stimulated rat peritoneal macrophages. This would suggest that the majority of IL-1 producing cells are Ia positive. Incubation of macrophages with indomethacin prevents this loss of Ia expression and IL-1 production (Steeg et al., 1982). It is unclear whether Ia is required for activation, synthesis or the release mechanism involved in IL-1 production. However, evidence suggests that Ia expression has a role in both antigen recognition and IL-1 production. Thus the roles of Ia expression, indomethacin and PGs in IL-1 production are, as yet, not well elucidated.

The inhibition by PGE_2 of IL-1 production by LGLs may partly explain the inhibitory effect of PGE_2 on a variety of lymphocyte functions in man. The findings are of even greater significance when one considers that PGs, particularly those of the E series, have been shown to be elevated in many human and animal tumours (Bennett et al., 1977a,b). Furthermore, mice bearing a number of malignancies, have been shown to produce elevated levels of PGE_2 (Lynch et al., 1978a, b). The results are interesting in the light of the observations that administration of indomethacin or asprin could inhibit tumour growth perhaps through restoration of NK cell reactivity (Lynch et al., 1978a).

The data presented here on the effect of PLC/PRF/5 supernatant on IL-1 is inconclusive. Firstly, although the previously observed effects of this culture supernatant are not attributable to PGs it is possible that these substances are present in the supernatant and that they inhibit not only IL-1 production but also mitogenesis of mouse thymocytes. This is presently being investigated using PLC/PRF/5 supernatants prepared from cells grown with indomethacin.

Secondly, the possibility that the PLC/PRF/5 supernatant simply inhibits mouse thymocyte responses to IL-1 is to be examined. This will be done by testing the pyrogenic effects of IL-1 (together with PLC/PRF/5 supernatants) on rabbit responses.

The third alternative that the supernatant binds to, degrades or interferes with the mode of action of IL-1 should be considered. Due to the length of time required for the effects of the supernatant to become apparent, it is not possible to pulse LGLs before stimulating IL-1 release, thereby excluding the supernatant from the final assay, it is therefore, extremely difficult to examine this question.

The effectiveness of NK activity is under the control of a complex regulatory network. Interferons have a major augmenting effect on NK cell activity and are probably important in maintaining this activity (Trinchieri et al., 1981). This could be explained by our previous findings that IL-1

production by LPS stimulated LGLs is increased by IFN treatment (Herman et al - submitted for publication). The finding that PGE_2, and possibly the PLC/PRF/5 supernatant, inhibit NK cells by suppressing IL-1 production indicates a central role for IL-1 in the augmentation and inhibition of NK cell activity.

CHAPTER 12

12. GENERAL DISCUSSION

12.1 Major findings

The major findings of this study are that:

1) Supernatants from the PLC/PRF/5 hepatocellular carcinoma cell line decreased the lytic capabilities of LGLs. This effect was found to be, in part, associated with a decrease in the ability of supernatant treated LGLs to bind to target cells. Supernatant treated LGLs were also shown to exhibit defective recycling capabilities.

2) Certain monosaccharides, namely mannose and methyl-mannoside, inhibited lysis of target cells by LGLs. The suppression was mediated at the effector cell level and resulted from decreased binding of effector and target cells. This finding suggests that glycoprotein receptors are important in recognition and lysis of target cells.

3) LGLs could be induced to release IL-1 in response to various stimuli.

4) Interferon accelerated the production and rate of release of IL-1 by stimulated LGLs and monocytes.

5) Patients with advanced malignant disease exhibited decreaed levels of NK cell activity. This was not a result

of reduced numbers of LGLs in the peripheral blood, but could be explained by the inability of these cells to produce IL-1. This latter defect could, to some extent be improved by treating the cells with IFN. Although the reason for decreased IL-1 production was not known, a similar defect in the ability of monocytes isolated from such patients was also demonstrated.

6) Besides acting synergistically with IL-2 and IFN to enhance NK cell lysis, IL-1 affected tumour target cells, such that the proportion of LGLs binding to them was increased. This effect depended upon normal RNA synthesis implying the development of receptors for LGL binding.

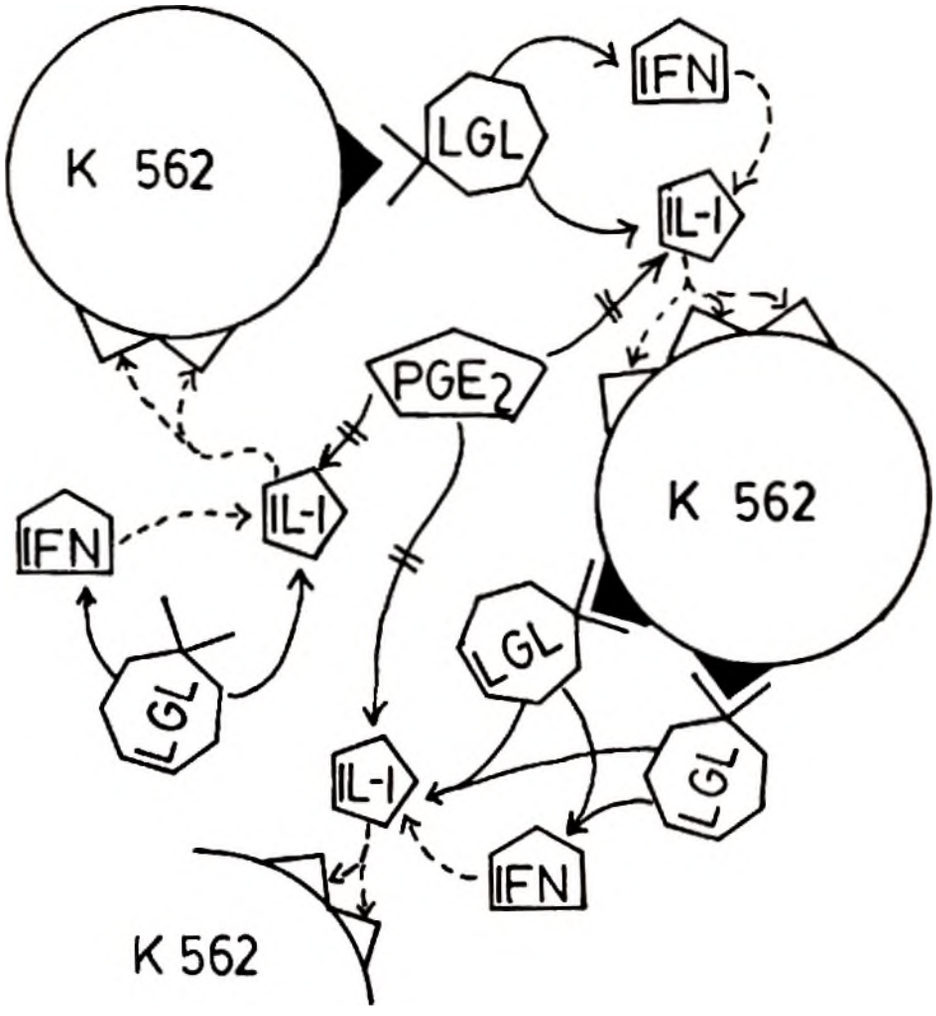
7) The amount of IL-1 produced by LGLs in response to stimulation by various cell lines was found to correate with the sensitivity of these target cells to NK cell cytotoxicity.

8) NK cell sensitive K562 cells could be made less sensitive to lysis by IFN treatment. This effect corresponded to a decrease in the ability of these cells to stimulate IL-1 release. The cause of this altered sensitivity is largely unknown.

9) PGE_2 was shown to have an inhibitory effect on NK cell lysis. This appeared to be mediated, at least in part, through inhibition of IL-1 production.

11) The observed decrease in cytolytic activity of PLC/PRF/5 supernatant treated LGLs was found to be mediated through inhibition of IL-1 production.

12.2 Proposed hypothesis



The interaction of LGLs with susceptible target cells results in the release of large quantities of IL-1 by the LGLs. This IL-1, acting on target cells stimulates their ability to bind increased numbers of LGLs. In addition it stimulates other, less susceptible, target cells to become more susceptible to LGL attack, resulting in an increase in cytotoxicity.

12.3 Areas of future research

The following experiments are currently being performed:

1) Data pertaining to the PLC/PRF/5 supernatants indicates that at least one of the mechanisms by which NK cell activity is depressed by this substance is by decreasing IL-1 production. Due to the fact that the culture supernatant affects the mouse thymocytes utilized in the assay, these results will be confirmed using different IL-1 assay systems. One method is to test the pyrogenic activity of such IL-1 containing supernatants. Another is the effect of IL-1 supernatants in a fibroblast proliferation assay detailed by Schmidt and his colleagues (1982).

2) Further investigation of membrane and intracellular effects of IFN on K562 cells is another area of interest. The possible "maturational" effects of IFN on these cells is to be examined.

3) PGE_2 is thought to mediate its effects on cytotoxicity, at least partially, by decreasing IL-1 production. It is known that many tumours release PGs (Karim, 1976; Seyberth et al., 1975; Demers et al., 1977). It is in the light of these findings that a study on the effects of indomethacin on LGL funetion in patients with advanced cancer is being performed. Preliminary results suggest a partial correction of the defect by indomethacin.

It is therefore, possible that excess PG production could be a factor contributing to the impaired NK cell function observed in patients with advanced malignant disease.

4) The possible importance of Ia in IL-1 production has been discussed (chapter 11). It could be that LGLs from patients with advanced malignant disease lack, or have diminished amounts of, cell surface Ia. Another alternative is that the IL-1 secreting cells have been constantly activated by the presence of large mass tumours and are no longer able to synthesise IL-1. These two possibilities are to be investigated.

5) IL-1 has been shown to enhance binding of target and effector cells. The mechanisms by which this occurs are currently under investigation. One line of research is the study of the effect of IL-1 on the density of transferrin receptors on K562 cells. Another aspect to be investigated is the determination of whether IL-1 treated K562 cells release factors which either attract LGLs or cause the K562 cell surfaces to become "sticky" thereby allowing greater numbers of LGLs to bind to them.

6) Another interesting area of research emerging from this study is the determination of whether virally infected cells (cells known to be susceptible to NK lysis) are capable of stimulating IL-1 production from LGLs.

7) Various sugars have been shown to inhibit effector-target cell binding. Further investigations into the effects of these sugars on effector cells will be performed, with particular reference to the effect of certain monosaccharides on LGL IL-1 production.

8) It has recently been shown that there are at least three phenotypically different subsets of LGLs (Kasahara et al., 1983). It is, therefore, important to determine, more closely, the subsets within the LGL population and to delineate functions of each cell type.

9) It is interesting to speculate on the observation that 100% lysis of susceptible target cells, such as K562 cells, is never achieved. One explanation could be that only target cells in certain stages of the cell cycle are susceptible to lysis by LGLs. Investigation into this phenomenon would involve inhibition of K562 cell growth at a certain point with agents such as colchicine. Lysis of such K562 cells by LGLs would then be studied. Another approach is to mix LGLs and K562 cells and to allow binding to occur. Unbound cells would then be removed. The ability of these K562 cells to stimulate IL-1 production from LGLs and to bind to LGLs would be examined.

10) K562 cells are known to differentiate under the stimulus of both sodium butyrate and hemin (Werkmeister et al.,

1982). K562 cells induced to differentiate under such conditions become relatively resistant to binding and lysis by NK cells. A study of differences in the cell-surface antigens of undifferentiated and differentiated cells could lead to greater understanding in the field of effector-target cell interactions.

APPENDIX

1. Sources of materials

Acetic acid	Hopkins and Williams Pty. Ltd. Johannesburg, South Africa
Actinomycin D	Sigma Chemical Company Missouri, USA.
Ammonium chloride	Merck, Daramstadt, Germany.
Anti-human Leu-7 monoclonal antibody	Becton Dickinson, Mountain View, California,USA.
Anti-Ia antibody	Dr. J. Kauffman, USA.
Anti-IL-1 antibody	Dr. C. Dinerallo, Boston, USA.
Chem-Fluor	Chemlab, Pretoria, South Africa.
Concanavilin A	Wellcome Reagents Ltd., Bechenham, England.
Endotoxin, bacterial lipopolysaccharide (*E.coli* 0127:B8)	Difco, Detroit, Michigan, USA.
Ethanol	Merck,Daramstadt, Germany.
Ficoll	Pharmacia, Uppsala, Sweden.
Fucose	Sigma Chemical Company, Missouri, USA.
Galactose	Sigma Chemical Company, Missouri, USA.
Gentamycin	Shering Corporation, Kenilworth, New Jersey, USA.

Giemsa stain and buffer	Coulter Diagnostics, Hialeah, Florida, USA.
Glutamine (L)	Mereck, Daramstadt, Germany.
Gluteraldehyde	BDH Chemicals, Poole, England.
Hanks balanced salt solution	Wellcome Reagents Ltd., Beckenham, England.
Heparin, preservative free	Evans Medical, Liverpool, England.
Hydrochloric acid	Mallinckrod Chemicel Works, St. Loius, Missouri, USA.
Hydrocortisone	Sigma Chemical Company, Missouri, USA.
Hypaque	Winthrop Laboratories, Durban, South Africa.
Indomethacin	Sigma Chemical Company, Missouri, USA.
Interferon - recombinant A	Roche, Johannesburg, South Africa.
Interferon	Prof. B. Schoub, NIV., Johannesburg, South Africa.
Maltose	Sigma Chemical Company, Missouri, USA.
Mannose	Sigma Chemical Company, Missouri, USA.
Medium, minimal essential	Polio Research Institute, Johannesburg, South Africa.
Medium,RPMI 1640	Polio Research Institute, Johannesburg, South Africa.

Methanol	Merck, Daramstadt, Germany.
Methyl-mannoside	Sigma Chemical Company, Missouri, USA.
N-acetyl-glucosamine	Sigma Chemical Company, Missouri, USA.
Orthoclone OKT3 pan, OKM1 monoclonal antibodies	Ortho Pharmaceutical Corporation, Ravitan, New Jersey, USA.
Percoll	Pharmacia, Uppsala, Sweden.
Phosphate buffered saline	Behring Institute, Behringwerker, Marburg.
Prostin E_2	Upjohn Pty, Ltd., Johannesburg, South Africa.
Rhamnose	Sigma Chemical Company, Missouri, USA.
Serum, foetal calf	Wellcome Reagents Ltd., Bechenham, England.
Sodium chloride	Hopkin and Williams Pty Ltd., Johannesburg, South Africa.
Sodium hydroxide	Merck, Daramstadt, Germany.
Sucrose	Sigma Chemical Company, Missouri, USA.
Trypsin-EDTA	Polio Research Institute, Johannesburg, South Africa.

Radiochemicals

Sodium chromate ($Na_2{}^{51}CrO_4$) 250-500 mCi/mg	Radiochemical centre, Amersham, England.

Tritiated thymidine (methyl-^{3}H) 17Ci/m.mol	Radiochemical centre, Amersham, England.

2. Sources of equipment

Centrifuge tubes (15 and 20 mℓ) Nos. 2095 and 2070.	Falcon Plastics, Division of Becton Dickinson, Mountain View, California, USA.
Gilson Pipettemans P1000, p200, p20.	Laboratory and Scientific, Johannesburg, South Africa.
LKB Rack-Beta liquid scintillation counter model 1217	LKB, Johannesburg, South Africa.
Microtitre plates round and flat bottomed	Greiner, Nurtingen, Germany.
Millipore filters (o.22µM 0.45µM)	Millipore Corporation, Bedford, Massachusettes, USA.
Multiple automatic harvester (MASH 11)	Microbiological Association, Betheseda, Maryland, USA.
Nylon fibre LP-1 Leoco-Pak leukocyte filter	Fenwal Laboratories, Morton Grove, Illinois, USA.
Packard Auto-gamma scintillation spectrometer	Packard Instrument Company, Downer Grove, Illinois, USA.
Plastic pipettes	Falcon Plastics Division, Oxnard, California, USA.
Scintillation vials	Packard Instrument Company, Downer Grove, Illinois, USA.
Syringes, plastic	Monoplast, Johannesburg, South Africa.

Tissue culture dishes 35x10mm, 60x10mm	Lux Scientific Corporation, Newburg Park, California, USA.
Tissue culture flasks ($44cm^2$, $75cm^2$)	Lux Scientific Corporation, Newburg Park, California, USA.
Tissue culture tubes (16x125mm, 17x100mm)	Falcon Plastics Division, Oxnard, California, USA.

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