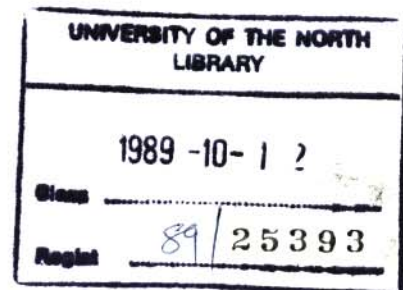


STUDIES ON MONOCYTES IN PATIENTS
WITH CANCER OF THE CERVIX

MOSEDI KEANETSE NAMANE

Submitted in part fulfilment of the requirements for the degree
of

MASTER OF SCIENCE



IN THE DEPARTMENT OF MEDICAL LABORATORY SCIENCES,
FACULTY OF MATHEMATICS AND NATURAL SCIENCES

UNIVERSITY OF THE NORTH

Supervisor : M Alberts



I declare that this thesis hereby submitted to the University of the North by me for the degree of Master of Science has not been submitted by me for a degree at another university and that it is my own original work.

Signed 
M.K.NAMANE

Dated 86-12-10

ACKNOWLEDGEMENTS

This work was supported in part by the CSIR

Thanks to:

Prof M Alberts

for promoting this thesis
and her advice

Staff members of the Department
of Medical Laboratory Science
of the University of the North

for their technical assistance

D Moeketsi

for his support

My parents Matshediso and
Serongoane Namane

for their encouragement

R Namane and M Hagen

for typing of this thesis

SUMMARY :

The purpose of this thesis was to investigate some of the changes which occur in the physiology of peripheral blood monocytes of patients with cancer of the cervix.

The immune status was assessed by:

- (1) Determining the percentages of monocytes, B-cells, total, helper and suppressor T-cells in the peripheral blood.
- (2) Performing the transformation assay - tritiated thymidine (TdR) uptake of peripheral blood mononuclear cells (PBMNCs) stimulated with phytohaemagglutinin (PHA).

The following functions of monocytes were determined:

- (1) secretion of interleukin 1 (IL 1);
- (2) lysing of K562 cells (cytotoxicity);
- (3) inhibition of proliferation of K562 cells (cytostasis).

The monocytes were stimulated with E. coli lipopolysaccharide (LPS).

The IL 1 produced was characterized by isoelectrofocusing and molecular weight determination. Commercial IL 1 was used as a control. The biological function of IL 1 was assessed by its ability to stimulate murine thymocytes.

Although the percentages of different subpopulations of peripheral blood immunocytes were similar in all individuals, the patients' cells appeared to be functionally impaired:

- (1) The monocytes produced less IL 1.
- (2) Cytotoxicity and cytostasis was decreased.
- (3) Tritiated thymidine (TdR) incorporation by PBMNCs was lower.

CONTENTS:

	<u>Page</u>
INTRODUCTION	1
MATERIALS	31
METHODS	33
Monocyte preparation	34
Thymocyte preparation	35
Identification of monocytes, B and T lymphocytes	36
Human IL 1 production	38
Determination of percentage B cells in PBMNCs	42
Determination of T helper and T suppressor cells	43
T cell transformation assay	44
Cytopathic assay	44
Cytostasis	44
Cytotoxicity	45
RESULTS	47
DISCUSSION	70
CONCLUSION	87
REFERENCES	89

INTRODUCTION

The immune response to tumours, like that to protein antigens and micro-organisms, is highly complex and several effector cells are known to participate in the anti-tumour response: natural killer cells (Roder et al., 1982 and Chang et al., 1985), cytotoxic T-cells (Larson et al., 1980) and activated macrophages (Taffet and Russel 1981, Kicza et al., 1985 and Lagadec et al., 1985).

Activated macrophages have been found during the development of an antitumour response in vivo. Such macrophage activation resembles that found during antibacterial immunity and is caused by lymphokines released by immune T-cells (Hammerström, 1979 and Ido et al., 1984).

The activated macrophage is cytotoxic or cytostatic to a variety of neoplastic cell types (Keller, 1973; Hammerström, 1979; Männel, 1980). Macrophages previously injected with Toxoplasma gondii or those given complete Freund's adjuvant have the capacity for non-specific destruction of a variety of tumour targets (Hibbs, 1971).

It has been observed that agents affecting macrophage activation also induce tumour regression in animals (Milas and Scott, 1978). Corynebacterium parvum (CP), was used to stimulate murine monocytes. Among the multiple effects produced were anti-tumour activity (Milas and Scott, 1978), increased bone-marrow monocyte production (Wolmaric and Fisher, 1974), reticuloendothelial stimulation and macrophage activation (Bomford and

Christie, 1975), enhanced antibody response (Slijic and Watson 1977), depression of T-cell immunological reactions and increased resistance to bacterial (Adam et al., 1972) and viral infections (Kirchner, 1977).

Macrophages may participate in the anti-tumour response by:

- (a) Presenting tumour antigens, thereby inducing cellular immunity:

Macrophages play a critical role in processing and effective presentation of immunologically active moieties to lymphocytes (Sullivan et al., 1984). There is evidence that macrophage-bound antigen is far more immunogenic than soluble antigen for both primary and secondary antibody responses (Sullivan et al., 1984). Recognition and subsequent response of T-lymphocytes to antigen occurs on the macrophage cell membrane. The specific T-cell is activated when antigen is presented to it on the surface of a macrophage bearing compatible Ia-molecules and cell interaction factors are postulated to undergo a transition to an "activated configuration" (Sullivan et al., 1984).

Macrophages affect the immunogenicity of antigen, not only by presentation, but also by processing (chemically altering antigen) (Fudenberg et al., 1984). For most interactions, however, there do appear to be genetic restriction i.e. both cell types must be identical in at least two regions of the major histocom-

patibility complex. In the case of T-cell dependent antigens, a specific subclass of macrophages that express Ia-antigens on their surface appears to be necessary for lymphocyte responses.

(b) Surveillance:

- (i) The response to an established progressive tumour includes an activation of the host's macrophage system as evidenced by a significant increase in its capacity for phagocytosing intravenously injected colloids (Walters, volume 2, 1978).
- (ii) Animals whose macrophage system has been activated by infections or other agents display an enhanced capacity for resisting the growth of tumour cells (Baraschi et al., 1984 and Neale and Matthews, 1980).
- (iii) Macrophages harvested from mice with an activated macrophage system display a striking capacity for destroying or inhibiting the growth of tumour cells in vitro (Walters, volume 2, 1978).

Macrophages may accumulate within tumours (Di Paulo et al., 1974) and in several tumour types the degree of infiltration has been shown to have a prognostic value (Gauci and Alexandra 1975, Lauder et al., 1977), while antimacrophage agents (silica, carageenan and cortisone) increase the incidence of metastases. (Chang et al., 1983).

An additional finding about macrophage activation in response to the growth of certain murine tumours was that macrophages so activated gave the host a strikingly enhanced capacity for resisting infections with microbial parasites (Walters, volume 2, 1978). This represents, therefore, the reciprocal of the general finding that a macrophage system activated by microbial infection can non-specifically express anti-tumour resistance.

Anti-tumour activity of macrophages is non-specifically mediated (Vose, 1978 and Keller, 1978). It is apparent, therefore, that activated macrophages, and, to a lesser extent, normal macrophages, can discriminate between neoplastic and normal cells in the absence of specific anti-tumour immunity (Walters, volume 2, 1978). This discriminative ability may give these cells the capacity for functioning as key agents in active anti-tumour surveillance. The relatively greater susceptibility of malignant cells to macrophages could be due to differences in normal and malignant cell membranes which would facilitate preferential binding of activated macrophages to malignant targets (Cameron, 1985). Membranes of malignant cells show many alterations in comparison to their normal counterparts. These include change in charge, loss of surface fibronectin and appearance of fucose-labelled glycopeptides with increased density of sialylation in these peptides (Cameron, 1985).

Indeed, a hypothesis of anti-tumour surveillance based on the discriminative and tumoricidal powers of macrophages would help to explain, for instance, why there is a lower than normal inci-

dence of spontaneous as well as chemically induced tumours in T-cell deficient mice, and why these mice also show increased resistance to the growth of tumour implants. These findings can be explained more easily on the basis of the revelation (Walters, volume 2, 1978) that T-cell deficient mice possess a highly activated macrophage system that compensates them for their immunodeficiency.

There is an increase in tumour incidence in animals and humans treated with immunosuppressive drugs. These drugs are capable of suppressing macrophage surveillance because of their cytotoxicity for replicating macrophage precursors in the bone-marrow (Fudenberg et al., 1980).

(c) There are several mechanisms whereby macrophages can damage tumour cells.

(i) Phagocytosis: Macrophages have been visualized, by means of electron microscopy, ingesting apparently intact cells of a chemically induced tumour and adenovirus induced tumour in syngeneic rates (Walters, volume 2, 1978). However, the contribution phagocytosis makes to restriction of tumour growth in these systems is not known. Macrophages could be induced to ingest allogenic lymphoma cells in vitro in the presence of alloantibody, but rejection of lymphoma in vivo seemed to involve contact cytotoxicity rather than phagocytosis as a primary event.

(ii) Antibody-dependent cell-mediated cytotoxicity (ADCC):

Macrophages can act as killer cells in collaboration with "lymphocyte-dependant antibody". Killing of this sort tends to be rapid and readily revealed by ^{51}Cr -release or similar assays (Uchida and Yanagawa, 1984), although macrophages are poor killers compared with K-cells. The absolute requirement for antibody should be reflected in obvious specificity (Uchida and Yanagawa, 1984).

(iii) Cytostasis and cytotoxicity: Contact between activated macrophages and target tumour cells in culture often appears necessary for both cytostasis and destruction (Hammerström, 1979 and Kiczka et al., 1985). The damage may be induced by a soluble mediator or as a consequence of some cellular transduction involving contact.

1.A. The development of macrophages.

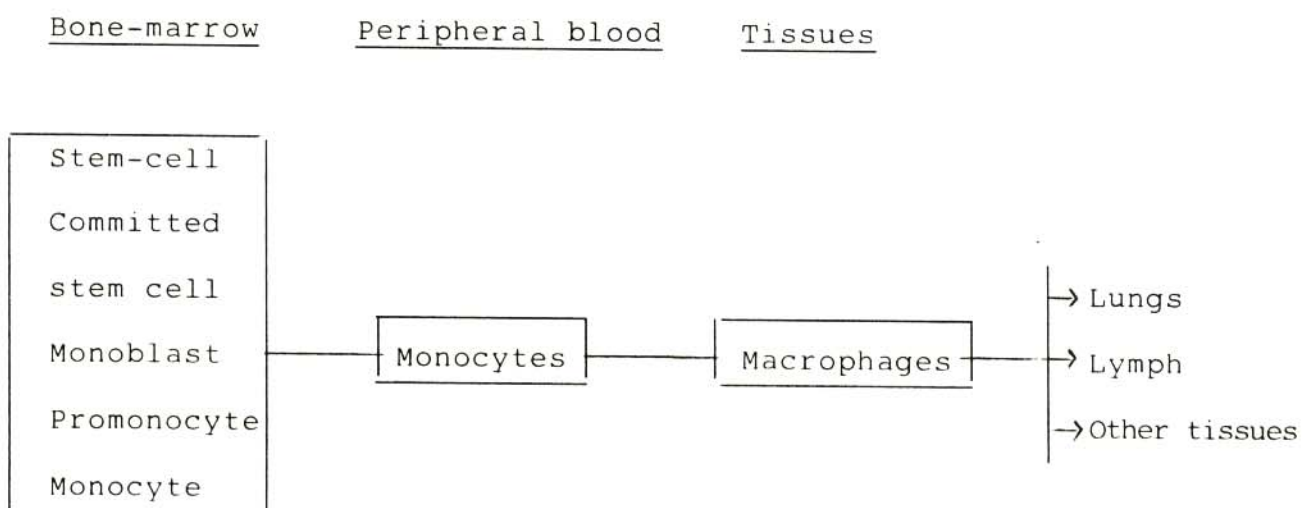
During ontogeny the haematopoietic stem cell, a cell of mesenchymal origin arises in the yolk sac (Van Furth, 1980). In the mouse, during the second week of gestation, stem cells migrate to the foetal liver, where immature mononuclear phagocytes develop; haematopoiesis in the liver does not cease until the second week of neo-

natal life (Van Furth, 1980). During the third week of gestation, haemotopoiesis commences in the bone-marrow, where pluripotent stem cells continue to give rise to monocyte precursors.

Monocytes originate in the bone-marrow from dividing precursor cells and enter the peripheral blood where they circulate until they leave to become macrophages in the tissues (Van Furth, 1980). The fate of macrophages in the tissues is uncertain: They may die in the tissues, migrate to local lymph nodes and die there, and/or migrate to other sites, e.g. the airspace in the lungs (Van Furth, 1980).

Table 1:

The origin, kinetics and fate of mononuclear phagocytes.



In culture human monocytes become macrophages after 6-10 days of in vitro culture as judged by their

morphology and by their biochemistry (Mori et al., 1984 and Cameron, 1985).

During the development of monocytes to macrophages the cells increase in size, in number of lysosomes and lipid vacuoles, and show an increase in the activity of 5' nucleotidase, acid phosphatase and phagocytosis, but decrease their production of hydrogen peroxide and oxygen (Mori et al., 1984).

In the second week of the in vitro culture of monocytes, adherent cells begin to appear attached to the surface of the culture flask and these cells persist throughout the life of the culture at approximately 20 percent of the total cell population. These adhered, non-dividing cells appear to be macrophages. The more immature replicating suspension cells, recognizable as monocytoïd cells, form clusters of 10-12 cells and sometimes loose clumps of 50-100 cells (Van Furth, 1980).

1.B. Morphological characterization of monocytes

Wright-Giemsa-stained cytopsin smears of the suspension cells allow recognition of four stages (Van Furth, 1980):

Stage 1: Immature monoblastic cells approximately 10-15 um in diameter, a high nuclear/cytoplasmic ratio (1:1), a large immature nucleus often containing distinct nucleoli, and a smooth basophilic cytoplasm

containing a few or no granules or vacuoles.

Stage 2: Slightly large monocytic cells having a reduction in nuclear/cytoplasmic ratio (1:2), the beginning of nuclear pyknosis, disappearance of nucleoli, reduced basophilic cytoplasm and emergency vacuolization.

Stage 3: Monocyte - macrophage cells of about 20-25 um in diameter, a low nuclear/cytoplasmic ratio (1:3), progressively higher nuclear pyknosis with a clear and often bi- or multi-nucleate eccentric nucleus, cytoplasm containing a number of irregular granules and vacuoles.

Stage 4: Mature macrophages with a stronger tendency to adhere to culture flask surfaces, low nucleus/cytoplasmic ratio with characteristics similar to stage 3 cells, a clear, highly vacuolized cytoplasm containing phagocytic vesicles, and an irregular cellular margin with numerous filaments.

All four monocyte stages can usually be found in any given culture, however, mitotic figures are primarily seen in stages 1 and 2.

Electron microscope studies

Less mature monocyte-macrophages: Rounded, relatively immature nucleus containing a distinct nucleolus and a normal chromatin distribution. The cytoplasm con-

tains a normal rough endoplasmic reticulum, mitochondria, numerous distinct lysosomal bodies, vacuoles and phagosomes (Van Furth, 1980).

More mature monocyte-macrophage: A mature nucleus without a detectable nucleolus, the cytoplasm contains fewer lysosomal bodies, numerous vacuoles and unfolding cytoplasmic membranes.

1.C Markers on the monocyte-macrophage serie

The monocyte-macrophages have no receptors for sheep red blood cells thereby differentiating them from T-lineage cells. The cells possess receptors for complement, Fc part of immunoglobulins (Stevenson et al., 1979) and fibronectin (Wirth and Kierszembraum, 1984). Receptors are present in all stages of macrophage development; but in immature cells (monoblasts and promonocytes) the percentage of cells with receptors may be lower than in mature monocytes and macrophages.

The macrophages do not possess surface-immunoglobulin or intracytoplasmic-immunoglobulin (Stevenson et al., 1979). The monocytes are positive for MO 2, UCHM 1 (Colotta et al., 1984) and OKM 1 (Rönblom et al., 1983) markers. All the developmental stages of the mononuclear phagocytes are positive for OKM1 whereas only peripheral blood monocytes are MO2 and MAC 120 positive (Stites et al., 1982).

1.D. Ia Antigens

The expression of Ia antigens is determined by genes located in the I-region of the H-3 complex which is also the location of Ir genes. (Thorsby et al., 1982 and Gonwa et al., 1982). Ia antigens may in fact be the product of Ir genes, although this has not been established.

In the human system, early members of the mononuclear phagocytes express Ia-like antigens (Beller and Ho, 1982).

However, only a small percentage of mature macrophages possess Ia-surface antigens. It may be that tissue macrophages are just like other tissue cells, i.e. they are able to express the classis, MCH-linked, K and D region specificities, but not Ia (Van Furth, 1980). Also, estimates are that 5-15 percent of mouse peritoneal macrophages stain or are killed by anti-Ia antibodies (Beller and Unanue, 1981). The Ia-antigen expression of macrophages in various tissues differs: for example, splenic (Koren et al., 1975), thymic (Beller and Unanue 1980) and liver (Richman et al., 1979), macrophages are mainly Ia⁺, whereas exudate macrophages are predominantly Ia⁻ (Koren et al., 1975 and Dort and Unanue 1978). (It can be hypothesized that in those tissues in which a high proportion of Ia⁺ macrophages resides, continued low-level production of an IFN- like mediator takes place which induces and maintains macrophages Ia antigen expression). The observation that mice reared in a specific patho-

gen-free environment have reduced macrophage Ia-antigen expression (Nussenzweig et al., 1981) suggests that background barrage of antigenic stimuli normally encountered may stimulate the continuous low-level of lymphokine production.

Recent evidence indicates that the Ia-Ag expression of macrophages, a principal class of accessory cells, is regulated by lymphokines. Steinman et al., (1980) have reported that culture-supernatants of Trypanosoma cruzi-activated spleen cells enhanced the synthesis and expression of Ia-antigens by murine macrophages in vitro. Steeg et al., (1980) observed that the culture supernatants of concanavalin-A stimulated spleen cells (Con A-SN) induced Ia-murine thioglycollate-elicited peritoneal macrophages to express Ia-antigens in vitro. Macrophages incubated with lymphokine-containing culture supernatants developed the capacity to initiate the mixed leukocyte reaction (MLR) (Steeg et al., 1980), antigen-specific activation of helper T-lymphocytes (Birmingham et al., 1982 and Walker et al., 1982) and intraperitoneal injections of supernatants of Listeria monocytogens - stimulated lymphocytes induced peritoneal exudates that are enriched in Ia macrophages.

The mediators responsible for the development of Ia-antigen share antigenic and biochemical characteris-

Monocytes and macrophages from both human and murine origin produce plasminogen activators (PA), and the secretion of these enzymes has been linked with the participation of mononuclear phagocytes in inflammatory reactions since peritoneal macrophages collected from inflammatory exudates synthesize and secrete such enzymes. (Van Furth, 1980).

Lysozyme, the major secretion of macrophages (approximately 25 percent of all extra-cellular protein) is produced continuously, and at a remarkably constant rate by all types of macrophages in culture and since its release is not disturbed by various stimuli known to activate macrophages, it may be considered a 'constitutive' secretion product of the macrophage (Van Furth, 1980). In contrast, high levels of plasminogen activator and other proteinase activities are only produced by macrophages after induction by various inflammatory, immunologic and endocytic stimuli and serve as useful markers of macrophage activation (Van Furth, 1980).

The levels of PA in macrophages may reflect their degree of differentiation. For instance, Weinberg et al., (1984) found that blood monocytes from normal humans had no activity immediately after isolation. However, after 6 days of incubation in 10 percent autologous, unheated serum (a procedure noted by some to cause maturation of monocytes to 'macrophages' (Weinberg et al., 1984), there was an increase in this activity.

1.F. Monokines and factors released by activated mononuclear phagocytes.

Macrophages can function not only as antigen-presenting cells in inducing immunity, but also as producers of hormone-like nonspecific factors. These soluble factors have a wide range of biological activities.

Natural killer cells which may be involved in resistance to cancer are susceptible to regulation by macrophages. As sources of, and responders to interferon (IFN), macrophages may augment NK cell activity, but as source of PG E₂ they can depress the activity (Djeu et al., 1979,; Tracey, 1979: Tracey and Adkinson, 1980 and Eriksen et al., 1985).

Human monocytes activated, in vitro with lymphokines and E. coli lipopolysaccharide release cytostatic protein factors that may be separated by ion-exchange chromatography in two fractions, termed cytostatic factors I and II (CF-II) (Nissen-Meyer and Seim, 1983). Both factors inhibited target cell (human non-adherent leukaemia K-562 cells) DNA synthesis as determined by (³H) thymidine uptake (Nissen-Meyer and Seim, 1983).

During inflammation tissue macrophages synthesize and secrete "factor increasing monocytopoiesis" (FIM) (Van Furth, 1980). FIM is then transported via the circulation to the bone-marrow where it stimulates monocyte production at the level of the monoblasts and promono-

cytes (Van Furth, 1980). The monocyte production inhibitor (MPI) is responsible for subsequent termination of the increased monocytopoiesis (Van Furth, 1980). MPI has an approximately 50K Dalton molecular weight and the site of production has not yet been established. FIM has a molecular weight of approximately 20K Dalton, is sensitive to inactivation by proteases but resistant to glycosidases, is cell-line specific (that is, it has no effect on the formation of granulocytes and lymphocytes), is not species-specific (that is, rabbit FIM can be active in mice and vice versa), is not related to complement factors or clotting factors, and has no chemotactic activity towards macrophages. FIM has no colony-stimulating activity, distinguishing it from the colony-stimulating factor (CSF) (Blyden and Handschumaker, 1977; Mizel et al., 1978; Gery et al., 1981; Mizel and Mizel, 1981).

One of the best characterized factors released by mononuclear phagocytes is IL 1, previously called lymphocyte-activating-factor (LAF) (Dinarello, 1984). Human IL 1 has molecular weight of 12 000 to 15 000 (Oppenheim and Gery, 1982; Toqaua et al., 1979; Wood, 1979). It augments thymocyte proliferation to sub-optimal levels of plant lectins (Unanue, 1980, Mizel, 1982; Maizel et al., 1981; Gery and Waksman, 1972). IL 1 concomittantly induces human B-lymphocyte immunoglobulin

production and the expression of surface immunoglobulin (Wood, 1976; Wood and Cameron, 1976; Wood, 1979). Originally called B-lymphocyte-activating-factor (BAF) it was later shown to co-purity with LAF. BAF is now considered to be identical to IL 1 (Wood, 1979). IL 1 can also partially restore pokeweed mitogen (PWM)- induced immunoglobulin production in cultures of macrophage-depleted human lymphocytes (Oppenheim and Gery, 1982). In some studies a 15 000 to 17 000 Dalton human monocyte product has been identified that caused a direct increase in the levels of antibody to sheep red blood cells released from splenocytes of nude mice (Wood et al., 1976). IL 1 is also an activator of T-cell function. It induces the production of a lymphocyte-derived mitogenic factor called T-cell growth factor (TCGF) or interleukin-2 (IL 2), Smith et al., 1980; Maizel et al., 1981; Rao et al., 1983). IL 2 is a true T-cell growth factor as it induced T-cell proliferation. On non-lymphocytic cells IL 1 has been found to have the following effects:

Table 2:

Effects of IL 1 on non-lymphocytic cells

Cell type	E f f e c t
Hypothalamic cell	Prostaglandin E ₂ production with resultant fever (Oppenheim and Gery, 1982). Also, IL 1 functions as a macrophage-derived leukocyte pyrogen (LP); Rossenwasser <u>et al.</u> , 1979; Murphy <u>et al.</u> , 1980; Saunder <u>et al.</u> , 1984).
Synovial cells, fibroblasts and chondrocytes	Proliferation and production of prostaglandin E ₂ and collagenase in response to a mononuclear derived factor (Oppenheim and Gery, 1982; Dinarello, 1984).
Hepatocytes	Production of acute phase proteins like serum amyloid A (Oppenheim and Gery, 1982; Turchik and Bornstein, 1980).

Experiments carried out by Rabson et al., (1983) suggests that IL 1 'primes tumour target cells for killing by NK cells.

Human epidermal cell-derived thymocyte activating factor (ETAf) is not only biochemically but also biologically similar to human IL 1; both factors activate thymocytes, induce fever, and are chemotactic for polymorphonuclear cells (PMNs), (Dinarello, 1984). Activated macrophages and monocytes also release colony stimulating factors (CSF) which promote the growth of polymorphonuclear

cells, macrophages, monocytes and NK cells (Fundenberg, 1982; Van Furth, 1980).

The supernatant from allogenic mixed leukocyte culture (MLC-SC) contains macrophage activating factor (MAF) together with lymphokines such as interleukin-2 (IL 2), interferon (IFN) and granulocyte-macrophages-colony stimulating factor (GM-CSF) (Kelso et al., 1982).

Supernatants of cultures of concanavalin A (Con A) stimulated spleen cells contain a lymphokine that maintains the cytolytic capabilities of activated macrophages (Taffet et al., 1981; Taffet and Russel, 1981). It is reported by Russel and Pace (1984) that one lymphokine that can mediate the effect is gamma interferon (IFN- γ). Not only is it involved in the induction of activation for tumour-cell killing, it also sustains killing in the fact of physiologic levels of prostaglandin E₂ (PGE) that would otherwise shut off the expression of cytolytic activity (Russel and Pace, 1984).

Activated macrophages themselves have been shown to be a rich source of PGE₂ (Taffet and Russel, 1981; Shaw et al., 1979), suggesting that activation may be autoregulated. Support for the hypothesis that endogenous PGE₂ is autoregulated in monocytes and macrophages is found since indomethacin, an inhibitor of prostaglandin synthetase, prevents both the secretion of PGE₂ by activated

macrophages and their loss of cytolytic activity (Taffet et al., 1984).

1.G. Tumour macrophage interaction.

It has been argued that macrophages could function in natural surveillance against neoplastic cells (Chang et al., 1983). If such a surveillance mechanism operates, it must include a mechanism for delivery of macrophages to the site of a newly arising tumour and, perhaps, for their appropriate activation or stimulation. The strongest evidence that macrophages may exert some natural resistance to neoplastic cells comes from experiments with rats whose apparently unstimulated macrophages are cytotoxic to tumour cells. Treatment of non-immune rats with anti-macrophage agents (carrageenan and silica) enhanced the growth of tumour isografts (Chang et al., 1983).

Macrophage activation apparently is a critical process preceding the development of cytostatic ability. Keller (1974) has suggested that since activated non-immune macrophages can inhibit proliferation of most replicating malignant cells, the macrophage can serve as a homeostatic regulatory cell. Evans and Alexander (1972), Lohmann-Matthen et al., (1972) and Fidler (1976) have shown that macrophages could be activated ("armed") by a factor released from immune lymphocytes. Evans et al., (1972) suggested that arming may be caused by a soluble T-cell factor, termed "specific macrophage arming factor" (SMAF).

The percentage of tumour-infiltrating cells identifiable as macrophages varied greatly from tumour to tumour. Some tumours with at least 50 percent macrophages still fail to regress spontaneously and it is possible that the presence of several additional types of mononuclear cells, lymphocytes, monocytes and histiocytes, as well as antibodies or other soluble factors may be obligatory for the induction of regression. The macrophages in human and animal tumours are quite heterogeneous in size (Evans and Alexander, 1970), representing both blood monocytes and tissue macrophages populations. For example Haskill and Koren, (1982), and Evans and Alexander (1970), separated Fc receptor (FcR)-positive macrophage populations in a number of human ovarian tissues. Haskill isolated, from the T1699 mammary adenocarcinoma, a population of effector cells with many properties of "armed monocytes" rather than "activated" macrophages. These effector cells have high levels of Fc receptors, are adherent, accumulate in the tumour of thymectomized irradiated marrow-reconstituted animals and are capable of demonstrating specific growth inhibition of the T1699 adenocarcinoma cell in vitro. An additional blood-borne marrow-derived monocyte was also present in the T1699 tumour and was activated either in an in vitro antibody-dependant cell-mediated cytotoxicity (ADCC) assay or against in vivo isolated tumour cells (Evans and Alexander, 1970).

Evans and Alexander, 1970, observed that 12 - 18 percent of the large macrophages in the T1699 tumour incorporate a one hour pulse of tritiated thymidine ($^3\text{H-TdR}$), suggesting that macrophage proliferation in situ may be an important source of inflammatory cells in the tumour.

Human monocytes activated in vitro by lymphokine-containing supernatants of autologous or allogeneic lymphocytes stimulated in vitro by Corynebacterium parvum expressed increased ability to suppress deoxyribonucleic acid (DNA) synthesis in a human tumour cell line (NH1K 3025). The cytostatic ability was described in a relatively long-term (24 hours) $^{51}\text{Chromium}$ -release assay (Hammerström et al., 1979). In animal system, Corynebacterium parvum has been reported to activate macrophages without lymphocyte co-operation.

Human monocytes activated with lymphokines and LPS release cytostatic proteins, namely cytostatic factors I and II and both factors inhibited target cell (human non-adherent leukaemia K562 cells) DNA synthesis (Nissen-Meyer and Seim, 1983).

The macrophage activating factor (MAF) contained in the supernatant from allogeneic mixed leukocyte culture (MLC-SN) activate peptone-induced peritoneal exudate cells or bone-marrow-driven macrophages (Kelso et al., 1982). The

activated macrophages can then lyse $^{51}\text{Chromium}$ -labelled tumour cells (Kelso et al., 1982).

Adherent cells isolated from human tumours had the characteristics of macrophages and showed cytotoxicity for tumour cells in an 18 hour $^{51}\text{Chromium}$ -release assay (Vose, 1978). The cytotoxicity was dose-dependant and nonspecific for tumour targets, but cells isolated from tumour-free lung areas appeared to be resistant to damage. Adherent cells from tumour-free lung areas also killed autologous tumour targets, but not normal lung cells.

Lymphocytes and monocyte-macrophages (MP) can destroy Herpes simplex virus (HSV)-infected Chang liver cells (CL) in the presence of specific antiviral antibody (antibody-dependant cell-mediated cytotoxicity _ ADCC) (Kohl et al., 1978). With each effector cell population, a high effector to target ratio (E:T) is necessary for optimal cytotoxicity.

The activation of mouse macrophages for tumour cell killing is negatively regulated by PGE_2 (Russel and Pace, 1984). However, a lymphokine IFN-r has been found not only to be involved in the induction of activation for tumour-cell killing (reviewed by Russel and Pace, 1984), but also to sustain killing in the face of physiologic levels of PGE_2 that would otherwise shut off the expression of cytolytic activity.

Mononuclear phagocytes can kill neoplastic cells in vitro, and this occurrence could represent an in vivo mechanism of antitumour resistance (reviewed by Colotta et al., 1984). The outcome of the interaction between mononuclear phagocytes and tumour cells is likely to depend, on the one hand, on the levels of cytotoxicity expressed by effector cells and, on the other hand, on the relative susceptibility of neoplastic cells.

Murine experimental tumours have been reported to vary in their relative susceptibility to macrophage cytotoxicity (Miner and Nicolson, 1983 and Gemsa et al., 1981), and tumour progression has been associated with the appearance of variants relatively resistant to macrophages (Urban and Schreider, 1983). Moreover, in human ovarian carcinoma, tumours from different patients have been shown to vary considerably in their susceptibility to killing by monocytes or macrophages (Peri et al., 1981 and Haskill et al., 1982). Hence the regulation of target cell resistance or susceptibility to mononuclear phagocytes may bear considerable importance to the role actually played by these cells in vivo.

Newborn mouse macrophage strongly suppress tumour cell growth and readily acquire cytolytic activity in comparison with adult macrophages. Embryonic macrophages were found to contribute greatly by eradicating denatured cells (Ido et al., 1984). Such embryonic macrophages remaining

in newborn mice have a high capacity to detect and clear away transformed cells as well as denatured cells.

1.H. Other cells which may be involved in surveillance.

(a) Natural killer cells (NK cells)

Natural killer cells are intermediate sized cells found in all fractions of peripheral blood mononuclear cells following elutriation (Stevenson et al., 1979). NK cells which may be involved in resistance to cancer are susceptible to regulation by macrophages. As sources of, and responders to, interferon, they may augment NK activity, but as sources of PGE₂ they can depress it (Chang et al., 1983).

The experimental data have indicated that in both peripheral blood mononuclear cells and macrophage-enriched populations, an appreciable proportion of the effector cells with cytolytic activity against adherant human or mouse tumour target cells (Natusch and TU-5 tumour cells) are NK cells as determined by monoclonal antibodies surface markers and morphology (Hoffman et al., 1983).

Rabson et al., (1983) observed that in normal individuals there are natural killer cells (NK cells) which can destroy tumour cells; some tumour-

cells stimulate NK cells to release IL 1, which enhances the cytotoxic activity of the NK cells; and finally, pre-treatment of tumour-cells with IL 1 before they were incubated with NK cells enhanced cytotoxicity, whereas pretreatment of NK cells IL 1 before adding tumour cells did not do so. From these observations Rabson et al., (1983) made the suggestion that IL 1 'primes' tumour target cells for killing by NK cells.

(b) Cytotoxic T-lymphocytes (CTL)

The initial evidence for the role of T-lymphocytes in controlling tumour growth came from findings that neonatally thymectomized rodents developed more primary neoplasms following infection with oncogenic viruses or treatment with chemical carcinogens (Ting and Law, 1976).

Lutz et al., (1981) reported that the generation of cell-mediated toxicity in mice against syngeneic tumour cells in vitro is suppressed by a subpopulation of nylonwool adherent T-cells, and that the suppression can be abrogated by treatment with anti-theta serum and complement (Cerottini and Brunner 1974). These cells are negative for surface membrane immunoglobulin (SmIg), unable to form rosettes with

sheep red blood cells, and specifically cytotoxic for autologous osteosarcoma cells (Lutz et al., 1981). The cytotoxic cells are not removed by procedures which eliminate highly adhesive cells or phagocytic cells, and CTLs have the electrophoretic mobility of T-cells (Anderson, 1973). This demonstrates that the central cytotoxic cells may belong to the T-cell lineage.

..

There is little direct evidence that CTLs as such are directly responsible for controlling existing tumours. There is, however, ample evidence of their capacity to function in transfer experiments.

Allison (1972) found that the polyoma virus-induced tumour growth could be prevented by the adoptive transfer of immune syngeneic lymphocytes and that pre-treatment of these cells with anti-theta serum and complement eliminated the protective effect.

Howell et al (1974), also using an adoptive transfer assay, found that T-cells were necessary to prevent the appearance of SV 40 tumours.

The CTLs can be generated in a mixed leukocyte-tumour cell cultures (MLTC) (Moretta et al., 1981). The cytolytic activity of these effector cells is drastically increased after culture of phytohaemagglutinin (PHA)-stimulated human spleen cells (Moretta, et al., 1981).

(c) Killer cells (K-cells)

It has been demonstrated that a small sub-population of lymphocytes which is non-'B', non-'T', with Fc receptors are effector cells in antibody-mediated cellular cytotoxicity (ADCC) (Fudenberg et al., 1982). These cells are called 'K' cells. The destruction of viral-infected target cells by K-cells and monocyte-macrophages (MP) in the presence of specific antiviral antibody has been described. With each effector cell population, a high effector to target ratio (E:T) is necessary for optimal cytotoxicity.

(d) Humoral immunity against tumour cells - B-cell participation.

Both humoral and cellular responses may have important co-operative as well as antagonistic interactions in host response to growing neoplasms. Tumour-specific humoral antibodies capable of binding to the tumour cell membrane have been demonstrated against some virus-induced murine neoplasms of the lymphoma-leukaemia group by cytotoxicity and immunofluorescent techniques (Slettenmark and Klein, 1962 and Klein and Klein, 1964). In contrast, humoral antibodies against MCA-induced neoplasms have not been demonstrated and immunity cannot be transferred by serum (Klein et al., 1960).

Complement and lymphocyte-dependant cytotoxic antibodies as well as precipitating antibody, reactive with a great variety of animal and human tumours, have been reported (Fudenberg et al., 1982). Such antibodies, may be directed against virally specified antigens, against phase-specific antigens, or against tumour-associated antigens of unknown etiology. The significance of such antibodies in the primary tumour host has not been established, although examples of enhanced tumour growth, regression of tumours, and clearance of serum-blocking factor (SBP) from the circulation have been reported following passive transfer to tumour-bearing hosts (Sjögren et al., 1972). The pattern of specificity and of cross-reactivity seen with anti-tumour antibodies may, as one would expect, be somewhat different from that of cellular immunity.

The serum of experimental animals or of patients bearing actively growing tumours frequently contain a component termed "serum blocking factor" (SBF) (Sjögren et al., 1972). SBF can also be eluted from the surface of these cells (Sjörgren et al., 1972). SBF is able, specifically, to subvert the antitumour cytotoxic potential of the hosts's own circulating lymphocytes. For example, Nelson et al., (1975) have detected an antibody in the supernatant of mouse spleen cells sensitized against methylcholantrene-

induced sarcoma which can block cell-mediated immunity against cultured cells of the same tumour. The specificity and cross-reactivity of SBF precisely mirrors that of anti-tumour cellular immunity.

SBF disappears rapidly from the circulation following surgical removal of the tumours (Nelson et al., 1975) and it is not found in the serum of experimental animals whose tumours are undergoing spontaneous regression (Nelson et al., 1975). Its reappearance in the circulation following removal of the primary tumour is a frequent signal of the imminent appearance of metastatic lesions (Nelson et al., 1975). Presently available evidence is compatible with the idea that the activity of SBF depends upon the presence of antigens released from the tumour cells and present either as free antigen or complexed with antibody (Nelson et al., 1975).

Many investigators have shown that mononuclear phagocytes may be important effector cells in host resistance against neoplasms. Since cancer of the cervix is very common in the Black populations in the Northern Transvaal, I have chosen the topic of my thesis to be **"STUDIES ON MONOCYTES IN PATIENTS WITH CANCER OF THE CERVIX"**.

MATERIALS

1. Antibiotics:
 - Crystapen 200 000 unit/l
Glaxo (Pty) Ltd.
Manchester
 - Streptomycin 200mg/l
Novo Industries
Johannesburg
2. Concanavalin A (Con A)
Wellcome Research
Labs., G.B.
3. Escherichia coli lipopoly
saccharide (E. coli LPS)
Sigma, St Louis
4. Ficoll-Paque
Pharmacia, Uppsala
5. Fluorescent isothiocyanate
(FITC) - conjugated anti-mouse
Wellcome Research
Labs., G.B.
6. Foetal calf serum (FCS)
Flow Laboratories
U.S.A.
7. HEPES
Sigma, St Louis
8. Human interleukin 1 (IL 1)
(Lot 0,72, specific
activity 8 units/pg protein)
Genzyme, Suffolk,
G.B.
9. Human Serum albumin (HSA)
National Blood
Fraction Centre,
South Africa
10. Monoclonal Antibodies (Abc)
OKM1, OKT3, OKT4, OKT8
Ortho diagnostics
11. Nylon wool, (Leucocyte filter)
Fenwald Laboratories,
12. Percoll (N17089101)
Pharmacia, Uppsala
13. Phytohaemagglutinin (PHA L9379)
Sigma, St Louis

- | | |
|---|--|
| 14. RPMI - 1640 | Gibco, Scotland |
| 15. Tritiated thymidine
(3H-TdR) | Amersham,
Amsterdam |
| 16. Microtiter plates | Sterilin, G.B. |
| 17. Sterile conical plastic
tubes and culture flasks | Falcon, Oxnard |
| 18. Vacutainers containing heparin | Radem Laboratory,
Wynberg, S Africa |

2. METHODS

2.1 Interleukin 1 (IL 1): Production and assay

2.1.1 Media: The medium used for culture was RPMI-1640 supplemented with 20mM HEPES, 10% foetal calf serum and antibiotics (Crystapen 200 000 units/l, and Streptomycin 200mg/l). The washing medium was phosphate buffered saline (PBS, pH 7,4). Isoosmotic percoll (285mosm) used for separating peripheral blood mononuclear cells was prepared by mixing 5,6 parts of RPMI-1640 and 4,4 parts of percoll solution (94 parts concentrated percoll and 6 parts (v/v)PBS 10X). Murine medium (RPMI supplemented with 5% heat-inactivated FCS, 200mM glutamine and 5×10^{-5} M mercapto-ethanol) was used for culturing mouse thymocytes in the IL 1-assay.

Cells: Monocytes were isolated from peripheral blood of 50 normal healthy subjects and 27 patients with cervical cancer. The normal subjects were students and workers from the University of the North, Pietersburg, while the patients were from Ga-Rankuwa hospital, Pretoria. Sixty

millilitres of blood was collected by venepuncture in a vacutainer containing heparin and was processed as soon as possible after collection. The source of thymocytes was 6 - 10 week old BALB/c mice.

2.1.2 Monocyte preparation: Fifteen millilitre of Ficoll-Paque was put into a 50ml sterile conical plastic tube and 20ml of blood was carefully layered over it using a 10ml sterile pipette. The resulting double layer was centrifuged in a Beckman model TJ- 6 at 2 000 rpm for 25 minutes at room temperature. This produced density gradient centrifugation and the peripheral blood mononuclear cells (PBMNCs) were found at the interface of the plasma and Ficoll-Paque layer. The plasma was removed carefully using a sterile Pasteur pipette and the mononuclear cell layer was transferred aseptically into another 50ml conical tube. The mononuclear cell were washed twice with PBS and once with RPMI-1640, using a 10 minute, 1 000 rpm centrifugation between each step. After the final wash the cells were resuspended in culture medium and a small aliquot removed

aseptically for counting using the Sysmcc microcell counter CC - 120.

Thirty to forty million cells were layered over 5ml isoosmotic percoll in 15ml conical tubes (Sterilin) and the tubes spun at 2300 rpm at 4°C for 30 minutes. Monocytes (floating) and lymphocytes (pellet) were suspended in PBS, washed twice and resuspended in RPMI-1640.

2.1.3 Thymocyte preparation: Two to three mice were sacrificed by cervical dislocation. The thymuses were removed aseptically and plated in a small sterile petri dish containing a few ml RPMI-1640. The organs were then teased apart with the tips of sterile Pasteur pipettes and the suspension aspirated into a tube. Large lumps were allowed to settle at unit gravity for 3-5 minutes after which the cells were placed in a new sterile tube and washed twice by gentle centrifugation (1 000 rpm) for 10 minutes. The cells were then resuspended in murine medium and transferred into 50ml Falcon plastic culture flasks. To remove macrophages

incubation for 1 hour in a humid chamber at 37°C with 5% CO₂ followed.

- 2.1.4 Viability test: The viability of the prepared cells was determined by mixing equal parts of the cells suspension and a 1% solution of trypan blue. The live cells excluded the dye as opposed to the staining of dead cells.
- 2.1.5 Characterization of peripheral blood immunocytes (monocytes, B and T lymphocytes): Cells were morphologically differentiated in May-Grünwald-Giemsa stained preparations. The mAb OKM1 was used to test for surface markers of monocytes by indirect immunofluorescence assay. OKT3 mAb was used in the complement dependent lysis of T-cells. The methods are described below.
- 2.1.5(a) Identification of monocytes: Cells were pelleted by centrifugation at 1 000 rpm for 10 minutes at 4°C. They were resuspended at a density of 5×10^6 cells/ml in culture medium supplemented with 5% (v/v) heat inactivated foetal calf serum. The cell suspension (200ul) was placed into

each of three 12 x 75 mm test tubes. Five microlitre of reconstituted monoclonal antibody solution was added to two of the test tubes. The third sample served as a reagent control for the antimouse immunoglobulin. After mixing the cell suspension was incubated in ice-water bath for 15-30 minutes. Agitation was performed every 10 minutes. The cells were washed twice with PBS at 1 000 rpm for 10 minutes at 4°C. One hundred microlitre of a 1:20 dilution of fluorescein-labelled antimouse immunoglobulin was added to the broken up cell pellet. After mixing the cell suspension was incubated again in ice-water bath for 15-30 minutes. Cells were agitated every 10 minutes and then washed twice. The pellets were resuspended in 1-2 drops of mounting medium (buffered glycerol pH 7,4) after which fluorescence microscopy was performed.

2.1.5(b) Identification of T-cells: Two hundred microlitre of 5×10^6 cells/ml were incubated with 5ul of reconstituted stock of OTK3 at room temperature for 1 hour (the control tubes contained cells only). The mixture was agitated every 5 minutes. After incubation, the cells were washed

by gentle centrifugation (1 000 rpm) for 10 minutes) twice with PBS and once with RPMI-1640. The supernatant was removed with a Pasteur pipette leaving approximately 100 ul of medium in the tube. The tubes were shaken to resuspend the cell pellets. Undiluted fresh or frozen rabbit serum (200 ul) was added as a source of complement and the tubes incubated at room temperature for an hour, with frequent gentle shaking at 5 minutes intervals. The cells were washed thrice with PBS then a drop of 10% trypan blue was added to the dry pellets.

After shaking to resuspend the cells a drop of the mixture was put on a microscope slide and the number of dead and living cells were counted (at least 200 cells) and the percentage of OKT3⁺ cells determined.

(For determination of % B cells see section 2.2.2(a)).

2.1.6(a) Human IL 1 production: The enriched monocyte fraction (section 2.1.2) was suspended in RPMI-1640 supplemented with 1mg/ml HSA

and adjusted to 1×10^6 cells/ml. E. coli LPS was added to a concentration of 10 ug/ml. Control cultures were not stimulated with LPS. After incubation at 37°C with 5% CO₂, the culture flasks were removed at 24 hour intervals for a period of 4 days. The cells were pelleted at 3 000 rpm for 5 minutes and the supernatant passed through 0,45 um millipore filter (Gelman) and stored at -20°C.

2.1.6(b)

Preparation of IL 1 supernatant (IL 1 - SN) fractions: Human IL 1 has been well defined and characterized and has been found to have a molecular weight of 12 000 - 15 000 (Dinarello, 1984). For isolation of IL 1 from culture supernatants, ultrafiltration employing a filter with a cut-off of 10 000 MW was used. The filtration system comprised an Amicon cell with a Diaflo ultrafiltration membrane PM10. Culture supernatants were concentrated 5 x and 50 x respectively. These fractions were referred to as IL 1-C. The filtrates (IL 1 - F) and the crude preparation (IL 1 - SN) were concentrated by freeze-drying overnight using the Freezemobile G.

2.1.7

IL 1 assay: The levels of IL 1 activity in

supernatants were determined according to their capacity to potentiate the response of murine thymocytes to different dilutions of PHA. Also a direct thymocyte proliferative assay was used. Thymus cells (10^7 /ml) from BALB/C mice were cultured for 72 hours in flat-bottom microtiter plates in triplicate in presence of 50 μ l of serial dilutions of monocyte supernatants (M ϕ -SN). In some experiments a submitogenic dose of PHA (1 μ g/ml) was added in order to determine IL 1 activity. One unit of IL 1 activity is defined as that amount required to double the proliferative response of mouse thymocytes stimulated with 1 μ g/ml PHA alone. Eighteen hours prior to harvesting 0,5 μ Ci 3 H-thymidine (3 H-TdR) was added per well. The radioactivity was determined in a scintillation counter. Purified T lymphocytes (section 2.2.2(b)) were suspended in medium and cultured at 2×10^5 cells/well using flat bottom microlitre plates in a volume of 200 μ l. PHA was added to a final concentration of 10 μ g/ml. Fifty microlitre of serial dilutions of M ϕ - SNs were added to some wells. The cells were cultured in an incubator (37°C, 5% CO $_2$) for 72 hours

and ^3H - TdR was added 18 hours before the end of the culture period. The radioactivity was expressed as counts per minute (cpm) of a triplicate culture.

2.1.8. Characterization of the IL 1 produced by monocytes: To identify the IL 1 produced by monocytes polyacrylamide gel electrophoresis was performed using commercial IL 1 of known molecular weight as standard. The material and apparatus used were:

Pharmacia Gradient Gels PAA 4/30

Pharmacia Gel Electrophoresis Apparatus
GE-2/4LS.

Pharmacia Gel Destainer GD 4.

The isoelectric point (pI) of the IL 1 was determined following the method in the LKB Instruction sheet 1804. The marker protein was commercial IL 1. Electrofocussing was performed on LKB Ampholine Polyacrylamide Gel plates (PAG plates 1804 - 101) using LKB Multiphor 2117 electrophoretic tank connected to Multitemp 2209 thermostatic circulator and LKB power supply 2103. After the run the pH gradient across the gel and the pI's the bands were measured and compared with

the band of commercial IL 1 by using
LKB 2117 - 222 surface electrode.

2.2 Determination of PBMNC fractions and
transformation assay:

2.2.1 Antibodies: OKT3, OKT4, OKT8 and FITC
conjugated sheep antihuman globulin.

Mitogens: PHA and Con.A.

2.2.2 Cell preparation: PBMNCs were prepared
as in section 2.1.2

2.2.2(a) Determination of percentage B cells
in PBMNCs: PBMNCs were suspended in
15 ml of culture medium with 10% FCS and
placed in a culture flask. Monocytes
were removed by incubating the cells at
37°C, 5% CO₂ for one hour. Nonadherent
cells were collected after careful shak-
ing and adjusted to 1×10^6 cells/ml.
One millilitre of the cell suspension
was placed in three 12 x 75mm test tube.
The cells were pelleted at 1 000 rpm and
4°C for 5 minutes. Hundred microlitre
of 1:20 dilution of FITC-conjugated sheep
antihuman globulin was added. This was
followed by incubation at 4°C for 20

minutes. The cells were washed 3 x with PBS. A drop of mounting medium was used for resuspension of cells. Fluorescent cells were counted using a Zeiss fluorescent microscope. The percentage B cells was determined by using the formula:

$$\frac{\text{Total number of fluorescent cells} \times 100}{\text{Total number of cells}}$$

- 2.2.2(b) Determination of T-helper (T_h) and T-suppressor (T_s) cells: The non-adherent cells prepared as in section 2.2.2(a) were purified into T- and B-enriched populations. The non-adherent cells were added to a nylon wool column at 10^8 cells/0,85g nylon wool and kept in 5% CO_2 at 37°C for one hour. The nonadherent cells were washed out with MEM 199. This population consisted mainly of T cells (section 2.1.5(b)). The ratio of $T_h:T_s$ cells was determined after employing OKT4 and OKT8 mAb using the method in section 2.1.5(b). T_h cells are $OKT4^+$ and T_s cells $OKT8^+$.
- 2.2.2(c) Estimation of the percentage of monocytes in PBMNCs: Five million cells of PBMNCs were processed as in section 2.1.5(a).

2.3 T-cell transformation assay:

PBMNCs and purified T-cells were stimulated with PHA (10 ug/ml) for 72 hours and kept in a humid chamber at 37°C with 5% Co₂. ³H-TdR was added 18 hours before the end of the culture period. DNA synthesis was determined by the amount of radioactive thymidine incorporated.

2.4 Cytotoxic assays:

The cytostatic and cytolytic effect of adherent cells on K562 cells was studied. The effector cells used were monocytes maintained in culture for 1 - 5 days. The K562 cells were kindly provided by Professor Dowdle (University of Cape Town) and Professor Rabson (University of the Witwatersrand).

2.4.1 Cytostatis:

2.4.1(a) Medium: RPMI - 1640 supplemented with 25 percent pooled human AB Rh-positive serum and 0,1 mM/L glutamine (HS-M).

2.4.1(b) Assay: Aliquots of 0,5 - 5 x 10⁶ mononuclear cells in HS-M were added to wells of flat-bottom microtiter plates. The non-adherent cells were aspirated after 90 minutes incubation, and the monolayers washed thrice with PBS with vigorous shak-

king of the plate. Unlabelled K562 ($10^4/0,5$ ml) were then added to plates containing adherent cells. DNA synthesis was assayed by adding 1 uCi 3 H-TdR per well for the last 5 hours of a 24 hour culture period of monocytes and target cells. Monocyte mediated cytostatis is expressed as percentage target cell inhibition

$$100 - \frac{\text{cpm (monocytes + K562)}}{\text{cpm (k562 plated alone)}} \times 100$$

Monocyte 5 H-TdR incorporated was ignored, as this did not exceed 5% of the cpm in target cell plates alone. Results are given as mean \pm SD.

2.4.2 Cytotoxicity:

2.4.2(a) Media:

(a) RPMI - 1640 supplemented with 10% horse serum

(RPMI - HS)

(b) RPMI - 1640 supplemented with 10% AB⁺ serum and 1% glutamine (RPMI-AB)

(c) RPMI - 1640 supplemented with 15% FCS and 1% L-glutamine (RPMI-FS).

(d) RPMI - 1640 with 0,25% trypsin (RPMI-T).

2.4.2(b) Assay: Monocytes were suspended in RPMI -HS at a concentration of 3, 2, 1 or 0,5 x 10⁶ cells/ml. Aliquots of 100 ul were added to Falcon microtitre plate wells and incubated for one hour at 37°C to allow adherence. The medium was removed using a sterile Pasteur pipette. Two hundred microlitre of RPMI-AB was added and incubation for 1 - 5 days at 37°C followed. Fifty microlitre of fresh RPMI-AB was added on the third day. The medium was removed and 150ul of RPMI-AB with 10ug/ml E. coli LPS was added. In control wells the medium added didnot have E. coli LPS.

Target cells were labelled with 20uCi ³H-TdR for 24 hours and then washed twice in RPMI and trypsinized with 4ml of RPMI-T for 5 minutes. After washing twice with 5ml of RPMI-FS, the cells were resuspended in the same medium at a concentration of 0,025 x 10⁶/ml.

The monolayers were overlaid with 200ul of a target cell suspension for 24 hours and incubated at 37°C 5% CO₂. Cells were harvested and the cytotoxic capacity of activated macrophages was determined using the following formula:

% Kill by activated macrophages:

1 - mean cpm (K562 + activated effector cells)

RESULTS:

The results of the determination of the percentages of T lymphocytes, B lymphocytes, monocytes and the ratio of helper to suppressor cells in the peripheral blood are presented in tables 3(a), 3(b) and 3(c).

Table 3(a):

Percentages of monocytes (OKM1 +ve), total (T3 +ve), helper (T4 +ve) and suppressor (T8 +ve) T lymphocyte and B lymphocyte (fluorescent +ve). Each reading is an average of three values.

Normal Subjects	OKM 1 (%) +ve	OKT3 (%) +ve	OKT4 (%) +ve	OKT8 (%) +ve	OKT4 +ve OKT8 +ve ratio	Fluorescent +ve %
1	8	62	50	33	1,52	18
2	10	68	53	25	2,12	21
3	8	71	54	36	1,50	20
4	7	72	48	24	2,00	18
5	9	73	52	33	1,58	16
6	8	72	52	21	2,48	16
7	6	76	54	21	2,57	17
8	9	73	58	33	1,76	18
9	9	68	54	26	2,08	18
10	8	69	51	22	2,32	20
11	8	72	48	32	1,50	22
12	6	73	57	30	1,90	20
13	11	66	54	21	2,57	19
14	7	71	49	20	2,45	19
15	8	73	50	33	1,52	19
16	8	76	53	21	2,53	18
17	8	71	58	21	2,76	16
18	10	69	48	26	1,85	17
19	9	72	51	21	2,43	18
20	6	73	52	30	1,73	20
21	7	71	54	36	1,50	21
22	12	64	53	22	2,41	23
23	8	73	54	20	2,70	18
24	6	74	50	32	1,56	20
25	9	72	48	31	1,55	20
26	9	68	46	28	1,64	20
27	9	62	49	29	1,69	24
28	8	72	51	32	1,59	20
29	8	72	56	33	1,70	18
30	7	71	51	31	1,65	17
31	6	73	54	26	2,08	17
32	8	76	52	27	1,93	16
33	8	79	48	20	2,40	16
34	10	68	47	21	2,24	18
35	11	62	49	21	2,33	20
36	12	61	53	33	1,61	22
37	9	67	56	30	1,87	21
38	9	73	49	29	1,67	19
39	9	66	58	36	1,61	19
40	6	69	53	31	1,71	19
41	8	71	50	29	1,72	19
42	8	66	48	26	1,85	19
43	10	68	48	27	1,78	20
44	6	71	50	33	1,52	20
45	8	64	51	30	1,70	22
46	9	70	53	29	1,83	22
47	9	67	49	22	2,23	23
48	8	71	60	33	1,82	16
49	8	70	48	21	2,29	17
50	8	69	49	24	2,04	14

RESULTS:Table 3(b)

Patients	OKM1 (%)	OKT3 (%)	OKT4 (%)	OKT8 (%)	OKT4 +ve OKT8 +ve ratio	Fluor- escent % +ve
1	6	72	46	30	1,50	11
2	9	69	49	29	1,69	21
3	8	73	52	32	1,63	18
4	8	62	40	27	1,78	19
5	7	72	50	26	1,92	16
6	5	73	49	29	1,69	20
7	10	68	57	34	1,38	24
8	11	69	46	29	1,59	20
9	9	71	50	33	1,52	18
10	8	68	57	36	1,58	18
11	10	73	55	29	1,90	17
12	11	66	57	30	1,90	19
13	6	64	50	31	1,67	23
14	6	71	50	22	2,27	21
15	12	66	48	28	1,21	20
16	10	65	47	21	2,24	18
17	9	66	49	22	2,23	18
18	9	67	56	21	2,67	19
19	9	68	56	32	1,75	18
20	6	69	55	31	1,78	19

Table 3(c) Statistical analysis of data in Tables 3(a) and 3(b).

Subjects	OKM1 +ve (%) OKM1 +ve (%)	OKT3 +ve (%) OKT3 +ve (%)	OKT4 +ve (%) OKT4 +ve (%)	OKT8 +ve (%) OKT8 +ve (%)	OKT4 +ve OKT8 +ve radio	Fluores- cent %
50 Healthy subjects	8,32±0,029	68,78±0,190	51,66±0,065	27,42±0,101	1,95±0,37	19,02±0,041
20 Patients	8,45±0,010 P>>0,5	69,10±0,141 P>>0,5	51,35±0,190 P>>0,5	28,32±0,217 P>0,317	1,82±0,31 P>>0,5	19,05±0,134 P>>0,5

1. The values represent MEAN ± SEM

2. P>0,05 in all cases. The difference is insignificant.

The percentages of different subpopulations of blood immunocytes were comparable between all subjects tested (patients and healthy subjects). In all cases P>0,05 implying the difference is insignificant (Table 3c).

The results of the transformation assay are presented in fig. 1 and table 4.

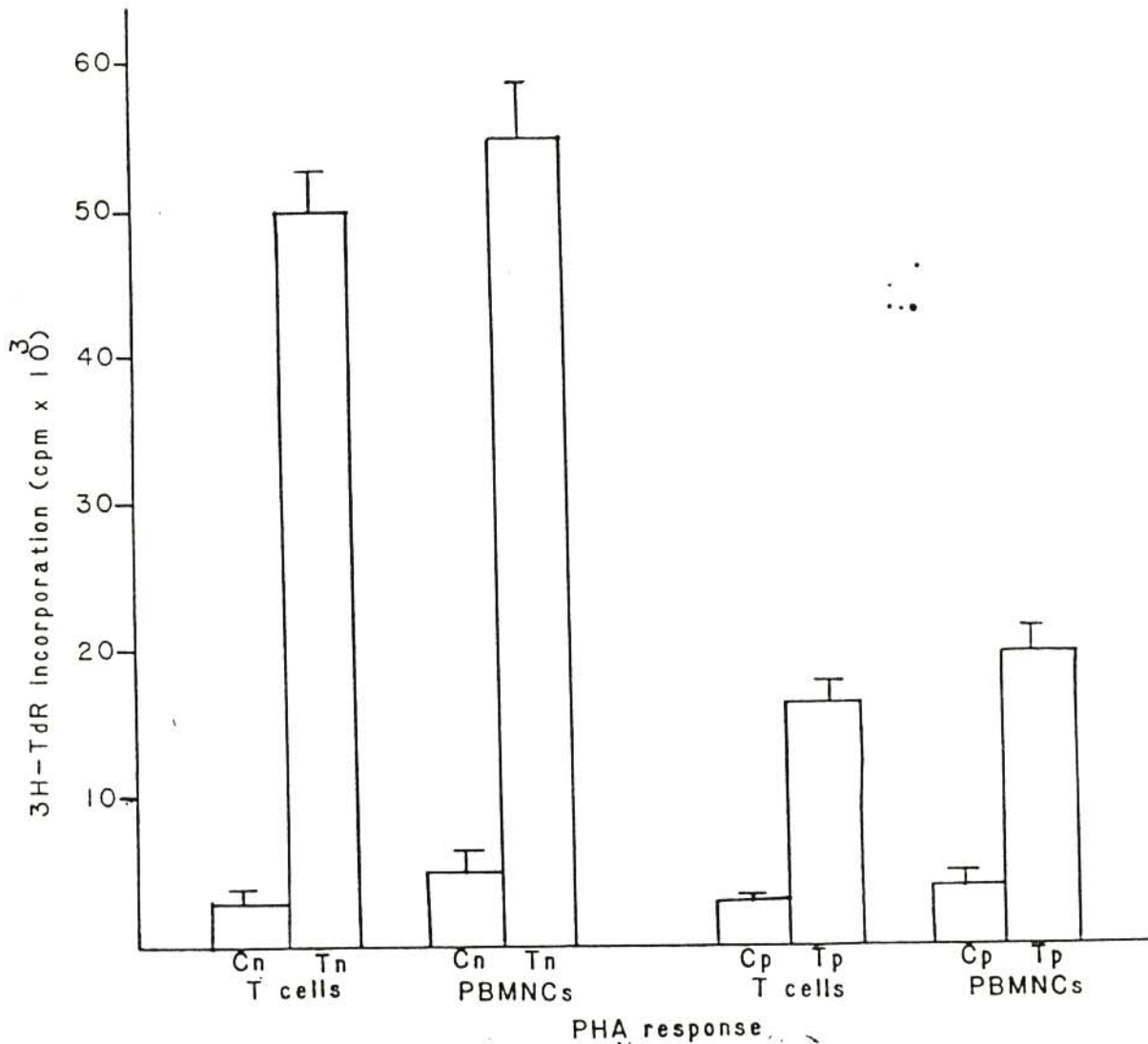


Fig. 1: Incorporation of $^3\text{H-TdR}$ at day 3 by normal (N) and patient (P) PBMNCs and T cells depleted by adherent cells, in the presence (T) or absence (C) of 10 ug/ml PHA. The cells were cultured at a density of 1×10^5 /well.

The $^3\text{H-TdR}$ uptake by cells from patients is less than that of cells from healthy subjects, particularly where cells are stimulated with PHA.

For T cells: T_p ($16\,500 \pm 1\,500$ cpm) T_N ($50\,000 \pm 3\,000$ cpm)

For PBMNCs: T_p ($20\,000 \pm 1\,000$ cpm) T_N ($55\,000 \pm 4\,000$ cpm)

TABLE 4: STATISTICAL ANALYSIS OF DATA ON FIG. 1

Subjects	T-cells (cpm x 10 ³) PHA 10ug/ml		P	PBMNBCs (cpr x 10 ³) PHA 10ug/ml		P
	Without PHA	PHA		Without PHA	PHA	
Controls	3,0 ⁺ ₋ 0,5	50,0 ⁺ ₋ 3,0	<< 0,01	5,0 ⁺ ₋ 1,5	55,0 ⁺ ₋ 4,0	<< 0,01
Patients	3,0 ⁺ ₋ 1,0	16,5 ⁺ ₋ 1,5	<< 0,01	4,0 ⁺ ₋ 1,0	20,0 ⁺ ₋ 1,7	<< 0,01
	P > 0,5	P < 0,01		0,02 > P > 0,01	P < 0,01	

1. The values are expressed as MEAN \pm SD

Statistical analysis of the results on transformation assay shows that the cells of normal subjects are stimulated more by PHA than cells of patients (Table 4). However in each case proliferation of test cultures (with PHA) is greater than proliferation of control cultures (without PHA) - $P < 0,01$.

∴

Production of IL 1. Freshly separated human mononuclear cells did not secrete IL 1 (Fig. 2). With time (24 to 96 hrs) the supernatants of monocytes stimulated with LPS showed a detectable IL 1 activity. The highest activity was detected in the 48 hour supernatant.

The optimum dilution of IL 1 is 1:4 (Fig. 3).

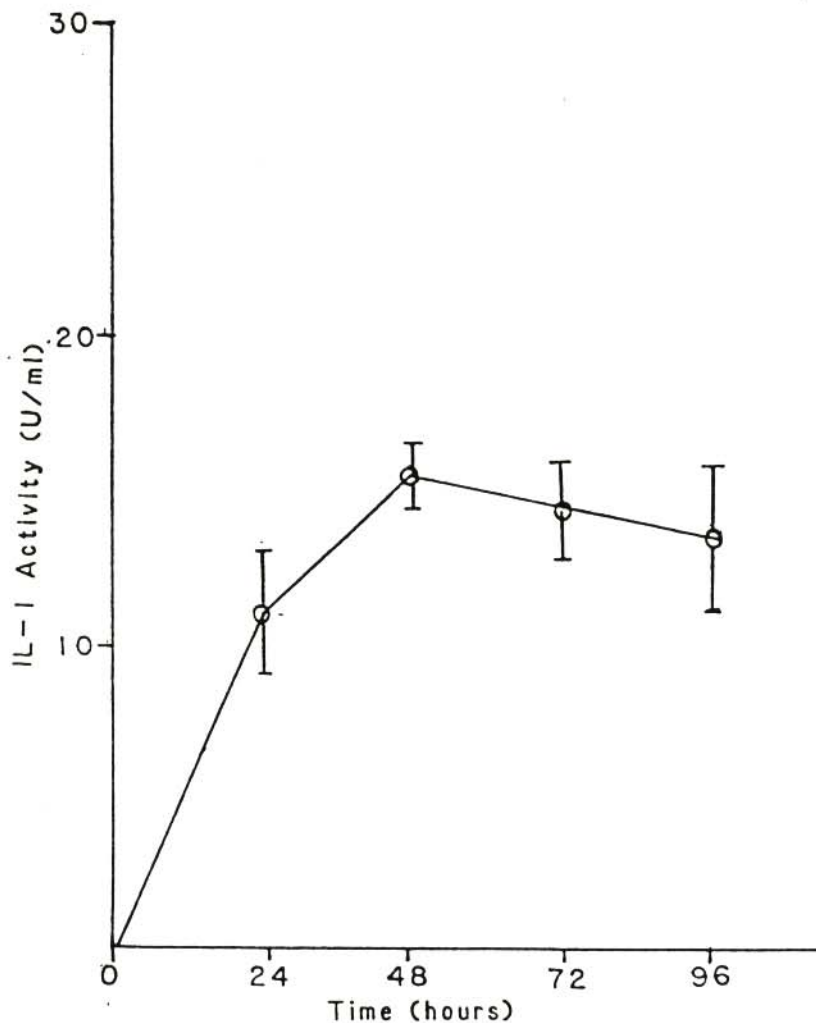


Fig. 2: One million human monocytes in 1mg/ml HSA cultured for 0, 24, 48, 72 and 96 hours with 20ug/ml LPS. The IL 1 activity was measured by a thymocyte-comitogenic assay as described under materials and methods. One unit of activity is defined as that amount of IL 1 required to double the proliferative response of mouse thymocytes stimulated with 1ug/ml of PHA alone. The results are a mean \pm SD of 20 normal subjects. Most of IL 1 activity is found in the 48 hour supernatant (15.5 ± 1 u/ml). The activity in the 0, 24, 72 and 96 hours

Determination of the optimal dilutions of monocyte
supernatant for proliferation of murine thymocytes

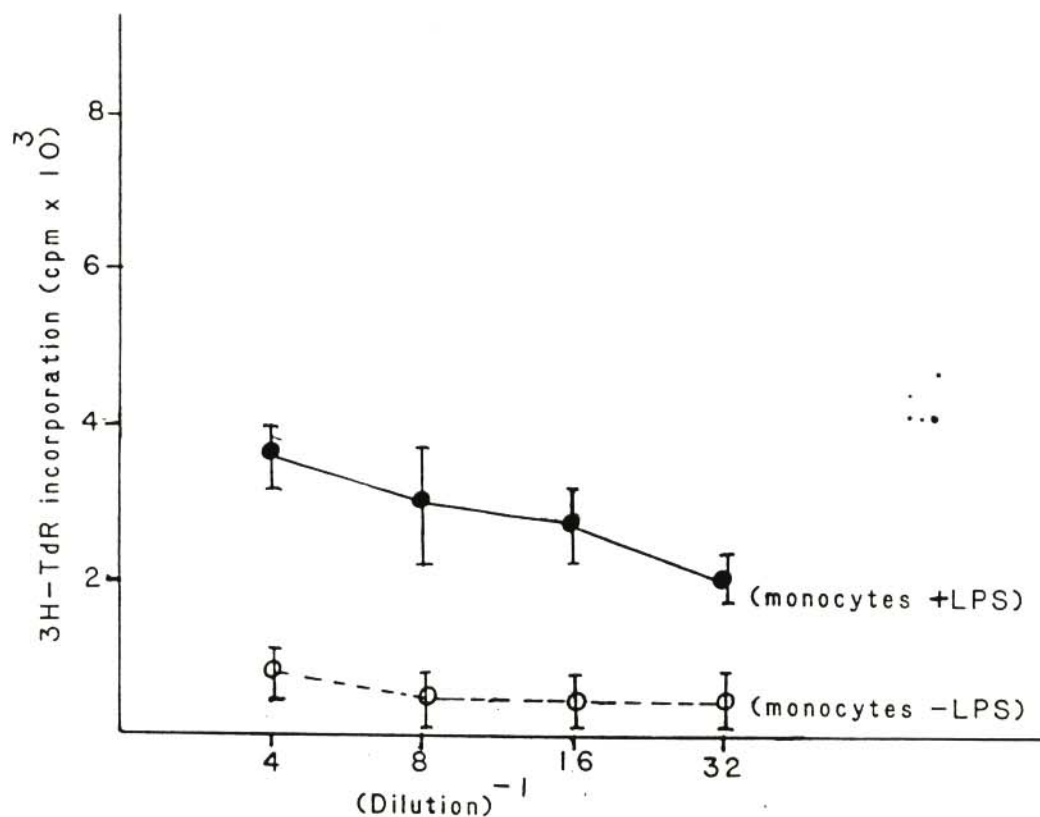


Fig. 3. One million cells from human monocyte - enriched fractions were cultured for 48 hours in 1ml RPMI - 1640 medium and 1mg/ml HSA with or without *E. coli* LPS. To measure the IL 1 activity in each culture supernatant, 1×10^6 cells/100ul were cultured for 72 hours with 10ug/ml PHA in the presence of serially diluted culture supernatant. Cultures were incubated overnight with 0,5 uCi ^3H -TdR/well. The results shown are a mean \pm SD of 20 normal subjects.

Dilution 1 in 4 represents 50ul of crude supernatant in a total culture volume of 200ul and has the highest proliferative effect, $3,6 \pm 0,4$ and $0,8 \pm 0,3 \times 10^3$ cpm for (●) and (○) respectively.

THYMOCYTE PROLIFERATION IN RESPONSE TO DIRECT TREATMENT WITH STIMULATING AGENTS:

Thymocytes proliferated in response to IL 1 alone, PHA alone or IL 1+PHA. All the other agents present in the monocyte cultures (HSA, LPS, FCS) did not have any mitogenic effect (fig. 4).

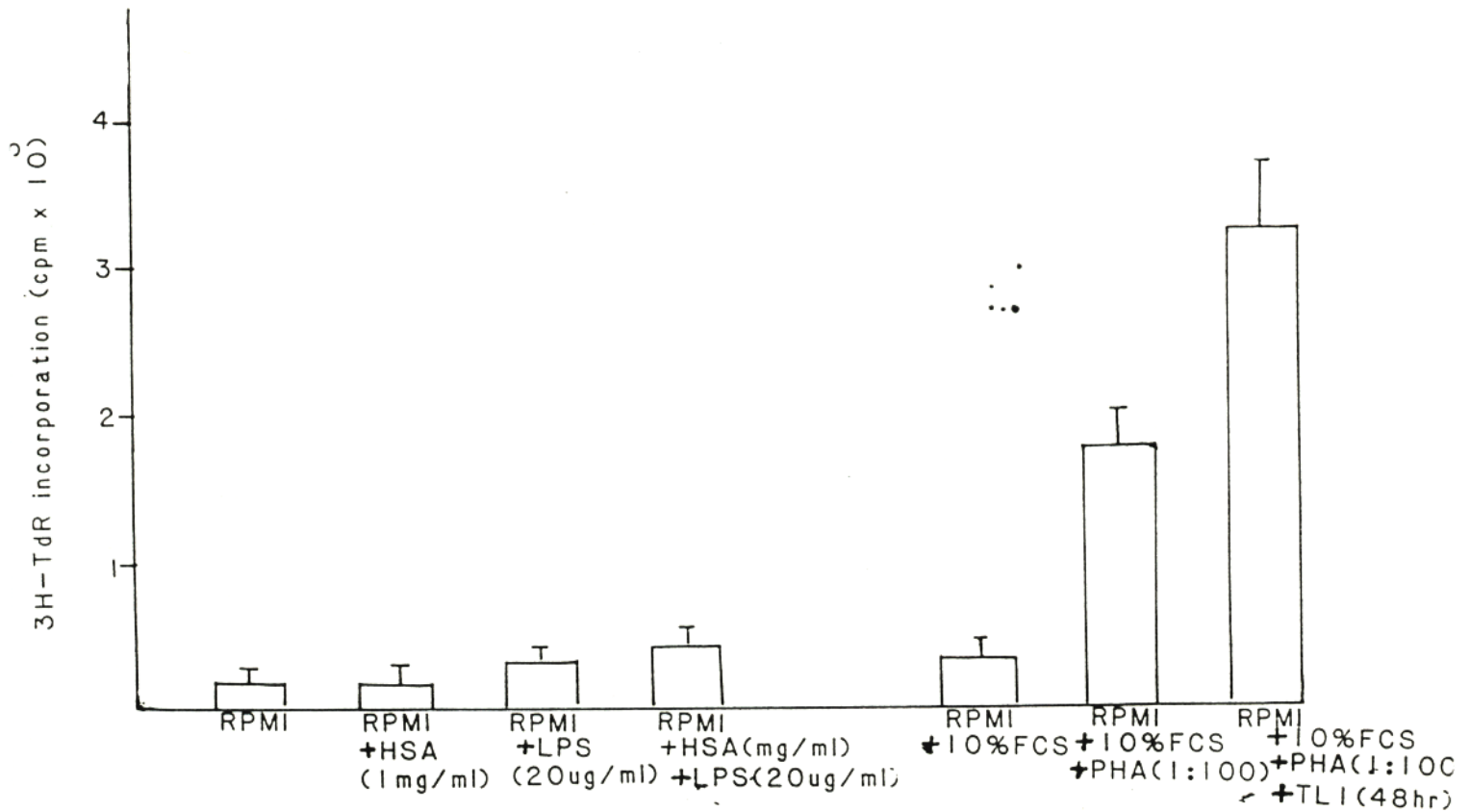


Fig 4. Murine thymocyte proliferation was determined by measuring the incorporation of ³H-TdR during the last 12 hours of a 72 hours incubation period. Each column is an average of twelve assays with an error bar representing one standard deviation.

HSA, LPS and FCS do not induce proliferation (at least at the concentrations used). PHA alone evokes proliferation (17 500[±] 223 cpm) and IL 1 enhances this proliferation (32 000[±] 460cpm).

Determination of optimum dilution of PHA for thymocyte proliferation in the IL assay.

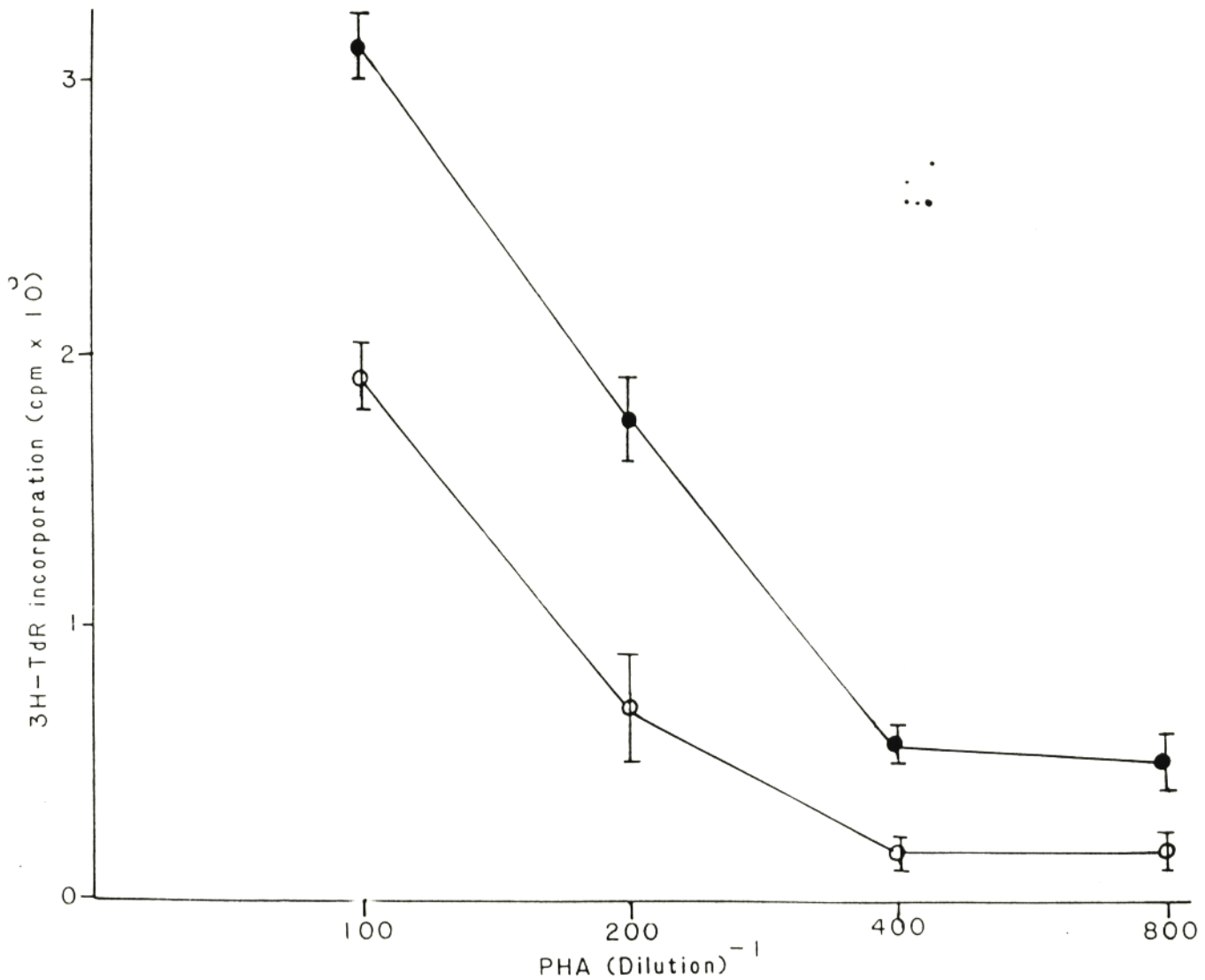


Fig. 5: Incorporation of ³H-TdR in murine thymocytes in the presence (●) or absence (○) of IL 1 containing supernatant from human monocytes. The thymocytes (10⁶ cells/well) were cultured in the presence of various doses of PHA. The cultures were harvested after 72 hours. The greatest proliferation is found in cultures with 1:100 PHA (31400 ± 112 cpm and 19300 ± 107 cpm for ● and ○ respectively).

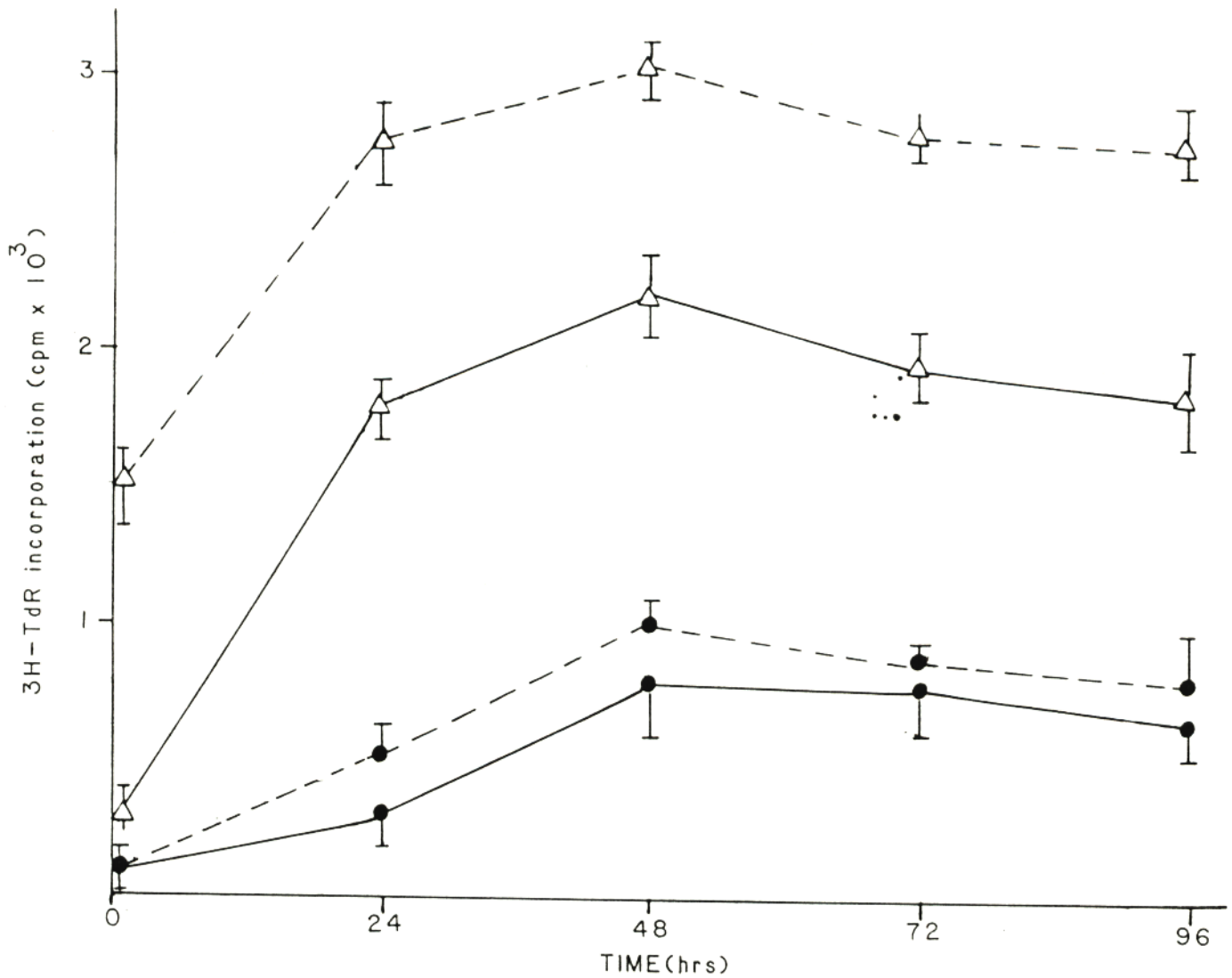


Fig. 6: Thymocyte proliferation in response to monocyte supernatants withdrawn at 0, 24, 48, 72 and 96 hours. Monocytes of normal subjects (Δ) and patients (o) in RPMI and 1mg/ml HSA were incubated with 20 ug/ml LPS. Each value is the mean \pm SD of 20 individuals. Broken lines represent cultures with optimal dose of PHA (50 ul of 1:100 PHA dilution in a total culture volume of 200ul).

48hour-SN of both patients and normal subjects has the most IL 1 activity. IL 1 also exhibit a synergistic effect when cocultured with PHA: $3\ 492 \pm 104$ cpm (Δ) and $1\ 003 \pm 89$ (\bullet).

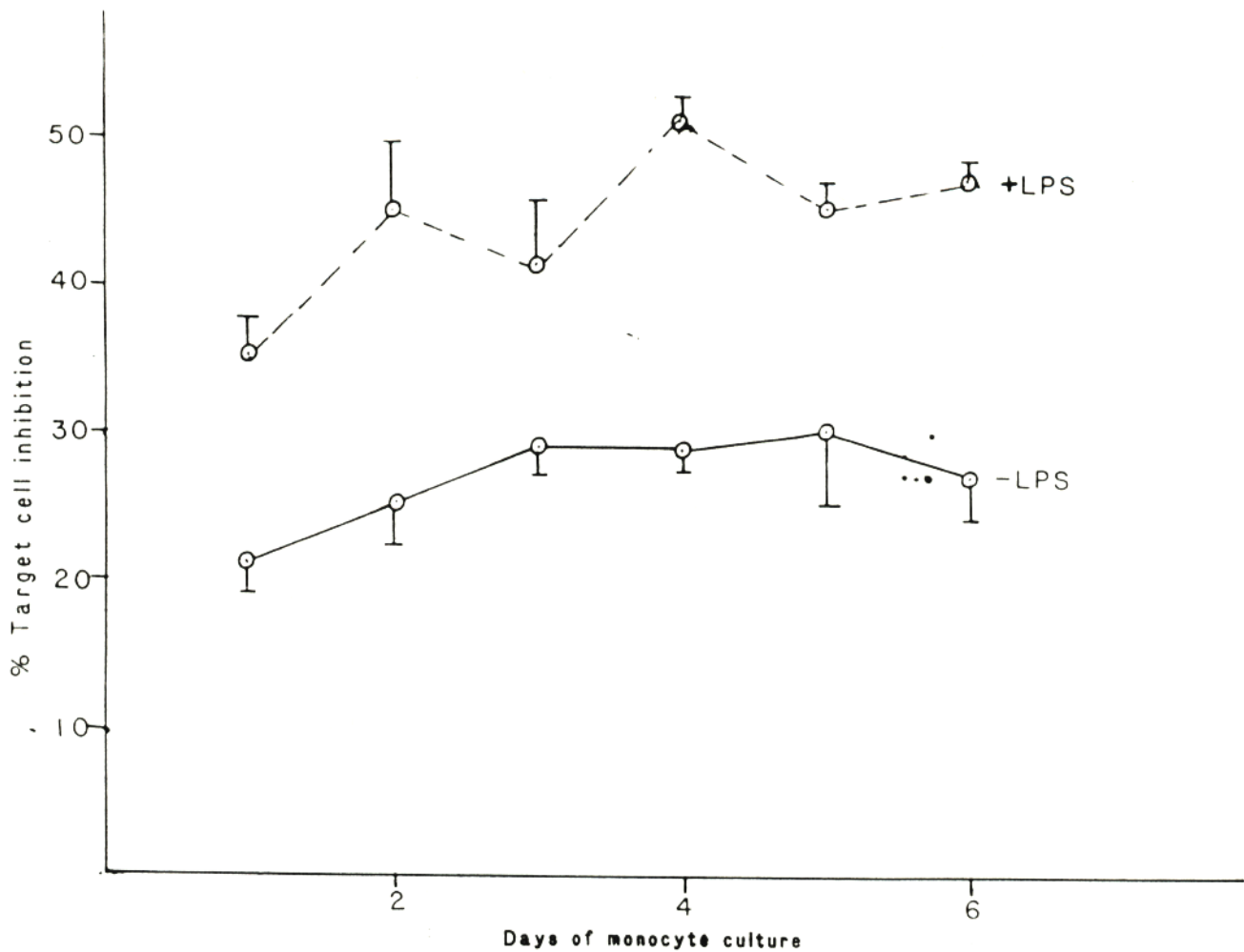
Cytotoxicity assay:

Fig. 7: Monocyte-mediated cytotoxicity of K562 cells during in vitro maturation of monocytes. Monocytes from the same donors (12) were cultured for up to 6 days. During the last 48 hours LPS was added to some of the cultures. During the last 24 hours of a culture period K562 target cells are added. E/T ratio 20;1. Each point represents mean \pm SD for 12 experiments.

Monocyte activation by LPS augments cytotoxicity. The most cytopathic effect is exhibited by 96 hour old monocytes. $38 \pm 4,0\%$ (+LPS) and $12 \pm 1,7\%$ (-LPS).

The results of the cytotoxic assay is presented in table 5.

TABLE 5:

<u>Controls</u>	<u>% cytotox- icity</u>	<u>Patients</u>	<u>% cytotox- icity</u>
1	15	1	12
2	23	2	6
3	10	3	12
4	8	4	9
5	24	5	10
6	29	6	3
7	33	7	11
8	28	8	5
9	23	9	4
10	24	10	3
11	30	11	21
12	33	12	11
13	31	13	7
14	17	14	3
15	19	15	4
16	27	16	8
17	31	17	8
18	35	18	12
19	26	19	9
20	8	20	18
21	17		
22	16		
23	19		
24	33		
25	31		
26	39		
27	28		
28	26		
29	34		
30	38		
MEAN \pm SD	25,17 \pm 8,38	MEAN \pm SD	8,80 \pm 4,7

The % cytotoxicity exhibited by monocytes from normal subjects is significantly greater than that by monocytes from patients. ($P < 0,001$).

The results of the cytostatic assay are presented in table 6.

TABLE 6 : Cytostatic activity of freshly isolated adherent cells of normal subjects and of patients with cervical carcinoma. Cytostasis by activated (+LPS) and non-activated (-LPS) monocytes was determined after 24 hours of coculture. E/T ratio 20:1. Each value is an average of triplicate experiments.

Controls	% cytostasis	
	-LPS	+LPS
1	20	52
2	30	40
3	15	27
4	16	31
5	18	27
6	22	42
7	32	47
8	33	56
9	30	35
10	28	60
11	26	40
12	19	39
13	24	27
14	29	49
15	31	49
16	33	62
17	36	72
18	35	70
19	21	51
20	23	38
21	24	52
22	27	50
23	31	49
24	33	71
25	19	28
26	17	25
27	18	29
28	31	45
29	34	49
MEAN \pm SD	26,04 \pm 6,35	45,59 \pm 13,13

Patients	% cytostasis	
	-LPS	+LPS
1	3	12
2	15	18
3	3	5
4	2	8
5	7	9
6	9	2
7	12	12
8	4	5
9	6	6
10	3	5
11	12	11
12	10	19
13	4	6
14	6	5
15	3	5
16	2	5
17	11	24
18	2	4
19	19	31
20	14	33
MEAN \pm SD	7,35 \pm 4,96	11,25 \pm 8,88

Cytostasis exhibited by activated monocytes is more than that exhibited by non-activated monocytes.

For normal subjects : $P \ll 0,01$

For patients : $0,1 > P > 0,5$

The percentage cytostasis exhibited by adherent cells from healthy subjects is significantly greater than that by cells from healthy subjects.

To determine if different ratios of effector cells to target cells influences the cytolytic activity an experiment was performed using monocytes from healthy subjects. Different numbers of monocytes were used while the number of target cells was constant. The results are presented in Fig. 8.

•
••

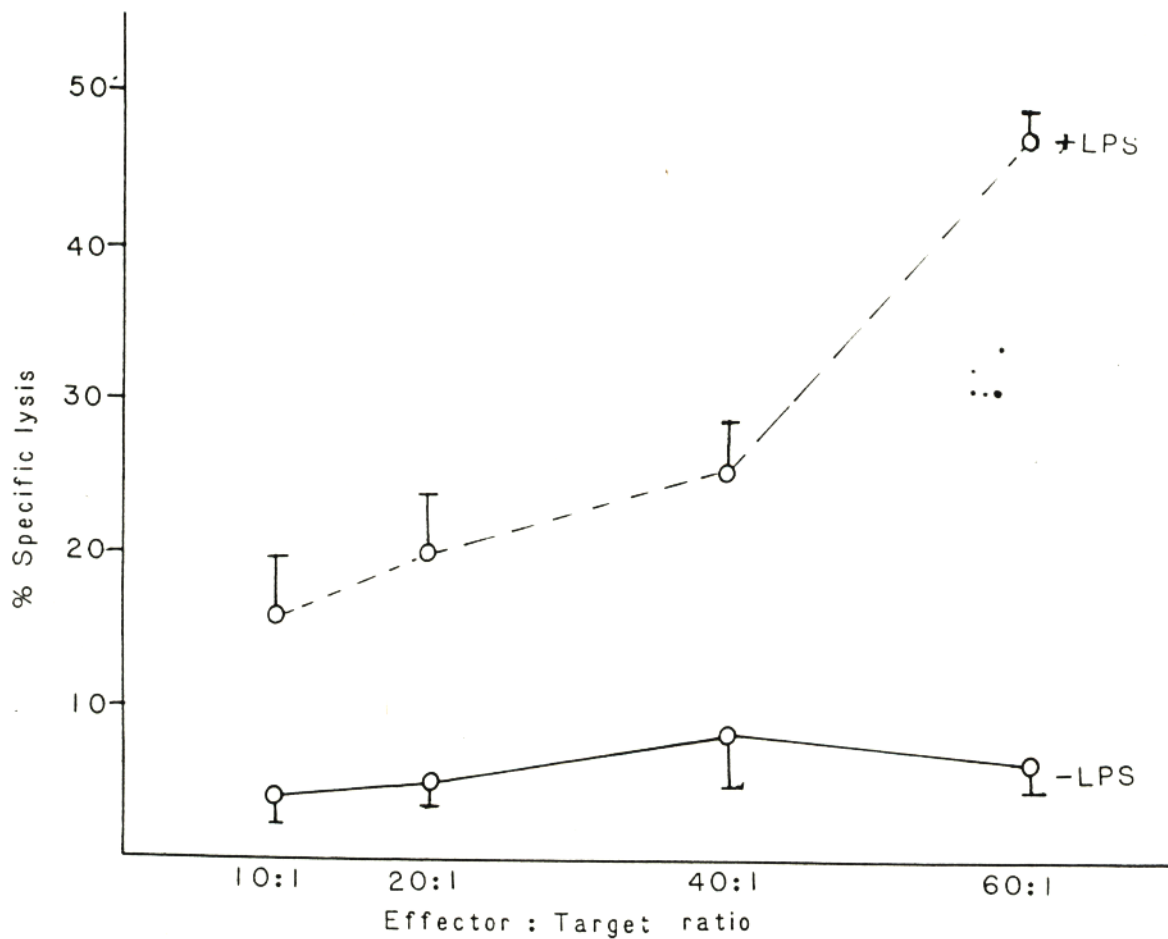


Fig. 8 The LPS activation procedure was carried out as described in Fig. 7. The ^3H -TdR prelabelled K562 cells were added on day 4 of monocyte culture. Each point is the Mean \pm SD of 10 experiments.

The cytolytic effect increased with increasing effector/target cell ratio $15,8 \pm 4,1$ with a ratio of 10:1 and $47,1 \pm 1,6$ with a ratio of 60:1.

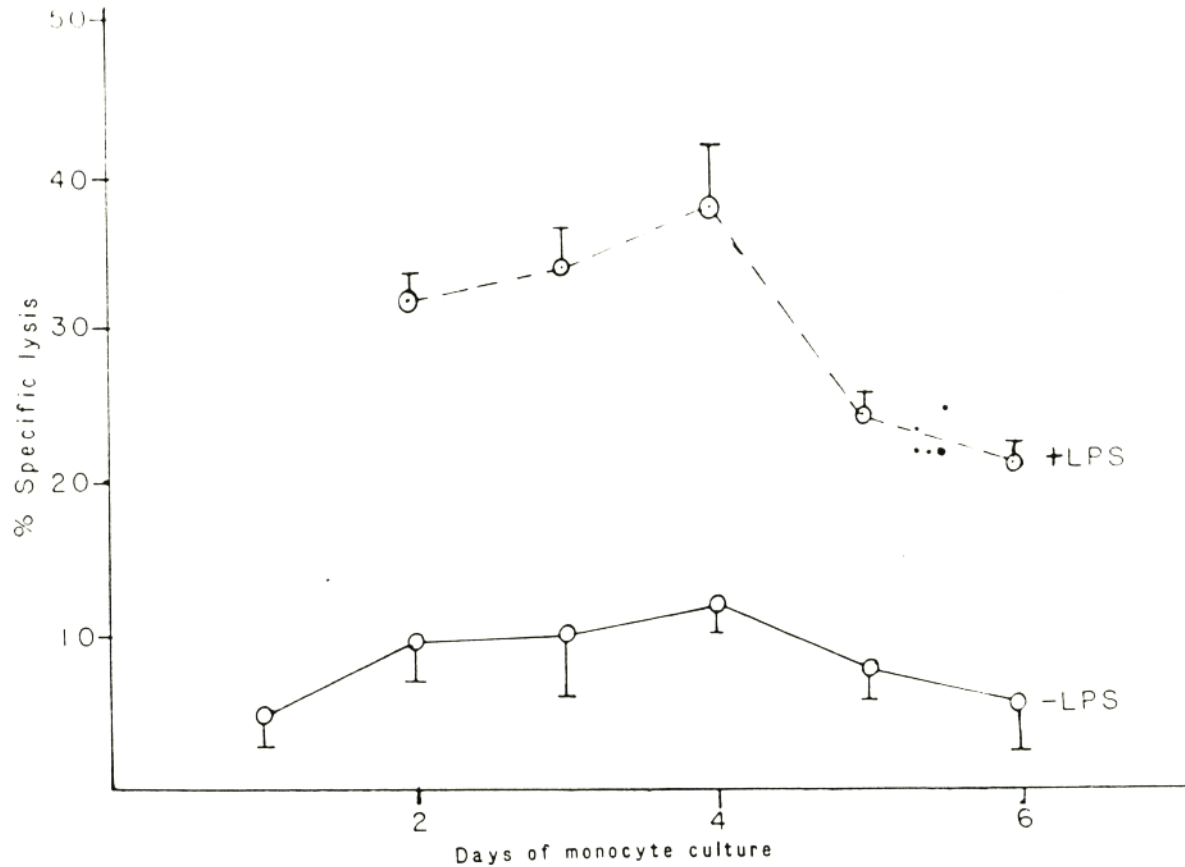


Fig. 9: Monocyte-mediated cytostatic activity during in vitro maturation of monocytes. Monocytes from the same donors (12) were cultured for up to six days and each day an aliquote was incubated with and without LPS for 24 hours before addition of unlabelled K-562 cells on the indicated day of monocyte culture. Cytostasis was determined after 24 hours of coculture. The effector/target cell ratio was 20:1. Each point represents Mean \pm SD of 12 experiments.

Spontaneous cytostasis is exhibited in all LPS negative cultures: $21,0 \pm 1,6\%$ in 1 day old cultures and $26,8 \pm 2,4\%$ in 6 day old cultures. The cytostatic activity was highest when LPS was added to the cultures for the last 48 hours.

The results of increasing the ratio of monocytes

K562 cells are shown in fig. 10.

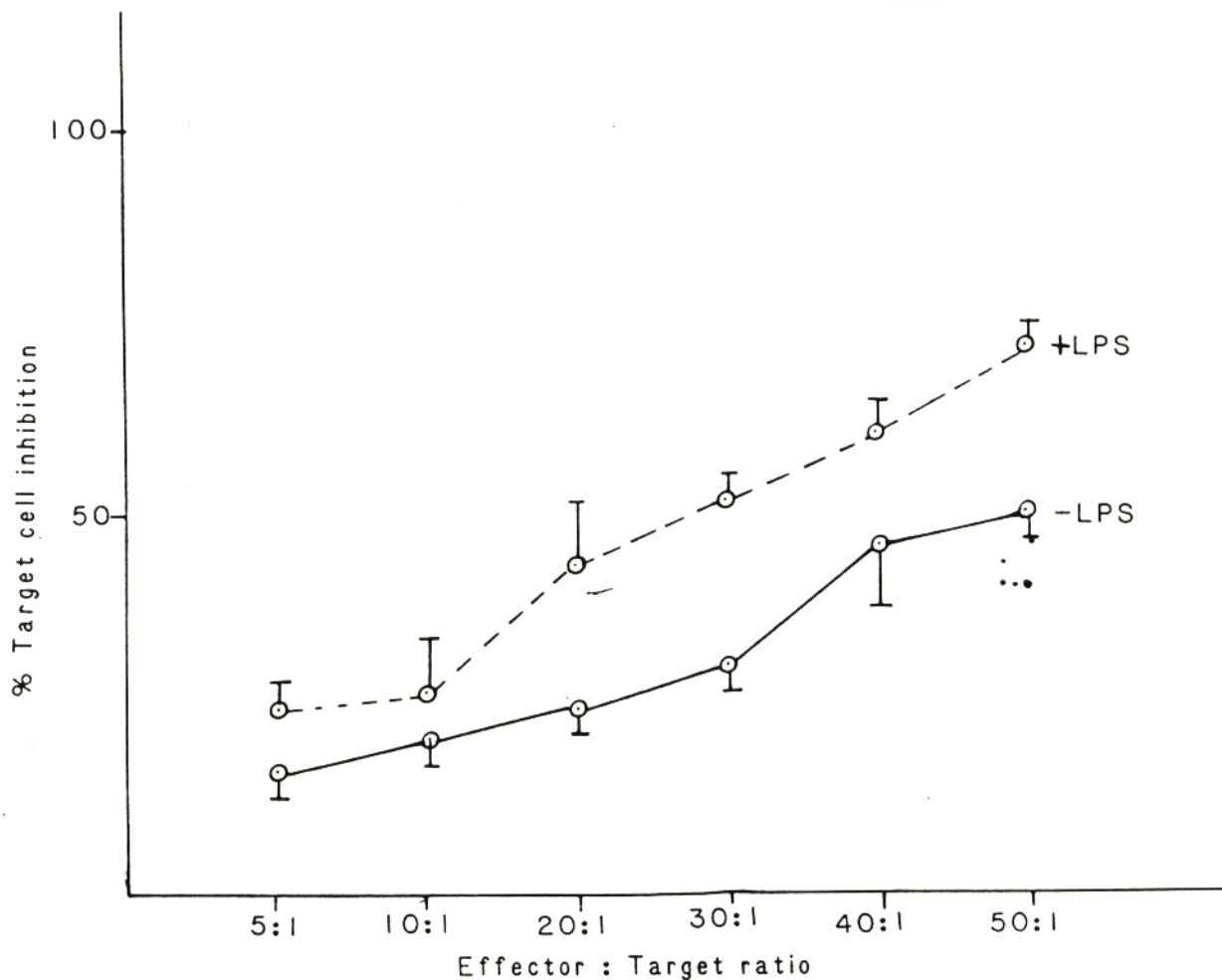


Fig. 10: Monocyte cultures with different densities were prepared by adding different numbers of mononuclear blood cells to the wells. Some of the cells were stimulated with LPS. Cytostasis was determined after 24 hours of co-culture with K562 cells. Each point represents Mean \pm SD of 12 experiments.

Increasing the E/T cell ratio increases cytostasis :
 $22,2 \pm 1,7\%$ (5:1 + LPS) and $70,9 \pm 2,0\%$ (50:1 + LPS).

The stimulated cells showed an increase in cytostasis compared with the unstimulated cells.

IL 1 Characterizationa) Molecular weight determination

Analysis of human monocytes IL1 fractions by polyacrylamide gradient gel PAA 4/30. The use of 5x concentrated IL 1 - C fractions in the IL 1 assay was stopped after it was found that the activity shown by various supernatants grossly differed and did not correlate (results not shown). However, meaningful results were obtained when using ordinary crude IL 1 supernatants.

.
..

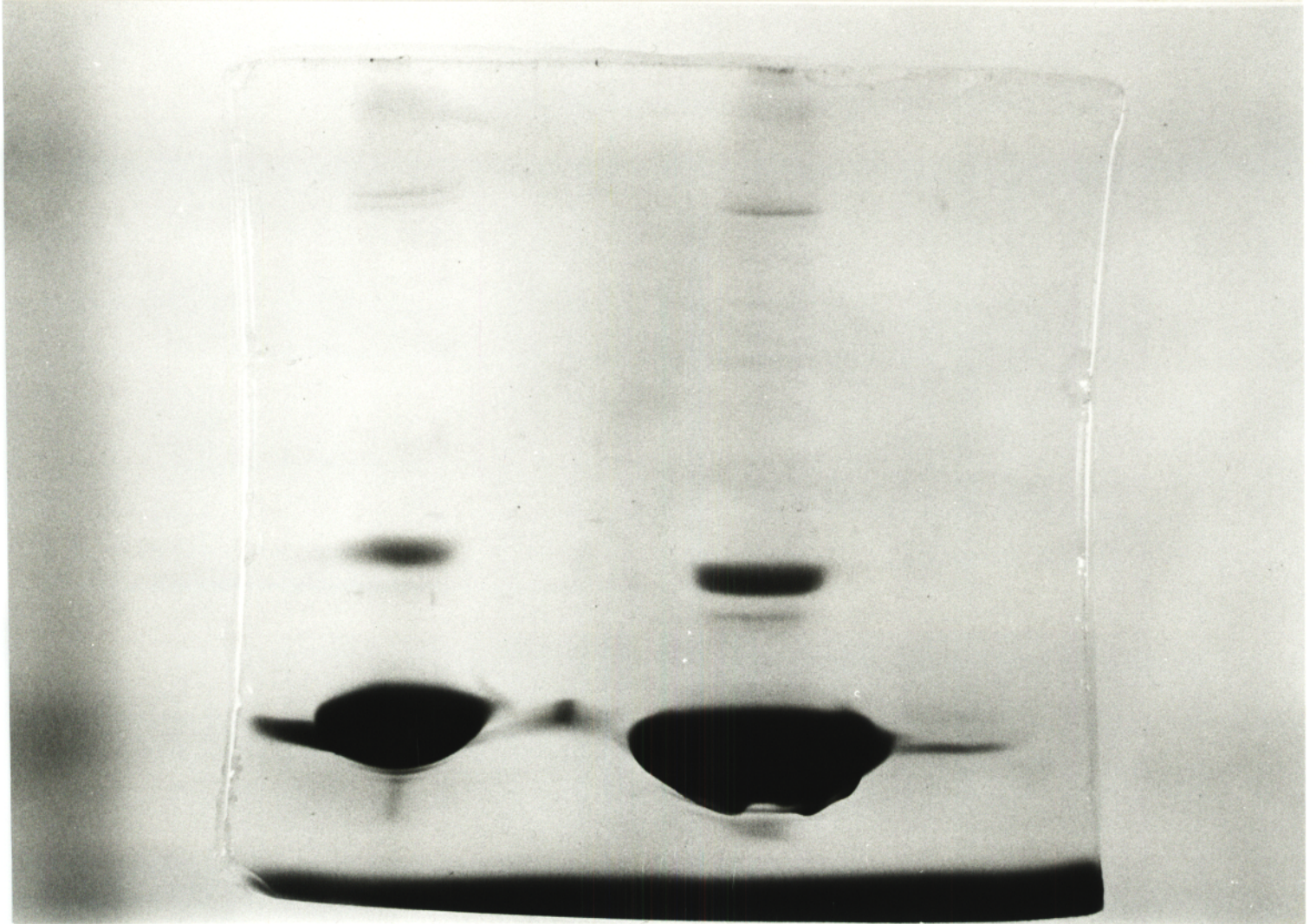


Fig. 11. Analysis of human monocytes IL-1 fractions on polyacrylamide gradient gel PAA 4/30. Samples (left to right): Commercial IL 1, IL 1-C, IL 1-F, IL 1-SN (10ul of each). Electrophoresis: 125 V, constant voltage 15 minutes pre-run; 70 V constant voltage 20 minutes pre-electrophoresis; 125 constant voltage 16 hours electrophoresis. Buffer

Tris (0,09 M); borate (0,08 M); Na₂EDTA (0,003 M) pH 8,35. Staining: 30 minutes 0,7% (w/v) Amido Black 10B in 7% (v/v) acetic acid. Destaining: electrophoretic gel destainer Gd-4, 36V, 40 minutes using 7% (v/v) acetic acid.

•
••

IL 1 is present in all fractions of the IL 1-SN.

The reasonable explanation for these findings could be that IL 1 is lost during the ultrafiltration process. Gearing et al. (1985) discovered that IL 1 is very hydrophobic and a substantial loss of activity occurred by binding to glass surfaces (Amicon cell) and filter membranes when the crude supernatant was ultrafiltered.

Although active IL 1 exists as a 12 000 - 15 000 MW substance, culture supernatants can contain IL 1 activity with a MW of 30 000 - 70 000 (Gearing et al., 1985). This probably explains why IL 1 moves with HSA, a protein of 70 000 MW (fig. 11).

An unexpected finding is that IL 1 activity is found in some IL 1-F (Fig. 11). This simply implies that the protein leaks through the ultrafilter MP10. This is the reason why some IL 1-F fractions augmented the proliferation of mouse thymocytes when they were least expected to (results not shown because they were very diverse).

b) pI determination

The IEF method used is described under MATERIALS AND METHODS Section 2.9.8. The pH meter indicated a pH of 5 - 6 for both prepared and commercial IL 1.

Microscopic observations and cell viability. The microscopic observations of cell cultures showed good cell appearance and viability throughout the period studied. Cell viability was monitored by trypan blue exclusion.

All the other experimental results are discussed under "Discussion" below.

DISCUSSION:The nature of the mitogenic signal

Mitogenic lectins bind strongly to carbohydrate compounds of cell membrane glycoproteins and glycolipids (Kay, 1980). Although different lectins are specific for different mono - or oligosaccharide residues, they all bind to many different membrane glycoproteins (Kay, 1980). It is not clear at present whether the effects of mitogens are due to their interaction with one specific receptor which controls the rate of proliferation or whether binding of a ligand to any or many of different glycoproteins would have similar consequences.

The binding of a mitogen to its receptor occurs within minutes of its addition to the culture medium, but the continuous presence of the mitogen for several hours is required before the lymphocytes can be committed to initiate DNA synthesis (Kay, 1980). If the mitogen is removed earlier, the metabolic changes which are established are gradually reversed and the cells revert to the unstimulated state.

The interaction of the mitogen with its receptor results in many changes in physical and functional properties of the cell membrane. The distribution of the glycoprotein receptors on the cell surface is altered and

they may become aggregated or even capped at one pole of the cell (Kay, 1980). Perhaps as a consequence, the fluidity of the membrane changes and there may be changes in the organisation of membrane-linked components such as microtubules (Kay, 1980).

Many investigators have observed that there are changes in the rate of transport of many ions and metabolites across the T-cell membrane after mitogen addition. Mills et al (1985) and Imboden et al. (1985) observed that the rate of uptake of calcium ions (Ca^{2+}) was increased resulting in high levels of cytoplasmic free Ca^{2+} . The latter appeared to be an intracellular messenger for the production of a factor called Interleukin 2 (IL 2) but not for the expression of IL 2 receptor (IL 1-Rc) (Mills et al., 1985). In contrast IL 2 production did not occur in the absence of extracellular free Ca^{2+} (Mills et al., 1985) and no proliferation took place.

Meuer et al., (1983) showed that monoclonal antibodies (C305) to antigen receptor (Ag-Rc) on T-cells could mimic the effects of antigen by inducing cellular proliferation and production of lymphokines. Ca^{2+} ionophores (ionomycin and A23187) could substitute for this antibody (Ab) in the activation by increasing cytosolic free Ca^{2+} (Imboden et al., 1985 and Kay, 1980). Antibodies to T_3 also acted like antibodies to Ag-Rc and ionophores (Weiss et al., 1984). Meuer and co-workers

12

(1983), after further investigations concluded that T_3 antibodies and Ag-Rc antibodies may activate the same receptor - complex (T_3 Ag-Rc) because the T_3 determinant appears to be noncovalently associated with the Ag-Rc on human T-cells. This is supported by studies which demonstrated that mutants of Jurkat (a T cell line) that do not express the T_3 Ag-Rc are not activated by C305, T_3 antibodies or PHA but do produce IL 2 activity in response to the combination of Ca^{2+} ionophores and phorbol myristate acetate (PMA) (Weiss and Stobo, 1984 and Weiss et al., 1984). Palacios (1985) believes that the T_3 complex is involved in the triggering process because, in addition to the fact that monoclonal antibodies against T_3 polyclonal activate T-cells, most mature T cells express the structure on their surfaces.

In the present study the medium (RPMI-1640 plus FCS) contained Ca^{2+} and proliferation was observed (Fig. 1) , when PHA was used as mitogen. Perhaps Ca^{2+} ions were also derived from intracellular stores as suggested by Imboden et al., (1985) who observed that there was an initial increase in the free intracellular Ca^{2+} ions in the absence of extracellular Ca^{2+} ions. These investigators also noted that although a sustained increase in cytoplasmic free Ca^{2+} required the presence of extracellular Ca^{2+} ions, a plasmic voltage-dependant Ca^{2+} - channel did not appear to be involved, because the increase was not affected by Ca^{2+} channel blockers.

75

In several cell systems, most notably in platelets, increases in cytoplasmic free Ca^{2+} and activated kinase C were observed to be clearly synergistic after activation of the cells (Michell, 1983). Michell thus hypothesized that a similar synergism was involved in T cell activation which identified a central role for the Ag-Rc in mediating the essential increase in cytoplasmic free Ca^{2+} . In support Palacios (1985) found that trifluoroperazine, an inhibitor of protein kinase C abrogated the growth of purified resting T cells induced by either OKT3 on beads plus IL1 or soluble OKT3 plus PMA. These results support the hypothesis that accessory cells and PMA may ultimately act via the same mechanism and also strengthen the view that protein kinase C plays a critical role in the transduction of signals in this system.

Possible mechanism by which accessory cells can contribute to the growth of human T cells and murine thymocytes.

PHA is mitogenic to purified human T cells and murine thymocytes (Fig. 1 and Fig 5 respectively). While there are many different agents known to cause cell proliferation, there are also many examples of lectins which bind to lymphocytes without being mitogenic. PHA and Con A bind equally well to B and T lymphocytes, but they only stimulate the latter (Kay, 1980). On the other hand antigens need

to be processed by macrophages before they can be presented to T helper cells and subsequently cause lymphocyte proliferation (Fudenberg et al., 1980 and Guttman et al., 1980).

Macrophages produce a factor called IL 1 which appears to be an important mediator of the immune system (Gery, 1982; Kurt-Jones et al., 1985; Roosnek et al., 1985; Kay et al., 1984; Palocio, 1985; Lepe-Zuniga and Gery, 1984; Mills et al., 1985; Imboden et al., 1985 and Gallily et al., 1985). Kay and co-workers (1984) demonstrated that the growth of a helper T cell line required both exogenously produced IL 1 and endogenously produced IL 1. Other experiments proved the primary role of IL 1 to be in the expression of surface IL 1 receptors (IL 2-Rc), rather than for promoting synthesis of IL 1 (Mills et al., 1985 and Kay, 1984). IL 1 production required an increase in free cytoplasmic Ca^{2+} which resulted from the activation of lymphocytes by mitogenic lectins (Mills et al., 1985; Weiss et al., 1984 and Michell, 1983).

In the present investigation PHA induced T cell proliferation in the absence of macrophages which is in agreement with Roosnek and co-workers (1985) who proposed that mitogens activate T cells to express IL 2-Rc. They believe that the second signal in proliferation was provided by IL 2 which was produced by another T cell, probably T helper cell. Roosnek and co-workers (1985) claim

that T helper cells have been proved to be IL 2 producing cells.

An alternative proliferation mechanism could be induced to cell aggregation caused by PHA. This hypothesis is supported by Palacios (1985) who believes that accessory cells help in proliferation by encouraging aggregation of T cells. In his study, Pal deduced that an antigen (OKT3-Ab) first binds to T cells and then to monocytes apparently via the Fc-portion. This means that the accessory cells provided the matrix that favoured cross-linking of T₃ complex on T cells. Palacios observed that the Fab fragments of OKT3-Ab were weakly, if at all, mitogenic and that depletion of monocytes prevented the mitogenic response of lymphocytes towards the intact antibody. Roosnek and co-workers (1985) also showed that high concentration of lectins were able to induce cell clustering in the absence of accessory cells. When T cells were stimulated by lectin doses that did not induce aggregation, a "helper" activity had to be added. This role was fulfilled by either accessory cells, thiols or PMA. The latter specifically binds to and activates protein kinase which acts synergistically with increased free Ca²⁺ in cytoplasm to activate T-cells (Rosenstreich and Mizel, 1979).

So it seems as if cell-cell contact is a prerequisite for T cell activation since aggregation could not be

observed with non-helper monocytic cells such as HL-60 or KG-1A, even in the presence of sub-optimal doses of Con A and PHA (Roosnek et al., 1985). Even so, the nature of this cell clustering phenomenon remains unclear. In contrast Kay et al., (1984) found that the antibodies cross-linking T cell Ag-Rc's caused abundant release of IL 2 but failed to induce growth. They hypothesized that the failure of antibody to induce growth was correlated with its failure to induce cell surface expression of IL 1 because in an overnight culture, a tenfold increase in IL 2-Rc occurred and the cells rapidly entered the S phase. So Kay and co-workers' findings suggested that cell-clustering on its own is not sufficient to cause cell proliferation. IL 1 (a product of macrophages) was needed as it appeared to be promoting the expression of IL 2 -Rc.

The results in Fig. 6 show that the human monocyte-derived IL 1 augmented the PHA proliferation response, indicating that IL 1 amplified the production of IL 2.

The direct mitogenic effect of IL 1 on mouse thymocytes which has been observed by previous investigators was confirmed in the present investigation. The proliferation is due to IL 2 release by IL 1 stimulated T cells, as monoclonal antibodies specific for IL 1 blocked the response (Oppenheim and Gery, 1982). Unlike human T-cells, mouse thymocytes proliferated in the presence of IL 1 and a low concentration of mitogenic lectins (Fig. 6). The low IL 1 activity observed in the supernatants of stimulated monocyte

from patients with cancer of the cervix (Fig. 7) suggests that the physiology of the monocytes is impaired. IL 1 has a wide range of biological activities both in vivo and in vitro, many of which are considered essential for the induction of an immune response (Dinarello, 1984). The most significant of these activities is the capacity to stimulate production of IL 2 by T cells, while at the same time, cause an increase in body temperature in vivo (Dinarello, 1984), which may help the immune response to bacteria and viruses by enhancing the effects of IL 1 on T cell proliferation (Dinarello, 1984). So it is possible that in patients with cervical cancer, these biological activities are defective.

With adequate stimulation other cell types (keratinocytes, astrocytes, mesangial cells, endothelial cells, large granular lymphocytes, Epstein Barr virus-transformed B lymphocytes and purified B cells) also produce IL1 (Matsushima et al., 1985; Gearing et al., 1985 and Oppenheim et al., 1984). The IL 1 produced by large granular lymphocytes of cancer patients was found to be low (Rabson et al., 1983), while IL 1 production by the other cell types has not been investigated. That accessory cell function is not confined to cells of monocytic origin has been proved by Roosnek and colleagues (1985). B cells were much more effective in acting as accessory cells in an assay where T cells were triggered by lectins (PHA and Con A). Addition of accessory cells caused a shift in the dose-response curve, resulting in strongly enhanced IL 2 production at low concen-

tration. Roosnek et al., (1985) also observed that the incapability to function as accessory cells was not due to lack of HLA CLASS II expression. Thus they proposed that the stimulus for all T cell responses was in fact not recognition of antigen plus MHC (Fudenberg et al., 1982, and Guttman et al., 1981), but some other property of the presenting cells. Endogenous lectins present on macrophages might be involved in this process and this might explain the autologous mixed lymphocyte reaction, they reasoned. . .

Kay et al., (1984) performed experiments which suggested that Ia molecules were not required in the process of proliferation. The following observations were made:

- (a) addition of anti I-A^b (against Ia molecules on H-2^b cells) in a culture of cells and H-2^b antigen presenting cells did not abrogate the proliferation.
- (b) Xenogenic rat spleen cells could act as accessory cells in proliferation.
- (c) Monoclonal antibodies (GK1,5) which recognize a 52 000MW T cell surface protein associated with recognition of class II MHC determinants, had no effect on the proliferative response of T helper cell-line.

However results are contradicting. T cell responses

have been described as monocyte independent and dependent. As a consequence of this, the mechanism of action of accessory cells is still unclear. The demonstration of apparent lack of involvement of Ia-Ag in proliferation confuse the matter further.

The low activity of IL 1 present in the supernatants of stimulated monocytes from patients with Ca of the cervix (Fig. 6) does not necessarily mean that the synthesis of IL 1 is low. It can be due to a defective release mechanism. Lepe-Zuniga et al., (1984) observed different patterns of production and release of IL 1 with time by monocytes. LPS raised preferentially intracellular levels whilst silica and PMA increased mainly the release of IL 1 while zymosan increased synthesis and release of IL 1. These findings suggest that synthesis and secretion of IL 1 by human monocytes are two distinct biological events.

The significance of PHA transformation assay.

The transformation assay is a measure of an immune status of an individual. In the present investigation, lymphocytes from patients with cancer of the cervix, responded less to PHA than lymphocytes of normal subjects (Fig. 1). T-lymphocytes have been shown to be primarily responsible for tissue rejection in transplantation and against antigenic tumours (see introduction). Since the distribution of lymphocytes was equal in the two groups, the low DNA

uptake in patients maybe due to some dysfunction of the lymphocytes.

Monocyte-macrophage cytophatic effects

Human macrophages, derived from peripheral blood monocytes, acquire an enhanced cytotoxicity for human tumour cells (K562) after incubation with E. coli LPS (Figs 7 and 8). Generally, the cytolytic effects of patients' macrophages were lower than that of macrophages of normal subjects. When the effector/target ratio was increased, the cytotoxicity was enhanced (Fig. 8), indicating that cytotoxicity was dose-dependent. However, the susceptibility to tumour growth in patients was found not to be due to low numbers in monocytes (OKM1+ve-Tables 3 a and b). The low cytotoxicity could be due to functional deficiencies of the cells. Freshly isolated blood monocytes did not lyse the K562 cells even when co-cultured with LPS. It appears that the response to LPS is acquired with the maturation of cells since four-day old cells cocultured with LPS showed increased cytotoxicity (Fig. 7).

It is not known how LPS cause macrophages to become tumoricidal. However, Cameron's study (1985) indicated that cell surface carbohydrates appear to be involved. In his experiment in which he incubated monocyte derived macrophages with α -L fucosidase a glycosidase with splits terminal α -L-fucose from oligosacchanides, the macrophages were no longer able to respond to LPS.

This experiment strongly suggests that -L fucose probably comprise an essential part of the macrophage membrane receptor for LPS.

The fact that cultivated macrophages killed K562 cells which were propagated in vitro, indicate that cytotoxicity is non-specific. This was also shown by Keller (1985) who found that rat and mouse macrophages from lung, peritoneum, marrow and spleen showed non-specific cytotoxicity. Spontaneous low cytolysis^{...} by macrophages not activated with LPS occurs with four-day old macrophages (Fig. 7). Cells older than four days showed a decrease in their cytolytic capacity (Fig. 7).

Freshly isolated adherent blood mononuclear cells are cytostatic to K562 cells (Fig. 10), although the cytostatis is depressed in monocytes from patients (Tables 4 a and b). Furthermore, human monocytes developed a strong cytostatic activity upon in vitro differentiation (Fig. 10).

Hammerström (1979) performed morphological studies on K562 cells co-cultured with adherent cells, when investigating the mechanism of lysis. He observed that a considerable number of K562 cells attach firmly to monocytes and were resistant to removal by washing. The target cells usually attach at the periphery of monocytes and develop unusually large blebs. Also, membrane "ghosts" could be seen attached to monocytes and proba-

bly represented the end stage of lysis.

In studies of natural killer cell activity of monocytes of healthy donors, Kiczka and co-workers (1985) observed their rapid binding to K562 cells. However, the formation of cytotoxic conjugates was less frequent. This agrees with the present finding in which spontaneous cytotoxicity was observed in four days old monocytes.

∴

In contrast, Uchida and Yanagawa (1984) demonstrated that monocytes from blood of normal individuals expressed natural cytotoxicity against K562 cells. In trying to analyse the mechanisms involved, the investigators generated some soluble factors by co-culturing monocytes with K562 cells. The culture supernatants had cytotoxic effects on K562 cells. The cytotoxic factors were referred to as monocyte cytotoxic factors (MCF).

Nissen-Meyer and Kildahl-Anderson (1984) also characterised human monocyte-released cytostatic factors (CF) which were liberated upon activation with lymphokines and LPS. The 30 000 - 40 000 dalton antitumour cytotoxin gives further evidence to the suggestion that monocyte-macrophages play an important role in host immune defence against tumours. Neale and Matthews (1980) purified a macrophage-derived cytotoxin (found

in high titer in the serum of BCG-primed rabbits) which killed malaria parasites and L929 tumour cells. The investigators named this cytotoxin, which had reduced stability after purification, a tumour necrosis factor (TNF). Abe et al., (1985) purified rabbit TNF. It was identified as a protein of 18 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). So it is not improbable that TNF is released when monocytes are activated and might be responsible for tumour death.

•
∴

Cytostasis appears to be an intermediate step in cytolysis. Adams and Marion (1981) studied the destructive changes in the target cells manifested by both cytostasis and cytolysis. They observed that target cells (neoplastic cells) underwent an almost complete reduction in cell division. Subsequently, a reduction in cellular motility was seen followed by degenerative morphology changes that included formation of membranous blebs, and cellular swelling. These changes, once underway, culminated within several hours, in massive and explosive lysis of the tumour cells.

Tumour development

In the present investigation patients with cervical cancer are immunocompromised, as reflected by the in vitro expressions of the immune activity (Fig. 1). If this is the case, perhaps human cancer can be controlled by

immunotherapy. However, disappointing results have so far been obtained with immunotherapy. These studies include non-specific immunisation particularly with BCG (Neale and Matthews, 1980), specific immunisation with syngeneic and allogeneic tumour cells and transfer of immunity with lymphoid cells or their products (Fudenberg, 1980). On the other hand immunologically deficient patients may not have been properly stimulated to kill or retard their tumours.

∴

If the normal immune response is capable of preventing or inhibiting tumour growth, the tumour may take three routes to ensure its success (Guttman et al., 1981). Firstly, the tumours may succeed by taking advantage of lapses in immune capacity. Examples are: stressful situations (general anaesthesia and surgery), viral infection and the common cold, age, immunologic deficiency diseases and immunosuppressive agents.

Purtilo and Merino (1985) estimated the frequency of malignancy in patients with X-linked lymphoproliferative syndrome (XLP)-an immunodeficiency syndrome - where 35% developed B-cell lymphoma. An increased risk of cancer patients with common variable immunodeficiency (CVID) was also calculated (Purtilo and Merino, 1985).

Secondly, a tumour may escape from the immune control, like in the case of non-immunogenic tumours, or a tumour may become established by suppressing the immune response. Szuro-Sudal and co-workers (1983) suppressed

macrophage antimicrobial activity. This microbicidal defect might be relevant to the incidence of opportunistic infections in patients with malignant disease (Ruskin and Remington, 1976).

On the other hand, the immune system appears to be totally irrelevant to tumour control. Nowadays, human papilloma virus (HPV) is strongly implicated as an aetiological agent of cervical cancer (Syrjänen et al., 1985; Evans-Jones et al., 1985 and Champion et al., 1985). Evidence includes detection of HPV 16 and 18 DNA sequences in lesions of invasive cervical cancer, integrated in the cancer cell genome.

Sexual behaviour has been regarded as an important factor in cervical carcinogenesis (Champion et al., 1985). Included in the sexual behaviour is: early age of first intercourse, multiple sexual partners, unstable marital relations and venereal disease. The latter is supported by evidence obtained by Champion and colleagues which suggests that HPV is a sexually transmitted agent since there was an increased risk of cervical neoplasia in consorts of men with penile condilomata acuminata.

Lately mental attitudes to cancer have been implicated as an additional prognostic factor (Pettingale et al., 1985). An association between mental attitudes and survival of patients with cancer of the breast has been observed. The psychological responses that were assessed

three months post-operatively were related to the outcome five years later. Recurrence free survival was significantly commoner among patients who reacted to cancer by denial or "fighting spirit" than among patients who responded with stoic acceptance or feelings of helplessness or hopelessness.

It appears that susceptibility to cancer may be genetically determined (Lagadec et al., 1985). Lagadec and co-workers observed that five pathogen-free rat strains exhibited in vitro strain - dependent ^{..}cytolysis against DHD/K12/TS cancer cells. The percentage of natural cytolysis varied from one rat to another, but was significantly different according to strain. Boraschi et al., (1984) discovered that macrophages from genetically defective mouse strains C3H/HeJ, A/J and P/J were unable to develop high levels of antitumour activities when stimulated either with immune recombinant IFN- or with non-immune IFN- and IFN- as compared to macrophages or normal C3H/HeJ mice.

Most of the patients who were studied in the present investigation were from the Eastern Transvaal, where cancer of the cervix is extremely common. According to the hospital authorities, two new patients are admitted every week. So it is possible that genetics may play a role in cancer susceptibility considering that intermarriages are a common practice.

CONCLUSION

The in vitro expressions of immune activity in patients with cervical carcinoma indicate that they are immunocompromised. The depressed lymphocyte stimulation in vitro which probably reflects the development of cytotoxic T cells in vivo, may explain failure in killing of neoplastic cells. Patients' accessory cell mediated cytostasis and cytolysis are also depressed. Probably this is an additional factor why tumour growth is facilitated. The possibility that immunodeficiency is triggered by malignancy rather than the other way round, is not excluded.

From the results obtained it appears as if there is no relation between the kinetics of monocyte-macrophage IL 1 production and of cytopathic effects. When during the ageing of in vitro monocytes from 24 - 96 hours, their IL 1 productive capacity is lowered, whereas the cytopathic capacities increase with maturation. IL 1 activity could be masked by suppressive factors such as PG E₂.

It is extremely difficult to know how relevant these in vitro measurements are to in vivo immunity. There is still hope that if the relationship between T cell proliferation, monocyte IL 1 production, cytostasis and cytotoxicity, and interactions with other components of the immune system is established then immunotherapy may be the answer to the cure of cancer. This belief

is strengthened when one thinks about immunosuppressive agents which will often increase the rate of growth of a tumour suggesting that it was at least being hampered by immune mechanism (Fudenberg, 1982). At this point in time, the world is aware of an association between immunodeficiency and cancer development: acquired immunodeficiency syndrome (AIDS) and Kaposi's sarcoma.

•
••

REFERENCES:

1. Abe S., Gatanaga T., Yawazaki M., Soma G. and Mizuno D. (1985). Purification of rabbit tumour necrosis factor. FEBS letters 180 (2) : 203.
2. Adam C., Broughton E.S. and Scott M.T. (1977). Enhanced resistance of mice to infection with bacteria following pretreatment with Corynebacterium parvum. Nature New Biol. 235:219.
3. Adams D.O., Kao K.J., Farb R. and Pizzo S.V. (1980). Effector mechanisms of cytolytically activated macrophages. 11. Secretion of a cytolytic factor by activated macrophages and its relationship to secreted neutral proteases. J. Immunol. 124:293.
4. Adams D.O. and Marino P.A. (1981). Activation of mononuclear phagocytes for destruction of tumour cells as a Model for study of Macrophage development. Departments of Pathology and Microbiology - Immunology, Duke University Medical Centre, Durham, North Carolina. 27720.
5. Allison A.C. (1972). Immunity and immunopathology in virus infections.
6. Andersson L.C. (1973). Size distribution of killer cells during allograft response. Scand. J. Immunol. 2:75.
7. Beller D.I. and H.K. (1982). Regulation of macrophage populations: V Evaluation of the control of macrophage IA expression in vitro. J Immunol 129:971.
8. Beller D.I. and Unanue E.R. (1980). Ia-antigens and antigen presenting function of thymic macrophages. J. Immunol. 124:1433.

- dependent tumour cell lines - contribution of natural killer-like cells. Scand. J. Immunol. 18:439.
18. Chang Y. and Yao C. (1979). Investigation of the human macrophage. 11. The in vitro cytotoxicity of macrophage. Eur. J. Immunol. 9:521.
 19. Cher M.D., Beller L. and Unanue E.R. (1980). Demonstration of a soluble mediator that induces exudates rich in Ia⁺ macrophages. J. Exp. Med. 152:1684.
..
 20. Christie G.H. and Bomford G.H. (1975). Mechanisms of macrophage activation by Corynebacterium parvum. 11. In vitro experiments. Cell. Immunol. 17:150.
 21. Christman J.K., Silverstein S.C. and Acs G. (1977). Plasminogen activators - proteinases in mammalian cells and tissues edited by Barrett, North Holland. Amsterdam.
 22. Colotta F., Peri G., Villa A and Mantovari A. (1984). Rapid killing of actinomycin D-treated tumour cells by human mononuclear cells. I. Effectors belong to the monocyte-macrophage lineage. J. Immunol. 132(2):936.
 23. Cowing C., Pincus S.H., Sachs D.H. and Dickler H.B. (1978). A subpopulation of adherent accessory cells bearing both I.A. and I-E or subregion antigens is required for antigen-specific murine T-lymphocyte proliferation. J. Immunol. 121:1680.
 24. Di Paulo M., Angelini L., Bertolitti A., Collizza, S. (1974). Host resistance in relation to survival in breast cancer. Br. Med. J. 11:286.

25. Djeu J.Y., Heinbaugh J.A., Holden H.T. and Herberman R.B. (1979). Role of macrophages in the augmentation of mouse Natural Killer cell activity by poly I:C and interferon. *J. Immunol.* 122:182.
26. Dorf M.E. and Unanue E.R. (1978). Subpopulations of peritoneal macrophages identified with anti-Ia sera. In *Ir genes and Ia antigens*. McDevitt H.O., editor. Academic Press Inc. New York 174.
27. Eriksen E.F., Richelsen B., Becknielsen H., Melsen F., Nielsen H.K. and Mosekilde L. (1985). Prostaglandin - E2 receptors in human peripheral blood monocytes. *Scand. J. Immunol.* 21:167.
28. Edelson P.J. (1981). Macrophage plasma membrane enzymes as differentiation markers of macrophage activation. *Lymphokines* 3:57.
29. Evans R. and Alexander P. (1970). Co-operation of immune lymphoid cells with macrophages in tumour immunity. *Nature* 228:620.
30. Evans R. and Alexander P. (1972). Mechanism of immunologically specific killing of tumour cells by macrophages. *Nature* 236:168.
31. Evans R., Grant C.K., Cox H., Steele K. and Alexander P. (1972). Thymus-derived lymphocytes produce an immunologically specific macrophage-arming factor. *J. Exp. Med.* 136-1318.
32. Evans-Jones J.C., Forbes-Smith P.A. and Hirschowitz L. (1985). Follow up of women with cervical Koilocytosis. *The Lancet* 1(8443): 1445.
33. Falk W., Harvath L., Leonard E.J., (1982). Functionally distinct subpopulations of human monocytes: receptors for

F-Met-Leu-Phe are expressed only on the chemotactically responsive cells in macrophages and Natural Killer cells. Regulation and Function. (Norman S.J. and Sorkin E., editors).

34. Fidler I.J. (1976). Macrophage deficiency in tumour-bearing animals: Control of experimental metastasis with macrophage activated in vitro in: The macrophage in Neoplasia (editor M. Fink) p. 245. Academic Press, New York.
35. Fruehauf P., Bonnard G.D. and Herberman R.B. (1982). The effect of lectins on production of Interleukin 1 by human monocytes.
36. Fudenberg H.H., Stites D.P., Caldwell J.L. and Wells J.V. (1982). Basic and Clinical Immunology (4th edition).
37. Gallily R., Zeira M. and Stain I. (1985). Thymus-derived macrophages in long-term culture: Release of Interleukin 1 activity by normal human peripheral blood B lymphocytes. J. Immunol. 132(2): 1132.
38. Gauci C.L., Alexandra P. (1975). The macrophage content of some human tumours. Cancer Lett. 1:29.
39. Gearing A.J.H., Johnstone A.P. and Thorpe R. (1985). Production and Assay of interleukins. J. Immunol. Methods 83:1.
40. Gemsa D. (1981). Stimulation of prostaglandin E2 release from macrophages and possible role in immune response. In Lymphokines (E. Pick, editor). 4:335. Academic Press, New York.
41. Gemsa D., Kramer W., Napierki I. Bärlin E., Till G. and Resch K. (1981). Potentiation of macrophage tumour cytostasis by tumour-induced ischitis. J. Immunol. 126:2143.

42. Gery I. (1982). Production and assay of Interleukin 1. In Isolation characterization and Utilization of T-lymphocyte clones. (Fathman G. and Fitch F.W., editor).
43. Gery I., Davies P., Derr J., Krett N. and Barranger J.A. (1981). Production and release of lymphocyte activating factor (Interleukin 1) by murine macrophages. I. Effects of various agents. *Cell. Immunol.* 64:293.
44. Gery I. Waksman B.H. (1972). Potentiation of the T-lymphocyte response to mitogens II. The cellular source of potentiating mediator(s). *J. Exp. Med.* 136:143.
45. Hammerström J. (1979). Human monocyte-mediated cytotoxicity to K562 cells : activation by lymphokines. *Scand. J. Immunol.* 10:575.
46. Haskill J.S., Häyry P. and Radör L.A. (1972). Systemic and local immunity in allograft and cancer rejection. *Contemporary topics in Immunobiology* 8:107. (Warner N.L. and Cooper M.D., editors).
47. Haskill S., Koren H., Becker S., Fowler W. and Walton L. (1982). Mononuclear cell infiltration in ovarian cancer. III. Suppressor-cell and ADCC activity of macrophages from ascitis and solid ovarian tumours. *Br. J. Cancer* 45:747.
48. Hayari Y., Kukulansky T. and Globerson A. (1985). Regulation of thymocyte proliferative response by macrophage-derived prostaglandin E2 and Interleukin 1. *Eur. J. Immunol.* 15:43.
49. Hibbs J.B., Jr., Lambert L.H., Jr. and Remington L. (1971). Resistance to murine tumours conferred by chronic infection of intracellular protozoa Toxoplasma gondii and Besnoitia jellisoni. *J. Inf. Dis.* 124:587.

50. Hodes R.J., Abraham G.B., Hathcock K.S., Dickler H.B. and Singer A. (1978). Cellular and genetic control of antibody responses in vitro. IV. Expression of Ia antigens on accessory cells required for responses to soluble antigens including a response under Ir gene control. J. Immunol. 121:1501.
51. Hofdfman T., Chano Z.L., Stevenson H.C., Trinchier G. and Herberman R.B. (1983). Characterization by monoclonal antibodies of the cytostatic effector cells reactive against anchorage-dependent tumour cell lines. Scand. J. Immunol. 18:451.
52. Ido K., Masuda T., Miyama-Inaba M., Aotsuka Y., Kura F., Komatsubara S., Ido M. and Muramatsu S. (1982). Ontogeny of "macrohage", function IV. Newborn mouse macrophage strongly suppress tumour cell growth and readily acquire cytolytic activity in comparison with adult macrophages. Immunol. S2. 307.
53. Imboden J.B., Weiss A. and Stobo J.D. (1985). The antigen-receptor on human T-cells line initiates activation by increasing cytoplasmic free calcium. J. Immunol. 134 (2) : 663.
54. Kaplan A.P., Castellini F.J., Collon D., Wiman B. and Taylor F.B. (1978). Molecular mechanism in fibrinolysis in man. Thrombos. Haemos. 39:263.
55. Kay J.E. (1980). The nature of mitogenic signal. Biochemistry of cellular regulation .1 (1)5 Clemens M.J., editor).

56. Kaye J., Gillis S., Mizel S.B., Shevach E.M., Malek T.R., Dinarello C.A., Lachman L.B. and Janeway C.A. Jr. (1984). Growth of a cloned helper T-cell line induced by a monoclonal antibody specific for antigen-receptor: Interleukin 1 is required for the expression of receptors for Interleukin 2. *J. Immunol.* 133 (3): 1339-1345.
57. Keller R. (1978). Macrophage-mediated natural cytotoxicity against various target cells in vitro. I. Macrophages from diverse anatomical sites and different strains of rats and mice. *Br.J. Cancer* 37:732.
58. Keller R. (1974). Modulation of cell proliferation by macrophages. A possible function apart from cytotoxic tumour rejection. *Brit. J. Cancer* 30:401.
59. Keller R. (1973). Cytostatic elimination of syngeneic rat tumour cells in vitro by non-specifically activated macrophages. *J. Exp. Med.* 138:625.
60. Kelley V. (1982). Increase in Ia positive macrophages in MRL autoimmune mice. *Fed. Proc.* 41:841.
61. Kelso A., Glasebrook A.L., Kanagawa O. and Brunner K.T. (1982). Production of macrophage-activating factor by the lymphocyte-clones and correlation with other lymphokine activities. *J. Immunol.* 129 (2) : 550.
62. Khansari I., Chou Y.K. and Fudenberg H.H. (1985). Human monocyte heterogeneity : Interleukin and prostaglandin E production by separate subsets. *Eur. J. Immunol.* 15:48.
63. Kiczka W., Szkaradkiewer A. and Flieger J. (1985). Studies on natural cytotoxicity of human monocytes in viral hepatitis. *Immunol. Letters* 9:87.

64. Kirchner H., Hirt H.M. and Munk K. (1977). Protection against Herpes simplex virus infection in mice by Corynebacterium parvum. *Infect. Immunol.* 16:9.
65. Klein E. and Klein G. (1964). Antigenic properties and lymphomas induced by the Moloney Agent. *J. Nat. Cancer Inst.* 32:547.
66. Klein G., Sjögren H.O., Klein E. and Hellström K.E. (1960). Demonstration of resistance against methylcholanthrene- induced sarcomas in the primary autochthonous host. *Cancer Res.* 20:1561. . .
..
67. Koren H., Handwerger B., Wunderlich J. (1975) Identification of macrophage-like characteristics in a cultured murine tumour line. *J. Immunol.* 114:894.
68. Kurt Jones E.A., Beller D.I. Mizel S.B. and Uranue E.R. (1985). Identification of a membrane associated Interleukin 1 in macrophages. *Proc. Nat. l Acad. Sci.* 82:1204.
69. Lagadec P., Jeannin J., Milan C. and Reissner D. (1985). Strain dependence on rat macrophage natural tumoricidal capacity and its stimulation by endotoxins. *Immunol. Letters* 9:117.
70. Larson E.L., Countinho A. and Martinez C. (1980). A suggested mechanism for T-lymphocyte activation: Implications on the acquisition of functional reactivities. *Immunol. Rev.* 51:61.
71. Lauder I., Aherne W., Stewart J., Sainsbury R. (1977). Macrophage infiltration of breast tumours: a prospective study. *J. Clin. Pathol.* 30:563.
72. Lepe-Zuniga J.L. and Grey I. (1984). Production of
- and extracellular Interleukin 1 (III) by

- human monocytes. Clin. Immunol. Immunopathol. 31:222.
73. Lombard G., Piccolella E., Vismara D., Colizzi V. and Asherson G.L. (1984). Candida albicans polysaccharide extract (MPPS) and PPD stimulate the production of Interleukin 1 and lymphocyte proliferation. Clin. Exp. Immunol. 58:581.
74. Lutz C.T., Glasebrook A.L. and Fitch F.W. (1981). Alloreactive cloned T cell lines IV. Interaction of alloantigen and T-cell growth factors (TCGF) to stimulate cloned cytolytic T-lymphocytes. J. Immunol. 127:391.
75. Maizel A.L., Menta S.R., Ford R.J. Lachman L.B. (1981). Effect of Interleukin 1 on mouse thymocytes and purified human T-cells. J. Exp. Med. 153:470.
76. Männel D.N., Moore R.N. and Mergenhagen S.E. (1980). Macrophages as a source of tumoricidal activity (tumour-necrotizing factor). Infect. Immunol. 30:523.
77. Matsushima K., Procopio A., Abe H., Scala G., Ortaldo J.R., Oppenheim J.J. (1985). Production of Interleukin 1 activity by normal human peripheral blood B lymphocytes. J. Immunol. 132(2):1132.
78. Meller G. (1982). Interleukins and lymphocyte activation. Immunol. Rev. p63.
79. Meuer S.C., Acuto O., Hussey R.E., Hodgdon J.C., Fitzgerald K.A., Schlossman S.E. and Reinherz E.L. (1983). Evidence for the T 3-associated 90 K heterodimer as the T-cell antigen receptor. Nature 303:808.
80. Michell B. (1983). Ca^{2+} and protein kinase C : two synergistic cellular signals. Trends Biochem. Sci. 8:263.

81. Milas L. and Scott M.T. (1978). Antitumour activity of Corynebacterium parvum. Adv. Cancer Res. 26:257.
82. Miner K.M. and Nicolson G.L. (1983). Differences in the sensitivities of murine metastatic lymphoma/lymphosarcoma variants to macrophage-mediate cytotoxicity and/or cytostasis. Cancer Res. 43:2063.
83. Mizel S.B. and Mizel D. (1981). Purification to apparent homogeneity of murine Interleukin 1. Immunol. 126:834.
84. Mizel S.B. Oppenheim J.J. and Rosenstreich D.L. (1978). Characterization of lymphocyte activating factor. J. Immunol. 118:1631.
85. Moretta A. Lolombatti M. and Chapnis B. (1981). Human Spleen cells as a source of T-cell growth factor. Clin. Exp. Immunol. 44:262.
86. Mori M. and Hayward A.R. (1984). Persistence of Ia antigen and antigen-presenting activity by cultured human monocytes. Clin. Immunol. Immunopathol 30:387.
87. Murphy P.A., Simon P.L. and Willough W.F. (1980). Endogenous pyrogens made by rabbit peritoneal exudate cells are identified with lymphocyte activating factor made by rabbit alveolar macrophages. J. Immunol. 124:2498.
88. Neale M.L. and Matthews N. (1980). Antimicrobial effects of a macrophage-derived cytotoxin from serum of BCG-primed rabbits (tumour necrosis serum). J. Med. Microb. 17(2):211.
89. Nelson D.S. (1981). Macrophages : progress and problems. Clin. Exp. Immunol. 45:225.

90. Nelson K., Pollack S.B. and Hellström K.E. (1975). In vitro synthesis of tumour specific factors with blocking and antibody-dependent cellular cytotoxicity (ADC) activities. Int. J. Cancer 16:932.
91. Nissen-Meyer J. and Kildahl- Andersen O. (1984). Purification of Cytostatic protein factors released from human monocytes. Scand. J. Immunol. 20:317.
92. Nissen-Meyer J. and Seim S. (1983). Effect of human monocyte-released cytostatic factors on target cell DNA synthesis. Protein synthesis and ATP content. Scand. J. Immunol. 18:465. ..•
93. Nussenzweig M.C. Steinman R.M., Unkeless J.C. Witmer M.D., Gutchinor B. and Cohen Z.A. (1981). Studies of the cell surface of mouse dendritic cells and other leukocytes. J. Exp. Med. 154:168.
94. Oppenheim J.J. and Gery I. (1982). Interleukin 1 is more than an interleukin. Immunol. Today 3(4):113.
95. Palacios R. (1985). Mechanisms by which accessory cells contribute in growth of resting T-lymphocytes initiated by OKT3 antibody. Eur. J. Immunol. 15:645.
96. Preble O.T., Black R.J., Friedman R.M., Klippel J.H. and Vileek J. (1982). SLE: presence in human serum of an unusual acid-labile leucocyte interferon. Science 216:429.
97. Rabson A.R. and S.A.M.R.C. Human Cellular Immunology unit (1983). The effect of Interleukin 1 on Natural killer cell-tumour cell interactins. S.A.I.M.R. Annual Report p.35.
98. Rao A., Mizel S.B. and Cantor H. (1983). Disparate functional properties of two interleukin 1 - responsive Ly-1⁺2⁻ T cell clones : distinction of T-cell

- growth factor activities. *J. Immunol.* 130:1743.
99. Richman L.K., Klingenstein R.J., Richman J.A., Strober W., and Berzofsky J.A. (1979). The murine kupffer cell Characterization of the cell serving accessory function in antigen - specific T-cell proliferation. *J. Immunol.* 123:2602.
100. Roder J.C., Helfand S., Werkmeister J., McGarry R. Beaumont T.J. and Duwe A. (1982). Oxygen intermediates are triggered early in the cytolytic pathway of human NK cells. *Nature* 298:569.
101. Römbloom L., Ramstedt U. and Alm G.V. (1983). Properties of human natural interferon - producing cells by tumour cell lines. *Eur. J. Immunol.* 13:471.
102. Roosnek E.E., Brouwer M.L., Aarden L.A. (1985). T-cell triggering by lectins. I. requirements for interleukin - 2 production, lectin concentration, determines the accessory cell dependency. *Eur. J. Immunol.* 15:652.
103. Rosenstreich D.L. and Mizel S.B. (1979). Signal requirements for T-cell activation. I. Replacement of macrophage-function with phorbol myristate acetate. *J. Immunol.* 123:1749.
104. Rossenwasser L.J., Dinarello C.A. and Rosenthal A.S. (1979). Adherent cell function in murine T-lymphocyte antigen recognition. IV. Enhancement of murine T-cell antigen recognition by human leukocyte pyrogens. *J. Exp. Med.* 150-709.
105. Russel S.W. and Pace J.L. (1984). Gamma interferon interferes with negative regulation of macrophage activation by prostaglandin E2. *Molecular Immunology* 21 (3):249.

106. Saunder D.N., Mounessa N.L., Katz S.I., Dinarello C.A. and Gallin J.I. (1984). Chemotactic cytokines: The role of leukocyte pyrogen and epidermal cell thymocyte activating-factor in neutrophil chemotaxis. *J. Immunol.* 132(2):828.
107. Schwartz R.H., David C.S., Sachs D.S. and Paul W.E. (1976). *J. Immunol.* 117:531.
108. Shaw J.O., Russel S.W., Printz M.P. and Skidgel R.A. (1979). Macrophage-mediated cell killing : Lack of independence on the cyclooxygenase pathway of prostaglandin synthesis. *J. Immunol.* 123:50.
109. Sjögren H.O., Hellstrom I., Bousal S.C., Warner C.A. and Hellstrom K.E. (1972). Elution of "blocking factors" from human tumours, capable of abrogating tumour-cell destruction by specifically immune lymphocytes. *Int. J. cancer* 9:274.
110. Sletten Mark B. and Klein E. (1962). Cytotoxic and neutralization tests with serum and lymphnode cells of isologous mice with induced resistance against lymphomas. *Cancer Res.* 22:947.
111. Slijic V.S. and Watson S.R. (1977). The adjuvant effect of Corynebacterium parvum. T-cell dependence of macrophage activation. *J. Exp. Med.* 145:45.
112. Smith H.R., Green D., Raveche E., Smathers P., Gershon R. and Steinberg A.D. (1982). The induction of autoimmunity in normal mice. *Fed. proc.* 41:954.
113. Smith K.A., Lachman L.B., Oppenheim J.J. and Favata M.F. (1986). The functional relationship of interleukins. *J. Exp. Med.* 151:1551.
114. Steeg P.S., Moore, R.N., Johnson H.M. and Oppenheim J.J. (1982). Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. *J. Exp. Med.* 156:1780.

115. Steeg P.S., Moore R.N. and Oppenheim J.J. (1980). Regulation of murine macrophage Ia antigen expression by products of activated spleen cells. *J. Exp. Med.* 152:1734.
116. Steeg P.S., Moore R.N. and Oppenheim J.J. (1981). Cytokine induces macrophage Ia antigen expression. *Fed. Proc.* 40:771.
117. Steinman R.M., Nogueira N., Witmer M.D., Tydings J.D. and Mellman I.S. (1980). Lymphokine enhances expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. *J. Exp. Med.* 152:1248.
118. Stevenson H.C., Katz P. and Fauci A.S. (1979). Purification of human monocyte-macrophages by a cell suspension culture technique. *Cell. Immunol.* 53:94.
119. Sullivan C.P., Kenny G. and Waldman H. (1984). The role of major histocompatibility complex in in vitro antibody responses, M.H.C. restriction in response involving linked recognition of antigen determinants is not solely consequent to T-cell accessory cell restrictions. *Immunology* 51:351.
120. Syrjänen K., De Villiers, E., Saari Koski S., Castren O., Vayrynen M., Mantyjärvi R. and Parkkinen S. (1985). Cervical papillomavirus infection progressing to invasive cancer in less than three years. *The Lancet* - 1 (8427):510.
121. Szuro-Sudol A., Murray H.W., and Nathan C.F. (1983). Suppression of macrophage antimicrobial activity by a tumour cell product. *J. Immunol.* 131: 384.
122. Taffet S.M., Pace J.L. and Russel S.W. (1981). Lymphokine maintains macrophage activation for tumour cell killing by interfering with negative regulatory effect of prostaglandin E₂. *J. Immunol.* 127:121.

123. Taffet S.M. and Russel S.W. (1981). Macrophage-mediated tumour cell killing : regulation of expression of cytolytic activity by prostaglandin E₂. J. Immunol. 126:424.
124. Ting R.C. and Law L.W. (1976). Thymic function and carcinogenesis. Prog. Exp. Tumor Res. 9:165.
125. Tracey D.E. (1979). The requirements for macrophages in the augmentation of NK cell activity by BCG. J. Immunol. 123 : 840.
126. Tracey D.E. and Adkinson N.F. Jr. (1980). Prostaglandin synthesis inhibitors potentiate the BCG induced augmentation of NK cell activity. J. Immunol. 125 : 136.
127. Turchik J.B., Bornstein D.L. (1980). Role of central nervous system in acute - phase responses to leukocytic pyrogen. Infect Immunol. 30:439.
128. Uchida A. and Vanagawa E. (1984). Natural cytotoxicity of human blood monocytes : Production of monocyte cytotoxic factors (MCF) during interaction with tumour cells. Immunology Letters 8:311.
129. Unanue E.R. (1980). The regulation of lymphocyte functions by macrophage. New Eng. J. Med. 303:1153.
130. Urban J.L. and Schreider H. (1983). Selection of macrophage resistant progressor tumour variants. J. Exp. Med. 157:642.
131. Van Furth R. (1980). Mononuclear phagocytes. Functional Aspects. Parts I and II.

132. Vose V.B. (1978). Cytotoxicity of adherent cells associated with some human tumours in lung tissues. *Cancer Immunol. Immunother.* 5:173.
133. Walker E.B., Lanrer L.L. and Warner N.L. (1982). Concomitant induction of the cell surface expression of Ia determinants and accessory cell function by a murine macrophage tumour cell line. *J. Exp. Med.* 152:1684.
134. Weinberg J.B., Hobbs, M.M. and Pizzo S.V. (1980). Micro-assay for the photometric quantitation of cell-associated plasminogen activator using a chromogenic tripeptide substrate. *J. Immunol. Meth.* 75:289.
135. Weiss A., Imboden J., Shoback D. and Stobo J. (1984). Role of T_3 molecules in human T-cell activation: T_3 dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. U.S.A.* 81:4169.