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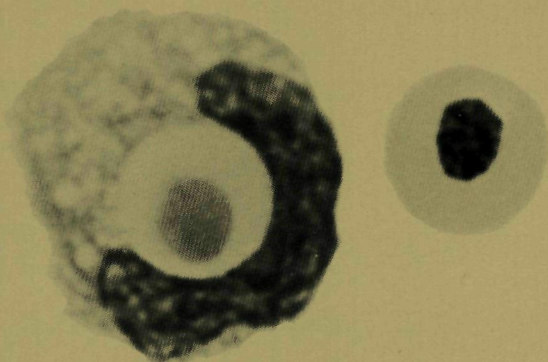

MONOCYTE FUNCTION IN HODGKIN'S DISEASE

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE AAN DE KATHOLIEKE
UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS
PROF. DR. J.H.G.I. GIESBERS
VOLGENS BESLUIT VAN HET
COLLEGE VAN DEKANEN IN HET
OPENBAAR TE VERDEDIGEN OP
VRIJDAG 10 JUNI 1983
DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

PIETER HENRI MARIA DE MULDER
GEBOREN TE HEERLEN

1983
DRUK: STICHTING STUDENTENPERS NIJMEGEN

The investigations presented in this thesis were performed in the Division of Hematology (head Prof. Dr. C. Haanen), Department of Medicine (head Prof. Dr. C.L.H. Majoor†, at present Prof. Dr. A. van 't Laar), University Hospital St. Radboud, Nijmegen The Netherlands.

The studies were supported by a grant (NUKC 79-10) from the Queen Wilhelmina Foundation, the Netherlands Organization against Cancer (KWF/NOK), the Maurits & Anna de Kock Foundation and the Ank van Vlissingen Foundation.

Aan Willemien en Huib

Aan mijn ouders

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CHAPTER 1

INTRODUCTION AND OUTLINE OF INVESTIGATION

INTRODUCTION

Since the presentation of Thomas Hodgkin's classical paper [1] entitled 'On some morbid appearances of the absorbent glands and spleen', and the meticulous description of the pathognomonic multinucleated giant cells by Sternberg [2] in 1898 and Reed [3] in 1902, this remarkable disease has challenged many clinicians and pathologists to disclose its nature and to contend with its previously fatal outcome. The results of these investigations, concerning the etiology, pathophysiology, classification, staging procedures, and treatment modalities were reviewed recently [4-11].
In this mosaic of facets relatively sparse attention was given to the role of the mononuclear phagocyte system in the pathophysiology of Hodgkin's disease (HD).

MONONUCLEAR PHAGOCYTE SYSTEM

It has been more than a decade since the concept of the mononuclear phagocyte system (MPS) was proposed to delineate the reticuloendothelial system, which is composed of monoblasts, promonocytes, monocytes and the structurally and functionally heterogeneous tissue macrophages [12]. This concept has been widely accepted. The components of MPS display a great variety of functions [13-18]. They collect the garbage of the body and are therefore involved in the debridement of wounds and the assimilation of senescent cells. The system is regarded as an important defense mechanism towards facultative and obligate intracellular parasites, such as the Mycobacterium Tuberculosis, Listeria Monocytogenes, Salmonella, and Legionella Pneumophilia. Another well defined function is their important accessory role in the induction and expression of humoral and cellular immunity. A vast burden of secretory products is found, such as enzymes, enzyme inhibitors, complement components, binding proteins, endogenous pyrogens, metabolites of nucleic acid metabolism, bioactive lipids and growth factors [15,19,20].
Animal studies suggest that the macrophage plays a part in the defensive reactions against neoplasia. However, direct and compelling evidence, that macrophages are important in human cancer is not yet available [21].

OUTLINE OF INVESTIGATION

The first aim of the study was the investigation of monocyte function in HD. Chapter 2 comprises a review of studies dealing with various aspects of MPS function in HD.

The most accessible cell of the MPS is the blood monocyte and therefore the

most studied component. All studies in HD were performed by using either mononuclear leukocyte suspensions or monocytes, purified on the basis of their adherent properties. The first objective of our study was the development of a monocyte purification method, resulting in good purity and yield, avoiding selection and activation. A recently described counterflow-centrifugation technique [22] formed the basis of the new separation procedure. The results have been presented and discussed in chapter 3.

As an important effector function the antibody-dependent cell- mediated cytotoxicity (ADCC) was evaluated using a DNA flow cytometric assay [23]. The results in HD have been described in chapter 4.

Whether the observed ADCC-pattern was HD-specific and/or possibly related with overt disease is dealt with in chapter 5.

An alternative approach of cell function analysis is the study of some aspects of cell metabolism. The intracellular activity of 3 enzymes of intermediary metabolism and 2 acid hydrolases were analysed in purified monocytes as well as in lymphocytes (chapter 6).

Chapter 7 deals with the results of the in vitro monocyte maturation in patients with HD, using two culture modalities.

In chapter 8 some results concerning the secretory capabilities of these monocyte derived macrophages are given.

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CHAPTER 2

ROLE OF THE MONONUCLEAR PHAGOCYTE SYSTEM IN THE PATHOPHYSIOLOGY OF HODGKIN'S DISEASE.

A review of the literature

P.H.M. de Mulder & B.E. de Pauw

Parts of this chapter are submitted for publication in 'Blut'.

INTRODUCTION

One of the most striking features in the histopathology of Hodgkin's disease (HD) is the absence of easily recognizable malignant cells, which form a tumor with invasive characteristics. The picture is dominated by cells apparently involved in tumor-host interactions, which are more prominent in HD than in any other neoplastic disease. Despite, or even because of, this presumed host reactivity at the site of the malignant process, the overall defence mechanisms in HD are frequently compromised even in the early stages of the disease [1-3].

This peculiar disease pattern has inspired many investigators to try to unravel the pathophysiology of HD. In most studies the role of the lymphocyte has been the primary object of study [4]. Scant attention has been given to the part played by the mononuclear phagocyte system (MPS), neither to its function in the afferent limb of the immune response nor to its effector capabilities.

The purpose of this paper is to review the data which have been reported concerning the function of the MPS in HD and the continuing controversy, regarding the macrophage origin of the supposed malignant cell.

KINETICS AND DISTRIBUTION OF MONONUCLEAR PHAGOCYTES IN HD

The MPS comprises cells which are predominantly derived from the granulocyte/macrophage progenitor cell in the bone marrow [5] and which are widely distributed all over the body.

Meuret et al. [6] demonstrated by means of in vitro ^{3}H - thymidine incorporation studies on bone marrow samples, a very significantly elevated monocyte production in 8 patients with HD, which returned to normal during long-term complete remissions. In non-Hodgkin lymphomas a diminished as well as normal or even increased monocyte production has been reported [6].

Bunting was the first to describe an absolute monocytosis in HD [7]. In Table 1 various studies are summarized which show counts ranging from low to elevated [6,8-10]. No relation with stage or activity of the disease was found. Abnormal monocytes (up to 30%), often resembling Reed-Sternberg cells, have been observed in the peripheral blood or in leukocyte concentrates of some patients with HD [11-13]. The appearance of these cells seemed to be associated with febrile periods [12.13].

Under normal circumstances peripheral blood monocytes marginate [14], i.e. adhere at some moment to the walls of the post capillary venules, and then leave the circulation and mature to macrophages in the various tissues. No information is available concerning the monocyte disappearance rate in HD. There is some evidence indicating a diminished in vitro yield of adherent monocytes from mixed mononuclear cell suspensions [15,16], which might

Author		% of patients with monocytes<500 mm³	n
Bunting	1914	100	25
Wiseman	1936	61	31
Levinson	1957	40	43
Meuret	1978	50	8
Kaplan	1980	37	100

Table 1. Absolute peripheral blood monocyte counts in Hodgkin's disease.

reflect adherence in vivo. On the other hand Urbanitz et al. [17] found a normal adherent cell yield, using enriched (up to 62%) monocyte suspensions. However, these studies provided no definite answer concerning monocyte adherent capacity, because the possible influence of the considerable lymphocyte admixture was not evaluated.
In vivo studies of skin-window macrophage cellularity provided conflicting results with a normal cell accumulation in adults [18] and a diminished accumulation in children with HD [19]. Katz [20] identified fewer macrophages in 44 specimens of uninvolved disaggregated tissues (lymph nodes or spleens) from patients with HD than in normal control tissues. None of the techniques employed have supplied reliable quantitative data about the macrophage distribution in several tissues in HD patients.

FUNCTION OF THE PERIPHERAL BLOOD MONOCYTE AND THE IN VITRO MONOCYTE-DERIVED MACROPHAGE

Afferent functions

Afferent monocyte functions in this section imply the influences of monocytes on antigen and mitogen induced lymphocyte responses.
In a few patients with advanced HD a normal antigen induced response was obtained, when purified allogeneic normal lymphocytes were incubated with precultured adherent patient monocytes [15]. These experiments with macrophages, which had been selected on basis of adhesiveness, may suggest a normal antigen presenting function of these cells. However, antigen presentation is an Ia restricted function and the observed lymphocyte response is therefore most likely induced by the remaining autologous monocytes in the 'purified' lymphocyte suspensions.
In several studies impairment of mitogen induced lymphocyte transformation was completely or partially reversed after depletion of adherent cells (i.e. adherent monocytes) from the mononuclear cell (MNL) suspension [21-27]. In

patients with a complete remission this impairment was less constantly observed [22-25,27]. Addition of adherent autologous monocytes resulted in a reinducement of the inhibition in patients with HD, at similar monocyte lymphocyte ratios this was not observed in normal controls. Macrophages of HD patients revealed no suppression on normal allogeneic lymphocytes [23]. This observation suggests an increased sensitivity of HD lymphocytes for eventual normal monocyte suppression. This recombination study was done with a very limited number of patients and controls.

In the supernatant of phytohaemagglutinin (PHA) stimulated MNL suspensions a fourfold higher prostaglandin-E_2 (PGE_2) level was found in comparison to normal controls [21]. Depletion of glass adherent cells resulted in a 80% reduction of PGE_2. The supposition regarding the inhibitory role of PGE_2 in mitogen induced lymphoblastogenesis was corroborated by the observation that indomethacin, a cyclooxygenase inhibitor, partially restored the impaired transformation [21,23,24,27]. Bockman [28] could actually prove an enhanced PGE_2 production by 24 h adherent monocytes in HD. The in vitro effect of indomethacin was observed also in the mitogen stimulated MNL suspensions from 2 out of 4 patients after oral use of indomethacin, whereas this had no effect on skin test responses [24]. In contrast with the earlier suggested possibility of an increased sensitivity of lymphocytes for monocyte suppression, no increased susceptibility of HD lymphocytes for exogenous supplemented PGE_2 was found [22].

Beside PGE_2, hydrogen peroxide appeared to be suppressive as well [27]. When catalase, promoting the reduction of hydrogen peroxide, was added to an indomethacin supplemented PHA stimulated MNL suspension of HD patients, an additional improvement of the proliferative response was observed [27]. Despite the abrogation of these two suppressor mechanisms, a subnormal response still remained, once more pointing towards a lymphocyte defect as well.

Twomey et al. [26] noted a positive correlation between the percentage of monocytes in the MNL and the level of suppression on B and T cell level. Since the monocyte admixture in MNL obtained from HD patients is quite often more pronounced than in normal controls [22,26], a quantitative, more than a qualitative effect of monocytes may be involved. This conclusion was partially in contrast with the findings of Schechter and Soehnlen [22], who demonstrated an equal amount of inhibition using either the original or half the number of monocytes in PHA driven lymphocyte blastogenesis. In favour of a quantitative effect was the observed relationship between a low lymphocyte/monocyte ratio in the peripheral blood, normally resulting in a higher percentage of monocytes in the MNL suspension, and the presence of suppression [22].

The main objection against most of these studies is the variable composition of the MNL suspension due to different separation procedures. Ideally one should work with the purified elements (i.e. lymphocytes and monocytes)

recombined at a constant ratio. The fact that none of the cultures showed an abolishment of the mitogen induced response after depletion of adherent cells (monocytes), suggests that the residual monocytes still could provide sufficient help, since pure lymphocytes have a low proliferative response to mitogens [30].

Only one study has been reported in which concanavalin-A induced blastogenesis was evaluated, using purified monocytes, selected by adherence, and lymphocytes, at a constant monocyte / lymphocyte ratio. No abnormal suppression could be demonstrated on both autologous (patient) and normal lymphocytes,

Also impairment of lymphocyte reactivity and transformation inducing capacity in mixed leukocyte cultures was found in HD [26,32-36]. Hillinger et al. [32] reported a significantly decreased base-line response by autologous mitomycin treated MNL in patients with active HD as well as in patients in remission. Monocyte and lymphocyte mediated suppression could be demonstrated. In the mixed lymphocyte reactivity test this suppression appeared to be positively related with the number of monocytes [26], however for HD lymphocytes a much lower monocyte concentration seemed to be required than for normal lymphocytes. This was corroborated by the finding of an increased sensitivity of peripheral blood lymphocytes in HD, to normal adherent suppressor cells [37].

The severely impaired T-cell proliferation induced by irradiated autologous non-T cells (autologous MLC) was shown to be monocyte independent [35], this suggests a dysfunction, a reduction of the responder T-cells [35] or a T-cell mediated suppression. A marked stage dependent reduction of T-colony formation was found, coinciding with an augmented PGE_2 production by adherent monocytes, whereas inhibition of prostaglandin synthesis only partially restored T-colony formation [28].

The in several in vitro systems found suppression, as discussed above, was neither disease specific nor was it demonstrated in all patients studied [22,31-36,38-42]. One should realize that the significance of this in vitro suppressor activity with regard to the etiology, clinical course, and prognosis of HD is unknown. Some effects may be even artificial due to laboratory bias. However, from the reviewed literature the conclusion seems justified, that a subpopulation of monocytes can exert suppressive effects on T-cells and maybe also on B cell activation in patients with HD. Furthermore it is not excluded that an intrinsic T-cell defect, lymphocyte mediated or serum factor dependent [43] suppression, may underly to the impaired T-cell proliferation in HD.

Effector functions

Monocyte chemotactic response and bactericidal activity were impaired in 6 out of 13 patients with advanced HD [44]. In 5 patients with stage II HD

Author	n patients	effector cell	microorganism	killing	Phagocytosis	Inhibition of growth
Cline '70	14	4-9 days cultured adherent monocytes	Listeria Monocytogenis	↑ **	nd	nd
King '75	4	MNL	Staphylococcus Aureus	N	nd	nd
Steigbigel '76	29	MNL	Escherichia Coli Listeria Monocytogenis	N	nd	nd
	9	7 days cultured adherent monocytes	Salmonella, Listeria Monocytogenis	nd nd	↑ * + ↑ * †	N ↓ * +
Leb '78	18					
	ST II 5	MNL	Escherichia Coli	N	nd	nd
	STIII-IV 13	MNL	Escherichia Coli	↓ *	nd	nd
Esteves '80	8	1 day adherent monocytes	Candida Pseudotropicalis	↓ *	↓ *	nd

* P < 0.05

** P < 0.01

† only significant after 1,5 h incubation, not after 3.5 h and 6 h.

N = normal ; nd = not done; ↑ = increased; ↓ = decreased; MNL = mononuclear leukocytes.

Table 2. Bacterial killing phagocytosis and growth inhibition by monocyte derived macrophages

monocyte chemotactic response appeared to be normal. Kitahara et al. [45] found a normal mean monocyte chemotaxis, although considerable variations were found. Studies concerning bacterial killing and phagocytosis by peripheral blood monocytes and derived macrophages are summarized in Table 2 [44,46-49]. No final conclusions can be drawn. In none of the studies freshly purified monocytes were used, and no correction was made for the variable admixture of lymphocytes in the MNL. The monocyte derived macrophages were all selected on basis of their adherent properties and, consequently, not representative for all circulating monocytes. Furthermore, correct quantitative interpretation of phagocytosis and killing is limited with the methods used [50].

Phagocytosis of IgG-coated human red blood cells by adherent monocytes was studied by Urbanitz et al. [17]. A significantly lower number of phagocytised red cells was observed in patients with advanced HD in comparison to normal controls. In stage II B patients the phagocytic capacity was enhanced instead of diminished. Holm et al. [31], applying a similar assay, observed a normal phagocytic activity in patients with HD.

Monocyte mediated antibody-dependent cellular cytotoxicity was evaluated in 3 studies and was found to be diminished [51], normal [31] and enhanced [52] as indicated in Table 3. Differences in target cells, source of antibody, effector cell population and the numbers of studied patients may have attributed to the diversity in results.

These results permit no conclusion regarding effector cell function of monocytes in patients with HD.

Metabolic aspects

Limited information is available concerning metabolic events in monocytes from patients with HD. One might assume that changes in afferent and efferent functions are reflected in an altered metabolic activity. However, it is difficult to delineate the complex metabolic backgrounds of certain biologic functions. After activation of macrophages, inducing a great variety of changes in stucture and function, an enhancement of the glucose metabolism, especially through the hexose monophosphate shunt (HMPS), is one of the first observed phenomena [53-55]. King et al. [56] showed an increased glycolysis, HMPS and Krebs's cycle activity in adherent monocytes of patients with malignant lymphoma, including 12 patients with HD, in comparison to normal control monocytes.

Nitroblue tetrazolium (NBT) reduction, normally found after phagocytosis [57,58] and reflecting the oxygen dependent oxidase activity [57], was shown to be increased without previous stimulation in 10 patients with HD in comparison to normal controls [17]. Zembala et al. [58] demonstrated that monocytes with an increased NBT reduction after zymosan phagocytosis, exhibited an augmented expression of Fc-receptors and a suppressive effect

Author	n patients	target cells	effector cells	h duration	result
Kohl '80	5	HSV infected chang liver cells coated with antiserum	ADH MO	18 h	↓
Pehamberger '80	23	B-HRBC coated with antiserum	MNL	20 h	↑ *
Holm '82	6	O-HRBC Rh D/D	ADH MO	18 h	N

* $P < 0.02$

HSV : Herpes Simplex Virus

HRBC: Human Red Blood Cells

ADH : adherent; MO: monocytes; MNL: mononuclear leukocytes; H = hours; N = normal; ↓ = decreased; ↑ = increased

Table 3. Monocyte and MNL mediated antibody-dependent cellular cytotoxicity

on mitogen induced lymphocyte responses. A relationship between 'state of activation' and NBT reduction was suggested.
Chemiluminescence (CL) occurs in granulocytes and monocytes after ingestion of zymosan or bacteria [45]. CL production by monocytes was shown to be significantly enhanced in malignant lymphoma [45]. All patients studied were either in complete remission or receiving chemotherapy. CL changes parallelled changes in the glucose metabolism [45].
As discussed before the PGE_2 production was shown to be increased in patients with HD [21,28]. The described metabolic events can support the presence of 'activated' monocytes in HD. The mechanisms of this activation are not understood; both tumor derived factors and lymphokines [53,55] may play a role.

FUNCTION OF TISSUE MACROPHAGES

The functional study of tissue macrophages is grossly hampered by the limited access to their anatomical sites, such as lymph nodes, spleen and liver. Furthermore, when in vitro studies are performed, these are only feasible after complex and damaging separation procedures, interfering with yield, function, and morphology. On the other hand, in vivo assays, selectively testing MPS function, are scarce. Sheagren et al. [59] found an increased clearance of ^{125} I labeled albumin-aggregates in 22 patients with HD, related to advanced stages of the disease and the presence of B-symptoms.
In a comparative study between adherent macrophages, recovered from malignant effusions of patients with HD, epitheloid cell tumors, non-Hodgkin lymphomas and benign conditions no clear functional differences were observed. This investigation included Fc- and complement-receptor density and phagocytosis [60]. A low number of pigment particles in skin window macrophages of patients with HD was interpreted as a diminished phagocytic capacity [18].
Tissue and plasma lysozyme might partially reflect macrophage function [61,62] and in the absence of monocytosis it is also related to granulocyte turnover [63]. Plasma lysozyme was significantly increased in patients with HD in a stage- and symptom-dependent manner [62], without relation to the presence or absence of lysozyme in the lesions [64].

THE HODGKIN CELL

There is growing evidence that the mononuclear Hodgkin cell and its bi-or multinucleated counterpart, the Reed-Sternberg cell, is the putative malignant cell in HD [65,66]. The origin of this neoplastic cell is still the subject of much debate and has recently been extensively reviewed [64-70]. It is always difficult to delineate whether certain characteristics of a

malignant cell exclude or establish its commitment to a known cell lineage. Malignant differentiation might be accompanied by the abrogation of normally present characteristic features. Analysis of Hodgkin cells is hampered by the difficulty of their isolation; to overcome this problem, many attempts have been undertaken to culture these cells. Establishment of HD cell-lines appeared extremely difficult, dependent on the source of the HD cells and stage of the disease [71,72]. Indicating that HD cells have to meet certain unknown characteristics before they are able to grow in vitro. Furthermore Harris et al. [72] could, using chromosome and isoenzyme analyses, demonstrate that several lines were not related to HD, but were non-human contaminants.

Valuable information about the Reed-Sternberg cell has been gleaned from clinical observations [73,74], ultrastructural [75-78], immunochemical [79-84], histochemical [85,86], functional [87], and cell culture studies [71,72,88-90]. A T- cell origin seems unlikely [70,71,8,86]. The presence of both surface bound and intra-cellular IgG gave rise to the supposition of a B-cell origin [91]. There is conflicting evidence concerning IgG being associated with free ribosomes and the endoplasmic reticulum [75,77]. The polyclonality [77,82], the observed internalization of exogenous IgG [87], and the lack of J-chains [80,83,92] do not support the B-cell hypothesis. Clinical observations provided evidence in favour of [74] and against [73] a relation with the B-lymphocyte lineage. The presence of a B-cell antigen on the surface of 50% of the cells from a HD derived cell line, demonstrated with use of the monoclonal antibody Tü-1, would be in favour of some relation with the B-lymphocyte lineage [71].

There are cumulating arguments in favour of a linkage with the MPS [71,73,76,78,79,81,82,84,87,88], and more recently attention has been focussed on the interdigitating reticulum cell, probably related to the MPS, as a possible condidate [70,86,87,93,94]. This interdigitating reticulum cell has spiny surface projectious, irregular shaped nuclei, lacks T-cell characteristics and is weakly acid phosphatase, and non-specific esterase positive. The cell is closely associated with T-lymphocytes and present in the thymic dependent regios of lymph nodes [94]. They are important accessory cells in the afferent limb of the immune response. Beckstead et al. [86] and Cadin et al. [91] found a weak acid phosphatase and non specific esterase activity in Hodgkin's cells, and Diehl et al. [71] observed a similar pattern in HD derived cell lines. Reed-Sternberg cells are predominantly found in the thymic dependent zones of lymph nodes [95], they display interactions with T-lymphocytes [78,86], the nucleus is strikingly irregular and the phagocytotic capacity is limited [87]. Moreover in a direct comparative study between Reed-Sternberg and interdigitating reticulum cells, Ia was shown to be present on both cell types and they lacked two common macrophage antigens (20.2 and 20.3) and 5'-nucleatidase [96]. In tissue biopsies and in HD derived cell lines this Ia positivity was also observed by others [71,83].

In a recent description of HD derived cell lines [71] a number of surface antigens normally found on immature cells of the myeloid pathway and on monocytes/macrophages were shown to be present. Furthermore, as a response to a specific phorbol-ester, functional properties such as weak phagocytosis and adherence were expressed, suggesting a differentiation along the monocytoid pathway. In addition, the supernatants of these lines contained a factor capable of suppression of spontaneous cell mediated cytolysis, and colony stimulating factor activity appeared to be present as well. Stein et al. [83] demonstrated with monoclonal antibodies (Tü5, Tu6, Tu9) also antigens of the granulopoiesis on Reed-Sternberg cells in fresh biopsy material.

A fascinating development is the generation of a monoclonal antibody against a Reed-Sternberg cell determinant (K1) [93]. No reactivity was observed in 50 cases of non-Hodgkin lymphomas. Up till now no reactivity has been found with peripheral blood cells, several types of macrophages, skin, liver, kidney, lung, or brain tissue. However, a small number of normal cells in tonsils, lymph nodes and bone marrow were K1 positive. Using monoclonal antibodies specific for T-cells, B- cells, macrophages, dendritic reticulum cells, interdigitating reticulum cells, cells of the granulopoiesis, erythropoiesis or thrombopoiesis, no cross reactivity could be demonstrated with these K1 positive cells in normal tissue.

In conclusion there still is no definite answer concerning the origin of the Hodgkin cell. Although the studies with monoclonal antibodies inclined to a cell with mono-myeloid and in a minority with B-cell characteristics, they did not fit to the morphological and histochemical features which tended more towards the interdigitating reticulum cell.

CONCLUDING REMARKS

The major part of the presented literature concerns the peripheral blood monocyte, the best accessible cell of the MPS, whereas MPS function is predominantly displayed by tissue macrophages. Therefore it is not possible to provide a balanced judgment concerning its function in HD.

The monocyte production was found to be enhanced in HD, which might have added to the often observed absolute monocytosis. No information is available in HD concerning the monocyte disappearance rate from the peripheral blood to the tissues. The more static results, such as numbers present at a certain time and place, allow no conclusions regarding turnover.

Adequate evaluations of in vitro function of tissue macrophages are due to technical reasons not available. The in vivo clearance of aggregated albumin indicates an enhanced MPS function at the tissue level.

The metabolic features of the peripheral blood monocyte, such as an increased PGE_2 production, the augmented CL and NBT reduction, and the

enhancement of glucose metabolism indicate a stimulated state. Its nature is unclear, it can be either the expression of the presence of a younger population monocytes, due to a higher turnover and therefore aspecific, or more directly related to the tumor itself.
The found monocyte-mediated suppression in vitro may be the expression of the presumed stimulation in vivo. Although monocyte mediated suppression on mitogen stimuled lymphocytes could be demonstrated in vitro, the significance for the in vivo antigen presentation and subsequent lymphocyte proliferation is not clarified.
The results of monocyte effector functions permit no final conclusion.
The main objection against the reports both those dealing with afferent and efferent functions, is the use of mixed cell populations, comprising monocytes and lymphocytes or the use of monocytes selected on the basis of functional (adherent) properties. The use of purified intact cell suspensions will provide more reliable data.
The 'enigma of the origin of the Reed-Sternberg and Hodgkin cell remains unsolved. The fact that the successfully established cellines were often derived from patients with endstage disease suggests a probably more 'malignant' character of these cells than the Hodgkin cells earlier in the disease.
The development of the K1 antibody offers new prospects for diagnosis, treatment and research.
In conclusion: no for HD specific alteration in MPS could be demonstrated so far. On the other hand many findings indicate an engagement of the MPS with probably tumor related processes and deserves therefore further study for a better understanding of the pathophysiology of HD.

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CHAPTER 3

MONOCYTE PURIFICATION WITH COUNTERFLOW CENTRIFUGATION MONITORED BY CONTINUOUS FLOW CYTOMETRY

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Published in: Journal of Immunological Methods 1981; 47:31-38

SUMMARY

Continuous monitoring of cell light scatter during counterflow centrifugation of a mononuclear cell suspension allows counting and size recognition of the cell types elutriated. With this method an optimal separation point between monocytes and lymphocytes, determined for each individual donor, can be established. With a constant flow of 15 ml/min this separation point is found at centrifugal velocities ranging from 2348 to 2444 rpm (n = 10). From 50 ml venous blood, 84.1% ± 4.1% (15.7 ± 8.6 x 10^6) of all elutriated monocytes, with a purity of 92.4% ± 1.4%, is collected in a volume of 50 ± 1 ml. In the same run, 92% ± 4.3% of the lymphocytes is gathered in one fraction with a purity of 98.9% ± 0.7%.
After counterflow centrifugation, 91.6 ± 10.5% of the cells loaded is recovered; viability exceeds 98%.

INTRODUCTION

Optimal study of immunological and non-immunological functions of monocytes requires purified monocyte suspensions. Conventional isolation techniques are based upon either their adherent properties [1,2,3]. or density. [4,5,6]. Monocyte isolation by counterflow centrifugation (elutriation) has recently been shown to give high purity, good recovery, and excellent viability and function [7,8,9,10]. Separation is based mainly on differences in cell size and to a much lesser extent on density. Meticulous regulation of flow, centrifugal force and temperature are essential to achieve optimal purification. After relatively 'blind' elutriation in fractions, screening of the effluent is usually performed by means of a Coulter counter. In this paper a new method is described whereby continuous monitoring of the elutriator output is obtained, allowing simultaneous counting and size quantification. In this way it is possible to determine the optimal separation point between the different mononuclear cell populations in each elutriator procedure, resulting in improved efficiency of counterflow centrifugation.

MATERIALS AND METHODS

Collection of blood

Approximately 50 ml venous blood from healthy volunteers, anti- coagulated with 6 IU/ml preservative-free heparin, is obtained by venapuncture. An aliquot is taken for total and differential cell counting.

Preparation of the mononuclear cell suspension (MNL)

The blood is immediately diluted 3-fold with calcium- and magnesium-free Hank's buffered balanced salt solution (HBBS), supplemented with 100 mg di-K-EDTA/l, final pH 7.3, 295 mosM/kg. Thirty-five ml samples are layered on 15 ml 1.077 g/ml Ficoll-Isopaque (pH 6.6, 272 mosM/kg). After centrifugation (400 x g; 30 min; 20°C) and removal of the supernatant, the interphase is collected, diluted with HBBS + 0.4% bovine serum albumin w/v (BSA), washed once (600 x g, 15 min, 4°C) and resuspended in 5-8 ml HBBS + 0.4% BSA. A fixed volume is taken for counting and differentiation. The remaining cells are introduced into the elutriator.

Elutriation

Elutriation system.
This consists of a J2-21C centrifuge equipped with the JE-6 elutriator rotor and standard separation chamber (Beckman Instruments, Palo Alto, CA, U.S.A.). The flow is maintained with a roller pump (Masterflex-Cole-Parmer Instruments, Chicago, IL, U.S.A.) and pulsations are flattened by a pulse suppressor chamber. Medium temperature is maintained at 10°C. The J2-21C centrifuge is modified by installing a fine scaled speed selector (one scale unit: 2.6 ± 0.1 rpm) and replacing the C102 condenser (0.022 UF) by a condenser of 47 pF; this results in improved stability of rotor speed. Rpm measurement was altered by using a high resolution counter (120 HMZ pH 6667 Philips, Eindhoven, The Netherlands). The elutriator effluent is collected in a small downflow cabinet. HBBS + 0.4% BSA w/v is used as elutriation medium.
Cell recognition device (Fig. 1).
Continuous sampling of the elutriator output is established by means of a T-drain. This sample stream (a, 0.2 ml/min) mantled in a sheath stream (b, 1.4 ml/min), is continuously analyzed for cell number and cell light scatter by the electro-optical peroxidase unit of the Hemalog-D (Technicon Instruments Corp., Tarrytown, U.S.A.). This unit, dismantled from the Hemalog-D, consists of a flow cell with optical assembly for focusing the light from a tungsten halogen light source and an absorption and scatter photodiode. The light scatter signals are accumulated and displayed on an ND-100 multichannel analyzer (Nuclear Data Inc., Schaumberg, IL, U.S.A.). The resulting histogram (fig. 2) indicates the number of cells as a function of cell size (scatter). When a new cluster of cells with a markedly different scatter signal is elutriated, a change in the histogram appears on the monitor. At any time the profile can be erased or stored in a memory, enabling direct comparison of elutriated cell populations.
Elutriation technique.
Prior to introduction of the cell suspension, entrapped air is removed, rotor speed adjusted to 2500 rpm, and medium flow established at 15 ml/min under

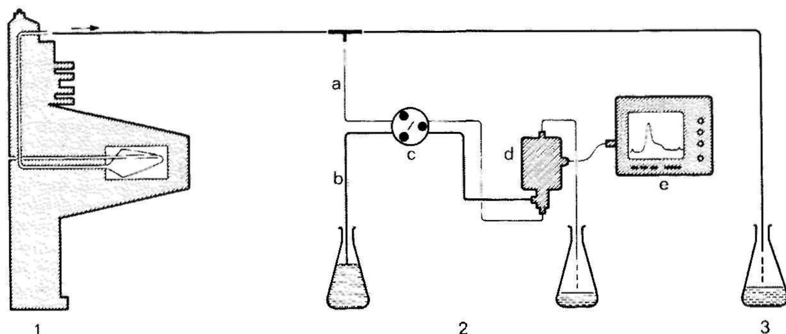

Fig. 1. Schematic representation of elutriator rotor (1) and the cell recognition device (2), consisting of: (a) sample stream directly derived from elutriator effluent; (b) sheath flow; (c) roller pump; (d) electro-optical unit; (e) ND-100 multichannel analyzer. Collection of elutriator output (3).

control of a flow-meter (Sho-rate flow-meter type No. R-2-25-B Brooks Instruments Inc.). By means of an infusor, the cell sample is introduced directly into the tubing line at a flow- rate of 0.3 ml/min. During this period monocytes and larger lymphocytes remain in the separation chamber, whereas smaller lymphocytes, erythrocytes and thrombocytes are eluted. When introduction of the cells is completed the rotor speed is lowered 26 ± 1 rpm every 2 min. The decrease of centrifugal force results in an outflow of lymphocytes with increasing diameter, while the number of cells eluted per time unit diminishes. At a certain rotor speed, indicated as the separation point, cells with a distinct scatter signal (monocytes) leave the elutriator (Fig. 2. profile 2). Since lymphocytes are still being washed out, a double peaked histogram is seen, representing the two cell populations. The rotor speed is then immediately increased 15 rpm in order to wash out the remaining lymphocytes and to retain the monocytes. Collection of fraction 2 is started. When the number of cells leaving the separation chamber falls below 100 cells/sec, the rotor is stopped and the effluent collected in one 50 ml tube (fraction 3). Immediately thereafter a 'rest' fraction is gathered (fraction 4). After washing with HBBS + 0.4% BSA 1(600 x g, 15 min, 4°C) the cells are resuspended and further analysis is performed. The whole procedure can be carried out within 1 h.

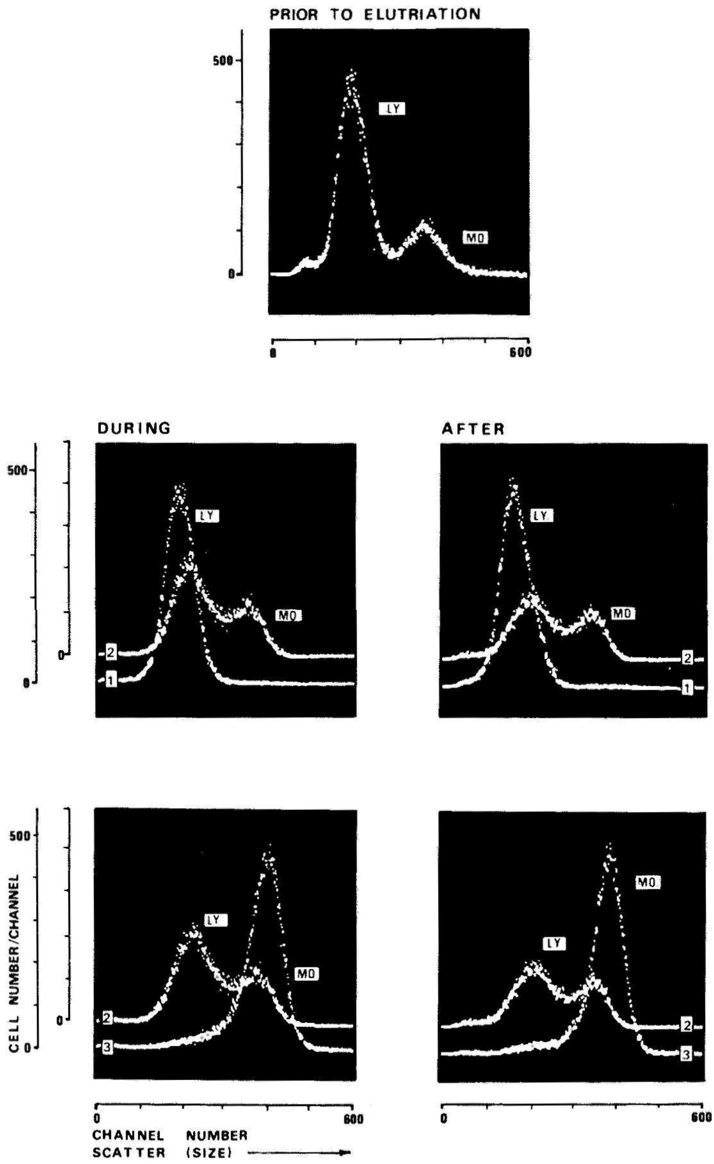

Fig. 2. Histograms as seen on the monitor of the ND-100 multichannel analyzer. Prior to elutriation: the mononuclear cell suspension. During elutriation: profile 1 represents lymphocytes, profile 2 represents the mixed population and profile 3 monocytes. After elutriation: scatter analysis of the entire lymphocyte, mixed, and monocyte population.

Cell counting and differentiation

Cell number in the starting material, MNL and elutriated fractions is measured with a Coulter counter. Cytocentrifuge preparations of the MNL and elutriated fractions are made according to Talstad and Gundersen [11] and stained with May- Grünwald-Giemsa and non-specific esterase, respectively [12,13]. Differential counting is performed on 400 cells.

Cell viability

Cell viability is assessed by two methods. (1) Cells suspended in a concentration of 3-5 x 10^5/ml are mixed with 20 µl fluorescein di-acetate/ml (5 mg FDA/ml acetone) and 20 µl ethidium bromide/ml (0.0025 mg EB/NaCL 35.8 mg/ml and Tris 12.1 mg/ml, pH 7.5). Viable cells are FDA-positive (green cytoplasmic fluorescence [14]) and dead cells ethidium bromide- positive (red DNA fluorescence). (2) Exclusion of trypan blue by viable cells.

RESULTS

If the rotor speed is continuously decreased and the flow-rate maintained constant, cells with a gradually increasing diameter will leave the elutriator. With the effluent monitored by the cell recognition device, measuring light scatter to analyse cell number and size, it is possible to record the distribution of these parameters in a mononuclear cell suspension (Fig. 3). In this particular experiment the monitoring was started after elutriation of the thrombocytes. The first and highest peak represents the lymphocytes, the second contains the monocytes. This form of cell scatter registration allows recognition of the different cells during elutriation. The possibility of immediate identification of cell type combined with low elutriator output between given cell populations is the basis of this separation method. The histograms registered during elutriation are comparable with those of the final separated fractions (Fig. 2). The results of 10 runs are listed in Table 1. Fraction 3 consists of elutriated monocytes of high purity with only slight admixture of lymphocytes (6.5 ± 1.6%), some basophils, and very few segmented neutrophils. The majority of the lymphocytes is present in the first fraction with a purity of 98.9 ± 0.7%, with only 1.1 ± 0.6% monocytes. In the second fraction a mixed population is found: 2.6 ± 4.3% of the lymphocytes and 6.2 ± 2.8% of the elutriated monocytes. Finally a less pure monocyte fraction (80.8 ± 5.6%) with 17.8 ± 4.5% lymphocytes has been gathered, representing only 5.5 ± 3.1 and 0.5 ± 0.3% respectively of the total eluted cell number. The rotor speed (rpm) at which, for the first time, two cell populations are to be seen, is defined as the separation point. This is found at 2392 ± 39.5 rpm with a range of 2348 - 2444 rpm. Viability of the elutriated cells is 97.7 ± 1.1% as

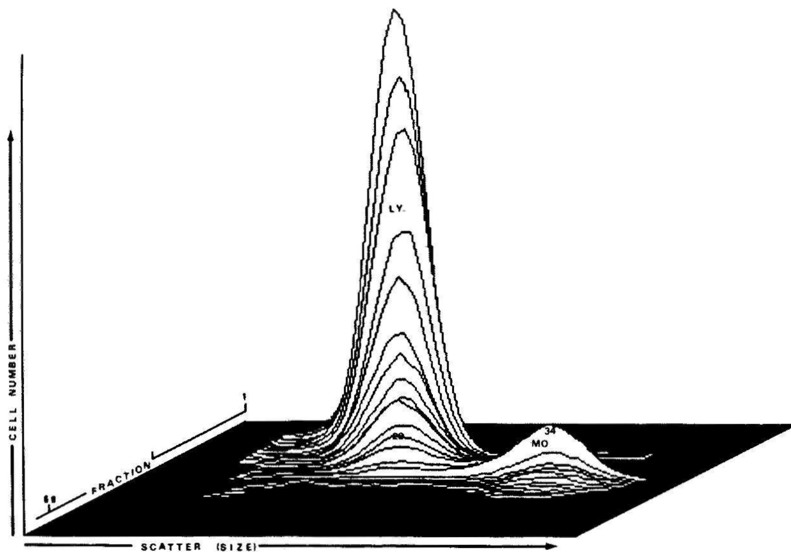

Fig. 3. Relationship between cell number and light scatter signal of 44 consecutively elutriated fractions. Each of the 44 histograms represents the number and the corresponding light scatter of the cells elutriated in 150 sec. At the same interval the rotor speed was diminished 26 ± 1 rpm, while the flow remained constant at 15 ml/min. Fraction 1 is comparable to histograms 1-29; fraction 2 to histograms 29-32; fraction 3 and 4 to histogram 32-44.

determined by EB/FDA and 98.6 ± 0.5% by trypan blue.
After elutriation , 91.6 ± 10.5% of the loaded cells are recovered. By the method described, 88.5 ± 17% of the monocytes and 64.8 ± 9% of the lymphocytes present in the peripheral blood sample are recovered in the elutriator effluent, of which 72% and 60% respectively are found in the purest fractions. The absolute number of monocytes and lymphocytes found in those fractions is 15.7 ± 8.6 x 10^6 and 63.9 ± 20 x 10^6 respectively.

Fraction	Monocytes		Lymphocytes	
	Purity (%)	Yield (%)	Purity (%)	Yield (%)
1	4.1 ± 1.8	1.1 ± 0.5	98.9 ± 0.7	92 ± 4.3
2	23.9 ± 13.8	6.2 ± 2.8	75.4 ± 13.7	6 ± 4.3
3	92.4 ± 1.4	84.1 ± 4.1	6.5 ± 1.7	1.5 ± 0.6
4	80.8 ± 5.6	5.5 ± 3.1	17.8 ± 4.5	0.5 ± 0.3

Table 1. Purity and yield of lymphocytes and monocytes in the 4 elutriated fractions (mean % ± S.D.). Yield is expressed as the relative distribution of lymphocytes and monocytes over the 4 fractions; purity as the composition within each fraction.

DISCUSSION

Purified monocytes are needed for study of their immunological and non-immunological functions. Isolation on the basis of functional properties means selection and possible changes in cellular metabolism induced by the isolation technique [15]. Counterflow centrifugation avoids this and provides an excellent method for cell separation. Sanderson et al. [7] were the first to report considerable purification of a small quantity of monocytes by elutriation. Weiner and Shah [8], Lionetti et al. [10], Contreras et al. [9] and Figdor et al [16] described the isolation of large quantities. A disadvantage of this technique, however, is the relatively blind separation in fractions obtained at flow-rates and rotor speeds based upon results of previous experiments. The use of a recognition device, continuously measuring elutriator output, solves this problem. Firstly, one can actually see when a cohort of cells is collected. In our experiments fraction 1 is collected in 456 ± 36 ml, fraction 2 in 116 ± 56 ml and fractions 3 and 4 each in 50 ± 1 ml. Secondly, and even more important, the technique allows immediate recognition of the eluted cell type. Contreras et al. [9] stated that the elutriation characteristics of each donor's mononuclear cell suspension are different, and each run has to be tailored individually for each donor. The relatively wide range of rpm (2348-2444) found as the separation point in our study confirms this and corroborates the advantage of a separation method involving direct visualization and recognition. On the other hand, Fogelman et al. [17] and Figdor et al. [16] documented highly reproducible monocyte isolation from run to run without individual adjustment. Variability of flow, rotorspeed and temperature influences, if not thoroughly standardized for each run, the elutriation characteristics considerably. In addition, biological spread in cell volume may account for the variation in separation

point observed in the present study and may be of importance, when dealing with patients' mononuclear cell suspensions. Using the isolation technique described, we achieved excellent purification of monocytes and lymphocytes with good recovery and viability. Moreover, the monocytes are gathered in a small effluent volume (50ml). The results reported here with a relatively small (50 ml) aliquot of peripheral venous blood as the source of the MNL suspensions and continuous identification of the eluted cell type, support the potential application of this purification technique in the study of monocyte function in patients. Preliminary results on blood from patients with Hodgkin's disease, non-Hodgkin lymphomas, and infectious mononucleosis are essentially similar to those reported here.

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CHAPTER 4

INCREASED ANTIBODY-DEPENDENT CYTOTOXICITY MEDIATED BY PURIFIED MONOCYTES IN HODGKIN'S DISEASE

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Published in: Clinical Immunology and Immunopathology. 1983; 26:406-414

SUMMARY

Monocyte antibody-dependent cytotoxicity was studied in 19 patients with Hodgkin's disease and 14 normal controls. This function was investigated after isolation of the monocytes by means of a modified elutriation technique. Direct sizing and counting of the cells, present in the effluent, enabled individual adjustment during each separation procedure. The absolute monocyte count in the peripheral blood of patients with Hodgkin's disease was higher ($P < 0.002$) than in normal controls. Nearly 90% pure monocyte suspensions, representing 82% of all elutriated monocytes, were obtained. The elutriation characteristics of the monocytes in both groups were essentially the same, irrespective of marked interindividual differences. Kill of antibody-coated chicken red blood cells was measured by DNA flow cytometry. In comparison to normal controls, a significantly increased ($P < 0.0004$), stage-independent, monocyte antibody-dependent cytotoxicity was found in patients with Hodgkin's disease. The percentage of kill in symptomatic patients tended to be higher than in the asymptomatic group; no correlation was found with the absolute number of circulating monocytes.

INTRODUCTION

In Hodgkin's disease (HD) a relative and/or absolute monocytosis, especially in advanced stages, is not infrequently noted [1-5]. Despite this observation, data concerning monocyte function in HD are scarce. Hitherto, most monocyte function studies have been performed on unfractionated mononuclear cells [6-8] or after purification using the adherent properties of monocytes [9]. However, purified cell suspensions, obtained by techniques providing high recovery and minimal alteration of function, are required for optimal investigation of different cell types. Kohl et al., studying adherent mononuclear cells taken from five asymptomatic HD patients, found an impaired antibody-dependent cellular cytotoxicity (ADCC) against herpes simplex virus-infected target cells [9]. On the contrary, Pehamberger et al. reported a significantly increased lysis of antibody-coated human red cells induced by unfractionated mononuclear cell suspensions [8]. In this study antibody-dependent cellular cytotoxicity (ADCC) was measured on monocytes obtained by a modified counterflow centrifugation (elutriation) technique. In this procedure fraction cutting is based on continuous monitoring of the elutriator output, hence optimizing purity and yield [10].
Monocyte ADCC was analyzed in 19 patients with HD and 14 normal controls employing a recently developed in vitro ADCC system [11] measuring kill of antibody-coated chicken red blood cells by means of DNA flow cytometry, using the differences in DNA content of the two cell populations involved.

PATIENTS AND METHODS

Patients and controls.

Peripheral blood monocytes of 15 consecutively diagnosed and untreated patients with biopsy-proven HD and 4 patients with relapses were included in this study. The histologic material was classified according to the Rye conference modification of the Lukes-Butler classification [12]. Staging procedures were carried out according to the criteria recommended at the Ann Arbor conference [13]. None of the patients had concomitant overt infectious disease. The clinical data are summarized in Table 1. A group of 14 healthy individuals of similar age and sex distribution served as controls; none of them was receiving any medication.

		Age (years)	
Patients	n=19	Mean	Range
Female	6	34	16 - 60
Male	13	29	19 - 55

Clinical stage					
Stage	I	II	III	IV	n
A	3	3	2	2	10
B	-	1	2	6	9
n	3	4	4	8	

Histology
14 Nodular sclerosis
2 Mixed cellularity
1 Lymphocytic predominance
1 Lymphocytic depletion

Table 1. Clinical data of the patient population

Monocyte isolation.

Monocyte suspensions were prepared as previously described (10). Briefly, approximately 50 ml venous blood obtained by venapuncture was heparinized, and subjected to Ficoll-Isopaque (1.077 g/ml) buoyant density centri-

fugation (400 x g; 30 min; 20°C), after threefold dilution with calcium- and magnesium-free Hank's buffered balanced salt solution containing 100 mg di-K-EDTA/liter (HBBS). The isolated mononuclear cell suspension was washed once and resuspended in 5-8 ml HBBS supplemented with 0.4% bovine serum albumin (BSA). This sample was introduced into the elutriator (Beckman J2-21 C centifuge equipped with a JE-6 elutriator rotor with a standard separation chamber). By gradually diminishing the centrifugal speed (rpm), cells were allowed to emerge from the separation chamber at a constant flow rate of 13.7 ml/min. An optimal separation for each sample was established by means of scatter analysis of the effluent, enabling continuous monitoring of size and numbers. The first appearance of monocytes in the effluent was used as the separation point between lymphocytes and monocytes. In each run four fractions were collected: a lymphocyte, a mixed lymphocyte and monocyte, a monocyte and a 'rest' fraction. Cytocentrifuged preparations of the elutriated fractions and peripheral blood smears were stained with May-Grünwald-Giemsa and with nonspecific esterase [14]. Differential counting was performed on 400 cells. Viability was assessed with fluorescein diacetate/Ethidium bromide and trypan blue exclusion as described previously [10].

Antibody-dependent cellular cytotoxicity assay (ADDC).

ADDC was measured according to Attallah et al. [11]. After ethidium bromide staining, chicken red blood cell (CRBC) targets can be distinguished readily from human effector cells with DNA flow cytometry, since the DNA content amounts to 2.5 and 6.0 pg per nucleus, respectively (Fig. 1).

Effector cells.

Isolated monocytes were resuspended in RPMI-Hepes-L-glutamine at a concentration of 4 x 10^6/ml.

Target cells.

CRBC were prepared freshly for each assay using the same chicken donor. Approximately 0.5 ml heparinized venous blood was washed five times in phosphate-buffered saline (PBS) and finally resuspended in RPMI-Hepes-L-glutamine at a concentration of 4 x 10^6 cells/ml.

Antibody.

A commercially available rabbit anti-CRBC lgG (R-anti CRBC, Unites States Biochemical Corp., Cleveland Ohio) served as antibody in a final dilution of 1:40,000.

Culturing.

Cultures were carried out in triplicate in 500µl round-bottom tubes (Luckham Ltd, Burgess Hill, Great Britain) with an effector to target (E/T) ratio of 2/1. The monocyte suspension (100 µl) and CRBC (50 µl) supplemented with either 50 µl medium or 50 µl R-anti-CRBC were incubated for 90 min at 37°C.

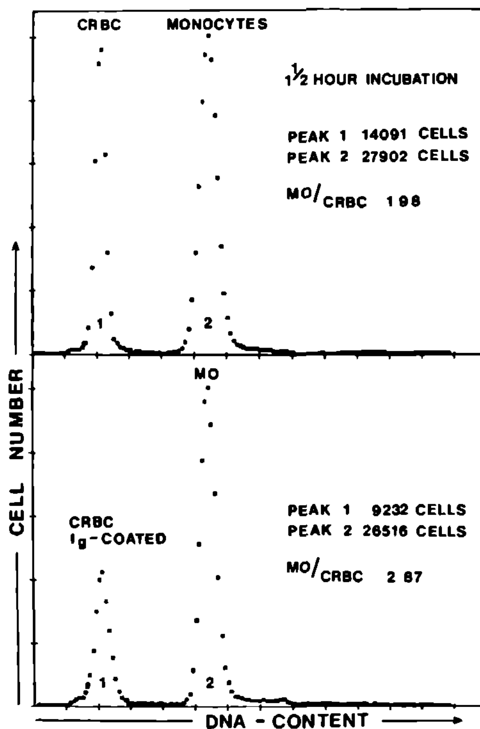

Fig. 1. Gould 5005 copy representing the histograms of the suspensions with (bottom) and without (top) antibody. The relative DNA content is indicated on the x axis and cell number on the y axis. The surface of each peak represents the cell number.

ADCC flow cytometry.

The incubation was stopped by adding 200 µl hypotonic ethidium bromide (EB: 0.1% trisodium citrate and 25 mg/liter ethidium bromide; 30 mosmol/kg, pH 7.6) supplemented with 0.1% Triton X- 100 (w/v) to each tube. After careful mixing and incubation for at least 15 min at 4°C, the suspensions were diluted further with 800 µl hypotonic EB/Triton and analyzed by means of flow cytometry (ICP-11) (15). The DNA histograms were recorded by a paper-tape punch unit, read by a PDP 11/45 computer, and E/T ratios calculated. The final results were provided in hard-copy form by a Gould 5005 unit (Fig. 1).

Calculations.

The percentage CRBC lysis due to monocyte cytotoxicity in the ADCC assay was calculated according to the formula:

$$\%\text{Kill} = 100 - \frac{\text{MO/CRBC - antibody}}{\text{MO/CRBC + antibody}} \times 100.$$

Statistics.

The Wilcoxon two-sample test was used to compare the results of ADCC between patients with HD and normal controls.

RESULTS

Relevant data concerning the composition of the peripheral blood are presented in Table 2. In 63% of the patients leukocytosis (leukocytes> 10,000/mm^3) was noted, mostly due to an increased number of polymorphs. The relative proportion of monocytes was normal, but the absolute number of circulating monocytes (68% of the patients had > 500 monocytes/mm^3) was significantly increased ($P < 0.002$). Likewise, the relative lymphocytopenia masked a normal mean number of cells per cubic millimeter, although 7 out of 12 patients with stage III and IV had lymphocyte counts of less than 1500/mm^3. Table 3 gives the results of the monocyte isolation using counterflow centrifugation. Recoveries and purities are similar in the two groups; the contaminating cells in the monocyte fraction were mainly lymphocytes, some basophils, and very few segmented neutrophils. The similarity of separation points, in view of the quite different composition of the peripheral blood, was striking.

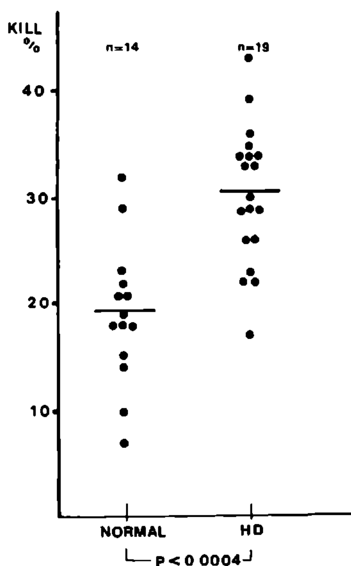

Fig. 2. Monocyte ADCC of the patient and control group

The lytic capacity of the isolated monocytes was significantly increased ($P < 0.0004$) in HD compared to normal controls (30.2 ± 6.5% versus 19.1 ± 6.6%; Fig. 2). A slight, though not significant, increase was noted with the presence of B symptoms (32.6 ± 6.1% versus 28.1 ± 6.4%; Fig. 3). Stage of disease was not related to killing capacity (Fig. 4) and no correlation was found between the percentage of kill and the number of circulating monocytes ($r = 0.16$).

	Normal	Hodgkin's disease	P
WBC/mm³	5300 ± 1300	13,400 ± 6400	< 0.0002
Monocytes (%)[a]	5.4 ± 1.7	4.9 ± 2	NS
Monocytes/mm³	287 ± 110	664 ± 366	< 0.002
Lymphocytes (%)[a]	36.7 ± 9.5	17.6 ± 16	< 0.0002
Lymphocytes/mm³	1966 ± 724	2052 ± 1982	NS

[a]*400 cells differentiated*

Table 2. Monocyte and lymphocyte numbers in the peripheral blood

	Normal	Hodgkin's disease
Purity of the monocyte fraction (%)	89.2 ± 2.5	88.1 ± 4.7
Relative yield of monocytes in the most enriched fraction (%)	76.7 ± 10	82.5 ± 8
Elutriation recovery (%)	89 ± 11	84 ± 16
Separation point (rpm)	2464 ± 161	2415 ± 141
Yield of monocytes ($x 10^6$)	16 ± 5.6	26.8 ± 16
Viability (%)	98	98

Table 3. Results of monocyte isolation in the patient and control group

DISCUSSION

The role of the mononuclear phagocyte system in HD is poorly understood. There are arguments for a monocyte-macrophage origin of the putative malignant cell in HD [16]. Besides lymphocytes, macrophages are important effector cells in the cell-mediated immune response, which is impaired in HD [17]. To study the mononuclear phagocyte system in vitro, the peripheral blood monocyte is the best accessible cell source. Available data from the literature concerning monocyte function in HD are limited and not conclusive. A possible explanation for the observed monocytosis in HD may be the increased medullary monocyte production as shown by Meuret et al. [18]. Earlier studies on chemotactic response [7], ADCC against virus- infected target cells [9], adherence [19], and phagocytosis [20] indicated a diminished monocyte function in HD; other investigations, however, revealed a normal [19] or increased activity of the monocytes [6,8,21,22]. The discrepancy between these results may be partly attributed to differences in the effector cell populations employed. Hitherto, study of monocyte function was partly hampered by the lack of an adequate, aselective method to obtain a sufficient number of monocytes without affecting the cell function. Counterflow centrifugation seems to solve this problem. Our findings on peripheral blood, isolated mononuclear leukocytes (data not shown), and monocyte yield after elutriation corroborate the presence of an increased absolute number of monocytes in HD in comparison to normal controls. The elutriation characteristics of monocytes in both the patients and the control group did not differ essentially. However, the considerable interindividual differences in the separation points observed in both series, as indicated by the range in Table 3, underline the necessity of elutriation with direct recognition of the cells leaving the separation chamber. We obtained nearly 90% pure monocyte suspensions representing 82% of all monocytes subjected to elutriation. The in vitro ADCC assay by means of DNA flow cytometry appeared to be an excellent method to assess the percentage of kill. Technical problems associated with labeling techniques are avoided and a substantial kill can be observed applying a low effector/target ratio, after a relatively short incubation period. Our results show an increased ADCC in HD compared to normal controls. The increase is not stage related but more pronounced in patients with B symptoms. The lack of correlation ($r = 0.16$) between the number of circulating monocytes and the percentage kill argues against a major shift in functionally different monocytes in HD on the basis of monocytosis alone. The increased monocyte ADCC does not support the hypothesis of a macrophage effector defect as a cause for the diminished cellular immunity and susceptibility to infections. Furthermore this increased cytotoxicity in vitro seems unable to prevent effectively the spread of the Hodgkin lesions. Other studies on ADCC in non-lymphomatous malignancies [23, 24] indicate that

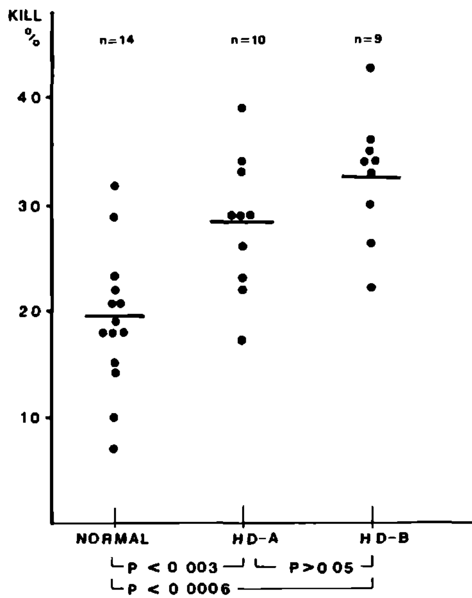

Fig. 3. Monocyte ADCC in relation to absence (A) or presence (B) of B symptoms.

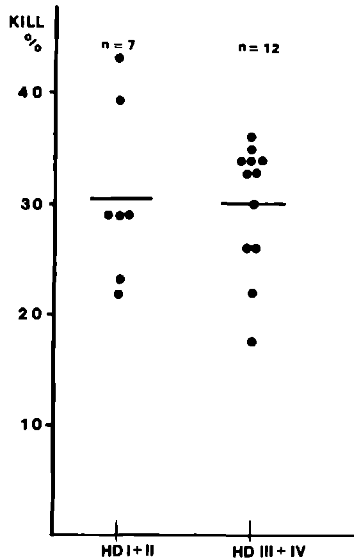

Fig. 4. Monocyte ADCC related to the stage of disease.

the increased lytic capacity of monocytes is not a HD-specific phenomenon. Katz [25] identified a diminished number of macrophages in uninvolved lymphoid tissue derived from patients with HD in comparison to normal controls. The increased production of leukocyte inhibitory factor in HD [26-28] may in part explain the lack of expression of this enhanced monocyte ADCC at tissue and tumor level.

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CHAPTER 5

MONOCYTE MEDIATED ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN MALIGNANT LYMPHOMA AND SOLID TUMORS.

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Accepted for publication in: Cancer.

SUMMARY

Monocyte-mediated antibody-dependent cellular cytotoxicity (MO- ADCC) was studied in 21 patients with Hodgkin's disease (HD), 15 patients with a long-lasting remission of HD, 11 patients with non-Hodgkin lymphoma (NHL), 11 patients with solid tumors, and 15 normal controls. Lymphocyte ADCC (LY-ADCC) was evaluated in 12 patients with HD and 9 normal controls. Monocytes and lymphocytes were isolated with cell-scatter monitored counterflow centrifugation providing high purity and yield. ADCC was evaluated by means of DNA flow cytometry, using antibody-coated chicken red blood cell targets (CRBC). In comparison with normal controls MO-ADCC was significantly increased in HD ($p < 0.0005$), NHL ($p < 0.005$) and solid tumors ($p < 0.005$). In patients in a long-lasting complete remission of HD MO-ADCC was in the normal range. LY-ADCC of 12 patients with HD was similar to that of 9 normal controls. In all experiments LY-ADCC was invariably lower than MO-ADCC of the same donor, indicating the monocyte as the most potent effector cell towards CRBC targets.
Our results indicate: 1. Purified cel suspensions of both lymphocytes and monocytes are essential to unravel their role as effector cells. 2. LY-ADCC in HD is similar to normal controls. 3. MO-ADCC enhancement is not uncommon in malignant lymphoma and several solid tumors. 4. Normal MO-ADCC in a group of succesfully treated patients with HD suggests a disease related induction of enhanced MO-ADCC.

INTRODUCTION

Antibody-dependent cellular cytotoxicity (ADCC) is a sensitive immunological defence mechanism participating in the inhibition of neoplastic growth [1,2], in allograft rejection [3], and parasite destruction [4]. Different cell types, lymphocytes, monocytes and granulocytes, all bearing membrane Fc-receptors, are known to possess ADCC properties [5,6]. The sensitivity of an effector cell depends on both target [7,8] (e.g. tumor cell lines, human red blood cells, chicken red blood cells) and on the type of antibody [8,9,10,11]. K- lymphocytes, for example, preferentially kill antibody-coated tumor cells and to a lesser degree erythrocyte targets such as chicken red blood cells (CRBC); whereas a total incapacity is observed towards human anti-B coated B-erythrocytes, which in turn are very sensitive targets for peripheral blood monocytes [11]. Dealing with a mixed effector cell population of varying composition, such as a mononuclear cell suspension, mutual interaction and varying killing capacity of cells and subsets should be taken into account. A prerequisite for optimal evaluation of ADCC is a well defined cell population.
In the present study we evaluated monocyte ADCC (MO-ADCC), using monocytes purified with cell-scatter monitored counterflow centrifugation (CFC),

minimizing selection and functional alteration [13]. ADCC was evaluated using antibody-coated CRBC targets by means of DNA flow cytometry, thus avoiding time consuming labeling techniques and enabling measurement after a relative short incubation period (90 min) [14,15]. In order to compare lymphocyte and monocyte ADCC towards antibody-coated CRBC, purified cells, obtained in one isolation procedure, were used as effectors.
In a previous report we showed an enhanced monocyte ADCC in Hodgkin's disease (HD) in comparison to normal controls [15]. To investigate whether this phenomenon is restricted to patients with overt HD or is likewise demonstrable in patients in clinical remission, we tested MO-ADCC in 15 patients in a long-lasting complete remission. Eventual disease specificity was studied by measuring ADCC in a group of 11 patients with malignant non-Hodgkin lymphomas and 11 patients with solid tumors.

MATERIALS AND METHODS

Patients and controls.

The individuals studied included 17 untreated patients with biopsy-proven HD, 4 HD in relapse, 15 HD in long-lasting remission, 11 previously untreated non-Hodgkin lymphomas (NHL), 11 with advanced solid tumors and 15 healthy individuals. The histologic classification of the patients with HD was made according to the Rye conference modification of the Lukes- Butler classification [16]. Staging procedures were carried out as recommended at the Ann Arbor conference [17]. The histologic classification of NHL was based on the criteria proposed by Rappaport [18] and clinical staging according to the criteria advised at the Ann Arbor conference [19]. The clinical data are summarized in Tables 1-3. All persons, both patients and normal controls were without medication and overt infectious disease at the time of the study.

Monocyte isolation.

Monocyte suspensions were prepared as previously described by means of counterflow centrifugation (CFC) [13]. Briefly: approximately 50 ml of heparinized peripheral blood was, after threefold dilution with either Hank's balanced salt solution or glucose phosphate buffer, subjected to Ficoll-Isopaque (1.077 g/ml) buoyant density centrifugation (400xg; 30 min; 20°C. After washing, the mononuclear cell suspension was introduced into the counterflow centrifuge (Beckman J2-21C equipped with a JE-6 elutriator rotor with standard separation chamber). The tubing system and chamber were previously sterilized by thorough rinsing with 70% ethanol. After loading the sample, cells emerge from the separation chamber mainly according to their size, by gradually diminishing the centrifugal speed at a constant

No	Age	Sex	Stage	PA	ADCC %
Hodgkin's disease					
1	19	M	PS IA	LP	29
*2	16	F	PS IA	NS	22
3	38	M	PS IA	LP	23
4	29	M	PS IIA	NS	39
5	21	M	PS IIA	MC	29
6	24	M	PS IIA	NS	29
7	21	M	PS IIB	NS	43
8	26	F	PS IIIA	NS	33
9	40	F	PS IIIA	NS	17
10	48	M	PS IIIA	LP	38
11	19	F	CS IIIB	NS	45
12	23	M	CS IIIB	NS	36
*13	26	M	CS IIIB	NS	22
14	21	F	CS IVA	NS	34
15	33	M	CS IVA	NS	26
16	25	M	CS IVB	NS	30
17	37	M	CS IVB	NS	34
*18	28	M	CS IVB	NS	34
19	55	M	CS IVB	MC	35
*20	42	F	CS IVB	NS	26
21	60	F	CS IVB	LD	33
Non-Hodgkin Lymphoma					
1	43	F	PS I E	Diff Hist.	9
2	60	M	PS IIA	Foll.Lymph.	31
3	58	M	PS IIE	Diff.Hist.	27
4	42	M	CS IIIB	Centrobl.	46
5	53	M	CS IVA	Diff.Lymph.	21
6	36	M	CS IVA	Diff.Lymph.	38
7	64	F	CS IVA	Foll.Lymph.	42
8	34	M	CS IVA	Foll.Lymph.	33
9	49	M	CS IVB	Diff.Hist.	37
10	60	M	CS IVB	Diff.Hist.	56
11	58	M	CS IVB	True Hist.	33

*Relapse of HD. PS: pathological stage; CS: clinical stage; NS: nodular sclerosis; LP: lymphocytic predominance; MC: mixed cellularity; LD: lymphocytic depletion; PA: pathological anatomy.

Table 1. Clinical data of the HD and the NHL group

counterflow rate. Continuous cell-scatter monitoring of the effluent enables direct recognition of both lymphocytes and monocytes and therefore determination of their optimal separation point, independent of cell number and composition of the mononuclear cell suspension. After elutriation of the lymphocytes, comprising 90% of all lymphocytes with a purity of 98%, a monocyte fraction is obtained, containing about 80% of the elutriated monocytes with an average purity of 90% in a 50 ml sample. Purity was established by differential counting of 400 cells in cytocentrifuge preparations, stained with May- Grünwald Giemsa and for non-specific esterase.

Antibody-dependent cellular cytotoxicity assay (ADCC).

MO-ADCC was measured as described previously [15]. Freshly prepared chicken red blood cells (CRBC) were used as targets. Since the DNA content amounts to 2.5 pg and human effector cells (i.e. monocytes and lymphocytes) to 6 pg, these cells can be distinguished easily, after staining with hypotonic ethidium bromide (EB), by means of flow cytometry. The effector to target ratio (E/T) was 2 : 1. Sensitization of CRBC was carried out with rabbit anti-CRBC IgG (United States Biochemical Corp. Cleveland/Ohio, USA) in a final dilution of 1 : 40.000. Incubations were carried out in triplicate or quadruplicate in a final volume of 200 µl in 500 µl round-bottom tubes. Incubation mixtures contained 100 µl monocyte suspension and 50 µl CRBC, both at a concentration of 4×10^6/ml, supplemented with either 50 µl rabbit anti-CRBC or medium (RPMI-Hepes-L- Glutamine) and incubated for 90 min at 37°C. The assay was stopped by adding 200 µl hypotonic ethidium bromide (30m Osmol/kg) supplemented with 0.1% Triton X-100 (v/v). After mixing and incubation for another 15 min at 4°C, DNA analysis was performed with a flow cytometer (ICP-11, Phywé Göttingen Germany). The DNA histograms were used to calculate MO/CRBC ratio.
ADCC was computed according to the formula:

$$\% \text{ kill} = 100 - \left(\frac{\text{MO/CRB} - \text{antibody}}{\text{MO/CRBC} + \text{antibody}} \right) \times 100$$

The same assay was used to determine ADCC of concomitantly isolated lymphocytes in a limited group of persons (n = 21) in order to compare ADCC capacity of lymphocytes and monocytes.
Most ADCC-assays of patients were combined with normal controls or with HD patients in long lasting complete remission.

No	Age	Sex	Stage	PA	Therapy	Years duration CR	ADCC %
1	28	F	PS IIA	NS	RT	1	20
2	49	F	PS IIA	NS	RT/CT	22	27
3	38	F	PS IIA	MC	RT	4	7
4	25	F	PS IIA	NS	RT	8	18
5	25	F	PS IIA	NS	RT	8	14
6	54	M	PS IIA	NS	RT	2	27
7	40	M	PS IIIA	NS	RT/CT	11	28
8	69	F	PS IIIA	NS	RT/CT	4	29
9	35	F	CS IIIB	NS	CT/RT	1	15
10	43	M	CS IIIB	MC	CT	4	20
11	49	F	CS IVB	LD	CT/RT	1	29
12	29	M	CS IVB	NS	CT/RT	1	26
13	45	M	CS IVB	LD	CT/RT	2	26
14	51	F	CS IVB	LD	CT/RT	0,7	9
15	25	F	CS IVB	MC	CT	8	7

RT: radiotherapy; CT: chemotherapy; CR: complete remission, PA: pathological anatomy.

Table 2. Clinical data of HD group in complete remission.

No	Age	Sex	Diagnosis	Stage	Localization	ADCC %
1	58	M	Squamous cell Ca	T3N1MO	ENT	44
2	66	M	Squamous cell Ca	T2N3MO	ENT	45
3	79	M	Squamous cell Ca	T3NOM1	FNT	17
4	53	M	Squamous cell Ca	T3N1MO	ENT	26
5	50	M	Anapl. Ca	T3NOM1	ENT	43
6	33	M	Squamous cell Ca	T4N3MO	ENT	41
7	53	M	Mesothelioma	T4NOMO	Abdomen	50
8	57	F	Undiff. Ca	CS III	Ovary	26
9	65	F	Hepatocell. Ca	-	Mediastinum	15
10	27	M	Anapl. seminoma	CS IIIB	Mediastinum	36
11	46	F	Adenocarcinoma	-	Duodenum	33

Ca: carcinoma; Anapl : anaplastic ; ENT: ear, nose and throat; TNM : tumor, nodus, metastasis classification; CS: clinical stage.

Table 3. Clinical data of the solid tumor group.

Statistics.

The Wilcoxon two-sample test was used to evaluate the statistic significance of differences. All results are given as means ± standard deviation. Possible influences of age and sex on the results were analyzed with anova (analyses of variance). $P < 0.01$ was considered significant.

RESULTS

It can be seen from Tables 1-3 that extensive disease was present in most cases. The sex and age distribution of the groups studied were not similar. As might be expected, the mean age of the HD patients was significantly lower than the NHL and the solid tumor groups (30.9 ± 12.2 versus 50.6 ± 10.4 and 53.4 ± 14.7 years). The elutriation recovery, purity and yield of the monocyte fractions were almost identical for the groups studied (Table 4). The mean separation point between monocytes and lymphocytes was, despite interindividual differences, comparable for the various groups studied. Lymphocyte purity was similar for both HD patients and controls (97.9 ± 2.6 and 98.6 ± 0.3% respectively), representing 88.8 ± 7.1 and 94.3 ± 2.8% of all elutriated lymphocytes. In order to establish the major effector cell in the ADCC system, lymphocyte and monocyte mediated ADCC was determined in 12 patients with active HD and 9 healthy controls. The mean lymphocyte-mediated ADCC (LY-ADCC) of the HD patients was 6.8 ± 4.9 versus 7.0 ± 4.0% for the normal controls. In each experiment monocyte-mediated ADCC (MO-ADCC) was invariably higher than LY-ADCC. The lowest MO-ADCC/LY-ADCC ratio found was 2 and the highest 33. Cytocentrifuge preparations of the incubated suspensions containing monocytes, CRBC, and antibody, revealed numerous engulfed CRBC in different phases of degradation (Fig. 1). The cytotoxic activity of monocytes from patients with HD was significantly increased in comparison with normal controls and patients in stable complete remission (31.3 ± 7.1 versus 19.1 ± 6.1 and 20.1 ± 8.1%; $P < 0.0005$ and $P < 0.001$ respectively) (Fig. 2). No difference was found between the group of healthy volunteers and patients in complete remission. Evaluation of the lytic capacity of monocytes obtained from patients with advanced NHL and solid tumors revealed a significantly enhanced activity in comparison to normal controls (33.2 ± 12.5 and 34.2 ± 11.7 versus 19.1 ± 6.4%; both $P < 0.005$). The results of MO-ADCC in HD, NHL and solid tumors were not significantly different. In view of the differences in age and sex distribution of the populations studied, the results of MO-ADCC were analysed regarding those parameters. Neither correlation with age nor significant differences between male and female were observed. The earlier reported tendency towards augmented ADCC in HD patients with B-symptoms in comparison with a-symptomatic patients was also observed in the NHL group (43.0 ± 10, versus 28.7 ± 11%).

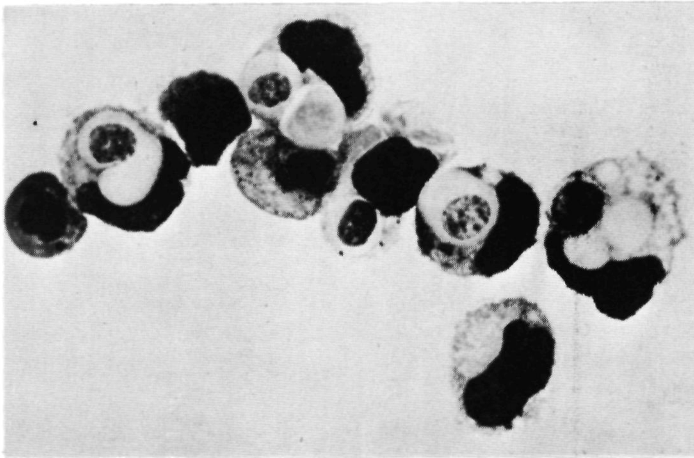

Fig. 1. Cytocentrifuge preparation of monocytes with antibody coated chicken red blood cells after 90 min incubation at 37°C.

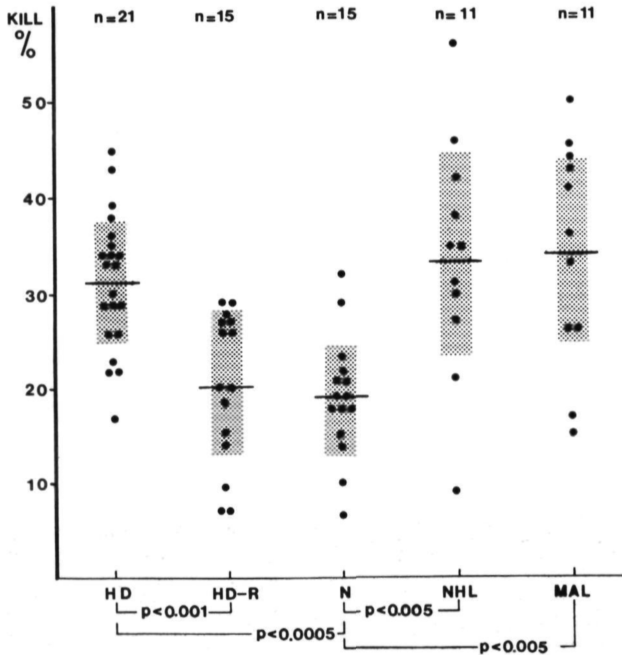

Fig. 2. Results of MO-ADCC in HD, HD in complete remission (HD-R), normal controls, NHL, and solid tumors (MAL) (Shaded area = standard deviation). Statistical analysis: Wilcoxon two-sample test.

	HD	HD-REM	NHL	Solid tumors	Normals
Monocyte purity (%)	89.4 ± 4.7	89.8 ± 2.2	89.2 ± 3.9	88.4 ± 4.8	89.5 ± 2.5
Relative yield in the purest fraction	82.5 ± 8	79 ± 8	78 ± 6	72 ± 4	76.6 ± 10
Elutriation recovery	84 ± 16	87 ± 11	88 ± 6	85 ± 15	89 ± 11
N = 73	21	15	11	11	15

HD-REM : Hodgkin's disease in complete clinical remission; NHL : non-Hodgkin lymphoma.

Table 4. Elutriation characteristics of monocyte separation with CFC.

DISCUSSION

Antibody-dependent cellular cytotoxicity (ADCC) in malignant lymphoma and non-lymphomatous malignancies was studied using homogeneous lymphocyte and monocyte effector cell populations. These well-defined cell fractions were obtained by counterflow centrifugation (CFC), which enables cell separation, mainly based upon differences in cell size [19,20]. In combination with continuous cell-scatter monitoring of the elutriator output a very reproducible isolation of monocytes and lymphocytes is obtained, both in purity and yield. Furthermore CFC provides, when compared with the most commonly used adherence technique, functionally non-selected and unaltered monocytes. In the 73 separations described in this study a mean monocyte purity of 89% was achieved representing almost 78% of all elutriated monocytes. For the lymphocyte purification in 21 experiments these figures were 98% and 91% respectively. ADCC in human cancer is most often studied using mononuclear cells as effectors [7,21,22,23,24,25,26]. Major effector cells were defined on the basis of correlations observed between a subset of cells present in those MNL suspensions and the percentage of kill [7,21,22,23]. A Fc-receptor positive non-adherent small lymphocyte was identified as the main effector cell towards CRBC targets, studying MNL-ADCC in human cancer [21]. Our results on ADCC with purified monocyte and lymphocyte suspensions do not support this observation. The monocyte appeared to be the most potent effector cell in all 12 HD and 9 normal controls which were studied. Although we did not screen the lymphocyte suspensions for Fc-receptor positivity, it is known from the literature that, in an almost identical separation procedure, this receptor was present in 18% of the purified lymphocytes [27]. The discrepancy may be explained by the difference in both cell separation technique and methodology of ADCC measurement. Cell destruction of sensitized erythroid targets by monocytes is probably due to extracellular cell lysis and phagocytosis [10,11]. This is also indicated by the cytocentrifuge preparation after incubation. Radiolabel- release assays may underestimate monocyte-mediated lysis, due to intracellular retention of the label after phagocytosis, which is therefore not detected in the supernatant. The results on LY-ADCC did not reveal any difference between HD patients and normal controls. This is in agreement with earlier reports. Using CRBC [28] and Herpes virus-infected Chang liver cell targets [29] with monocyte depleted lymphocyte suspensions no increment was observed.
In contrast with this observation, MO-ADCC of patients with HD was significantly increased in comparison to a normal control group and a group of patients in a long lasting complete remission. To our knowledge no previous study has demonstrated normal MO-ADCC after successful treatment of HD patients. The scarce data on MO-ADCC in HD in the literature are conflicting. Two earlier publications on MO-ADCC, employing respectively human red blood cells (HRBC) [30] and herpes virus-infected Chang liver

cells [29] as targets, reported a normal [30] and decreased ADCC [29]. In both studies purified monocytes were used, obtained by adherence [29] and albumin density gradient centrifugation [30]. On the other hand, with use of MNL as effector cells towards HRBC an augmented activity was found [22].
In NHL a significantly increased MO-ADCC was found in comparison to normal controls. MO-ADCC in patients with symptoms was higher in comparison to the asymptomatic group. For statistical analysis the group was too small. This finding is in contrast with Holm et al. [30], who detected no augmentation of MO-ADCC in 11 untreated patients with NHL. In order to investigate whether this MO-ADCC increment was specific for lymphomatous malignancies, a control group of patients with solid tumors was tested for MO-ADCC activity. Although remarkable variation was found, the mean ADCC was significantly increased compared to normal controls, and no essential difference was seen in comparison to HD and NHL. Data concerning ADCC mediated by purified monocytes in non- lymphomatous malignancies are scarce. Kragballe et al found in various forms of acute myeloid leukemia a depressed antibody-dependent killing capacity [31]. Reports on MNL-ADCC in solid tumors indicate enhanced [21,23], normal [21,24], and also diminished activity [25]. In view of a recent study pointing to a relation between age and MNL-ADCC towards antibody-coated HRBC [20] we analysed the influences of sex and age on MO-ADCC for the entire group. No significant influence was found, which is in agreement with other studies [20,24,25]. The enhanced MO- ADCC in both malignant lymphomas and in a variety of solid tumors as presented in this study, suggests that this is a feature not uncommon in malignant disease. This enhancement of MO-ADCC might be due to an increased number of Fc-receptors [32,33] after activation. Products of mixed leukocytes cultures are shown to stimulate the expression of Fc-receptors on human macrophages [33]. Other studies indicate that MO-ADCC and macrophage ADCC can be induced or augmented by lymphokines from antigen and mitogen-stimulated lymphocyte cultures [34,35]. Furthermore fibronectin [36] and lysozyme [37] enhance monocyte-mediated tumoricidal activity. Lysozyme was found to be elevated in HD especially with the presence of constitutional (B) symptoms [38]. This parallels our observation of more pronounced MO-ADCC in HD [15]. In NHL, however, no elevated lysozyme is observed [39]. Although the precise activation mechanism is unknown, it is tempting to assume that a disease related, immune or non-immune stimulus initiates monocyte activiation in malignant lymphoma and in various solid tumors.

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CHAPTER 6

ALTERED INTRACELLULAR ENZYME ACTIVITY OF MONOCYTES AND LYMPHOCYTES IN HODGKIN'S DISEASE

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Published in: The European Journal of Cancer and Clinical Oncology 1983; 19:333-337.

SUMMARY

To evaluate metabolic functionality of monocytes and lymphocytes in Hodgkin's disease (HD) we studied 3 enzymes of the intermediary metabolism, G-6-PDH, PHI, ICDH, and the acid hydrolases, NAG and ACP. These enzymes were measured in purified cell fractions of 9 patients with advanced disease and 11 normal controls. The cells were isolated with cell scatter monitored counterflow centrifugation. Enzymes were measured in the cell lysates by means of fluorimetric microassays. In the monocytes of HD patients a significantly increased G-6-PDH activity was found ($P < 0.01$), indicating an enhanced activity of the hexose monophosphate shunt (HMPS). The other enzymes showed no clear differences compared to normal controls. The lymphocytes of HD patients showed a significantly augmented activity of both G-6-PDH ($P < 0.001$) and PHI ($P < 0.01$), pointing to an increased HMPS and glycolytic activity. These findings are in support of an enhanced metabolic activity of both monocytes and lymphocytes in HD.

INTRODUCTION

Although the impairment of cell-mediated immunity in Hodgkin's disease (HD) is well established [1,2], data concerning lymphocyte and monocyte function are not in mutual concurrence. For both lymphocytes and monocytes, increased [3-7] and diminished [1,2,8-10] activities have been described. In a recent study we could clearly show an enhanced monocyte- mediated antibody-dependent cytotoxicity in HD patients [11], indicating an increased function of these cells.

Since it may be assumed that any functional change will be a reflection of alterations in the underlying metabolic patterns, we measured the activity of five key enzymes in monocytes and lymphocytes of 9 patients with HD and 11 normal controls. Monocytes and lymphocytes were isolated with cell-scatter monitored counterflow centrifugation (elutriation) [12] and enzyme measurement made by means of microfluorimetric techniques. The enzymes studied were: glucose-6-phosphate dehydrogenase (G-6-PDH) of the hexose monophosphate shunt (HMPS), the glycolytic enzyme phosphohexose isomerase (PHI), the Krebs cycle enzyme isocitrate dehydrogenase (ICDH) and two lysosomal enzymes N-acetyl-ß-glucosamidase (NAG) and acid phosphatase (ACP).

MATERIALS AND METHODS

Materials

Materials used for the monocyte and lymphocyte isolation were described previously [12]. Glucose-6-phosphate, nicotinamide adenine dinucleotide

phosphate (NADP) and NADPH, fructose-6-phosphate (grade I), G-6-PDH (type XV), isocitric acid (type I), L (+) lactic acid (grade I), 4-methyl-umbelliferone (4-MU) and linked derivatives (4-MU-P, 4-MU-NAG) were all obtained from the Sigma Chemical Co, St. Louis, U.S.A. The fluorochrome 4',6-diamidino-2-phenylindole. 2HCL (DAPI) was purchased from Boehringer Mannheim. Demineralized bovine serum albumin (BSA) (Povite, Amsterdam, The Netherlands). All other materials, as listed in Table 2, were of analytical purity and obtained from Merck (Darmstadt, Germany).

Patients and controls

Eight newly diagnosed biopsy-proven patients with Hodgkin's disease and one patient with a relapse after radiotherapy and chemotherapy were included in this study. The histological material was classified according to the Rye conference modification of the Lukes Butler classification [13]. Staging procedures were carried out according to the criteria recommended at the Ann Arbor conference [14]. None of the patients had concomitant overt infectious disease. The clinical data are summarized in Table 1. A group of 11 healthy volunteers (10 men and 1 woman, age 30 ± 5 yr) served as controls.

	Stage of disease				
	I	II	III	IV	n*
A	1	-	1	1	3
B	-	1	1	4	6
n	1	1	2	5	

Total = 9 patients age 37 ± 14 yr sex all male, histology
4 nodular sclerosis, 4 mixed cellularity and 1 lymphocytic predominance

Table I. Patient population

Monocyte and lymphocyte isolation

The suspensions were prepared as previously described [12]. Briefly, a mononuclear cell suspension was obtained from 50 ml heparinized venous blood after Ficoll-Isopaque (1.077 g/ml) buoyant density centrifugation. After one wash step the sample was introduced into the elutriator (Beckman J2-21 C centrifuge equipped with a JE-6 elutriator rotor with standard separation chamber). By gradually diminishing the centrifugal speed at a constant counterflow rate, cells emerge from the separation chamber mainly according to their size. Continuous cell scatter monitoring enables optimal

separation for each sample. Four fractions were gathered, a lymphocyte fraction containing approximately 90% of all elutriated lymphocytes with a purity of 99%, a mixed lymphocyte and monocyte fraction, a monocyte fraction with about 80% of the elutriated monocytes with an average purity of 90% and, after flushing the tubing system, a rest fraction. After washing, the lymphocyte and monocyte fractions were further analyzed. Differential counting of May-Grünwald-Giemsa and non- specific esterase [15] stained cytocentrifuge preparations was performed on 400 cells.

Biochemical assays

The three enzymes of intermediary metabolism (G-6-PDH, PHI and ICDH) were measured by fluorimetric determination of NADPH generated in the presence of appropriate substrates using either direct of coupled systems. The acid hydrolases (NAG and ACP) were assayed by fluorimetric determination of 4-MU released from the corresponding 4-MU-linked derivatives. The individual assay methods are detailed in Table 2; in all cases, preliminary experiments were carried out to verify the linearity of the system with respect to incubation time and enzyme activity. Cells (about 10^6) were lysed in 500 µl of aqueous bovine serum albumin solution (1 mg/ml; BSA) by one cycle of freeze-thawing followed by 2 x 10 sec ultrasonication at 0^oC. The lysate was centrifuged (5 min, 3000 x g) and appropriate dilutions of the supernatant made in BSA. Duplicate 20 µl aliquots of the diluted lysate were added to either 100 µl (G-6-PDH, PHI and ICDH) or 20 µl (NAG or ACP) of the pre- mixed reagent. After incubation at 37^oC, the reaction was stopped by cooling in ice and dilution and the fluorescence determined using a 'Fluorispec' fluorimeter (Baird Europe B.V., The Hague, The Netherlands). After subtraction of appropriate blanks (BSA in place of lysate), activity was calculated as nmol/min.µg DNA by reference to NADPH or 4-MU standards. Preliminary experiments revealed that lysates showed no change in enzyme activities for at least 3 months at - 80^oC; all samples were therefore assayed within this period after isolation of the cells. The DNA content of the lysate was determined by fluorescence of the complex with DAPI [16] using a modification described by Mier et al. [17].

Statistics

The Wilcoxon two-sample test was used to compare the results, wich are given as means ± standard deviation.

Enzyme	Reaction conditions				Stopped by	Fluorescence	
	Composition	Volume (µl)	pH	Time (min)		λ ex	λ em
G-6-PDH	Tris, 5 µmol $MgCl_2$, 1 µmol EDTA, 0.5 µmol G-6-P, 20 nmol NADP, 10 nmol	120	7.6	30	0.2 M Na_2CO_3 pH 10.5, 0.5 ml	342	455
PHI	Tris, 5 µmol $MgCl_2$, 1 µmol EDTA, 0.5 µmol F-6-P, 20 nmol NADP, 10 nmol G-6-PDH, 0.1 IU	120	7.6	30	0.2 M Na_2CO_3 pH 10.5. 0.5 ml	342	455
ICDH	Tris, 5 µmol $MgCl_2$, 1 µmol $MnCl_2$, 1 µmol EDTA, 0.5 µmol Isocitrate, 20 nmol NADP, 10 nmol	120	7.6	60	H_2O, 0.5 ml	342	455
NAG	Lactate, 4 µmol 4 MU-NAG, 20 nmol	40	4.3	60	0.2 M Na_2CO_3 pH 10.5, 1 ml	362	450
ACP	Lactate, 2 µmol 4 MU-P, 4 nmol	40	3.6	60	0.2 M Na_2CO_3 pH 10.5, 1 ml	362	450

ex = extinction

em = emission

Table 2. Assay systems for the individual enzymes

RESULTS

After elutriation the mean purities of lymphocytes for the HD group and normal controls were 97 ± 3.7 and 99 ± 0.6% respectively. For the monocyte suspensions the corresponding purities were 87.0 ± 5.8 and 88.2 ± 3.3%. The results of the enzyme measurements are presented in Figs 1 and 2. The G-6-PDH activity of the monocytes from patients with HD (1.65 ± 0.29 nmol/min µg DNA) was significantly increased ($P < 0.01$) in comparison to normal control monocytes (1.34 ± 0.19). No differences were found in PHI, ICDH, NAG and ACP activity. In the lymphocytes of HD patients both G-6-PDH and PHI activity were significantly increased: G-6-PDH for HD was 0.67 ± 0.13, and for the normal controls was 0.45 ± o.1 ($P < 0.001$). PHI activity in HD and normals was respectively 5.1 ± 1.1 and 3.9 ± 0.7 ($P < 0.01$). Although ICDH and the lysosomal enzymes NAG and ACP in HD lymphocytes tended to be higher, the differences did not reach $P = 0.01$.

DISCUSSION

The increased intracellular level of G-6-PDH in purified lymphocytes and monocytes of 9 patients with HD indicates an enhanced hexose monophosphate shunt (HMPS) activity, reflecting ribose and NADPH production, essential for DNA, RNA and protein synthesis. In view of studies pointing to an augmented HMPS activity in lymphokine-activated monocytes [18] and in PHA- stimulated lymphocytes [19], these findings are in support of an increased lymphocyte and monocyte activity in HD. No correlation between age and G-6-PDH activity in monocytes, as described by King et al. [20], was observed in our study. Using radiolabeled glucose, King et al. [21] registered an increased HMPS activity in adherent monocytes from male patients with malignant lymphoma, and they also noted an augmented glycolytic and Krebs cycle activity. However, the results in our study concerning the activity of PHI of the glycolytic pathway and IDCH of the Krebs cycle did not confirm this observation for monocytes from patients with HD. In the lymphocytes, next to the increased HMPS activity, the observed level of PHI is in agreement with an increased function of the glycolytic pathway, which is supposed [22] to be involved in lymphoblastic transformation. The occurence of increased spontaneous lymphocyte transformation in patients with HD [4] seems to corroborate the validity of this observation. No alteration in the Krebs cycle enzyme ICDH was found in HD lymphocytes. There is, to our knowledge, no information available concerning NAG and ACP activity of monocytes of HD patients. These enzymes are important for the hydrolytic degradation of glycoproteins, mucopolysaccharides and glycolipids. The results of this study reveal a comparable level for monocytes of both HD patients and controls. The activity of several lymphocytic lysosomal enzymes are shown to be both increased [23,24] and decreased in lymphoproliferative diseases

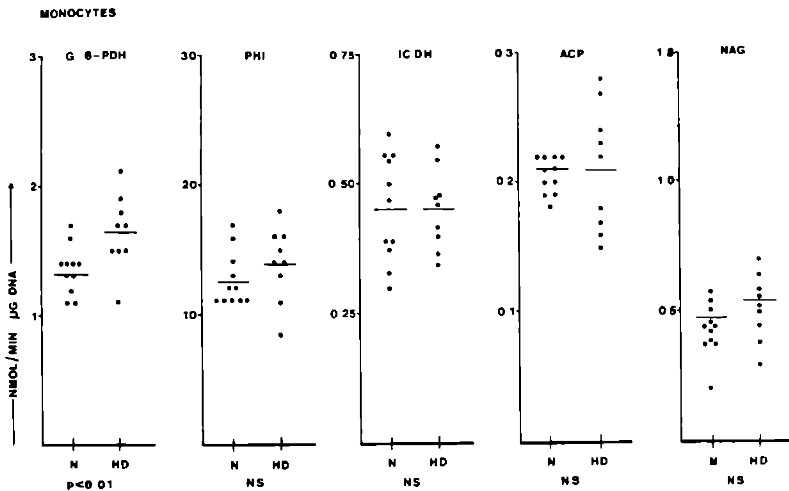

Fig. 1. Activity of G-6-PDH, PHI, ICDH, ACP and NAG in monocytes from patients with HD and normal controls.

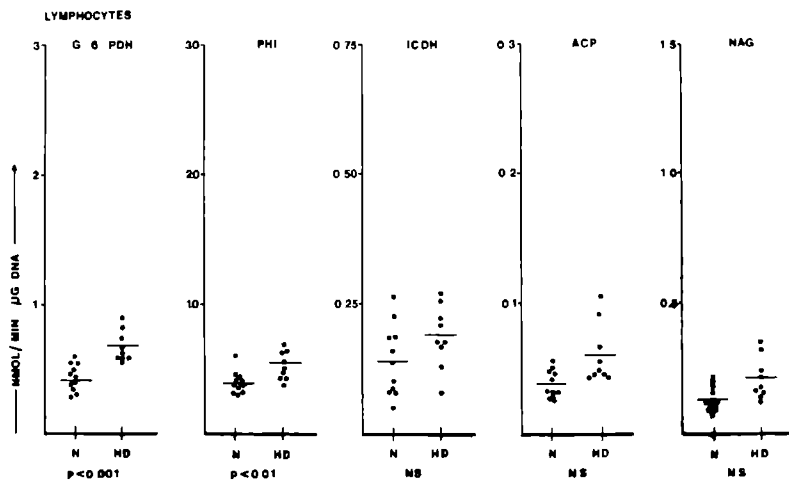

Fig. 2. Activity of G-6-PDH, PHI, ICDH, ACP and NAG in lymphocytes from patients with HD and normal controls.

[25]. Woessner [26], studying the lysosomal enzyme β- glucuronidase in lymphocytes from patients with HD, found values within or above the normal level. Our figures for NAG and ACP activity tended to be increased in HD lymphocytes without reaching a statistically significant level (P = 0.01). In conclusion, our findings are in support of an increased function of monocytes and lymphocytes in HD.

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CHAPTER 7

CHARACTERIZATION OF MONOCYTE MATURATION IN ADHERENT AND NON-ADHERENT CULTURES AND ITS APPLICATION TO STUDY MONOCYTE DIFFERENTIATION IN HODGKIN'S DISEASE

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Accepted for publication in: Clinical and Experimental Immunology

SUMMARY

Monocytes purified with cell scatter monitored counterflow centrifugation were cultured in plastic (adherent) and in teflon culture bags (non-adherent). Maturation was monitored during 15 days by measuring intracellular activity of 3 enzymes of intermediary metabolism: glucose-6-phosphate dehydrogenase (G-6-PDH), phosphohexose isomerase (PHI) and isocitrate dehydrogenase (ICDH), and the two acid hydrolases: acid phoshatase (ACP) and N-acetyl-ß-glucosaminidase (NAG). In the adherent macrophages G-6-PDH showed an increment of 26 times the 2 h value, for PHI and ICDH was this 3 and 5 times respectively. For NAG and ACP a 20 and 47 fold increase was found. In teflon grown macrophages a significantly lower G-6-PDH activity was seen after 15 days in comparison to plastic adherent macrophages ($p < 0.0002$), indicating activation by adherence. For the other enzymes similar values for both culture modalities were found. The significantly, cycloheximide insensitive, higher values for G-6-PDH, PHI and ICDH in 2 h adherent monocytes in comparison with non-adherent monocytes, suggest a relationship between adherent capacity and the level of intermediary metabolism. The overall yield of adherent macrophages was 35% in contrast with 89% for the in suspension cultured macrophages. This corroborates the existence of adherent and non-adherent monocytes, both capable of maturation to macrophages. In 14 patients with advanced Hodgkin's disease (HD) and 14 normal controls monocyte maturation was studied applying both culture modalities. The enzyme levels, reflecting growth and intermediary metabolism, were similar for both groups, in non-adherent as well as in adherent grown macrophages. The adherent capacity and yield, both in plastic and in teflon, after 15 days was comparable for both groups. It was concluded that monocyte maturation in the presence of autologous serum was qualitatively and quantitatively normal in advanced HD; this is in favour of an intrinsically normal function of monocytes in HD.

INTRODUCTION

The blood monocyte is derived from the granulocyte/macrophage progenitor cell in the bone marrow. This cell type is a transit form in the development to the various types of tissue macrophages, together forming the mononuclear phagocyte system (MPS) [1]. The cells of this system exhibit distinct morphological features, and different functional and metabolic characteristics at various levels of differentiation and maturation [2,3,4,5,6,7]. Several investigators have shown that during in vitro culturing of monocytes, cellular peroxidase activity decreases [5,6] 5'-nucleotidase activity as well increases [4,6] as decreases to low levels after 7 days [4], and that the lysosomal enzymes acid phosphatase (ACP) [3,4] and N-acetyl-ß-glucosaminidase (NAG) [4,7] increase.

We studied intracellular key enzyme activities as parameter of monocyte maturation. Monocytes were isolated with cell scatter monitored counterflow centrifugation [8,9] and cultured in plastic tubes for 15 days, enabling the study of both adherent capacity and the maturation pattern of the adherent cells. Culturing in hydrophobic teflon bags [10] permitted the determination of monocyte maturation in suspension. Cultures were monitored by measurement of the intracellular levels of two lysosomal enzymes: ACP and NAG as markers for cell growth, and three enzymes of the intermediary carbohydrate metabolism: glucose-6-phosphate dehydrogenase (G-6-PDH), phosphohexose isomerase (PHI) and isocitrate dehydrogenase (ICDH). G-6-PDH is the first enzyme in the hexose monophosphate shunt (HMPS), generating NADPH the major source of reducing power in the cell and ribose-5-phosphate a component of important biomolecules such as RNA, DNA and ATP. Glucose utilization through this pathway is shown to increase during activation of macrophages [11,12]. PHI and ICDH are enzymes of the glycolytic pathway and the Krebs' cycle respectively, and reflects energy supply as ATP formation.
Recent publications indicate a correlation between a diminished in vitro maturation and a poor prognosis in patients with solid tumours [13,14]. In Hodgkin's disease (HD) no information is available concerning quantitative and qualitative monocyte maturation in vitro. There is conflicting indirect in vivo evidence pointing towards either a diminished [15,16] or normal monocyte maturation [17]. In 14 patients with advanced HD and 14 normal controls this differentiation proces was evaluated in vitro using both culture modalities.

MATERIALS AND METHODS

Patients and controls.

The patient group studied consisted of 13 patients with newly diagnosed HD and 1 patient in relapse; 4 women and 10 men, ranging in age from 16 to 68 years (mean age, 30 years). Table 1 summarizes clinical stage, disease activity and histology. Staging procedures were carried out according to the criteria recommended at the Ann Arbor conference [18]. The histological material was classified according to the Rye conference modification of the Lukes-Butler classification [19]. A group of 14 healthy volunteers served as normal controls. None of the patients and normal controls were taking any medication or had overt infectious disease.

Monocyte isolation.

Monocytes were isolated as described previously with the use of cell scatter monitored counterflow centrifugation [8,9,20]. An average purity of 89%, representing almost 80% of all elutriated monocytes was achieved for both patients and normal controls.

		n	
PS	II	A	2	(NS,MC)
PS	II	B	1	(MC)
CS	III	B	5	(4NS,1MC)
CS	IV	A	1	(NS)
CS	IV	B	5	(1LD,4NS)

NS Nodular Sclerosis, MC Mixed Cellularity, LD Lymphocytic depletion , PS Pathological Stage, CS Clinical Stage

Table 1. Clinical data of 14 patients with HD

Adherent monocyte culturing.

Freshly isolated monocytes were resuspended at a concentration of 2 x 10^5/ml in light protected, RPMI-hepes-L-glutamine (Gibco, cat. no. 430-1800, Grand Island Biologic Company, New York, U.S.A.) and 10% autologous serum. One ml of this suspension was seeded in plastic flat bottomed tubes (Costar, no 3393, 16 x 93 mm, Cambridge, Mass. U.S.A.) and cultured at 37°C for different time intervals (2 h, 1,3,5,7,10 and 15 days). No antibiotics were added nor was the tube opened or the medium refreshed during the culture period. Cultures were performed in duplo. Culturing was stopped by decanting the supernatant and non-adherent cells. Loosly adherent cells were removed by rinsing (5 times) with 1 ml phosphate buffered saline (PBS) 0,5% v/v human serum albumin (Albumine Merieux 20%, Rhône-Poulenc, Charbonnieres-les-Bains, France), and the adherent cells were lysed with water/BSA solution (1 mg bovine serum albumin/ml water) to release their intracellular enzyme content. After thorough mixing of the lysate the samples were stored at -80°C, for measurement of enzyme activity and DNA content.

Monocyte culturing in suspension (non-adherent).

Culturing was performed in hydrophobic teflon bags (TCB) (Dupont de Nemours and Co., Switzerland; gauge 25 mm, according to van der Meer et al. [10]. Freshly isolated monocytes were resuspended in bicarbonate buffered RPMI-1640 (Flow Laboratories, Irvine, Scotland) containing 1 % glutamine (Micro Biological Associates, Bethesda, Maryland, U.S.A.) at a concentration of 3 x 10^5/ml in the presence of 10% autologous serum. Five ml of this cell suspension was injected gently into the TCB, and after diathermically sealing of the opening, cultured with 10% CO_2 in air in a humidified atmosphere at 37°C. Culturing was stopped after 15 days by aspiration of the cell suspension after gentle kneading of the TCB. After one wash step the cells were resuspended in PBS. Samples were taken for counting, cyto-

centrifuge preparations and enzyme studies. Cytocentrifuge preparations were stained for May-Grunwald-Giemsa and for non — specific esterase. Enzyme measurements were done on 2×10^5 cells, after pelleting, lysis in 0.5 ml H_2O/BSA solution and storage at - 80^oC.

Estimation of macrophage yield.

Cell recovery in the plastic cultures was evaluated after 2 hours and 15 days by measurement of the DNA content in 1 ml lysed uncultured cell suspension and in the lysate of the adherent cells after 2 hours and 15 days culturing. Recovery was expressed as percentage and calculated from the ratio:

$$\frac{\text{DNA g/ml at 2 h}}{\text{DNA g/ml at t = 0}} \quad \text{(2h recovery)}$$

and

$$\frac{\text{DNA g/ml at 15 days}}{\text{DNA g/ml at 2h}} \quad \text{(15 days recovery)}$$

Macrophage yield from the teflon cultures was calculated from:

$$\frac{\text{cells} \times 10^6 \text{ in TCB at 15 days}}{\text{cells} \times 10^6 \text{ initially injected.}}$$

Cell countings were performed with a coulter counter model ZF. Viability was tested by means of trypan blue exclusion.

Biochemical assays.

Enzyme measurements were performed with a fluorimetric micro assay as described previously [20]. The three intracellular enzymes of intermediary metabolism, G-6-PDH, PHI and ICDH were measured by fluorimetric determination of NADPH generated from NADP in the presence of appropriate substrates using either direct or coupled systems. The acid hydrolases, NAG and ACP, were assayed by fluorimetric determination of 4- methylumbelliferone released from linked derivatives. After thawing of the samples, they were subjected to 2 x 10 sec. ultrasonication at 0^oC and after centrifugation twenty µl of the lysate was incubated in duplo with the appropriate substrates and fluorescense determined. Activity was expressed in nmol/min. ug DNA.

The DNA content of the lysate was measured by fluorescense of the complex with the fluorochrome 4'6-diamidino-2-phenylindole- 2-HCL.

Statistics.

The Wilcoxon two sample test was used to evaluate the results. Numbers are given as mean ± standard deviation.

RESULTS

Enzyme changes during culturing of plastic-adherent monocytes.

Monocyte maturation and metabolic activity were quantified by the determination of enzyme activities at different time intervals. The relative and absolute changes of intracellular enzyme activities of plastic adherent monocytes are depicted in Fig 1. In all experiments (n = 6) a similar pattern was to be seen. G-6-PDH showed an increment of 26 times the level observed after two hours adherence, for PHI and ICDH this was 3 and 5 times respectively. The acid hydrolases ACP and NAG revealed an increase of 47 and 20 times their basic level. After 7 days the curve of all enzymes flattened, but a still further increase was observed with prolonged culturing. This change in enzyme activity paralelled the light microscopical (phase contrast) changes in most cells such as increase in size, and the appearance of cytoplasmic granulae.

Enzyme activity in non-adherent (teflon) cultured monocytes.

After 15 days no significant differences were found in PHI, ICDH, NAG and ACP activity between plastic and TCB-cultured macrophages. However, a significantly higher G-6-PDH activity was observed in the plastic-adherent macrophages in comparison with the non-adherent teflon grown macrophages (53.7 ± 15.5 versus 31.9 ± 13.6 nmol/min. ug DNA; $p < 0.0002$). May-Grunwald-Giemsa and non-specific esterase stained cytocentrifuge preparations consisted of 95% or more macrophages (Fig. 2). The Number of bi-or multinucleated cells was 1% or less.

Influence of adherence on enzyme activity

The intermediary metabolism representing enzymes, G-6-PDH, PHI and ICDH, of 2 h adherent monocytes, were markedly elevated in comparison with freshly isolated monocytes (Table 2). No such differences were found for NAG and ACP. This increased activity of intermediary metabolism might be due to either activation, selection or both. Activation would imply denovo synthesis of these enzymes, because release or activation of inactive forms is never demonstrated. To abolish protein synthesis, cycloheximide (2 ug/ml) was added to the cell suspensions in 5 experiments. The results are shown in Table 3. For all enzymes both lysosomal and of the intermediary

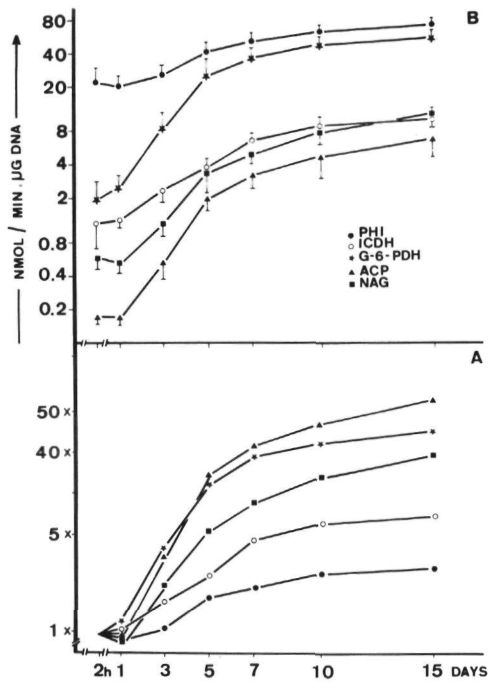

Fig. 1. Relative (A) and absolute (B) changes in enzyme activity in plastic adherent macrophages during 15 days culturing.

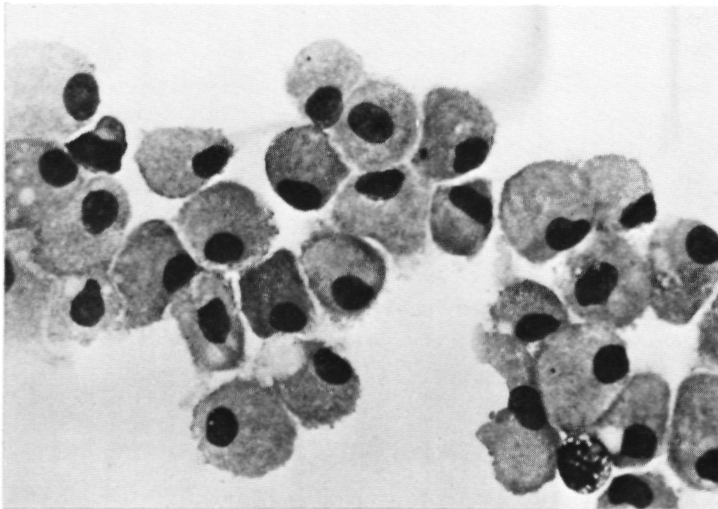

Fig. 2. May-Grünwald-Giemsa stained cytocentrifuge preparation of teflon grown macrophages (x 400).

metabolism, no differences in activity were found between the adherent cells cultured in the presence of cycloheximide and those without. Evaluation of enzyme activity in the non- adherent cells revealed very low non-overlapping values for G- 6-PDH, PHI and ICDH in comparison with the adherent cell population (Table 4). No such differences were observed for the acid hydrolases NAG and ACP.

Macrophage yield in adherent plastic (n = 28) and non-adherent (teflon; n = 22) cultures.

The number of adherent monocytes after 2 h was 50 ± 12% of those originally seeded. The yield of macrophages after 15 days was 70 ± 20% of the number adherent after 2 h. Macrophage yield from the TCB after 15 days was 89 ± 13%. Viability was 93 ± 2%.

Monocyte maturation in patients with HD and normal controls.

In 14 patients with HD and 14 normal controls monocyte adherence and subsequent changes in enzyme content over a 15 day culture period were measured. In 11 patients and 11 normal controls also teflon grown macrophages were studied. The majority of the patients was suffering from advanced active disease (71% III/IV B; Table 1). The adherent capacity of monocytes after 2 h was for both groups comparable (48 ±12, normal controls versus 51 ± 11% for the HD group) After 15 days a similar adherent macrophage yield was found (73 ± 27% and 74 ± 23% respectively). The pattern of enzyme changes in the adherent monocytes as shown in Fig 1. was for both groups identical. No significant differences in enzyme activities were found (P < 0.01)(Table 5), although G-6-PDH and ICDH values in the HD group tended to be slightly higher (Fig 3). This tendency was not observed in the suspension grown macrophages. The 3 enzymes of intermediary metabolism and the 2 acid hydrolases in the teflon grown macrophages were similar for both groups. Macrophage yield in the suspension cultures was for both HD patients and normal controls identical (89 ± 13% and 89 ± 14% respectively). G-6-PDH activity of freshly isolated monocytes from 20 patients with HD (this included 9 patients of chapter 4) revealed a significantly higher activity in comparison with those from 24 normal controls (1.67 ± 0.44 and 1.26 ± 0.31 nmol/min. ug DNA respectively, P < 0.0006). No such difference, however was observed in the adherent population after 2 hours (2.42 ± 1.2 and 1.90 ± 0.78 nmol/min. μg DNA respectively, P = 0.19).

DISCUSSION

The exact role of the circulating monocyte is not yet elucidated and is still an object of investigation. It is not clear whether the monocyte has a major

	G-6-PDH	PHI	ICDH	NAG	ACP	n
t = 0*	1.39 ± 0.51	14.4 ± 3.7**	0,47 ± 0,15***	0.48 ± 0.13	0.12 ± 0.02	31
t = 2h	2.38 ± 1.03	21.3 ± 8.8	1.68 ± 0.81	0.55 ± 0.22	0.11 ± 0.02	31

* values of earlier experiments as described in chapter 6 are included n = 20

** $P < 0.001$

*** $P < 0.0005$

Activity : nmol/min.µg DNA

Table 2. Enzyme activity of freshly isolated monocytes and after 2 h adherence.

	G-6-PDH	PHI	ICDH	NAG	ACP	n
cycloheximide -	2.91 ± 1.36^{ns}	24.9 ± 8.5^{ns}	0.75 ± 0.30^{ns}	0.75 ± 0.20^{ns}	0.12 ± 0.01^{ns}	5
cycloheximide +	2.20 ± 1.40	21.6 ± 7.0	1.30 ± 0.40	0.63 ± 0.16	0.09 ± 0.02	5

activity : nmol/min.µg DNA

ns : not significant $P > 0.01$

Table 3. Influence of cycloheximide on enzyme activity after 2 h adherene.

	G-6-PDH	PHI	ICDH	NAG	ACP	n
non-adherent	0.27 ± 0.08*	5.8 ± 1.7*	0.20 ± 0.15*	0.60 ± 0.27	0.08 ± 0.02	8
adherent	2.03 ± 0.68	17.9 ± 3.6	1.18 ± 0.22	0.66 ± 0.11	0.10 ± 0.02	8

* $P < 0.01$

activity : nmol/min.µg DNA

Table 4. Influence of adherence on enzyme activity after 2 h.

	G-6-PDH	PHI	ICDH	NAG	ACP	n
N - ADH	51.3 ± 13.6	69.4 ± 14.6	8.2 ± 3.2	10.2 ± 4.9	5.5 ± 3	14
N - SUSP	32.9 ± 11.5*	70.8 ± 13.9	7.4 ± 3.5	14.3 ± 4.5	6.5 ± 2.7	11
HD - ADH	62.5 ± 25.8	71.7 ± 21.7	10.4 ± 4	11.9 ± 5.2	6.9 ± 2.8	14
HD - SUSP	28.1 ± 17.1*	70.1 ± 9.9	5.0 ± 2.9	17.9 ± 8.5	8.4 ± 5.7	11

* $P < 0.01$

N = Normal

HD = Hodgkin's Disease

ADH = adherent

SUSP = suspension

Activity : nmol/min.µg DNA

Table 5. Enzyme activity after 15 days in both adherent and suspension cultures.

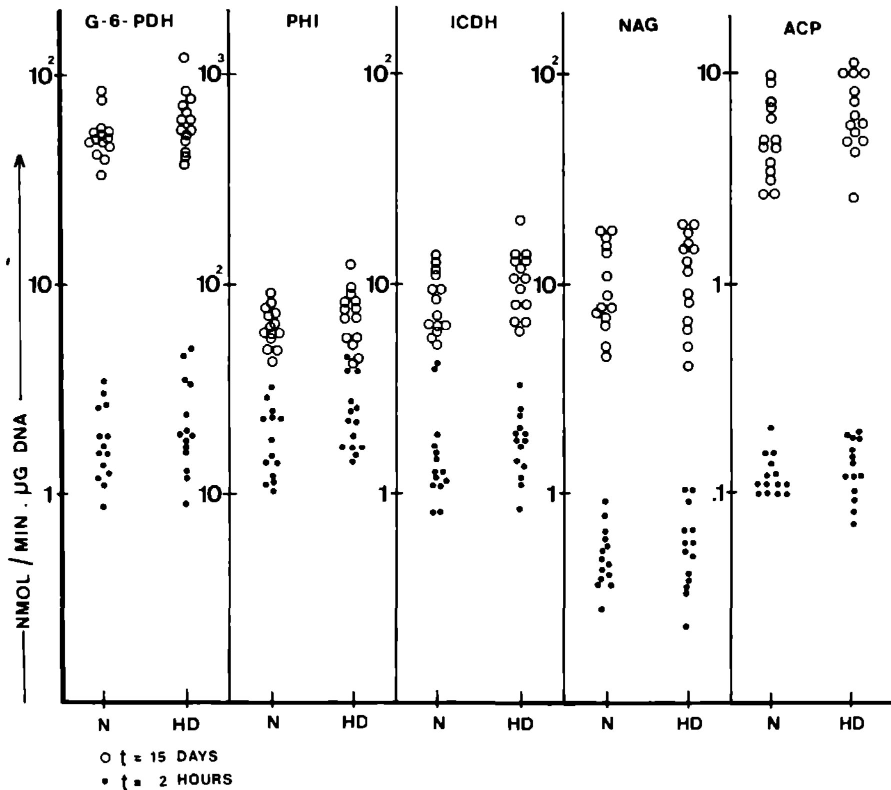

Fig. 3. Changes in enzyme activity in 14 patients and 14 normal controls.

role as circulating macrophage or is just an immature cell of the MPS, which displays a great variety of functions after extravascular maturation and/or activation. To evaluate the role of MPS in health and disease it seems preferable to study the mature exponents of this system, however limited access to their anatomical sites in humans hampers this kind of work. An approach to overcome this limitation is the study of in vitro maturation of peripheral blood monocytes. This process of maturation is extensively described using morphological, functional, enzyme and receptor alterations as parameters [2,3,4,5,6,7,21]. In most studies monocytes adherent to plastic, glass or coated surfaces were investigated. These monocytes were recovered from a mixed cell suspension on the basis of their adherent properties, which implies selection and activation.

In the present study we investigated the plastic-adherent capacity and subsequent maturation of CFC purified monocytes and compared those results with the maturation process observed in suspension cultures. To our knowledge no information is available concerning alterations in the intermediary metabolism during maturation. G-6-PDH activity was markedly increased (26 times), PHI and ICDH, on the other hand, showed much lesser changes (3 and 5 times respectively). This predominant increase in G-6-PDH activity indicates an enhanced HMPS glucose utilization and is most likely reflecting an augmented cellular need for NADPH and ribose-5-phosphate during maturation.

A remarkable finding was the significantly lower G-6-PDH in the teflon grown macrophages after 15 days in comparison with the plastic-adherent cells. Bodell et al [22] could demonstrate a rapid appearance of peroxidase activity in the rough endoplasmic reticulum after adherence. It was postulated that surface stimulation altered metabolic events. Our finding corroborates this supposition, although some influence of the differences in the culture conditions in our study cannot be excluded (bicarbonate buffered versus hepes buffered RPMI-1640, humidified atmosphere versus closed tubes). The observed alterations in NAG and ACP are in accordance with the literature The differences in G-6-PDH, PHI and ICDH activity between adherent and non-adherent monocytes after 2 h indicates a relationship between adherent capacity and intermediary metabolism. The fact that cycloheximide did not influence the 2 h enzyme levels makes denovo synthesis unlikely and points towards selection. The high recovery of teflon grown macrophages (88%) in comparison to the plastic-adherent (35%) implies that the non-adherent monocytes are likely to give rise to mature macrophages. In preliminary experiments (data not shown) we could actually prove this, by growing mature macrophages in TCB from both adherent (after detachment) and non-adherent monocytes.

After characterization of maturation in both suspension and adherent cultures we evaluated this process in 14 patients with predominantly advanced HD and 14 normal controls. It is clearly shown in this study that, in

the presence of 10% antologous serum, the differentiation from monocyte to macrophage in HD is undisturbed. The adherent capacity of the purified monocytes was for both groups identical. Measurement of the DNA content, instead of microscopical counting, facilitates accurate quantitation of adherent cells. There are two earlier reports describing, in contrast with our results, low adherent cell yield in patients with HD [23,24]. A close relationship was observed between a diminished PHA induced blastogenesis and a decreased number of adherent macrophages in unstimulated cultures in HD in comparison to normal controls [24]. On the other hand Urbanitz et al. [25] found a for HD and normal controls similar percentage of monocytes attached after 50 min. (26 and 24% respectively). In patients with solid tumours also a marked decrease in adherent macrophage yield was found after 7 days culturing [13,14,26,27]. In all studies mononuclear cell suspensions, containing monocytes, platelets and lymphocytes, were cultured in the presence of autologous serum. The non-adherent cells remained in culture. The influence of autologous serum factors was excluded in two studies and the existence of an intrinsic monocyte defect suggested [26,27]. However, the maturation of non-adherent monocytes to macrophages as we could demonstrate in our study, and the influence of non-adherent cells (direct or exerted by mediators) on both adherence and differentiation was not considered. Lymphokines of antigen stimulated lymphocytes promoted human monocyte adherence in vitro [11,28]. Musson and Henson [29] could demonstrate and inhibition of monocyte adherence by plasma and serum factors which was counteracted by platelets. Lee et al. [30] showed that interferon-1 can strongly inhibit monocyte maturation as measured with biochemical and morphological parameters. Except for the serum factors, all other influences were excluded in our study and permits therefore a more definite judgment concerning adherence and in vitro monocyte maturation.

It was concluded that the in vitro monocyte differentiation was undisturbed in advanced HD, despite the well established presence of leukocyte function inhibiting serum factors. [31,32,32]. This argues against an intrinsic monocyte defect in HD.

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CHAPTER 8

LYSOZYME AND COLONY STIMULATING FACTOR SECRETION BY MONOCYTE DERIVED MACROPHAGES IN HODGKIN'S DISEASE

P.H.M. de Mulder, J.M.C. Wessels, T. de Witte, E. van de Ven, A. van Erp and C. Haanen

INTRODUCTION

It has recently become evident that the macrophage had major secretory capabilities [1,2]. Known secretory products of mononuclear phagocytes are enzymes, enzyme inhibitors, binding proteins, reactive metabolites of oxygen, nucleic acid and metabolites, bioactive lipids, growth factors and endogenous pyrogens [1].
Lysozyme constitutes the bulk secretory product of macrophages [1,3], but is also present in the polymorponoclear leucocyte, however, it is not synthesized by the latter, end stage cell [4].
Serum lysozyme levels were found to be increased in patients with HD [5,6]. This lysozyme appeared to be positively correlated with the stage of the disease and the presence of B-symptoms [5]. A relationship with tumor mass was suggested [5,7]. Because it has been shown that monocyte-derived macrophages produce and secrete lysozyme [3], these cells may be responsible for the elevated serum levels observed in HD. Colony stimulating factor (CSF) is produced by a variety of normal cell types including mononuclear phagocytes [8,9,10], T-Lymphocytes, and vascular endothelial cells [11]. Besides spontaneous release, a merked enhancement of CSF production can be initiated by endotoxin [9]. Furthermore the mononuclear phagocyte seems to play an important role in the regulation of CSF release by other cell types such as T-lymphocytes and endothelial cells [11].
The aim of the present study was the evaluation of some secretory properties of in vitro monocyte-derived macrophages as parameter of their function in patients with Hodgkin's disease. Lysozyme and spontaneous colony stimulating factor secretion were evaluated in 12 and 10 patients respectively.

MATERIALS AND METHODS

Patients and controls

Lysozyme activity was studied in 12 patients, 4 women and 8 men, mean age 42.2 ± 19 years (range: 19-73 years). The clinical data are depicted in Table 1. Twelve healthy volunteerse were used as controls (3 women, 9 men; mean age 31.5 ± 7.1 years, range: 24-50 years).
Colony stimulating factor production was measured in 10 patients, 2 women and 8 men, age 28.1 ± 6.6 years (range: 21-42 years). Stage and pathology are shown in Table 2. Seven healthy volunteers served as controls (2 women and 5 men, mean age 22.6 ± 9.3 years; range 22-33 years). The histological classification was according to Lukes et al. [22], staging procedures were carried out as recommended at the Ann Arbor conference [13].

Stage	n	PA
IA	3	(1 NS, 2 MC)
IIA	2	(2 MC)
IIIA	1	(NS)
IIIB	1	(MC)
IVA	3	(2 NS, 1 MC)

Table 1. Clinical data of HD patients tested for serum and monocyte lysozyme activity

Stage	n	PA
IIA	5	(2 MC, 3 NS)
IIB	2	(NS, LP)
IIIB	2	(2 MC)
IVA	1	(NS)
IVB	2	(NS,MC)

NS nodular sclerosis
MC mixed cellularity
LP lymphocytic predominance

Table 2. Clinical data of HD patients tested for monocyte CSF production

Monocyte isolation

Monocytes were isolated by means of cell scatter monitored counterflow centrifugation as described previously [14,15]. In both patients and normal controls an average purity of 89% was obtained, representing 80% of all elutriated monocytes.

Monocyte-derived macrophage lymsozyme secretion

Monocytes were cultured in hydrophobic teflon bags (TCB) according to Van der Meer et al. [16]. Freshly isolated monocytes were resuspended at a concentration of 3 x 10^5/ml in RPMI-1640 supplemented with 4mM glutamine, and 10% autologous serum. Five ml of this cell suspension was gently injected into the TCB, and after diathermically sealing, cultured for 7 and 15 days in a fully humidified atmosphere of 5% co_2 in air at 37°C. At the end of the culture period, after gentle kneading of the TCB, the cell suspension was recovered, centrifuged (600 x 9, 10 min, at 20°C) and the supernatant stored at -20°C for lysozyme measurements.

Lysozyme assay

Lysozyme activity was measured in both serum and culture supernatant, according to the method described by Prockop et al. [19]. Enzyme activity was evaluated with a Beckman DB-G grating spectophotometer by measuring the decrease of turbidity, due to lysozyme induced desaggregation of the cell wall of the Micrococcus Lysodeickticus (Testomar-Lysozyme kit, Behring). Activity was expressed as mg human lysozyme/liter (Hl/l).

Preparation of monocyte conditioned medium (MCM)

Monocytes were cultured according to a modification of the method described by Chervenick and Robuskio [8]. After isolation, the monocytes were washed for 10 min, 400 x 9 at 20°C. Cells were resuspended in bicarbonate buffered RPMI-1640 (Boehringer) supplemented with 20% (v/v) foetal calf serum (FCS, Gibco) and 4mM glutamine (Flow) at a concentration of 2 x 10^6/ml. Three ml of this suspension was seeded in 50 ml plastic flasks (Costar) and incubated at 37°C for 7 days in a fully humidified atmosphere of 5% CO_2 in air. At the end of the culture period the supernatant was decanted, centrifuged (10 min, 600 x 9 at 20°C) and after filtration through a 0,45u filter (Millipore) stored at -20°C.

Granulocyte-macrophage colony forming assay

In vitro culture assays were performed as described previously [17]. Briefly: cultures were done in 35 mm plastic petri dishes (Costar). Dulbecco's modified Eagles medium (Flow) was used with 20% heat inactivated FCS from a preselected batch in 0,3% Bacto-agar (Difco). Nucleated cells (2 x 10^5) were seeded with 10% v/v. MCM obtained from the various monocyte cultures. All cultures were done in duplicate using the same normal bone marrow donor. As reference source, CSF derived from human placenta conditioned in the presence of endotoxin was used. Incubations were performed at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 10 days. Cultures were scored at 20-40 x magnification. Aggregates consisting of 40 and more cells were scored as colonies, aggregates consisting of < 40 cells as clusters, and < 20 cells as small clusters.

Cell counting and differentiation

Peripheral blood cell counts were performed with a Coulter counter model ZF. Cell differentiations were done on smears after staining for May-Grunwald-Giemsa and by means of the Hemalog D (Technicon).

Statistics

The Wilcoxon two sample test was used for statistical analysis of the results. Numbers are given as mean ± standard deviation.

RESULTS

Lysozyme measurements

Mean serum lysozyme activity in HD patients was significantly increased in comparison to normal controls (2.41 ± 0.47 and 1.32 ± 0.43 mg Hl/l respectively; $p < 0.01$). Mean relative and absolute monocyte and granulocyte numbers are presented in Table 3. The mean absolute monocyte and granulocyte count in HD group was higher in comparison to the normal group, however the differences were not statistically significant ($p < 0.05$). A significant positive correlation was found between the absolute peripheral blood granylocyte count and the serum lysozyme content (R: 0.47, $p < 0.05$). This was not observed between the monocyte count en serum lysozyme level. In a preliminary experiment a lysozyme increase from 0.50 mg Hl/l ($t = 0$) to 3.7 mg Hl/l ($t = 18$ days) was found in the supernatant of the moncyte cultures. This rise of lysozyme was almost exponential.
Lysozyme secretion by monocyte derived macrophages form 12 patients with HD and 12 normal controls, evaluated at day 7 and 15 was not essentially different (Table 3).

CSF production by monocyte derived macrophages

CSF activity of the supernatant from 7 days cultured purified monocytes was measured in 10 patients with predominantly asymptomatic HD. The results are shown in Table 4. Both the number of colonies and clusters obtained with the MCM from patients with HD tended to be lower, however the differences were not statistically significant ($p > 0.05$).

DISCUSSION

Serum lysozyme in the HD group was significantly increased in comparison to the normal control group. This is in agreement with earlier observations [5,6]. No significant correlation was found with the absolute number of circulating monocytes which corroborates the finding of Corberand et al. [6]. The absolute granulocyte number showed a moderate, but statistically significant correlation with the serum lysozyme level of the whole group studied. Hansen et al. [20] found a strong correlation between both granulocyte turnover and total blood granulocyte pool, and serum lysozyme activity (R: 0.88 and R 0.81 respectively). In the present study the monocyte derived macrophages of

	HD	normal controls
Relative monocyte count (%)	6.4 ± 3.8	5.5 ± 1.8
Absolute monocyte count/mm³	474 ± 205	377 ± 155
Relative granulocyte count (%)	66.2 ± 12.9	52.3 ± 11.5
Absolute granulocyte count/mm³	5638 ± 2807	3723 ± 1720
Serum lysozyme mg Hl/l	2.41 ± 0.47*	1.32 ± 0.43
7 days supernatant lysozyme	0.98 ± 0.27	0.96 ± 0.19
15 days supernatant lysozyme	2.58 ± 0.66	2.40 ± 1.05

* $p < 0.01$

Table 3. The granulocyte and monocyte values in the peripheral blood 12 patients with HD and 12 normal controls. Results of lysozyme measurements.

	colonies	clusters	
		< 20	< 40
without supernatant	0	0	0
placenta CSF	83	41	41
mono-supernatant normals	5.6 ± 7.3	137.1 ± 41.5	93 ± 47
mono-supernatant HD	2.8 ± 4	134 ± 73	55 ± 49

Table 4. Results of CSF production by 7 days cultured monocytes in 10 patients with HD and 7 normal controls.

both groups secreted almost indentical amounts of lysozyme measured at two different time intervals. One should realize that no information is available concerning the monocyte transit time in the peripheral blood compartment and that the absolute blood monocyte number is not only reflecting turnover. The possibility that an increased macrophage mass in HD may account for the observed elevated serum lysozyme levels in not excluded. Our observations on monocyte-derived macrophages makes it unlikely that the elevated serum lysozyme level is due to an increased production by single macrophages, which is in agreement with a studies in other malignancies [21].
The mononuclear phagocyte system plays an important modulatng role in hematopoiesis by releasing CSF and other factors which stimulate CSF production by other cell types [11,22,23]. Moore et al. [22] observed a close positive correlation between monocyte number in the peripheral blood and detectable CSF activity in the serum of a cyclic neutropenic patient.

Besides stimulatory factors, monocytes release inhibitors of hematopoiesis, such as prostaglandin E_2 [10,24,25]. For these reasons MCM contains stimulators and inhibitors and in fact a netto effect is evaluated. The MCM in our study induced much less colonies than optimal CSF obtained from human placenta. The results with MCM of 10 predominantly asymptomatic patients with HD indicate a lower mean number of colonies and clusters in comparison to 7 normal controls, however the differences were not statistically significant. Monocyte PGE_2 production has been shown to be increased in HD patients [26]. This might in part explain the observed tendency of diminished CSF activity of MCM in the present study.

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CHAPTER 9

SUMMARY AND GENERAL CONCLUSIONS

Chapter 1 presents a short introduction and an outline of the investigations.

Chapter 2 reviews the function of the mononuclear phagocyte system (MPS) in the pathophysiology of Hodgkin's disease (HD). Few studies are available dealing with the function of the tissue macrophages. All the studies on monocyte function described in this review, were performed either on mixed cell populations or adherent monocytes.
So far no specific alteration in the MPS function could be demonstrated in HD.

In chapter 3 a new method for monocyte isolation has been described. Continuous monitoring of cell light scatter during counterflow centrifugation allowed counting and size recognition of the cells. For each donor an optimal separation point between lymphocytes and monocytes could be determined, resulting in a monocyte purity of more than 90%, with 84% of all elutriated monocytes present in this enriched fraction. The cell recovery after elutriation (or counterflow centrifugation) was 90% and the viability was excellent.

In chapter 4 the results of antibody-dependent cellular cytotoxicity mediated by purified monocytes (MO-ADCC) have been presented. A new and sensitive ADCC assay was used, avoiding time consuming labeling techniques. The method is based on the difference in DNA content between the chicken red blood cell (CRBC) target and the monocyte effector cell. Analysis was performed by means of DNA flow cytometry. In HD a significantly increased MO-ADCC was found in comparison to normal controls. No correlation was found between the absolute monocyte number in the peripheral blood and the level of MO-ADCC. This argues against a shift in functionally different monocytes on the basis of monocytosis, which was present in 50% of the patients studied. There was no relation with the stage of the disease; however, in patients with B-symptoms MO-ADCC tended to be higher than in the asymptomatic group.

Chapter 5 outlined the results of MO-ADCC in a group of patients with HD in remission, a group with non-Hodgkin lymphomas, and a group with solid tumors. MO-ADCC appeared to be significantly increased in both non-Hodgkin lymphomas and patients with solid tumors. MO-ADCC was established to be normal in patiens with HD in complete remission. Furthermore the monocyte appeared to be a more potent effector cell towards CRBC than concomitantly isolated lymphocytes.

In chapter 6 the results of intracellular enzyme analyses of monocytes obtained from HD patients and normal controls, this is compatible with an enhanced function of the hexose monophosphate shunt, and reports of increased glucose utilization through this shunt after activation.

Monocyte maturation was studied in HD patients and normal controls using adherent and non-adherent (suspension) culture conditions. Cell growth was evaluated by measuring the intracellular content of two acid hydrolases, and glucose utilization by measuring 3 enzymes of intermediary metabolism. The results are presented in chapter 7.
The yield of macrophages after 15 days in the non-adherent cultures was 89%, in contrast to 35% in the adherent cultures. After 2 hours, monocyte adherence was accompanied by a significantly higher acitvity of 3 enzymes of intermediary metabolism, in comparison to the activity in non-adherent monocytes
Monocyte maturation in both adherent and in suspension cultures was undisturbed in HD. The adherent capacity of monocytes from HD patients appeared to be comparable with normal control monocytes.

In chapter 8 the results of some secretional properties of monocyte derived macrophages have been reported. Lysozyme secretion by macrophages from patients with HD, measured at two time intervals, was comparable to that observed in normal controls. On the other hand serum lysozyme activity was significantly increased in HD patients. No correlation with the absolute monocyte number in the peripheral blood was found. the elevated serum lysozyme level might be due either to an augmented granulocyte turnover, or related to an increased mass of MPS in HD.
Colony stimulating factor activity in the supernatant of 7 days cultured monocytes tended to be lower in HD patients in comparison to normal controls.

Till now all studies on monocytes have been performed on unseparated or functionally selected cells. In our study atraumatically purified monocytes have been used with only minimal selection and avoiding activation, and the results are therefore more representative for the circulating blood monocyte. The separation procedure was, thanks to the cell recognition device, very reproducible and well applicable to the purification of monocytes from patients mononuclear cell suspensions. The advantage of this isolation method is once more emphasized by the finding that monocytes are heterogeneous in their adherent capacity, and that prolonged adherence coincides with hexose monophosphate shunt activation.
Both our findings on effector function activity and intermediary metabolism are in accordance with a higher activity of monocytes obtained from patients with HD. The increased effector function was related with overt disease, but

also present in patients with non-Hodgkin lymphomas and solid tumors. Monocyte maturation and adherent capacity in HD was normal, which is in contrast with reports by investigators using mixed cell populations. Lysozyme production by in vitro grown macrophages also indicated a normal function in HD. The measured activity of colony stimulating factor in the supernatant is in fact the result of a balance between stimulators and inhibitors, both produced by monocytes and macrophages.
The suppression of lymphocyte proliferation by monocytes from patients with HD, described in the literature, is not in contrast with our findings of an activated monocyte function, in view of the known higher suppressive capabilities of monocytes after activation.

In conclusion, the monocyte appears to be functionally intact in patients with HD. The number of monocytes in HD is frequently elevated and their function appears to be enhanced. These alterations are related with overt disease, but HD specificity could not be demonstrated.

ALGEMENE SAMENVATTING

De ziekte van Hodgkin is een bijzondere vorm van kwaadaardige lymfeklier ziekte, die voor het eerst werd beschreven door Thomas Hodgkin in 1832. Pas de laatste 15-20 jaar is er enig inzicht verkregen omtrent de aard en de oorzaak van deze ziekte, die zonder behandeling, fataal verloopt. Veel aandacht is besteed aan de bij patienten met de ziekte van Hodgkin zo opvallend verhoogde gevoeligheid voor virale en mycotische infecties. Met name werd veel onderzoek verricht naar de mogelijke rol van de lymfocyt in dit immunologische defect.
Recent is ook belangstelling ontstaan voor de betekenis van het mononucleair fagocyterend systeem (MPS) in de pathofysiologie van de ziekte van Hodgkin. Het MPS bestaat uit voorloper cellen in het beenmerg, die uitgroeien tot de monocyten in het perifere bloed en vervolgens uitrijpen tot een grote verscheidenheid aan weefsel macrofagen. Het MPS speelt een belangrijke rol bij het opruimen van afstervende cellen en bij het ontstaan en effectueren van afweer reacties, verder worden zeer veel stoffen geproduceerd en uitgescheiden, die betrokken zijn bij talrijke biologische processen, welke onder meer samenhangen met proliferatie en maturatie van cellen.

In Hoofstuk 1 wordt een kort overzicht gegeven van het doel en werkplan van de studie. Het onderzoek van het MPS is geconcentreerd op de relatief eenvoudig te verkrijgen bloed monocyt.

Hoofdstuk 2 gaat in op de in de literatuur vermelde waarnemingen betreffende de functie van het MPS bij de ziekte van Hodgkin. Er is tot nu toe geen voor de ziekte kenmerkende afwijking in de functie van het MPS vastgesteld. Het overgrote deel van deze onderzoekingen is gedaan met onzuivere cel populaties (lymfocyten en monocyten) en slechts enkele met gezuiverde monocyten. Deze laatste waren echter niet aselectief verkregen, aangezien de zuiveringsmethode berustte op de adhererende eigenschap van de monocyt. Over de functie van macrofagen is weinig bekend, daar deze niet in voldoende mate intact voor onderzoek te verkrijgen zijn.

Gezien het ontbreken van onderzoek met aselectief gezuiverde monocyten bij de ziekte van Hodgkin werd een nieuwe isolatie methode ontwikkeld, waarmee monocyten kunnen worden verkregen met een hoge zuiverheid (90%), met een goede opbrengst (84%) en viabiliteit (98%). De scheiding vindt plaats met behulp van een tegenstroom centrifuge, gekoppeld aan een systeem dat de grootte van de cel continue kan bepalen. Op deze manier kan voor iedere donor het optimale scheidingspunt tussen lymfocyten en monocyten worden vastgesteld (Hoofdstuk 3).

Als belangrijke effector functie werd het antilichaam afhankelijke cel dodende vermogen van de monocyt onderzocht (MO-ADCC) (Hoofstuk 4).
Voor het meten van deze functie werd een nieuwe en gevoelige methode gebruikt, gebaseerd op het verschil in DNA gehalte tussen de kippe rode bloed cellen (CRBC) en de monocyten. De cel-dood, gemeten aan verlies van het DNA-gehalte van de met antilichaam gecoate CRBC, geschiedde met behulp van DNA- flowcytometrie. Bij patienten met de ziekte van Hodgkin bleek de MO-ADCC significant hoger te zijn dan bij de normale controle groep. Er werd geen correlatie gevonden tussen het absolute aantal monocyten in het peripere bloed en het percentage celdood. Dit pleit tegen een eventuele functieverandering tengevolge van een toegenomen aantal monocyten in het bloed. In 50% van de onderzochte patienten werd een absolute monocytose gevonden. Er was geen relatie met het stadium van de ziekte en de hoogte van de MO-ADCC; patienten met B-symptomen tendeerden echter tot een hogere MO-ADCC dan de asymptomatische patienten.

Hoofdstuk 5 bevat de resultaten van MO-ADCC van een groep patienten met de ziekte van Hodgkin na het bereiken van een complete remissie,van een groep met een non-Hodgkin lymfoom en van patienten met een solide tumor. Zowel bij patienten met een non-Hodgkin lymfoom als bij patienten met een solide tumor werd een significant verhoogde MO-ADCC gevonden. Bij patienten, die met goed resultaat behandeld waren voor de ziekte van Hodgkin bleek de MO-ADCC in het normale gebied te liggen. Deze resultaten geven aan dat de verhoogde monocyten activiteit bij een grote verscheidenheid van kwaad-aardige ziekten aanwezig is, en na effectieve behandeling, zoals aangetoond bij de ziekte van Hodgkin, normaliseert.
Hoewel de lymfocyt ook effectief een ADCC tot expressie kan brengen in het gebruikte testsysteem, bleek dit vermogen bij de monocyt in alle onder-zochte gevallen sterker.

Een andere manier om de eventueel verhoogde activiteit van de monocyt bij de ziekte van Hogkin aan te tonen is de bestudering van enige metabole aspecten. Dit is weergegeven in Hoofstuk 6.
Monocyten van patienten met de ziekte van Hodgkin bleken een significant hogere glucose-6-fosfaat dehydrogenase (G-6-PDH) activiteit te vertonen in vergelijking met monocyten van een normale controle groep. Dit past bij een activatie van de hexose monofosfaat shunt. In de literatuur is beschreven dat de metabolisering van glucose door deze shunt toeneemt indien monocyten en macrofagen gestimuleerd worden door bijvoorbeeld lymfokinen.

De monocyt groeit na het verlaten van de bloedbaan, in de weefsels uit tot macrofaag en ondergaat tijdens dit proces een groot aantal veranderingen. In Hoofdstuk 7 zijn de karakteristieken van de twee gebruikte in vitro kweekmethoden aangegeven en wordt het uitrijpingsproces van monocyten van

patienten met de ziekte van Hodgkin en van normale controle personen beschreven. Monocyten werden zowel adherent (vastgehecht aan plastic) als non-adherent (in teflon) gekweekt. De opbrengst aan adherente macrofagen na 15 dagen was 35% in vergelijking tot 89% voor de non-adherent gekweekte. Deze laatste hadden na 15 dagen een significant lagere G-6-PDH activiteit dan de adherent gekweekte macrofagen, hetgeen wijst op stimulatie en selectie door de hechting aan plastic.
De monocyten, die na 2 uur niet hechtten, bleken een beduidend lager glucose metabolisme te bezitten in vergelijking met de monocyten die dit vermogen wel bezaten.
Het uitrijpings proces, vervolgd aan de hand van 2 lysozomale enzymen, bleek voor monocyten van patienten met de ziekte van Hodgkin vergelijkbaar met dat van normale monocyten. Het vermogen tot hechten was voor beide groepen eveneens gelijk. Deze bevindingen wijkt af van de waarnemingen beschreven in de literatuur, waarbij de uitrijping van de adherente monocyten afkomstig van patienten met solide tumoren sterk verminderd zou zijn.

De resultaten betreffende de uitscheiding van lysozyme en kolonie stimulerende activiteit door in vitro gekweekte macrofagen worden beschreven in Hoofdstuk 8.
De lysozyme uitscheiding gemeten op 2 tijdstippen bleek voor patienten met de ziekte van Hodgkin en normalen gelijk. Het serum lysozyme gehalte was echter aanzienlijk hoger bij Hodgkin patienten dan bij normale controle personen. Er werd geen correlatie gevonden met het absolute aantal monocyten in het perifere bloed. Het verhoogde lysozyme gehalte in het serum kan afkomstig zjn van een verhoogde granulocyten turnover of samenhangen met een toegenomen massa van het MPS. Over dit laatste aspect zijn echter geen gegevens beschikbaar. De kolonie stimulerende activiteit in het kweek supernatant bleek bij patienten met de ziekte van Hodgkin wat lager dan bij de normale controle groep.

Alle tot nu toe in de literatuur beschreven onderzoeken betreffende de monocyten functie bij patienten met de ziekte van Hodgkin zijn verricht met niet gezuiverde monocyten, waardoor interactie met andere cellen niet kon worden uitgesloten, evenmin als functie veranderingen ten gevolge van de op adherentie gebaseerde isolatie methode. In tegenstelling daarmee zijn de hier beschreven waarnemingen verricht met atraumatisch gezuiverde monocyten, waarbij de selectie minimaal was en activering door bijvoorbeeld hechten is vermeden. De verkregen gegevens mogen daarom ook als meer representatief voor de perifere bloed monocyt beschouwd worden. De scheidingsprocedure is, dankzij het cel herkenningssysteem, zeer reproduceerbaar.
Het voordeel wordt geaccentueerd door de waarneming dat de monocyten

heterogeen zijn ten aanzien van hun vermogen zich te hechten aan vreemde oppervlakken en het feit dat zij door dit laatste metabool geactiveerd worden.
Zowel de toegenomen effector functie (ADCC) als de gevonden verandering in het intermediaire metabolisme passen bij een toegenomen activiteit van de monocyt bij de ziekte van Hodgkin. Deze toeneming lijkt samen te hangen met de aanwezigheid van de ziekte, maar is eveneens aantoonbaar bij patienten met een non- Hodgkin lymfoom en patienten met een solide tumor. Dit wijst op een waarschijnlijk aspecifieke beinvloeding van de monocyten functie door dergelijke aandoeningen. De in vitro monocyten uitrijping was ongestoord hetgeen een belangrijk argument voor normaal funktioneren is. De gevonden lysozyme productie door in vitro gekweekte macrofagen wijst eveneens in die richting. De licht verlaagde kolonie stimulerende activiteit is in feite het resultaat van zowel stimulatoren als remmers, welke beide door de monocyt/ macrofaag worden geproduceerd. De in de literatuur beschreven onder-drukking van de lymfocyten proliferatie door de monocyt, is niet strijdig met onze resultaten, die wijzen op een geactiveerde functie. Het is namelijk bekend dat de suppressieve eigenschappen van de monocyt toenemen na stimulatie.
Samenvattend blijkt uit dit onderzoek dat de monocyt bij de ziekte van Hodgkin functioneel intact is, maar dat veelal zowel het aantal als hun activiteit is toegenomen. Deze quantitatieve en qualitatieve veranderingen van monocyten hangen samen met het manifest zijn van de ziekte, maar is als zodanig niet specifiek voor de ziekte van Hodgkin.

Woorden van dank

Velen hebben meegeholpen bij het tot stand komen van dit proefschrift. Op de eerste plaats de vele medewerkers van het Laboratorium Bloedziekten, die mij hebben ingewijd in de mij destijds ontbrekende specifieke laboratorium vaardigheden. Met name wil ik noemen Elly Geestman als docente 'steriel werken', John Smeulders als 'ficoll' specialist en Aart Plas, die mij op de voor insiders bekende wijze vertrouwd maakte met de elutriator en tijdens de gehele onderzoek periode voor dit onderdeel als een onmisbare 'trouble shooter' is opgetreden.

Voor de uitvoering van de vele scheidingen, kleuringen en bepalingen heb ik dagelijks mogen samenwerken met Erik van de Ven en in de beginfase met Gerdien Rosenbrand. Arie Pennings was behulpzaam met het opzetten van cytotoxiciteits test en Jan van Egmond ontwikkelde het juiste computer programma.

Mariet Hillegers-Ewals maakte de vele honderden liters buffer nodig voor de elutriator experimenten.

Vruchtbaar was de samenwerking met de werkgroep Biochemie van de afdeling Dermatologie (hoofd P.D. Mier, Ph D), alwaar Helga van Rennes op inventieve en accurate wijze de vele enzym bepalingen verichtte en Mieke Bergers behulpzaam was bij de start van de monocyten kweek. Aan de patienten en de vele normale proefpersonen, die belangeloos hun medewerking verleenden, ben ik veel dank verschuldingd.

De medewerking van vele collegae in de regio was, voor het verkrijgen van voldoende patienten, onontbeerlijk. Met name wil ik noemen Drs. G. Boetius, Dr. W. Breed, Dr. F. van Dam, Drs. A. Dolman, Drs. G. Hoogendoorn, Drs. L. Eekhout, Dr. R. Kurstjens, Drs. H. Scheerder en Drs. J. Steenbergen.

De afdeling medische fotografie (hoofd: A. Reijnen) was behulpzaam met het drukklaar maken van de tabellen, foto's en de omslag. Paul Mier vond steeds tijd voor het bijschaven van het Engels en de laatste kritische en nauwgezette correctie van het manuscript geschiedde door de heer J. de Pauw.

De heer de Graaff en zijn medewerkers waren altijd zeer behulpzaam bij het verzamelen van de literatuur.

Angela van den Broek en Loes Baadenhuijsen verzorgden op deskundige en geautomatiseerde wijze het typewerk en drukklaar maken van het manuscript. Marjan Janssen verzorgde de tabellen.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 28 mei 1949 te Heerlen. In 1967 werd het eindexamen HBS-B behaald op het St.Dominicus College te Nijmegen. Aansluitend studeerde hij geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1971 was hij 3 maanden werkzaam als Extern in het Somerset Hospital, Somerville, New Jersey, USA.
Het doctoraal examen werd in 1973 en het artsexamen in 1975 behaald. De dienstplicht werd vervuld, als bataljon's arts bij de Koninklijke Landmacht. In 1976 werd begonnen met de opleiding tot internist aan de Universiteits Kliniek voor Inwendige ziekten te Nijmegen (hoofd destijds Prof.dr.C.L.H. Majoor † en sinds januari 1980 Prof.dr.A. van 't Laar). Medio 1979 werd gestart met het onderzoek (KWF-subsidie NUKC 79-10) op de afdeling Bloedziekten (hoofd Prof.dr.C.Haanen). In de toekomst zal hij werkzaam zijn op de afdeling Medische Oncologie (hoofd Prof.dr.D.J.Th. Wagener).
Hij is getrouwd met Willemien Mertens en zij hebben een zoon, Huib.

Stellingen
behorende bij het proefschrift van
P.H.M. de Mulder

I

Voor de bestudering van eigenschappen van een specifieke celpopulatie is een aselectieve en atraumatische celscheiding een essentiële voorwaarde.

Dit proefschrift.

II

Reproduceerbare scheiding van mononucleaire celsuspensies, bestaande uit per donor variabele aantallen trombocyten, lymfocyten en monocyten, is optimaal mogelijk met behulp van tegenstroom centrifugatie, indien de uitstroom, met behulp van lichtverstrooiing, continu op celaantal en grootte geanalyseerd wordt.

Dit proefschrift.

III

De hexose-monofosfaatshunt activiteit, de maturatie, de lysozyme secretie en de antilichaam afhankelijke cytotoxiciteit van monocyten van patiënten met de ziekte van Hodgkin is normaal tot toegenomen. Bij patiënten met een non-Hodgkin lymfoom of solide tumor wordt eveneens frequent een verhoogd cytotoxisch vermogen gevonden.

Dit proefschrift.

IV

Adherente macrofagen representeren niet het totale aantal precursors (monocyten) in het perifere bloed.

Dit proefschrift.

V

Monocyten zijn functioneel te onderscheiden op basis van hun adhererend vermogen. Parallel hiermee blijkt het glucose metsbolisme veranderd.

Dit proefschrift.

VI

De aanwezigheid van dermatopatische lymfadenopathie bij patiënten met een T-cel lymfoon van de huid moet als uiting van generalisatie worden beschouwd. Men dient naast locale behandeling een systemische therapie te overwegen.

Bunn P.A., Huberman M.S., Whang-Peng J. et al., Prospective staging evaluation of patients with cutaneous T-cell lymphonas. Demonstration of a high frequency of extracutaneous dissemination. *Ann. Intern. Med.* 1980; 93: 223.

VII

Interval-verlenging tussen de cytostatische kuren bij de behandeling van de ziekte van Hodgkin en non-Hodgkin lymfomen vermindert de kans op het bereiken van een complete remissie. Uitstel op andere gronden dan een beperkte beenmerg tolerantie moet daarom vermeden worden.

VIII

Voor de prognose van patiënten met een non-Hodgkin lymfoom is het histologisch beeld meer indicatief dan het stadium.

IX

Indien bij een patiënt met een gesupprimeerd immunologisch systeem neurologische symptomen ontstaan, moet de goed behandelbare Toxoplasma Gondii infectie van het centrale zenuwstelsel overwogen worden, en bij de uitsluiting van duidelijke andere oorzaken dient de patiënt als zodanig behandeld te worden.

Townsend J.J., Wolinsky J.S., Baringer J.R., Johnson P.C., Acquired toxoplasmosis. A neglected cause of treatable nervous system disease. *Arch. Neurol.* 1975; 32: 335.

Eigen waarneming.

X

Bij 'idiopathisch' hypoproteinemisch oedeem op basis van een hypertrofische gastritis (ziekte van Ménétrier) kan een spontaan herstel van het serum eiwitgehalte gezien worden. Het verdient daarom aanbeveling, indien voor deze indicatie een maagoppervlak verkleinende operatie wordt overwogen, een afwachtende houding aan te nemen.

XI

De spontane in vitro groei van burst forming units uit geaspireerd beenmerg is het beste diagnostische criterium voor een polycythemia vera.

Lacombe C., Cadaderall N., Varet B., Polycythemia vera: in vitro studies of circulating erythroid progenitors. *Br. J. Haematol* 1980; 44: 189.

Th. de Witte, persoonlijke mededeling.

XII

Hematoporfyrine derivaten (HPD) vormen, onder invloed van licht, sterk cytotoxische zuurstof radicalen. Door de gevonden verhoogde retentie van HPD in maligne en prémaligne weefsels lijkt 'fototherapie' een nieuwe en beloftevolle behandelingsmodaliteit tegen kanker te kunnen worden.

Dougherty I.J., Grindey G.B., Fiel R. et al., Photoradiation therapy: II cure of animal tumors with haematoporhyrin and light. *J. Nat. Cancer Inst.* 1975; 55: 115.

Forbes I.J., Cowled P.A., Leong A.S.Y. et al., Phototherapy of human tumours using haematoporphyrin derivative. *Med. J. Aust.* 1980; 2: 489.

XIII

Het ontbreken van een citatie index voor universitaire onderwijs activiteiten komt de prioriteit die dit onderwijs verdient niet ten goede.

XIV

Het zou aanbeveling verdienen tenminste één van de maatschappijvakken voor het eindexamen in het voortgezet onderwijs verplicht te stellen.

XV

Inhoudelijk wordt op de arts-patiëntrelatie vooral een beroep gedaan als het medisch technische resultaat niet aan de verwachtingen beantwoordt.

Nijmegen, 10 juni 1983 P.H.M. de Mulder