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in vitro modulation by cytokines

and anticancer drugs

Rob van Schie

HUMAN MONOCYTES

IN VITRO MODULATION BY CYTOKINES AND ANTICANCER DRUGS

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen, in het bijzonder de Geneeskunde

Proefschrift ter verkrijging van de graad van doctor aan de Katholieke Universiteit Nijmegen, volgens besluit van het College van Decanen in het openbaar te verdedigen op maandag 28 september 1992, des namiddags te 1.30 uur precies

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Cover illustration May-Grunwald Giemsa stained cytocentrifuge preparation of 9-day cultured human peripheral blood monocytes, containing phagocytozed Rhesus D+ erythrocytes sensitized with human alloserum against Rhesus D (Original magnification x 400)

The research presented in this thesis was performed in the Divisions of Medical Oncology (head Prof Dr D J Th Wagener), and Hematology (head Prof Dr T J M de Witte), Department of Internal Medicine (head Prof Dr R A P Koene), University Hospital Nijmegen, The Netherlands (Fax +31-80-540788)

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ABBREVIATIONS

ACP	acid phosphatase
ADCC	antibody-dependent cell mediated cytotoxicity
Bleo	bleomycin sulphate
BRM	biological response modifier
BSA	bovine serum albumin
CD	cluster of differentiation
***cis*-DDP**	cisplatin
^{51}Cr	radionuclide chromium-51
CSF	colony-stimulating factor
Dox	doxorubicin hydrochloride
E	erythrocytes
EA-IgG	E *sensitized with IgG*
EdSm	ethyldeshydroxysparsomycin
E:T ratio	effector to target cell ratio
FCS	fetal calf serum
FcγR	receptor for the Fc moiety of IgG
5-FU	flourouracil
GM-CSF	granulocyte macrophage CSF
G-6-PDH	glucose-6-phosphate dehydrogenase
h	human
HR	high-responder (to mIgG1)
^{125}I	radionuclide iodine-125
ICAM-1	intercellular adhesion molecule-1
ICDH	isocitrate dehydrogenase
IF	immunofluorescence
IF buffer	PBS / 1 0% BSA / 0 1% NaN_3
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
K_a	equilibrium association constant
LFA-1	leukocyte function adhesion-1 antigen
LPS	lipopolysaccharide
LR	low-responder (to mIgG1)
m	murine
mAb	monoclonal antibody
M-CSF	macrophage CSF
MHC	major histocompatibility complex
MPS	mononuclear phagocyte system
NAG	*N*-acetyl-β-glucosaminidase
4-OHCY	4-hydroxycyclophosphamide
PBS	phosphate-buffered saline
PHI	phosphohexose isomerase
r	recombinant
RAS	*right angle scatter*
RFS	red forward scatter
RIA	radioimmunoassay
TFB	Teflon foil bag
TNF	tumor necrosis factor
U	unit

GENERAL INTRODUCTION

INTRODUCTION

In the last few years the efforts to improve cancer therapy have been directed at stimulating the natural immune system by means of biological response modifiers (BRM) or cytokines Genetic engineering has resulted in a growing number of recombinant BRM becoming available, including interferons (IFN), interleukins (IL), colony-stimulating factors (CSF), and tumor necrosis factors (TNF) In the past decade the impact and results of immunotherapy have been such that, in addition to more conventional treatment such as surgery, radiation therapy and chemotherapy, immunotherapy can be considered as the "Fourth Modality of Cancer Treatment" (1) High doses of single agent recombinant cytokines have resulted in antitumor effects both in murine models and in the treatment of humans with metastatic cancer (2-9) Unfortunately, high doses of cytokines were toxic when administered systemically, while the observed responses have been rather transient Since effectiveness appeared to be dependent on the tumor load, a logical next step was to combine this approach with chemotherapy Consequently improvements in experimental cancer therapy have been made using recombinant cytokines to augment the action of anticancer drugs and reduce the doses necessary to achieve antitumor effects (10-16)

Most anticancer drugs known were generally considered to exert their effects through antiproliferative and cytotoxic actions (17) but it has now become apparent that chemotherapy regimens can induce both immunological depression and specific immune activation of antineoplastic host defenses (18) The activity of the immune system depends upon the participation of a large number of highly specialized cells in the blood and tissues, each type performing a unique function Monocytes and macrophages play a central role in the immune response and are a first line of defense against the development and spread of tumors (19-21) Anticancer drug interactions with human monocyte/macrophage functions are complex and have been the object of limited investigation (22-29) The *in vitro* examination of the influence of cytokines and/or anticancer drugs on human monocytes at different stages of maturation *in vitro* is described in this thesis This knowledge is necessary for a better understanding of combined approaches to cancer treatment that may lead to further therapeutic advances

MONOCYTES AND MACROPHAGES

Origin, ontogeny and distribution. Human macrophages are the final differentiated cells of the mononuclear phagocyte system (MPS) and are derived from peripheral blood monocytes via promonocytes and monoblasts that originate during bone marrow hematopoiesis (30-34) Newly formed monocytes move from the marrow into the

peripheral blood stream within a day (35) and distribute themselves between marginating and circulating pools (36) having a transit half-time in the circulation of approximately 70 h (37,38). In some severe infections, monocytes may differentiate in the peripheral blood to become macrophages (39) In general, this transformation takes place after diapedesis and migration into various extravascular tissues (40) Up to now, there is no evidence that monocytes are programmed for any particular tissue destination However, both the quantity and velocity of monocyte recruitment to a particular organ may be strongly influenced by the production of several chemotactic factors as cytokines, growth factors, *N*-formylmethionyl peptides and complement products which are induced by inflammatory foci (41-44) Once in the tissues, monocytes develop into macrophages characteristic of the different tissues (Table I)

Table I.

Localization of monocytes and different types of macrophages throughout the human body

Cell Type	Localization
Monocyte	bone marrow, peripheral blood
Macrophage	bone marrow, endocrine organs, gastrointestinal tract, genitourinary tract, lymph node (free and fixed), mucosa associated lymphoid tissue, thymus
Alveolar macrophage	lung
Epithelioid cell	inflammatory tissue
Exudate macrophage	inflammatory tissue
Histiocyte	connective tissue, skin
Kupffer cell	liver
Langerhans cell	skin
Microglial cell	central nervous system
Multinucleate giant cell	inflammatory tissue
Osteoclast	bone
Peritoneal macrophage	serous cavity
Pleural macrophage	serous cavity
Red pulp macrophage	spleen (free and fixed)
Synovial type A cell	synovium
Tumor-associated macrophage/ Tumor infiltrating macrophage	solid tumor

The cell's morphology is influenced by innumerable tissue-specific stimuli which not only induce augmentation of lysosomal enzymes and granules but also increase the overall metabolic and functional potential in general (45,46) Macrophages can also be further induced to alter their metabolism and function (e g , phagocytosis and chemo-

taxis) by many stimuli This additional activation is characterized by the presence of certain markers (47) Moreover they increase in size and their adherence and secretory capacity are enhanced (48) The functional heterogeneity associated with macrophages is, to a large extent, a reflection of their differentiation state (49,50)

Monocyte differentiation *in vitro*. The peripheral blood monocyte is the most accessible cell of the MPS and differentiation *in vitro* of this cell type has been widely used as a model system to study macrophage development Many of the changes that accompany the process of monocyte differentiation *in vitro*, appear to resemble the alterations observed *in vivo* (51 60) Cultured monocytes (i e , monocyte-derived macrophages) seem to be largely representative of the tissue macrophages
Isolation. Pure monocyte suspensions are recommended when investigating the biological properties of monocyte differentiation Obviously, any cell separation procedure should not affect the viability or function of the cells In contrast to conventional isolation techniques [e g , adherence (61), density (55), phagocytosis (62)], monocyte isolation by counterflow centrifugation (elutriation) has been shown to give large numbers of highly purified cells (>95%), good recovery (>95%), and excellent viability (>98%) In addition, this method has the advantage that the separation time is relatively short, and that functional properties are not adversely affected by the procedure (63-66)
Culture. Culture systems impose major limitations when investigating the differentiation and functional properties of cultivated monocytes Monocytes readily become firmly attached to plastic or glass surfaces which impedes their activation Suspending the cells requires physical manipulation by a rubber policeman, or chemical treatment with trypsin or chelating agents Any of which might well lead to a reduced recovery and loss of viability or function Culturing monocytes in Teflon foil bags overcomes these problems since cells grow in suspension and can be recovered for experimentation at any stage of maturation Furthermore, this culture system has many other important advantages Teflon foil bags are cheap and disposable, they can be quickly made to any size, the risk of infection is reduced, the gas exchange is guaranteed and the cells can be easily harvested with minimal damage Therefore recovery is almost complete, the cells are not functionally impaired, and activation is minimal (56,57,61,66-70) Although the precise signals responsible for the initiation of monocyte maturation *in vitro* are largely unknown, good results are obtained using medium supplemented with between 1 and 25% heat inactivated human serum (57,60,70)
Morphology. Alterations in cell morphology are obvious during monocyte differentiation *in vitro* although the structure, extent and rate of morphological changes may vary with the cultural conditions (e g , serum source and concentration, addition of BRM) However, in general, monocytes loosely attach to the Teflon surface and the majority

of cells appear rounded During culture they increase greatly in size and develop a higher cytoplasm to nucleus ratio Furthermore, the eccentrically placed horseshoe shaped nucleus differentiates into a spherical centrally located nucleus (56,57,60,70,71) (for covering photographs see chapter 5, page 84)

Functional aspects and cell characteristics of monocytes and macrophages.

Monocytes and macrophages can be regarded as multifunctional cells which play a central role in the host defense against neoplasia (19-21) A full account of the abundant functions executed by the monocyte/macrophage is beyond the scope of this thesis Therefore only those that are of immediate relevance will be discussed

Antigen presentation. Monocytes and macrophages assist in initiating and facilitating cell mediated immune responses After internalization, antigens are processed by intracellular proteolysis and presented as peptides in association with Major Histocompatibility Complex (MHC) class II molecules on the cell surface (72) The quantity of these MHC class II molecules (e g , HLA DR) on the surface of monocytes/macrophages is of paramount importance to the initiation of an immune response that proceeds efficiently (73) The highly specific interaction of T-lymphocytes with class II/antigenic peptide-complexes in the presence of IL-1 will ultimately induce proliferation and differentiation of T-cells (74)

CD14. Ninety percent of circulating blood monocytes (75) and the vast majority of macrophages (76) possess the differentiation-associated antigen CD14 (77) This is a receptor for the complex formed of an acute phase serum lipopolysaccharide (LPS) binding protein and LPS of Gram-negative bacteria (78,79) In addition, CD14 is involved in LPS-induced TNF production (79) The gene for CD14 is mapped to the long arm of chromosome 5, band q32, a region encoding for growth factors and receptors (80) Furthermore, the cellular consequences of CD14 ligation are not yet clear but it is likely to be an important mechanism for the clearance of Gram-negative bacteria from the bloodstream and interstitial fluids

Secretion of biologically active constituents. Monocytes and macrophages are capable of secreting a large number of well defined molecules (81) which include complement components, coagulation factors, cytokines, enzymes, enzyme inhibitors, growth factors, hormones and reactive oxygen intermediates Constituents are released either continuously (constitutive secretion), or after stimulation (regulated secretion) (82,83) allowing the cells to orchestrate a wide spectrum of activities (e g , destruction of tumor cells, secretion) of other cell types (e g , lymphocytes, granulocytes, natural killer cells) Alternatively they may become the target of a positive feedback loop when activated by their own secretory products (e g , IL-1, TNF) (84)

Mechanisms of cytotoxicity. Monocyte and/or macrophage-mediated lysis of tumor cells may be most efficient when cell-cell contact is established and the initial step in destroying neoplastic target cells occurs by selective recognition of the target. The effector cell may recognize either neoplastically transformed cells expressing a tumorigenic phenotype (85,86), or tumor cells opsonized with IgG antibodies (87-89). It is remarkable that the specificity of the latter event is determined by the antibody rather than any direct programming by the monocyte/macrophage itself. This second mechanism of lysis, known as antibody-dependent cell-mediated cytotoxicity (ADCC), is mediated via receptors capable of binding the Fc moiety of IgG (FcγR) (Fig. 1).

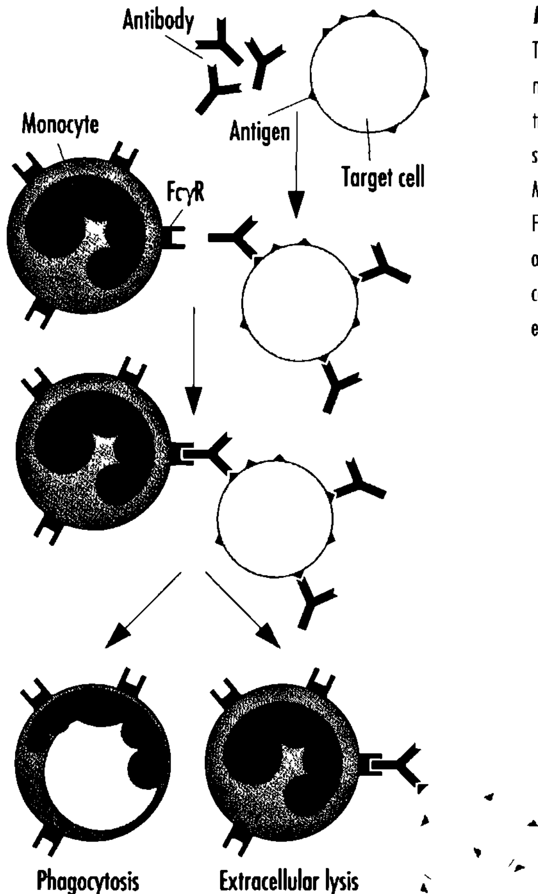

Figure 1
The process of antibody-dependent cell-mediated cytotoxicity (ADCC) is illustrated. Specific antibody binds to cell surface antigens on the target cell. Monocytes possessing FcγR bind to the Fc moiety of these cell associated IgG antibodies, and kill the attached target cells by means of either phagocytosis or extracellular lysis.

Three major classes of FcγR have been identified in man FcγRI, FcγRII, and FcγRIII (for reviews 90-92) Both freshly isolated monocytes and macrophages express FcγRI (CD64) and FcγRII (CD32) In addition, human macrophages and a small subpopulation of monocytes (<10%) express a subclass of FcγRIII (CD16), i e , FcγRIIIa (90,93,94) *In vitro*, FcγRIII (CD16) will be expressed on monocytes after at least 4 days of culture (95) and remarkable progress has been made in characterizing the protein and cDNA levels The genes for all FcγR classes have been cloned (96-98), and mapped to the long arm of chromosome 1, bands q23-24 (96) FcγRI, a 72 kDa glycoprotein, is the only human FcγR class with high affinity for both monomeric and complexed human (h)IgG FcγRII (40 kDa) has negligible affinity for monomeric hIgG, low affinity for hIgG complexes or hIgG-coated particles, and cross-reacts with murine (m)IgG1 (99-101, for review 102)

Table II.

General characteristics of human monocyte/macrophage IgG FcR[a]

Receptor class	FcγRI	FcγRII[b]		FcγRIII
Transcripts[c]	FcγRIa	FcγRIIa[HR]	FcγRIIa[LR]	FcγRIIIa
CD design	CD64	CD32	CD32	CD16
Molecular mass (kDa)	72	40	40	50-80
Receptors/cell (x 1000):				
- monocytes	15-40	30-60	30-60	1-5[d]
- macrophages	50-100	30-80	30-80	40-100
Affinity for monomeric IgG	high	low	low	medium
(K_a)	(~10^8-10^9 M^{-1})	(<10^7 M^{-1})	(<10^7 M^{-1})	(~2 3 x 10^8 M^{-1})
IgG isotype ligands[e]:				
- human	3>1>4>>>2	1,3>>2>4	3>1,2>>>4	1,3>>>2,4
- mouse	2a,3>>>1,2b	1>2b>>>2a,3	2b>>>1,2a,3	2a>2b>>>1
Anti-FcγR mAb[f]	10 1, 197, 32.2	IV.3	IV.3	3G8, Leu 11b
ADCC	Yes	Yes	Yes	Yes

[a] *references 90,92,94,106,107*

[b] *polymorphism of FcγRII on monocytes/macrophages FcγRIIa[LR] fails to support induction of T-cell proliferation by mIgG1 anti-CD3 mAb, whereas FcγRIIa[HR] supports this T-cell mitogenesis (references 99,100)*

[c] *transcripts relevant to the scope of this theses*

[d] *present on a small subpopulation of monocytes (<10%)*

[e] *specificities derived from studies of one or a few cell types (may not be generally applicable)*

[f] *anti-FcγR mAb used in the studies described in this thesis*

Two allotypic variants of this receptor are distinguished with respect to their ability to bind hIgG and mIgG complexes either strongly or weakly [i e , high-responders (FcγRIIa^{HR}) and low-responders (FcγRIIa^{LR})] (99,100,103-106, for review 107) FcγRIIIa, a glycoprotein of 50-80 kDa, is a transmembrane receptor with a medium affinity for hIgG (90) Furthermore, all three classes of FcγR have been shown to mediate ADCC (108) The general characteristics of FcγR expressed on human mono cytes and macrophages are listed in Table II

'Activation' of monocytes and macrophages. A remarkable feature of monocyte/macrophage mediated tumor cytotoxicity is that these cells must be appropriately activated for such lysis to occur (109) Adams and Hamilton (110) defined the activation of monocytes and macrophages being the "*acquisition of competence to complete a complex function*" Performing a specific function (e g , ADCC) is dependent upon the acquisition of the requisite capacities (e g , FcγR), and activation may be either an enhanced or suppressed expression of genes encoding for proteins cardinal to the function being activated (111) *In vivo*, a resting tissue macrophage may come in contact with many inductive or suppressive compounds as cytokines, colony-stimulating factors, growth factors, and LPS (or other bacterial products) which induce activation However, it is important to note that an activated macrophage will return to the original basal state when the inductive signal either disappears or is exchanged by a suppressive signal

Monocytes and macrophages can be activated both *in vivo* and *in vitro* by recombinant glycoproteins (IL, IFN, CSF, TNF) (110) In the more natural context, these BRM are involved in regulating the functional state of the cells, and will selectively affect phenotype, and function (72,81,110,112-119), depending on the stage of maturation of these cells (118) Furthermore, different mediators may affect similar functions of monocytes For instance, both IFN-γ, and IL-4 enhance expression of MHC class II molecules on monocytes (70,112) However, the cell surface expression of FcγR is increased when monocytes are cultured with IFN-γ, and decreased when exposed to IL-4 (113,119)

AIM OF THE STUDIES

The modulation by cytokines and anticancer drugs of *in vitro* cultured human monocytes and monocyte-derived macrophages is the subject of the current thesis Monocyte-derived macrophage FcγRI-mediated cytotoxicity is investigated in chapter 2, and results presented show a profound effect of IFN-γ on both the inhibition by monomeric human IgG and the mechanism of FcγRI-mediated cytolysis Influences of IFN-γ on the expression and function of the most widely distributed receptor for IgG, FcγRII, are reported in chapter 3 Remarkably, effects on both allotypic variants of FcγRIIa, $Fc\gamma RIIa^{HR}$ and $Fc\gamma RIIa^{LR}$, are observed to occur only in an early stage of monocyte differentiation Chapter 4 deals with the effects of doxorubicin on human monocytes in adherent and non adherent cultures In an alternative approach of cell function analysis, attention is focussed on some aspects of cell metabolism by measuring three enzymes of the intermediary metabolism and two acid hydrolases as indicators of monocyte maturation Chapter 5 describes *in vitro* studies on the influence of anticancer drugs in combination with cytokines on the maturation and differentiation process of human monocytes The observations indicate that doxorubicin does not negatively influence the activation state of monocytes/macrophages, induced by IFN-γ In the next two chapters, the immunomodulatory effects induced by either various cytokines (chapter 6), or major classes of anticancer drugs (chapter 7), on morphology, phenotype, FcγR-mediated cytotoxicity, and cytokine secretion at different stages of maturation during *in vitro* culture of human monocytes have been described

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EFFECT OF RECOMBINANT IFN-γ (rIFN-γ) ON THE MECHANISM OF HUMAN MACROPHAGE IgG FcRI-MEDIATED CYTOTOXICITY.

rIFN-γ DECREASES INHIBITION BY CYTOPHILIC HUMAN IgG AND CHANGES THE CYTOLYTIC MECHANISM.

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ABSTRACT

Different classes of receptors for the Fc moiety of IgG (FcγR) have been defined on human monocytes and macrophages FcγRI, FcγRII, and FcγRIII All three classes are capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC) FcγRI, which binds monomeric human IgG (hIgG) with high affinity, was shown an effective cytotoxic trigger molecule on different types of cells *In vitro*, the inhibition of FcγRI-mediated ADCC by hIgG is well documented The low affinity receptor classes, FcγRII and FcγRIII, are not blocked by monomeric hIgG Because monomeric hIgG is present at high concentrations in plasma and interstitial fluids it has been postulated inhibitory *in vivo*

We investigated the effect of rIFN γ on macrophage FcγRI-mediated ADCC in the presence of low doses hIgG With human erythrocytes (E) sensitized with hIgG as target cells, FcγRI was studied selectively We found that rIFN-γ enhances both expression and cell surface density of FcγRI on cultured peripheral blood monocytes Furthermore, this cytokine partially reversed the inhibitory effect of monomeric hIgG on ADCC More interestingly, we found that the cytolytic mechanism of monocyte-derived macrophages changed completely after prolonged culture with rIFN-γ Monocytes cultured for 9 days in control medium mediate predominantly phagocytosis After long-term rIFN-γ stimulation (9 days), monocyte-derived macrophages almost completely lost the capacity to perform phagocytosis Interestingly, they became highly efficient in mediating extracellular lysis of human E sensitized with hIgG Short-term rIFN-γ stimulated monocyte-derived macrophages (for the last 40 h of culture) were found to mediate both phagocytosis and extracellular lysis

Our findings suggest that *in vivo* rIFN-γ stimulated macrophages may be most efficient in FcγRI-mediated cytolysis as a consequence of a changed cytolytic mechanism in combination with enhanced FcγRI density

INTRODUCTION

FcγR provide an important link between humoral and cellular branches of the immune response Three major classes of FcγR have been identified in man FcγRI, FcγRII, and FcγRIII (reviewed in Refs 1-3) Both monocytes and macrophages express FcγRI (CD64) and FcγRII (CD32) In addition, human macrophages and a small subpopulation of monocytes (<10%) express a subclass of FcγRIII (CD16), i e , FcγRIIIa (1,4) The genes for all FcγR classes have been cloned (5,6), and mapped to the long arm of chromosome 1, bands q23-24 (5) FcγRI has a molecular mass of 72 kDa, binds both monomeric and complexed hIgG with high affinity, and is recognized by different

mAb including 197, 32.2, and 10.1 (7-9). FcγRII is a 40 kDa glycoprotein, has hardly affinity for monomeric hIgG, and low affinity for polymeric hIgG (hIgG-coated particles or hIgG complexes). FcγRIIIa, a glycoprotein of 50 to 80 kDa, is a transmembrane receptor with a medium affinity for hIgG (1).

Mononuclear phagocytes can be activated *in vivo* and in cell cultures by various cytokines (10). Incubation with rIFN-γ results in increased numbers of FcγRI (11-13), and enhanced ability to mediate ADCC (13-15). The expression of FcγRII and FcγRIII is hardly affected by incubation of cells with rIFN-γ (1,4,16,17).

In vivo, IgG-mediated effector responses most likely occur in an environment with excess hIgG. *In vitro*, inhibition of FcγRI-mediated activities by monomeric hIgG is well documented (18,19). It has been hypothesized that this phenomenon may support an important physiological role for FcγRII, because this receptor is not blocked by monomeric hIgG, and proteolytic enzymes can significantly increase the affinity of this low affinity receptor (20). The question how monocytes and macrophages can overcome monomeric hIgG inhibition of FcγRI mediated functions has only been partially resolved (19).

In ongoing studies on the influence of rIFN-γ on human macrophage FcγRI-mediated cytotoxicity, we noted that cytophilic hIgG only blocked ADCC activity mediated by untreated cells. Furthermore, we recently described that rIFN-γ treated human monocytes, besides modulation of FcγRI expression, underwent alterations in morphology (13). Now we studied two FcγRI mediated processes in more detail, ADCC and antibody-dependent phagocytosis, in the presence of limited amounts of monomeric hIgG. We observed a profound effect of rIFN-γ on both the inhibition by monomeric hIgG and the mechanism of FcγRI mediated cytolysis.

MATERIALS AND METHODS

Monocytes. Monocytes were isolated as described previously, by using cell scatter monitored counterflow centrifugation (21). Briefly, mononuclear cells obtained from buffy coats, or cytapheresis of healthy volunteers, were isolated by Percoll (Pharmacia, Uppsala, Sweden) centrifugation (δ=1.075 g/ml) at 4°C. Monocytes were purified from mononuclear cells by counterflow centrifugation. Monocyte fractions were over 95% pure (as evaluated in cytocentrifuge preparations after staining for nonspecific esterase and May-Grunwald-Giemsa), and viability was higher than 98%, as assessed by trypan blue dye exclusion. Isolated cells were either cultured immediately or were cryopreserved (22), and stored in liquid nitrogen until use. Recovery and viability of cryopreserved monocytes were >70% and >95%, respectively.

Culture and stimulation of monocytes with rIFN-γ. Freshly isolated or thawed cryopreserved monocytes were cultured at concentrations of 3 x 10^5 cells/ml in RPMI 1640 medium with 20 mM HEPES (Flow Laboratories, Irvine, UK), 2 mM L-glutamine (GIBCO, New York), 1 mM pyruvic acid (Sigma, St Louis, MO), 40 µg/ml gentamicin (Boehringer, Mannheim, FRG), and 10% heat-inactivated pooled human serum, in a humidified incubator with 5% CO_2 in air at 37°C Teflon foil bags (Dupont de Nemours and Co , Switzerland) were used for nonadherent culture of monocyte suspensions (23)

Human rIFN-γ (specific activity 10 U/ml) was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands) Monocytes were cultured with rIFN-γ (150 U/ml) by adding it to the culture directly at day 0, or at day 7 This concentration of rIFN-γ was chosen after the performance of pilot experiments which indicated optimal FcγRI induction with minimal effect on cell viability at this dose (data not shown)

Harvesting of monocyte-derived macrophages. After 9 days, cells were recovered by needle aspiration after cooling Teflon foil bags to 4°C for 1 h and gentle kneading, and washed twice in RPMI 1640 medium (without serum) The cells exhibited a macrophage like appearance with increased cell size and a higher cytoplasm to nucleus ratio (13) There was no difference in yield (>90%) or viability (>98%) between control cultures and cells cultured with rIFN-γ for 9 days or 40 h Morphology was studied in May-Grunwald-Giemsa-stained cytocentrifuge preparations Cell size was estimated of ~5 x 10^6 cultured cells from 3 independent donors by forward light scatter by continuous flow cytometry and a calibration curve of monosized polymer particles (Dyno particles, Lillestrom , Norway) (13) To remove cell associated hIgG, collected cells were further incubated in polypropylene tubes for 1 h at 37°C in RPMI 1640 medium This additional washing procedure was performed in all experiments except when indicated otherwise (Figs 1 and 4) After incubation, tubes were cooled on iced water for 15 min, and adherent cells were obtained by using a rubber policeman (Costar, Cambridge, MA), and washed thrice in RPMI 1640 medium Further incubation at 37°C in hIgG free medium did not result in exposure of additional IgG binding sites

Anti-FcγR mAb. Specific murine anti-FcγR mAb used were 197 (CD64 purified IgG)(Medarex, W Lebanon, NH), which binds to an epitope outside the ligand-binding site on FcγRI (7), 10 1 (CD64 culture supernatant)(generously provided by Dr Nancy Hogg, ICRF, London, UK), IV 3 (CD32 purified IgG)(Medarex), and 3G8 (CD16 purified IgG)(Medarex) which compete with the FcγRI, FcγRII and FcγRIII ligand-binding sites, respectively (9,24,25) mAb 197 was used in both immunofluorescence and inhibition studies (10 µg/ml), mAb 10 1 (1 4), IV 3 (5 µg/ml) and 3G8 (7 µg/ml) were employed in inhibition analyzes

Immunofluorescence studies. Monocyte-derived macrophages were suspended at 2 x 10^6 cells/ml in PBS / 1.0% BSA / 0.1% NaN_3 (=IF buffer). Cell suspensions of 100 µl were incubated on ice for 45 min with an equal volume of mAb. Cells were washed once with IF buffer, supplemented with 10% heat-inactivated pooled human serum (to avoid nonspecific binding of antibodies). Cells binding the mAb were detected with FITC-conjugated goat $F(ab)_2$ anti-mouse IgG (H and L chain) (Cappel, Malvern, PA), diluted in IF buffer supplemented with 10% heat-inactivated pooled human serum. After an additional incubation for 45 min at 4°C in the dark, cells were washed twice and analysed on an Ortho 30H flow cytometer. The fluorescence intensity of 5000 cells was analyzed. For all analyses, gatings (red forward and right angle scatter) were set around the macrophage population and the mean fluorescence intensity (expressed in arbitrary fluorescence units/cell) was calculated (linear) from the histograms (26).

ADCC assays. ADCC capacity of monocyte-derived macrophages from different donors was studied using a ^{51}Cr release assay as described previously (27), in which antibody-coated human E were used as target cells. Briefly, a quantity of 10^8 E were suspended in 0.3 ml PBS containing 100 µCi ^{51}Cr (sodium chromate, Radiochemical Centre, Amersham, UK), and incubated at 37°C. After 30 min, the incubation volume was increased with PBS to 1 ml, and an equal volume of sensitizing antibody, or PBS / 0.1% BSA alone (control for nonspecific binding), was added. Rhesus D-positive ^{51}Cr-labeled E were sensitized with human alloserum against Rhesus D (Merz & Dade, Dudingen, Switzerland). Antibodies were used at various dilutions, resulting in E sensitized with a range of IgG molecules per cell (EA-hIgG), as described elsewhere (28). After incubation for 30 min, cells were washed three times with PBS / 0.1% BSA, suspended in RPMI 1640 medium plus 2 mM L-glutamine, 40 µg/ml gentamicin and 10% heat-inactivated FCS, and used immediately. Cytotoxicity assays were performed in U-bottomed microtiter plates (Costar, Cambridge, MA). Equal volumes (100 µl) of effector and target cells were mixed in the plates, in the absence or presence of graded amounts of either hIgG or mAb 10.1, 197, IV.3, or 3G8, centrifuged (2 min, 50 x g, room temperature) and incubated for 18 h (or for different times, indicated in the text) in a humidified incubator at 37°C.

In some experiments we used the assay described by Boot et al. (29) with minor modifications. Briefly, 10^5 effector cells (50 µl) and 2.5 x 10^5 ^{51}Cr-labeled sensitized E (50 µl) were mixed. The plates were centrifuged (2 min, 50 x g) at room temperature and incubated for 90 min in a humidified incubator at 37°C. Then ^{51}Cr release from damaged E was promoted by addition of 0.1 ml of hypotonic (17 mM) NaCl for 2 min. Plates were centrifuged (10 min, 400 x g, room temperature), and half the supernatant was removed for estimation of ^{51}Cr release by an LKB gamma counter. All tests were

carried out in triplicate The results were calculated as follows % cytotoxicity = (C - S)/(T - S) x 100, where C = mean cpm of test sample S = mean cpm of spontaneous ^{51}Cr release (i e , ^{51}Cr release by labeled target cells in medium only), and T = mean cpm of the maximal ^{51}Cr release, obtained by addition of 20% saponin (Coulter, Dunstable, UK) to the target cells The average spontaneous release of E was 3 ± 1% (mean ± SD), and release from unsensitized E incubated with effector cells never exceeded spontaneous release

IgG preparations. Monomeric hIgG was purified from human serum by ammonium sulphate precipitation and DEAE Sephadex (Pharmacia, Uppsala, Sweden) chromatography (27) After isolation, all fractions were depleted of aggregates by ultracentrifugation (1 h at 105 000 x g, 4°C) The proteins were rapidly snap-frozen in small aliquots in liquid nitrogen, stored at 20°C, and thawed once, just before use Aggregated hIgG was obtained by heating hIgG for 30 min at 63°C

^{125}I-hIgG binding assays. Binding of monomeric hIgG1 was studied on different cell types We used thawed cryopreserved monocytes, and monocyte-derived macrophages cultured in the absence or presence of rIFN-γ After removing all cell-associated hIgG, as described above, cells were resuspended in IF buffer

Purified hIgG1 myeloma proteins were generously donated by Dr Arjen Vlug (CLB, Amsterdam, The Netherlands) After labeling with ^{125}I (Amersham) by a mild oxidative method with Protag (J T Baker, Deventer, The Netherlands) followed by purification on Sephadex G-25 (Pharmacia), ^{125}I-hIgG1 was ultracentrifuged (1 h, 105,000 x g, 4°C) and stored at 4°C until use One hundred microliters of cells (~14 x 10^6 cells/ml) were incubated with increasing concentrations of ^{125}I-hIgG1 at 4°C for 3 h to reach equilibrium The amount of cell-associated ^{125}I-hIgG1 was determined after washing twice with PBS/BSA (0 1%) Total activity was evaluated separately by counting total ^{125}I input Nonspecific binding was defined as cell-associated activity in the presence of a 500 fold excess of unlabeled hIgG Data were analyzed using the method described by Scatchard (30)

Phagocytosis assay. A ^{51}Cr-based radioactive assay and microscopic assay were performed to assess phagocytic capacity of monocytes and monocyte-derived macrophages (unstimulated or stimulated with rIFN-γ) from different donors Effector and target cells were prepared similarly as for ADCC (see above)

In the radiometric method, equal volumes (100 μl) of 10^5 effector cells and 2 5 x 10^5 target cells (optimally sensitized with hIgG), were mixed in polypropylene tubes centrifuged (2 min, 50 x g, room temperature) and incubated for 0 (control), 0 5,

1, 2, 3, or 4 h in a humidified incubator at 37°C. Nonengulfed EA-hIgG were lysed by addition of 3 ml warm (37°C) lysis buffer (8.29 g NH_4Cl, 37 mg K_2EDTA and 1 g/liter $KHCO_3$, pH 7.3), and cells were washed twice with PBS / 0.1% BSA. Cell pellets containing intact effector cells and phagocytozed ^{51}Cr-labeled E served to quantify radioactivity. All tests were carried out in triplicate. The results were calculated as follows: % phagocytosis = 100 x (C - t_0)/T, where C = mean cpm of test sample, t_0 = mean cpm of phagocytozed ^{51}Cr labeled E at t=0 (Control), and T = mean cpm of maximal ^{51}Cr release obtained by addition of 20% saponin (Coulter) to the target cells. The average t_0 ± SD of EA-hIgG was 6 ± 1%, and phagocytosis of unsensitized E incubated with effector cells never exceeded phagocytosis at t=0.

The microscopic assay of phagocytosis was performed in U-bottomed microtiter plates (Costar), in which 10^5 effector cells (100 µl) and 2.5 x 10^5 unlabeled sensitized E (100 µl) were mixed. Plates were centrifuged (2 min, 50 x g) at room temperature and incubated in a humidified incubator at 37°C, for 0 (control), 0.5, 1, and 2 h. After different incubation times, cell suspensions containing intact effector cells and phagocytozed unlabeled EA-hIgG, were fixed and stained (May-Grunwald-Giemsa) on cytocentrifuge preparations. To determine the percentage of phagocytic effector cells, 100 cells on each slide were counted in randomly chosen fields and scored for the number of engulfed EA-hIgG.

RESULTS

FcγRI-mediated ADCC activity. FcγRI and FcγRII can be independently assayed in experiments by employing E sensitized with hIgG, or murine IgG1, respectively. The specificity of these indicators for the two FcγR classes has been characterized previously in rosetting assays (31), modulation analyses (32), and ADCC experiments (13,27). Monocytes were cultured for 9 days and cytotoxicity towards EA-hIgG was evaluated. The optimal E:T ratio was estimated to be 0.4. With this ratio we analyzed the effect of rIFN-γ on FcγRI-mediated ADCC activity.

Human peripheral blood monocytes were cocultured with rIFN-γ either for 9 days, or for the last 40 h (to 7-day cultured monocyte-derived macrophages). E used as targets were sensitized at different dilutions of hIgG anti-Rhesus D alloserum. This resulted in sensitizations varying from ~1.4 to 19.3 x 10^3 molecules per target cell (28). ADCC activity mediated by the unstimulated monocyte-derived macrophages resulted in a half maximal cytotoxicity to EA-hIgG, when target cells contained approximately 2.5 x 10^3 hIgG molecules. ADCC activity of both cell populations cultured with rIFN-γ was significantly enhanced (Fig. 1).

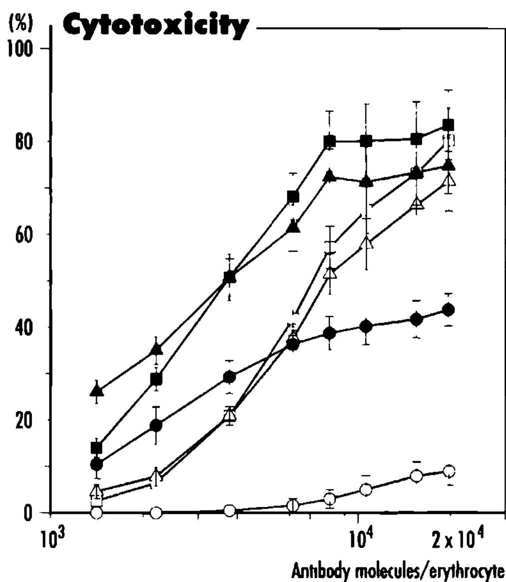

Figure 1.
Effect of rIFN-γ on FcγRI-mediated long-term (18 h) ADCC activity in the absence or presence of cytophilic hIgG ADCC activity was measured after culturing monocytes for 9 days in medium alone (●, ○), or in medium supplemented with 150 U/ml of rIFN-γ, either for 9 days (■, □), or for the last 40 h (▲, △) ADCC tests (18 h) were performed using target E sensitized with variable numbers of hIgG molecules/cell, in the absence (*closed symbols*) or presence (*open symbols*) of cytophilic hIgG [no special efforts were taken to remove this cell-bound hIgG, prior to the ADCC experiment (*see Materials and Methods*)] Results represent data from 9 to 13 individual experiments with cells from different donors

The specificity of ADCC was evaluated in inhibition studies with an unique anti-FcγRI mAb, 10 1, which reacts in/near the ligand-binding site (9), and with mAb 197, which binds to an epitope outside the ligand-binding site (7) This last anti-FcγRI mAb is of the mIgG2a isotype, a subclass which binds avidly to hFcγRI (1,33) This mAb can selectively inhibit ligand binding to this receptor via its Fc-part ("Kurlander phenomenon") (1) In two individual experiments, with cells from different donors, 9-day cultured effector cells and optimally sensitized EA-hIgG were incubated with blocking mAb and subsequently tested for cytotoxicity ADCC activity mediated by monocyte-derived macrophages cultured without, and with rIFN-γ, either for 9 days, or for the last 40 h was strongly inhibited by mAb 10 1 [96 3 ± 5 2% (without rIFN-γ), 83 4 ± 5 9% (long-term rIFN-γ), and 97 8 ± 0 7% (short-term rIFN-γ) inhibition, respectively] Incubation of the three different effector populations with mAb 197 resulted in complete (100%) inhibition of cytotoxicity to EA-hIgG Anti-FcγRII (mAb IV 3) mAb had only a marginal inhibitory effect on ADCC activity [4 6 ± 3 6% (without rIFN-γ), 5 5 ± 3 6% (long-term rIFN-γ), and 1 5 ± 1 3% (short-term rIFN-γ) inhibition, respectively], and anti-FcγRIII (mAb 3G8) mAb did not inhibit cytotoxicity mediated by any of the three effector populations Taken together, these results indicate that lysis of the EA-hIgG target cells occurs predominantly via FcγRI In control experiments, we observed that

FcγRII-mediated ADCC activity towards EA-murine IgG1, was strongly inhibited by the same concentration of mAb IV 3, and was unaffected by the same amount of anti-FcγRI mAb 197 (data not shown)

Effect of monomeric IgG on FcγRI-mediated cytotoxicity. Cytotoxicity mediated by unstimulated monocyte-derived macrophages to EA-hIgG is sensitive to low doses of monomeric hIgG Cytophilic hIgG still present on monocytes after culture in the presence of human serum (when no special precautions were taken to remove this cell-bound hIgG, see *Materials and Methods*) almost totally blocked ADCC activity mediated by the untreated population ADCC activity via FcγRI of the rIFN-γ stimulated monocyte-derived macrophages, was decreased only to a minor extend by the presence of cell-bound hIgG (Fig 1)

We next studied the effect of different amounts of hIgG on FcγRI-mediated cytotoxicity In the presence of monomeric hIgG, half maximal inhibition was observed for unstimulated monocyte-derived macrophages, and 9 day rIFN-γ stimulated populations, at concentrations of 11, and 33 μg/ml hIgG, respectively An almost complete inhibition was found at concentrations of 100, and over 300 μg/ml hIgG (Fig 2) Similar inhibition curves were found with monomeric hIgG1, or hIgG3 myeloma proteins, aggregated hIgG, or with 'cold' sensitized targets (EA-hIgG) (data not shown) These results indicated that the affinity for hIgG did not change dramatically after culture of cells with rIFN-γ

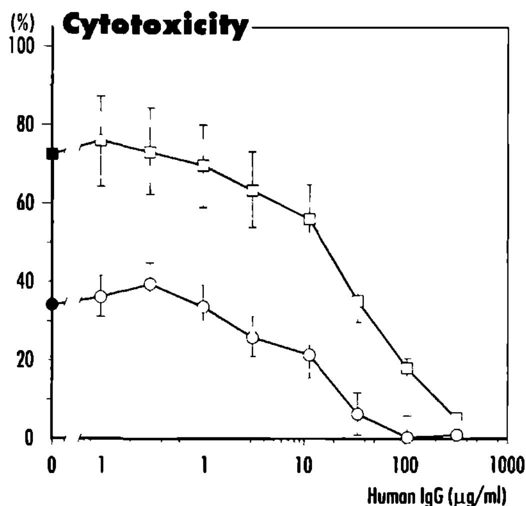

Figure 2.
FcγRI-mediated cytotoxicity in the presence of hIgG Monocyte-derived macrophages cultured for 9 days in the absence (●, ○), or presence (■, □) of rIFN-γ (150 U/ml) were incubated with target E, sensitized with ~8 x 10^3 hIgG molecules/cell, in the absence (*closed symbols*) or presence (*open symbols*) of different amounts of hIgG Results represent mean ±SD from three individual experiments with cells from different donors

Effect of rIFN-γ on monocyte-derived macrophage morphology, and FcγRI expression. To further analyze the effect of rIFN-γ on cultured monocytes, we estimated cell sizes, FcγRI surface expression, and FcγRI receptor density We observed alterations in morphology, size, and FcγRI surface expression of monocyte-derived macrophages after culture for 9 days with rIFN-γ (150 U/ml) After short-term rIFN-γ stimulation, FcγRI expression increased dramatically (short-term long-term ratio is 1 6), nevertheless receptor densities after both stimulation modalities were in the same range (Table I)

Table I.

Effect of rIFN-γ on cell size and expression and density of FcγRI

Cell type[a]	Cell Diameter (μm)[b]	Surface Area (μm^2)[c]	Relative FcγRI Expression[d]	Relative FcγRI Density[e]
Macrophages	30±4	2827	1	1
Macrophages/rIFN γ day$_{7-9}$	30±4	2827	5 8±3 2	5 8
Macrophages/rIFN γ day$_{0-9}$	24±4	1809	3 6±2 5	5 6

[a] *Monocyte-derived macrophages were obtained by culture for 9 days with or without 150 U of rIFN-γ/ml*

[b] *Cell diameter was estimated of ~5 x 10^6 cultured cells from three independent donors, by forward light scatter using a flow cytometer as described in Materials and Methods (data represent mean ± SD)*

[c] *Surface area (μm^2) was calculated from the mean cell diameter ($4\pi\rho^2$), assuming that the cells were spherical*

[d] *Expression of FcγRI was evaluated on monocyte derived macrophages by immunofluorescence with mAb 197 (data represent mean ± SD, from five separate experiments) Relative expression of FcγRI was calculated as follows Mean fluorescence intensity of rIFN-γ cultured cells/mean fluorescence intensity of cells cultured in control medium*

[e] *Relative density of FcγRI was obtained by dividing relative expression of FcγRI by surface area (μm2)*

Binding of ^{125}I-labeled hIgG1 to the different effector cells. We then checked affinities and number of binding sites for monomer hIgG1 on monocytes, and monocyte-derived macrophages, cultured in the absence or presence of rIFN-γ Specific binding of radiolabeled monomeric hIgG1 to all cell types under equilibrium conditions was saturable Scatchard plots of the data were linear (Fig 3), indicating that there is only one class of binding sites, and binding affinities of the different cell populations were in the same range (Table II)

Furthermore, the number of binding sites ranged on individual monocytes from 19,000 to 37,000 sites/cell The increase in binding sites on monocyte-derived macrophages after culture, in the absence or presence of rIFN-γ for 40 h, or 9 days was ~3-, 18-, and 12-fold, respectively (Table II) The number and affinity of binding sites on rIFN-γ stimulated monocyte-derived macrophages could not provide an explanation for the observed decreased inhibition by cytophilic hIgG Therefore, we next analyzed the mechanism of cytolysis

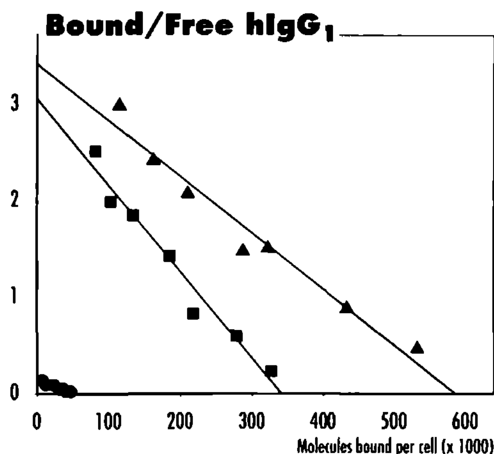

Figure 3.
Effect of rIFN-γ on the binding of monomeric ^{125}I labeled hIgG1 to monocyte-derived macrophages Monocytes were cultured for 9 days in medium alone (●), or medium supplemented with 150 U/ml of rIFN-γ, either for 9 days (■), or for the last 40 h (▲) Binding studies were performed with 1 4 x 10^6 cells as described in *Materials and Methods* Binding data were plotted according to Scatchard (30) Bound and free hIgG1 on the ordinate are in counts/min The equilibrium association constant (K_a) was calculated from the slope of the straight line The K_a for monomeric hIgG1 binding were 1 6 x 10^8 M^{-1}, 3 5 x 10^8 M^{-1}, and 5 3 x 10^8 M^{-1} for *circles*, *triangles* and *squares*, respectively One representative experiment out of three is shown

Table II.

Binding of monomeric ^{125}I-hIgG1 to various types of cells

Cell type[a]	K_a (x 10^8 M^{-1})[b]	Receptors/Cell (x 1000)	n[c]
Monocytes	5.4±2.0	24.6±8.4	4
Macrophages	2.7±1.4	78 5±58.5	4
Macrophages/rIFN-γ $day_{7\text{-}9}$	5.0±2.3	437.7±137.2	3
Macrophages/rIFN-γ $day_{0\text{-}9}$	7.0±0.7	285.5 ±17.3	3

[a] *Monocytes from five different donors were used either directly after thawing, or after culture for 9 days with or without 150 U of rIFN-γ/ml*

[b] *The data were plotted according to Scatchard (30) to calculate affinities and number of binding sites per cell Results represent mean ± SD*

[c] *Number of individual experiments*

Effect of rIFN-γ on the kinetics of lysis. To investigate the influence of rIFN-γ on the mechanism of cytolysis, short term assays (90 min) were carried out in order to decrease the contribution of phagocytosis (34) Monocyte-derived macrophages stimulated with rIFN-γ showed to be active in extracellular lysis of EA hIgG, whereas the unstimulated population was hardly at all capable of mediating cytolysis over this time period (Fig 4) Furthermore, cytophilic hIgG did not significantly inhibit extracellular lysis of EA-hIgG target cells

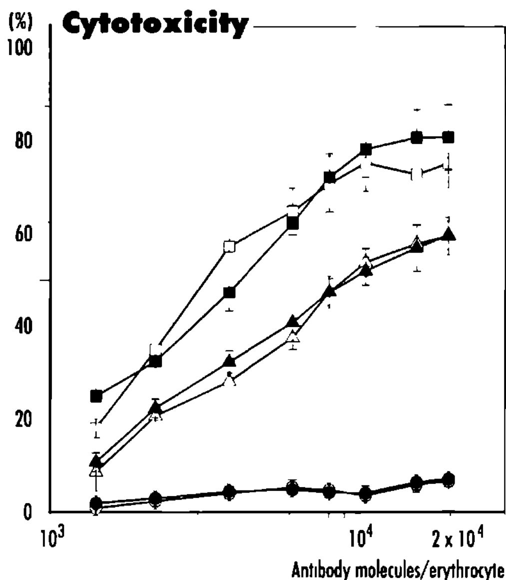

Figure 4.

Effect of rIFN-γ on FcγRI mediated short term (90 min) ADCC activity in the absence or presence of cytophilic hIgG Monocytes were cultured for 9 days in medium alone (●,), or medium supplemented with 150 U/ml of rIFN-γ, either for 9 days (■,), or for the last 40 h (▲, △) ADCC tests (90 min) were performed using target E sensitized with variable numbers of hIgG molecules/cell, in the absence (*closed symbols*) or presence (*open symbols*) of cytophilic hIgG [no special efforts were taken to remove this cell-bound hIgG prior to the experiment (*see Materials and Methods*)] Results represent data from three individual experiments with cells from different donors

To get more insight in the differences between the 18 h (Fig 1) and the 90 min (Fig 4) ADCC assays, we performed time course experiments (over 18 h) Measurable soluble radioactivity in supernatants may be derived from both extracellular cytolysis, and exocytozed products from digested E Figure 5 shows that monocytes stimulated continuously with rIFN-γ are most efficient in killing of EA-hIgG After 2-2 5 h the ^{5}Cr concentration in the medium reached 50%, and optimal lysis was reached by 7 h (84 ± 7%) In the unstimulated monocyte-derived macrophage population we noted a slow increase of soluble radioactivity in the supernatant in time However, even after 3 h the ^{51}Cr activity in the medium was still only marginal compared to rIFN-γ stimulated

monocyte-derived macrophage populations After 18 h, 36 ± 7% of the total amount of ^{51}Cr labeled E, appeared as extracellular medium-associated radioactivity ADCC activity of the short-term rIFN-γ stimulated monocyte-derived macrophage population showed the same pattern as the long term stimulated monocytes The 50% value was reached after 3 h incubation, and maximal effect (72 ± 2%) was not reached before ± 18 h

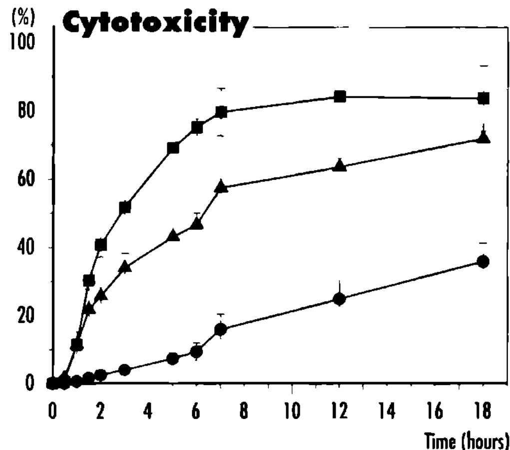

Figure 5. Kinetics of FcγRI mediated ADCC Monocytes were cultured for 9 days in medium alone (●), or medium supplemented with 150 U/ml of rIFN-γ, either for 9 days (■), or for the last 40 h (▲) ADCC tests were run for variable times, using optimally sensitized target E Results represent data from two individual experiments with cells from different donors

Effect of rIFN-γ on FcγRI phagocytosis. We next studied phagocytosis mediated by the different effector cell populations A time course experiment was performed over 4 h Monocyte-derived macrophages appeared highly active in phagocytosis, target cells were taken up rapidly and after 2 h the amount of ingested ^{51}Cr was nearly maximal In contrast, phagocytic capacity of cells cultured for 9 days with rIFN-γ was low, even at 4 h of incubation Phagocytic activity of monocyte-derived macrophages exposed to rIFN-γ for 40 h was significantly lower than that of unstimulated cells (Fig 6)

Monocyte-derived macrophages from different donors, cultured in the absence or presence of rIFN-γ, were also assayed microscopically for their ability to exhibit FcγRI-mediated phagocytosis of optimally sensitized E Table III shows that not all cells participate in the phagocytic process Most striking was the difference between the phagocytic activity mediated by the unstimulated and the long-term rIFN-γ stimulated monocyte-derived macrophages After 2 h, 21 ± 2% of the unstimulated effector cells engulfed 1 3 target cells, and 29 ± 5% phagocytozed ≥4 target cells Of the monocyte-derived macrophages continuously incubated with rIFN-γ, 95 ± 1% did not engulf any of the maximally sensitized E, and in only a small subpopulation (5 ± 1%) we observed one to three E intracellulary Phagocytic capacity decreased slightly (compared to unstimulated macrophages) when monocyte-derived macrophages were incubated with rIFN-γ for 40 h

Table III.

Phagocytosis of EA-hIgG mediated by different types of cells

Cell type[a]	No. of E ingested	Percent of effector cells at time at which phagocytosis of EA-hIgG was scored: 30 min		1 h		2 h	
		A	B	A	B	A	B
Macrophages	0	74	77	80	59	46	56
	1	14	4	12	16	7	4
	2	10	8	6	9	8	9
	3	1	7	2	4	7	6
	≥4	1	2	0	12	32	25
Macrophages/rIFN-γ day$_{7,9}$	0	90	89	61	75	56	57
	1	6	8	12	7	18	14
	2	4	3	9	6	6	9
	3	0	0	3	3	2	1
	≥4	0	0	15	9	18	19
Macrophages/rIFN-γ day$_{0,9}$	0	97	96	88	87	94	96
	1	3	4	9	9	2	4
	2	0	0	3	4	2	0
	3	0	0	0	0	2	0
	≥4	0	0	0	0	0	0

[a] *Monocyte-derived macrophages, cultured for 9 days with or without 150 U/ml of rIFN-γ from two donors (named A and B) were used The E T ratio was 0 4 for all effector populations After various times of incubation, cells were fixed and stained (May-Grunwald-Giemsa) on cytocentrifuge preparations*
To determine the percent of phagocytic effector cells, 100 cells on each slide were counted in randomly chosen fields and scored for the number of engulfed EA-hIgG

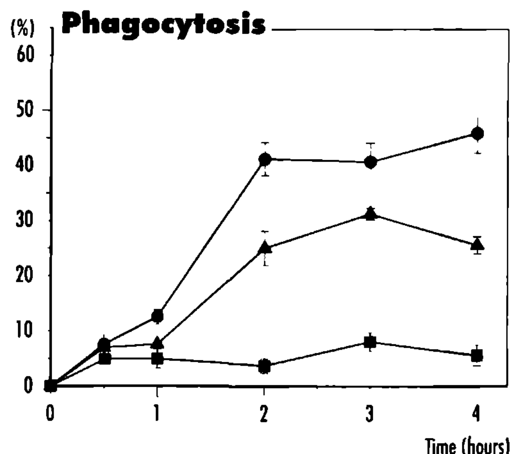

Figure 6.
Kinetics of FcγRI mediated phagocytosis Monocytes were cultured for 9 days in medium alone (●), or medium supplemented with 150 U/ml of rIFN-γ, either for 9 days (■), or for the last 40 h (▲) After different periods of time, cells were harvested and phagocytosis was evaluated using ophmally sensitized target E Results represent data from two individual experiments with cells from different donors

DISCUSSION

In this report we studied the effect of rIFN-γ on monocyte-derived macrophage FcγRI-mediated cytotoxicity against EA-hIgG in the absence and presence of monomeric hIgG Inhibition analyses with anti-FcγRI, anti-FcγRII, and anti-FcγRIII mAb showed that cytotoxicity against EA-hIgG (both before and after culture with rIFN-γ) was mediated via FcγRI We isolated large numbers of highly purified monocytes from mononuclear cells by counterflow elutriation, and cultured the cells in suspension in Teflon foil bags to minimize activation Our data confirmed that the expression of FcγRI (11-13) and FcγRI-mediated ADCC is strongly enhanced by culture with rIFN-γ (13-15) Shen et al (14) showed that the enhanced IgG antibody-mediated killing appears to be due to both increased FcγRI expression, and activation of the cellular mechanism of lysis In this study we found that prolonged (9 days) rIFN-γ treatment of monocytes resulted in a significantly smaller cell size, and lower FcγRI expression, than the short-term (40 h) treated monocyte-derived macrophages With both Scatchard, and flow cytometric analyses, we found that receptor densities were similar for cells treated either for 9 days, or 40 h with rIFN-γ In addition we did not observe differences in affinity for monomeric hIgG between these situations Our results may, therefore, point to the relevance of receptor density next to number of FcγRI molecules for cytotoxic activity

We noted that rIFN-γ partially reverses the inhibitory effects of monomeric hIgG on ADCC In studies with increasing concentrations of monomeric or aggregated hIgG preparations, we found inhibition of ADCC without changes in slopes or forms of the curves between rIFN-γ stimulated and unstimulated cells (Fig 2) This supports an

effect of rIFN-γ on the number of receptors involved, and not on their affinity A mathematical model for multivalent binding of ligand to effectors, proposed by Segal et al (19), predicts that the interaction between hIgG-coated target cells and FcγRI will be inhibited by monomeric hIgG, but that inhibition can be counteracted by increasing the density of receptors on the surface. In other words, the presence of monomeric hIgG in plasma and interstitial fluids may increase the threshold levels of antibody on target cells, and FcγRI density on effector cells, to trigger effectors

Number and affinity of FcγRI on rIFN-γ stimulated monocyte-derived macrophages did not adequately explain the observed increases in ADCC activity, and the diminishing inhibitory effects of hIgG. The much smaller cell size and the ~40% lower FcγRI expression, after long-term, vs short-term rIFN-γ stimulation, focused our attention on the mechanism of cytolysis It has been demonstrated that macrophage-mediated ADCC activity, measured by the release of ^{51}Cr is an end result of both extracellular lysis, and phagocytosis (35,36) The first appearance of ^{51}Cr in the supernatant after engulfment of EA-associated ^{51}Cr was found after only ~2 h (34) In an attempt to dissociate extracellular and intracellular lysis we performed a 90 min assay (29), in addition to an 18 h cytotoxicity assay (27) and a specific phagocytosis test In combination, these assays clarified the lytic mechanisms of monocyte derived macrophages. Freshly isolated monocytes mediate both extracellular lysis, and phagocytosis (data not shown). We demonstrate here that prolonged culture of monocytes (9 days), either with or without rIFN-γ, ultimately results in populations of monocyte-derived macrophages with different lytic mechanisms Short-term exposure to rIFN-γ leads to increased ADCC activity and decreased phagocytosis of EA-hIgG by monocyte-derived macrophages, which was also observed by Ruegg and Jungi (15). However, after 9-day (long-term) exposure to rIFN-γ, a marginal phagocytosis and abundant extracellular lysis pattern was found (Fig. 5 and Fig 6) Our study suggests that after *in vitro* culture extracellular lysis and phagocytosis are mediated by different populations of monocyte-derived macrophages This may reflect the maturation and activation state of individual monocytes Three arguments may support this hypothesis First, culture without rIFN-γ results in a population which mediates predominantly phagocytosis, with marginal capacity for extracellular lysis. Second, short-term activation with rIFN-γ of monocyte-derived macrophages produces a population which mediates both phagocytosis and extracellular lysis Third, culture with rIFN-γ for a longer period (9 days) results in a more efficient killer cell, because extracellular lysis is no longer combined with the slower process of phagocytosis.

Microscopic determination of the phagocytic capacity supported that not all cells participate in phagocytosis As our monocyte fractions were over 95% pure (and after 9 days of culture viability was still >98%), interference of a contaminating nonphago-

cytic/nonviable subpopulation is unlikely After 2 h, when phagocytic capacity was optimal (Fig 6), ~50% of the unstimulated monocyte-derived macrophages were capable of engulfing one or more EA hIgG However, when stimulated with rIFN-γ for the whole culture period, only 5% of the effector population was mediating phagocytosis Connor et al (37) showed recently that only ~58% of human peripheral blood monocytes mediates extracellular lysis (i e , ADCC) A significant increase in the percentage of monocytes mediating ADCC could be observed after incubation with rIFN-γ Connor et al did not analyze whether the population active in extracellular lysis was also active in intracellular lysis of sensitized E (i e , phagocytosis) Our experiments now show that the population of unstimulated monocyte-derived macrophages predominantly mediates phagocytosis and that the long-term (9 days) rIFN-γ stimulated effector cells were incompetent in this respect However, populations of freshly isolated monocytes (data not shown) and monocyte derived macrophages stimulated with rIFN-γ for the last 40 h were executing both types of killing It is unknown, however, *whether these events are mediated by the same or by different cells* Our study underlines the existence of a monocyte population that is heterogeneous with respect to cytotoxicity as a consequence of maturation and activation

A possible explanation for the change in cytotoxicity against EA hIgG after prolonged rIFN-γ treatment may be production of different FcγRI-isoforms Previous reports showed the existence of two different transcripts for FcγRI, one with a size of 1 7 kb, and another of 1 6 kb, evident only after culture of cells with rIFN-γ (38-40) In addition, evidence was obtained recently for the existence of multiple FcγRI genes (6) Miettinen et al (41) described that the murine FcγRII-b2 isoform in contrast to FcγRII-b1, efficiently mediates internalization of bound ligand An alternate FcγRI form induced by rIFN γ may possibly provide an explanation for the differences observed after rIFN-γ stimulation of monocyte-derived macrophages in their capacity to mediate FcγRI-phagocytosis

A critical role has been postulated for FcγRI *in vivo* by Ruiz et al (42), who showed that dysfunction of macrophage FcγRI in patients with end-stage renal disease contributes to high prevalence of infection Furthermore, administration of rIFN-γ is an established and effective therapy to reduce the frequency of serious infections in patients with chronic granulomatous disease (43) Induction of FcγRI on neutrophils from these patients *in vivo* was found (44) It is tentative to speculate that our *in vitro* study with prolonged rIFN γ stimulation of monocytes may provide an explanation for the effectivity of rIFN-γ therapy in patients with chronic granulomatous disease

In vivo, FcγRI-mediated biological responses seem to be restrained by hIgG However, rIFN-γ may alter the threshold of hIgG inhibition as well as the mechanism of lysis via FcγRI This receptor may be an important effector molecule with respect to

cytotoxicity towards solid tumors Steplewski et al (45) previously demonstrated FcγRI-mediated killing of tumor cells *in vitro*, and Herlyn and Koprowski (46) and Adams et al (47) showed FcγRI-mediated cytotoxicity of tumor cells *in vivo* Furthermore, these last two studies underline the importance of FcγRI-mediated cytotoxicity, since the most effective destruction of tumor cells was seen with tumor-specific mAb of the mIgG2a subclass [which interact with FcγRI (1-3)] (45-47)

Macrophages may become efficient in FcγRI mediated ADCC at tumor sites as a consequence of increased FcγRI density after activation by rIFN-γ The mechanisms of extracellular and phagocytic cytotoxicity and the increased resistance to the inhibitory effects of monomeric hIgG clearly require further investigation Furthermore, knowledge of molecular details will be indispensable to understand the role of FcγRI on monocytes and macrophages

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EFFECT OF rIFN-γ ON ANTIBODY-MEDIATED CYTOTOXICITY VIA HUMAN MONOCYTE IgG Fc RECEPTOR II (CD32).

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ABSTRACT

Human monocytes and macrophages express an isoform of IgG Fc receptor II (FcγRII), FcγRIIa Two allotypic variants of this receptor could be distinguished with respect to their ability to bind murine (m)IgG1 complexes either strongly or weakly, defined as high-responder (HR) and low-responder (LR), respectively

We investigated the effect of recombinant (r)IFN-γ on the ability of freshly isolated monocytes, and those cultured for 40 h and 9 days, to mediated antibody-dependent cell-mediated cytotoxicity (ADCC) Using human erythrocytes (E) sensitized with mIgG1 as target cells, FcγRII was studied selectively Cells which had been cultured for 40 h exhibit a significantly decreased FcγRII expression, and FcγRII-mediated ADCC activity as compared to freshly isolated monocytes Co culture with rIFN-γ (40 h) reversed this decrease Short-term rIFN-γ cultured cells, and fresh cells express similar numbers of FcγRII, and exhibit comparable FcγRII-mediated ADCC activity Phagocytic activity was not affected Prolonged culture of monocytes for 9 days, co-cultured with rIFN-γ either from day 0 or from day 7, did not affect expression or functional activity of FcγRII Furthermore, the effects were observed in both HR and LR individuals

Our results show that rIFN-γ has strong effects on FcγRII-mediated responses specifically during the early stages of monocyte maturation, most likely by affecting receptor expression levels

INTRODUCTION

Cell surface receptors for the Fc fragment of IgG (FcγR) provide an important bridge between cellular and humoral immune responses Expressed on cells of the mononuclear phagocyte system, FcγR mediate multiple biological functions, e g , phagocytosis (1), extracellular lysis of antibody-coated targets (2), and induction of human anti-CD3 T-cell proliferation (3) Different classes of FcγR are recognized based on variation in size, specificity and affinity for ligands, mAb reactivity, and at protein and cDNA levels Human monocytes and macrophages express three distinct classes of FcγR FcγRI (CD64), FcγRII (CD32), and a subclass of FcγRIII (CD16), i e , FcγRIIIa (for general reviews see refs 4 and 5) FcγRI, a 72 kDa glycoprotein, is the only human FcγR class with high affinity for both monomeric and complexed human (h)IgG FcγRII (40 kDa) has negligible affinity for monomeric hIgG, low affinity for hIgG complexes or hIgG-coated particles, and cross-reacts with murine (m)IgG1) (6 8) FcγRIIIa, a glycoprotein of 50-80 kDa, is a transmembrane receptor with a medium affinity for hIgG (4) Remarkable progress has been made in characterizing FcγR at

protein and cDNA levels The genes for all FcγR classes have been cloned (9,10), and mapped to the long arm of chromosome 1, bands q23-24 (9)

FcγRII is encoded by three different genes (FcγRIIA, B, and C) (4,9) For transcripts of FcγRIIA, a genetically determined polymorphism was initially recognized by the failure of human monocytes of 30% of Caucasian individuals [low-responders (LR)] to support induction of T-cell proliferation by mIgG1 anti-CD3 mAb, whereas monocytes from the other individuals [high-responders (HR)] supported this T-cell mitogenesis (6,7) Similar numbers of FcγRIIa molecules are expressed on monocytes from HR and LR (11,12), but isoelectric focussing analysis of the receptor molecules suggested the existence of two allelic forms of FcγRIIa (11) Recently, the molecular basis of this structural polymorphism for FcγRIIa was elucidated In the LR form, one amino acid in the second Ig-like domain was found critical for the strongly diminished binding of mIgG1-sensitized E (13,14)

Mononuclear phagocytes can be activated *in vivo* and culture by various cytokines (15) Incubation with recombinant (r)IFN-γ results in increased numbers of FcγRI (16,17), and enhanced ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (17,18) Furthermore, we recently described that rIFN-γ treated human monocytes, besides modulation of FcγRI expression underwent alterations in morphology (19), and cytolytic mechanism (17)

In this study we evaluated the effect of rIFN-γ on FcγRII-mediated cytotoxicity of monocytes, short-term cultured monocytes, and 9-day cultured monocyte-derived macrophages Monocytes were obtained both from HR and LR individuals We observed a profound effect of rIFN-γ on both cytotoxicity and FcγRII expression of short-term cultured monocytes

MATERIALS AND METHODS

Monocytes. Monocytes were isolated as described previously, by using cell scatter monitored counterflow centrifugation (20) Briefly, mononuclear cells obtained from buffy coats, or cytapheresis of healthy volunteers, were isolated by Percoll (Pharmacia, Uppsala, Sweden) centrifugation (δ=1 075 g/ml) at 4°C Monocytes were purified from mononuclear cells by counterflow centrifugation Monocyte fractions were over 95% pure (as evaluated in cytocentrifuge preparations after staining for non-specific esterase and May-Grunwald-Giemsa), and viability was higher than 98%, as assessed by trypan blue dye exclusion Isolated cells were either cultured immediately or were cryopreserved (21), and stored in liquid nitrogen until use Recovery and viability of cryopreserved monocytes were >70%, and >95%, respectively

Anti-CD3 T-cell proliferation. To identify HR and LR individuals, T-cell proliferation induced by the mIgG1 anti-CD3 mAb WT31 was measured as described previously (6). Briefly, mononuclear cells were cultured for 72 h together with mAb WT31. During the last 18 h, cells were pulsed with [^{3}H]thymidine (Amersham, UK). Proliferation tests were performed in triplicate. Stimulation indices were calculated by dividing [^{3}H]thymidine incorporation obtained in the presence of mAb WT31, by background [^{3}H]thymidine incorporation obtained in the absence of mAb. HR were defined as having stimulation indices > 5.

Culture and stimulation of monocytes with rIFN-γ. Freshly isolated or thawed cryopreserved monocytes were cultured at concentrations of 3 x 10^5 cells/ml in RPMI 1640 medium with 20 mM HEPES (Flow Laboratories, Irvine, UK), 2 mM L-glutamine (GIBCO), 1 mM pyruvic acid (Sigma), 40 μg/ml gentamicin (Boehringer), and 10% heat-inactivated pooled human serum, in a humidified incubator with 5% CO_2 in air at 37°C. Teflon foil bags (Dupont de Nemours and Co., Switzerland) were used for non-adherent culture of monocyte suspensions (22).

Human rIFN-γ (specific activity 10^7 U/ml) was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands). Monocytes were cultured with rIFN-γ (150 U/ml) by adding it to the culture either at day 0, or at day 7. This concentration of rIFN-γ was chosen after performing pilot experiments, which indicated optimal FcγRI induction with minimal effect on cell viability at this dose (data not shown).

Harvesting monocyte-derived macrophages. After culture for either 40 h, or 9 days, cells were recovered by needle aspiration after cooling Teflon foil bags to 4°C for 1 h and gentle kneading, and washed twice in RPMI 1640 medium. The cells exhibited a macrophage-like appearance with increased cell size and a higher cytoplasm to nucleus ratio (19). There was no difference in yield (>90%) or viability (>98%) between control cultures and cells cultured with rIFN-γ for either 40 h, or 9 days. Morphology was studied in May-Grunwald-Giemsa stained cytocentrifuge preparations.

Anti-FcγR mAb. Specific murine anti-FcγR mAb used were: 197 (CD64: purified IgG) (Medarex, W. Lebanon, NH), which selectively inhibits ligand binding to FcγRI via its Fc-part ("Kurlander phenomenon") (4), IV.3 (CD32: purified IgG) (Medarex), and 3G8 (CD16: purified IgG) (Medarex) which block FcγRII, and FcγRIII ligand-binding sites, respectively (23,24).

Immunofluorescence studies. Expression of FcγRII and HLA-DR on monocytes and cultured monocytes was assayed by indirect immunofluorescence as described (17).

using anti-FcγRII mAb IV 3 (IgG2b), anti-HLA-DR mAb OK-DR (Ortho, Westwood, MA), and FITC-conjugated goat F(ab')2 anti-mouse IgG (H and L chain) (Cappel, Malvern, PA) Fluorescence intensity of 5000 cells was measured with an Ortho 30H flow cytometer For all analyses, gatings (red forward and right angle scatter) were set around the monocyte population and the mean fluorescence intensity (expressed in arbitrary fluorescence units/cell) was calculated (linear) from the histograms Relative expressions of FcγRII were calculated as follows
$(M_c - M_n)/(M_u - M_n) \times 100\%$, where M_c = mean fluorescence intensity of cultured monocytes (in the absence or presence of rIFN-γ), M_n = mean fluorescence intensity of negative control, and M_u = mean fluorescence intensity of uncultured monocytes

ADCC assays. ADCC capacity of monocyte-derived macrophages from different donors was studied using a ^{51}Cr release assay as described previously (8) in which antibody-coated human E were used as targets Briefly, E were suspended in PBS containing ^{51}Cr (sodium chromate, Amersham), and incubated at 37°C After 30 min, mIgG1 mAb directed against glycophorin A, or PBS / 0 1% BSA alone (control) was added Antibody was used at various dilutions, resulting in E sensitized with a range of mIgG1 molecules per cell (EA-mIgG1), as described elsewhere (25) After incubation for 30 min, EA-mIgG1 were washed thrice, suspended, and mixed with equal volumes of effector cells, in the absence or presence of graded amounts of either mAb 197, IV 3, or 3G8 After incubation for 18 h at 37°C, half the supernatant was removed for estimation of ^{51}Cr release All tests were carried out in triplicate The results were calculated as follows % cytotoxicity = $(C - S)/(T - S) \times 100$, where C = mean cpm of test sample, S = mean cpm of spontaneous ^{51}Cr release (i e , ^{51}Cr release by labeled target cells in medium alone); and T = mean cpm of the maximal ^{51}Cr release, obtained by addition of 20% saponin (Coulter, Dunstable, UK) to the target cells The average spontaneous release of E was 3 ± 1% (mean ± SD), and release from unsensitized E incubated with effector cells never exceeded spontaneous release

Phagocytosis assay. A ^{51}Cr-based radioactive assay was performed to assess phagocytic capacity of monocytes and 40 h cultured monocytes (unstimulated or stimulated with rIFN-γ) from different donors, as described previously (17) Briefly, effector and target cells were prepared similarly as for ADCC (see above) Equal volumes (100 μl) of 3 75 x 10^5 effector and 2 5 x 10^5 target cells (sensitized with mIgG1 1 250), were mixed, centrifuged and incubated for 0 (control), 0 5, 1, 2, 3, or 4 h in a humidified incubator at 37°C Non-engulfed EA-mIgG1 were lysed, and cells were washed twice with PBS / 0 1% BSA Cell pellets containing intact effector cells and phagocytozed ^{51}Cr-labeled E served to quantify radioactivity All tests were carried out in triplicate

The results were calculated as follows % phagocytosis = 100 x (C - t_0)/T, where C = mean cpm of test sample, t_0 = mean cpm of phagocytozed ^{51}Cr-labeled E at t=0 (Control), and T = mean cpm of maximal ^{51}Cr release obtained by addition of 20% saponin (Coulter) to the target cells The average t_0 ± SD of EA-mIgG was 6 ± 1%, and phagocytosis of unsensitized E incubated with effector cells never exceeded phagocytosis at t=0

RESULTS

FcγRII-mediated ADCC activity. Using E sensitized with mIgG1 (EA-mIgG1), or hIgG (EA-hIgG) as target cells, the cytolytic activity mediated by either FcγRII, or FcγRI, can be assayed independently The specificity of these types of indicators for the two FcγR classes has been characterized previously in ADCC experiments (8,17,19), rosetting assays (26), and modulation analyses (27) Monocytes and 9-day cultured monocyte-derived macrophages expressing either FcγRIIa^{HR}, or FcγRIIa^{LR}, were evaluated for cytotoxicity towards E sensitized with a 1 500 dilution (ascites) of mIgG1 anti-glycophorin A mAb Figure 1 shows that the optimal effector to target cell ratio (E T ratio) for both HR and LR individuals was similar The optimal E T ratio of monocytes, and 9-day cultured monocyte-derived macrophages was estimated to be 1 5, and 0 5, respectively These results showed that monocytes retain the functional HR/LR polymorphism of FcγRII in ADCC during culture

Previously, it was shown that the degree of E sensitization obtained with anti-glycophorin A mAb gives rise to large differences in FcγRII-mediated ADCC activity (8,9) Since the degree of target cell sensitization directly affects ADCC, we used E sensitized at different dilutions of mIgG1 anti-glycophorin A, which results in sensitizations varying from ~12 to 419 x 10^3 mIgG1 molecules per target cell (25)

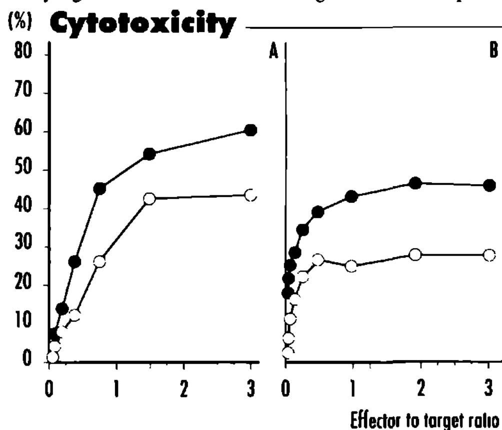

Figure 1. Polymorphism in cytotoxic activity of monocytes from different individuals towards EA-mIgG1 Monocytes were obtained from HR (*closed symbols*), or LR (*open symbols*) individuals ADCC activity of freshly isolated monocytes (A), or after 9 days in culture (B) was tested Targets were human E sensitized with mIgG1 mAb against glycophorin A (1 500 dilution of ascites) Results are average data from 3-4 experiments with cells from different donors SD were all smaller than 10%

The availability of specific anti-FcγRII mAb IV 3, which reacts in/near the ligand-binding site (23), enabled us to test the specificity of ADCC Using 9-day cultured monocyte-derived macrophages and EA-mIgG1 (at an optimal E T ratio of 0 5, and 1 250 dilution of anti-glycophorin A ascites), ADCC activity was strongly inhibited by mAb IV 3 (Fig 2) In this study, two more mAb were used, which selectively block either FcγRI, or FcγRIII Neither anti-FcγRI (mAb 197), nor anti-FcγRIII (mAb 3G8) mAb affected ADCC activity against E sensitized with mIgG1 Furthermore, no qualitative differences were found between HR and LR individuals (Fig 2) This implies that, under the test conditions, lysis of EA-mIgG1 targets occurs primarily via FcγRII Additionally, we have previously demonstrated that FcgRI-mediated ADCC activity against E sensitized with hIgG, was completely inhibited (100%) by mAb 197, marginally inhibited (4 6 ± 3 6%) by mAb IV 3, and unaffected by mAb 3G8 (17)

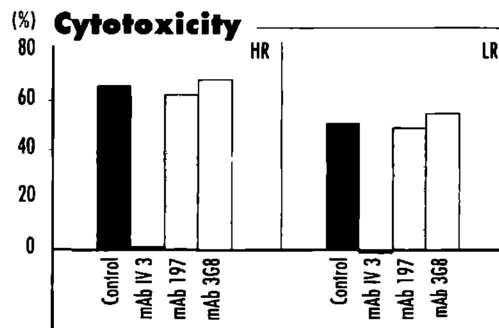

Figure 2.
Identification of FcγR involved in cytotoxicity towards EA-mIgG1 Monocyte-derived macrophages, cultured for 9 days, and optimally sensitized EA mIgG1, were incubated in medium alone (control), or in medium containing either anti-FcγRII mAb IV 3 (5 mg/ml), anti-FcγRI mAb 197 (10 mg/ml), or anti FcγRIII mAb 3G8 (7 mg/ml) Results are data from one HR, and one LR

Effect of rIFN-γ on freshly isolated, and short- and long-term cultured monocytes.

Differences between monocytes and macrophages are the result of their maturational or activation state (28) When monocytes are cultured *in vitro*, the cells differentiate and after 7 days of culture they acquire a macrophage-like appearance (19,29) In our experiments we evaluated the effect of rIFN-γ on three stages of maturation freshly isolated monocytes, short-term (40 h) cultured monocytes, and long-term (9 days) cultured monocytes

Using short-term cultured monocytes at an optimal E T ratio of 1 5, we analyzed the effect of rIFN-γ on both $Fc\gamma RIIa^{HR}$ and $Fc\gamma RIIa^{LR}$-mediated ADCC activity, and the expression of FcγRII Figure 3 shows that ADCC activity was significantly reduced when monocytes are cultured (40 h) in the absence of rIFN-γ When monocytes were cultured (40 h) in the presence of rIFN-γ, the decrease of FcγRII-mediated ADCC activity was not observed Compared to freshly isolated monocytes, half-maximal cytolysis

by unstimulated 40 h cultured HR and LR monocytes occurred only with targets which contained ~120%, and ~25% higher numbers of mIgG1 molecules/E, respectively (Table I)

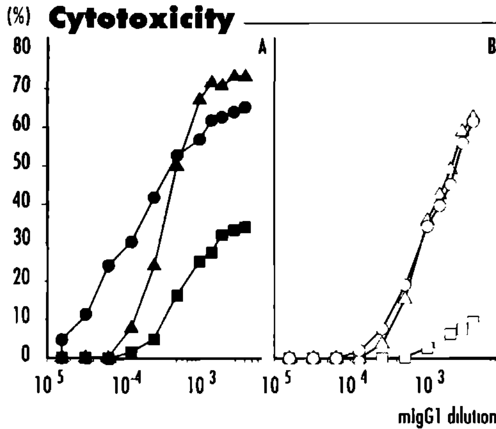

Figure 3.

Effect of short term culture and rIFN-γ on monocyte FcγRII-mediated ADCC activity Freshly isolated monocytes (*circles*) of HR (A) and LR (B) donors, monocytes cultured for 40 h in either medium alone (*squares*), or medium supplemented with 150 U/ml of rIFN-γ (*triangles*), were evaluated for cytotoxic activity ADCC tests were performed as described in *Materials and Methods*, using target E sensitized with variable amounts of mIgG1 anti-glycophorin A mAb (optimal E T ratio of 1 5) Results are average data from individual experiments using cells from (● = 12, ■ = 7, ▲ = 4) different HR, and (○ = 8, □ = 3, △ = 3) different LR donors SD were all smaller than 8%

Table I.

Target cell sensitization at half-maximal cytotoxicity with HR and LR monocytes

	mIgG1 molecules/E (x 1000)[c] at half-maximal cytotoxicity with cells from.	
Effector cells[a b]	High Responders	Low Responders
freshly isolated monocytes	90±15	320±30
40 h cultured monocytes	200±45	400±38
40 h cultured monocytes+ rIFN-γ	134±33	340±31
monocyte-derived macrophages	85±18	360±25
monocyte-derived macrophages/rIFN-γ day_{0-9}	42±16	190±21
monocyte-derived macrophages/rIFN-γ day_{7-9}	80±21	250±26

[a] *Monocytes were cultured for 40 h with or without 150 U/ml of rIFN-γ*

[b] *Monocyte-derived macrophages were obtained by culturing monocytes for 9 days with or without 150 U/ml of rIFN-γ, either for 9 days, or for the last 40 h*

[c] *Sensitization of E with mIgG1 was performed as described previously (25)*

Next, we examined whether reduced FcγRII-mediated ADCC activity was paralleled by lowered FcγRII expression. Cells from both FcγRII responder types express similar levels of FcγRII molecules (11,12). As a consequence of the 40 h culture period, the relative expression of FcγRII decreased dramatically to 53.0 ± 6.7% (mean ± SD of ten experiments) of the control value, and co-culture with rIFN-γ resulted in comparable relative FcγRII expression levels (112.1 ± 12.6%), as compared with freshly isolated monocytes (mean ± SD of ten experiments). In Figure 4 one representative experiment out of ten is shown. In contrast, 40 h of culture resulted in a 2.3 ± 0.8-fold increase in the expression of HLA-DR (mean ± SD of six experiments), indicating that culture conditions did not cause a general reduction in membrane antigens. These data showed that reduction of both FcγRII-mediated ADCC activity, and FcγRII expression levels, could be reversed by culture of monocytes with rIFN-γ.

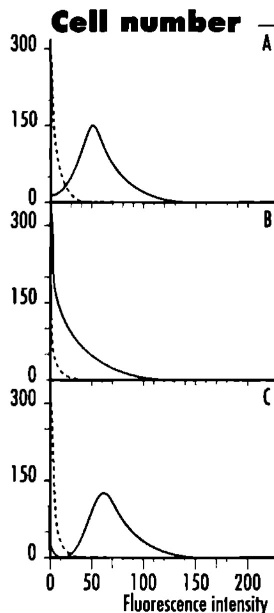

Figure 4.
rIFN-γ culture prevents decrease in FcγRII expression on short-term cultured monocytes The expression of FcγRII on freshly isolated monocytes (A), monocytes cultured for 40 h in either medium alone (B), or medium supplemented with 150 U/ml of rIFN-γ (C) was measured by immunofluorescence analysis using anti-FcγRII mAb IV 3 The fluorescence intensity (arbitrary units) was recorded on the abscissa (linear scale) Negative control values are represented by dashed-lines One representative experiment out of ten is shown.

Next, we studied the effect of rIFN-γ on FcγRII-mediated ADCC activity, and the expression of FcγRII using long-term cultured HR and LR monocytes. Monocytes were cultured for 9 days. rIFN-γ was added either for 9 days, or for the last 40 h of culture. Monocyte-derived macrophages that had been cultured in the presence of rIFN-γ, showed a slight enhancement of ADCC activity against EA-mIgG1, at an optimal E:T ratio of 0.5 (data not shown). The time at which rIFN-γ was added to effector cells did not result in a significantly different FcγRII-mediated cytolysis.

When rIFN-γ was added at the start of the 9-day culture period, the target cell sensitization required for half-maximal cytolysis was decreased by about 50% (for both HR and LR monocytes) The presence of rIFN-γ only during the last 40 h of culture did not dramatically affect the sensitization required for half maximal cytolysis (Table I)

In contrast to the 40 h rIFN-γ cultured monocyte population, the relative FcγRII expression of monocyte derived macrophages was not influenced by co-culture with rIFN-γ for either 9 days (82 4 ± 17 9%), or for the last 40 h (89 7 ± 11 9%) (mean ± SD of three individual experiments)

FcγRII phagocytosis mediated by short-term cultured monocytes. Recently, we showed with respect to FcγRI mediated cytolysis, that 9-day cultured monocyte-derived macrophages predominantly mediate phagocytosis, and that long-term (9 days) rIFN-γ stimulated effector cells were more active in extracellular lysis (17) We evaluated whether in addition to FcγRII mediated ADCC activity, also phagocytic capacity of monocytes was impaired by short-term culture A time course experiment was performed over 4 h with freshly isolated monocytes, and monocytes cultured for 40 h in the absence or presence of rIFN-γ All tested populations exhibited a comparable phagocytic activity (Table II) Target cells were taken up rapidly and after 3 h the amount of ingested ^{51}Cr was nearly maximal This suggested that the impaired ADCC activity observed after short-term culture was mainly caused by a decrease in monocyte FcγRII-mediated extracellular lysis

Table II.

FcγRII-mediated phagocytosis of EA-mIgG1

	Percentage phagocytosis of EA mIgG1[b] after 3 h with cells from	
Cell type[a]	**High Responders**	**Low Responders**
freshly isolated monocytes	30 8±6 1	16 3±3 9
40 h cultured monocytes	29 5±4 5	17 5±4 2
40 h cultured monocytes/rIFN γ	28 0±3 3	17 0±3 1

[a] Monocytes were either used directly after thawing, or after culture for 40 h with or without 150 U of rIFN-γ/ml

[b] Phagocytic capacity of all effector populations was assessed as described in Materials and Methods, at an E T ratio of 1 5 Data represent mean ± SD from three HR, and three LR

DISCUSSION

In this study we investigated the effects of rIFN-γ on a biological function mediated by FcγRII, on short- (40 h), and long-term (9 days) cultured monocytes. Inhibition studies with specific anti-FcγR mAb confirmed that lysis of the EA-mIgG1 target cells was specifically mediated via FcγRII. We show that short-term cultured monocytes (40 h) exhibit a significantly decreased FcγRII expression, and FcγRII-mediated ADCC activity towards EA-mIgG1 as compared to freshly isolated human peripheral blood monocytes. Remarkably, this decrease in FcγRII expression and FcγRII-mediated ADCC activity was reversed by co-culture of monocytes with rIFN-γ.

Cytokines are known to affect the activation state of mononuclear phagocytes, and rIFN-γ is one of the strongest modulators (15). It has been observed that rIFN-γ enhances FcγRII-mediated cytotoxicity, without affecting the expression of FcγRII (2). In this report we demonstrate that under some conditions rIFN-γ also affects FcγRII expression levels (Fig. 4). However, *modulation of FcγRII expression and activity by* rIFN-γ seems to depend critically on the stage of monocyte maturation. We now show that, compared to freshly isolated monocytes, short-term (40 h) cultured cells exhibit a significantly lower FcγRII expression, and a reduced ADCC activity against EA-mIgG1. Both short-term (40 h) rIFN-γ cultured monocytes, and freshly isolated monocytes express similar numbers of FcγRII, and exhibit a comparable FcγRII-mediated ADCC activity. These observations indicate that rIFN-γ may affect FcγRII expression and furthermore, that receptor density correlates with FcγRII-mediated function, since freshly prepared monocytes, and monocytes cultured in the presence of rIFN-γ have a similar capacity to lyse EA-mIgG1. With a specific phagocytosis test, we focused our attention on the mechanism of cytolysis mediated via FcγRII. Surprisingly, monocyte phagocytic activity was not impaired after 40 h of culture. ADCC as measured in our assay may result from both extracellular lysis, and phagocytosis. Therefore, we deduce from the phagocytosis data that the observed impaired ADCC activity in short-term cultured cells is the result of decreased FcγRII-mediated extracellular lysis. The observed rIFN-γ modulation of FcγRII expression, is confirmed by a study of Becker and Daniel (29). These authors showed that rIFN-γ treated 40 h cultured monocytes expressed twice as many FcγRII molecules compared to unstimulated cells. The mechanism by which rIFN-γ affects FcγRII expression, and ADCC, however, remains unclear up to now.

We also compared the two allotypic variants of FcγRIIa (HR and LR) for cytotoxic activity. Donor heterogeneity as found in the anti-CD3 T-cell proliferation assay, was also reflected in the ADCC assays. Cells expressing the FcγRIIa^{LR} allotypic form needed a much higher degree of sensitization of E with mIgG1 anti-glycophorin A mAb for half-maximal lysis than the FcγRIIa^{HR} monocytes. However, alterations in FcγRII-

mediated ADCC activity, as a consequence of culture, either in the absence or presence of rIFN γ, were not different between the the FcγRIIa polymorphic forms Recently, two findings were reported which may be of clinical relevance with respect to the FcγRIIa polymorphism First, pronase or trypsin-treatment changes the affinity of both FcγRIIa^{HR}, and FcγRIIa^{LR} for hIgG on monocytes (30) Second, the FcγRIIa^{LR} allotypic form was found, as the sole human FcγR class, to bind hIgG2 complexes effectively (31)

Until now little is known about the physiological significance of FcγRII *In vivo*, monocyte and macrophage mediated effector responses most likely occur in an environment with excess hIgG The presence of hIgG will not be of a hindrance to FcγRII-mediated cytotoxicity in contrast to FcγRI mediated activities (32) It has been hypothesized that this phenomenon may support an important physiological role for the most widely distributed class of FcγR, FcγRII (33) Recently we reported that rIFN-γ alters the threshold of hIgG inhibition, as well as the mechanism of lysis via FcγRI expressed on mature macrophages, which may be an important feature in areas of inflammation (17) In this respect it is of interest that FcγRII expression on short-term cultured freshly isolated monocytes, in contrast to more mature monocyte derived macrophages, was highly sensitive to rIFN-γ treatment It is tempting to speculate that during immune responses, an immunologically active dose of IFN γ may induce alterations in FcγRII expression and FcγRII-mediated cytotoxicity, especially on monocytes which have recently left the peripheral blood circulation where they reside a short time in the tissues before becoming tissue macrophages FcγRII expression on resident macrophages may be less affected by this cytokine

In conclusion in this report we document a strong effect of rIFN γ on the expression and function of FcγRII Remarkably, this effect was observed to be temporal and occurred only in the early stages of monocyte differentiation, revealing a new level of complexity in regulation of the receptor levels of this molecule

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EFFECTS OF DOXORUBICIN ON MATURATION OF HUMAN MONOCYTES IN ADHERENT AND NON-ADHERENT CULTURES.

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ABSTRACT

Purified human monocytes were cultured for 2 h, 88 h, and 10 days in plastic tubes (adherent) and for 10 days in Teflon foil bags (non-adherent). Monocytes were incubated with doxorubicin by two short-term exposures (750 or 1500 ng/ml) for 1 h or by continuous exposure (75 ng/ml). Maturation was monitored by measuring the intracellular activity of three metabolic enzymes and two acid hydrolases. Expression of receptors for the Fc moiety of immunoglobulin G (FcγRI, FcγRII, FcγRIII), CD14, and HLA-DR was assayed by indirect immunofluorescence with monoclonal antibodies. In the presence of doxorubicin, the adherent capacity, the yield, and the enzyme activities reflecting growth and intermediary metabolism were similar to the control groups. However, doxorubicin reduced the expression of FcγRI (32-45%), FcγRII (10-26%), CD14 (20-37%), and HLA-DR (25-34%) on the monocyte-derived macrophages. Expression of FcγRIII was not detectable after 10 days of culture.

INTRODUCTION

Monocytes and macrophages, part of the mononuclear phagocyte system, are a first-line defence against the development and spread of tumors (1,2). Doxorubicin is active against a wide range of tumors, including leukemia, lymphoma, sarcoma, and breast, small cell lung, and ovarian cancers (3,4). In view of the limited progress so far with the use of cytostatic agents in metastatic disease, non-specific stimulation of the immune system by biological response modifiers has been studied (5). Cytotoxicity was synergistic when doxorubicin was combined with interferon (6,7). However, cancer chemotherapy can both immunosuppress and activate the mononuclear phagocyte system (8).

We have investigated *in vitro* the effects of doxorubicin on the maturation and differentiation of human monocytes. We assayed five enzyme activities as indicators of monocyte maturation: glucose-6-phosphate dehydrogenase (G 6 PDH), phosphohexose isomerase (PHI), isocitrate dehydrogenase (ICDH), acid phosphatase (ACP), and *N*-acetyl-β-glucosaminidase (NAG). We also examined the expression of three distinct classes of receptors for the Fc moiety of immunoglobulin G [FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16)] which have been identified on human mononuclear phagocytes (9,10) and allow killing of antibody-coated target cells (11-13). We also studied gp55 (CD14), a membrane protein which is expressed in abundance on the surface of mature monocytes and macrophages (14,15), and HLA-DR which is expressed on antigen-presenting cells (16).

MATERIALS AND METHODS

Peripheral blood monocytes. Monocytes were purified from mononuclear cells of healthy volunteers with counterflow centrifugation monitored by continuous flow cytometry (17). Monocyte fractions were over 95% pure and viability was more than 98%.

Monocyte cell cultures. Freshly isolated monocytes were cultured at 3 x 10^5/ml in RPMI 1640 (Dutch modification) with 20 mmol/l HEPES buffer (Flow) supplemented with freshly added 2 mmol/l L glutamine (GIBCO), 1 mmol/l pyruvic acid (Sigma), 40 µg/ml gentamicin (Boehringer) and 10% heat inactivated pooled human serum in a humidified incubator with 5% CO_2 in air at 37°C. For adherent culturing 1 ml of the cell suspension was seeded in plastic flat bottomed tubes (Costar no 3393, 16 x 93 mm, Cambridge) and harvested at different times (2 h, 88 h, and 10 days). Cultures were done in duplicate. The supernatant and the non adherent cells were decanted and the loosely adherent cells removed by rinsing six times with 1 ml phosphate buffered saline/1% bovine serum albumin (PBS/BSA) at 37°C. By addition of 0.5 ml 2 mmol/l EDTA/0.1% BSA the adherent cells were lysed to release their enzyme content. The lysates were stored at -80°C for later measurement of enzyme activity and DNA content. Teflon foil bags (Dupont de Nemours) were used for culturing 10 ml non-adherent monocyte suspensions (18). After 10 days cells were recovered by needle aspiration after cooling the bags to 4°C for 1 h and gentle kneading. The cells were washed and resuspended in PBS. Samples were taken for enzyme measurements (10^6 cells), and stored at -80°C after pelleting and lysing in 1 ml EDTA/BSA.

Doxorubicin exposure. Doxorubicin was purchased from Laboratoire Roger Bellon (Neuilly sur Seine, France) and diluted in sterile saline. Two schedules were used: continuous exposure for 10 days and a short term exposure for 1 h. For continuous exposure, monocytes were added directly to culture medium containing doxorubicin 1-1000 ng/ml. For the short term exposure, 10^6 monocytes per ml were incubated with doxorubicin 10-10 000 ng/ml. Culture without doxorubicin served as control. Incubation tubes were cooled on iced water, the cells were collected with a rubber policeman and washed four times with culture medium before seeding them into the plastic tubes or Teflon foil bags. After culture for 10 days we measured the doxorubicin and doxorubicinol concentrations in the culture supernatants of the cells grown in Teflon foil bags by high-pressure liquid chromatography (19).

Flow cytometry. Flow cytometry was done on an Ortho 30H flow cytometer (Ortho instruments, Westwood, Massachusetts) and the data were recorded linear in list mode

on a MINC computer using linear converters (Digital Electronic Company, Maynard, MA). Because doxorubicin autofluoresces it is possible to measure doxorubicin assimilation by monocytes and macrophages. We measured uptake of this drug in short-term incubated monocytes before and after 10 days' culture, and in cells co-cultured with doxorubicin for 10 days. Binding of specific monoclonal antibodies (mAbs) to FcγRI, FcγRII, FcγRIII, CD14, and HLA DR on doxorubicin-treated cells was assayed by indirect immunofluorescence (IF) with 197 (anti-FcγRI, Medarex, West Lebanon, New Hampshire), IV.3 (anti-FcγRII, Medarex), anti Leu 11b (anti-FcγRIII, Becton Dickinson), WT14 (which reacts strongly with human monocytes and macrophages and belongs to the CD14 cluster of differentiation) (15), and OK-DR (anti HLA-DR, Ortho). After culture in Teflon foil bags monocytes were harvested, washed and suspended (3 x 10^6/ml) in IF buffer (PBS/BSA 1.0% containing 0.1% sodium azide). Hundred μl cell suspension was incubated with an equal volume of saturation levels of mAb for 30 min at 4°C. Cells binding the mAb were detected with goat anti-mouse Ig (heavy and light chain) conjugated to fluorescein isothiocyanate (Cappel, Malvern, Pennsylvania), diluted 1:50 in IF buffer. After 30 min at 4°C in the dark, the cells were washed twice and fixed with 1% paraformaldehyde for analysis. The fluorescence intensity from 5000 cells was measured. For all analyses, gatings (red forward and right angle scatter) were set around the macrophage population and the mean fluorescence intensity (expressed in arbitrary fluorescence units/cell) was calculated (linear) from the histograms (20).

Enzyme and DNA measurements. G-6-PDH, PHI, and ICDH were assayed by fluorimetric measurement of NADPH generated from NADP in the presence of appropriate substrates with either direct or coupled systems, NAG and ACP were measured by fluorimetric assay of 4-methylumbelliferone released from linked derivatives (21). Preliminary experiments verified the linearity of the system with respect to incubation time and enzyme activity. After subtraction of appropriate blanks (2 mmol/l EDTA/ 0.1% BSA), activity was expressed in nmol/min per μg DNA. The DNA content of the lysate was measured by fluorescence of the complex with fluorochrome 4,6-diamidino-2-phenylindole-2-HCl (22).

RESULTS

Intracellular doxorubicin in non-adherent monocytes. After both short-term exposure to 10-10,000 ng/ml and in cells continuously exposed for 10 days to lower doses (1-1000 ng/ml) cellular fluorescence was linearly correlated with the concentration of doxorubicin. The mean fluorescence of the short-term exposed monocytes, before and after culturing, was similar to that of the cells which were continuously cultured (data not shown).

Extracellular doxorubicin in supernatants of non-adherent monocytes. No drug metabolites were found in the supernatants when monocytes were continuously exposed to doxorubicin at 1-250 ng/ml. However, at 500-1000 ng/ml, we measured doxorubicinol concentrations of 15.6-32.1 ng/ml (i.e. 3.2 ± 0.1%) of the originally added doxorubicin concentration. The residual amount of parent compound after 10 days of continuous exposure to 1-1000 ng/ml was, for all measured points, still 24 ± 2% of the original quantity. In the short-term exposures to 10-10,000 ng/ml, no derivatives were found, and the doxorubicin concentrations in the culture supernatants were always less than 0.25% of the original.

Doxorubicin and cell recovery. When doxorubicin was used at concentrations above 200 ng/ml in continuous exposure, and above 2000 ng/ml in the short-term, cell recovery was halved, and most (over 70%) of the harvested cells were dead. The monocytes had not matured into large macrophages but remained small with vacuoles (data not shown). For further studies we used doxorubicin concentrations that were comparable to those clinically, i.e. 75 ng/ml for continuous exposure, and 750 or 1500 ng/ml for the short-term.

Compared with control cells, no significant changes were seen in adherence capacity of the plastic-cultured monocytes after the short-term or continuous exposures (Table I). In the controls, 19.2% of the seeded monocytes adhered after 2 h of culturing and with doxorubicin, adherence ranged from 17.4% to 20.5%. Thus, doxorubicin did not affect cell recovery in adherent cultures. The total amount of cells harvested after 10 days was over 100% at all concentrations of drugs studied. Cell recovery and viability of the non-adherent monocytes were 83,6 and over 95%, respectively (Table I).

Table I.

Percentage cell recovery after doxorubicin exposure in adherent and non-adherent cell cultures

	Adherent			Non-adherent
	2 h	88 h	10 days	10 days
Control	19.2±3.9	27.7±5.1	31.8±12.1	83.6±5.0
Short-term 750 ng/ml	20.5±2.7	28.5±4.3	30.7±9.4	84.4±6.3
Short-term 1500 ng/ml	17.1±6.2	23.9±2.2	24.0±5.5	86.9±7.0
Continuous 75 ng/ml	17.4±3.3	22.9±2.6	23.9±8.2	78.1±9.0

Recovery = DNA content in lysate/content in 1 ml lysed uncultured monocytes for adherent, and harvested cells/cells initially seeded for non-adherent. Results represent mean ± SD from six individual experiments using cells from different donors.

Effects of doxorubicin on enzyme activities. After culture without doxorubicin for 10 days in plastic tubes, G-6-PDH increased 25 times compared with the level observed after 2 h culture The corresponding figures for PHI and ICDH were 6 and 4 times respectively ACP and NAG activity increased 33 and 10 times Compared with 1 h non-adherent incubation (polypropylene tubes, 37°C), enzyme activities of monocytes cultured in Teflon foil bags after 10 days were increased as follows G-6 PDH, 27 times, PHI, 5 times, ICDH, 8 times ACP, 19 times, and NAG, 28 times (Table II) In the presence of doxorubicin, in short-term exposure (750 and 1500 ng/ml) or continuous exposure (75 ng/ml), enzyme activities of adherent or non-adherent monocytes were similar to the control values (Table II)

Effect of doxorubicin on FcγR, CD14, and HLA-DR expression. We characterized the cultured macrophages, which were continuously exposed to doxorubicin 75 ng/ml or underwent short-term exposure to 750 or 1500 ng/ml, before culturing All schedules and doses of doxorubicin diminished the expression of FcγRI (32-45%), FcγRII (10-26%), CD14 (20-37%), and HLA-DR (25-34%) (Fig 1) The reductions were all significant (*t* test) at $P < 0.05$, with exception of the effect on the FcγRII expression after continuous exposure ($P < 0.154$) Expression of FcγRIII was not affected, because this receptor was not present on our 10 day cultured macrophages

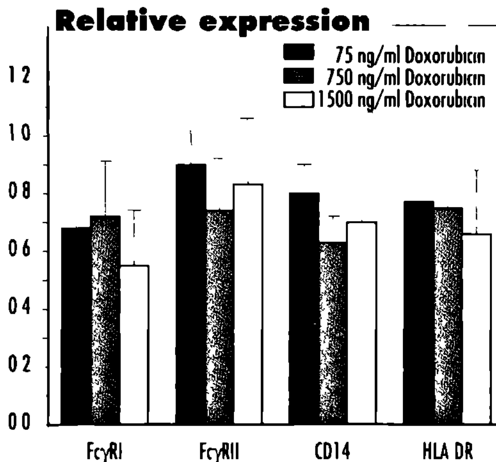

Figure 1.
Expression of FcγRI, FcγRII, CD14, and HLA DR on monocyte-derived macrophages Monocytes were cultured for 10 days in Teflon foil bags without drug or with continuous exposure to doxorubicin 75 ng/ml, or after short term exposures to 750 or 1500 ng/ml Relative expression = doxorubicin-treated non treated ratio Results represent mean ± SD from five individual experiments using cells from different donors

Table II.

Effects of doxorubicin on enzyme activities in adherent and non-adherent cultured monocytes

		Doxorubicin (ng/ml)			
		Short-term		Continuous	
Enzyme	Control	750	1500	75	Total
G-6 PDH					
adherent					
2 h	4.47±0.56	4.65±0.86	4.94±0.81	5.21±0.80	4.80±0.82
88 h	41.1±8.5	39.6±14.1	38.6±15.8	35.1±10.6	38.3±13.2
10 days	112±23	113±23	116±21	104±30	112±25
non-adherent					
0 h	1.38±0.32	–	–	–	1.38±0.32
1 h	3.14±0.90	4.08±1.70	3.36±0.76	3.41±0.99	3.51±1.14
10 days	83.3±16.5	79.1±12.6	74.9±12.5	79.8±13.3	79.2±12.4
PHI					
adherent					
2 h	25.5±3.1	28.1±5.0	27.9±10.9	33.9±3.5	28.9±6.8
88 h	62.8±18.1	56.8±19.5	61.5±22.8	59.2±17.6	60.1 ±19.7
10 days	157±61	148±49	162±52	156±65	156±57
non-adherent					
0 h	8.73±3.95	–	–	–	8.73±3.95
1 h	32.1±9.9	42.8±18.3	35.9±6.2	36.4±9.6	36.9±11.4
10 days	154±27	147±25	137±22	142±25	143±22
ICDH					
adherent					
2 h	2.27±0.35	2.22±0.17	2.73±0.43	2.99±0.95	2.53±0.64
88 h	4.95±0.85	4.51±1.30	4.61±1.24	5.24±1.14	4.83±1.19
10 days	9.46±2.91	8.52±2.28	9.18±1.81	9.03±2.66	9.05±2.47
non-adherent					
0 h	0.30±0.04	–	–	–	0.30±0.04
1 h	0.93±0.45	1.32±0.89	0.91±0.69	1.12±0.58	1.08±0.60
10 days	7.21±1.74	6.94±1.79	6.27±1.49	6.31±1.75	6.69±1.51

(continued)

Enzyme	Control	Doxorubicin (ng/ml) Short-term 750	1500	Continuous 75	Total
ACP					
adherent					
2 h	0.22±0.02	0.22±0.03	0.22±0.04	0.23±0.03	0.22±0.03
88 h	3.04±1.03	3.17±1.16	2.90±0 83	2.84±0.75	2.99±0.97
10 days	7.23±2.78	6.92±2.06	6.93±1.75	6.01±1.61	6.77±2.15
non-adherent					
0 h	0.18±0.03	–	–	–	0.18±0.03
1 h	0.29±0.03	0.35+0.07	0.37±0.05	0.31±0.03	0.33±0.05
10 days	5.50±1.11	5.73±1.37	5.75±1.96	5.48±1.52	5.61±1.32
NAG					
adherent					
2 h	1.20±0.35	1.12±0.31	1.18±0.40	1.32±0.56	1.20±0.42
88 h	3.63±1.09	3.52±1.26	3.59±1.60	3.51±0.91	3.56±1.24
10 days	11.6±2.6	12.5±1.8	14.2±2.1	14.6±3.2	13.2±2.8
non-adherent					
0 h	0.60±0.11	–	–	–	0.60±0.11
1 h	0.78±0.05	0.98±0.21	0.94±0.13	0.79±0.05	0.87±0.14
10 days	21.7±7.9	23.9±9.2	26.5±11.1	23.1±11.6	24.0±8.9

Enzyme activities were measured in the lysates of the adherent cell cultures after 2 h, 88 h, and 10 days. Non-adherent cells were determined for enzyme activity by measuring lysates of cryopreserved monocytes, 1 h incubated monocytes, and at day 10 harvested cells Activities are expressed in nmol/min per µg DNA. Results represent mean ± SD from six individual experiments using cells from different donors.

DISCUSSION

As part of the investigation of the influence of cytostatic agents on the immune system, we evaluated the effects of doxorubicin on human monocytes in adherent and non-adherent cultures We measured extracellular and intracellular drug concentrations, because doxorubicin can be rapidly metabolized into doxorubicinol and doxorubicinone (23) Since doxorubicinone is not cytotoxic (24) and the cytotoxic potential of doxorubicinol is only a tenth that of the parent compound (25), we needed to know whether formation of these derivatives could be excluded In our systems, no metabolites were found In many *in vitro* experiments cell death is related to cellular doxorubicin concentrations (26) It is not clear whether free radical formation, intercalation of doxorubicin into DNA, induction of DNA breaks and chromosomal aberrations, or drug-induced alterations of cell membranes actually kill the cell

At high concentrations of doxorubicin obtainable only *in vitro*, negative effects were seen on cell recovery, viability, and morphology after 10 days of culture To mimic the clinical situation, we used two schedules of non-toxic incubation continuous exposure to doxorubicin 75 ng/ml (akin to continuous infusion), and short-term exposure to 750 or 1500 ng/ml before culture (akin to bolus infusion)

Doxorubicin did not influence the adherent capacity or yield of the cultured monocytes Our observation that adherence was unaffected was different from the results described by Athlin and Domellof (27) They described a significant reduction of monocyte glass attachment one week after patients had been treated with combination chemotherapy of fluorouracil, doxorubicin, and mitomycin However, these discrepancies can be explained by the different drugs administered, and *in vivo* versus *in vitro* conditions Another point is that, one week after treatment, Athlin and Domellof were looking at a monocyte population that was not present in the peripheral blood at the time of the combination chemotherapy When these patients were treated, this cell population were still precursor monocytes Patients with solid tumors also showed a marked decrease in yield of adherent macrophages after a week of culture (28,29) In our study, the amount of adhered cells increased slightly with the time of culture independently of the doxorubicin treatment After 10 days the overall yield of suspension-cultured macrophages was more than 2 5 times higher than plastic-cultured macrophages These observations indicate the existence of adherent and non-adherent monocytes, both capable of maturation to macrophages, and suggest that at least some monocytes can become adherent macrophages as a consequence of maturation and age Our finding that after 2 h nearly a fifth of the plastic-seeded monocytes adhered contradicts our earlier report (18) Previously, we found about half the cells adhered after 2 h, however, the yield of adherent macrophages after 15 days was 70 ± 20% of those originally seeded,

which is similar to our yield after 10 days (31,8 ± 12 1%) It is not easy to explain these differences because we used the same conditions on both occasions Depending on the conditions there is considerable variation in the adherent yield, resulting in marked differences in the relative amounts of adherent and non-adherent cells Results of studies of monocyte function should be critically regarded in this respect Monocyte isolation by counterflow centrifugation (17) has an advantage here since selection is minimal

There are reports of changes in enzymes and receptors as indicators of the *in vitro* maturation of non-treated monocytes (18,21,30-33) No information is, however, available about the effects of doxorubicin on the maturation as reflected in the enzyme levels of highly purified and *in vitro* cultured monocytes We found that in the presence of doxorubicin, the intracellular level of G-6-PDH was unaltered, which indicates that hexose monophosphate shunt activity was similar to unexposed cells The activities of PHI, of the glycolytic pathway, and of ICDH, of the Krebs' cycle, were also unaffected NAG and ACP activity, important for the hydrolytic degradation of glycoproteins, mucopolysaccharides, and glycolipids, remained the same when cells were exposed to doxorubicin continuously or in a short-term schedule

Tritton and Yee (34) showed that doxorubicin can act solely by interaction with the cell surface, so it may be important to consider the cell surface as a target We examined possible alterations of the cell membrane by doxorubicin by study of the expression of FcγRI, FcγRII, FcγRIII, CD14, and HLA-DR FcγRs and HLA antigens can be affected by biological response modifiers (35,36) Modulation of CD14, a membrane protein expressed on the surface of mature monocytes and macrophages (14,15), has not been previously described We found that doxorubicin caused a slight but significant decrease in FcγRI, FcγRII, CD14, and HLA-DR expression on *in vitro* cultured monocytes (with the exception of FcγRII in the continuous exposure experiments) That FcγRIII expression was not measurable in our study can be explained by the observation of Clarkson and Ory (12), that FcγRIII, which is not present on monocytes, will appear on macrophages in measurable quantities after 14 days of culture How doxorubicin modulates the expression of surface molecules and whether it will affect function are not known Our preliminary experiments indicate that doxorubicin does not influence the FcγRI and FcγRII mediated antibody-dependent cellular cytotoxicity Alterations in the membrane molecules will be counteracted by the *de novo* synthesis of the original membrane molecules, unless inhibited by DNA intercalation

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IN VITRO STUDIES ON THE INFLUENCE OF DOXORUBICIN IN COMBINATION WITH RECOMBINANT INTERFERON-GAMMA ON HUMAN MONOCYTES.

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ABSTRACT

The maturation and differentiation process of human monocytes and human monocyte-derived macrophage cytotoxicity mediated by receptors for the Fc moiety of immunoglobulin G (FcγRs) were studied *in vitro* using the chemotherapeutic drug doxorubicin in combination with the biological response modifier (BRM) recombinant interferon-gamma (rIFN-γ) Human monocytes were cultured for 9 days and treated with doxorubicin before, after, or on day 7 of the culture rIFN-γ was added continuously on day 0 or on day 7 of the culture, either alone or in combination with doxorubicin In the presence of doxorubicin, all measured intracellular enzyme activities were at the same level as the controls and the rIFN-γ treated cells However, when monocytes were co-cultured for 9 days with rIFN-γ alone or in combination with doxorubicin, enzyme levels decreased to between 45% and 61% of the controls In control cultures, antibody-dependent cell-mediated cytotoxicity (ADCC) activities against maximally sensitized hRBC mediated by FcγRI and FcγRII were 41 7 ± 8 8% and 42 7 ± 11 6%, respectively When monocytes were exposed to rIFN-γ continuously or 40 h before harvesting, FcγRI ADCC activity increased to 78 9 ± 9 8% and 68 1 ± 8 1%, respectively, and FcγRII ADCC activity to 56 3 ± 8 4% and 55 4 ± 7 3%, respectively The addition of doxorubicin to monocyte cultures in the presence or absence of rIFN-γ did not influence the lysis of the two types of sensitized hRBC These observations indicate that doxorubicin does not negatively influence the activation state of monocytes/ macrophages, induced by rIFN-γ

INTRODUCTION

During the past decade, renewed interest in immunotherapy has been aroused The main reasons are the availability of highly purified biological response modifiers (BRMs), and the limited progress recently made with the use of chemotherapeutic agents in many neoplastic diseases Under certain conditions BRMs and chemotherapeutic agents could be combined, resulting in additive or synergistic tumoricidal cytotoxicity (1-6)

Because chemotherapeutic agents may have detrimental effects on the immune system, knowledge regarding the influence of chemotherapeutic agents as well as of BRMs on the mononuclear phagocyte system (MPS) is compulsory for a better understanding of this combined approach We have investigated whether chemotherapy can inhibit activations of the MPS elicited by BRMs We studied the *in vitro* effects of doxorubicin on recombinant interferon-gamma (rIFN-γ)-induced activation of human monocyte-derived macrophages The evaluated parameters were morphology, maturation, differentiation, and functional processes rIFN-γ is a strong activator of the MPS that induces

a o the expression of receptors for the Fc moiety of immunoglobulin G (FcγR) and of class II MHC antigens on monocytes (7-9)

Furthermore it possesses a strong antiviral activity (10), antitumor activity, and is an important cytokine released upon treatment with IL-2 and TNF (11) Doxorubicin is a chemotherapeutic agent with antitumor activity against a wide spectrum of malignancies (12-14) In a recent study with human monocytes we could show that doxorubicin treatment significantly impaired the expression of the surface markers FcγRI, FcγRII, CD14, and HLA-DR, which are involved in cellular immunoregulation Monocyte maturation was qualitatively and quantitatively normal (15)

In the present work we assayed three enzymes of the intermediary carbohydrate metabolism glucose-6-phosphate dehydrogenase (G-6-PDH), phosphohexose isomerase (PHI), and isocitrate dehydrogenase (ICDH), and two lysosomal enzymes acid phosphatase (ACP) and *N*-acetyl-β-glucosaminidase (NAG) as markers for the cell growth We examined the expression of FcγRI (CD64) and FcγRII (CD32) which have been identified on human mononuclear phagocytes (16,17), and are important mediators in immune defense processes (18,19) We also studied the antibody-dependent cell-mediated cytotoxicity (ADCC) against human red blood cells (hRBC) mediated by FcγRI and FcγRII of human monocyte-derived macrophages

MATERIALS AND METHODS

Peripheral blood monocytes. Monocytes were isolated as described previously with the use of cell scatter monitored counterflow centrifugation (20) Briefly, mononuclear cells were obtained from buffy coats or by cytapheresis of healthy volunteers and isolated by Percoll (Pharmacia, Uppsala, Sweden) centrifugation (δ=1 075 g/ml) at 4°C Monocytes were purified from mononuclear cells with counterflow centrifugation monitored by continuous flow cytometry Monocyte fractions were over 95% pure (evaluated in cytocentrifuge preparations after staining for non-specific esterase and with May-Grunwald-Giemsa), and viability was more than 98%, as assessed by trypan blue dye exclusion

Monocyte cell cultures. Freshly isolated monocytes were cultured (singular) at a concentration of 3 x 10^5/ml in RPMI 1640 medium (Dutch modification) with 20 mM hepes buffer (Flow Laboratories, Irvine, UK) supplemented with freshly added 2 mM L-glutamine (GIBCO Laboratories, NY), 1 mM pyruvic acid (Sigma Chemical Co , St Louis, MO), 40 μg/ml gentamicin (Boehringer, Mannheim, FRG), and 10% heat-inactivated pooled human serum, in a humidified incubator with 5% CO_2 in air at 37°C Teflon foil bags (TFB)(Dupont de Nemours and Co , Switzerland) were used for culturing 10 ml non-adherent monocyte suspensions (21)

Harvesting of the monocyte-derived macrophages. After 9 days, matured cells were recovered by needle aspiration after cooling to 4°C for 1 h and gentle kneading Morphology was studied in May Grunwald Giemsa stained cytocentrifuge preparations Cell size was estimated with forward light scatter by continuous flow cytometry and a calibration curve of monosized polymer particles (Dyno particles, Lillestrom, Norway) To remove all cell-associated human IgG from the surface, collected cells were washed twice and incubated for 60 min at 37°C in RPMI medium (Dutch modification), in polypropylene tubes After incubation, the tubes were cooled on iced water for 15 min, adherented cells were scraped off with a rubber policeman (Costar, Cambridge, MA), and washed thrice in RPMI medium (Dutch modification)

Samples were taken for enzyme and DNA content measurements (10^6 cells), and stored at 80°C after pelleting and lysing in 1 ml 2mM EDTA supplemented with 0 1% bovine serum albumin (BSA) (Cohn fraction V Sigma, St Louis, MO)

Doxorubicin and rIFN-γ exposure. Doxorubicin was purchased from Laboratoire Roger Bellon (Neuilly sur Seine, France), dissolved in water and diluted in sterile saline Two schedules of doxorubicin exposure were used a long-term from day 0 or 7, and a short-term for 1 h before or after culture We used doxorubicin concentrations comparable to the serum concentrations found in the clinical setting, i e 75 ng/ml for the long-term exposure (added directly to the culture), and 750 ng/ml for the short-term exposure (10^6 monocytes per ml) The short-term incubation tubes were cooled on iced water, the cells were collected with a rubber policeman and washed 4 times with culture medium before being seeded into the TFB, or washed once before being used in further experiments Cultures without doxorubicin served as a control Human rIFN-γ was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands) Monocytes were co-cultured with rIFN-γ (150 U/ml) by adding it directly to the culture at day 0 or 7

Enzyme and DNA measurement. The three intracellular enzymes of intermediary metabolism, G-6-PDH, PHI and ICDH, were assayed 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 measured by fluorimetric determination of 4-methylumbelliferone released from linked derivatives Enzyme measurements were performed with fluorimetric micro-assays, which have been described in detail (22) Preliminary experiments verified the linearity of the system with respect to incubation time and enzyme activity After subtraction of appropriate blanks (2 mM EDTA/BSA 0 1%), activity was expressed in nmol/min per μg DNA

The DNA content of the lysate was determined by fluorescence of the complex with the fluorochrome 4'6-diamidino 2-phenylindole-2-HCl (23)

Cell-surface immunofluorescence. Binding of specific monoclonal antibodies (mAbs) to the FcγRs was assayed by indirect immunofluorescence (IF) with 197 (anti FcγRI, Medarex, West Lebanon, NH), and IV 3 (anti-FcγRII, Medarex) Cultured monocytes were harvested, washed and suspended (3 x 10^6/ml) in IF buffer [phosphate buffered saline (PBS)/BSA 1,0% containing 0 1% sodium azide] Cell suspensions of 100 µl were incubated on ice for 45 min with equal volumes of the various mAb The cells were then washed once with 2 ml IF buffer Cells binding the mAb were detected with goat anti-mouse Ig (heavy and light chain) conjugated to fluorescein isothiocyanate (Cappel, Malvern, PA), diluted 1 200 in IF buffer After an additional 45 min at 4°C in the dark, the cells were washed twice and fixed with 1% paraformaldehyde for analysis

Flow cytometry. Cells were analysed on a flow cytometer (30H Cytofluorograf, Ortho Diagnostic Systems, Westwood, MA) The 488 nm line of a 5-W Argon laser at 0 4 W output was used for excitation and right angle scatter (RAS) A 0 8 mW HeNe laser was used for red forward scatter (RFS) and a band-pass filter 515/30 for green fluorescence (GFL) The fluorescence intensity from 5,000 cells was analysed at a flow rate of 100 cells/s, and recorded linear in list mode on a MINC computer using linear converters (Digital Electronic Company, Maynard, MA) For all analyses, the gatings (RFS and RAS) were set around the macrophage population and the mean fluorescence intensity (expressed in arbitrary fluorescence units/cell) was calculated from the histograms (24)

Cytotoxicity assay. ADCC capacity of monocyte-derived macrophages from different donors was studied using a ^{51}Cr release assay as described previously (25), in which antibody-coated hRBC were used as target cells Briefly, a quantity of 10^8 hRBC were suspended in 0 3 ml PBS containing 100 µCi ^{51}Cr (sodium chromate, Radiochemical Centre, Amersham, UK), and incubated at 37°C After 30 min, the incubation volume was increased with PBS to 1 ml, and an equal volume of sensitizing antibody or, as a control for nonspecific binding, with PBS/BSA 0 1% alone, was added To sensitize the 51Cr labeled hRBC, two types of antibodies were used Rhesus D-positive hRBC were sensitized either with human alloserum against rhesus D (Merz & Dade, Dudingen, Switzerland) or with mouse IgG1 mAb specific for glycophorin A on hRBC (a kind gift of M Bos from the Depart-ment of Immunohaematology, University of Amsterdam, The Netherlands) Both types of mAb were used in various dilutions, resulting in hRBC sensitized with a range of IgG molecules per cell (EA-IgG), as has been described in detail (26) After incubation for 30 min, cells were washed thrice with PBS/BSA 0 1%, suspended in RPMI 1640 medium plus 2 mM L-glutamine, 40 µg/ml gentamicin and 10% heat-inactivated FCS and used immediately Cytotoxicity assays were performed in U-bottomed microtiter plates (Costar, Cambridge, MA) Equal volumes (100 µl) of

10^5 effector cells and 2.5 x 10^5 target cells were mixed in the plates, centrifuged (2 min, 50 x g, room temperature) and incubated for 18 h at 37°C. After incubation, plates were centrifuged (10 min, 400 x g, room temperature), and half the supernatant was removed for estimation of ^{51}Cr release in a LKB gamma counter. The results were calculated as follows: % cytotoxicity = (E - S)/(T - S) x 100 where E = mean cpm of test sample; S = mean cpm of spontaneous ^{51}Cr release (i.e. ^{51}Cr release by labeled target cells in medium only); and T = mean cpm of the maximal ^{51}Cr release that was obtained by addition of 20% saponin (Coulter, Dunstable, UK) to the target cells. The average spontaneous release ± SD of hRBC was 3 ± 1%, and release from unsensitized hRBC incubated with effector cells did not exceed spontaneous release.

Statistics. The Mann-Whitney two sample test is used to test the statistical significance of differences in the relative enzyme activities between the means of the untreated and the with doxorubicin and/or rIFN-γ treated cells. Significance is accepted at the $P < 0.05$ level.

RESULTS

Effects of doxorubicin and rIFN-γ on size and morphology of cultured monocytes.

Monocytes cultured in TFB loosely attach to the surface and the majority of cells appear rounded, increase greatly in size (30 ± 4 μm) and develop a higher cytoplasm to nucleus ratio (Fig. 1B). After doxorubicin-treatment the morphology does not differ from that of monocytes cultured in the absence of doxorubicin. Co-culture with rIFN-γ (150 U/ml) for 9 days causes homotypic monocyte adhesion which ultimately results in big clumps of cells being in suspension, and in addition cell size was increased to a lesser extent (24 ± 4 μm). Beside the smaller cell size, the horseshoe shaped nucleus does not differentiate into a round shaped centrally located nucleus, but in a flattened lobed and more juxta membranal located nucleus (Fig. 1C). When rIFN-γ is added on day 7 of the culture, a plurality of monocytes acquire a dendritic-like appearance, however, cell morphology (cytocentrifuge preparations) and size are not altered. After their initiate state of spreading, we could also observe clumping on day 12, when the culture period of the monocyte-derived macrophages stimulated on day 7 with rIFN-γ was prolonged after day 9.

Effects of doxorubicin and rIFN-γ on enzyme activities of cultured monocytes.

Monocytes cultured in the presence or absence of doxorubicin and or rIFN-γ were tested for the intracellular enzyme activities of G-6-PDH, PHI, ICDH, ACP, and NAG. Monocytes co-cultured with rIFN-γ for 9 days showed a reduced level in their relative

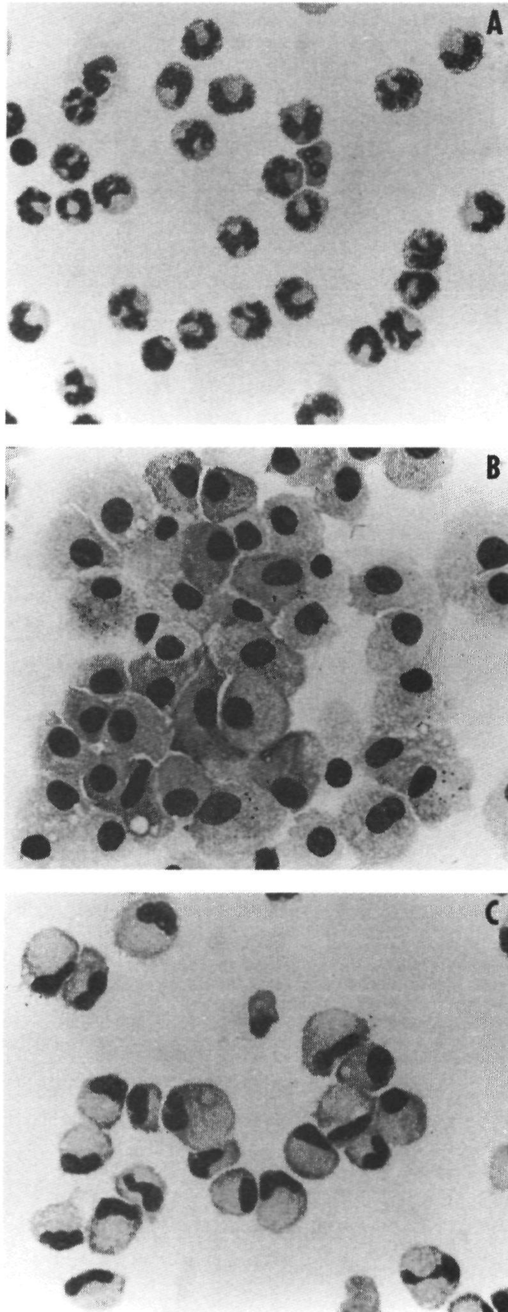

Figure 1.

May-Grünwald Giemsa stained cytocentrifuge preparations of freshly isolated monocytes (A), monocytes cultured for 9 days in Teflon bags with RPMI 1640 medium (B) or co-cultured for the whole culture period with 150 U rIFN-γ/ml (C). (Magnification x 400)

enzyme activities. Compared with the control cultures, the relative enzyme activities decreased to G-6-PDH, 46.6 ± 12.6%, PHI, 44.8 ± 8.8%; ACP, 61.0 ± 10.6%; and NAG, 57.3 ± 10.6%. The enzyme activity of ICDH was not affected (Fig. 2). The reductions were all significant at $P < 0.001$ (n=8). When rIFN-γ was added on day 7, only the at day 9 measured intracellular ACP activity diminished with significance ($P < 0.05$). The relative values are : G-6-PDH, 87.9 ± 11.8%, PHI, 87.4 ± 15.1%; ACP, 78.1 ± 13.3%; NAG, 89.7 ± 9.4%; and ICDH, 110.2 ± 30.0% (Fig. 2). In the presence of doxorubicin, in long-term or short-term exposures, neither the enzyme activities of the control cells nor the alterations in enzyme activities provoked by rIFN-γ significantly changed (Fig. 2).

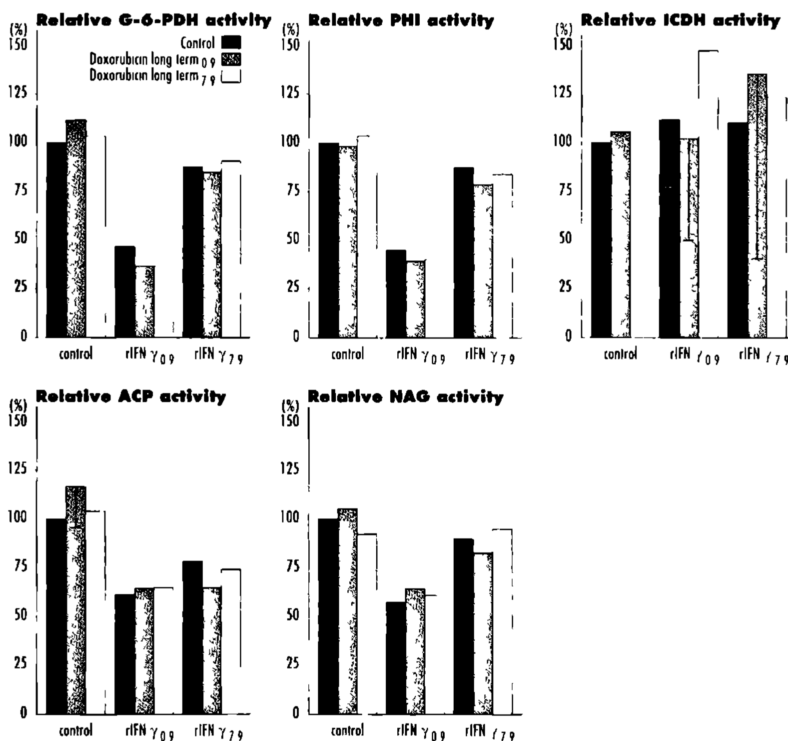

Figure 2. Effects of doxorubicin and/or rIFN-γ on the intracellular levels of G-6-PDH, PHI, ICDH, ACP, and NAG in human monocyte-derived macrophages. Monocytes were cultured for 9 days in RPMI 1640 medium or co-cultured for 9 days or the last 40 h with 150 U rIFN-γ/ml. Doxorubicin treatment (75 ng/ml) was in long-term schedules for 9 days or the last 40 h. Enzyme activity was measured in nmol/min per μg DNA. Results show the mean ± SD (n=8) of relative enzyme activities obtained by treated non-treated activity ratios

Effects of doxorubicin and rIFN-γ on FcγRI and FcγRII expression by cultured monocytes. Expression of the surface markers FcγRI and FcγRII is diminished after both long-term and short-term culture in doxorubicin (15). We cultured monocytes for 9 days in the presence of doxorubicin and/or rIFN-γ. Co-culture with 150 U/ml rIFN-γ from day 0 and from day 7 induced a 3.6 ± 2.5- *versus* a 5.8 ± 3.2-fold increase in the expression of FcγRI, as compared with control cultures (mean ± SD of 7 experiments). The expression of FcγRII was not influenced by rIFN-γ. Short-term and long-term exposures to doxorubicin did not affect the expression of either of the FcγRs on the human monocyte-derived macrophages activated with rIFN-γ.

Effects of doxorubicin and rIFN-γ on ADCC activities of cultured monocytes. To determine whether doxorubicin and rIFN-γ, alone or in a combined approach, alter functional Fc-mediated activity of the cultured monocytes, we investigated for cytotoxicity against two types of sensitized hRBC. In control cultures ADCC activity against maximally sensitized hRBC mediated by FcγRI and FcγRII was 41.7 ± 8.8% and 42.7 ± 11.6%, respectively (Fig. 3). When monocytes were co-cultured with rIFN-γ (from day 0), ADCC activity against maximally sensitized EA-hIgG increased to 78.9 ± 9.8% and for macrophages (stimulation at day 7) cytotoxicity increased to 68.1 ± 8.1%. Co-culture with rIFN-γ caused an increase to a lesser extent in the cytotoxicity mediated by FcγRII against maximally sensitized EA-mIgG1. ADCC activity rose to 56.3 ± 8.4% and 55.4 ± 7.3%, respectively (Fig. 3).

The short-term and long-term exposures, to doxorubicin did not affect the ADCC activity mediated by either of the FcγRs of the control cells, nor of the human monocyte-derived macrophages activated with rIFN-γ (Fig. 3).

DISCUSSION

Biological treatment may be considered the "Fourth Modality of Cancer Treatment" (27). The past decade of research has demonstrated that there is indeed a role for biological agents in cancer treatment. Over the next decade, the combination of BRMs with chemotherapeutic agents is one of the modalities that could lead to advances in cancer treatment (1-6).

As part of our investigations on the influence of cytostatic agents in combination with BRMs on the MPS, we evaluated possible modulations induced by doxorubicin on the activation of human monocytes provoked by rIFN-γ. rIFN-γ appears to be the major monocyte-activating lymphokine (7-9). Doxorubicin is active against a variety of malignancies (12-14); it can act solely with the cell surface (28), and it is therefore important to consider the cell surface as a target.

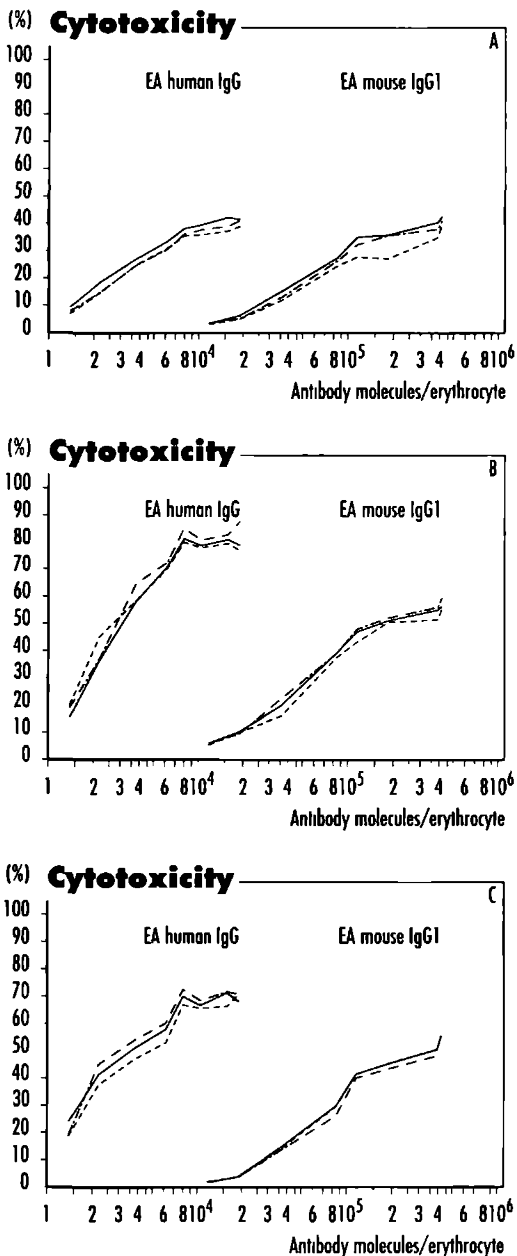

Figure 3.

Effects of doxorubicin on ADCC of human monocyte-derived macrophages to two types of sensitized erythrocytes Monocytes were cultured for 9 days with RPMI medium (A), co-cultured for 9 days with 150 U rIFN-γ/ml (B) or for the last 40 h with 150 U rIFN-γ/ml (C) Monocytes were cultured without (——), or with doxorubicin (75 ng/ml) in long term schedules for 9 days (– –) or the last 40 h (- - -) Results represent data from 4 8 individual experiments using cells from different donors SD were all smaller than 10%

In our experiments rIFN-γ but not doxorubicin treatment, induced morphological changes Most strikingly was the smaller cell size when monocytes were cultured in the presence of rIFN γ from the start of culture Previously, Becker (29) found that human monocytes, isolated by adherence and cultured with 5% autologous serum in plastic culture plates, slightly increased in volume by IFN-γ during the first 3 days of culture, whereas later in the maturation process the volume of the IFN-γ treated macrophages was smaller than that of the non-treated macrophages In our studies, both adherent and non adherent monocytes were cultured as we isolated highly purified monocytes from mononuclear cells with counterflow centrifugation Furthermore the culture took place in suspension in the presence of 10% pooled human serum Except for the serum factors, all other influences were excluded in our study which therefore allows a more definite judgement to be made concerning the cell size of the monocytes cultured with rIFN-γ

The phenomenon of monocyte clumping which appears when they are cultured in the presence of rIFN-γ for 9 days can be explained by an upregulation of adhesion molecules Weinberg et al (30) and later Mentzer et al (31) reported that IFN γ selectively enhances the leukocyte function adhesion-1 antigen (LFA-1) and its ligand the intercellular adhesion molecule 1 (ICAM-1), which mediate homotypic monocyte adhesion We could also demonstrate an increased presence of these molecules in this study (data not shown)

There are reports of changes in enzymes and receptors as indicators of the *in vitro* maturation of non-treated monocytes (21,23,32 35) Some information has been published on the effects of IFN-γ on enzyme levels of mouse peritoneal macrophages (36,37) No information is, however, available on the effects of doxorubicin in combination with rIFN-γ on maturation, as reflected on enzyme levels of highly purified and *in vitro* cultured monocytes In an earlier report we found that the intracellular levels of the key enzymes G-6-PDH, PHI, ICDH, ACP and NAG remained the same when cells were exposed to doxorubicin in both short-term and long-term schedules (15) In this study we found that the intracellular enzyme levels were also unaltered when cells treated with rIFN γ were exposed to doxorubicin in the long-term or the short-term schedule This shows that doxorubicin exposures do not affect the rIFN γ induced monocyte activation However, when monocytes were co-cultured for 9 days with rIFN-γ in the presence or absence of doxorubicin, all measured enzyme levels, with the exception of the ICDH activity, decreased significantly as compared with the controls G 6-PDH and PHI are both enzymes of the intermediary metabolism, since we measured the enzyme activities in nmol/min per μg DNA, this phenomenon can be explained by the decreased cell volume of the monocytes co-cultured continuously with rIFN-γ, because these enzyme activities were reduced in a comparable ratio However, the bulk enzymes NAG and ACP, important for hydrolytic degradation of mucopolysaccharides, glyco-

proteins, and glycolipids, decreased in enzyme activity to a lesser extent, and a change in volume is therefore not the only possible explanation The anomalous behavior of the ICDH activity may in all likelihood be caused by variations of the very small amounts of the ICDH activities measured Monocytes co-cultured with rIFN-γ from day 7 showed a tendency towards slightly decreasing enzyme levels for G-6-PDH, PHI, and NAG Only ACP activity diminished significantly These results are in contradiction with observations of Pierangeli et al (36), who showed an increase in ACP content per cell of IFN-γ treated murine peritoneal macrophages, with a maximum at 24 h of incubation Moreland et al (37) described initially a rise and thereafter a drop in ACP content when murine peritoneal macrophages were treated with IFN γ for 48 -72 h The disparity observed between the results of Pierangeli et al (36) and the present study with rIFN-γ co-cultured from day 7 could be the consequence of 1 the origin of the cell population human peripheral blood monocytes *versus* murine peritoneal macrophages, 2 the way in which the cells were obtained isolation of the whole monocyte population by means of elutriation *versus* the selective isolation of peritoneal macrophages via plastic adherence, 3 the duration of stimulation 40 h *versus* 24 h All our measurements were expressed in nmol/min per μg DNA not per cell and a difference based on methodological factors is unlikely

It is well known that IFN-γ increases both the number of FcγRI (8,38) and the ability of monocytes to perform ADCC of red cell targets through this receptor (39) The finding that monocytes co cultured with rIFN-γ for the last 2 days, showed a 1 6 fold higher increase in FcγRI expression and a 10 8% lower ADCC activity *versus* the monocytes co-cultured for 9 days with rIFN-γ seems to be in disaccordance with this theory However, cell size after continuous co-culture with rIFN-γ was increased to a lesser extent (24 μm) as compared with the monocytes cultured from day 7 with rIFN-γ (30 μm), which ultimately results, assuming that the cells were spheres, in a FcγRI surface density comparable to the ADCC activity

We have previously published that doxorubicin caused a slight but significant decrease in FcγRI expression on *in vitro* cultured monocytes (15) When combined with rIFN-γ, we could not observe any modulation induced by doxorubicin in both the number of FcγRI or FcγRII expressions, or in functional activities mediated by those receptors

Finally, these observations indicate that in a combined approach, doxorubicin does not negatively influence the activation state of monocytes/macrophages induced by rIFN γ The results obtained appear to allow the combination of optimal doxorubicin administration with rIFN-γ immunotherapy

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MODULATION BY CYTOKINES OF PHENOTYPE AND FcγR-MEDIATED CYTOTOXICITY OF *IN VITRO* CULTURED HUMAN MONOCYTES.

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CHAPTER 6

ABSTRACT

We evaluated the capacity of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, macrophage colony stimulating factor (M-CSF), granulocyte-macrophage (GM)-CSF, and IL-2 to modulate freshly isolated monocytes at different stages of maturation during *in vitro* culture, i.e., long-term (9 days) and short term (40 h) cultured.

When long-term (9 days) cultured monocytes were incubated with IFN-γ for the last 40 h of culture, IgG Fc receptor (FcγR)I expression was found to be increased dramatically, whereas FcγRII and FcγRIII were not affected. In cultures with IL-4 or TNF-α, reduced expression of all three FcγR classes was observed. With exception of the TNF-α-cultured cells, the altered FcγRI and FcγRII expression was paralleled by the antibody-dependent cell-mediated cytotoxicity (ADCC) of the long-term cultured monocytes. Short-term (40 h) cultured monocytes exhibited a strongly decreased FcγRI and FcγRII expression and ADCC as compared to freshly isolated monocytes. FcγRIII expression was hardly detectable. When co-cultured with cytokines, IFN-γ could reverse this decrease in both FcγRI and FcγRII expression. Interestingly, GM-CSF and TNF-α were capable of reversing the decrease only in FcγRII expression. IFN-γ and IL-4 were the only cytokines that influenced the 40-h cultured monocyte FcγRII-mediated ADCC activity. Furthermore, all tested cytokines up-regulated the HLA-DR expression on 40-h cultured monocytes. Supernatants of both short- and long-term (cytokine) cultured monocytes were negative for IL-1β, and TNF-α activity.

These results show strong effects of cytokines on the expression of various cell surface antigens, and FcγR-mediated cytotoxicity. Specific effects were varying with the stage of monocyte differentiation.

INTRODUCTION

Immunotherapy with recombinant cytokines is a promising treatment modality for metastatic cancer (1,2). Human macrophages probably have an important contribution in such therapies, because of their central role in the immune response. Tissue macrophages are known to derive from peripheral blood monocytes, which are intermediate cells in terms of differentiation. These easy accessible monocytes are developing to mature macrophages when cultured under appropriate conditions *in vitro* (3-5). Both freshly isolated human monocytes, and mature monocyte-derived macrophages express IgG Fc receptor (FcγR)I (CD64), and FcγRII (CD32) (reviewed in refs. 6,7). *In vitro*, FcγRIII (CD16) will be expressed on monocytes after at least 4 days of culture (8). All these classes of FcγR have been shown to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (9). The amount of MHC class II molecules (HLA-DR) on the

monocyte/macrophage membranes is crucial for the initiation of an immune response (10), and CD14, a differentiation antigen expressed on mature monocytes, is a receptor for lipopolysaccharide (LPS) complexed with LPS binding protein (11)

Precise signals involved in regulating the functional state of monocytes/macrophages are incompletely understood Recombinant glycoproteins, which include interleukins (IL), interferons (IFN), colony-stimulating factors (CSF), and tumor necrosis factors (TNF) selectively affect phenotype, and function of monocytes/macrophages (12-15), merely depending on the stage of maturation of these cells (16-18) Different mediators may affect similar functions of monocytes For instance, both IFN γ, and IL-4 enhance expression of class II antigens on monocytes (19,20) However, the cell surface expression of FcγR is increased when monocytes are cultured with IFN-γ, and decreased when exposed to IL-4 (21,22) Furthermore, we recently described that IFN γ treated human monocytes also underwent alterations in morphology (3), and cytolytic mechanism (17)

For the present study, we have investigated to what extent various cytokines, to be used *in cancer immunotherapy, affect the above-mentioned phenotypic and functional* characteristics of freshly isolated monocytes in an early stage of maturation *in vitro* (40 h), and of monocyte-derived macrophages *in vitro*

MATERIALS AND METHODS

Isolation and culture of human monocytes. Monocytes were isolated as described (23) Briefly, mononuclear cells obtained from buffy coats, or cytapheresis of healthy volunteers, were isolated by Percoll (Pharmacia, Uppsala, Sweden) centrifugation Monocytes were purified from mononuclear cells by cell scatter monitored counterflow centrifugation Monocyte fractions were over 95% pure (as evaluated by staining for non-specific esterase and May-Grunwald-Giemsa), and viability was higher than 98%, as assessed by trypan blue dye exclusion Isolated cells were either cultured immediately or were cryopreserved (24), and stored in liquid nitrogen until use Recovery and viability of cryopreserved monocytes were >70%, and >95%, respectively

Freshly isolated or thawed cryopreserved monocytes were cultured for 40 h or 9 days at concentrations of 3 x 10^5 cells/ml in RPMI 1640 medium with 20 mM HEPES (Flow Laboratories, Irvine, UK), 2 mM L-glutamine (GIBCO, NY), 1 mM pyruvic acid (Sigma, St Louis, MO), 40 μg/ml gentamicin (Boehringer, Mannheim Germany), and 5% heat-inactivated pooled human serum, in a humidified incubator with 5% CO_2 in air at 37°C Cytokines were added to cultures during 40 h, either directly at day 0 (40-h cultured population), or at day 7 (9-day cultured population) Teflon foil bags (Dupont de Nemours and Co , Switzerland) were used for non-adherent culture of monocyte suspensions (25)

Harvesting monocyte-derived macrophages. After culture for either 40 h, or 9 days, cells were recovered by needle aspiration after cooling Teflon foil bags to 4°C for 1 h and gentle kneading After removal of the culture supernatant (collected for determination of IL-1β and TNF-α), the cells were washed twice in RPMI 1640 medium (without serum), and kept on ice until use There was no difference in the number (>90%) or viability (>98%) between cells recovered after culture with cytokines and those recovered from control cultures For the benefit of FcγRI-mediated ADCC experiments, an additional washing procedure at 37°C in RPMI 1640 medium was performed in order to remove cytophilic human IgG (hIgG), as described (17)

Human recombinant cytokines. Human granulocyte-macrophage (GM)-CSF, and human IL-4 were a kind gift of Dr P Trotta (Schering-Plough Research) Human macrophage (M)-CSF was generously donated by Cetus (Emeryville, CA) Human IL-2 produced by Biogen was a kind gift of Glaxo (Geneva, Switzerland) Human TNF-α and human IFN-γ were kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands) Monocytes and 7-day cultured monocytes were incubated with 150 U IFN-γ/ml, chosen following the performance of pilot experiments (17) (data not shown) Optimal concentrations of the other cytokines were determined by adding these cytokines at three concentrations to 7-day cultured monocytes The ultimately concentrations were chosen to give optimal phenotypic responses with minimal effects on cell viability of the at day 9 harvested monocyte-derived macrophages

Immunofluorescence studies. Expression of FcγRI, FcγRII, FcγRIII, CD14, and HLA DR on cultured monocytes was assayed by indirect immunofluorescence as described previously (17) using specific mAb 32 2 [CD64 purified murine (m)IgG1] (Medarex, W Lebanon, NH), 197 (CD64 purified mIgG2a) (Medarex), which bind to different epitopes outside the ligand-binding site on FcγRI (26), IV 3 (CD32 purified mIgG2b) (Medarex), which competes with the FcγRII ligand-binding site (27), 3G8 (CD16 purified mIgG1) (Medarex), and Leu 11b (CD16 purified mIgM) (Becton Dickinson, Mountain View, CA), which identify different epitopes close to the FcγRIII ligand-binding site (28), WT14 (CD14 culture supernatant mIgG1) (provided by Dr W J M Tax, University Hospital Nijmegen, The Netherlands) which reacts strongly with CD14, expressed selectively on human monocytes and macrophages (29), anti HLA-DR (purified mIgG2a) (Becton Dickinson) detects HLA-DR, a common determinant of the MHC class II antigen, and FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H and L chain) (Cappel, Malvern, PA) Fluorescence intensity of 5,000 cells was measured with an Ortho 30H flow cytometer For all analyses, gatings (red forward and right angle scatter) were set around the monocyte/macrophage population Relative

expressions were calculated as follows $(M_c - M_n)/(M_m - M_n)$, where M_c = mean fluorescence intensity (arbitrary units) of cytokine cultured monocytes, M_n = mean fluorescence intensity of negative control, and M_m = mean fluorescence intensities of monocytes cultured in control medium.

FcγR-mediated cytotoxicity. ADCC capacity of monocyte-derived macrophages from different donors was studied using a ^{51}Cr release assay as described previously (17,18), in which antibody-coated human erythrocytes (E) were used as targets. Briefly, E were suspended in PBS containing ^{51}Cr (sodium chromate, Amersham, UK), and incubated at 37°C. After 30 min, sensitizing antibody or PBS / 0.1% BSA alone (control), was added. To sensitize the ^{51}Cr labeled E two types of antibodies were used. Rhesus D$^+$ ^{51}Cr-labeled E were sensitized either with human alloserum against Rhesus D (Merz & Dade, Dudingen, Switzerland) or with ascites of mIgG1 mAb directed at glycophorin A. Both types of antibodies were used at various dilutions, resulting in E sensitized with a range of IgG molecules per cell (EA IgG), described in (30). After incubation for 30 min, cells were washed thrice, suspended, and 2.5 x 10^5 target cells were mixed with equal volumes of effector cells (freshly isolated monocytes/40-h cultured monocytes = 3.75 x 10^5 cells, 9-day cultured monocytes = 1.25 x 10^5 cells). After incubation for 18 h at 37°C, half the supernatant was removed for estimation of ^{51}Cr release. All tests were carried out in triplicate The results were calculated as follows: % cytotoxicity = (C - S)/(T - S) x 100, where C = mean cpm of test sample, S = mean cpm of spontaneous ^{51}Cr release (i.e., ^{51}Cr release by labeled target cells in medium alone), and T = mean cpm of the maximal ^{51}Cr release, obtained by addition of 20% saponin (Coulter, Dunstable, UK) to the target cells. The average spontaneous release of E was 3 ± 1% (mean ± SD), and release from unsensitized E incubated with effector cells never exceeded spontaneous release.

Radioimmunoassays (RIA) for IL-1β and TNF-α. The concentrations of extracellular IL-1β and TNF α in the supernatants of monocytes cultured for 40 h or 9 days, either in the absence, or presence of cytokines were measured by specific RIA, as described previously (31-33). Briefly, on day one samples of the monocyte-culture supernatants were incubated with rabbit allosera against human IL-1β or TNF-α (room temperature). On the same day, radioiodinated cytokine tracer was added, and finally sheep anti-rabbit IgG antiserum on day 4. Antibody-antigen complexes were precipitated by centrifugation. Radioactivity was determined in a gamma counter and expressed as a percentage of the total binding of IL-1β, or TNF α. Cytokine concentrations were calculated with a four parameter method, obtained with serial dilutions of known standards in monocyte culture medium.

RESULTS

Modulation by cytokines of surface expression of 9-day cultured human monocyte-derived macrophages. The result of *in vitro* monocyte/macrophage activation by cytokines may depend on the duration of cell culture (34). In our study we first screened a number of cytokines, at various concentrations, for their ability to modulate the phenotype of long term cultured monocytes.

Monocyte derived macrophages were stimulated after 7 days of culture for 40 h, with IFN-γ at one concentration, and with TNF-α, IL-4, M-CSF, GM-CSF and IL-2 at three different concentrations. On day 9, cell size and viability of monocyte-derived macrophages cultured with the various cytokines did not differ from those cultured in the absence of cytokine. IFN-γ appears to increase FcγRI expression dramatically (575 ± 318%) (Fig. 1). A dose-dependent but small decrease in expression of FcγRIII was observed when monocyte-derived macrophages were treated with GM-CSF. Both IL-4 and TNF-α reduced the membrane expression of all three FcγR classes. In addition, IL-4 also decreased CD14 expression in a dose dependent manner. However, enhancement of HLA-DR antigen expression on 9-day cultured monocyte-derived macrophages which were treated with IL-4, indicated that IL-4 did not cause a general reduction in expression of antigens.

Effect of cytokines on FcγR-mediated cytotoxicity by 9-day cultured human monocyte-derived macrophages. We investigated whether effects on FcγRI and FcγRII expression were paralleled by functional activities of the cells. ADCC activity of FcγRI and II can be independently assayed using E sensitized with hIgG or mIgG1, respectively. In previous studies, the specificity of monocyte and macrophage FcγRI and FcγRII-mediated cytotoxicity has been evaluated, using unique anti-FcγR mAb (17,18,35).

Monocytes were cultured for 9 days in medium alone, or with cytokine for the last 40 h, and cytotoxicity towards EA-hIgG and EA-mIgG1 targets was evaluated. Both FcγRI and FcγRII-mediated ADCC activity of 9-day cultured monocytes were significantly reduced after IL-4 (100 U/ml) treatment (FcγRI: from 40.5 ± 3.9% to 19.6 ± 1.4%, FcγRII: from 45.4 ± 3.7% to 28.0 ± 2.8%) (Fig. 2). Monocyte-derived macrophages that had been cultured in the presence of IFN-γ (150 U/ml) showed an enhancement of ADCC activity against EA-hIgG (to 71.1 ± 5.8%). Contrary to expectation, where TNF-α strongly reduced FcγR expression, ADCC activity via FcγRI and FcγRII was not affected. Except for TNF-α, these results suggested that for 9 day cultured monocyte-derived macrophages FcγR expression and ADCC activity are strongly related.

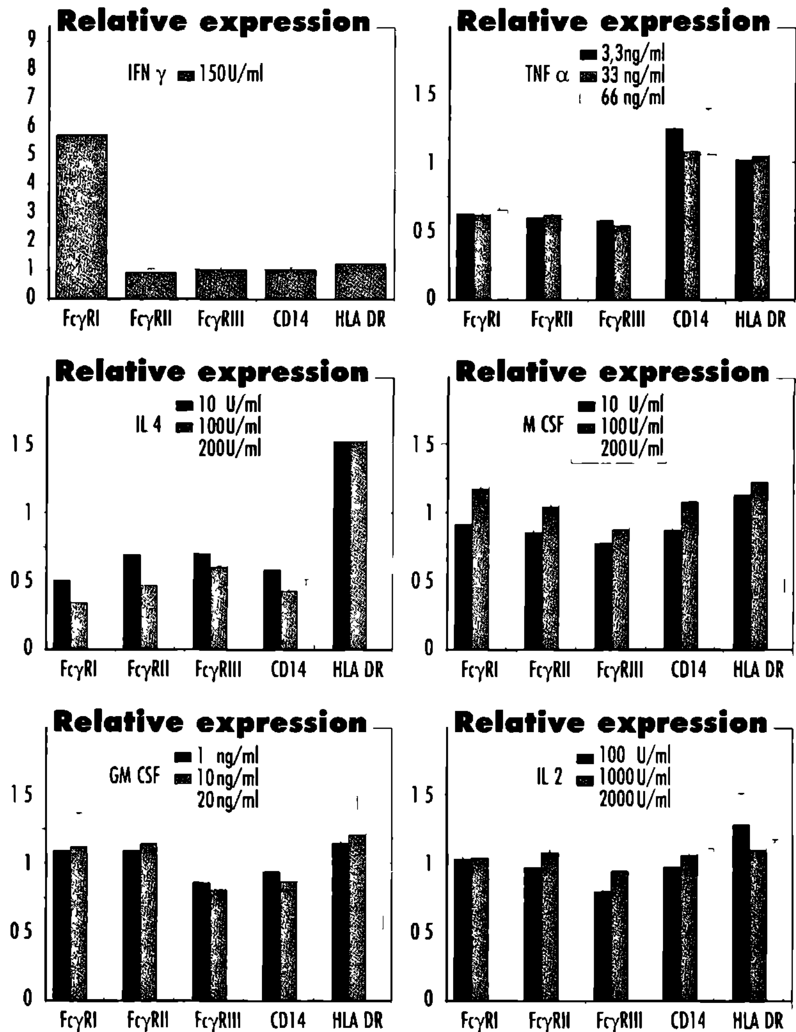

Figure 1.

Dose effect of cytokines on 9-day cultured monocytes Monocyte-derived macrophages were obtained by culturing monocytes for 9 days in medium alone (control), or in medium supplemented with three different concentrations of the indicated cytokines for the last 40 h of culture Expression of FcγRI, FcγRII, FcγRIII, CD14, and HLA DR was evaluated by immunofluorescence staining with specific mAb Relative expression of the surface molecules was calculated as described in *Materials and Methods* Results represent mean ± SD from three experiments with cells from different donors

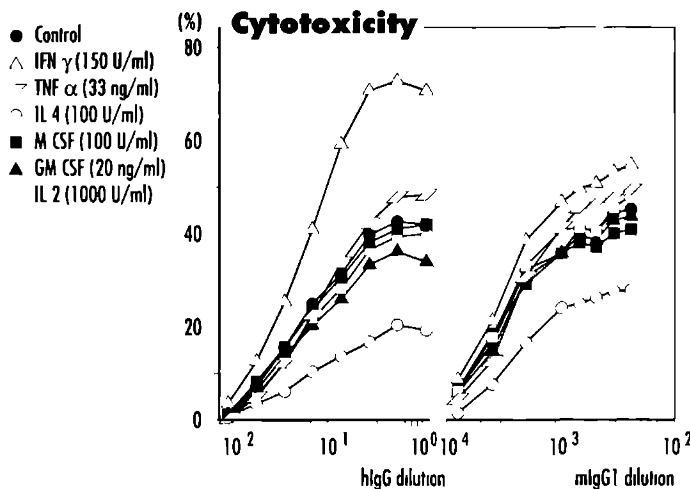

Figure 2.
Effect of cytokines on monocyte-derived macrophage FcγR mediated ADCC activity ADCC activity was assessed after culturing monocytes for 9 days in medium alone (control), or in medium supplemented with the indicated cytokines for the last 40 h of culture ADCC tests were performed as described in *Materials and Methods*, using target E sensitized with variable amounts of either hIgG anti-Rhesus D alloserum or mIgG1 anti-glycophorin A mAb (optimal E T ratio of 0 5) Results represent data from three individual experiments with cells from different donors SD were all smaller than 10%

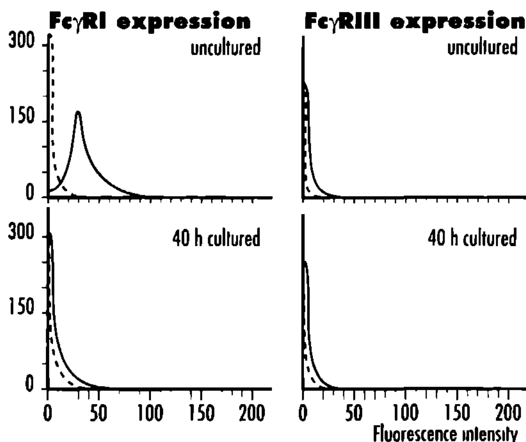

Figure 3.
Expression of FcγRI and FcγRIII on short term cultured monocytes The expression of FcγRI and FcγRIII on freshly isolated monocytes, and monocytes cultured for 40 h in medium alone, was measured by immunofluorescence analysis using specific anti FcγR mAb The fluorescence intensity (arbitrary units) was recorded on the abscissa (linear scale) Negative control values are represented by dashed-lines One representative experiment out of six is shown using anti-FcγRI mAb 32 2, and anti-FcγRIII mAb Leu 11b

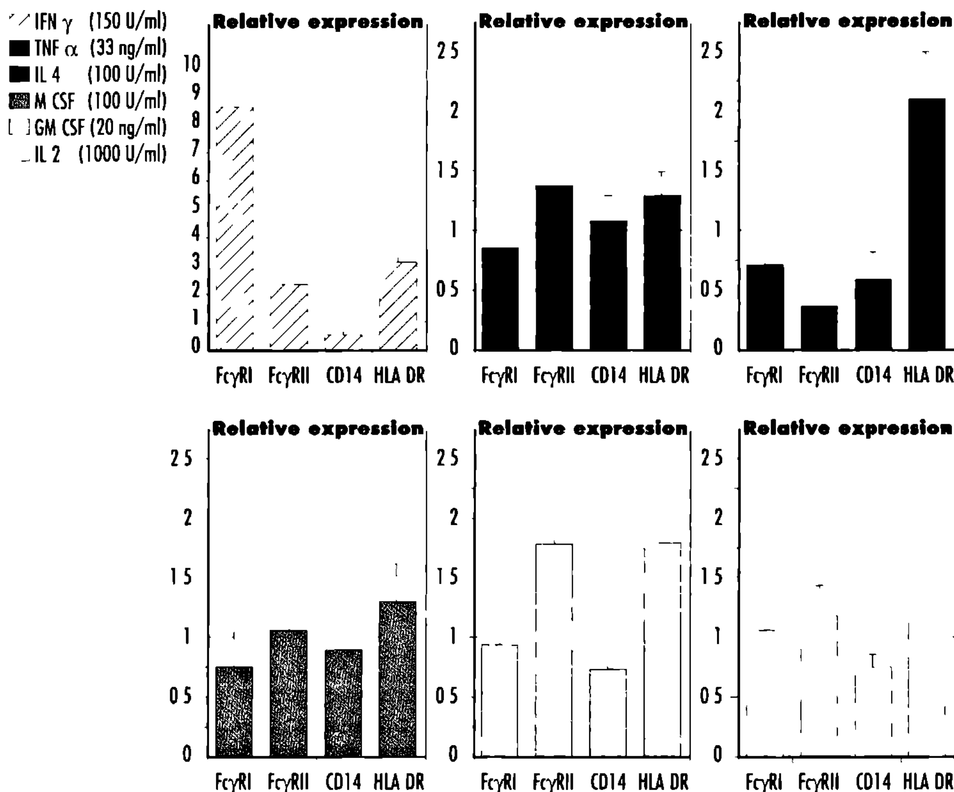

Figure 4.

Effect of different cytokines on 40-h cultured monocyte surface antigen expression Monocytes were cultured for 40 h in medium alone (control), or in medium supplemented with the indicated cytokines Expression of FcγRI, FcγRII, CD14, and HLA DR was evaluated by immunofluorescence staining with specific mAb Relative expression of the surface molecules was calculated as described in Materials and Methods Results represent mean ± SD from three experiments with cells from different donors

Modulation by cytokines of surface expression of 40-h cultured monocytes. We previously demonstrated that, as a consequence of culture, FcγRII expression on 40-h cultured monocytes was decreased (18) In six separate experiments with cells from different donors, we also compared freshly isolated to 40-h cultured monocytes for FcγRI and FcγRIII expression With the use of specific anti-FcγRI mAb (197 and 32 2), we showed that FcγRI expression on 40-h cultured monocytes decreased dramatically to 25 8 ± 10 7% (mean ± SD), as compared to freshly isolated monocytes Using two anti-FcγRIII mAb (3G8 and Leu 11b), which recognize different CD16 epitopes, we

showed that FcγRIII on 40-h cultured monocytes was still hardly detectable (Fig 3)

Next, we evaluated the effects of IFN γ (150 U/ml), TNF-α (33 ng/ml), IL-4 (100 U/ml), M-CSF (100 U/ml), GM-CSF (20 ng/ml), and IL-2 (1000 U/ml) on the phenotype of monocytes at the short-term cultured (40 h) maturation stage Cell size and viability of cytokine-treated, and control monocyte populations was found to be similar The preexistent low expression after 40 h of culture did not permit a reliable assessment of FcγRIII modulation by cytokines As depicted in Figure 4, IFN-γ and IL-4 had antagonistic effects on the expression of both the classes of FcγR Compared to the 40-h cultured control monocyte population, FcγRI and FcγRII expression were increased when cultured in the presence of IFN-γ, and decreased when cultured with IL-4, respectively Surface expression of FcγRII was also increased by TNF-α and GM-CSF With exception of TNF-α-treated cells, all cytokine-treated populations exhibited a decreased CD14 expression, although, the influence of M-CSF was minimal On the other hand, all tested cytokines up-regulated the expression of HLA-DR molecules on 40-h cultured monocytes

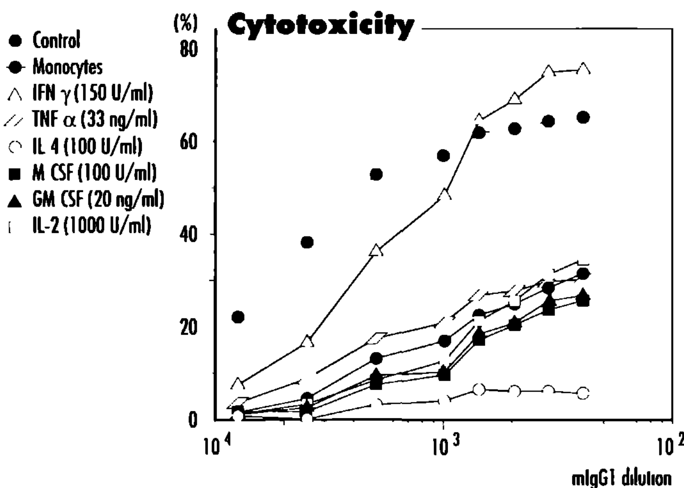

Figure 5.
ADCC activity of short term cytokine-cultured monocytes against mIgG1-sensitized human E Freshly isolated monocytes, or monocytes cultured for 40 h in either medium alone (control), or medium supplemented with the indicated cytokines, were evaluated for cytotoxic activity ADCC tests were performed as described in *Materials and Methods*, using target E sensitized with variable amounts of mIgG1 anti-glycophorin A mAb (optimal E T ratio of 1 5) Results are mean data from three individual experiments with cells from different donors SD were all smaller than 8%

Effect of cytokines on FcγR-mediated cytotoxicity by 40-h cultured human monocytes. Next, we investigated whether the found FcγR expressions are manifested in FcγR-mediated cytotoxicity of the short term cultured monocytes Freshly isolated, and 40-h (cytokine) cultured monocytes were evaluated for cytotoxicity towards EA-hIgG and EA-mIgG1, using an optimal effector to target cell ratio 1 5 FcγRI-mediated ADCC activity by monocytes cultured for 40 h, either without (control), or with TNF-α (33 ng/ml), IL 4 (100 U/ml), M-CSF (100 U/ml), GM-CSF (20 ng/ml), or IL-2 (1000 U/ml), never exceeded a 10% cytotoxicity level towards optimally with hIgG sensitized E However, when monocytes were cultured for 40 h in the presence of IFN-γ (150 U/ml), FcγRI-mediated ADCC activity was enhanced to 70 ± 6 3% (not shown) In Figure 5 we show that monocytes treated with IL-4 (100 U/ml) during the 40 h culture period, exhibited a reduced FcγRII-mediated ADCC activity against EA-mIgG1 (from 31 5 ± 2 1% to 6 2 ± 2 8%) When cultured in the presence of IFN-γ (150 U/ml), this activity was strongly enhanced (to 75 6 ± 5 7%) However, when the 40-h IFN-γ-cultured monocytes are compared with freshly isolated monocytes (65 1 ± 4 7), lysis of EA-mIgG1 was almost similar (Fig 5)

Effect of cytokines on the production of IL-1β and TNF-α by cultured monocytes. Production and secretion of IL-1β and TNF-α by monocytes and monocyte-derived macrophages is not constitutive but is induced by a variety of endogenous and exogenous stimuli In this study, strict precautions were taken with respect to the isolation (Percoll centrifugation and elutriation), and the *in vitro* culture (non-adherent) of highly purified monocytes to minimize IL-1β and TNF-α synthesis Under these conditions, it was possible to induce a profound IL-1β and TNF-α synthesis/release via stimulation with LPS (unpublished results) Monocytes were incubated in suspension for 40 h or 9 days, either in the absence, or presence of recombinant cytokines, as described in Materials and Methods Compared with the recombinant cytokine-free populations, neither IFN-γ (150 U/ml), IL-4 (100 U/ml), M-CSF (100 U/ml), GM-CSF (20 ng/ml), nor IL-2 (1000 U/ml) induced any IL-1β or TNF-α secretion by monocytes at different stages of maturation, as detected in the various culture supernatants by the specific RIA (Table I) The concentration of TNF-α measured in the supernatant was always lower than originally added to the cell cultures On the contrary, in two out of three supernatants of 40-h cultured monocytes stimulated with TNF-α (33 ng/ml), a slight increase in the concentration of IL-1β was detected

Table I.

Analysis of TNF-α and IL-1β production by short-, and long-term cytokine cultured monocytes

Donor	control –	IL-2 (1000 U/ml)	IL-4 (100 U/ml)	TNF-α (33 ng/ml)	IFN-γ (150 U/ml)	M-CSF (100 U/ml)	GM-CSF (20 ng/ml)
TNF-α present in supernatant of 40-h cultured monocytes[a] (ng/ml)							
A	1.24[c]	1.07	<0.19	25.2	1 08	0.84	1.38
B	<0.19	<0.19	<0.19	26.76	0.36	<0.19	0.39
C	0.43	0.36	<0.19	21.0	–	0.29	0 52
IL-1β present in supernatant of 40-h cultured monocytes (ng/ml)							
A	0.43	0.25	0.10	1.40	0.40	0 27	0.66
B	0.13	0.15	0.13	0.43	0.19	0.17	0.11
C	0.12	<0.08	<0.08	0.10	–	<0.08	0.10
TNF-α present in supernatant of 9-day cultured monocyte-derived macrophages[b] (ng/ml)							
D	0.26	0 22	0 25	19 6	–	0.27	<0.19
E	0.65	0 59	0 70	26 5	–	0.48	0.27
F	0.24	0.33	0.25	19.3	–	<0.19	0.39
IL-1β present in supernatant of 9-day cultured monocyte-derived macrophages (ng/ml)							
D	0.09	0.12	0.17	0.12	–	0.19	0.11
E	0.53	0 31	0 35	0.23	–	0.43	0.25
F	<0.08	0.15	0 10	<0.08	–	<0.10	0.15

[a] *Supernatant was obtained from monocytes (3 x 10^5/ml) cultured in suspension for 40 h with or without cytokine*

[b] *Supernatant from monocyte-derived macrophages was obtained by in suspension culturing of monocytes (3 x 10^5/ml) for 9 days in medium, or medium supplemented with cytokine for the last 40 h of culture*

[c] *Concentrations of IL-1β and TNF-α in the supernatants of cultured cells were measured by specific RIA's, as described previously (31-33)*

DISCUSSION

It is of relevance to assess the putative effects of cytokines on various differentiation stages of human monocytes Differences between monocytes, and monocyte-derived macrophages are incontestable the result of their maturational or activation state (5,34). Therefore, our studies have focused on the process of monocyte differentiation at different stages of maturation, i e , freshly isolated monocytes, short- (40 h) and long-term (9 days) cultured monocytes In the present report, immunomodulatory effects induced by IFN-γ, TNF-α, IL-4, M-CSF, GM-CSF, and IL-2, on phenotype, FcγR-mediated cytotoxicity, and cytokine secretion during *in vitro* culture of human monocytes were studied. We first screened the modulatory effects of the indicated cytokines on long-term cultured monocytes, in the second part of our study we monitored on short-term cultured cells.

Monocyte-derived macrophages showed sensitivity for modulation of the quantitative expression of some cell surface molecules when stimulated, for the last 40 h of culture, with IFN-γ, TNF-α, or IL-4. Most obvious are the effects caused by IFN-γ on the expression of FcγRI (increased), and the decreased expression of all three FcγR classes after culture with either IL-4, or TNF α. Relevant, and most intriguing are the consequences, of the by the cytokines changed quantitative expression of FcγR molecules, for akin FcγR-mediated ADCC activity. For both the with IFN-γ and IL-4 cultured monocyte-derived macrophages there was a good relationship between the altered membrane expression of the FcγR, and the capacity to lyse EA-IgG (Fig. 2). In contrast, similar FcγRI- and FcγRII-mediated ADCC activities were observed with both untreated, and TNF-α-treated monocyte-derived macrophages, although the latter cells express less FcγR. These results suggest that provoked changes in either FcγRI, or FcγRII expression levels are not always related to FcγR-mediated functional activities. A possible interpretation for this discrepancy in the putative 'action-reaction' model may be the initiation of additional changes in mechanisms which are not affiliated directly with the number of FcγR by which the function is assumed to be attained. Another example is the stimulation by IFN-γ of the FcγRII-mediated cellular cytotoxicity of neutrophils, monocytes and myeloid cell lines without increasing FcγRII expression (9).

A changing monocyte phenotype is inherent to culture. Recently, we compared freshly isolated monocytes with 40-h cultured monocytes for the expression of FcγRII and HLA-DR (18). As a consequence of the 40 h culture period, FcγRII expression on monocytes was halved, whereas the amount of HLA-DR antigens doubled. These data imply that the appropriate culture conditions did not cause a general reduction in membrane molecules. Others showed that FcγRI expression was down regulated on monocytes after 1 day of culture (36), and that expression of FcγRIII on freshly isolated monocytes is negligible but will increase with length of time in culture (8). Before studying any modulatory effects of cytokines on 40-h cultured monocyte FcγR expression, we therefore first measured FcγRI and FcγRIII expression on both freshly isolated monocytes, and on *in vitro* differentiated, unstimulated 40 h cultured monocytes. Also a strong reduced FcγRI expression on 40-h cultured monocytes was observed (Fig. 3), and this seemed to correlate well with the observed decrease in FcγRI-mediated ADCC activity against E sensitized with hIgG. Until now, the phenomenon of the "FcγR disappearing" remains unclear. Tripathi et al. (36) supposed that there is a mechanism of rapid internalization of the FcγR. However, knowledge about recycling of human FcγR is limited. In contrast to the recycling capability of FcγRII (37) Jones et al. (38) demonstrated that human FcγRI, neither on the U937 cell line, nor on monocytes recycles

Next, we will discuss effects of cytokines on short-term cultured (40 h) monocytes. Highly remarkable were our observations with respect to the effects of IFN-γ on the expression levels of FcγRI and FcγRII. When 40-h IFN-γ-cultured cells are compared with freshly isolated monocytes for FcγR expression, FcγRI expression increased two times, and the expression level of FcγRII was unchanged (18). In addition we show that under these defined conditions, TNF-α-, and GM-CSF-cultured monocytes express also higher FcγRII levels than their unstimulated counterpart (Fig. 4). Our data clearly demonstrate that the reduction of FcγRII expression levels could be reversed completely by culturing monocytes with IFN-γ, or GM-CSF, and was reversed marginally when cultured in the presence of TNF-α. Furthermore, modulation of FcγRII expression by these cytokines seems to depend critically on the stage of monocyte maturation, revealing a new level of complexity in regulation of FcγR expression levels. The mechanism by which cytokines affect FcγR expression remains unclear up to now.

Freshly prepared monocytes, and monocytes cultured in the presence of IFN-γ have an almost equal capacity to lyse EA-mIgG1. However, 40-h cultured monocytes (control), and monocytes cultured in the presence of GM-CSF, or TNF-α are comparable in lysing EA-mIgG1 (Fig. 5). As described above, these observations also show that an altered FcγRII expression level not necessarily has functional consequences.

All tested cytokines, IFN-γ, TNF-α, IL-4, M-CSF, GM-CSF, and IL-2, positively affected the level of HLA-DR expression. The cytokine-induced increase in this antigen expression is in fact cumulative to the previously described doubling of HLA-DR antigens, as a consequence of the 40 culture period only (18). The amount of HLA-DR antigens on the monocyte is crucial for the antigen presenting cell function of this cell type.

The capacity of normal human monocytes, either short-term or long-term cultured, to produce and secrete cytokines such as IL-1 and TNF is regulated by various stimuli including endotoxin and exogenous pyrogens. We quantified the IL-1β (secreted form of IL-1) and TNF-α (by monocytes secreted form of TNF) concentration in supernatants of monocytes incubated with multiple cytokines, by specific RIA to avoid confusing factors introduced by the use of bioassays. In this study, under the conditions as described, in medium cultured monocytes did induce neither IL-1β, nor TNF-α secretion. This finding is of importance, because both IL-1 and TNF induce monocytes to produce CSF and other cytokines (39), which in turn may interfere with the originally added specific cytokine.

In conclusion, in this report we document strong effects of cytokines on the expression of various cell surface antigens, and FcγR-mediated cytotoxicity. These effects were different, depending on the stage of monocyte differentiation.

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PLEIOTROPIC EFFECTS OF ANTICANCER DRUGS ON BOTH PHENOTYPE AND FUNCTION OF HUMAN MONOCYTE-DERIVED MACROPHAGES

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CHAPTER 7

ABSTRACT

We evaluated the effects of various classes of cytostatics (antibiotics, alkylating agents, antimetabolites, protein synthesis inhibitors, and miscellaneous agents) on human monocytes at different stages of maturation *in vitro*, i.e., long-term (9 days) and short-term (40 h) cultured.

During culture in Teflon foil bags, monocytes flattened and spreaded out on the surface. When long-term cultured, and incubated for the last 40 h with cisplatin (*cis*-DDP), the flattening and spread out of cells disappeared. In addition, the protein synthesis inhibitor ethyldeshydroxysparsomycin (EdSm), and fluorouracil (5-FU) induced homotypic monocyte adhesion. When short-term cultured with *cis*-DDP, 4-hydroxycyclophosphamide (4-OHCY), EdSm, or 5-FU, monocytes lost spreading capabilities. Expression of immunoglobulin G Fc receptor (FcγR)I and FcγRIII decreased when long-term cultured monocytes were treated with *cis*-DDP, 4-OHCY, EdSm, or 5-FU. Moreover, EdSm reduced the expression of FcγRII, CD14, and HLA-DR. Short-term EdSm co-cultured monocytes were reduced in FcγRI, FcγRII, and CD14 expression. 4-OHCY, however induced an increase in CD14 expression. The antibody-dependent cell-mediated cytotoxicity via FcγRI and FcγRII of long-term cultured monocytes was unchanged after culture with anticancer drugs. Interestingly, EdSm was the solely drug that could reduce FcγRII-mediated ADCC activity of 40 h cultured monocytes.

Our results indicate that specific effects of chemotherapeutic agents on monocyte morphology, differentiation-associated antigens, and FcγR-mediated cytotoxicity are depending on the class of drugs, and the stage of monocyte differentiation.

INTRODUCTION

Human monocytes and macrophages contribute in initiating and regulating host defense responses against the development and spread of neoplasia (for review see 1). The maturation stage along the monocyte/macrophage-lineage, and environmentally interactions (e.g., cytokine treatment, inflammatory processes) may critically affect the expression of cell surface molecules, and functional activities mediated by these cells (2,3). Both freshly isolated human monocytes, and monocyte-derived macrophages express immunoglobulin G Fc receptor (FcγR)I (CD64), and FcγRII (CD32) (reviewed in refs. 4,5). *In vitro*, FcγRIII (CD16) will be expressed on monocytes after at least 4 days of culture (6). All these classes of FcγR have been shown to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (7). The amount of MHC class II molecules (HLA-DR) on the monocyte/macrophage membrane is crucial for the initiation of an immune response (8), and CD14, a differentiation antigen expressed on mature

monocytes, is a receptor for lipopolysaccharide (LPS) complexed with LPS binding protein (9).

Cancer immunotherapy is increasingly important. However, the effectiveness of this treatment modality is more or less dependent on the tumor load. Therefore it is likely that this approach will be combined with chemotherapy. Chemotherapy may however affect the functional integrity of the immune apparatus. Anticancer drug interactions with human monocyte/macrophage functions are complex, and have been studied to some extent, but the available information in this respect is generally limited (10-20). Morphology and function of monocytes can be influenced on one hand by a host of factors pertaining to the classes of anticancer drugs employed, and on the other hand the stage of monocyte development. Although until recently it was thought that anticancer drugs induce only immunosuppression because of their antiproliferative and cytotoxic actions, it is now well established that some of them can also cause selective and rather specific immunoaugmentation (for review see 21).

We concentrated on compounds of major classes of chemotherapeutic agents. Possible mechanisms of antineoplastic drugs at play are numerous. Doxorubicin hydrochloride (Dox) and bleomycin sulphate (Bleo) belong to the antibiotics. The effects of Dox include intercalation with DNA, effects on topoisomerase, and formation of toxic-free radicals (22). The molecular target of Bleo is DNA (23), and its cleavage is dependent on oxygen and ferrous ions (24). Cisplatin (*cis* DDP), a miscellaneous agent, kills tumor cells in culture by damaging the DNA template (25). Cyclophosphamide, an alkylating agent, is not active per se, but requires to be 'activated' with the formation of metabolites (26). We used the metabolite 4-hydroxycyclophosphamide (4-OHCY). Fluorouracil (5 FU), a pyrimidine antimetabolite, is cytotoxic by inhibiting DNA synthesis, incorporating into nuclear RNA and DNA (27). Ethyldeshydroxysparsomycin (EdSm), an analogue of sparsomycin (28) is a strong inhibitor of the protein synthesis (29).

For the present study, we have investigated to what extent the above-mentioned anticancer drugs, to be used in cancer chemotherapy, affect morphologic, phenotypic and functional characteristics of freshly isolated monocytes in two stages of maturation *in vitro* (i.e., cultured for 40 h and 9 days).

MATERIALS AND METHODS

Isolation and culture of human monocytes. Monocytes were isolated as described (30). Briefly, mononuclear cells obtained from buffy coats, or cytapheresis of healthy volunteers, were isolated by Percoll (Pharmacia, Uppsala, Sweden) centrifugation. Monocytes were purified from mononuclear cells by cell scatter monitored counterflow

centrifugation Monocyte fractions were over 95% pure (as evaluated by staining for non-specific esterase and May-Grunwald-Giemsa), and viability was higher than 98%, as assessed by trypan blue dye exclusion Isolated cells were either cultured immediately or were cryopreserved (31), and stored in liquid nitrogen until use Recovery and viability of cryopreserved monocytes were >70%, and >95%, respectively

Freshly isolated or thawed cryopreserved monocytes were cultured for 40 h or 9 days at concentrations of 3 x 10^5 cells/ml in RPMI 1640 medium with 20 mM HEPES (Flow Laboratories, Irvine, UK), 2 mM L-glutamine (GIBCO, NY), 1 mM pyruvic acid (Sigma, St Louis, MO), 40 µg/ml gentamicin (Boehringer, Mannheim Germany), and 5% heat inactivated pooled human serum, in a humidified incubator with 5% CO_2 in air at 37°C Anticancer drugs were added to cultures during 40 h, either directly at day 0 (40-h cultured population), or at day 7 (9-day cultured population) Teflon foil bags (Dupont de Nemours and Co , Switzerland) were used for culture of monocyte suspensions (11)

Harvesting monocyte-derived macrophages. After culture for either 40 h, or 9 days, cells were recovered by needle aspiration after cooling Teflon foil bags to 4°C for 1 h and gentle kneading After removal of the culture supernatant [collected for determination of interleukin (IL)-1β and tumor necrosis factor (TNF)-α], the cells were washed twice in RPMI 1640 medium (without serum), and kept on ice until use There was no difference in the number (>90%) or viability (>90%) between cells recovered after culture with the finally selected drug concentration, and those recovered from control cultures For the benefit of FcγRI-mediated ADCC experiments, an additional washing procedure was performed in order to remove cytophilic human (h)IgG, as described (2)

Anticancer drugs. Dox (Adriblastina®, Farmitalia Carlo Erba, France), Bleo (Lundbeck, Denmark), 4-OHCY (Asta Germany), and EdSm [provided by the Dept of Organic Chemistry, University of Nijmegen, The Netherlands, and synthesized as described (28)] were obtained as lyophilized powders, and reconstituted sterile with distilled H_2O *Cis*-DDP (Cyanamid, UK), and 5-FU (Hoffmann-La Roche, Switzerland) were purchased in solution All drugs were further diluted aseptically with phosphate-buffered (PBS) to stock solutions Dox, Bleo, and 4-OHCY were stored deep frozen at -20°C, *cis*-DDP and 5-FU at room temperature, and EdSm at 4°C Prior to incubation, the anticancer drugs were diluted with growth medium to working concentrations as indicated

We did strive after drug concentrations comparable to the serum concentrations found in the clinical setting Concentrations were calculated as described previously (32) (C x S)/(V x W), where C = concentration of the drug in blood or plasma per m^2,

S = surface area (2 m^2) which is related to mean body weight and length. V = total body volume of distribution at steady state (liters/kg), and W = body weight (76 kg). Calculated concentrations for *in vitro* use were Dox (40 ng/ml), Bleo (2 µg/ml), *cis*-DDP (5 µg/ml), 4-OHCY (27 µg/ml), EdSm (1 µM), and 5-FU (63 µg/ml). Anticancer drugs were added at three to five concentrations, between 10^{-1} to 10^{1} times the calculated value, to 7-day cultured monocytes. For further studies, ultimately concentrations were chosen as optimally tolerated doses, with minimal effects on cell viability (>90%) of the at day 9 harvested monocyte-derived macrophages.

Immunofluorescence studies. Expression of FcγRI, FcγRII, FcγRIII, CD14, and HLA-DR on cultured monocytes was assayed by indirect immunofluorescence as described previously (2) using specific mAb: 197 [CD64: purified murine (m)IgG2a] (Medarex, W. Lebanon, NH), which bind to an epitope outside the ligand-binding site on FcγRI (33), IV.3 (CD32: purified mIgG2b) (Medarex), which competes with the FcγRII ligand-binding site (34), 3G8 (CD16: purified mIgG1) (Medarex), which identifies an epitope close to the FcγRIII ligand-binding site (35), WT14 (CD14: culture supernatant mIgG1) (provided by Dr. W.J.M. Tax, University Hospital Nijmegen, The Netherlands) which reacts strongly with CD14 molecules, expressed selectively on human monocytes and macrophages (36), anti HLA-DR (purified mIgG2a) (Becton Dickinson) detects HLA-DR, a common determinant of the MHC class II antigen, and FITC-conjugated goat F(ab')2 anti-mouse IgG (Heavy and Light chain) (Cappel, Malvern, PA). Fluorescence intensity of 5000 cells was measured with an Ortho 30H flow cytometer. For all analyses, gatings (red forward and right angle scatter) were set around the monocyte/macrophage population and the mean fluorescence intensity (expressed in arbitrary fluorescence units/cell) was calculated (linear) from the histograms. Relative expressions were calculated as follows: $(M_d - M_n)/(M_m - M_n)$, where M_d = mean fluorescence intensity of anticancer drug-cultured monocytes, M_n = mean fluorescence intensity of negative control, and M_m = mean fluorescence intensities of monocytes cultured in control medium.

FcγR-mediated cytotoxicity. ADCC capacity of monocyte-derived macrophages from different donors was studied using a ^{51}Cr release assay as described previously (2,37), in which antibody-coated human erythrocytes (E) were used as targets. Briefly, E were suspended in PBS containing ^{51}Cr (sodium chromate, Amersham, UK), and incubated at 37°C. After 30 min, sensitizing antibody, or PBS / 0.1% bovine serum albumin alone (control), was added. To sensitize the ^{51}Cr labeled E, two types of antibodies were used: Rhesus D^+ ^{51}Cr-labeled E were sensitized either with human alloserum against Rhesus D (Merz & Dade, Dudingen, Switzerland) or with ascites of mIgG1 mAb directed at

glycophorin A. Both types of antibodies were used at various dilutions, resulting in E sensitized with a range of IgG molecules per cell (EA-IgG), as described (38). After incubation for 30 min, cells were washed thrice, suspended, and 2.5 x 10^5 target cells were mixed with equal volumes of effector cells (freshly isolated monocytes/40-h cultured monocytes = 3.75 x 10^5 cells, 9-day cultured monocytes = 1.25 x 10^5 cells). After incubation for 18 h at 37°C, half the supernatant was removed for estimation of ^{51}Cr release. All tests were carried out in triplicate. The results were calculated as follows: % cytotoxicity = (C - S)/(T - S) x 100, where C = mean cpm of test sample, S = mean cpm of spontaneous ^{51}Cr release (i.e., ^{51}Cr release by labeled target cells in medium alone), and T = mean cpm of the maximal ^{51}Cr release, obtained by addition of 20% saponin (Coulter, Dunstable, UK) to the target cells. The average spontaneous release of E was 3 ± 1% (mean ± SD), and release from unsensitized E incubated with effector cells never exceeded spontaneous release.

Radioimmunoassays (RIA) for IL-1β and TNF-α. The concentrations of extracellular IL-1β and TNF-α in the supernatants of monocytes cultured for 40 h or 9 days, either in the absence, or presence of anticancer drugs were measured by specific RIA, as described previously (39-41). Briefly, on day one samples of the monocyte-culture supernatants were incubated with rabbit allosera against human IL-1β or TNF-α (room temperature). On the same day, radioiodinated cytokine tracer was added, and finally sheep anti-rabbit IgG antiserum on day 4. Antibody antigen complexes were precipitated by centrifugation. Radioactivity was determined in a gamma counter and expressed a percentage of the total binding of IL-1β, or TNF-α. Cytokine concentrations were calculated with a four parameter method, obtained with serial dilutions of known standards in monocyte culture medium.

RESULTS

Viability and morphology of anticancer drug-cultured monocytes. Preliminary to the study on the modulation by anticancer drugs of phenotypic and functional properties of human monocytes, we first scrutinized cell viability and morphology. Monocyte-derived macrophages were stimulated at day 7 of culture, for 40 h with Dox, Bleo, *cis*-DDP, 4-OHCY, EdSm, and 5-FU at concentrations between 10^{-1} to 10^{1} times an optimally calculated value (see *Materials and Methods*). On day 9, viability of recovered cells, either cultured in medium alone, or being exposed to various concentrations of Dox, Bleo, or 5-FU, was always higher than 90%. However, the for *cis*-DDP, 4-OHCY, and EdSm optimally and tenfold concentrations resulted either in at least 20% or 90% cell death, respectively (Table I).

Table I.

Effect of anticancer drugs on viability of 9-day cultured human monocytes

Cell population[a]	Concentration anticancer drugs	Percentage viability[b]
Control	–	>90
Dox	4, 40*, 400 (ng/ml)	>90
Bleo	0 2, 2*, 20 (μg/ml)	>90
cis-DDP	0 5, 1, 2 5 (μg/ml)	>90
	5* (μg/ml)	70
	50 (μg/ml)	0
4-OHCY	2 7, 5 4, 10 8 (μg/ml)	>90
	27* (μg/ml)	60
	270 (μg/ml)	10
EdSm	0 1, 0 3, 0 6 (μM)	>90
	1* (μM)	80
	10 (μM)	0
5-FU	6 3, 63*, 630 (μg/ml)	>90

[a] *Monocyte-derived macrophage populations, cultured for 9 days in medium alone, or with different anticancer drugs for the last 40 h of culture (*comparable to serum concentration found in clinical setting, as described in Materials and Methods)*

[b] *Cells recovered after culture were assessed for viability by trypan blue dye exclusion To determine the percent of viability, 100 cells of each population were counted in randomly chosen fields*

Next we studied effects of anticancer drugs on cell morphology during the last 40 h of a long-term (9 days) culture period, using those drug concentrations which resulted in at least 90% viable cells During culture in Teflon foil bags, a subpopulation (11) of monocytes loosely attach to the surface, and the majority of these cells flatten and spread out Pending the culture with Dox (40 ng/ml) or Bleo (2 μg/ml), morphology of the monocyte-derived macrophages was similar to cells during culture in medium alone When cultured with *cis*-DDP (2 5 μg/ml), cells normally kept attached to the Teflon surface, but the flattening and spread out disappeared The majority of monocyte-derived macrophages co-cultured with 4-OHCY (10 8 μg/ml) went in suspension, and co-culture with FdSm (0 6 μM) or 5-FU (63 μg/ml) for the last 40 h of the culture period, caused homotypic monocyte adhesion which ultimately resulted in big clumps of cells being in suspension

When short-term cultured (40 h), we observed that before harvesting, monocytes were in a state of spreading However, when co-cultured with *cis*-DDP (2 5 μg/ml), 4-OHCY (2 7 μg/ml), EdSm (0 6 μM), or 5-FU (63 μg/ml), monocytes lost their spreading capability, and appeared rounded towards the end of the culture period

Furthermore, after treatment with 4-OHCY (10 8 μg/ml), viability of the monocyte population was halved A 90% monocyte survival was reached when cultured with 4-OHCY at an optimal concentration of 2 7 μg/ml

Modulation by anticancer drugs of receptor expression on 9-day cultured human monocytes. Parallel to the above described viability study the same long term cultured drug treated monocyte populations were screened for induced phenotypic alterations By culturing monocytes with Dox (4 ng/ml) expression of FcγRI increased to 143 0 ± 37% (mean ± SD), and the expression of FcγRIII to 146 5 ± 31 2% (Fig 1) Bleo did not alter the quantitative expression of surface molecules on long term cultured monocytes A small decrease in expression of FcγRI and FcγRIII was observed when cells were treated either with *cis* DDP (2 5 μg/ml), or 4 OHCY (10 8 μg/ml) FdSm (all concentrations) did, more or less reduce the membrane expression of all three FcγR classes, CD14, and HLA DR In addition co culture for the last 40 h with 5 FU decreased FcγRI and FcγRIII expression in a dose-dependent manner and slightly reduced HLA DR antigen expression Finally, the most striking reductions in antigen expression observed were induced at concentrations of *cis* DDP 4 OHCY, and EdSm, which also detrimentally affected cell viability (Table I) Furthermore, these harmful effects did vary with the different tested surface molecules (Fig 1)

Effect of anticancer drugs on FcγR-mediated cytotoxicity by 9-day cultured human monocytes. Next we investigated whether FcγRI and FcγRII expression is manifested in functional activities of 9 day cultured monocyte derived macrophages ADCC activity of FcγRI and FcγRII can be independently assayed using E sensitized with hIgG or mIgG1, respectively In previous studies the specificity of monocyte and macrophage FcγRI and FcγRII mediated cytotoxicity has been evaluated, using unique anti FcγR mAb (2, 37, 42)

Monocytes were cultured for 9 days in medium alone or co-cultured for the last 40 h with Dox (40 ng/ml), Bleo (2 μg/ml) *cis* DDP (2 5 μg/ml) 4 OHCY (10 8 μg/ml), EdSm (0 6 μM), or 5 FU (63 μg/ml) Cytotoxicity towards EA hIgG and EA-mIgG1 targets was evaluated with at least 90% viable effector cells In Figure 2 it is depicted that, despite a decreased receptor expression, both FcγRI and FcγRII mediated ADCC activity was not affected after culture with cytostatics

Modulation by anticancer drugs of receptor expression on 40-h cultured human monocytes. Next we evaluated the effects of anticancer drugs on monocyte phenotype in an early stage of maturation Expression of FcγRI, FcγRII, CD14, and HLA-DR was analyzed on short term cultured monocytes (40 h) which were co cultured with Dox

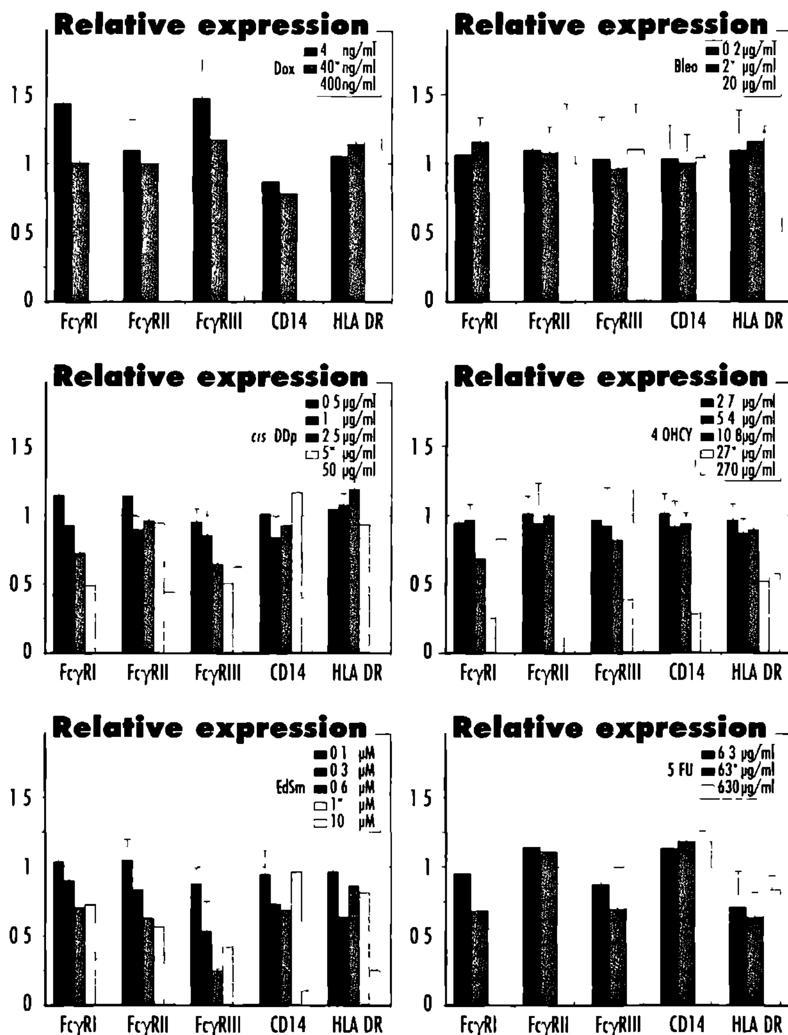

Figure 1.

Dose effect of anticancer drugs on 9-day cultured monocytes Monocyte-derived macrophages were obtained by culturing monocytes for 9 days in medium alone (control), or in medium supplemented with different concentrations of the indicated anticancer drugs for the last 40 h of culture (* concentration comparable to the serum concentrations found in the clinical setting) Expression of FcγRI, FcγRII, FcγRIII, CD14, and HLA DR was evaluated by immunofluorescence staining with specific mAb Relative expression of the surface molecules was calculated as described in *Materials and Methods* Results represent mean ± SD from three experiments with cells from different donors

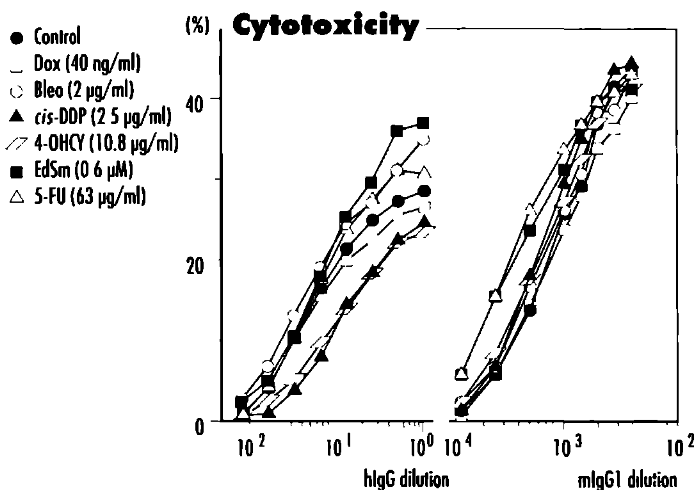

Figure 2.
Effect of anticancer drugs on monocyte-derived macrophage FcγR mediated ADCC activity ADCC activity was assessed after culturing monocytes for 9 days in medium alone (control), or in medium supplemented with the indicated anticancer drugs for the last 40 h of culture ADCC tests were performed as described in *Materials and Methods*, using target E sensitized with variable amounts of either hIgG anti-Rhesus D alloserum, or mIgG1 anti-glycophorin A mAb (optimal E T ratio of 0 5) Results *represent data from three individual* experiments with cells from different donors SD were all smaller than 10%

(40 ng/ml), Bleo (2 µg/ml), *cis*-DDP (2.5 µg/ml), 4-OHCY (2.7 µg/ml), EdSm (2 µM), or 5-FU (63 µg/ml). Effect on FcγRIII expression was not evaluated, because this receptor was expressed at a negligible level on 40 h cultured monocytes (43).

As shown in Figure 3, EdSm cultured monocytes were decreased in the expression of FcγRI (to 68.7 ± 13.7%), FcγRII (to 40 6 ± 6.5%), and CD14 (68.7 ± 10 2%), when compared with 40-h cultured control monocytes. On the other hand, EdSm treatment did not influence the expression of HLA-DR molecules Monocytes responded to 4-OHCY with an increase in expression of CD14 molecules (to 145.1 ± 26.5%). Dox, Bleo, *cis*-DDP, and 5-FU did not influence the quantity of surface molecules expressed on short-term cultured monocytes.

Effect of anticancer drugs on FcγR-mediated cytotoxicity by 40-h cultured human monocytes. Freshly isolated, and 40-h (anticancer drug) cultured monocytes were evaluated for cytotoxicity towards EA-hIgG and EA-mIgG1, using an optimal effector to target cell ratio 1.5 When uncultured monocytes were tested for FcγRI-mediated ADCC activity against optimally with hIgG sensitized E, cytotoxicity was 52 ± 6%. However, when cultured for 40 h, either without drug (control), or with Dox (40 ng/ml), Bleo (2 µg/ml), *cis*-DDP (2.5 µg/ml), 4-OHCY (2.7 µg/ml), EdSm (0.6 µM), or 5-FU (63 µg/ml), FcγRI-mediated ADCC activity never exceeded a 10% cytotoxicity level (not shown).

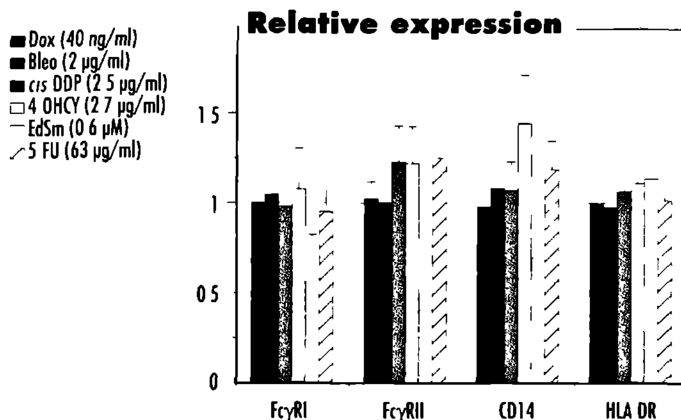

Figure 3.
Effect of different anticancer drugs on 40 h cultured monocyte surface antigen expression Monocytes were cultured for 40 h in medium alone (control), or in medium supplemented with the indicated anticancer drugs Expression of FcγRI FcγRII, CD14, and HLA DR was evaluated by immunofluorescence staining with specific mAb Relative expression of the surface molecules was calculated as described in *Materials and Methods* Results represent mean ± SD from three experiments with cells from different donors

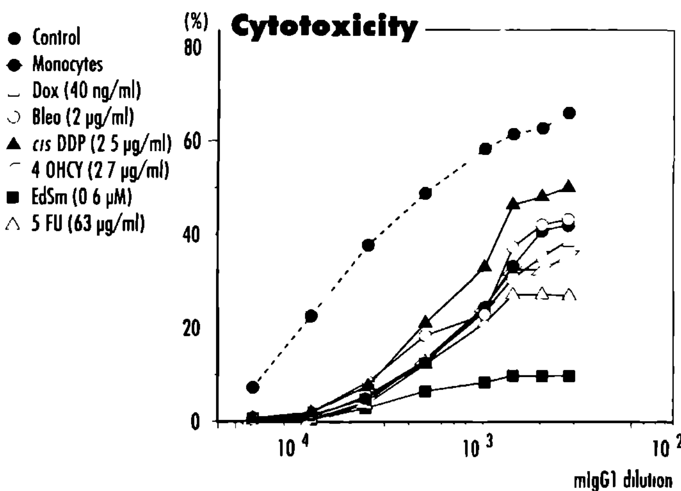

Figure 4.
ADCC activity of short term anticancer drug cultured monocytes against mIgG1 sensitized human E Freshly isolated monocytes, monocytes cultured for 40 h in either medium alone (control), or medium supplemented with the indicated anticancer drugs, were evaluated for cytotoxic activity ADCC tests were performed as described in *Materials and Methods*, using target E sensitized with variable amounts of mIgG1 anti-glycophorin A mAb (optimal E T ratio of 1 5) Results are mean data from three individual experiments with cells from different donors SD were all smaller than 8%

Monocyte FcγRII-mediated ADCC activity against optimally sensitized EA-mIgG1 was reduced from 65 8 ± 5 1% to 41 7 ± 3 3 %, as a consequence of short-term culture in medium alone, and was further reduced to 9 7 ± 0 7% when co cultured with EdSm (Fig 4)

Monocytes cultured in the presence of Dox, Bleo, or 4 OHCY showed an almost similar lysis of target cells when compared with untreated 40 h cultured monocytes *Cis*-DDP and 5-FU treated effector populations were slightly affected in the FcγRII mediated ADCC activity, by either an increase from 41 7 ± 3 3 % to 50 1 ± 3 9 %, or a decrease to 27 3 ± 2 1% cytotoxicity, respectively

Table II.

Analysis of TNF-α and IL-1β production by short-, and long-term anticancer drug cultured monocytes

Donor	Control –	Dox (40 ng/ml)	Bleo (2 μg/ml)	*cis* DDP (2 5 μg/ml)	4 OHCY (2 7 μg/ml)	EdSm (0 6 μM)	5 FU (63 μg/ml)
TNF α present in supernatant of 40 h cultured monocytes[a] (ng/ml)							
A	0 50[c]	0 44	0 29	0 30	<0 19	<0 19	<0 19
B	0 24	<0 19	<0 19	0 20	<0 19	<0 19	<0 19
C	<0 19	<0 19	<0 19	<0 19	<0 19	<0 19	<0 19
IL 1β present in supernatant of 40 h cultured monocytes (ng/ml)							
A	0 17	0 14	0 13	0 16	<0 08	0 13	0 16
B	0 11	0 11	0 16	0 11	0 12	0 11	0 14
C	0 14	0 32	<0 08	0 11	<0 08	0 09	<0 08
TNF α present in supernatant of 9 day cultured monocyte derived macrophages[b] (ng/ml)							
D	0 30	0 42	0 27	0 25	0 24	0 28	0 33
E	0 42	0 42	0 44	0 48	0 41	0 55	0 36
F	1 67	–	–	–	1 50	1 75	1 77
G	0 93	0 68	0 80	0 87	–	–	–
IL 1β present in supernatant of 9 day cultured monocyte derived macrophages (ng/ml)							
D	0 11	0 11	0 08	0 20	0 14	0 14	0 09
E	0 19	0 21	0 21	0 22	0 16	0 32	0 15
F	1 99	–	–	–	1 75	1 96	1 91
G	0 53	0 47	0 39	0 40	–	–	–

[a] Supernatant was obtained from monocytes (3 x 10^5/ml) cultured in suspension for 40 h with or without anticancer drug

[b] Supernatant from monocyte-derived macrophages was obtained by in suspension culturing of monocytes (3 x 10^5/ml) for 9 days in medium or medium supplemented with an anticancer drug for the last 40 h of culture

[c] Concentrations of IL 1β and TNF α in the supernatants of cultured cells were measured by specific RIA's, as described previously (39 41)

Effect of anticancer drugs on the production of IL-1β and TNF-α by cultured human monocytes. Production and secretion of IL-1β and TNF-α by monocytes and monocyte-derived macrophages is not constitutive but is induced by a variety of endogenous and exogenous stimuli Under appropriate conditions, it was possible to induce a profound IL 1β and TNF-α synthesis/release via stimulation with LPS (unpublished results) Monocytes were incubated in suspension for 40 h or 9 days, either in the absence, or presence of anticancer drugs, as described in *Materials and Methods* Compared with the anticancer drug-free populations, neither Dox (40 ng/ml), Bleo (2 μg/ml), *cis*-DDP (2 5 μg/ml), 4 OHCY (monocytes 2 7 μg/ml, monocyte-derived macrophages 10 8 μg/ml), EdSm (0 6 μM) nor 5-FU (63 μg/ml) induced any IL-1β or TNF-α secretion by cultured monocytes at different stages of maturation, as detected in the various culture supernatants by the specific RIA (Table II)

DISCUSSION

Interactions of anticancer drugs with cells of the immune system, and the influence of such interactions on the fate of functional activities mediated by these cells, are of therapeutic significance Most of the anticancer drugs are generally considered to exert their effects through antiproliferative and cytotoxic actions Biochemical characteristics of cytostatics are incontestably determinative for their selectivity of action on only certain cell types, or on cells at only certain stages of development (21) As a part of our investigations on the influence of cytostatics in combination with biological response modifiers (e g , interleukins, interferons, colony-stimulating factors) on the mononuclear phagocyte system, we assessed in this study modulatory effects of anticancer drugs on human blood monocytes, which represent a first-line defense against the spread of neoplastic cells (1) We studied the effects of major groups of anticancer drugs on monocyte differentiation at two stages of *in vitro* maturation, i e , on 40-h, and 9-day cultured monocytes

Monocytes cultured *in vitro* under appropriate conditions differentiate into mature macrophages (10,44,45) Normally, when placed in culture in Teflon foil bags, a subpopulation of monocytes (11) loosely attach to the surface, and the majority of these cells flatten and spread out It is apparent that different cytostatics vary in their capacity to affect Teflon-attachment of monocytes during culture *in vitro* Adherence capacity of cells was affected more or less, by co-culture with *cis*-DDP, 4-OHCY, EdSm and 5-FU Effects varied from the loss off spreading and adhering capacities when short-term cultured Cell clumping was observed when long-term cultured monocyte-derived macrophages were co-cultured with EdSm or 5-FU Previously, we already

described the phenomenon of monocyte clumping during prolonged co-culture (9 days) with rIFN-γ (10) The selective enhancement of adhesion molecules by rIFN-γ turned out to be responsible for the induced homotypic monocyte adhesion Especially, the EdSm induced clumping of cells is difficult to explain by a putative increase in the expression of newly formed integrins, as this drug is a potent inhibitor of ribosomal protein synthesis

Many anticancer drugs are known to suppress and/or augment the host defense system (21) Immunomodulation *in vitro* by chemotherapeutics may be dependent on the type of 'target' and immune function, variation in dose, time of administration, or nature (structure) of the drug The 'target' in use in this study is a highly versatile cell type *In vivo*, an active dose of anticancer drugs may either affect monocytes which have recently left the peripheral blood circulation, and reside since a short time in the tissues, and monocytes already differentiated into tissue macrophages Therefore, immunomodulation was studied on phenotype and function of both short-, and long-term cultured monocytes A slight augmentation of FcγRI and FcγRIII expression on 9-day cultured cells was observed when co-cultured for the last 40 h with a suboptimal dose of Dox Previously, this drug has been described to augment the host defense system under suboptimal response conditions only (46) The mechanism of Dox is not fully understood, and it is not clear which of the multiple effects of Dox in target cells are responsible for immunomodulation or antitumor actions (22) *Cis*-DDP and 4-OHCY were observed to reduce FcγRI and FcγRIII expression on 9 days cultured monocyte-derived macrophages. In addition, the antimetabolite 5-FU reduced both the expression of FcγR and HLA-DR molecules However, *cis*-DDP, 4-OHCY, and 5-FU did not influence all these cell surface molecules, indicating those drugs did not cause a general non-specific reduction of receptor expression In contrast, EdSm caused a reduction in the amount of all measured cell surface molecules Most intriguing were the unchanged FcγR-mediated ADCC activities of the with anticancer drug cultured monocyte-derived macrophages These results suggest that lowered FcγR expression levels do not have to be reflected in a changing functional activity of the long-term cultured monocytes

With respect to the effects of anticancer drugs on short term cultured (40 h) monocytes, it is important to note that freshly isolated human monocytes express only FcγRI and FcγRII (6,43) It has been previously shown that, upon 40 h of culture, both FcγRI and FcγRII expression levels were decreased with 75% and 50%, respectively (37,43) The drop in the specific ADCC activity against EA-hIgG and EA-mIgG1 was related with the observed changes in FcγR expression levels Expression of FcγRIII was still negligible The mechanism by which short-term culture reduced the expression of FcγR remains unclear up to now EdSm was the only of the tested drugs which could further decrease FcγRI expression An effect on the expression of FcγRII was observed when

short-term co-cultured with EdSm (a 60% decrease), and *cis*-DDP, 4-OHCY, or 5-FU (a 20% increase) In contrast to long-term cultured monocytes, effects of EdSm and *cis*-DDP on short-term cultured cells were paralleled by an equivalent alteration in FcγRII-mediated cytotoxicity Furthermore, in spite of a small increased FcγRII expression on 40 h cultured monocyte, co culture with 4-OHCY or 5-FU slightly decreased FcγRII mediated ADCC activity From the drugs tested, only *cis*-DDP could positively influence ADCC activity (Fig 4) Some authors showed already that *cis*-DDP could stimulate spontaneous human monocyte-mediated cytotoxicity directly (12,47,48) No information is, however, available on the mechanism by which EdSm influences phenotype and function of highly purified, and *in vitro* cultured monocytes

Under the conditions described in this study, monocytes cultured in medium induced neither IL 1β, nor TNF-α secretion This finding is of importance, because both IL-1 and TNF induce monocytes to produce colony-stimulating factors and other cytokines (49), which in turn may interfere with the added anticancer drug

In conclusion, the results indicate that *in vitro* co-culture of monocytes or monocyte-derived macrophages with anticancer drugs, to be used in cancer chemotherapy, can be heterogeneous for the effects on phenotype and function The observed influences cannot always be explained by pharmacokinetic data With respect to FcγRII-mediated ADCC activity, it is of interest that short-term cultured freshly isolated monocytes, in contrast to mature monocyte-derived macrophages, may be sensitive to drug treatment

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GENERAL DISCUSSION

concluding remarks and recommendations

The studies presented in this thesis describe several new aspects of the *in vitro* modulation by cytokines and anticancer drugs of human peripheral blood monocytes at various stages in the differentiation to mature macrophages (i e , monocyte-derived macrophages) Monocytes and their immature and mature counterparts have been identified to be potent effectors of natural and specific immune responses against tumors (1-5) The earliest step in the specific destruction of neoplastically transformed cells may occur under the condition that monocytes/macrophages selectively recognize tumor cells either directly via a tumorigenic phenotype (5,6) or indirectly via opsonized immunoglobulin (Ig) Specific sensitization with antitumor antibodies causes neoplastic cells to become susceptible to antibody dependent cell-mediated cytotoxicity (ADCC) (7) It is noteworthy that the specificity of the ADCC event will be determined in the first place by the antigen binding site of the antibody Secondly, the heavy-chain isotype of a specific antitumor antibody is defining the Fc receptor that will be cross-linked and initiated for its mode of action Three closely related classes of human receptors for the Fc moiety of IgG (FcγR) have been defined, FcγRI, FcγRII and FcγRIII, each of which is able to mediate ADCC (8,9)

A part of these studies focused on the monocyte/macrophage FcγRI- and FcγRII-mediated ADCC activity, with special curiosity about the mechanism of cytolysis Macrophage-mediated ADCC activity, measured by the release of ^{51}Cr, is the end result of both extracellular lysis and phagocytosis (10,11) In chapter 2, a new and highly intriguing phenomenon of monocyte/macrophage mediated ADCC was described, which suggests the heterogeneity of monocyte populations with respect to cytotoxicity due to maturation and activation While freshly isolated monocytes mediated both extracellular lysis and phagocytosis, those maintained for 9 days in basal medium resulted in a population which exhibited phagocytosis, with only marginal extracellular lysis Short term (40 h) activation of monocyte-derived macrophages at day 7 of culture with recombinant interferon (IFN)-γ reversed this, yielding a population which mediated both phagocytosis and extracellular lysis However culture with IFN γ for a longer period (9 days) resulted in a population which displayed extracellular lysis In addition, cytospin preparations indicated that, although the population of monocyte-derived macrophages exhibits phagocytosis, almost half the cells do not participate This could not be explained by mixed culture or loss of viability since cell fractions were over 95% pure and more than 98% viable Moreover both freshly isolated monocytes and those exposed for a short time to IFN-γ mediated both extracellular lysis and phagocytosis Whether or not these activities involve the same cells or different cells requires further investigation Another striking feature of FcγRI was observed when either monocytes, or their derived macrophages were cultured in the presence of IFN γ (chapter 2) The results clearly showed that IFN-γ is able to reverse the inhibitory effects of mono-

meric human (h)IgG on FcγRI-mediated ADCC *In vivo*. IgG-mediated effector responses occur in an environment with excess hIgG, and since FcγRI is the only class of FcγR with high affinity for monomeric hIgG, this receptor will be continuously saturated with serum IgG (12) Indeed FcγRI may have an important physiological role since inhibition by monomeric IgG is overcome after culture with IFN-γ It is therefore possible that the responses of both circulating monocytes and resting tissue macrophages which are executed via FcγRI will be hindered by high amounts of hIgG Thus, monocytes/macrophages appear initially 'restrained' from attacking every opsonized antigenic particle, cell or microorganism However, at any site of inflammation or tumor, T-lymphocytes might produce a number of polypeptides (e g , IFN-γ), which induce activation of the relatively dormant tissue macrophages (13) After prolonged stimulation by IFN γ, the IgG inhibition can be counteracted most probably by increasing the density of FcγRI on the surface, and the cytolytic mechanism will be also subject to alteration Consequently, macrophages may become very efficient 'killers' of neoplastically transformed cells, because extracellular lysis has no longer to be combined with the slower process of phagocytosis

Until now, it was generally accepted that IFN-γ could potentiate FcγRII-mediated cytotoxicity, without altering receptor levels (14) However, this phenomenon appears to be related to their age and the length of time cells were exposed to IFN-γ (chapter 3) Compared to freshly isolated monocytes, short-term (40 h) cultured cells exhibit a significantly lower FcγRII expression, and a reduced ADCC activity However, co-culture with IFN-γ (40 h) completely reverses this decrease in both receptor level expression and FcγRII-mediated cytotoxicity When analyzing the mechanism of cytolysis mediated via FcγRII, it was observed that uncultured monocytes, and those cultured for 40 h either in the absence or presence of IFN-γ exhibited a comparable phagocytic capacity (chapter 3, Table 2) Therefore the impaired ADCC activity in short-term control cultured cells may be due to a decrease in extracellular lysis Remarkably, in contrast to the effect of IFN-γ on the FcγRI expression level (chapter 2), the cytokine only prevented a decrease in the number of receptors expressed on monocytes when cultured for a short time Since FcγRII expression on monocyte-derived macrophages is less amenable to modulation by IFN-γ, an immunologically active dose of this cytokine might induce alterations in FcγRII expression and function, especially on monocytes which have recently entered the tissue before becoming full-grown macrophages

An obvious inference from these observations is that further investigation of the initial signals transmitted to the cell via these FcγR is necessary Moreover, it is imperative to clarify the molecular biology by employing cDNA probes to examine the modulation of FcγRI and FcγRII mRNA by physiologic and pharmacologic signals (e g , IFN-γ, anticancer drugs) Recently such probes encoding for FcγRI (15), FcγRII (16)

and FcγRIII (17) have been isolated and cloned. Time course experiments using inhibitors of protein synthesis, together with Northern blot analysis and flow cytometry might help elucidate the kinetics of receptor level regulation. However, as monocytes and (monocyte-derived) macrophages are heterogeneous with respect to phenotype and function (18-20, chapter 2,4), it is crucial to evaluate the modulation of both monocyte/macrophage mRNA and protein transcripts at the single cell level. Hybridohistochemistry (i.e., RNA *in situ* hybridization) combined with immunohistochemistry may offer more refined information and will be complement the examination of single cells for both the expression of mRNA and protein transcripts on the cell surface (21,22).

Doxorubicin was investigated in chapter 4 to assess its influence on the maturation and differentiation of monocytes. Initially, we wondered whether or not non-dividing monocytes/macrophages really had the capability to escape cell death after they had been treated, since uptake of doxorubicin appears to be cell-cycle dependent. Monocytes were cultured by adherence to plastic flat-bottomed tubes and in suspension using Teflon foil bags. Two different non-toxic incubation schedules were employed to mimic both continuous and bolus infusion reflecting the current dosages and schedules used clinically. However, the optimal schedule with respect to tumor cytotoxicity and dose-limiting side effects has never been investigated in a prospective, randomized manner (23). The capacity to adhere, the yield and enzyme activities that reflect growth and intermediary metabolism were similar under all conditions. However the number of adherent monocytes increased proportionally with duration of culture (chapter 4). This phenomenon corroborates the concept that the monocyte/macrophage lineage is a heterogeneous population (19), and that functional heterogeneity is, to a large extent, a reflection of their state of differentiation (24,25). For this reason it is important to ascertain that a cell population, isolated by adherence (26), represents only a selection or subpopulation of the monocyte/macrophage lineage. Isolation by counterflow centrifugation is ideally suited to this task since selection is minimal (27).

The effects of cytokines and anticancer drugs on the immune system must be known before combining in a clinical setting. IFN-γ appears to be the major cytokine that activates monocytes (28,29, chapter 2,3), and doxorubicin has a very broad antitumor spectrum, compared with other anticancer drugs (23). The modulation induced by doxorubicin on the activation of human monocytes provoked by IFN-γ was the subject of investigation in chapter 5 which focused on putative alterations in the cellular morphology, intracellular enzyme activities, FcγR expression, and cytotoxicity. The results indicated that neither the control populations, nor the IFN-γ activated monocyte-derived macrophages were significantly influenced by either short- or long-term exposure to doxorubicin. On the contrary, IFN-γ appeared responsible for the extraordinary alterations in morphology (e.g., smaller cell size, flattened lobed and more juxta membranal

located nucleus, homotypic monocyte clumping) as well as a significant decrease in most enzyme levels This gave a clear indication that the combined approach did not negatively influence the activation of the monocyte/macrophage population induced by IFN-γ

A common characteristic of monocytes/macrophages is that these cells need to be appropriately activated to optimally fulfil their function (30) Monocytes and macrophages can be activated both *in vivo* and *in vitro* by various biological response modifiers (BRM) (31,32) The BRM used in the study described in chapter 6, seem to be involved in regulating the functional state of the cells The effects observed varied with the type of cytokine and depended upon the stage of maturation of the human monocytes Culture for 9 days showed that changes in FcγRI and FcγRII expression levels were not related per se to FcγR-mediated functional activities The most obvious explanation for this phenomenon must be the initiation of additional changes in mechanisms which are independent of the number of FcγR which was thought responsible for function Studying short-term cultured (40 h) monocytes initially demonstrated that the FcγRII expression level is decreased as a consequence of culture This was reversed either completely by culturing monocytes with IFN-γ (see also chapter 3) and GM CSF, or partially, when cultured in the presence of TNF-α However, in contrast to the culture with IFN-γ, GM-CSF and TNF-α did not restore ADCC activity The mechanism by which cytokines affect FcγR expression remain unclear

Chapter 7 dealt with the immunomodulatory effects induced by anticancer drugs on human monocytes cultured *in vitro*, using a design framework similar to that presented in chapter 6 The results suggested that the drugs used for cancer treatment might specifically affect the morphology, phenotype, and function of monocytes cultured *in vitro* The effects depended mainly on the class and dose of the drugs but the stage of monocyte differentiation seemed also to play a principal part, since (functional) immunomodulation occurred mainly in short-term cultured monocytes Furthermore, immunomodulation is unlikely to be elicited optimally using anticancer drugs at concentrations close to the maximal tolerated dose since this will invariably result in cell death Remarkably, ethyldeshydroxysparsomycin (EdSm) (33,34) was the only experimental anticancer drug to cause homotypic cell adhesion (clumping) and a profound decrease in the number of FcγR ADCC activity of the short term cultured monocytes was impaired and there was downregulation of the cell surface expression of CD14 This drug may prove valuable as an inhibitor of protein synthesis for investigating the biology of the monocyte/macrophage

Clearly, much remains to be done and many more questions have been raised than could be answered Nevertheless, it is hoped that the work presented here will form the basis of a continuing program of research which will make a valuable contribution to both our knowledge and understanding of the biology of the immune response to cancer and the ability to improve its treatment

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SUMMARY

Stimulation of the immune system by means of biological response modifiers (BRM) or cytokines is becoming increasingly important in the treatment of metastatic cancers. In spite of this, complete remissions remain limited, and are related to the tumor load. During the past few years experimental therapy of different types of cancer, however, improved by combining immunotherapy with conventional chemotherapy. As a consequence the question was raised, whether or not chemotherapeutic drugs could detrimentally affect the human immune system. This system depends on the participation of a large number of highly specialized white blood cells in both peripheral blood and tissues such as lymphocytes, granulocytes, natural killer cells, monocytes, and macrophages. Cells of the monocyte/macrophage lineage play a central role in both the afferent and efferent arms of the immune system. A role of importance in the body's defense against neoplasms was postulated. Monocytes/macrophages may destroy tumor cells upon establishment of cell-cell contact. Binding occurs either via selective recognition of tumorigenic antigens, or via receptors that bind immunoglobulins present on target cells. For this purpose, human monocytes and macrophages possess three classes of receptors for the Fc moiety of IgG (FcγRI, II, and III) which specifically bind the Fc part of IgG antibodies. The latter mechanism may be followed up by cytolysis, known as antibody-dependent cell mediated cytotoxicity (ADCC), and was selected for further investigations. The purpose of this thesis was to study the *in vitro* effects of cytokines and anticancer drugs on human peripheral blood monocytes, at various stages in the differentiation to mature macrophages.

The background to the studies is presented in **Chapter 1** (General Introduction). Human peripheral blood monocytes originate in the bone marrow, and become mature macrophages upon migration into extravascular tissues and body cavities. In this thesis monocyte cultures were used as a model system to study macrophage development, as monocytes differentiated *in vitro* largely resemble tissue macrophages. Large numbers of monocytes were isolated from peripheral blood by counterflow centrifugation, and consecutively cultured in Teflon bags. Due to both the isolation procedure, and the hydrophobicity of the culture system, it was possible to establish suspension cultures with over 95% pure cells which were easily recoverable at different differentiation stages. Upon harvesting the monocyte-derived macrophages were of excellent quality, with a viability higher than 98%. Although the precise signals responsible for initiation of monocyte maturation *in vitro* remain largely unknown, preliminary examinations showed that best results were obtained using a culture medium supplemented with 5-10% heat-inactivated (pooled) human serum. Cytotoxic activity of both monocytes and *in vitro* differentiated macrophages were tested in ^{51}Cr-release assays in which antibody-coated human erythrocytes served as targets.

The study described in **Chapter 2** focused on macrophage FcγRI-mediated ADCC activity, cultured in the absence or presence of low doses human (h)IgG It is well known that the *in vivo* IgG-mediated effector responses occur in an environment with excess hIgG, and since FcγRI is the solely class of FcγR with high-affinity for monomeric hIgG, this receptor may be continuously saturated with serum IgG However, a striking observation was made for FcγRI when monocytes, or macrophages were cultured in the presence of the cytokine IFN-γ The experiments showed that prolonged stimulation by IFN-γ, both enhanced the cell surface density of FcγRI, and reversed the inhibitory effects of monomeric hIgG on FcγRI-mediated ADCC In addition, the mechanism of monocyte/macrophage FcγRI-mediated cytotoxicity was analyzed. Freshly isolated monocytes were found to mediate both extracellular lysis and phagocytosis, while cells maintained for 9 days in culture medium exhibited predominantly phagocytosis, and only a marginal level of extracellular lysis Short-term (40 h) activation of monocyte-derived macrophages at day 7 of culture with IFN-γ reversed this mechanism of cytotoxicity, and resulted in cells which mediated both phagocytosis, and extracellular lysis Prolonged stimulation with IFN-γ for 9 days resulted in a cell population which displayed predominantly extracellular lysis In conclusion, the results showed macrophages to become quite efficient in FcγRI-mediated cytotoxicity upon culture with IFN-γ

In **Chapter 3**, effects were studied of IFN-γ on monocyte/macrophage FcγRII expression levels and ADCC Furthermore, the study focused on specific effects of this cytokine at various stages of differentiation (i e , freshly isolated monocytes, and those cultured for 40 h and 9 days) The effects on FcγRII expression levels and ADCC activity induced by IFN-γ were found to be less striking, than those on FcγRI In addition, the shift in the mechanism of cytolysis observed for FcγRI did not occur for FcγRII Compared with freshly isolated monocytes, short-term (40 h) cultured cells exhibited a significantly lower FcγRII expression, and reduced ADCC activity However, co-culture with IFN-γ (40 h) completely reversed the decrease of receptor expression levels, and FcγRII-mediated cytotoxicity The impaired ADCC activity in short-term control cultured cells was found to be entirely associated with decreased FcγRII-mediated extracellular lysis Two allotypic variants of FcγRIIa were recognized with respect to their ability to bind murine IgG1 complexes Cells from different donors bound these complexes either strongly [high-responders ($Fc\gamma RIIa^{HR}$)] or weakly [low-responders ($Fc\gamma RIIa^{LR}$)]. For both allotypic forms similar results were observed In conclusion, IFN-γ may affect FcγRII expression and function of monocytes which have recently entered the tissue before becoming full-grown macrophages.

In **Chapter 4** the effect was evaluated of doxorubicin as a prototypic anticancer drug on maturation and differentiation of monocytes In the first studies it was verified

whether the (non-dividing) monocytes/macrophages were escaping cell death upon culture with doxorubicin Monocytes were cultured by adherence to plastic tubes, and in suspension using Teflon bags Two different incubation schedules were used to mimic continuous, and bolus infusion, reflecting the dosages and schedules currently used in the clinic The adherence capacity, yield, and enzyme activities that reflect growth and intermediary metabolism were found similar under all conditions evaluated Remarkably, independently of doxorubicin, the number of adherent monocytes increased proportionally with duration of culture

The above studies (Chapters 2 to 4) served to characterize our monocyte culture system, and provided a rationale for further investigations (Chapters 5 to 7) to evaluate the extent cytokines and anticancer drugs affect phenotypic and functional characteristics of monocytes at various stages of maturation *in vitro*

The effects of cytokines and anticancer drugs on the immune system need to be known before combining them in a clinical setting As IFN-γ appears to be the major monocyte activating cytokine, and doxorubicin possesses a wide spectrum of antitumor activity (compared with other anticancer drugs), the combination of these two compounds was assessed in **Chapter 5** The data on cellular morphology, intracellular enzyme activities, FcγR expression, and cytotoxicity indicated that neither the control populations, nor the IFN-γ activated monocyte-derived macrophages were significantly affected by exposure to doxorubicin However, culture with IFN-γ resulted in some extraordinary alterations in morphology (e g , a smaller cell size, a flattened and more juxta membranally located nucleus, homotypic monocyte clumping), and significant decreases in most enzyme levels These results provided a clear indication that in the combined approach, doxorubicin did not negatively affect the effects of IFN-γ on the monocyte/macrophage population

Monocytes/macrophages require activation for optimal functioning **Chapter 6** deals with the modulation by cytokines, such as IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, macrophage colony stimulating factor (M-CSF), granulocyte-macrophage (GM) CSF, and IL-2 Co-culture resulted in both strong and minimal effects on expression of cell surface antigens Specific modulation varied with the type of cytokine used, and depended upon the stage of maturation of monocytes The most profound effects on either phenotype and functional activities were induced by co-culture of cells with IFN-γ and IL-4 Changes in FcγRI and FcγRII expression levels were not always found to be reflected in FcγR-mediated ADCC When monocytes were cultured for 40 h, FcγRII expression levels decreased dramatically This reduction reversed either completely by culturing monocytes with IFN-γ and GM-CSF, or partially, when cultured in the presence of TNF-α However, in contrast to the cells cultured with IFN-γ, both GM-CSF- and TNF-α cultured monocytes did not exhibit a restored ADCC

In the past, anticancer drugs were thought to exert their effects through antiprolif erative and cytotoxic actions Nowadays, it has become apparent that they can induce both immunosuppression, and relatively specific immunoaugmentation **Chapter 7** deals with the immunomodulatory effects induced by various classes of cytostatics, such as antibiotics, alkylating agents, antimetabolites, and protein synthesis inhibitors, on *in vitro* cultured human monocytes A similar design as that presented in Chapter 6 was used The results show that effects of chemotherapeutic agents on monocyte morphology, phenotype, and function, to be dependent on the class of drugs, and the stage of monocyte differentiation Most obvious was the detrimental effect induced by the protein synthesis inhibitor ethyldeshydroxysparsomycin (EdSm) on FcγRII-mediated cytotoxicity of 40 h cultured monocytes

In **Chapter 8** a general discussion is presented, including recommendations for further research on analyzing specific effects of cytokines and anticancer drugs on *in vitro* cultured human monocytes

SAMENVATTING

De behandeling van kankerpatiënten waarbij de primaire (oorspronkelijke) tumor reeds is gemetastaseerd (uitgezaaid) baart de behandelende medici veel zorgen. Op het moment dat een tumor wordt gediagnostiseerd, is bij een groot aantal patiënten al sprake van dergelijke metastasen. Terwijl een primaire tumor meestal nog via chirurgie verwijderd, of door middel van radiotherapie bestreden, kan worden, is het gewoonlijk uiterst moeilijk eenzelfde therapie toe te passen op metastasen omdat deze op verschillende en soms lastig bereikbare plaatsen terechtkomen. De belangrijkste vorm van behandeling van patiënten met uitgezaaide tumoren is chemotherapie, waarbij ze een of meerdere keren behandeld worden met één of meerdere cytostatica (stoffen die remmend werken op snel delende tumorcellen). Helaas worden hierbij vaak lang niet alle tumorcellen gedood. Veel factoren spelen hierbij een rol, zoals het ontwikkelen van resistentie tegen de gebruikte cytostatica.

Onder andere hierom wordt op dit moment de rol van immunotherapie, ofwel stimulatie van het immuunsysteem (afweersysteem) door middel van *cytokinen* uitgebreid onderzocht bij patiënten met kanker. Cytokinen zijn eiwitten die door lichaamscellen worden geproduceerd om de onderlinge communicatie en stimulatie van witte bloedcellen te verzorgen die betrokken zijn bij het afweer van het lichaam tegen infecties en mogelijk ook tegen tumorcellen. Deze eiwitten kunnen tegenwoordig ook in het laboratorium geproduceerd worden via de *recombinant DNA technologie*. Hierdoor zijn ze nu in zuivere vorm en grote hoeveelheden beschikbaar gekomen voor zowel klinische toepassingen, als voor onderzoeksdoeleinden. Cytokinen worden meer recent ook gebruikt in combinatie met, of voorafgaand aan chemotherapie. De toepassing van deze behandelingswijze roept echter de vraag op, in hoeverre de gebruikte cytostatica nadelige effecten hebben op de goede werking van het immuunsysteem. Binnen het immuunsysteem bevinden zich een groot aantal witte bloedcellen zoals lymfocyten, granulocyten, 'natural killer' cellen, monocyten en macrofagen, die ieder een eigen rol vervullen bij de afweerreacties. De monocyten en macrofagen behoren tot een type van afweercellen dat op veel fronten actief is (**Hoofdstuk 1**). De meest algemeen bekende activiteit van deze cellen is het opruimen van 'afval'. Daarnaast wordt verondersteld dat ze een belangrijke rol spelen bij de vernietiging van tumorcellen. Om een kankercel onschadelijk te kunnen maken, moet er eerst een cel-cel contact tot stand komen tussen de monocyt of macrofaag en de te vernietigen doelcel. De binding kan ontstaan, enerzijds via een selectieve herkenning van een antigeen dat specifiek voorkomt op de tumorcel, anderzijds via receptoren die binden met immunoglobulinen (Ig), welke aanwezig kunnen zijn op de doelcel. Voor dit doeleinde beschikken monocyten en macrofagen over drie klassen van receptoren voor het Fc deel van IgG (FcγRI, FcγRII en FcγRIII).

De laatstgenoemde binding kan lysis (uiteenvallen) van de doelcel tot gevolg hebben. Aan dit proces van antilichaam afhankelijke celdoding (ADCC) werd in dit onderzoek uitgebreid aandacht besteed (voor begeleidende illustratie zie blz 11). Het doel van dit proefschrift was het bestuderen van de effecten die cytokinen en cytostatica kunnen veroorzaken op de functies van humane (van de mens afkomstige) monocyten uit het perifere bloed. Daarbij was er speciale belangstelling voor de verschillende stadia bij de ontwikkeling van monocyt tot macrofaag.

De perifere bloed monocyten vinden hun oorsprong in het beenmerg. Nadat ze hun weg gevonden hebben naar de diverse weefsels en organen van het lichaam, groeien ze uit tot macrofagen. In deze studies werd de celkweek als modelsysteem gebruikt, om deze ontwikkeling te bestuderen. Dit is verantwoord omdat de ontwikkeling van mono cyt tot macrofaag in een kweeksysteem veel gelijkenis vertoont met de ontwikkeling, zoals die zich in de weefsels van het menselijk lichaam afspeelt. Uit het perifere bloed van gezonde vrijwilligers werden, door middel van tegenstroomcentrifugatie grote aantallen monocyten geisoleerd, die vervolgens gekweekt werden in Teflon zakjes. Dankzij de gebruikte scheidingstechniek en het hydrofobe karakter van het Teflon was het mogelijk om 95% zuivere monocytenpopulaties in suspensie (zwevend) te kweken die, op elk gewenst moment van ontwikkeling, op eenvoudige wijze te oogsten waren. De zo verkregen macrofagen waren na het oogsten van uitstekende kwaliteit, met een levensvatbaarheid van 98%.

Tot op heden is het nog steeds onduidelijk welke signalen in de monocytenkweek verantwoordelijk zijn voor de ontwikkeling tot macrofagen. Inleidende studies wezen uit dat de beste resultaten werden verkregen door gebruik te maken van een kweekmedium waaraan 5 tot 10% humaan serum was toegevoegd.

Het celdodend vermogen van zowel monocyten als van de in kweek ontwikkelde macrofagen, werd bepaald in een ADCC test waarbij met IgG antilichamen bedekte humane rode bloedcellen als doelcel fungeerden.

In de studie, die beschreven staat in **Hoofdstuk 2**, werden macrofagen onderzocht op de via FcγRI verlopende ADCC activiteit. De testen werden uitgevoerd in zowel afwezigheid als aanwezigheid van een lage dosis IgG, afkomstig uit humaan serum. Het is een algemeen bekend gegeven dat humaan IgG, in feite de antilichamen, in hoge concentraties aanwezig is in het bloed en de weefselvloeistoffen. Omdat FcγRI de enige klasse van FcγR is met een hoge affiniteit voor humane IgG moleculen, zal deze receptor hiermee voortdurend verzadigd zijn. Het gevolg van deze verzadiging is dat de via FcγRI verlopende ADCC-activiteit altijd geblokkeerd zal zijn, eenvoudigweg omdat de bindings plaatsen voor IgG bezet worden gehouden. Een opvallende waarneming werd gedaan met betrekking tot FcγRI nadat monocyten gekweekt waren in de aanwezigheid van het cytokine interferon (IFN) γ. De experimenten lieten duidelijk zien dat, als gevolg

van een langdurige stimulatie met IFN-γ, zowel het aantal FcγRI sterk toenam en tevens de remming van de ADCC-activiteit was verdwenen. Bovendien werd het mechanisme bestudeerd waarmee FcγRI de rode bloedcellen kan lyseren. Vers geïsoleerde monocyten bleken deze doelcellen zowel uitwendig (extracellulaire lysis), als inwendig (fagocytose) te kunnen lyseren, terwijl de gedurende 9 dagen gekweekte monocyten voornamelijk bleken te fagocyteren. Indien op de 7^e dag van een kweek alsnog gedurende 40 uur IFN-γ werd toegevoegd, dan leverde dit een populatie van macrofagen op die, merkwaardig genoeg, weer in staat was tot de beide vormen van lysis. De monocytenkweek met IFN-γ, gedurende de totale kweekperiode van 9 dagen resulteerde in een celpopulatie die bijna alleen nog maar in staat bleek tot extracellulaire lysis. De resultaten laten zien dat, na stimulatie met IFN-γ, de macrofagen uiterst efficiente celdoders werden.

In **Hoofdstuk 3** werden de effecten van IFN-γ bestudeerd op het expressie niveau en ADCC van FcγRII. Dit onderzoek was met name gericht op verschillende ontwikkelingsstadia van de monocyten (vers geïsoleerde, 40 uur en 9 dagen gekweekte cellen). De effecten van IFN-γ, op de expressie en ADCC van FcγRII waren minder uitgesproken dan die op FcγRI. Bovendien, een verandering in het mechanisme van cytolysis zoals die werd waargenomen voor FcγRI vond niet plaats voor FcγRII. In vergelijking met de vers geïsoleerde monocyten was, bij de gedurende 40 uur gekweekte monocyten, de hoeveelheid FcγRII en de ADCC gehalveerd. Echter, indien IFN-γ constant aanwezig was tijdens de kweekperiode van 40 uur, dan werd de verlaging van zowel het receptor aantal als van de ADCC helemaal tenietgedaan. De verminderde celdodingscapaciteit van de 40 uur gekweekte monocyten bleek uiteindelijk gekoppeld te zijn aan een verlaging van de extracellulaire lysis. Omdat er geen effecten van IFN-γ op het aantal FcγRII en ADCC werden waargenomen bij de 9 dagen gekweekte macrofagen, zou geconcludeerd kunnen worden dat er in het menselijk lichaam alleen veranderingen plaatsvinden bij perifere bloed monocyten en cellen die kort daarvoor gestart zijn met de ontwikkeling tot volwassen macrofagen.

In **Hoofdstuk 4** werden de effecten geevalueerd die het cytostaticum doxorubicine had op de ontwikkeling van monocyten. In eerste instantie werd aan de hand van een monocytenkweek bekeken of deze cellen in staat waren om zich aan de celdodende werking van doxorubicine te onttrekken. Monocyten werden zowel in plastic buisjes (hechtend), als in Teflon zakjes (in suspensie) gekweekt. Bij de experimenten met doxorubicine werd er naar gestreefd om, zowel met de dosis, als de toedieningswijze, een klinische situatie na te bootsen. De hechtingscapaciteit van monocyten/macrofagen aan plastic, de cel opbrengst na kweken en de enzymactiviteiten die een afspiegeling vormen van zowel de groei als het metabolisme, bleken onder alle omstandigheden gelijk te zijn. Wel is het opmerkelijk dat, los van de behandeling met doxorubicine, het aantal adherente monocyten toenam naarmate er langer gekweekt werd.

De hiervoor beschreven studies (Hoofdstukken 2 t/m 4) zijn een karakterisering van het gebruikte kweeksysteem Ze vormden de uiteindelijke basis voor een verdere evaluatie (Hoofdstukken 5 t/m 7) van de mate waarin fenotype en functionele karakteristieken van monocyten, in verschillende stadia van de ontwikkeling tot macrofagen, beinvloed kunnen worden door zowel cytokinen als cytostatica

Het is van belang dat de effecten die cytokinen en cytostatica op het immuunsysteem kunnen uitoefenen bekend zijn, alvorens ze in combinatie met elkaar toe te passen Omdat IFN-γ het belangrijkste cytokine is voor de activatie van de monocyt en doxorubicine (vergeleken met andere cytostatica) een breed antitumorspectrum heeft, werd de combinatie van deze stoffen onderzocht op de mogelijke effecten die ze teweegbrengen bij monocyten (**Hoofdstuk 5**) De resultaten met betrekking tot de morfologie (vorm) van de cellen, de intracellulaire (binnenin de cel) enzymactiviteiten, de expressie van FcγR en de celdodingscapaciteiten duidden erop dat noch de controle populaties noch de met IFN-γ geactiveerde macrofagen beinvloed werden na blootstelling aan doxorubicine Echter, indien afzonderlijk met IFN-γ werd gekweekt, leidde dit tot uitzonderlijke morfologische veranderingen (o a kleiner, platter, kern meer tegen de celwand gelegen, celklontering) en duidelijk verminderde enzymactiviteiten Gesteld kan worden dat bij deze combinatie doxorubicine geen veranderingen aanbrengt in de effecten die IFN γ uitoefent op de monocyten/macrofagen populatie

Om bepaalde functies optimaal te kunnen uitvoeren moeten monocyten/macrofagen geactiveerd worden **Hoofdstuk 6** gaat over deze activatie door middel van cytokinen, zoals IFN-γ, tumor necrose factor (TNF)-α, interleukine (IL)-4, macrofaag kolonie-stimulerende factor (M-CSF), granulocyt-macrofaag (GM)-CSF en IL-2 Monocytenkweken met daaraan toegevoegd diverse soorten cytokinen, resulteerden in nogal sterk wisselende effecten op de expressie van oppervlakteantigenen Specifieke effecten varieerden met de gebruikte cytokinen en de leeftijd van de gekweekte monocyt De meest uitgesproken effecten werden veroorzaakt door IFN γ en IL-4 De na 40 uur kweken gehalveerde expressie van FcγRII (zie ook beschrijving van hoofdstuk 3) kon, behalve met IFN-γ, ook in zijn geheel worden opgeheven met GM-CSF en gedeeltelijk met behulp van TNF-α Verder was het opmerkelijk dat de verminderde celdoding onveranderd laag bleef, ook nadat monocyten 40 uur in de aanwezigheid van deze laatste twee genoemde cytokinen gekweekt waren

In het verleden werd altijd verondersteld dat cytostatica hun werking te danken hadden aan de remming van de celdeling en hun giftige uitwerking op de cellen Tegenwoordig is het duidelijk geworden dat ze ook in staat zijn om het immuunsysteem specifiek te beinvloeden **Hoofdstuk 7** handelt over de beinvloeding van monocyten door verschillende klassen van cytostatica zoals antibiotica, alkylerende middelen, antimetabolieten en een eiwitsyntheseremmer Evenals voor de effecten van de

cytokinen, geldt voor de verschillende cytostatica dat hun uitwerking op de monocyten sterk afhangt van het soort cytostaticum, de gebruikte concentraties en het stadium van ontwikkeling waarin de monocyt/macrofaag zich bevindt op het moment van de blootstelling Meest in het oog springend waren de nadelige effecten die veroorzaakt werden door de eiwitsyntheseremmer ethyldeshydroxysparsomycine (EdSm), op de via FcγRII verlopende ADCC van 40 uur gekweekte monocyten.

Een algemene discussie en aanbevelingen voor verder onderzoek zijn beschreven in **Hoofdstuk 8**

DANKWOORD

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CURRICULUM VITAE

Rob van Schie werd geboren op 26 juni 1955 te Eindhoven Na het behalen van het MAVO diploma in 1972 (Titus Brandsma-MAVO) en het HAVO diploma in 1974 (Eckart-College), bracht hij een jaar door op de Pedagogische Academie in zijn geboorteplaats Vervolgens werd van 1975 tot 1977 het Instituut voor Hoger Beroeps Onderwijs Eindhoven bezocht, alwaar hij de A-opleiding tot medisch analist, specialisatie microbiologie, met gunstig gevolg heeft afgelegd Na een colloquium doctum in 1978, begon hij aansluitend met de studie biologie aan de Universiteit van Amsterdam In juni 1981 werd het kandidaatsexamen B1' (Medische Biologie) afgelegd In februari 1985 behaalde hij het doctoraal examen Medische Biologie met als hoofdvak Cellulaire Immunologie en de bijvakken Anatomie/Embryologie en Algemene- en Vakdidaktiek Tevens werd de 1^{e} graads lesbevoegdheid biologie toegekend

In de jaren 1985 en 1986 was hij onder meer werkzaam als kandidaat-assistent en voluntair bij de vakgroep Histologie en Celbiologie van de Universiteit van Amsterdam en als assistent wetenschappelijk medewerker bij de vakgroep Gastroenterologie van de Universiteit van Amsterdam In februari 1987 volgde een aanstelling als Assistent in Opleiding bij de Katholieke Universiteit Nijmegen Op de afdeling Medische Oncologie (hoofd Prof Dr D J Th Wagener) van het Academisch Ziekenhuis Nijmegen werd tot februari 1992, onder leiding van Dr P H M de Mulder, gewerkt aan het onderzoek waarvan de resultaten in deze dissertatie beschreven staan

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STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT
"HUMAN MONOCYTES *IN VITRO* MODULATION BY CYTOKINES AND ANTICANCER DRUGS"

Voor het optimaal uitoefenen van een antilichaam afhankelijke cytotoxische activiteit door monocyten is de dichtheid van de Fcγ receptoren op het celoppervlak belangrijker dan het aantal.
dit proefschrift

Monomeer immunoglobuline G, dat aanwezig is in het plasma en de interstitiële vloeistoffen, speelt een belangrijke rol bij de homeostatische regulatie van de activiteiten van Fcγ receptor I.
Segal et al. *Mol Immunol 20:1177, 1983*

Non-adherente monocyten kunnen als gevolg van *in vitro* maturatie veranderen in adherente macrofagen.
dit proefschrift

Bij het meten van de antilichaam afhankelijke cytotoxiciteit (ADCC) van monocyten en macrofagen, met behulp van een ^{51}Cr-release assay, verdient het aanbeveling om een duidelijk onderscheid te maken tussen fagocytose en extracellulaire lysis.
dit proefschrift

De komende jaren zullen combinaties van cytokinen en cytostatica een belangrijke rol gaan spelen bij de behandeling van patiënten met uitgezaaide tumoren.

De verklaring voor de gunstige effecten die gezien worden bij patienten met chronische granulomateuze ziekte na behandeling met IFN-γ, moet mede gezocht worden in een quantitatieve toename van Fcγ receptor I op de monocyt.

Het ontwikkelingsstadium waarin monocyten verkeren, is sterk bepalend voor de effecten die kunnen optreden na stimulatie met cytokinen en of cytostatica.
dit proefschrift

Lymfocyten spelen de muziek maar de macrofaag geeft de toon aan.
Solbach et al. *Immunol Today 12:4, 1991*

Heeft men voorlopig niet genoeg waarnemingen, maar mochten deze later nog gedaan worden, dan moet men aan deze waarnemingen meer geloof schenken dan aan de theorie en deze laatste slechts, als zij tot hetzelfde resultaat voert als de verschijnselen.
Aristoteles (384-322 voor Christus)

“You have the world, leave us the forest”.
Magoh, Penan headman (Sarawak, Borneo)

Uit het feit, dat kranten dagelijks berichten over verkeersongevallen veroorzaakt door zowel dronken als nuchtere verkeersdeelnemers en nimmer melding maken van ongelukken waarbij mensen betrokken zijn na gebruik van marihuana, zou men kunnen afleiden dat dit genotmiddel een zeker gunstig effect heeft op de verkeersveiligheid.

Politiek Den Haag begint voorzichtig bepaalde vormen van celdeling te accepteren.

Nijmegen, 28 september 1992
Rob van Schie