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ESTABLISHMENT OF A HUMAN CELL LINE (MONO MAC 6) WITH CHARACTERISTICS OF MATURE MONOCYTES

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A monocytic cell line, termed Mono Mac, was established from peripheral blood of a patient with monoblastic leukemia. Two clones, designated Mono Mac 1 and Mono Mac 6, were isolated and both were assigned to the monocyte lineage on the basis of morphological, cytochemical and immunological criteria. Most importantly, the clones express NaF-sensitive non-specific-esterase, produce reactive oxygen and stain with MAb My4. Mono Mac 6, in addition, constitutively exhibits phagocytosis of antibody-coated erythrocytes in 80% of the cells and reacts with a panel of MAbs that are specific for mature monocytes, *i.e.*, M42, LeuM3, 63D3, Mo2 and UCHM1. By contrast, the monoblastic cell lines U937 and THP-1 are negative for all these markers. Only expression of My4 could be detected after differentiation induced by interferon-γ (IFN-γ). Similar treatment of Mono Mac 1, however, resulted in staining with all the monocyte-specific MAbs mentioned above, while IFN-γ treatment of Mono Mac 6 enhanced antigen expression. In addition, the cells showed an increased frequency of multinucleated cells with a rise from 4.8% to 21.9%. Mono Mac 6 appears to be the only one of the cell lines studied to constitutively express phenotypic and functional features of mature monocytes.

Cell lines established from transformants of a variety of tissues provide an excellent tool for studying various questions related to malignancy as well as the properties of the normal cellular counterpart in a homogenous cell population.

Very few cell lines of the monocyte lineage have been described (Sundström and Nilsson, 1976; Tsuchiya *et al.*, 1980; Ohno *et al.*, 1986; Ohta *et al.*, 1986). The best characterized lines, U937 and THP-1, are representatives of the early stage of monocytic differentiation. These lines might be considered as monoblasts since they must be induced to differentiate in order to develop some properties peculiar to mature monocytes (Koren *et al.*, 1979), such as antibody-dependent cell-mediated cytotoxicity and phagocytosis and expression of My4-defined antigen (Herrmann *et al.*, 1985). The ability of induced U937 cells to ingest antibody-coated erythrocytes and the concomitant use of the term "monocyte/macrophage" lead to the belief that U937, after differentiation induction, might serve as a tool for studying properties of mature monocytes and macrophages in humans (Nilsson, 1981). Our analysis using well-established MAbs directed against peripheral blood monocytes, however, revealed that even after differentiation induction no significant expression of the 63D3, M42, Mo2, LeuM3, UlHM1 markers could be detected.

In the present report we describe the cell line Mono Mac, one clone of which (Mono Mac 6) appears to represent mature monocytes on the basis of morphological criteria, observed by light and electron microscopy, of immunological phenotype including the expression of a set of MAb-defined cell surface markers, and of the ability of the cells to constitutively phagocytise antibody-coated erythrocytes.

MATERIAL AND METHODS

Patient history and establishment of the cell line

In 1981, myeloid metaplasia was diagnosed in a 60-year-old male patient on the basis of bone-marrow biopsy. He was repeatedly treated with corticosteroids until, in February 1985, he presented with multiple skin infiltrations and a leukocyte count of 35,000/mm^3 with 70% leukemic cells. Since the leukemic cells were positive for NaF-sensitive non-specific esterase and for the MAb-defined cell surface marker 63D3, monoblastic leukemia was diagnosed. The patient was treated with multiple cycles of Vincristine and etoposide, resulting in temporary improvement and remission of blood-cell leukemia, but no sustained and complete remission was achieved. After relapse, the patient died in August 1985. Mononuclear cells were isolated by density-gradient centrifugation from a blood sample taken in July 1985 and a culture was started in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 20% selected fetal calf serum, non-essential amino acids (043–1140, Gibco), oxalacetate (1 mM; 0–4126, Sigma, Munich, FRG), pyruvate (1 mM; No. 15990, Fluka, Buchs, Switzerland), penicillin-streptomycin (Gibco) and L-glutamine (2 mM; Gibco). The culture grew immediately and was split after 10 days. Two weeks after initiation of the culture, a first series of 2 sequential clonings at 0.5 cells/well in 96-well microtiter plates was started which finally gave rise to the 63D3$^-$ clone Mono Mac 1. A second series was started from bulk culture 3 months after initiation and resulted in a 63D3$^+$ clone designated Mono Mac 6.

HLA-typing revealed the presence of the following antigens: A3, A26, Bw4, Bw6, Cw7, DRw6, DRw52 for Mono Mac 1 and A3, Bw4, Bw6, B51, B7, Cw7, DR1, DRw6, DQw1 for Mono Mac 6.

Karyotyping on synchronized cells (Yunis *et al.*, 1981) was done on log-phase cells after 3 hr of Colcemid (Gibco). Mono Mac 1 and 6 had a modal number of 47.8 ± 1.3 and 42.3 ± 1.1, respectively. Both lines exhibited a trisomy 3 and a monosomy 12 and 17. In addition, there were characteristic marker chromosomes for each clone.

Other cell lines

The U937 line (Sundström and Nilsson, 1976) was kindly provided by Dr. K. Nilsson. The THP-1 line (Tsuchiya *et al.*, 1980) was obtained through the courtesy of Dr. R. Munker, Munich. Before initiation of the studies, both lines were cloned once and one clone each was used after phenotypic analysis (63D3, class II and Fc receptors) had shown that they gave the same pattern of staining as the respective maternal cell line. For the experiments reported herein, cells were cultured in 2-ml volumes in 24-well tissue culture plates at starting densities of 1–2 × 10^5 cells/ml.

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Abbreviations: EBNA, Epstein-Barr virus nuclear antigen; IFN, interferon; Il-1, Interleukin-1; MAb, monoclonal antibody; PAS, periodic-acid-Schiff; POX, peroxidase; SRBC, sheep red blood cells; TEM, transmission electron microscopy; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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TABLE I - MONOCLONAL ANTIBODIES USED

MAbs	Source	Reference
63D3	ATCC hybridoma	Ugolini *et al.* (1980)
M42	E.P. Rieber	Ziegler-Heitbrock *et al.* (1986)
Mo2	Coulter	Todd *et al.* (1981)
My4	Coulter	Griffin *et al.* (1981)
LeuM3	Becton-Dickinson	Dimitriu-Bona *et al.* (1983)
UCHM-1	P. Beverly	Hogg *et al.* (1984)
M522 (CD11)	E.P. Rieber	Lohmeyer *et al.* (1981)
T151, T310 (CD4)	E.P. Rieber	Rieber *et al.* (1984)
L243 (class II DR)	ATCC hybridoma	Lampson and Levy (1980)
Leu10 (class II DQ)	Becton-Dickinson	Chen *et al.* (1984)
OKT3 (CD3)	Ortho	Kung *et al.* (1979)
OKT 11 (CD2)	Ortho	Verbi *et al.* (1982)
B1 (CD20)	Coulter	Stashenko *et al.* (1980)

Cytochemistry

Staining for PAS, POX and esterases (Naphthol-AS-acetate, naphthylacetate and chloroacetate as substrates) was performed after appropriate fixation according to standard laboratory procedures.

Immunofluorescence

The MAbs used are listed in Table I. All were employed at optimum dilutions and analyzed in comparison to appropriate isotype controls which were obtained from the same manufacturer who provided the specific MAb. Isotype controls for antibodies purified and biotinylated in our laboratory were commercially purified reagents (MOPC21, UPC10, FLOPC21) from Sigma. All procedures were done in immunofluorescence buffer (phosphate-buffered saline with 2.5% FCS and 0.02% NaN_3). After incubation for 10 min with aggregated human Ig, cells were reacted in the cold for 30 min with mouse MAbs, either unconjugated or conjugated with biotin or FITC. After 2 washes cells were reacted either with goat anti-mouse Ig FITC diluted 1:50 (Tago, Burlingame, CA) for another 30 min in the case of unconjugated MAb, or with avidin-phycoerythrin diluted 1:2.5 (Becton Dickinson, Mountain View, CA) in the case of biotinylated MAbs; cells were left untreated in the case of FITC-conjugated MAbs. After 2 final washes, cells were fixed in 1% paraformaldehyde. Flow cytometry was performed with an EPICS V (Coulter, Hialeah, FL) equipped with an argon ion laser. The laser was operated with 350 mW at 488 nm and light scatter signals were used to gate out dead cells. Fluorescence signals were calibrated at channel 210 of a logarithmic scale with 6.25% bright beads (Coulter) with voltages of around 1,200 V for the green and 1,100 V for the yellow photomultiplier. Fluorescence signals were directed by a 550-nm long-pass dichroic mirror to the green photomultiplier equipped with a 525-nm bandpass filter and to the yellow photomultiplier equipped with a 575-nm bandpass filter. At least 1×10^4 viable cells were analyzed per sample, and histograms acquired were analyzed either with the 3/1 software of the EPICS V MDADS system or with specially designed software (kindly provided by P. Rohwer, Erlangen, FRG) on a SAM 68k computer (KWS, Ettlingen, FRG). If there was an overlap between staining of the specific MAb and the irrelevant control MAb of the same isotype at the same concentration, we applied a subtraction mode program (Coulter).

Electron microscopy

Suspended cells were treated with Karnovsky fixative (Karnovsky, 1965) for 2 hr at room temperature and the cell pellet was post-fixed in 1% OsO_4 for 1 hr at 4°C. Thin sections from epon-embedded samples were stained with lead citrate and examined under a Siemens EMscope 1.

Phagocytosis

Phagocytosis of antibody-coated sheep erythrocytes was done with sheep red blood cells (SRBC) that were pre-stained for endogenous peroxidase using carbazole in order to improve reading of final results. These SRBC were then coated with anti-SRBC anti-serum (Ambozeptor, Behringwerke, Marburg, FRG) at sub-agglutinating dilutions. One hundred μl of the antibody-coated SRBC at 5×10^8/ml were added to 10^6 monocytes/ml and cultured for 4 hr at 37°C. Non-ingested SRBC were lysed with ammonium chloride buffer. Pappenheim-stained cytospin preparations were examined under light microscopy, and the percentage of cells with phagocytosis was determined by counting the number of cells with 1 or more erythrocytes in the cytoplasm.

FIGURE 1 - Pappenheim staining of Mono Mac 6. Note the presence of multiple cytoplasmic granules. Scale bar represents 10 μ.

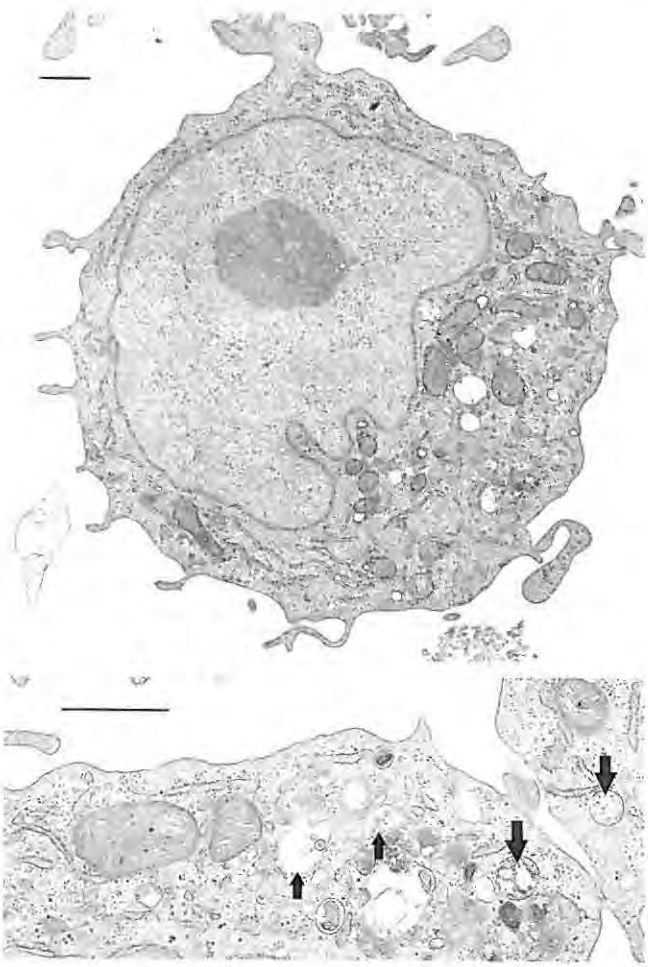

FIGURE 2 – TEM of Mono Mac 6. Scale bar represents 1 μ. The cytoplasm is rich in mitochondria, rough endoplasmatic reticulum, endocytic vesicles, multivesicular bodies and lysosomes. Small arrows indicate multivesicular bodies, large arrows point to structures with characteristics of lysosomes.

Treatment with interferon and phorbolester

Cells were cultured at 2×10^5/ml in wells of 24-well plates (3524, Costar, Cambridge, MA). Recombinant IFN-γ kindly provided by Dr. W. Wolf, Bioferon, Laupheim, FRG was added to cultures at 1,000 U/ml final concentration. TPA (Sigma), dissolved in acetone, was added at 1.6 nM final concentration, while control cultures received acetone only (0.001%).

Miscellaneous procedures

Measurement of Il-1 activity was kindly performed by Dr. M. Blumenstein, Munich. Epstein-Barr virus-associated nuclear antigen determination was kindly done by Dr. H. Wolf, Munich. Terminal transferase was determined by standard immunofluoresence, and lysozyme was measured with a commercial photometric assay (Behring-Werke). Reactive oxygen production was determined by flow cytometry with dichlorofluorescein-diacetate (Szejda *et al.*, 1984) using TPA trigger of 10 nM for 15 min.

RESULTS

Assignment of Mono Mac 1 and 6 to the monocyte lineage

The 2 clones Mono Mac 1 and Mono Mac 6 grow in suspension with a doubling time of 50 hr. Mono Mac 6 cells may loosely attach to the plastic surface of the tissue culture vessel, but the cells can readily be suspended by pipetting. Under light microscopy, after staining with Pappenheim, cells of both types have a diameter of approximately 16 μ and they show a round or indented nucleus with lacy chromatin, and sometimes 1 or 2 nucleoli. Both clones have a basophilic to light-blue cytoplasm, while fine granules are sometimes seen in Mono Mac 6 (Fig. 1). Cells with 2–4 nuclei are observed in 4.8 $\pm$ 1.9% of the cells in Mono Mac 6. In TEM, both clones exhibit a villous cell surface and a nucleus rich in euchromatin with prominent nucleoli. The cytoplasm contains many mitochondria, numerous rough endoplasmatic reticulum cysternae, a prominent Golgi complex, lysosomes, coated vesicles, endocytic vesicles and multivesicular bodies (Fig. 2). Upon incubation of Mono Mac 6 cells with immunoglobulin-gold conjugate, these particles could be demonstrated in the endocytic structures by TEM (not shown).

Both clones were assigned to the monocyte lineage on the basis of the absence of features associated with other lineages of the lymphohematopoietic system, and on the basis of the presence of monocyte-associated properties (Table II). Mono Mac 1 and Mono Mac 6 were negative for erythrocyte-associated glycophorin, granulocyte-associated peroxidase, lymphocyte-associated PAS and TdT, B-cell-associated B1 and EBNA, and T-cell associated T3 and T11 antigens, but they are positive for markers associated with monocytes, *i.e.* NaF-sensitive esterase, lysozyme and the cell-surface marker My4 (Table II). Further, both clones were able to produce reactive oxygen. Phagocytosis of antibody-coated erythrocytes is minimal in Mono Mac 1 (10.7 $\pm$ 6.5%) while in Mono Mac 6 79.3 $\pm$ 10.3% of the cells phagocytosis with up to 9 erythrocytes per cell was seen (Fig. 3). For comparison, phagocytosis was absent in U937 and minimal in THP1 (15.7 $\pm$ 3.8).

Expression of MAb defined monocyte-associated cell-surface molecules

Our findings suggest that both clones bear monocyte features. We thus tested the cells with a panel of MAb-defined cell-surface molecules that are associated with monocytes. For comparison we also stained the U937 and THP-1 cell lines. It appears from Figure 4 that U937 as well as THP-1 are negative for all of the 5 MAbs tested. In contrast, as mentioned earlier, Mono Mac 1 is positive for My4, and Mono Mac 6, in addition, is positive for LeuM3, Mo2, 63D3 and M42. Percentages of positive cells calculated from these histograms are

TABLE II – LINEAGE ASSIGNMENT OF MONO MAC 1 AND MONO MAC 6

Marker[1]	Mono Mac 1	Mono Mac 6
Glycophorin	–	–
TdT	–	–
CD 2 (OKT 11)	–	–
CD 3 (OKT 3)	–	–
EBNA	–	–
CD 20 (B1)	–	–
PAS	–	–
Peroxidase	–	–
Esterase		
Naphthol AS acetate	+	+
Naphthyl acetate (NaF-sensitive)	+	+
Chloracetate	–	–
Acid phosphatase	–	+
Il-1	+	+
Lysozyme	+	+
My4	+	+
Reactive oxygen	+	+
Phagocytosis (EA)	+ (10.7$\pm$6.5)	+ (79.3$\pm$10.3)

[1]Erythrocyte-associated glycophorin was studied with the MAb A6; TdT = terminal transferase.

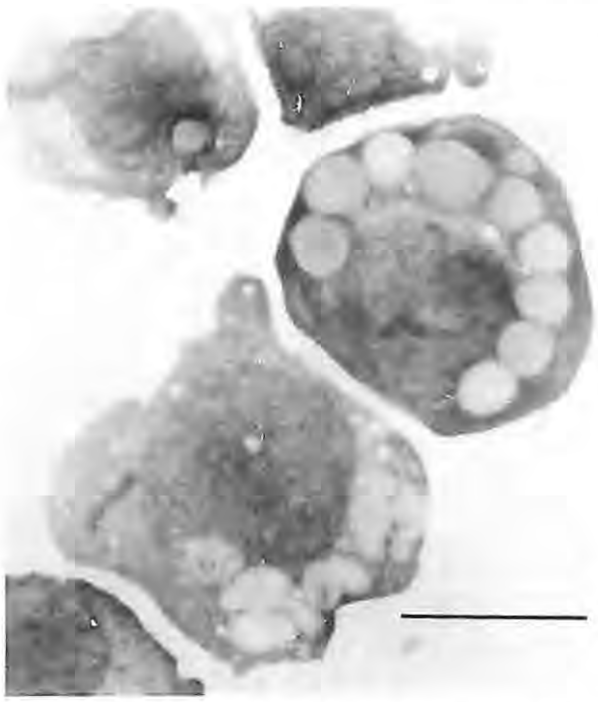

FIGURE 3 – Phagocytosis of antibody-coated erythrocytes by Mono Mac 6. Scale bar represents 10 μ.

given in Table III which summarizes data from 2 to 5 independent experiments. Values for U937 and THP-1 were less than 15% for any of the MAbs, staining of Mono Mac 1 for My4 was 75.5% while percentages of the other 5 markers of mature monocytes were below 15%. By contrast, more than 40% of the Mono Mac 6 cells are positive for LeuM3, Mo2, 63D3 and M42 and approximately 30% of the cells are positive for UCHM1.

Additional molecules studied were CD11, CD4, Fc-receptor and MHC class-II antigens. Table III indicates that CD11 is strongly expressed on U937, while TPH-1, Mono Mac 1 and Mono Mac 6 show low staining. CD4 similarly is strongly expressed on U937, and also clearly on THP-1, while Mono Mac 1 and Mono Mac 6 show negligible staining with either MAb 310 or MAb 151 which recognize different epitopes on the CD4 molecule. MHC class II is not detected on U937 but is present on THP-1, Mono Mac 1 and Mono Mac 6. In Mono Mac 6, however, DQ expression, as detected with the Leu10 MAb, is lacking, while all 4 cell lines are positive for Fc-receptors. Thus it appears that Mono Mac 6 expresses many markers (My4, LeuM3, Mo2, 63D3, M42, UCHM1) which are restricted to mature cells of the monocyte lineage while Mono Mac 1 is positive only for My4, and U937 and THP-1 express negligible amounts of these monocyte markers, or none at all.

Differentiation induction with IFN-γ

The pattern of MAb reactivity indicates that U937 and THP-1 are the least mature of this set of 4 cell lines followed, by increasing degree of maturation, by Mono Mac 1 and Mono Mac 6. We therefore asked whether the different cells could be induced to a higher level of maturation by means of IFN-γ treatment. Treatment of U937 for 3 days with 1,000 U/ml of IFN-γ resulted in a clear appearance of My4 staining while M42, 63D3, LeuM3 and Mo2 staining remained negligible. The same was true for THP-1. Further, treatment of both cell lines with TPA, a more potent inducer of differentiation, gave the same pattern (data not shown). Treatment of Mono Mac 1 with IFN-γ however, resulted in the appearance of M42, 63D3, LeuM3 and Mo2 (Fig. 5). The full expression of these monocyte markers in Mono Mac 1, as exemplified by LeuM3, required 3 days of IFN-γ stimulation, while on day 1 only a slight staining began to appear (data not shown). This indicates that Mono Mac 1 can be induced to differentiate with respect to MAb-defined cell surface phenotype to the level of Mono Mac 6. In Mono Mac 6 the expression of this set of MAb-defined monocyte markers did not increase after IFN-γ treatment with respect to percentage of positive cells, while there was an increase in fluorescence intensity (data not shown). Morphologically, however, there was an increase of multinucleated cells from 4.8 ± 1.9% to 21.9 ± 3.1%, with 5.7 ± 2.5% cells having 3 or 4 nuclei (Fig. 6).

DISCUSSION

With our current knowledge, the 2 clones Mono Mac 1 and Mono Mac 6 can be assigned with confidence to the monocyte lineage. This conclusion is based on the presence of a large panel of monocyte-associated and monocyte-specific markers and on the absence of markers associated with other bone-marrow-derived cell lineages (Table I). The leukemic sample used to establish the cell lines was positive for the 63D3-defined antigen, but when the initial bulk culture was cloned, such $63D3^+$ clones were only found with a moderate frequency (5 of 17 clones tested). There might be a growth disadvantage for more mature monocytic cells, which probably explains why other workers have so far been unable to establish human cell lines with characteristics of mature monocytes.

The cell lines U937 and THP-1, for instance, do not express any monocyte-associated markers such as M42, LeuM3, 63D3, Mo2, My4 or UCHM1 (Fig. 1, Table III). Even after induced differentiation of U937 and THP-1 using either TPA or IFN-γ, we could not detect significant expression of any of the

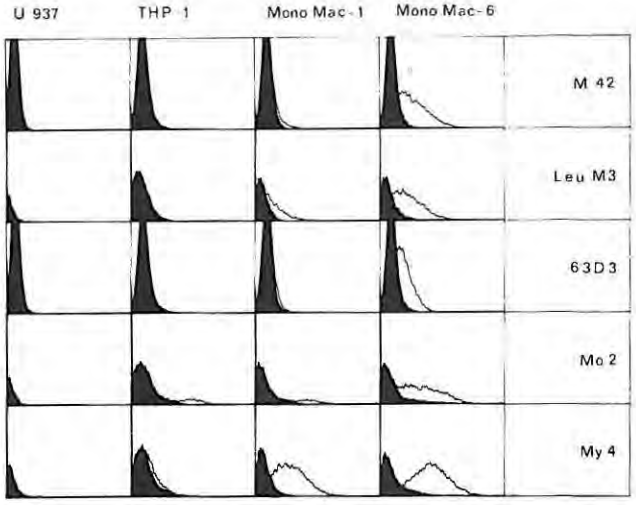

FIGURE 4 – Flow cytometry analysis of monoctye-associated cell-surface markers. The cell lines were stained with the respective MAb and analyzed with 20,000 cells per sample using log amplification. The respective isotype control is given in black.

TABLE III - COMPARISON OF CELL-SURFACE PHENOTYPE OF MONO MAC 1 AND 6 TO U937 AND THP-1

	Cells positive for (%)[1]				
	M42	LeuM3	63D3	Mo2	My4
U937	1.0±1.2	2.5±2.3	1.0±0.8	2.6±3.8	9.5±9.2
THP-1	3.8±5.6	3.1±1.9	3.8±3.4	13.0±7.8	7.5±1.7
Mono Mac 1	14.8±6.0	14.4±4.4	9.8±5.1	11.2±3.3	75.5±12.3
Mono Mac 6	61.5±10.5	49.7±11.7	61.5±10.5	45.0±2.8	82.0±7.2

	Cells positive for (%)[1]					
	UCHM1	CD11	CD4	agg. IgG[2]	DR	DQ
U937	7.0±5.7	68.3±5.9	83.6±12.5	58.9±0.2	0	0
THP-1	0	6.0±8.7	30.0±0	81.6±4.9	69.5±27.6	8.5±2.1
Mono Mac 1	12.0±16.9	13.0±4.0	4.1±8.0	76.0±24.0	83.5±4.9	29.0±9.9
Mono Mac 6	28.0±8.5	28.2±10.2	7.8±3.3	87.5±4.9	73.5±20.5	0

[1]Results are mean ± SD from 2 to 5 experiments.–[2]Heat-aggregated Ig for detection of Fc-receptors.

monocyte-specific markers except My4. My4 has also been induced in U937 (Herrmann *et al.*, 1985), but it was the only marker tested in that study. The absence of the Mo2 antigen from U937 cells has also been reported (Trinchieri *et al.*, 1987; Cannistra *et al.*, 1987). This does not, however, exclude the possibility of obtaining an Mo2-positive derivative from the original U937 line.

My4 is constitutively expressed by the Mono Mac 1 cell line and the other monocyte-specific markers were lacking, but they could be induced by differentiation induction. Appearance of the LeuM3-defined antigen required 2–3 days of culture, indicating neosynthesis rather than transport from a cytoplasmic pool or conformational changes in a molecule already present. This set of data is consistent with the notion that My4 is a cell-surface marker that appears earlier in differentiation than any of the other MAb-defined structures. Cotransfection experiments of monocyte DNA in murine fibroblasts indicate that My4, LeuM3 and 63D3 may recognize epitopes on the same 55-kDa molecule (Ashmun *et al.*, 1987). Molecule cloning, however, is required to conclusively demonstrate the molecular relationship of these reagents.

Consistent with their monocyte nature, both Mono Mac 1 and Mono Mac 6 express class-II antigens. The CD4 antigen detectable in low amounts on blood monocytes was not found in Mono Mac 1 and 6. Both clones express Fc receptors but only in Mono Mac 6 can the majority of the cells phagocytise antibody-coated erythrocytes.

The hallmark of Mono Mac 6, the expression of monocyte-associated antigens, like LeuM3, was stable in culture for at least 6 months. In one instance, after prolonged culture, LeuM3 expression was lost from the majority of cells and cultures were started again from initially frozen samples. Hence, as with any other cell line, the stability of properties of interest will have to be monitored for Mono Mac 1 and 6, as well.

TEM showed the Mono Mac clones to contain all compartments required for protein export and for endocytosis, such as lysosomes and endocytotic vesicles, features that have not been reported for either U937 or THP-1. These results, together with the ability of Mono Mac 6 to readily phagocytise antibody-coated erythrocytes, further support the notion that Mono Mac 6 is a representative of mature monocytes.

It would thus be of interest to examine whether features of tissue macrophages could be induced upon differentiation. Preliminary studies using IFN-γ and TPA suggest the appearance of multi-nucleated cells in Mono Mac 6, reminiscent of giant cells seen, for instance, in granuloma. Multinucleated

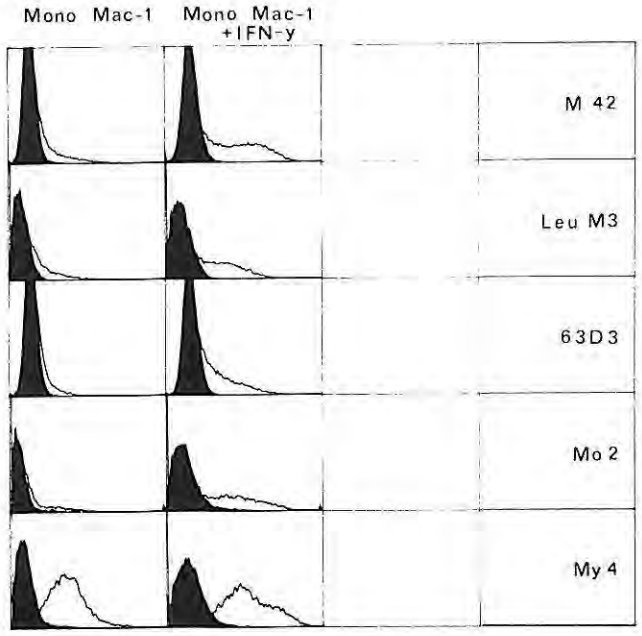

FIGURE 5 - Flow cytometry analysis of monocyte-associated cell-surface markers with Mono Mac 1 after IFN-γ treatment. Mono Mac 1 was cultured for 3 days with or without 1,000 U IFN-γ/ml and then processed as in Figure 4.

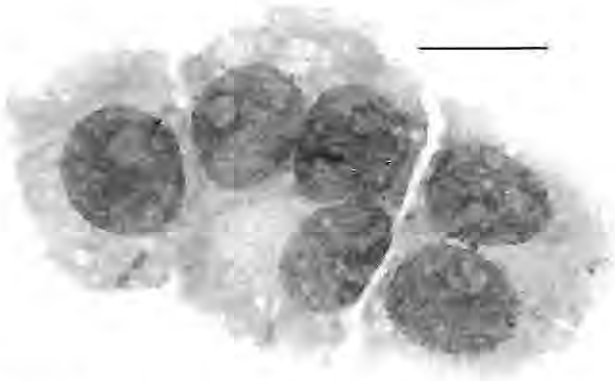

FIGURE 6 – Pappenheim stain of Mono Mac 6 after IFN-γ treatment. Scale bar represents 10 μ.

cells were also observed in IFN-γ-treated monocytes and in IFN-γ-treated HL60 cells (Weinberg *et al.*, 1984, 1986). The number of nuclei in such polykaryons (> 100 nuclei per cell) was much higher in the IFN-γ-treated monocytes compared to IFN-γ-treated Mono Mac 6, an average of 2.5 nuclei. Experiments with various inducers are currently under way in order to study pathways of differentiation in Mono Mac 6. Even without differentiation induction, however, the Mono Mac 1 and Mono Mac 6 cell lines appear to be an informative set of cells for analysis of monocyte-related properties at the clonal level.

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