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Impaired *in vitro* survival of monocytes from patients with HIV infection

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SUMMARY

In vitro survival of monocytes (MO) was studied in 59 patients with HIV infection of different clinical stages. MO from 61 donors and 12 healthy seronegative homosexual men were also examined. Compared with the number of MO seeded, the percentage of adherent monocyte-derived macrophages (MDM) present after 10 days was significantly lower in patients with HIV infection than in the controls. However, the number of viable, non-adherent MO/MDM was similar in patients and controls. Our data indicate markedly decreased *in vitro* survival of MO from patients with HIV infection. After 10 days, the MDM population in the patient cultures was significantly less differentiated than the control cells, assessed by immunocytochemical staining with monoclonal antibodies against differentiation antigens. Reduced *in vitro* survival of MO/MDM was associated with low numbers of $CD4^+$ and $CD8^+$ lymphocytes in blood, reduced lymphocyte mitogen responses, presence of HIV p24 antigen in serum and advanced clinical stage. Decreased *in vitro* survival of MO/MDM may be associated with HIV replication in the cells. Although the level of HIV replication in the cultures was low as assessed by measurement of HIV p24 antigen in culture supernatants and staining of MO/MDM for HIV antigens, cytopathogenic effects of HIV or HIV products cannot be ruled out.

Keywords monocytes macrophages HIV *in vitro* survival

INTRODUCTION

The importance of the monocyte-macrophage system in the pathogenesis of HIV infection has been established during the last few years. Monocytes and macrophages can be infected by HIV *in vitro*, and HIV can be recovered from monocytes and macrophages obtained from blood and various organs of HIV-infected individuals (Levy *et al.*, 1985; Gartner *et al.*, 1986; Ho, Rota & Hirsch, 1986; Nicholson *et al.*, 1986; Salahuddin *et al.*, 1986). Various functional abnormalities of monocytes and macrophages have been reported in patients with HIV infection, including abnormal secretion of monokines (Fauci, 1988; Wright *et al.*, 1988), defective chemotaxis (Smith *et al.*, 1984; Poli *et al.*, 1985), and reduced microbial activity (Estevez *et al.*, 1986; Eales, Moshtael & Pinching, 1987).

In the present study, we have examined the ability of MO from patients with different clinical stages of HIV infection to survive and differentiate in culture. The association between several immunological, virological and clinical parameters and *in vitro* monocyte survival was also studied.

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MATERIALS AND METHODS

Patients

Fifty-nine adult patients (30 homosexual men, 18 intravenous drug abusers, seven with heterosexual transmission and four haemophiliacs) with HIV infection were studied. All patients had antibodies to HIV as measured by ELISA (Organon Teknika, Boxtel, The Netherlands) and confirmed by Western blot (DuPont, Wilmington, DE). The patients were clinically classified according to the Centers for Disease Control criteria (1986) as shown in Table 1. Classification in CDC group IV C1 was based on *Pneumocystis carinii* pneumonia in 13 patients, and on other opportunistic infections in two patients. One patient in CDC group IV D had Kaposi's sarcoma. None of these patients received antiviral treatment against HIV. As control groups, 12 healthy homosexual men as well as 61 blood donors were selected. All were HIV-antibody negative by ELISA testing.

Monocyte culture

Mononuclear cells (MNC) from heparinized blood samples were obtained by isopaque–Ficoll (Lymphoprep, Nycomed AS, Oslo, Norway) gradient centrifugation (Bøyum, 1968) within 2 h after blood sampling. Cells were washed twice in Hanks

Table 1. Clinical data and laboratory information

	Number	Age	White blood cells	Monocytes	$CD4^+$ Lymphocytes	$CD8^+$ Lymphocytes
			($\times 10^6$/l)			
Blood donors	61	38 (30–42)	3800 (3300–4700)	430 (320–530)	810 (700–980)	410 (340–510)
HIV-seronegative homosexual men	12	33 (29–37)	4600 (3700–6700)	480 (380–1000)	760 (570–1030)	680* (590–840)
CDC II/III	44	32 (27–36)	4200 (3300–5300)	500 (290–660)	430*·† (300–610)	830* (570–1080)
CDC IV C1/D	15	36 (26–42)	2800*·†·‡ (2400–3600)	230 (170–410)	80*·†·‡ (50–190)	480†·‡ (230–800)

Data are given as medians and 25–75 per centiles.
* $P < 0.05$ *versus* blood donors.
† $P < 0.05$ *versus* seronegative homosexual men.
‡ $P < 0.05$ *versus* CDC II/III.
$CD4^+$ and $CD8^+$ lymphocyte counts in blood donors are given according to Brinchmann *et al.* (1989).

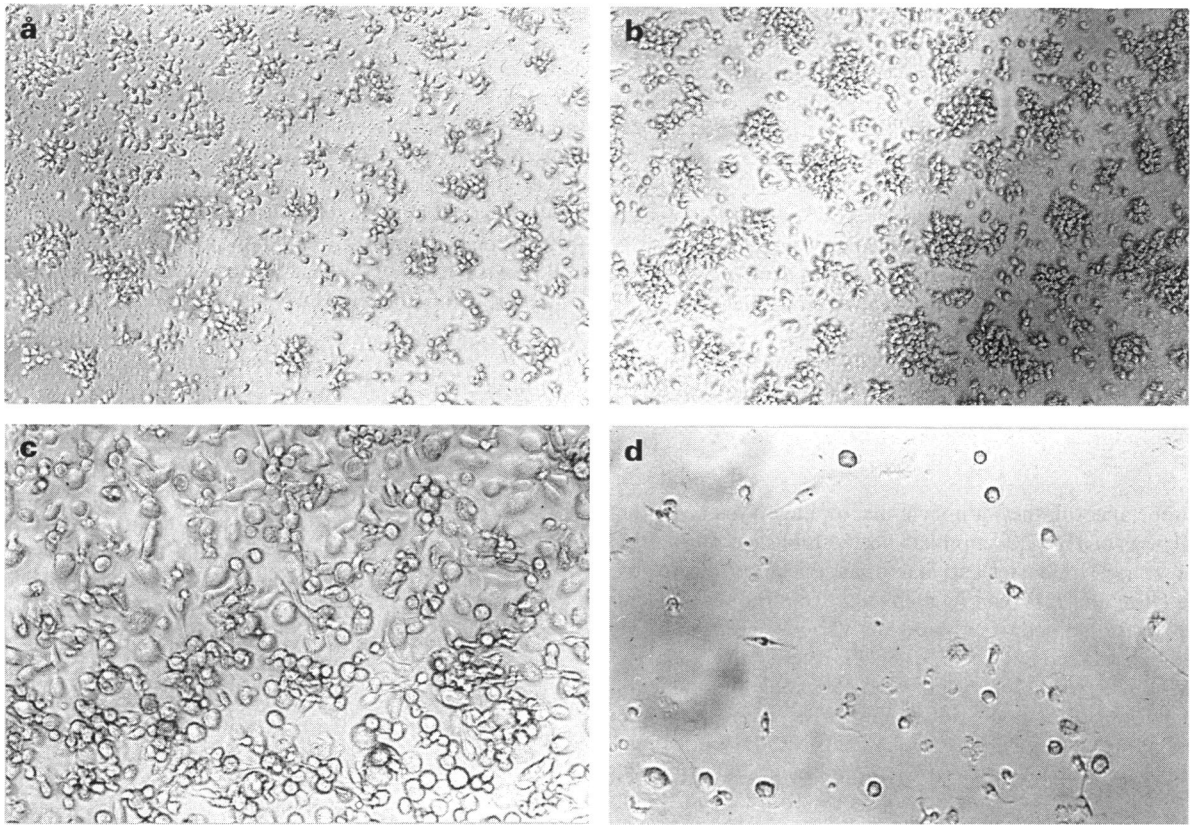

Fig. 1. Culture of monocytes. Representative cultures from a blood donor (left) and from a patient with AIDS (right) are shown after 1 (a & b) and 10 (c & d) days (× 65). Note the similar cell density after one day. After 10 days, a striking decrease in the cell density is seen in the culture from the AIDS patient compared with the blood donor.

balanced salt solution (HBSS, Gibco, Paisley, UK) and resuspended to $2.5–3 \times 10^6$ cells/ml in RPMI 1640 with glutamine (Gibco), supplemented with gentamycin (40 μg/ml), amphotericin B (0·25 μg/ml), (hereafter referred to as medium) and 10% heat-inactivated human AB serum.

The endotoxin content, tested by the *Limulus* amoebocyte lysate test, was < 0·1 ng/ml in medium with 10% AB serum.

Cytospins of MNC were stained with May–Grünwald–Giemsa (MGG) to evaluate granulocyte contamination, (median 1·4%; range, 0–2·9%). To determine the number of MO

seeded, cytospin preparations of MNC were also stained for non-specific esterase (NSE) as described by Yam, Li & Crosby (1971).

Aliquots of 200 μl of the cell suspensions were incubated in flat-bottomed, 96-well microtitre plates (Costar, Cambridge, MA) for 1 h to allow adherence of MO. In addition, cells (1-ml aliquots) were incubated on 14-mm cover slips in 24-well plates (Costar). Non-adherent cells were removed by washing three times with pre-warmed HBSS, and medium with 20% AB serum was added. The non-adherent cells were counted and stained for NSE. When cultured for 10 days, medium with 10% AB serum was replaced at day 3 and 7. Patient cells were always cultured in the same microtitre trays as control blood donor cells, and only experiments where the control cells grew to continuous monolayers (Fig. 1c) were included in the study.

Quantification of monocytes/macrophages

Cover slips harvested after 1 and 10 days were stained for NSE. On day 1, the median percentage of monocytes was 89% (range, 81–96%) in patient cultures *versus* 93% (range, 90–97%) in cultures from the blood donors. After 10 days, the median was 94% (range, 89–95%) and 97% (range, 91–99%), respectively. The esterase-negative cells were morphological lymphocytes. Viability of adherent cells was determined by acridine orange/ethidium bromide staining (Sigma, St Louis, MO) according to Parks *et al.* (1979) and was routinely greater than 95% on day 1 and 10 in cultures from both patients and controls.

After 10 days of culture, adherent cell nuclei were counted as described by Unkeless & Eisen (1975). Multi-nucleated cells were seldom seen, the median nucleus-to-cell ratio on day 10 was 1·05 (range, 1·02–1·16) and 1·08 (range, 1·03–1·14) for patient and control cultures, respectively. Adherent cell protein was measured according to Lowry *et al.* (1951) using bovine serum albumin as standard. The detection limits of these assays were 10^4 nuclei/well and 1 μg protein/well (data not shown).

On days 3, 7 and 10, the number of non-adherent cells in the cultures were counted and the percentage of MO/MDM determined by morphology and acridine orange staining according to Allison, Harrington & Birbeck (1966). Also, viability of the non-adherent cells was determined.

Monoclonal antibodies (MoAb)

MoAb 1D5 (IgG1) has been shown to react with an antigen expressed on all MO cultured on glass, but the antigen is progressively lost as MO differentiate to MDM *in vitro* (Kaplan & Gaudernack, 1982). MoAb 3D10 (IgG1, G. Gaudernack) which recognizes a different antigen than 1D5, has shown a similar reactivity as 1D5 on MO cultured for 1 and 10 days (unpublished observations).

MoAb against the HIV proteins p15, p24 and gp120 (all IgG1; DuPont) were used in combination to detect HIV antigens expressed by MO/MDM. As a positive control, MDM infected by HIV strain IIIB (Maehlen & Degré, 1989) were stained by the same procedure.

Immunocytochemical procedures

Cover slips harvested on day 1 and 10 were washed once with HBSS, air-dried, fixed in acetone at 20°C for 10 min and stored at −70°C. Immediately before staining, cover slips were thawed and re-fixed in acetone for 10 min at 20°C. Cover slips were stained by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method as described elsewhere (Müller, Frøland & Brandtzaeg, 1989).

A non-reactive control antibody (anti-keratin, clone K92, IgG1, Dakopatts, Denmark) was included in cover slips from all patients on day 1 and 10.

Due to the small number of monocytes obtained from most patients with AIDS, cover slips from only four of the AIDS patients were included in the immunocytochemical studies.

Virological studies

All HIV-positive patients and seronegative homosexual men were examined by ELISA for HIV p24 antigen in serum (Organon). Culture supernatants harvested at day 3 and 10 from 36 patients with HIV infection were also assayed for HIV p24 antigen. In the p24 antigen ELISA, the detection limit was 20 pg/ml. In addition, staining of cells for HIV antigens was performed by the APAAP procedure.

Lymphocyte studies

The number of $CD4^+$ and $CD8^+$ lymphocytes was determined by immunomagnetic quantification (Brinchmann *et al.*, 1988). $CD4^+$ and $CD8^+$ lymphocytes were not counted in the blood donors in this material. Quantification of the lymphocyte subsets in blood donors at our hospital has previously been done (Brinchmann *et al.*, 1989) and these data are given in Table 1.

Lymphocyte mitogen responses were measured in response to phytohemagglutinin (PHA), concanavalin A (ConA) and pokeweed mitogen (PWM) as described elsewhere (Bentdal, Frøland & Larsen, 1989). Mitogen responses were classified as normal (all responses greater than 50% of controls), depressed PWM response only (less than 50% of control) or all three responses depressed (less than 50% of controls). Controls were blood donor MNC run at the same time as patient cultures.

Statistical analysis

When groups of patients were compared, the Wilcoxon rank sum test (two-tailed) or the Kruskal–Wallis test was used. If a significant difference was found using the Kruskal–Wallis test, Fisher's least significant difference was computed on the ranks (Conover, 1980) to determine differences between pairs of groups. Correlation was determined by Spearman rank correlation.

RESULTS

Number of monocytes in peripheral blood

The number of monocytes in peripheral blood was at the same level in blood donors and patients of stage CDC II/III (Table 1). In the AIDS patients, the monocyte count tended to be lower, but not significantly so. This was in contrast to the reduction of $CD4^+$ lymphocytes seen in CDC II/III and even more in CDC IV C1/D (Table 1).

In vitro *survival of monocytes*

After adherence to plastic for 1 h, the number of viable, non-adherent MO was similar in patients and controls (data not shown).

When examined by inverse-phase contrast microscopy, patient and control cultures appeared similar for the first 3–4 days of culture (Fig. 1a). Thereafter, the cell density in cultures

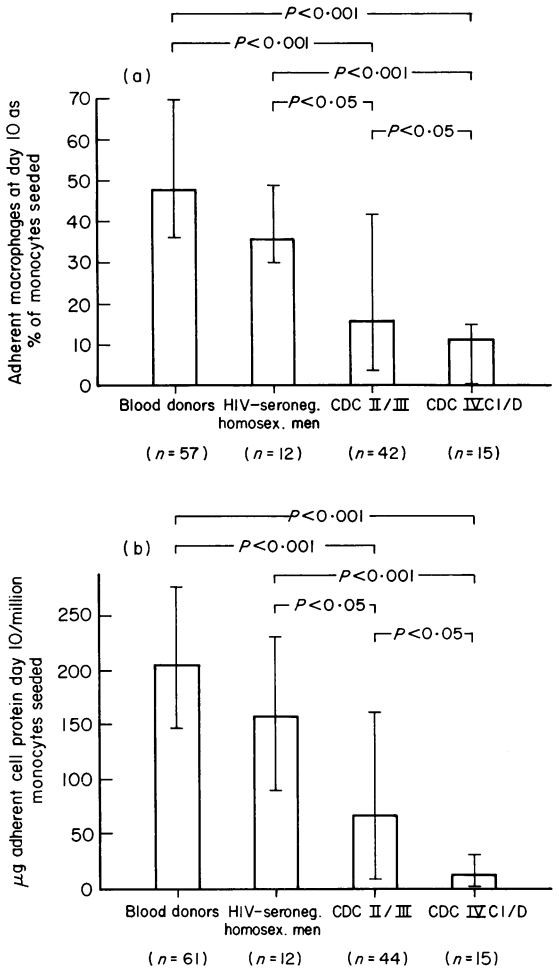

Fig. 2. *In vitro* survival of monocytes. Survival in culture was assessed as (a) adherent macrophages after 10 days as per cent of monocytes seeded and (b) amount of adherent cell protein after 10 days per million monocytes seeded. Results are given as medians and 25–74 percentiles.

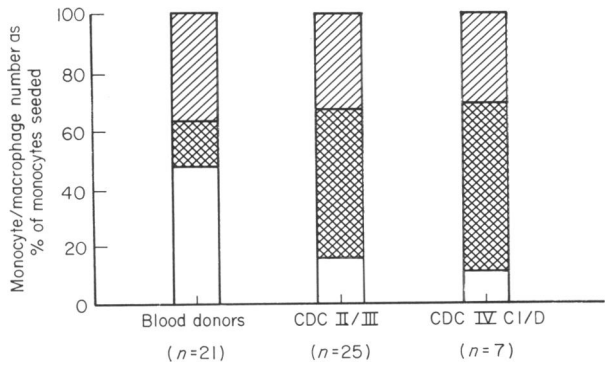

Fig. 3. *In vitro* survival of monocytes. □ viable, adherent macrophages after 10 days of culture as per cent of MO seeded. ■ viable, non-adherent monocytes/macrophages detected during cell culture as per cent of monocytes seeded. ▩ loss of monocytes/macrophages during culture determined as the difference between the number of monocytes seeded and the sum of adherent and non-adherent monocytes/macrophages counted during culture. Results are given as medians.

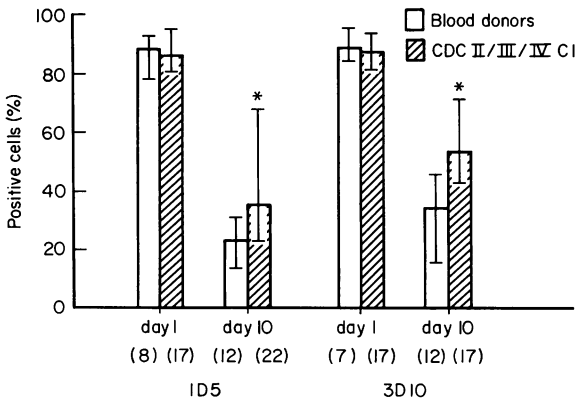

Fig. 4. Monocyte differentiation in culture. Expression of 1D5 and 3D10 antigens on MO/MDM cultured for 1 and 10 days from patients with HIV infection (CDC II/III/IV C1) and blood donors. The number of individuals included is given below the bars. Results are given as medians and 25–75 percentiles. * $P < 0{\cdot}05$ *versus* blood donor cells at day 10.

from most HIV-positive individuals became substantially lower than in the controls (Fig. 1d).

After 10 days of culture, the number of adherent cells and amount of adherent cell protein (in proportion to the number of MO seeded) were significantly reduced in patients with HIV infection compared with the controls. This reduction was most pronounced in patients with AIDS (Fig. 2). The correlation coefficient between cell counts and the protein assay was 0·88.

The percentage (compared with the number of MO seeded) of viable non-adherent MO/MDM counted during cell culture did not differ between patients and controls (Fig. 3). The viability of the non-adherent MO/MDM was routinely >80%, both in patients and controls.

To determine the consistency of *in vitro* MO/MDM survival patterns, 10 patients were selected for a second MO culture after a mean period of 7 months (range, 4–10 months). During this period, the clinical condition and $CD4^+$ lymphocyte count were stable in these patients. When the results from the first and second culture were compared, the correlation coefficient was 0·76 for adherent cell protein and 0·92 for the number of adherent cells after 10 days of culture.

In the HIV-seronegative homosexual men, the *in vitro* survival of MO tended to be lower than in the blood donors, but not significantly so.

Macrophage differentiation in culture

In the cultures from patients with HIV infection, the percentage of cells expressing the antigens recognized by the antibodies 1D5 and 3D10 was significantly higher on day 10 compared with controls, (35% *versus* 23% for 1D5 and 53% *versus* 34% for 3D10) indicating a less mature cell population. On day 1, approximately 90% of MO expressed the 1D5 and 3D10 antigens both in patients with HIV infection and the controls. (Fig. 4).

Association between in vitro *survival and other immunological and virological parameters*

Patients with HIV infection were grouped according to numbers of $CD4^+$ and $CD8^+$ lymphocytes, lymphocyte mitogen re-

Table 2. *In vitro* survival of monocytes/macrophages from patients with HIV infection in relation to (a) HIV p24 antigen concentration (b) $CD4^+$ and (c) $CD8^+$ lymphocyte counts and (d) lymphocyte mitogen responses

	Number	μg adherent protein: day 10/10^6 monocytes seeded	% adherent cell nuclei: day 10/monocytes seeded
(a) HIV p24 antigen in serum			
< 20 pg/ml	47	74 (0–158)	15 (8–39)
> 20 pg/ml	12	0* (0–25)	0* (0–10)
(b) $CD4^+$ lymphocytes ($\times 10^6$/l)			
> 400	29	86 (13–177)	29 (6–47)
< 400	29	12* (0–39)	12* (0–18)
(c) $CD8^+$ lymphocytes ($\times 10^6$/l)			
> 800	27	81 (12–196)	18 (8–41)
< 800	31	13* (0–86)	11 (0–26)
(d) Lymphocyte mitogen responses			
Normal	26	101 (1–220)	34 (4–51)
PWM depressed	23	21* (0–81)	11 (0–22)
All depressed	10	21* (0–35)	13 (6–18)

Data are given as medians and 25–75 percentiles.
(a) * $P < 0.01$ *versus* individuals with HIV p24 < 20 pg/ml in serum.
(b) * $P < 0.05$ *versus* patients with $> 400 \times 10^6$ $CD4^+$ lymphocytes/l.
(c) * $P < 0.05$ *versus* patients with $> 800 \times 10^6$ $CD8^+$ lymphocytes/l.
(d) * $P < 0.05$ *versus* individuals with normal mitogen responses.

sponses and HIV p24 antigen status. *In vitro* survival was significantly lower in patients with low numbers of $CD4^+$ cells, $CD8^+$ cells, depressed lymphocytes mitogen responses and HIV p24 antigen in serum (Table 2).

Virological studies

In supernatants harvested after 3 and 10 days from monocyte cultures of 36 HIV-positive patients (28 CDC II/III, 8 CDC IV C1/D), the concentration of HIV p24 antigen was below 20 pg/ml in all but one supernatant.

In MO/MDM cultures examined after 1 ($n = 14$) and 10 ($n = 18$) days, less than 1 of 5000 cells expressed HIV antigens.

DISCUSSION

In the present study we observed that monocytes from most patients with HIV infection showed decreased survival in culture. When cultured for 10 days, only a small number of adherent macrophages were present, whereas blood donor monocytes grew to continuous macrophage monolayers.

Others have cultured monocytes from patients with HIV infection under apparently similar culture conditions, but have not reported decreased *in vitro* survival (Eales *et al.*, 1987; Murray *et al.*, 1987). From the published descriptions of culture methods, we find it difficult to discern any important differences in experimental conditions which might explain the discrepancy in monocyte viability *in vitro*. Our culture system is apparently adequate for culturing monocytes from healthy individuals. Even if our methodology does not ensure optimal *in vitro* conditions for monocytes, this may be advantageous for our purpose, enabling us to uncover biologically relevant defects in survival and differentiation of monocytes from patients with HIV infection.

When monocytes were cultured for 1 day, most cells expressed the antigens 1D5 and 3D10, both in patient and control cultures. After 10 days, however, a higher proportion of cells expressed these antigens in the patient cultures than in the controls, suggesting that the remaining population of MO/MDM was less differentiated in the former. Possibly, monocyte-to-macrophage differentiation was impaired in cultures from patients with HIV infection. Alternatively, a selective loss of differentiated macrophages, due to poorer *in vitro* survival compared with less-differentiated cells, could have explained our findings. Our data do not permit any definite conclusion concerning the mechanism.

Based on enzymatic activities in MO and MDM, Offen-

berger *et al.* (1986) have also reported that the differentiation of MO from patients with HIV infection is impaired. However, their data are difficult to interpret because they have assumed that the number of MO was constant during the culture period, which may not have been the case.

As monocytes and macrophages are known to be infected by HIV *in vivo*, the decreased *in vitro* survival of MO/MDM might be due to active HIV replication in the cultures. One possibility is that a low level of HIV replication in a high proportion of cells could contribute to the reduced *in vitro* survival. Also, HIV-derived proteins and other cell products released from a limited number of infected cells might contribute to the decreased MO/MDM *in vitro* survival. Alternatively, growth and maturation of MO precursor cells in bone marrow might be affected, resulting in functionally deficient MO with decreased vitality during culture.

The poorest MO/MDM survival was seen in patients with serious immune dysfunction measured as low numbers of $CD4^+$ and $CD8^+$ lymphocytes and depressed lymphocyte mitogen responses. Also, HIV p24 antigen in serum and advanced clinical stage were associated with poor *in vitro* survival of MO/MDM.

The biological and clinical significance of our *in vitro* results are unknown. They suggest, however, that the function of the monocyte-macrophage system may be impaired *in vivo* as has been shown by others (reviewed by Roy & Wainberg, 1988). This would contribute to the profound immune deficiency essential for the pathogenesis of HIV disease in man.

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