

Unique monocyte subset in patients with AIDS dementia

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Summary

Background 15–30% of patients infected with HIV will develop a debilitating dementia. Whilst HIV enters the brain soon after infection, presumably within monocyte-derived macrophages, not all patients with HIV become demented. Blood monocytes probably cross the blood-brain barrier and give rise ultimately to parenchyma macrophages. We looked for a specific monocyte subset in AIDS patients with dementia.

Methods Peripheral blood monocytes from three groups were compared: AIDS patients with (n=12) and without (n=11) dementia, and ten HIV seronegative healthy controls. We used flow cytometry to analyse monocytes, and cell lysis and apoptosis assays to examine monocyte effects on human brain cells in vitro.

Findings We found a unique subset of monocytes in patients with AIDS dementia. These monocytes were more dense and granular and expressed CD14/CD16 and CD14/CD69. Means (SD) for CD14/CD16 in HIV-negative controls and in AIDS non-dementia and AIDS dementia patients were 6.5% (4), 16% (13), and 37% (21), respectively (p=0.008 between the two groups of patients). The corresponding means for CD14/CD69 were 7% (6), 8% (10), and 69% (18) (p<0.0001).

Interpretation CD69 is a member of the natural-killer-cell gene complex that is expressed after activation. Supernatants from cultures containing these dense cells can trigger apoptosis of human brain cells in vitro. The monocyte subset we found in patients with AIDS dementia might enter the brain and expose neural cells to toxic factors.

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Introduction

Although the CD4 T-cell is the main peripheral-blood target of HIV, the macrophage is the cell predominantly infected in the brain. The monocyte, unlike the CD4 lymphocyte, is infected in a chronic non-lytic manner. The HIV-infected monocyte-derived macrophage may enter into the brain early in infection;¹ however, dementia occurs late in most patients, especially in severe immunosuppression. Investigators disagree about the mechanism(s) responsible for the cognitive dysfunction associated with AIDS, although most believe it is an indirect mechanism associated with macrophages.^{2–4} There is a stronger correlation between the presence and number of macrophages in the brain in AIDS dementia than the number of cells positive for HIV-1 antigen.² Soluble factors produced from HIV-infected monocyte-derived macrophages are neurotoxic in vitro.^{3,4} Specific factors produced from chronically activated and/or HIV-infected macrophages include tissue necrosis factor α (TNF α),^{5,6} platelet-activating factor,^{7,8} arachidonic acid metabolites,^{7,9} and quinolinic acid.^{10,11} Only TNF α and quinolinic acid are increased in the brains of patients with AIDS dementia.¹² We have examined peripheral-blood mononuclear cells from HIV-infected patients to determine whether a specific monocyte subset occurred more often in patients with dementia. We also studied whether soluble products from a monocyte subset caused neural-cell death in vitro.

Participants and methods

Blood samples

Individuals with AIDS dementia were recruited from St Mary's Medical Center Dementia Ward (San Francisco), and clinics of the University of California, San Francisco. Exclusion criteria included history of head injury, seizures, or multiple sclerosis, active opportunistic infection, active opportunistic central-nervous system infection or lymphoma, cerebrovascular disease, major psychiatric illness, other known causes of dementia, or pre-existing cause of brain disorder. Participants or their representatives gave informed consent. Individuals with CD4 counts below 200/ μ L (defined as AIDS) were selected from patients at San Francisco General Hospital. Blood was obtained in accordance with the Committee on Human Research. HIV-1 seronegative controls were recruited from laboratory personnel and gave informed consent.

Flow cytometry for monocyte subsets

5 mL blood was obtained from patients and controls. 100 μ L whole heparinised blood was stained with fluorescein-conjugated anti-CD14 (DAKO Corp) and with phycoerythrin-conjugated anti-CD16 and CD69 (Becton Dickinson). Red-blood cells were lysed by the addition of FACSLYSE solution (Becton Dickinson). The cell suspensions were centrifuged and the cell pellets resuspended in phosphate-buffered saline containing sodium azide and paraformaldehyde and stored at 40°C. To stain intracellularly for TNF α , cell pellets were resuspended in "Permeabilising Solution" (Becton Dickinson). Buffer was added to each tube, followed by centrifugation. Cell pellets were resuspended in buffer and stained with phycoerythrin-conjugated TNF α . The cells were washed, fixed, and stored as above.

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Normal controls		CD4 <200/ μ L			
%CD14/ CD16	%CD14/ CD69	AIDS		AIDS dementia	
		%CD14/ CD16	%CD14/ CD69	%CD14/ CD16	%CD14/ CD69
7	4	24	8	58	46
8	4	12	6	30	96
9	14	41	34	39	72
6	0	21	21	57	96
8	20	2	3	41	80
8	6	4	8	72	76
0	12	19	9	27	48
2	0	8	2	13	74
2	7	2	3	49†	65†
15	2	1	2	39	64
—	—	28	1	16	43
—	—	31	3	0	—
6.5 (4)*	7 (6)	16 (13)	8 (10)	37 (21)	69 (18)

*Mean (SD). †Patient in figure 2.

CD14/16 and CD14/69 cells in individual participants

Cells were analysed with a FACSCAN flow cytometer (Becton Dickinson). At least 2000 cells per sample were analysed. Negative staining was defined as the area of dot plots that contained over 99% of isotype-stained (DAKO) "control cells".

Another measure of density is the degree of granularity as measured by flow cytometry. We used light-scatter analysis on CD14-positive cells from non-demented HIV-infected patients, control patients, and patients with dementia.

Separation of peripheral blood monocytes

We had previously separated peripheral blood monocytes on Ficoll.⁴ We found that a standard Ficoll Hypaque separation gave lower yields of monocytes from demented patients than from HIV-infected controls (44 vs 67%, respectively). Several demented patients yielded under 10% of predicted, suggesting that a subset of monocytes was being missed. We then used Percoll gradient separation, which allows for denser cells to be captured. A two-step gradient was prepared in 15 mL conical tubes: bottom layer of 1.087 density Percoll, overlaid with 1.077 density. 1.5 mL whole heparinised blood was mixed with an equal volume of isotonic saline. This blood/saline was layered over the gradient and centrifuged. Cells from the 1.077 and 1.087 interfaces were collected and washed in 5 volumes of RPMI 1640. Cell pellets were resuspended and counted by haemocytometer.

Preparation of mononuclear cell supernatants

To collect a maximum number of monocytes (both heavy and light), we used a one-step Percoll protocol. 1.5 mL whole heparinised blood was mixed with an equal volume of isotonic phosphate-buffered solution and layered over 5 mL Percoll at 1.087. The cells were centrifuged, washed, and resuspended in RPMI 1640 supplemented with 10% fetal calf serum at 10⁶/mL. To separate non-adherent lymphocytes from adherent monocytes, culture dishes were washed with RPMI after overnight incubation. The adherent cells were re-fed with RPMI and cultured for 7 days to obtain supernatants. Supernatants were clarified by centrifugation at 90 000 *g* over 25% sucrose overnight. The supernatant was filtered through a 0.22 μ m syringe filter and stored at -70°C.

Brain aggregate cultures and supernatant treatment

Human fetal brain tissue from between 16 and 18 weeks' gestation was obtained from elective abortions for preparation of brain cell aggregates.¹³ These aggregates contain all the cells of the central nervous system including neurons, astrocytes, oligodendrocytes with accompanying myelin, and microglial cells. After 10 days in culture and before experimentation, aggregates were tested for viability by trypan-blue exclusion. Aggregates were divided into flasks at 50 mg wet-weight in 2 mL exchange medium (DMEM with 15% fetal calf serum). Aggregates were

treated with mononuclear cell supernatants (20%) for 3 days. The flasks contents were centrifuged; the supernatant was used for lactate dehydrogenase assay and the aggregate pellet for DNA fragmentation (programmed cell death) assay.

Quantification of cell death by ELISA

Lactate dehydrogenase is released on cell lysis. We used a cytotoxicity ELISA kit (Boehringer Mannheim). The brain aggregate supernatant was reacted with yellow tetrazolium salt and absorbance was read at 490 nm. The DNA fragmentation assay kit (Boehringer Mannheim) measures cytosolic oligonucleosome-bound DNA. The brain aggregate pellet was solubilised and used for this assay.

Electron microscopy

Brain cell aggregates were treated with 20% supernatant from cultured CD14 cells from a demented AIDS patient. Treatment was for 72 h; after treatment, the aggregates were washed and fixed in Karnovsky's solution. The cells were post-fixed in 1% osmium tetroxide dehydrated for staining with uranyl acetate and lead citrate. Thin sections were examined in a JEOL 100SX electron microscope.

Results

Forward-angle light-scatter analysis between the various populations of monocytes was similar, suggesting that the overall size of the monocytes between the various groups was the same. However, with side-scatter analysis, there was a significant increase in scatter among monocytes from patients with AIDS dementia compared with non-demented AIDS patients ($p=0.001$) (data not shown). Most cells from patients with dementia had side-scatter histograms over 1000.

To study whether the monocytes with increased granularity were activated, cells were co-stained with antibodies to CD14 and TNF α . Monocytes from seronegative controls had a mean of 1.5% (SD 2) positive cells while ten AIDS patients had a mean of 14% (SD 16) and 22 demented AIDS patients with under 200 CD4 cells per μ L had a mean of 21% (21). There was no significant difference between the percentages in patients with AIDS dementia and patients with AIDS.

Another marker for more differentiated monocytes is the cell-surface molecule CD16, an antigen that represents a type of Fc receptor¹⁴ and which expresses high levels of cytokines,¹⁴⁻¹⁷ indicative of a maturer tissue macrophage. Healthy HIV seronegative controls had low CD16 monocytes (table). Patients with AIDS had a mean of 16% (13) while AIDS dementia patients had a mean of 37%. There was a significant difference between the AIDS dementia and AIDS groups ($p=0.008$).

CD69, another marker of monocyte activation and a member of the natural-killer-cell gene family^{18,19} was also examined. The percent of CD69-expressing cells in normals (7 [6]) and AIDS patients (8 [10]) was similar, suggesting this is not a non-specific marker for activation. Patients with AIDS dementia had significantly elevated CD69 monocytes (69[18]) (table). The CD69-expressing cells all had high side-scatters (>500); many were over 1000.

If this subset of dense, activated CD69 monocytes is the peripheral link to damage in the brain parenchyma, it would be significant to demonstrate neurotoxicity in vitro from these cultured cells. Percoll-isolated monocytes from several patients with AIDS dementia and controls used in the studies in the table were cultured by adherence for 7 days. The supernatants were incubated (20%) with

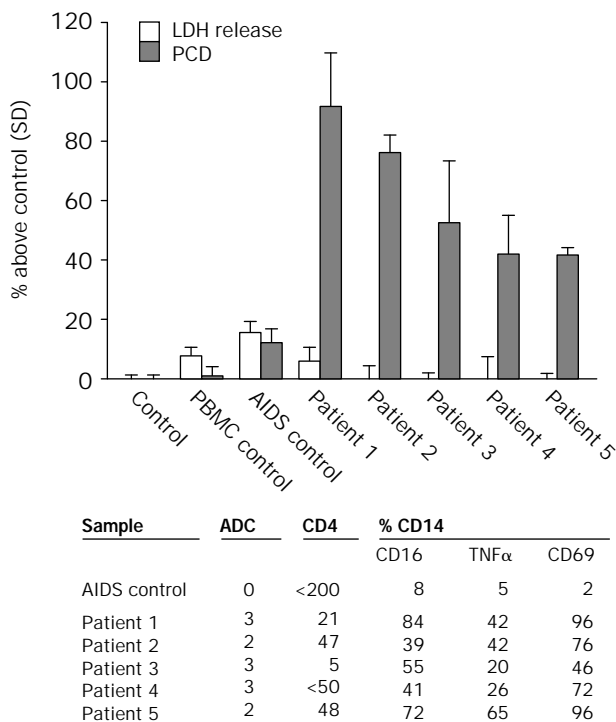


Figure 1: Effect of supernatants from cultured monocyte-derived macrophages of demented AIDS patients on human neural cells

Percoll-separated monocytes from five AIDS dementia patients were cultured for 7 days and supernatants (20%) used to treat human brain aggregates for 72 h. Controls included supernatant from peripheral blood monocytes of HIV-seronegative individual (peripheral-blood mononuclear cell [PBMC] control), cultured monocytes from non-demented AIDS patient (AIDS control), and untreated brain aggregates (control). Neural cell necrosis was measured by lactate dehydrogenase (LDH) release in supernatant and DNA fragmentation (programmed cell death, PCD), was measured by ELISA on brain aggregate pellet. Table describes patients and percent peripheral CD14 subsets. ADC refers to Memorial Sloan Kettering staging scheme for AIDS dementia complex.²⁰

human brain cell aggregates for 72 h followed by analysis for apoptotic versus necrotic cell death (figure 1). These supernatants caused significant apoptosis as measured by DNA fragmentation and electron microscopy (figure 2). Necrotic death, as measured by release of lactate dehydrogenase and trypan-blue exclusion (data not shown) was low to absent.

Discussion

Investigators have looked in the cerebrospinal fluid and serum for markers of immune activation specific for HIV neurological involvement, such as neopterin, β_2 -microglobulin, and quinolinic acid. These three are elevated in HIV encephalitis, although none showed a strong correlation with severity of HIV neurological involvement.²⁰ Quinolinic acid is increased in the cerebrospinal fluid and serum of patients with HIV infection and neurological involvement.²¹ Neopterin and β_2 -microglobulin are elevated in the cerebrospinal fluid of patients with HIV infection and central-nervous-system involvement; however, the results were non-specific and raised levels of neopterin were not related to monocytic pleocytosis.^{22,23} These products of immune activation were increased in the cerebrospinal fluid and serum of patients with AIDS.²⁴ Increased β_2 -microglobulin in cerebrospinal fluid, not in serum, was the only significant predictor of AIDS dementia.

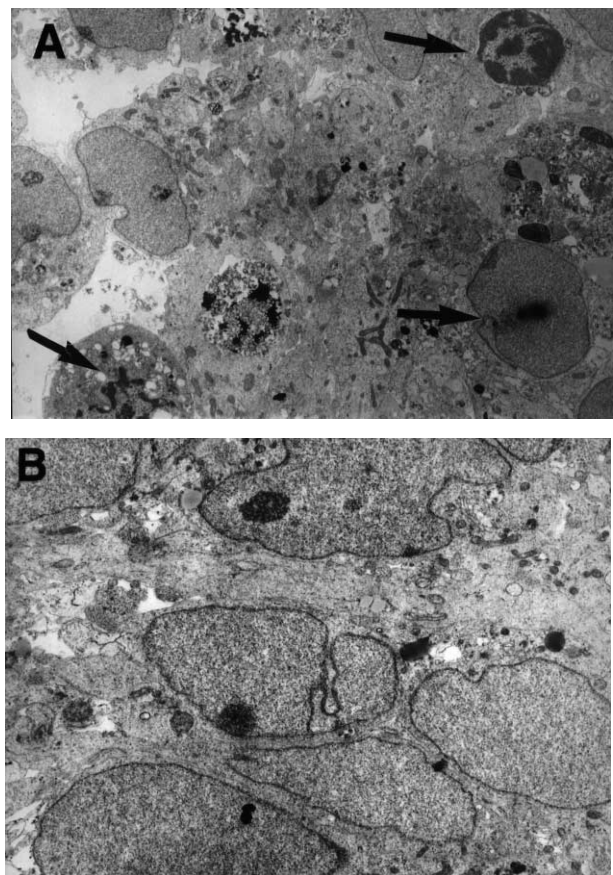


Figure 2: Transmission electron microscopy of neural cells within human brain aggregates

(A) Brain aggregates treated for 72 h with supernatant from monocyte-derived macrophages cultured from patient identified in table. Note neural cells undergoing characteristic stages of apoptosis including chromatin condensation and margination (arrows). Cell on bottom left appears to be undergoing nuclear apoptosis and secondary cytoplasmic necrosis (\times about 3000). (B) Brain aggregates treated for 72 h with supernatant from monocyte-derived macrophages of AIDS non-demented patient. Cells appear to be neurons and astrocytes and do not display characteristics of apoptosis or necrosis (\times about 5000).

We have looked at whether soluble factors produced from peripheral blood mononuclear cells could predict AIDS dementia.²⁵ We concluded that such factors were significantly more neurotoxic than those from healthy seropositive individuals. Production of TNF α and interleukin-6 was not a significant predictor.

Rather than look for specific cerebrospinal-fluid or serum products of immune activation, we were interested in whether a specific peripheral-blood subset of activated monocytes was elevated in patients with AIDS dementia. TNF α is increased in brains of patients with AIDS; however, there was no significant difference between circulating monocytes from AIDS patients and AIDS patients with dementia expressing this cytokine. The CD16 monocyte has properties consistent with a mature macrophage, including the expression of TNF α , HLA-DR, CD64, and CD11 antigens.^{15-17,26} The activated CD14/CD16 monocyte subset is increased during HIV infection and in AIDS.²⁶ This subset is further increased in patients with AIDS dementia.

We found that a monocyte subset marker, CD69, was significantly elevated in patients with AIDS dementia compared with those patients without dementia matched for CD4 count. This monocyte subset may enter the brain and chronically expose surrounding neural cells to toxic

factors leading to cellular dysfunction and apoptosis. The demented patients that have been studied thus far have all had moderate to severe (Memorial Sloan Kettering ≥ 2) dementia. Future experiments need to quantify the percent of CD14/CD69-expressing cells in patients with mild-to-moderate dementia. Patients with other types of dementia and with opportunistic infections of the central nervous system need to be studied to determine whether this increase is specific for HIV-associated dementia.

CD69 antigen is a member of the natural-killer gene complex and is inducible in T cells, B cells, natural-killer cells, monocytes, neutrophils, and eosinophils.¹⁹ Crosslinking of CD69 receptors on monocytes induces extracellular calcium influx, prostaglandin E₂, nitric oxide, and arachidonic acid metabolites,¹⁸ all potential neurotoxins implicated in AIDS dementia.^{7,12,27}

Neuronal loss and possibly atrophy associated with AIDS dementia is at least partly due to apoptosis.^{28,29} Apoptotic cell death is an active process requiring modulation in gene expression and protein synthesis. We found that supernatants from the dense activated monocyte-derived macrophages of demented patients caused significant neural-cell apoptosis in vitro. If these monocyte-derived macrophages traffic in and out of the brain, they may be the initiator of an apoptotic mechanism.

Investigators studying AIDS dementia need an objective marker to be used with neuropsychological testing and neuroimaging to screen new agents and possibly use in animal studies. A unique monocyte subset may also be a peripheral marker for therapeutic efficacy. Future studies need to determine whether and how this monocyte subset preferentially enters the brain parenchyma and what neurotoxic factors/cytokines these cells produce.

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