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Abnormal *in vitro* proliferation and differentiation of T colony forming cells in AIDS patients and clinically normal male homosexuals

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SUMMARY

T cell colonies were generated from the peripheral blood and bone marrow of 11 patients with acquired immune deficiency syndrome (AIDS), 17 normal male and female heterosexuals and seven clinically normal male homosexuals. Mononuclear cells were cultured in methylcellulose both in the absence and presence of interleukin-2 (IL-2) containing conditioned medium. Clinically normal homosexuals showed a low number of $T4^+$ ($P < 0{\cdot}01$) but not $T8^+$ cells. The number of T cell colony forming cells (T-CFC) from both AIDS patients and homosexuals was significantly ($P < 0{\cdot}01$) reduced compared to T-CFC from normal heterosexuals. In seven and four out of 11 AIDS patients, T-CFC from peripheral blood and bone marrow, respectively, were able to generate colonies in the absence of added growth factors and/or mitogenic stimulation. Pooled spontaneous and induced colonies from AIDS patients as well as induced colonies from normal homosexuals were composed of immature cells bearing the $T3^+$, $T4^+$, $T6^+$ $T8^+$ surface phenotype, unlike colonies from normal heterosexuals which displayed mature cells bearing the $T3^+$ $T4^+$ $T6^-$ and $T3^+$ $T8^+$ $T6^-$ surface phenotype. Moreover, most T-CFC from primary spontaneous and induced colonies had lost their self-renewal capacity either in the absence or the presence of added growth factors. These results suggest that early impairment of T-CFC may play a predominant role in the pathogenesis of AIDS.

Keywords AIDS T cell colonies T-CFC T cell differentiation

INTRODUCTION

The physiology of haemopoietic precursors has greatly benefited from the development of *in vitro* techniques to detect and quantify the stem cells of each cell lineage. Over the past several years, a variety of methods have been developed for the growth of T lymphocyte colonies from human peripheral blood leucocytes in semi-solid media (Fibach, Gerassi & Jachs, 1976; Riou *et al.*, 1976). These have allowed for enumeration of T colony forming cells (T-CFC) in normal or pathological states (Sutherland, Dalton & Wilson, 1976).

In vitro colony formation from normal peripheral blood and bone marrow T-CFC requires

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stimulation with phytohaemagglutinin (PHA) and/or the addition into the culture of interleukin-2 (IL-2) containing conditioned medium (Triebel *et al.*, 1981a, 1981b). These colonies are composed of well differentiated mature ($T3^+$ $T4^+$ $T6^-$ and $T3^+$ $T8^+$ $T6^-$ cells.

Patients with acquired immune deficiency syndrome (AIDS) have significant qualitative and quantitative T lymphocyte modifications including: (a) decreased absolute numbers of $T4^+$ cells resulting in the inversion $T4^+/T8^+$ ratio (Fauci *et al.*, 1984); (b) reduction of T cell proliferative responses to alloantigens and mitogens; (c) decreased IL-2 production (Fauci *et al.*, 1984; Gupta & Safal, 183; Stahl *et al.*, 1982; Talal & Shearer, 1983) and (d) the proportion of circulating T lymphocyte expressing IL-2 receptors after PHA stimulation is also reduced (Kwong, Fundenberg & Galbraith, 1984). Finally, at advanced stages of the disease a severe lymphopenia affecting all T cell subsets is usually observed (Fauci *et al.*, 1984; Stahl *et al.*, 1982; Talal & Shearer, 1983).

These qualitative and quantitative abnormalities of T lymphocytes and the evolution of the disease seem to indicate a primary involvement of T cell precursors in the pathogenesis of AIDS. We therefore studied the proliferation and differentiation capacity of T-CFC in clinically normal male homosexuals and AIDS patients.

MATERIALS AND METHODS

Patients. Heparinized peripheral blood and bone marrow were obtained from 11 AIDS patients fulfilling the criteria defined by the Center for Disease Control (CDC) (1982a, 1982b). All patients were male homosexuals, 29–50 years old and they were studied before any treatment was applied. Peripheral blood was also obtained from 17 male and female normal subjects and seven clinically normal male homosexuals consulting for various reasons such as an AIDS partner and/or having multiple sexual partners.

Cell separation. Peripheral blood (PBMC) and bone marrow (BMMC) mononuclear cells were obtained after Ficoll-Hypaque (Pharmacia Fine Chemicals, Sweden) density centrifugation ($d = 1{\cdot}077$). Interface cells were washed with Hank's balanced salt solution (HBSS) and resuspended in growth medium (α-MEM, GIBCO, USA) at 10^6/ml.

T cell colony assay. Five hundred thousand cells/ml were seeded in 0·8% methylcellulose (Fluka, Bucks, Switzerland) in α-MEM supplemented with 20% (vol./vol.) heat-inactivated fetal calf serum (FCS, GIBCO), 2 mM L-Glutamine and antibiotics, in the presence or absence of 20% (vol./vol.) of a 7 day supernatant from PHA stimulated (PHA-M 1%, vol./vol. DIFCO Lab., USA) normal PBMC (10^6 cells/ml in MEM supplemented with 10% FCS and 2 mM L-glutamine). This supernatant (PHA-LCM) contained 0·3 u/ml of IL-2 activity when tested in an IL-2-dependent human T cell line unresponsive to PHA (Bertoglio *et al.*, 1982). One tenth of a millilitre of the methylcellulose–cell preparation was seeded per well in flat bottomed well microtest plates (Nunc, Denmark) and incubated for 5–7 days at 37°C in 5% CO_2 in air. Aggregates containing more than 50 cells were counted as colonies under an inverted microscope.

Self-renewal capacity. The clonogenic capacity of T-CFC was studied by two culture procedures. First, PBMC or BMMC (10^6 cells/ml) were incubated in growth medium supplemented with 10% FCS and 2 mM L-glutamine for 4 days at 37°C in 5% CO_2 in air. Five hundred thousand washed viable cells/ml were seeded in methylcellulose as described above (delayed plating). The second method was to pick individual primary spontaneous colonies, pool and dissociate them by pipetting. After washing, 5×10^4 viable cells/ml were seeded as for primary colony growth, in the absence or presence of PHA-LCM.

Cell characterization. PBMC and BMMC were phenotyped using T3 (pan-T cell), T4 (helper/inducer) and T8 (suppressor/cytotoxic) monoclonal antibodies (MoAb) (Ortho, Raritan, New Jersey) and fluoresceinated goat anti-mouse immunoglobulin (Cappel Labs, Cochranville, USA) as a second reagent. Positive cells were determined using a spectrum III Cell Analyser (Ortho). Cells from pooled colonies were also phenotyped with the T series MoAb by indirect immunofluorescence. Because of the small numbers of cells recovered, a minimum of 50–100 cells were scored for each determination. Non-specific fluorescence was always lower than 5%. Slides

Table 1. Clinical, haematological, immunological characteristics and induced colony growth from peripheral blood and bone marrow T-CFC of AIDS patients, clinically normal, lymphopenic homosexuals and normal heterosexuals

Patient (age/sex)	Diagnosis*	Total lymphocytes ($\times 10^9$/l)	Number of cells/ml of peripheral blood				Bone marrow Number of colonies§ (5×10^4 cells)
			PBMC† ($\times 10^5$)	T4$^+$ ($\times 10^3$)	T8$^+$ ($\times 10^3$)	T-CFC‡	
1 (50/M)	KS	0·64	1·8	39·6	115·2	1,152±68	0
2 (33/M)	KS	2·05	3·25	71·5	143	2,314±98	236±17
3 (30/M)	KS	1·14	0·28	2·8	12·2	114±12	120±19
4 (37/M)	TG	0·74	1·63	9·8	81·7	725±105	252±12
5 (32/M)	CS	1·05	1	9	65	966±58	570±31
6 (29/M)	PCP	0·5	0·33	5·0	23·3	710±55	191±18
7 (29/M)	KS+SAL	1·08	3·73	51·5	233·5	2,225±21	8±1
8 (37/M)	KS+PCP+CAND	1·5	2	20	136	168±12	35±7
9 (37/M)	KS+PCP	0·83	0·12	0·01	6·9	166±9	40±6
10 (35/M)	PML	0·8	2·05	1·02	123	0	100±21
11 (35/M)	TG	1·3	2·47	74	76·5	858±79	0
Mean±s.d.		1·06±0·44	1·7±1·19	25·8±28·4	92·4±67·4	854±798	
Control homosexuals	1·9±0·4	6·5±3·8	251±175	178±139	919±342	ND¶	
Normal heterosexuals	2·4±0·5	10·2±3·9	510±196	155±98	7,548±140	246±86**	

* KS = Kaposi's sarcoma, TG = *Toxoplasma gondii*, CS = cryotosporidiosis, SAL = salmonellosis, CAND = generalized candidiasis, PCP = *Pneumocystis carinii* pneumonia, PML = progressive multifocal leukoencephalopathy.

† PBMC were obtained after Ficoll-Hypaque density centrifugation.

‡ Five hundred thousand PBMC/ml were seeded in methylcellulose with an IL-2 containing conditioned medium (PHA-LCM) and incubated as described in Materials and Methods. After 5–7 days, aggregates containing more than 50 cells were counted as colonies. Results are expressed as mean±s.d. of circulating T-CFC/ml of peripheral blood from triplicate wells.

§ BMMC (5×10^5/ml) were seeded in methylcellulose under the same condition as PBMC. Results are expressed as mean±s.d. of colonies/5×10^4.

¶ ND = not done.

** Mean values reported by Triebel *et al* (1981a, 1981b).

were also prepared by cytocentrifugation and stained with May–Grunwald–Giemsa for morphological examination.

Statistical analysis. Statistical comparison of the mean percent positivity for a given surface marker was done using the Student's *t*-test.

RESULTS

Haematological and immunological profile of AIDS patients

As shown in Table 1, six patients had Kaposi's sarcoma that was associated in three cases with opportunistic infections. Severe opportunistic infections were also observed in four additional patients without Kaposi's sarcoma and one patient had progressive multifocal leukoencephalopathy. As expected, AIDS patients showed low numbers of PBMC/ml in peripheral blood, lymphopenia, and a dramatic decrease of $T4^+$ cells. The number of $T8^+$ cells was also low except in patient No. 7. The same trend of decreased numbers of PBMC and $T4^+$ ($P < 0{\cdot}05$) but not $T8^+$ cells was observed in the peripheral blood of clinically normal male homosexuals as compared with the controls.

T cell colony growth

The number of peripheral blood T-CFC from all AIDS patients was significantly reduced when compared to normal heterosexuals ($P < 0{\cdot}001$). Their numbers showed a linear correlation with that of $T4^+$ ($r = 0{\cdot}75$, $P < 0{\cdot}01$) and $T8^+$ cells ($r = 0{\cdot}64$, $P < 0{\cdot}05$). In patient 10 (Table 1), no colony growth could be obtained from PBMC whereas BMMC generated a significant number of colonies. The reverse was observed in patients 1 and 11 (Table 1). It is noteworthy that clinically normal homosexuals also presented a decreased absolute number of peripheral blood T-CFC. In seven and four out of 11 AIDS patients some clonogenic cells from the peripheral blood and bone marrow, respectively, generated colonies in the absence of added growth factors (Table 2). Small aggregates, containing about 10 cells, were detected on day 2 of the culture and continuously grew, to form discrete colonies on the surface of the methylcellulose reaching their maximum size (about 200 cells) on day 5–7. No spontaneous colonies could be obtained from PBMC of normal controls and clinically normal homosexuals.

Table 2. Spontaneous colony growth from peripheral blood and bone marrow T-CFC of AIDS patients

Patient	Number of T-CFC/ml of peripheral blood*	Number of colonies/5×10^4 BMMC*
1	65 ± 7	0
2	754 ± 91	46 ± 4
3	34 ± 6	0
4	376 ± 46	316 ± 12
5	60 ± 6	830 ± 43
6	360 ± 19	0
7	824 ± 6	0
8	0	0
9	0	0
10	0	0
11	0	0
Control homosexuals	0	ND†
Normal heterosexuals	0	0

* PBMC and BMMC (5×10^5/ml) were seeded in methylcellulose in the absence of added growth factors or mitogenic stimulation and incubated as described in Materials and Methods. Results are expressed as mean $\pm$ s.d. of T-CFC and number of colonies for the PBMC and BMMC, respectively, from triplicate wells.

† Not done.

Table 3. Phenotypic characterization of peripheral blood and bone marrow derived spontaneous and induced T cell colonies

		Spontaneous colonies*					Induced colonies*				
Patient	Origin of colonies	T11	T3	T4	T6	T8	T11	T3	T4	T6	T8
1	PBMC		NCG†				65	67	61	67	64
2	PBMC	87‡	70	76	74	67	54	67	64	70	65
	BMMC	72	80	60	70	71	75	87	50	80	71
3	PBMC	ND§	ND	80	71	71	ND	ND	80	75	67
	BMMC		NCG				ND	ND	79	80	73
4	PBMC	89	65	77	47	87	81	59	72	78	71
	BMMC	75	68	52	59	57	ND	52	31	44	80
6	PBMC	60	50	50	60	58	ND	ND	ND	ND	ND
7	PBMC	ND	ND	56	63	60	ND	ND	80	87	73
8	PBMC		NCG				57	76	64	60	70
	BMMC		NCG				60	75	72	ND	42
	Mean ±s.d.	76±12	66·6±10·8	64±12	63±9·3	67±10·5	65±10·6	69±11·6	65·3±5	72±12	67·6±10
Control homosexuals	PBMC		NCG				80±8	79±9	60±11	50±14	48±15
Normal heterosexuals	PBMC		NCG				76±6	75±6	47±13	17±5	35±4

* Pooled spontaneous and induced colonies were dissociated, washed with culture medium and colony cells were phenotyped with MoAb by indirect immunofluorescence.

† No colony growth.

‡ Percentage of positive colony cells.

§ Not determined.

Table 4. Delayed plating efficiency of peripheral blood T-CFC from AIDS patients

Patients	Immediate plating*		Delayed plating†	
	Spontaneous	Induced	Spontaneous	Induced
2	116±17	356±27	0	18±3
3	60±7	202±19	0	0
4	115±21	222±31	0	228±21
7	120±19	324±28	0	0
8	0	42±5	0	0
Control homosexuals ($n=3$)	0	203±46	0	0
Normal subjects ($n=3$)	0	370±179	0	185±86

* PBMC (5×10^5/ml) were seeded in methylcellulose immediately after Ficoll-Hypaque separation and incubated as described. Results represent the mean number of colonies ±s.d. from triplicate wells.

† PBMC (10^6/ml) were incubated in α-MEM with 10% FCS and L-glutamine for 3 days as described in Materials and Methods. Five hundred thousand viable cells/ml were seeded in methylcellulose, in the absence and the presence of IL-2 containing conditioned medium. Results are expressed as the mean number of colonies ±s.d. from triplicate wells.

Colony characterization

T cell colonies from normal subjects were composed of mature lymphoblastoid cells bearing the $T11^+$, $T3^+$, $T4^+$ or $T8^+$ phenotype. The percentage of $T6^+$ cell detected in the colonies was only 17% (Table 3). PBMC and BMMC derived induced colonies from AIDS patients were composed of significantly more $T4^+$ (50–80%, $P<0.01$), $T6^+$ (44–87%, $P<0.01$) and $T8^+$ (42–80%, $P<0.001$) cells than colonies from normal subjects. Interestingly, PBMC derived colonies from clinically normal homosexuals were also composed of significantly more $T6^+$ (50–77%, $P<0.01$) and $T8^+$ (40–60%, $P<0.05$) but not $T4^+$ cells. Pooled spontaneous colonies were also composed of lymphoblastoid cells bearing surface markers of the T cell lineage. The phenotypic characterization revealed 50–80% $T4^+$, 47–87% $T6^+$ and 57–87% $T8^+$ cells. In the majority of the patients (Nos 2, 3, 4, 7; Table 3) the phenotype of cells from spontaneous colonies was not significantly different from that of induced colonies.

Self-renewal capacity

Cells from pooled primary spontaneous colonies were replated in methylcellulose in order to evaluate their self-renewal capacity in the absence and the presence of added growth factors. In five patients studied (cases 2, 3, 4, 7, 8), no secondary colony growth could be obtained (data not shown). The proliferative capacity of peripheral blood T-CFC was also tested after a 4 day liquid culture of PBMC in the absence of added growth factors or/and mitogen. This delayed plating efficiency has been proposed as a measure of the proliferative capacity of the clonogenic cells (Nicola *et al.*, 1981). In four AIDS patients and three control homosexuals no delayed spontaneous colony growth could be obtained. Similarly, in four out of five AIDS patients and in three control homosexuals delayed colony formation induced wth PHA-LCM was significantly decreased (Table 4). Conversely, in normal heterosexuals the delayed plating yielded the half of the observed immediate colony number (Table 4).

DISCUSSION

The primary intent of these investigations was to study quantitatively and qualitatively the peripheral blood and bone marrow T-CFC of AIDS patients. In normal subjects, T-CFC have

either an immature (E-T3$^-$) or presumably mature (E$^+$) phenotype (Triebel *et al.*, 1981a, 1981b; Claesson *et al.*, 1977; Rozenszajn, Shohaw & Kalecheman, 1975; Georgoulias *et al.*, 1984).

In AIDS patients, as it has been previously reported by many investigators (Fauci *et al.*, 1984; Talal & Shearer, 1983), we found an absolute lymphopenia with a marked decrease of T4$^+$ and T8$^+$ cells in peripheral blood. Moreover, patients displayed a dramatically decreased T cell colony growth. The absolute number of T-CFC was significantly correlated with that of T4$^+$ and T8$^+$ lymphocytes, suggesting mainly a depletion of E$^+$ clonogenic cells. However, we cannot exclude, at the present time, that inhibitory mechanisms could also account for the decreased plating efficiency.

Some peripheral blood and bone marrow clonogenic cells from AIDS patients were able to generate T cell colonies in the absence of added growth factors. This phenomenon has not been observed in lymphopenic clinically normal homosexuals or normal heterosexuals. The biological significance of these spontaneous colonies, apparently independent of added growth factors, is not yet known. We have previously observed spontaneous colonies in two hemopoietic malignancies. (1) In human T cell neoplasias, T-CFC can generate spontaneous colonies (Georgoulias, Auclair & Jasmin, 1984). This spontaneous proliferation of T-CFC could be explained by the constitutive release of some growth factor(s) (T cell colony promoting activity; T-CPA) leading to an endogenous activation and proliferation of T-CFC (Georgoulias, Allouche & Jasmin, 1984). These spontaneously proliferating T-CFC possessed a great self-renewal capacity in the absence of added growth factors. Conversely, spontaneously proliferating T-CFC from AIDS patients have lost their self-renewal capacity, which is a property of primitive stem cells.

(2) We have also described that susceptible mice inoculated with the Myeloproliferative sarcoma virus (MPSV) show a spontaneous growth of granulo-macrophagic precursors (GM-CFU) which have lost self-renewal capacity in the absence of colony stimulating activity (CSA) (Klein *et al.*, 1982). It is therefore probable that such spontaneous GM-CFU represent cycling and fully committed cells. The spontaneous proliferation of T-CFC without self-renewal capacity in AIDS patients is compatible with this model.

In AIDS patients pooled spontaneous and induced T cell colonies were composed of a high proportion of cells bearing the T4$^+$ T6$^+$ T8$^+$ surface phenotype. Conversely, colonies generated from normal subjects displayed less than 20% and 40% T6$^+$ and T8$^+$ cells, respectively (Triebel *et al.*, 1981a, 1981b; Georgoulias *et al.*, 1984). Since a similar proportion of colony cells could be stained with the T4, T6 and T8 MoAb it could be assumed that most of them expressed, at the same time, all these antigenic determinants. However, double staining studies are needed to verify this hypothesis. The increased expression of T6$^+$ antigen which characterizes immature lymphocytes (common thymocytes; Reinherz & Schlossman, 1980), indicates a block of the *in vitro* differentiation of patients T-CFC. This abnormal differentiation of T-CFC was revealed in both induced and spontaneous colonies, indicating that this phenomenon is independent of added IL-2. This observation strongly suggests that the *in vivo* observed reduced T4$^+$/T8$^+$ ratio in AIDS patients is not due to the impaired IL-2 production by patients' lymphocytes (Fauci *et al.*, 1984; Stahl *et al.*, 1982; Talal & Shearer, 1983). However, since pooled colonies were used for phenotypic studies it is unclear whether all colonies were composed of immature T cells or whether some of them were composed of more differentiated cells.

T4$^+$ but not T8$^+$ cells were decreased in the peripheral blood of clinically normal male homosexuals as previously described (Pinching *et al.*, 1983). The observation that peripheral blood T-CFC from these subjects were dramatically reduced (Table 1), may be due either to decreased T-CFC numbers or/and to inhibition of their proliferation. It is noteworthy, that T-CFC also showed impaired self-renewal capacity and abnormal *in vitro* differentiation as in AIDS patients. These findings might indicate that abnormalities of T-CFC could represent early events in the establishment of an immunosuppressed status of male homosexuals (Pinching *et al.*, 1983). Recent studies strongly suggest the involvement of retrovirus (LAV: lymphadenopathy associated virus and HTLV III: human T leukaemia/lymphoma virus), in the causation of AIDS (Barre-Sinoussi *et al.*, 1984; Klatzman *et al.*, 1984; Sarngdharan *et al.*, 1984). Indeed, these viruses may specifically lyse T4$^+$ cells although it cannot be excluded that LAV and/or HTLV III can also infect clonogenic T-CFC, blocking their differentiation and/or lysing them. Although it is likely that failure of antibody production could account for seronegative results, it can be reasonably assumed that

AIDS patients had been infected with LAV/HTLV III. The virological results, obtained in our patients indicated that only three out of 11 AIDS and two out of seven clinically normal homosexuals had serum antibodies against LAV (not shown). Moreover, two seropositive as well as four out of eight seronegative patients with AIDS but none of the normal male homosexuals generated spontaneous colonies. These findings do not allow any definitive conclusion on the role of LAV infection in the generation of spontaneous colonies.

These abnormalities of T-CFC proliferation and differentiation in AIDS patients and normal male homosexuals suggest a pathophysiological scheme of AIDS. In an early asymptomatic phase, the causative agent(s) may either affect directly T-CFC inducing an abnormal differentiation or/and lysing mature $T4^+$ but not $T8^+$ lymphocytes. These hypotheses are supported by our findings in clinically normal male homosexuals. As the proportion of peripheral blood mature T lymphocytes decreases, there may be a compensatory proliferative commitment and activation of the most primitive and quiescent (Georgoulias *et al.*, 1984) T lymphocyte precursors, which can proliferate in the absence of added growth factors. During the evolution of the disease, T-CFC become progressively exhausted and/or colony inhibitory mechanisms could be activated. Finally, at the terminal phase of the disease, the exhaustion of T-CFC could lead to a complete lymphoid aplasia. Additional prospective biological and clinical studies concerning the constitutive production of IL-2 and/or other T cell growth factor(s) as well as the capacity of patients' T-CFC to respond to them during the different clinical phases are under way further elucidate the pathophysiology of AIDS.

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