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Impairment in proliferation, lymphokine production and frequency distribution of mitogen-responsive and interleukin-2-producing cells in Hodgkin's disease

Rajendra N. Damle[1], Suresh H. Advani[2], and Sudha G. Gangal[1]

[1] Cancer Research Institute, and [2] Tata Memorial Hospital, Tata Memorial Centre, Parel, Bombay-400012, India

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Summary. In this paper, we have correlated the ability of peripheral blood lymphocytes (PBL) from Hodgkin's Disease patients to proliferate in response to a mitogen, phytohaemagglutinin (PHA), with production of lymphokines interleukin-2 (IL-2) and interferon γ (IFNγ), accumulating in the activated lymphocyte culture supernatants. We have also studied the frequency distribution of PHA-responsive and IL-2-producing T cells from PBL using limiting-dilution analysis. We observed that the levels of IL-2 and IFNγ in the supernatants of activated lymphocytes from patients with Hodgkin's disease were significantly reduced compared to those of healthy donors. Substage-B patients showed marked reduction in the ability to produce IFNγ. Levels of IL-2 and IFNγ in the culture supernatants of PBL from Hodgkin's disease patients correlated positively with proliferative responses, when analysed by linear regression ($r = 0.79$ and $r = 0.60$ respectively). However, production of the two lymphokines by activated lymphocytes from the same patients did not correlate ($r = +0.04$). Further, the frequencies of PHA-responsive cells and IL-2-producing cells in the PBL of patients with Hodgkin's disease (ranges 1/111–1/554 and 1/3009–1/6709 respectively) were also less than those of the healthy donors (ranges 1/80–1/181 and 1/761–1/1828 respectively). Proliferation, IL-2 production in bulk cultures and frequencies of PHA-responsive and IL-2-producing cells correlated well in individual healthy donors. Whereas, one patient (BC 11214) with a frequency of PHA-responsive cells within normal limits had a very low frequency of IL-2-producing cells. Taken together, the results indicate abnormalities in cytokine production and frequency distribution of cells required for amplification of immune response in patients with Hodgkin's disease.

Key words: Hodgkin's disease – T cell hyporesponsiveness – Lymphokines – Limiting-dilution analysis

Introduction

Hodgkin's disease (HD), a malignancy of the lymphoreticular system, is characteristically associated with defective T lymphocyte functions [8, 27]. We have earlier demonstrated depressed responses of HD lymphocytes to mitogens [17], lowered colony-forming ability of activated T cells [20] and insufficient restoration of these defects by supplementation with exogenous interleukin-2 (IL-2) [21]. We have also demonstrated abnormalities in IL-2 production, IL-2 receptor (Tac antigen) expression and high- and low-affinity IL-2 receptor status on activated lymphocytes from HD patients [4, 13].

Lymphocytes, upon activation with mitogens and antigens, undergo a cascade of events, which include activation of genes encoding lymphokines and their receptors, responsible for triggering of the cellular machinery involved in clonal expansion [14, 25]. IL-2 is the authentic G_1 progression factor for the T cells [28], while recently it has been reported that interferon γ (IFNγ) also acts as a growth-promoting factor for T cells [15]. The effective immune response needs a critical number of immunocompetent cells capable of recognising the antigen/mitogen stimulus and generating IL-2 required for proliferation of immune cells. Limiting-dilution assays are sensitive microculture techniques used to estimate the frequency distribution of immunocompetent cells with specific, measurable functions [16, 29].

In this paper, we have studied the ability of peripheral blood lymphocytes (PBL) from HD patients to proliferate in response to phytohaemagglutinin (PHA), and to generate IL-2 and IFNγ. We have also studied the frequency distribution of PHA-responsive cells and IL-2-producing cells in HD. The results showed that activated lymphocytes from HD patients are deficient in all the parameters investigated.

Offprint requests to: S. G. Gangal

Materials and methods

Lymphocyte donors

The studies were conducted on 40 untreated HD patients having all stages and grades of the disease, aged between 20 and 57 years. As controls, 25 healthy donors (patients' relatives or laboratory personnel) aged between 21 and 45 years with a similar proportion of male volunteers were studied.

Proliferation and lymphokine production

Peripheral blood lymphocytes (PBL) were separated from heparinized blood using a Ficoll/Hypaque gradient [3]. The PBL were suspended in Dulbeccos modified Eagle's medium (Gibco, USA) supplemented with antibiotics, glutamine (4 mM), 2-mercaptoethanol (0.05 mM) and 10% human AB blood-group serum, at a concentration of 1×10^6 cells/ml. Lymphocytes were cultured in microtest plates (200 µl/well, round-bottom, Nunc, Denmark) in replicates of six with or without PHA-M (Gibco, USA 0.5% v/v). After 24 h, 100-µl samples of supernatants collected from control and "stimulated" cultures were pooled separately and stored at −20° C for further quantification of IL-2 and IFNγ. A parallel set of cultures was used for proliferation, assayed by tritium-labelled thymidine ([^{3}H]dT; Board of Radiation and Isotope Technology) incorporation, as described before [4].

Interleukin-2 assay

Mouse cytotoxic T lymphocyte (CTLL) cells, which are dependent on IL-2 for their growth, were used for the assay [12]. Six replicates of CTLL microcultures received 25% v/v lymphocyte culture supernatants to be tested for IL-2, while six replicates were left as controls. The cells were incubated for 48 h, in the last 16–18 h of which they received 0.5 µCi [^{3}H]-dT/well. Results are expressed as net [^{3}H]dT (cpm) incorporated by CTLL cells.

Interferon γ assay

IFNγ activity in the culture supernatants was assessed as previously described [2, 22]. Briefly, WISH cells (Wistar Institute, Susan Hayflick) suspended in Iscovés modified Dulbecco's medium (IMDM) (Gibco, USA) supplemented with 10% fetal calf serum were grown in 96-well flat-bottom microtest plates. Monolayers of WISH cells in microtest plates were incubated with serially diluted culture supernatants (12 dilutions) for 18 h. One row served as control. The washed microtest cultures were challenged with appropriately diluted vesicular stomatitis virus for 20–24 h. The washed plates were treated with neutral red (Sigma, USA, final dilution 0.06% v/v in Hank's balanced salt solution) for 2 h at 37° C. The dye taken up by the cells was eluted using 50% ethanol containing 100 mM acetic acid, and the absorbance was read at 540 nm (Organon Technica Microtest plate reader, Belgium). Results were expressed as the amount of interferon (U) in culture supernatants, derived from the standard curve obtained from serially diluted standard IFNγ (NIH, USA).

Limiting-dilution analysis

PHA-responsive cells. T lymphocytes were separated from non-adherent PBL on nylon-wool columns (Fenwal Labs., USA). About 90% of the resultant cells were OKT3-positive. The T cells were seeded in limiting numbers ranging from 2000–31 or 50 cells/200 µl medium in 24 replicates along with 10^4 irradiated autologous PBL (30 Gy using a ^{60}Co source) as feeders, in 96-well round-bottom plates. Equal number of replicates, which did not receive responder T cells, served as controls. PHA-M (0.5% v/v) was added to all wells and the cultures were incubated for 3 days in a CO_2 incubator. Samples of 100 µl supernatant from all cultures were replaced with 100 µl fresh medium containing 2 U rIL-2 (Cetus Corporation, USA). The plates were further incubated for a period of 48 h, and [^{3}H]dT incorporated in the last 16–18 h was measured. The cultures showing radioactivity more than the mean +3 SD of controls were scored as "responders". The frequencies were calculated according to the method of Taswell [29] based on the Poisson equation.

IL-2-producing cells. Feeder layers containing 10^4 irradiated PBL were plated in each well in round-bottom microtest plates and incubated overnight in a CO_2 incubator. Since standardization experiments showed that the supernatants of the feeder cells contained a measurable IL-2 activity, in further experiments, 75% of each supernatant was discarded before nylon-wool-eluted T cells of the same donor were added. Microtest plates received T cells in the range of 12000–400 cells/well in 24 replicates with 0.5% v/v PHA-M. The cultures were incubated for 24 h and 100 µl supernatants were transferred well-by-well to another set of microtest plates, which already contained 10^4 washed CTLL cells/well, for IL-2 assay. The frequencies of IL-2-producing cells were determined as mentioned above.

Statistical analysis

Statistical validity of the data was determined by Student's *t*-test. Correlation between two parameters was determined as described earlier [24].

Results

Correlation between lymphokine production and proliferative response

Activated lymphocytes from HD patients as a group showed significantly less proliferative response and lower amounts of bioactive IL-2 and IFNγ in the culture supernatants as compared to those from healthy donors, ($P < 0.001$; Table 1). Like the proliferative responses, the IL-2 content in the supernatants showed gradation in activity depending on the stage of the disease (Table 1). However, proliferation as well as IL-2 production by lymphocytes from substage I + II A patients were also significantly less than healthy donors.

Patients with substage I + II B of the disease showed significantly lower amounts of IFNγ accumulating in the activated lymphocyte culture supernatants, than those with substage I + II A. The proliferative response correlated positively with the production of IL-2 ($P < 0.001$, Fig. 1) and IFNγ ($P < 0.05$, Fig. 2) when these parameters were studied using PBL from the same patients. However, when the levels of IL-2 and IFNγ produced by activated lymphocytes from the same patients were compared, the parameters did not correlate ($r = 0.04$, data not shown).

Frequency analysis

With the assay procedure used by us, the frequency distribution of PHA-responsive cells in 10 healthy donors ranged between 1/80 and 1/181 (Table 2). Whereas, in 8 HD patients studied, the frequency of PHA-reactive cells was much lower ($P < 0.001$), varying between 1/111 and 1/554. The stage of the disease did not affect the proportion of circulating, PHA-responsive cells.

Table 1. Proliferation and cytokine production by phytohaemagglutinin(PHA)-activated peripheral blood lymphocytes (PBL)

Study group	Proliferation (net mean cpm) ± SE	Levels of cytokines in supernatants	
		IL-2 (net mean cpm) ± SE	IFNγ (U/ml)
Healthy donors	88 374 ± 7286 ($n = 20$)	28 156 ± 3910 ($n = 13$)	785 ± 175 ($n = 16$)
Hodgkin's disease patients	37 012 ± 4331 ($n = 24$; $P < 0.001$)*	8853 ± 1641 ($n = 24$; $P < 0.001$)*	293 ± 74 ($n = 12$; $P < 0.02$)*
Stage I + II A	42 577 ± 5516 ($n = 14$)	11 150 ± 2330 ($n = 14$)	411 ± 84 ($n = 8$)
Stage I + II B	33 350 ± 8068 ($n = 7$; NS)**	6513 ± 2036 ($n = 7$; NS)**	59 ± 6 ($n = 4$; $P < 0.01$)**
Stage I + II A + B	39 502 ± 4654 ($n = 21$; $P < 0.001$)*	9605 ± 1761 ($n = 21$; $P < 0.001$)*	293 ± 74 ($n = 12$; $P < 0.02$)*
Stage III + IV B	19 582 ± 4843 ($n = 3$; $P < 0.01$)***	3590 ± 1627 ($n = 3$; $P < 0.02$)***	ND

NS, not significant; ND, not done
Compared to healthy donors
** Compared to stage I + II A
*** Compared to stage I + II A + B

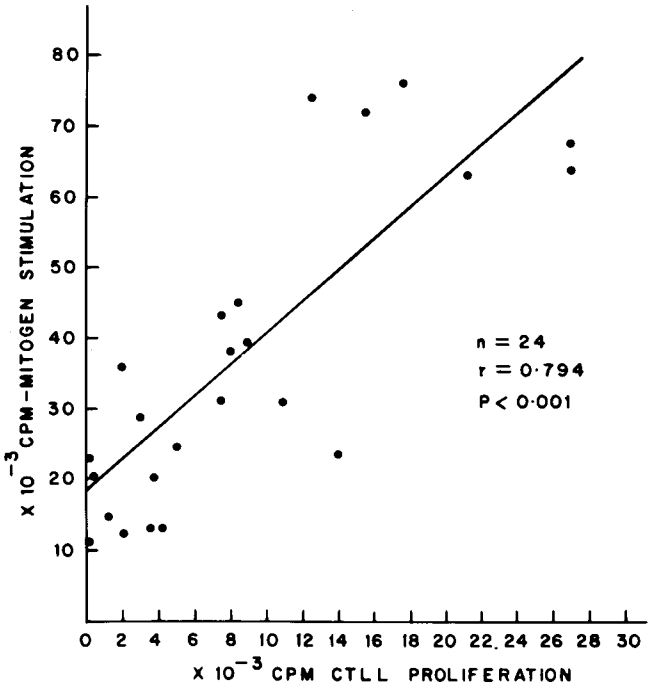

Fig. 1. Correlation of proliferation with interleukin-2 production by mitogen-activated lymphocytes from Hodgkin's disease patients

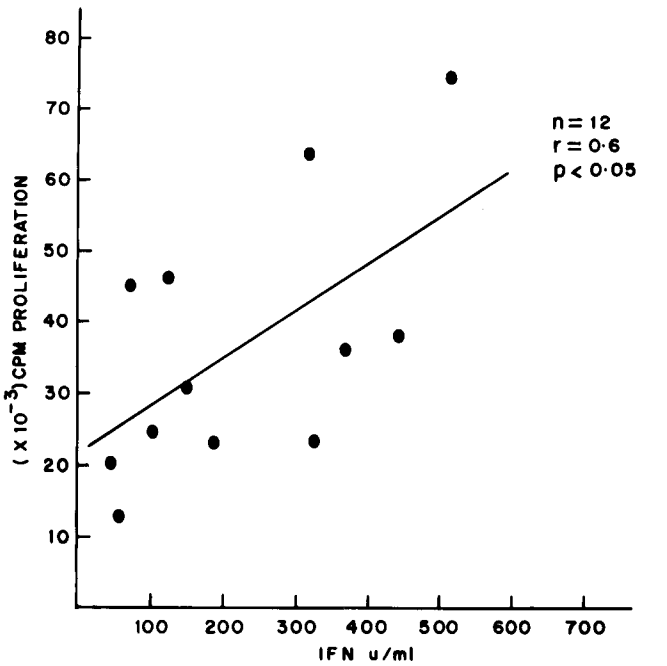

Fig. 2. Correlation of proliferation with interferon γ production by mitogen-activated lymphocytes from HD patients

The frequency of IL-2-producing cells was studied in 5 HD patients and 5 healthy donors (Fig. 3). The frequency distribution of T cells from PBL of HD patients, screened under conditions of limiting dilution adopted by us, varied between 1/3009 and 1/6709, and were significantly lower than those for healthy donors ($P < 0.01$). These 5 HD patients belonged to stages I and III, which shows that there was no correlation between the progression of the disease and frequency of IL-2-producing cells.

From amongst the group of healthy donors and HD patients studied for frequency analysis, 5 healthy donors were studied for proliferation and IL-2 production in bulk cultures as well as for frequencies. Whereas, 3 HD patients were studied for both frequencies (but not bulk cultures) (Table 3). It could be seen that, in the case of healthy donors, the frequencies of PHA-responsive and IL-2-producing cells compared well with each other, and with the proliferation and IL-2 production in bulk cultures. On the other hand, although 1 of the 3 HD patients studied had PHA-responsive cells within the normal range, a large number of these cells were incapable of accumulating adequate levels of IL-2 in 24 h, as reflected in the lower frequency distribution of IL-2-producing cells.

Table 2. Frequency distribution of PHA-responsive cells in the PBL of Hodgkin's disease (HD) patients and healthy donors

HD patients				Healthy donors		
Case no.	stage/grade	Frequency	95% Confidence limits^{-1}	No.	Frequency	95% Confidence limits^{-1}
BC 11214	IB/MC	1/111	94–141	1	1/90	63–162
BC 11717	IIIB/MC	1/217	136–555	2	1/120	71–391
BC 14191	IA/MC	1/240	161–303	3	1/151	91–465
BC 14832	IIIB/LP	1/321	193–989	4	1/80	42–119
BC 16083	IA/LP	1/554	428–800	5	1/138	126–154
BC 16435	IA/MC	1/548	488–633	6	1/181	139–261
BC 17070	IIIB/NS	1/329	286–392	7	1/112	94–150
BC 17631	IIIB/MC	1/386	295–469	8	1/128	106–162
				9	1/159	133–200
				10	1/139	89–173
Mean ± SE		1/265 ± 1/1292 (P <0.001)			1/122 ± 1/1532	
Range		1/111–1/554			1/80–1/181	

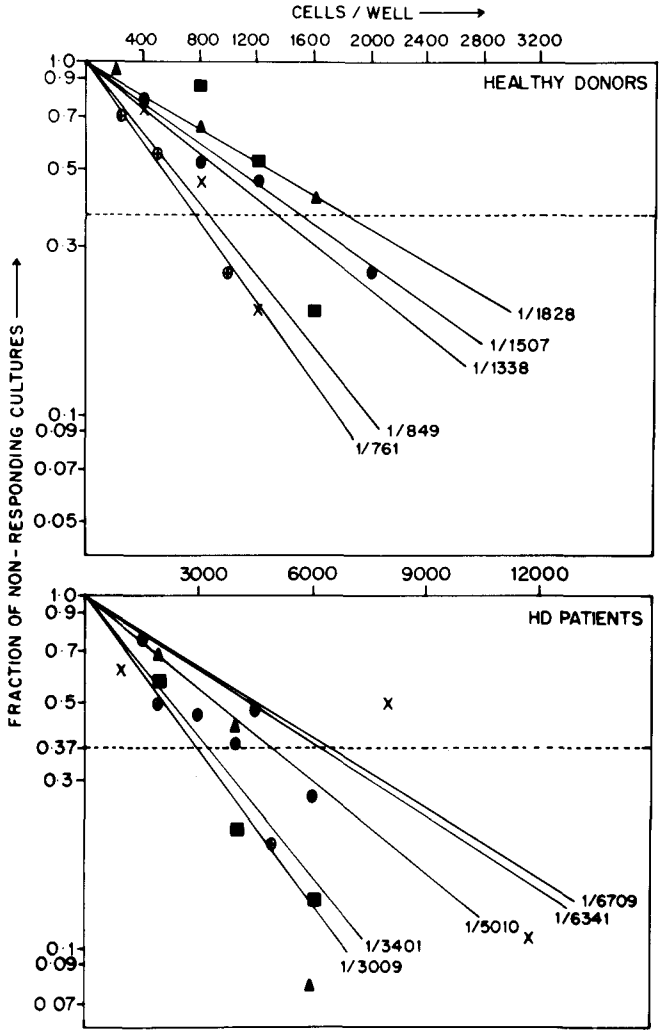

Fig. 3. Frequency distribution of interleukin-2-producing cells in healthy donors and HD patients

Table 3. T cell responses in bulk culture and at single-cell level

Donor	Frequency: PHA-responsive cells	Proliferation (net mean cpm)	Frequenccy: IL-2-producing cells	IL-2 production (net mean cpm)
Healthy donors				
1	1/90	96025	1/761	32092
2	1/181	75165	1/1338	29327
3	1/159	144490	ND	41963
4	ND	68279	1/1507	13095
5	1/80	63220	1/849	29685
HD patients (Case no.)				
BC 11214	1/111	ND	1/6709	ND
BC 14191	1/240	ND	1/5010	ND
BC 16435	1/548	ND	1/6341	ND

ND, not done

Discussion

A number of parameters involved in T cell activation, expansion and differentiation have been investigated in HD, in order to provide the rationale for mechanisms underlying the compromised T cell functions. These include impairment in IL-2 production in spite of normal IL-1 levels, increased production of prostaglandins, reduced IL-2 receptor (Tac antigen) expression, impaired status of high-affinity IL-2 receptor, partial restoration of proliferation with exogenous IL-2 and the presence of inhibitory factors in the sera [1, 4, 8, 13, 17, 21, 23, 27, 32]. A common feature in all these studies appears to be the low proliferative response and decreased ability of PBL from HD patients to accumulate IL-2 in the culture supernatants after stimulation with PHA. In this paper, we have tried to correlate the proliferative function with lymphokine production, and have tried to assess the frequency of T cells responding to PHA and producing IL-2, using limiting-dilution analysis.

The role of IL-2 in T cell proliferation is well documented. Interferons are known to act as differentiating

signals for many cells involved in the immune system [10]. IFNγ can act on T lymphocytes by enhancing their proliferation and functional differentiation [15], by increasing production of IL-2 by T helper cells [9] and by increasing IL-2 receptor expression [31]. We observed that production of both the cytokines IL-2 and IFNγ was impaired in activated PBL from HD patients. IFNγ production was markedly reduced in patients with B symptoms, which could be associated with reduced antiviral function in these individuals leading to viral infections accompanied with B symptoms. It has been suggested that an IL-1 signal is essential for production of IFNγ as well as IL-2 [7]. IL-1 production appears to be normal in HD as demonstrated frequently [1, 8, 32]. Therefore, it is possible that impairment in signals other than IL-1 may be responsible for reduced IFNγ and IL-2 production in HD.

Using linear regression analysis, correlation could be shown in proliferation and IL-2 production, and proliferation and IFNγ production in individual HD patients. However, when tested for IL-2 and IFNγ production, in individual patients, no correlation could be established. Although 5′-flanking regions of IL-2, IL-2 receptor and interferon genes share a consensus sequence [11] it is known that they can also be activated independently, perhaps because of the existence of other regulatory elements specific for each gene [5]. Therefore, it is likely that independent activation of IL-2 and interferon genes may occur in activated lymphocytes from HD patients. This could be further qualified by studying the mRNA for IL-2 and IFNγ in activated lymphocytes from HD.

Since the demonstration of the use of limiting-dilution analysis to assess the frequency distribution of functional and precursor cells, the method has been extensively used in immunology. The studies reported deal with frequency distribution of various cells such as cytotoxic alloantigen-reactive precursors [26], mitogen-reactive cells [19] and IL-2-secreting cells [18, 30]. Most of these reports assess the reactivity of cells from healthy donors. In the present study we have attempted to compare the frequency distribution of mitogen-responsive and IL-2-producing cells in healthy donors and HD patients.

Moretta et al. [19] have demonstrated that every T cell from human PBL, stimulated with PHA, has a clonogenic potential. In our studies the frequency of PHA-responsive cells, even in healthy donors, is comparatively low. The main difference between these assay procedures is that they have maintained the limiting-dilution microcultures in the conditioned medium from PHA-stimulated human spleen cells. The microcultures received IL-2-containing medium and fresh irradiated feeder cells for 2–3 weeks, before they were subjected to the frequency analysis of proliferating and cytotoxic cells. Essentially, this method gave an estimation of clonogenic potential of the precursor cells and this estimate might include other cells with IL-2 receptors capable of expanding in IL-2-containing medium.

On the other hand, our method estimated the frequency of mitogen-responsive cells, before they had a chance to proliferate in the presence of IL-2. Short exposure to IL-2, in our experiments, helped accumulation of cells in S phase. Using this estimate we found that, in healthy donors, the range of T cells responding to mitogen stimulus alone is between 1/80 and 1/181, while this frequency in HD patients ranged from 1/111 to 1/554, which was significantly less.

Similarly, very high frequencies of IL-2-producing cells have been reported by Moretta [19], and Vie and Miller [30]. In Moretta's experiments, again the microcultures were allowed to expand in the presence of spleen-cell-conditioned medium and fresh irradiated feeders each week, for 2–3 weeks. The cells from each well were then used for frequency analysis of IL-2-producing cells. On the other hand, in the method adopted by Vie and Miller, Epstein-Barr-virus transformed allogeneic B cell lines have been used as feeders. These feeders helped in providing an IL-1-like signal and also absorbed inhibitory factors such as prostaglandin E_2 creating an environment conducive for IL-2 production.

Our score of IL-2-producing cells, assayed 24 h after mitogen stimulation of naive cells, worked out to be 1/761–1/1828 in healthy donors and 1/3009–1/6709 in HD patients, the difference being statistically significant. In our assays, perhaps while attempting to remove the preformed IL-2 from feeder cells, we also removed the IL-1 signal required for IL-2 production, which may have been partly responsible for our lower estimates. However, since the same method was adopted for healthy donors and HD patients, the difference in the responder frequency should be real.

The studies reported here show concordance between bulk proliferation, bulk IL-2 production and single-cell estimates of these functions in healthy donors. In HD, apparently, fewer microcultures responding by proliferation secreted measurable quantities of IL-2 in the first 24 h of culture, compared to healthy donors. It will be essential to compare the time kinetics of IL-2 accumulation in HD PBL vis-a-vis PBL from healthy donors.

Association of T cell hyporesponsiveness with impaired delayed-type hypersensitivity responses to skin test antigens and with infections was shown a long time ago [6]. In our clinic, we have noted that most of the patients with impaired T cell responses, especially those with B symptoms show frequent infections with *Candida*, herpes virus and tuberculosis.

We feel that these studies give grounds for further investigation of whether the depression in proliferative responses and cytokine production in HD could be reflected at the level of reduced frequency of responding cells.

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