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Serum suppressive activity of HIV seropositive patients

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SUMMARY

The mechanisms by which HIV induces immunosuppression are still poorly understood so far. Several pathways of CD4 cell destruction are known, including cytolysis with or without syncitium formation and killing by cytotoxic effectors of HIV infected or non-infected CD4 cells. However, a discrepancy exists between the small number of actually infected cells *in vivo* and the extent of HIV-related immunodeficiency. Among other possible immunosuppressive factors, serum blocking factors have been reported, but only in AIDS-related opportunistic infections (OI), i.e. in a quite specific type of full-blown HIV disease. The purpose of this work was to determine whether serum blocking activity was unique to this group of patients, or if it was also expressed in other clinical presentations and, moreover, at earlier stages of the disease. We also attempted to delineate the nature of these seric factors. In order to do so, we assessed serum suppressive activity of 50 HIV seropositive patients, seven with OI, eight with Kaposi's sarcoma (KS), and 35 with no clinical AIDS. Our results confirm the existence of serum inhibiting factors in AIDS, and demonstrate their presence at earlier stages of the disease. They also highlight the fact that the level of serum suppression does not correlate with patients clinical status, but increases with the severity of the disease. The lower the CD4 count, the higher the suppression exerted. Furthermore, we showed that the suppression was at least partly mediated by small size molecules, which are not complement-mediated or directly lymphocytotoxic. On the other hand, this activity does not correlate with the serum level of p24 HIV core protein. The possible relation with other viral components is discussed. The relevance of these data to prognosis and pathogenesis of HIV disease deserves further investigation.

Keywords HIV related disorders serum suppressive activity

INTRODUCTION

The mechanisms by which HIV induces immunosuppression are still poorly understood so far. The progressive and profound depletion of the CD4 cell subset observed in this disease certainly plays an important role in this phenomenon. In HIV seropositive patients, CD4 cells are in fact exposed to destruction through different mechanisms, including the cytopathogenic effect of HIV itself with (Sodroski *et al.*, 1986) or without (Somasundaran & Robinson 1987) syncitium formation. Moreover, HIV infected cells have been reported to be the targets of anti-HIV cytotoxic effectors (Walker *et al.*, 1987), while others have suggested that in HIV disease lymphoid cells could be the targets of effectors directed towards self-determinants (Andrieu, Even & Venet, 1986; Ziegler & Stites 1986). However, a large discrepancy exists between the apparent small number of lymphocytes actually infected *in vivo* and the extent of immune dysfunctions which characterize the disease, suggesting that other cellular and humoral factors are likely to participate in this immunodeficiency. Furthermore, in several animal models, and particularly the Feline Leukemia Virus (FeLV) system in the cat, retroviral structural components have been proved to strongly impair the immune functions (Orosz *et al.*, 1985). Finally, several humoral immunosuppressive candidates have been described in AIDS, such as cytotoxic antilymphocyte antibodies (Ozturk *et al.*, 1987; Stricker *et al.*, 1987), as well as serum blocking factors (Cunningham-Rundles, Michelis & Masur, 1983; Siegel *et al.*, 1985). Only the latter have been reported exclusively in AIDS-related opportunistic infections (OI) final clinical step of the disease characterized by an end-stage immunodeficiency.

The purpose of this work was first to determine whether such factors could also be demonstrated in other types of CDC-defined AIDS and/or, more importantly, at earlier stages of the disease. Secondly, we attempted to give some information about the nature of these factors. In order to do so, we evaluated serum suppressive activity of 50 HIV seropositive patients (pts), 15 with full-blown AIDS and 35 with no clinical AIDS.

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MATERIALS AND METHODS

Study population

We studied 50 HIV seropositive patients (confirmed by ELISA and Western blot analysis). Fifteen had a CDC-defined AIDS (eight opportunistic infections, seven Kaposi's sarcomas), and 35 had no clinical AIDS (non-AIDS patients). There were altogether 5 females and 45 males, belonging to the following risk groups: sexual transmission (40), needle sharing (9), transfusions (1).

We classified them according to the Laennec Working Staging System (LWSS). Briefly, we defined three stages according to the CD4 cell count (I: CD4 > 600/μl, II: 300–600, III: CD4 < 300/μl) and 3 clinical classes (A: asymptomatic, B: persistent lymphadenopathy, C: constitutional symptoms, such as weight loss (> 10% baseline), diarrhoea, fever and/or intense night sweating, (with no other cause than HIV). For the sake of clarity it should be indicated that class A is equivalent to CDC group II, B to III and C to IV A.

Four male homosexuals seronegative for HIV served as controls, as well as four subjects from the laboratory staff at no risk for AIDS.

Serum from all patients and controls was kept frozen in aliquots at −20°C after heat-inactivation at 56°C for 60 min.

Suppressive assay

Peripheral blood lymphocytes (PBL) from four normal subjects different from the eight normal donors of serum were isolated by Ficoll-Hypaque centrifugation. Proliferative responses of these cells were tested in the presence of mitogens, with or without addition of serum from patients or controls. PBL from each of the four normal cell donors were plated in triplicate in U-shaped microtitre plates with 10^5 PBL per well in RPMI 1640 supplemented with 1% glutamin and 20% heat-inactivated pooled normal serum (PNHS). We established the cultures in PNHS instead of autologous patient serum in order to actually evaluate the effect of test serum on PBL proliferation. This precluded the possibility of only demonstrating test serum failure to support proliferation. A fourth well was established in the same condition and served as a cell viability control (Trypan blue test) at the end of the culture period. Serum from either patients or seronegative controls was added at different dilutions (10, 5, 2 or 1% final concentrations) at initiation of the cultures. Two different mitogens were used: PHA-P (Difco) and Concanavalin-A (Con A, Sigma) at both optimal and suboptimal concentrations (PHA 10^{-3} and 10^{-4} v/v, and Con A 50 and 20 μg/ml, respectively). All final volumes were adjusted to 200 μl/well. Cultures were maintained at 37°C, 95% air 5% CO_2 for 72 h, and pulsed for the last 18 h with 1 μCi of tritiated thymidine. Cells were harvested onto glass-fibre filters by the aid of an automatic cell harvester. The radioactivity incorporated into the cells was counted in a liquid scintillation counter, and expressed in counts per minute (ct/min). For each of the four normal cell donors, we denoted ct/min obtained in the presence of mitogen alone, with no addition of test or control serum, as baseline counts (B), and those obtained in the presence of additional serum as experimental counts (E). An individual suppression index (SI) was calculated in each case as $B - E/B \times 100\%$. Each test serum was tested on the four sources of normal PBL. As the suppression exerted by one given serum on these four controls was quite homogeneous, we expressed individual SI as the mean ± standard deviation (s.d.) of these four measurements. Finally, results from all patients in each biological stage and clinical class were pooled and expressed as the mean ± s.d. of all individual SI within this given stage or class. SI observed on PHA or on Con A stimulated cultures are referred to as PHA-SI and Con A-SI respectively.

Table 1. Suppression index of HIV$^+$ patients

	CD4 Stages			Clinical classes		
	I	II	III	A	B	C
Non-AIDS	18 ± 10%	31 ± 11%	41 ± 15%	25 ± 19%	32 ± 12%	31 ± 12%
		*	*		(NS)	(NS)
AIDS						
OI			47 ± 14%	(NS)		
KS			44 ± 13%			

I CD4 > 600/μ^3, II CD4 between 300 and 600/μ^3, III CD4 < 300/μ^3.
A Asymptomatic, B persistent lymphadenopathy, C constitutional symptoms.
OI Opportunistic infections, KS Kaposi's sarcoma
* $P < 0{\cdot}05$ NS not significant

Statistical analysis

Results are expressed as mean ± standard deviation (m ± s.d.). Comparisons were made using Student's *t*-test or the Mann–Whitney test for unpaired series.

RESULTS

Study population according to the LWSS classification

Our 50 patients were distributed as follows: class A: $n = 11$ (22%), B: $n = 17$ (34%), C: $n = 7$ (14%), AIDS: $n = 15$ (30%). It is noteworthy that all AIDS patients, whether with opportunistic infections (OI, $n = 8$) or Kaposi's sarcoma (KS, $n = 7$) belonged to stage III. Furthermore, most patients from stage III ($n = 25$) did present with full blown AIDS (60%). Finally, the great majority of non-AIDS patients (72%) belonged to stages I and II.

Effect of serum addition on normal PBL mitogen-induced proliferation

Results of experiments using optimal conditions. These experiments were carried out with PHA 10^{-3} or Con A 50 μg/ml, and a 10% final concentration of all additional serum.

The addition of serum from the eight seronegative controls, whether or not at risk for AIDS, exerted no suppression whatsoever. In contrast, sera from HIV seropositive patients had a marked suppressive effect (Table 1). Altogether, sera from non-AIDS patients (pooling SI from classes A, B and C patients) exerted a 29 ± 15% suppression on PHA induced proliferation compared to a 46 ± 13% suppression obtained with sera from AIDS patients ($P < 5 \times 10^{-4}$). With Con A, the same phenomenon was observed: SI = 35 ± 11% in non-AIDS subjects, vs 45 ± 9% in AIDS ($P < 5 \times 10^{-4}$). In any case, cell viability assessed at the end of all cultures was always > 85%, indicating that the lack of proliferation was not due to a lymphocytotoxic effect of serum.

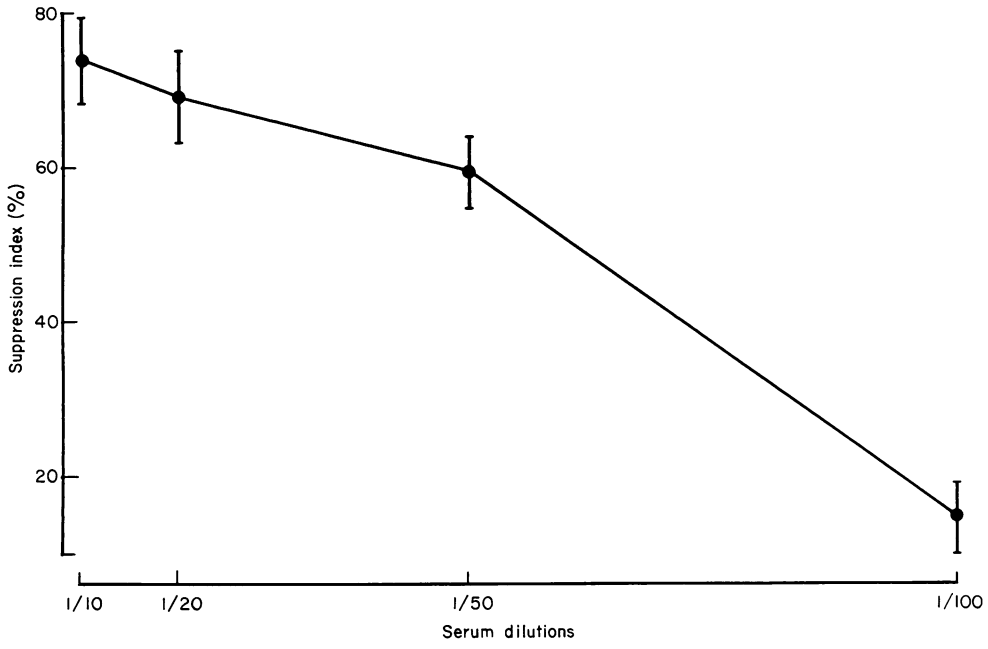

Fig. 1. Effect of serum dilution on the suppression index. Results reported here are the means ± standard deviation of experiments using sera from nine stage III patients.

Interestingly, inside the AIDS group suppression exerted by patients with KS was not different from that due to those with OI (PHA-SI = 44 ± 13% and 47 ± 14%, Con A-SI = 47 ± 9% and 42 ± 17% respectively). Moreover, inside the non-AIDS group, PHA-SI of the three clinical classes were not statistically different: 25 ± 19, 32 ± 12 and 31 ± 12% respectively in classes A, B and C. With Con A, SI were 32 ± 12, 36 ± 10 and 36 ± 10% in A, B and C respectively.

Contrasting with these data, a marked difference was observed according to the patients CD4 stages (Table 1). Indeed, PHA-SI were 18 ± 10%, 31 ± 11% and 41 ± 15% in stages I, II and III respectively ($P < 0{\cdot}05$, all comparisons). Similarly, Con A-SI were 29 ± 11%, 38 ± 9% and 44 ± 9% in stages I, II and III respectively. Furthermore, we have also found a significant correlation between individual CD4 counts and SI values ($r = -0{,}36$, $P < 0{\cdot}01$).

Thus, serum suppressive activity of non-AIDS patients is clearly not correlated with their clinical presentation but rather with their CD4 cell counts. In other words, the existence of lymphadenopathy, as could be expected, makes no difference to the level of suppression observed. More interestingly, constitutional symptoms usually regarded as markers of disease severity, do not indicate a higher level of serum suppression in patients with comparable CD4 counts. Finally, it is noteworthy that when all stage III patients are taken as a whole, no statistical differences are observed between PHA-SI of non-AIDS (35 ± 15%) and AIDS patients (45 ± 13%), nor between Con A-SI of both groups (40 ± 10% vs 46 ± 9% respectively). Again, whether clinical AIDS has yet occurred or not in patients with markedly low CD4 counts makes no difference to the level of suppression observed.

Results of experiments using suboptimal concentrations of mitogens. These experiments were performed in 21 non-AIDS patients (stage I:4 subjects, II:9, III:8), and we compared SI obtained with suboptimal concentrations of mitogens (PHA: 10^{-4} and Con A 20 μg/ml) to those obtained with optimal ones. SI were 50 ± 17% vs 83 ± 7% for PHA 10^{-3} and PHA 10^{-4} respectively ($P < 0{\cdot}01$) and 53 ± 20% vs 70 ± 20% for Con A 50 μg/ml and 20 μg/ml respectively ($P < 0{\cdot}01$). We will discuss later the fact that suppressive activity is best expressed in suboptimal experimental conditions.

Effect of serum dilution

We chose nine sera previously shown to be highly suppressive at the usual final concentration of 10% and compared their effect at 5, 2 and 1%.

Figure 1 shows that the suppressive activity can indeed be diluted out. Moreover, it is noteworthy that this activity is still present at a test serum concentration as low as 1%.

Effect of serum dialysis

In an attempt to delineate the nature of the factor(s) responsible for suppression, we evaluated their molecular weight using extensive dialysis against RPMI 1640 of the nine sera referred to in the above paragraph through 6,000–8,000 mol. wt. pores, and compared PHA-SI using native and dialysed sera in parallel experiments. We observed that PHA-SI markedly decreased from 52 ± 3% to 11 ± 5% after dialysis ($P < 0{\cdot}01$), leading to the conclusion that suppression was at least partly mediated by small molecules.

DISCUSSION

Our results confirm previously reported data on factors present in the serum of patients with AIDS-related opportunistic infections, and inhibiting normal PBL proliferation (Cunningham-Rundles, Michelis & Masur, 1983; Siegel *et al.*, 1985). We also demonstrate that such suppressive factors are not specifically present in this group of patients, since they can also be found in patients with Kaposi's sarcoma as well. More

interestingly, HIV seropositive non-AIDS subjects also clearly display a serum suppressive activity, which to our knowledge has never been reported before. Intensity of this inhibitory activity increases along with the severity of the disease, reflected by the depletion of the CD4 cell pool. In that respect it is noteworthy that if a significant difference is observed between AIDS and non-AIDS groups, this should not be related to differences in clinical characteristics, but rather to the fact that 100% of the subjects in the former group belong to stage III, whereas 72% of the last one belong to stages I and II. Of course correlation between suppression intensity and CD4 counts does not establish a causative relationship between these two parameters. It indicates simply that the level of suppression exerted by serum somehow parallels the disease evolution. This phenomenon is likely to participate, concurrently with the depletion of CD4 cells, in the immunosuppression observed in late stages of the disease.

In addition it is noteworthy that serum from patients with normal CD4 cell counts, who are clinically asymptomatic, also exhibits a slight but consistent immunosuppressive capacity.

It is noteworthy that the highest suppression is observed when suboptimal concentrations of mitogens are used, confirming the data reported by Siegel et al. 1985. This observation could be quite relevant to actual *in vivo* situations. Indeed, the antigenic challenges observed *in vivo* lead to immune responses which are much weaker than those due to the use of optimal doses of mitogens *in vitro*, and are probably much better reflected by suboptimal experimental conditions. Anyhow, the effect of sera on antigen driven proliferation is currently being evaluated in our laboratory.

As to the nature of blocking factors, this point is still questionable. We showed that they did not act through complement-mediated lysis, since all sera were heat-inactivated before use. Moreover, they are not directly lymphocytotoxic as assessed by good cell viability in our experimental conditions. In addition, the fact that at least part of the suppression is dialysable argues against the immunoglobulinic nature of these factors.

On the other hand, inhibition could be related to HIV itself or to some of its components. In this respect, it has to be stressed that seronegative subjects, whether or not at risk for AIDS, display no suppressive activity. Furthermore, evidence exists in the literature as to the immunosuppressive properties of HIV viral preparations, using whole inactivated HIV (Wainberg, Blain & Spira, 1987; Amadori *et al.*, 1988) or banded preparations (Pahwa *et al.*, 1985). Purified HIV envelope protein gp 120 has also been shown to inhibit PBL proliferation (Mann *et al.*, 1987), as well as several synthetic peptides (Chanh *et al.*, 1987; Chanh, Kennedy & Kanda, 1988). Other experimental models have demonstrated the immunosuppressive properties of infected cell culture supernatants (Laurence, Gottlieb & Kunkel, 1983; Laurence & Mayer, 1984). In all our patients, viremia was evaluated by the assessment of serum p24 antigen, using an immunocapture assay. Thirty two patients were p24 negative and 18 p24 positive. SI were respectively $35 \pm 17\%$ and $34 \pm 14\%$, indicating that suppression is clearly not correlated with this particular viral antigen. This of course does not preclude the viral origin of suppressive factors, particularly in view of the fact that strong immunosuppressive properties have been reported for peptides corresponding to sequences of transmembrane glycoproteins, p15 E in FeLV (Ciancolo *et al.*, 1985), and two peptides of the gp41 in HIV (Chanh, *et al.*, 1988). Furthermore, an immunosuppressive factor inhibting monocyte chemotaxis and sharing epitopes with gp41 has very recently been reported in HIV seropositive patients (Tas, Drexhage & Goudsmit, 1988). Efforts should concentrate on searching for circulating components of HIV transmembranous envelope protein.

Finally, we should note the fact that an immunosuppressive activity has also been detected in other diseases of the immune system, not only in HTLV I infection (Shirakawa *et al.*, 1986), knowing that FeLV p15 E is homologous to a sequence of the transmembrane envelope glycoprotein of HTLV I, but also in Hodgkin's disease (Sugden & Lilleyman, 1980), whose retroviral origin has recently been suggested (Lesser *et al.*, 1987). Comparisons between pathogenesis of these human retroviral-induced immunodeficiencies certainly deserve further study.

Other crucial questions clearly remain to be answered as to HIV, the nature of blocking factors, their exact cellular sources and targets, as well as their mechanisms of action. Finally, their relevance to clinical management and particularly to prognosis evaluation is a worthwhile issue, currently under investigation in our institution by serially assessing biological and clinical parameters in a long-term follow-up of our cohort of 500 HIV seropositive patients.

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