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Correlation between expression of antibodies to histone H2B and clinical activity in HIV-infected individuals

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SUMMARY

Levels of autoantibodies specific for the histone, H2B, were measured in individuals with HIV infection. In comparison with normal (uninfected) controls, infected patients, particularly those with symptomatic disease, had significantly elevated titres of anti-H2B antibodies. Longitudinal studies confirmed that levels of these antibodies were highest in patients with lymphadenopathy and declined with the development of AIDS. In preliminary experiments designed to determine the biological significance of the anti-histone antibodies, H2B was shown to be immunologically cross-reactive with an 18-kD antigen on the surface of HIV-infected or mitogen-activated $CD4^+$ cells. Protein sequencing of the 18-kD antigen has since shown complete homology with histone H2B. Because the titres of H2B autoantibodies were found to parallel the numbers of circulating CD4 cells, it is possible that these antibodies are involved in the destruction of the helper/inducer T lymphocyte population.

Keywords HIV autoimmunity anti-lymphocyte antibodies histones

INTRODUCTION

Patients with AIDS, ARC and related disorders associated with HIV invariably have serious abnormalities of T lymphocyte subpopulations, including reduced numbers of helper/inducer (CD4) cells and a decreased ratio of helper/inducer to cytotoxic/suppressor cell (see [1] for review). The mechanism by which the virus causes the destruction of the CD4 T cell population is poorly understood, especially as only one in 1000 cells or fewer in peripheral blood are infected with HIV [2]. Other studies have indicated that there is a greater frequency of HIV infection in the lymphoid tissues [3,4], and that the destruction of the CD4 population cannot be attributed to viral load alone.

Several studies have indicated that HIV infection may induce anti-lymphocyte autoantibodies (ALA), although in the initial reports it was unclear as to whether they were subset-specific [5–9]. Other investigations have attempted to characterize potential molecular targets on T cells with which ALA are reactive. Stricker *et al*. [10,11] found that sera from most patients with ARC and AIDS have autoantibodies which bind to an 18-kD antigen present on HIV-infected or mitogen-activated $CD4^+$ T cells. These antibodies were shown to be capable of suppressing the proliferative reactions of $CD4^+$ T cells *in vitro* and were cytotoxic for these cells in the presence of complement [10]. A high degree of correlation was found between the presence of anti-18-kD antibodies and the clinical status of infected individuals. The autoantibody was virtually absent in asymptomatic, HIV^+ individuals, yet almost always detected in patients with ARC or AIDS. Further studies have indicated that the 18-kD antigen is a molecule which serologically resembles histone H2B (R. B. Stricker, unpublished observations), and in chimpanzees infected with HIV and macaques and mangabeys infected with simian immunodeficiency virus (SIV), the levels of anti-H2B antibodies in the serum proved to be a good indicator of clinical activity [12,13]. Recent biochemical analysis which involved N-terminal sequencing of the 18-kD antigen revealed complete amino acid homology with histone H2B (R. M. Comacchio *et al*., manuscript in preparation).

The aims of this study were: (i) to determine whether HIV-infected individuals have detectable levels of anti-histone H2B antibodies in their sera; (ii) to establish whether the presence of anti-H2B antibodies correlated with clinical status; and (iii) to determine if there was any relationship between anti-H2B antibodies and the 18-kD reactive anti-lymphocyte antibodies described in previous studies.

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PATIENTS AND METHODS

Study design and sampling

Two hundred homosexual men at risk for AIDS were recruited in

San Diego between May and September 1984, and have since been continuously monitored. For the purpose of this study most subjects were examined every 6 months. At the time of the study patients were classified clinically asymptomatic or as having persistent lymphadenopathy (LAS), ARC or AIDS. This system was used for patients enrolled into the San Diego cohort study since the mid 1980s and for the sake of consistency we have not used the more recent 1993 CDC classification [14]. Effectively patients with LAS would fall primarily into the current category A, while patients with ARC or AIDS could be placed in categories B or C, respectively. Our sample groups were of insufficient size to break down into subcategories based on $CD4^+$ T cell numbers. From this cohort two longitudinal case control studies were constructed to examine the correlation of anti-histone H2B expression and disease progression: 22 HIV^+ individuals who progressed to ARC and AIDS were matched with 22 clinically stable seropositive controls.

Laboratory evaluations

At every patient visit the following routine laboratory tests were conducted: complete blood count, blood chemistry panel, lymphocyte subsets, total immunoglobulin, anti-HIV antibodies and levels of serum p24 *gag* antigen.

Western blot analysis of sera

The human-derived $CD4^+$ H9 cell line used in this study was either phytohaemagglutinin (PHA)-transformed [10] or was chronically infected with the HIV IIIB isolate (the HXB line). Either mitogen-activated or HIV-infected T cells (5×10^5) were solubilized with 2% SDS, and resolved (5 μg per lane) on a 12% SDS–PAGE gel (Novex, San Diego, CA) under non-reducing conditions [10,15]. Resolved cell lysates were transferred electrophoretically to nitrocellulose sheets [16]. Non-specific binding of antibodies to nitrocellulose sheets was reduced by pre-incubation in a blocking solution of 2% bovine serum albumin (BSA; Sigma, St Louis, MO) diluted in PBS containing 0·05% Tween 20 (Sigma) (PBS–T) for 1 h at room temperature with constant agitation. Sera from HIV^+ individuals were diluted 1 : 1000 in PBS–T and incubated with blotted nitrocellulose sheets for 1 h at room temperature with constant agitation. Non-bound serum proteins were removed by three 10-min washes with PBS–T with constant agitation. Antibodies bound to resolved cell lysate were detected with a biotinylated, affinity-purified, anti-human immunoglobulin reagent (Southern Biotech, Birmingham, AL) diluted 1 : 1000 in PBS–T, and incubated for 1 h at room temperature with constant agitation. Following three 10-min wash steps, horseradish peroxidase (HRP)-labelled streptavidin (Southern Biotech) diluted 1 : 1000 in PBS–T was incubated for 1 h at room temperature with constant agitation. Bound antibodies were visualized with 4-chloro-1-napthol substrate (Sigma).

Anti-histone H2B ELISA

Polystyrene 96-well microtitre plates were coated with histone H2B (Worthington Biochemical Corp, Freehold, NJ) by overnight incubation at 4°C with a solution (5 μg/ml) of H2B in 0·1 M carbonate/bicarbonate buffer pH 9·2. After this time the coating solution was discarded and the plates were blocked with 0·1 M phosphate buffer pH 7·4, containing 0·15 M NaCl and 1 mg/ml gelatin (Sigma), for 2 h at 4°C. Excess blocking agent was removed by two washes with PBS–T. Test and control serum diluted 1 : 100 with 0·1 M phosphate buffer pH 7·4, containing 0·15 M NaCl, 1 mg/ml gelatin, 0·5 mg/ml bovine γ-globulin (Sigma), 0·1 mg/ml BSA and 0·05% Tween, were incubated for 2 h at 37°C. The plates were then washed five times and HRP-conjugated goat anti-human immunoglobulin (Southern Biotech) (diluted 1 : 4000) was added to each well and incubated for 1 h at 37°C. Finally the plates were washed five times and *o*-phenylenediamine peroxidase substrate (Sigma) was added and incubated for 20 min. Plates were read in a Molecular Devices (San Jose, CA) ELISA reader at 490 nm.

Human monoclonal antibody selection and testing

Human MoAb-secreting hybridomas were prepared by our group using standard techniques according to the protocol of Shoenfeld *et al*. [17]. Supernatants and purified antibody were screened by ELISA for reactivity with histones H1, H2A, H2B, H3 and H4 by the technique of Muzellec *et al*. [18]. Supernatants reactive with H2B in ELISA were tested by Western blot analysis (as previously described) for reactivity with SDS–PAGE-resolved cell lysates of PHA-stimulated or HIV-infected $CD4^+$ H9 cells, and non-PHA-stimulated H9 cells [10]. Both BEN27b and a control human MoAb, VAR4, of identical isotype (which does not bind DNA, histones, cardiolipin or several other autoantigens), were also tested for reactivity with SDS–PAGE-resolved purified calf thymus histone H2B. Serum from HIV^+ individuals was run in parallel.

RESULTS

The levels of anti-H2B antibodies in asymptomatic HIV^+ individuals and patients with LAS, ARC and AIDS were considered to be elevated if they were above a level (OD = 0·38) established as the mean + 2 s.d. of 50 healthy HIV^- blood donors. Only 6/22 (27%) asymptomatic seropositive individuals possessed elevated levels of antibodies binding to H2B, and these were at relatively low titres. In contrast, 31/58 (53%) patients with LAS had elevated levels of anti-H2B antibodies, with substantially higher titres. The incidence of H2B binding above the normal range of patients with ARC and AIDS was 5/14 (36%) and 11/24 (46%), respectively. The results are shown in Fig. 1.

The longitudinal study of HIV^+ patients showed those who progressed to develop disease exhibited a decrease in anti-H2B antibody titres approximately 12 months before AIDS diagnosis. Clinically stable seropositive individuals maintained higher levels of anti-H2B antibodies (Fig. 2). While titres of anti-H2B antibodies did not correlate strongly (as measured by regression analysis) with $CD4^+$ cell numbers in any of the clinical categories (ARC patients showed the highest level of association: $r = 0{\cdot}4$, $P = 0{\cdot}04$), anti-H2B antibody levels did fall in parallel with CD4 cell numbers in the group of individuals where disease progressed (Fig. 3).

Of 72 human MoAbs screened for reactivity, one, designated 'BEN27b', was shown by ELISA to bind predominantly to histone H2B, although reactivity with other histones was noted at a far lower level (data not shown). No reactivity with ssDNA or dsDNA could be detected. BEN27b was found to bind an 18-kD antigen in the lysate of PHA-stimulated H9 cells (Fig. 4, lane 4). It also bound an 18-kD band on a lysate from HIV-infected H9 cells (Fig. 4, lane 5). In contrast, BEN27b did not bind to an unstimulated H9 lysate (Fig. 4, lane 3). The monoclonal also bound to 18-kD bands in unpurified calf thymus histone (Fig. 4, lane 2) and commercially

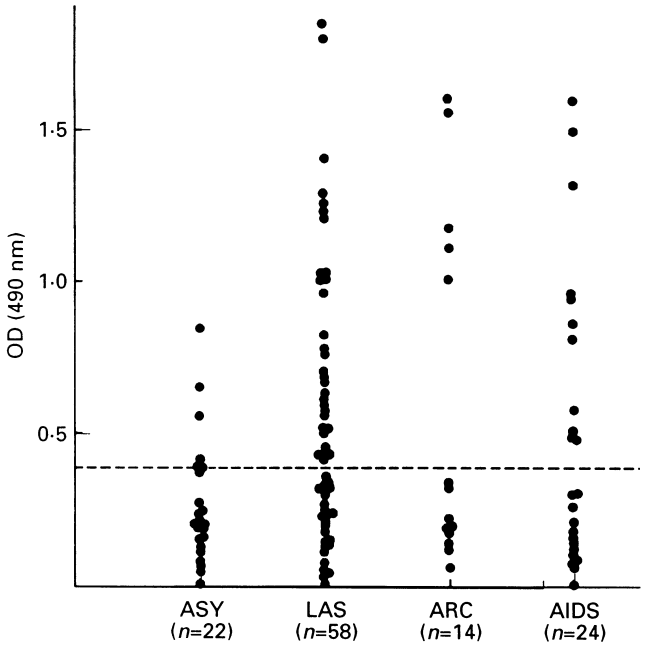

Fig. 1. Levels of anti-H2B antibodies in asymptomatic HIV$^+$ individuals (ASY), patients with lymphadenopathy syndrome (LAS), ARC and AIDS. Numbers in parentheses indicate samples in each group. Dotted line indicates normal range established as the mean + 2 s.d. of H2B binding in 50 healthy HIV$^-$ blood donors.

available histone H2B (Worthington Biochemical Corp.) (Fig. 5, lane 4). This staining pattern was indistinguishable from that of serum from an HIV$^+$ individual who had high titres of anti-H2B antibodies as detected by ELISA (Fig. 5, lane 1). The control MoAb, VAR4, showed no binding (Fig. 5, lane 2). VAR4 showed no binding to lectin-stimulated, HIV-infected or unstimulated H9 lysates.

DISCUSSION

The results of this study demonstrate clearly that individuals infected with HIV develop antibodies against histone H2B.

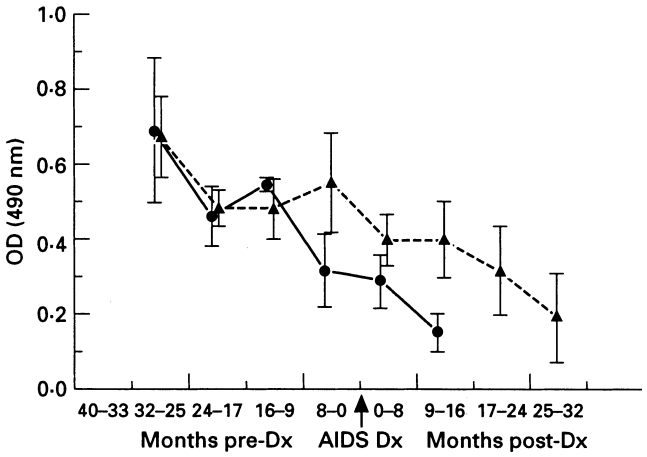

Fig. 2. Anti-H2B antibody levels (shown as mean OD levels) in HIV-infected, clinically unstable individuals who progress to ARC and AIDS (AIDS progressors, ●) and HIV-infected, clinically stable matched case control group (▲). (Vertical bars represent s.e.m.)

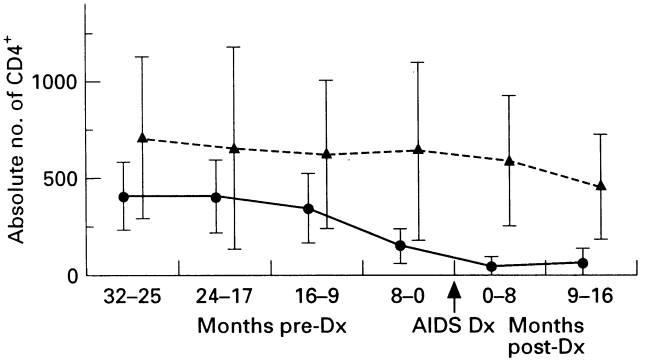

Fig. 3. Numbers of CD4$^+$ cells in HIV-infected, clinically unstable individuals who progress to ARC and AIDS (AIDS progressors, ●) and HIV-infected, clinically stable matched case controls (▲). (Vertical bars represent s.e.m.)

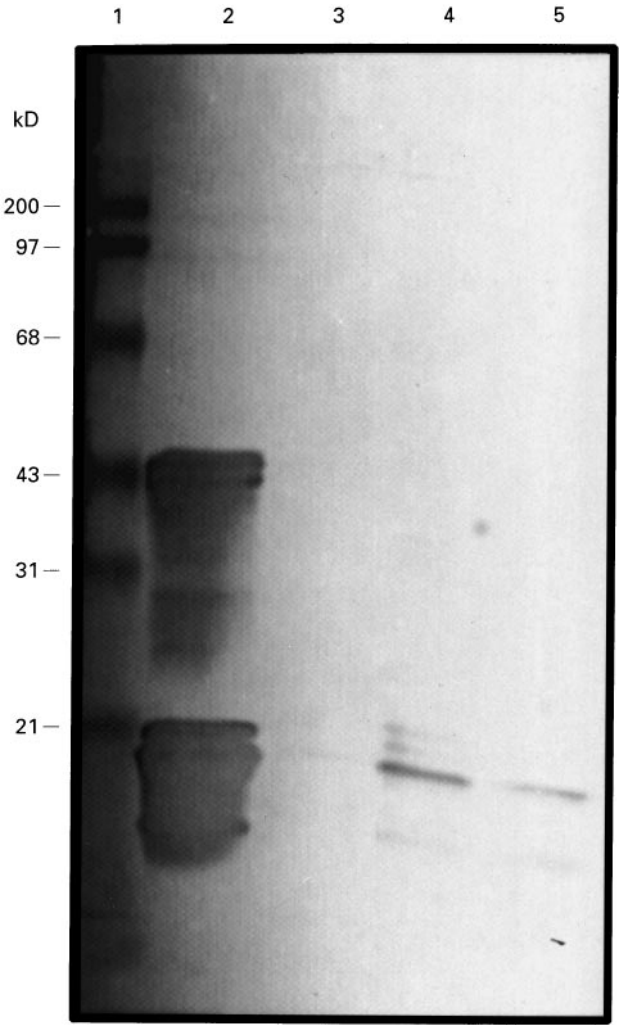

Fig. 4. Western blot performed with the BEN27b human anti-histone MoAb. Lane 1, pre-stained molecular weight markers; lane 2, unpurified calf thymus histone. The specificity of BEN27b for a common histone epitope is quite evident; lane 3, lysate prepared from resting (not phytohaemagglutinin (PHA)-stimulated) H9 CD4$^+$ T cells; lane 4, lysate prepared from PHA-stimulated H9 cells; lane 5, lysate prepared from HXB cells (H9 cell line infected with the HIV IIIB isolate).

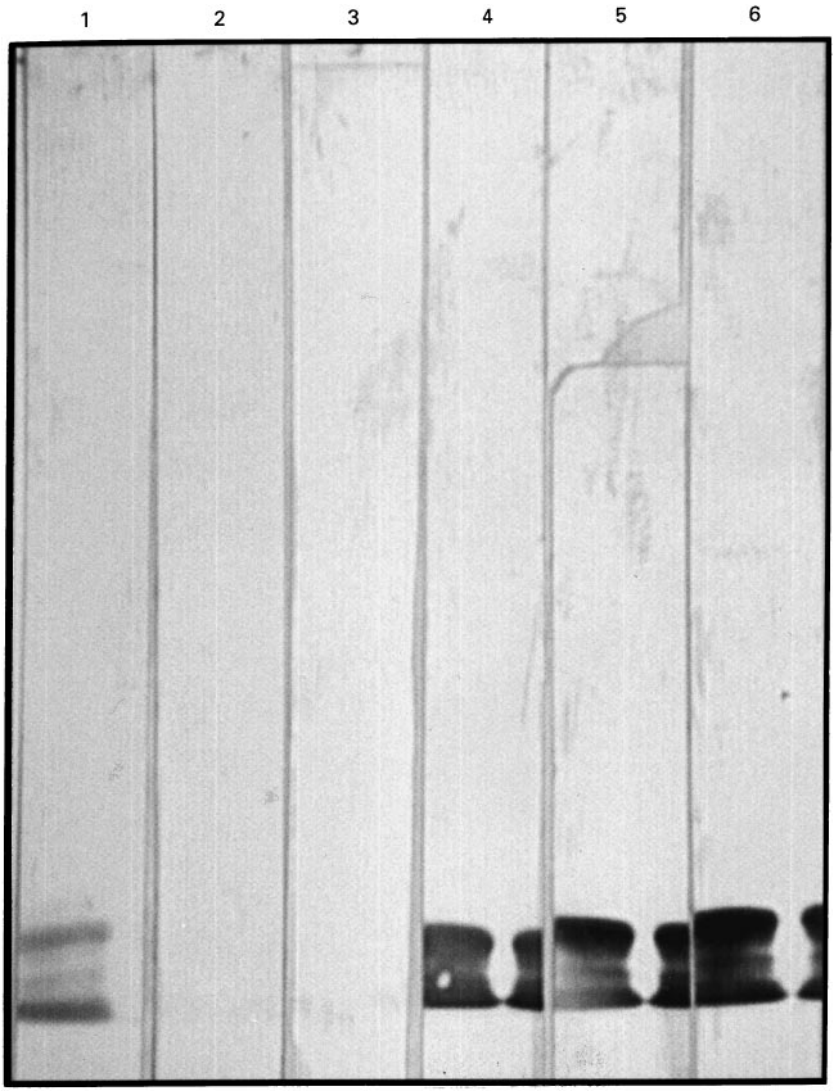

Fig. 5. Immunoblot showing anti-histone H2B reactivity of: lane 1, serum from an HIV^+ individual; lane 2, VAR4 human MoAb (negative control); lane 3, supernatant from a culture of GM4672 cells used as fusion partners (negative control); lanes 4–6, varying dilutions of BEN27b.

These antibodies were found mostly in patients with symptomatic disease, although titres decreased in patients with fulminant AIDS. In longitudinal studies, levels of the antibody appeared to parallel the numbers of $CD4^+$ cells. In previous studies, we have shown that a population of antibodies directed against activated or HIV-infected $CD4^+$ cells react with an 18-kD antigen [10]. We have demonstrated that sera containing antibodies to the 18-kD antigen react with histone H2B, and that both these molecules have a molecular mass of 18 000. The fact that the anti-histone human MoAb BEN27b reacts strongly with the 18-kD band, further suggests that this antigen is H2B. Furthermore, both BEN27b and a murine anti-H2B MoAb [19] were shown to react with PHA-activated human CD4 cells by flow cytometry (data not shown). In addition, N-terminal amino acid sequencing of 18-kD material obtained from Western blots indicated that the band is histone H2B (Comacchio RM *et al*., manuscript in preparation).

While the correlation between anti-H2B antibody titres and total serum IgG levels was not performed in this study, observations in autoimmune diseases suggest that anti-histone levels may vary independently of serum IgG levels, anti-nuclear antibodies, disease-associated idiotype levels or anti-tetanus toxoid antibody [20–22]. In systemic lupus erythematosus (SLE) anti-histone H2B antibodies may be detected in more than 60% of patients [20], and the occurrence of lymphocytotoxic antibodies (LCA) is common. Furthermore, anti-lymphocyte antibodies (those capable of cell surface binding) of IgG and IgM isotype were detected in 14% and 43% of SLE patients, respectively [23]. In this latter study there was a poor correlation between LCA or ALA and anti-DNA antibodies. Although anti-histone antibody levels were not examined, it is tempting to speculate that a correlation between the presence of anti-histone antibodies and lymphocytotoxicity exists in SLE.

Anti-histone antibodies have been reported in patients with connective tissue diseases without the striking loss of T cells found in late stage AIDS patients. For example, in chronic liver disease

anti-histone H3 antibodies are the most prominent autoantibodies, while in chronic cirrhosis antibodies reactive with histones H1 and H3 are most frequently detected [24]. In chronic active hepatitis, anti-H2B antibodies were the most prevalent [24]. In this and other studies the most commonly detected anti-histone antibodies in the healthy control group were those reactive with histone H2B, albeit at far lower frequency (approximately 5%) [20,24]. Recent studies with overlapping peptides representative of histone amino acid sequences have shown that anti-histone antibodies may recognize specific amino acid sequences [25–27], thus the fine specificity of anti-histone antibodies, including those reactive with H2B, may be important in different diseases. Therefore the cytopathic effects of anti-histone antibodies may be initiated by their recognition and binding to specific amino acid sequences. Such serological specificity has been shown for antibodies reactive with histone H1 in SLE patient sera [28]. It is also possible that circulating anti-histone antibodies may bind epitopes on histones which are only accessible after processing and presentation in a precise orientation, for example at the cell surface.

Traditionally, histones are thought to be associated exclusively with cell nuclei, and the question of why they are apparently associated with the cell membrane remains to be addressed. In fact, there is substantial evidence to suggest that histones can be found on the cell surface. Horneland *et al*. described a population of antinuclear antibodies that also bound to the plasma membrane of vascular endothelial cells [29]. In addition, it has been shown that serum-derived and monoclonal antibodies specific for histones and DNA are capable of binding to 'homologous structures' expressed on the surfaces of several cell types, including lymphocytes [30,31]. More recently, similar reactions have been observed in murine systems [32] and with human cells [33]. It has also been shown that histones H3 and H2B may be expressed on lymphocyte surfaces after PHA stimulation [31]. In the last few years several groups have attributed the cytopathic effect of HIV to be associated with programmed cell death (apoptosis) [34–37]. During the course of virus infection, large quantities of histones were observed to be generated in $CD4^+$ cell lines infected with HIV; a process which these authors associated with the DNA fragmentation characteristic of apoptosis. Such a mechanism would also explain why histones are presented to the immune system during the course of HIV infection. In fact, studies by Argov and colleagues [38] and Muller *et al*. [39] have described a high incidence of autoantibodies to nuclear proteins, including histones, occurring in HIV-infected individuals.

In addition to these findings in human subjects, the presence of anti-18-kD antibodies was also demonstrated in Rhesus monkeys infected with SIV, which developed an AIDS-like disease [11,40], but not in Sooty mangabeys, which do not become sick following infection with the virus. Furthermore, asymptomatic HIV-infected chimpanzees do not have anti-18-kD antibodies in their sera, although they were detected in one animal with profound lymphopenia [41].

High titre anti-histone antibodies have been demonstrated in animal models of autoimmune disease [19] and graft-*versus*-host disease (GVHD) [42]. The parallel between AIDS and GVHD is interesting, as both involve immune activation and self reactivity. Indeed, Habeshaw *et al*. have advanced the hypothesis that gp120 mimics a portion of an MHC-like protein, and thus can further potentiate an autoimmune GVHD-like reaction [43]. A variation of this hypothesis has been advanced by Hoffmann and colleagues [44,45], who have used the observation that patients with HIV infection have high levels of anti-collagen antibodies as evidence for a GVHD reaction [46].

The results of the longitudinal study of HIV^+ patients showed those who progressed to develop disease exhibited a decrease in anti-H2B antibody titres. It is conceivable that this phenomenon occurs because of increased antigen expression which results in immune complex formation and consequently reduces the levels of free autoantibody. Alternatively, the production of anti-18-kD antibodies may be regulated by antigenic feedback: the loss of $18\,kD^+$, $CD4^+$ cells results in a concomitant loss of autoantibodies. A final possibility is that the decrease in autoantibody titres associated with fulminant AIDS is a reflection of the overall destruction of the immune system.

There is now compelling evidence to suggest that HIV triggers a diverse range of autoimmune phenomena which frequently manifest as clinical disease (see [47–49] for review). The mechanism(s) promoting these immunopathological consequences are complex and beyond the scope of this study, but as discussed above, the presence of anti-histone antibodies in HIV^+ individuals has now been reported by several groups [38,39]. These autoantibodies may result from a breakdown of regulatory function, and consequently generalized activation of the immune system occurs. A similar phenomenon (specifically the production of anti-histone antibodies) in animals infected with the BK virus [50] has been reported. From these initial observations we conclude that the anti-H2B antibodies present in HIV^+ individuals have an antigenic specificity related to the 18-kD antigen found in activated CD4 cells. The role of these antibodies is unclear, but it is very likely that they are intimately involved in the sequence of events that leads to the dysfunction and elimination of T cells in HIV-infected individuals.

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