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# Inhibition of histone/anti-histone reactivity by histone-binding serum components; differential effect on anti-H1 *versus* anti-H2B antibodies

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## SUMMARY

IgG fractions were purified on a protein G-agarose column from sera of both systemic lupus erythematosus (SLE) patients and healthy donors. All IgG fractions, after elution with 0·5 M acetic acid, reacted with histones in an anti-histone ELISA assay, and IgG anti-histone activity was in all instances higher in the IgG fraction than in the corresponding whole serum. This was shown to be due to the presence in serum of histone-binding components that inhibited IgG binding to histones. Both normal human and SLE patients' sera had these histone-binding components, and disparity between serum-positive and -negative anti-histone antibody (AHA) tests was not dependent on differences in the blocking capacity but on IgG antibody levels and avidity. Interaction of normal serum IgG fraction with all five histones was of low avidity, whereas interaction of IgG from AHA-positive SLE sera with both H1 and H2B had high avidity. Low-affinity antibodies to every histone fraction, but also high-affinity anti-H1 antibodies, were preferentially inhibited. Our data indicate that several serum protein components are inhibiting histone/anti-histone interaction and may play a protective role against both high-affinity anti-H1 antibodies present in SLE patients, and natural, low-affinity, anti-histone antibodies. As some acute phase proteins, notably C-reactive protein, bind to histones, it is conceivable that they play such a role. High-affinity anti-H2B antibodies, present in some SLE patients, and not inhibited by these serum components, may, on the other hand, participate in the pathogenesis of the disease.

**Keywords** autoimmunity autoantibodies anti-histone antibodies systemic lupus erythematosus

## INTRODUCTION

Histones are small, highly conserved, extremely cationic, DNA binding proteins [1]. They are one of the major constituents of cell nucleus chromatin, and can be found in the circulation, mainly in the form of nucleosomes [2]. Although histones are weakly immunogenic due to their high degree of conservation, anti-histone antibodies (AHA) are among the most prevalent antinuclear antibodies, being most frequently found in drug-induced lupus and systemic lupus erythematosus (SLE). However, they have attracted relatively little attention compared with other antinuclear antibodies (anti-DNA and antibodies binding to several nuclear extractable antigens). Recently, interest in them has increased due to reports showing histone affinity for glomerular basement membrane [2] and cell surfaces [3], that have raised the exciting possibility that histone–antibody complexes play an important pathogenic role in SLE [4,5].

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Solid-phase assays have greatly facilitated AHA measurements. Histones readily adsorb to polystyrene, and antibody binding can be quantified with a class-specific enzyme- or radiolabelled anti-immunoglobulin [1]. However, disparate results have been reported concerning the specificities and frequency of AHA in SLE [6–9]. These discrepancies could be due to the technique employed and the patients studied, but also to the tendency of histones to aggregate and to interact with other molecules (DNA, $\alpha_2$-macroglobulin, C-reactive protein, nucleolin, actin, myosin . . .) [1,10–14].

Measuring IgG AHA using an ELISA technique, we obtained awkward results when serial dilutions of individual sera were tested. This prompted us to purify IgG by affinity chromatography from normal and SLE sera. Unexpectedly, we observed that binding to histones of IgG isolated from other serum proteins was increased. We noted this was due to competitive inhibition by histone-binding proteins present in all sera. As binding to these proteins is different for the different histone classes, the blocking effect for antibody reactivities in serum depends on their specificity.

## PATIENTS AND METHODS

*Serum samples*

Sera were obtained from 22 SLE patients. All of them fulfilled at least four of the revised American Rheumatism Association criteria for SLE [15]. When tested for AHA by the routine ELISA assay (i.e. performed with total histones in serum samples diluted 1:100) used for clinical purposes, 11 out of the 22 SLE sera were negative (called AHA-negative sera and numbered 1–11), and 11 positive (called AHA-positive sera and numbered 12–22). Forty sera from normal, healthy volunteers were used as controls. Blood was allowed to clot (1 h at 37°C). Serum was then recovered, and stored at −40°C in aliquots with sodium azide (0.1%) until assayed.

*Measurement of anti-histone activity*

The ELISA, used for measuring antibody binding to total histones or purified fractions, was carried out as previously described [10] with minor modifications. Ninety-six-well polystyrene flat-bottomed ELISA plates (Costar, Cambridge, MA) were incubated overnight at 4°C with total calf thymus or isolated histones H2A, H2B, H3, H4, or H1 (Boehringer Mannheim, Mannheim, Germany) at 5 μg/ml in PBS (0·01 M phosphate, 0·15 M NaCl, pH 7·2). Previously, purity of each histone fraction had been checked by SDS–17% PAGE stained with coomassie blue (Bio Rad Labs, Richmond, CA). Batches used did not show any contamination. Serum samples were diluted 1:100 in PBS, 1% bovine serum albumin (BSA), 0·1% Tween 20 (PBS-BSA-Tween). Bound antibodies were detected with peroxidase-conjugated monospecific $F(ab')_2$ antibodies to IgG (Fc) or IgM (μ-chain) (Pel-Freez, Rogers, AR). After a 1-h incubation, wells were washed and substrate dilution (0·05% $H_2O_2$, 0·02 M *o*-phenylenediamine, 0·1 M citrate buffer pH 5·5) was added. The enzymatic reaction was stopped with 2 N HCl. The absorbance values were measured at 492 nm (STL 210; Kontron, Grödig, Austria).

Upper limit of normal was defined in each assay at 3 s.d. above the mean value of 40 healthy controls. Measurements were done in duplicates. Intra-assay co-efficient of variation (CV) was always <6% and interassay CV <15%. When inhibition studies were carried out, samples to be compared were always retested in the same assay.

*Isolation of IgG fractions*

IgG fractions were purified from human sera by absorption onto a protein G-agarose column (Gammabind Genex, Gaithersburg, MD). Serum (0·5 ml) was passed onto a column of 2 ml of protein G-agarose, equilibrated with PBS. Protein G binds to all human IgG isotypes, and these can be eluted afterwards with 0.5 M acetic acid pH 3. The eluted IgG (neutralized with 1 M Tris) was dialysed against PBS. Protein concentration of the IgG fraction was determined by absorbance at 280 nm or by ELISA with anti-human γ chain conjugates, as described below. IgG recovery in the eluate was always between 80% and 95%, and no traces of IgG were found in the effluent.

*Isolation of histone-binding serum components*

Histone-binding serum components (HBSC) were isolated by affinity chromatography. Total calf thymus histones (Boehringer Mannheim) were immobilized on cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by the supplier. Effluents from a protein G column (2 ml with 5 mg/ml of protein from control sera, or 3 ml with 2·5 mg/ml of protein from SLE sera) were passed onto this histone–Sepharose column. After washing with PBS until no protein could be detected in the effluent (non-HBSC fraction) by measurement of absorbance at 280 nm (10-fold the column volume), retained components were eluted with 2 M NaSCN. Eluted fractions were dialysed against PBS overnight and concentrated by lyophilization.

*PAGE*

Proteins eluted (20 μg/ml) from the histone-CNBr Sepharose 4B column were resolved in a SDS–10% polyacrylamide gel [16] and silver stained (BioRad). Their molecular weights were estimated using a commercial protein standard (BioRad).

*IgG and IgM measurements*

IgM and IgG levels in sera and other samples were measured by ELISA. Wells of microtitre plates (Costar) were coated with goat antiserum to human IgG (Kallestad, Austin, TX) or goat anti-human μ chain (Tago, Burlingame, CA) at 5 μg/ml in PBS and postcoated with PBS–1% BSA. Samples diluted 1:200 000 were added and peroxidase-coupled antibodies to human IgM or IgG (Tago) were used as detecting reagents. The ELISA was then continued as described above for AHA detection. A standard curve was generated, to convert OD values into μg/ml by using a human standard serum (Janssen Biochimica, Geel, Belgium).

*Inhibition of IgG binding to histones*

The effect of serum effluent from a protein G column or HBSC on IgG reactivity against histones was tested. Plates coated with either total histones or each histone fraction were incubated with isolated IgG (50 μg/ml) in the presence of protein G effluent, HBSC or non-HBSC. After 1 h incubation, the AHA test was carried out further as previously described. Inhibition was calculated by comparing IgG binding in the presence or absence of any of the inhibitors. In some experiments, an inhibition curve with increasing concentrations (from 80 ng/ml to 80 μg/ml of protein) of these three samples was performed.

*Isolation of IgM from serum proteins*

In some instances we removed IgM from serum to study its effect on IgG binding to histones. An anti-human μ chain antiserum (Tago) was coupled to CNBr-activated Sepharose 4B (Pharmacia), according to the supplier. After washing with PBS, 3 ml (100 μg/ml of IgM) of protein G effluent (effluent A) from an SLE serum (no. 3) or from NHS serum were added. Only 0·5% of the IgM passed was detected in the flow-through fraction (effluent B) that was used as IgM-free in experiments of inhibition of IgG binding to histones.

*Measurement of the IgG anti-histone avidity*

We studied the avidity or relative affinity of isolated IgG from 22 SLE and six healthy volunteers' sera for the five histone fractions. This study was performed, by ELISA, taking advantage of the ability of NaCl to interfere with antigen–antibody binding in a concentration-dependent manner [17,18]. This technique allows the estimation of average antibody avidity based on the molarity of NaCl needed to decrease maximum antibody binding by 50% (M $NaCl_{50}$). IgG, diluted in PBS–1%

BSA, was incubated in the plates coated with the different histones, under standard conditions (0.15 M NaCl) or in the presence of increasing NaCl concentrations (0·3–2·4 M). NaCl was allowed to inhibit the antigen–antibody interaction for 1 h at room temperature, and then the ELISA was carried out further according to the standard procedure. A dilution curve of each IgG was included, to calculate the value for a 50% decrease in OD and the NaCl molarity that produced this decrease.

*Statistical analysis*

Data were analysed using Microstat, an Ecosoft computer program. Experimental differences were analysed by hypothesis test for means, pooled estimate of variance. $P < 0{\cdot}05$ was considered significant.

## RESULTS

*IgG anti-histone activity in whole serum and in affinity-purified IgG*

IgG fractions were purified from 22 SLE sera (11 positive and 11 negative in the routine AHA assay) and from six normal human sera, by affinity chromatography in a protein G-agarose column. Protein-containing fractions from the eluate were pooled and adjusted to 1:100 dilution (100-fold the serum volume passed onto the column). As shown in Fig. 1, significantly higher levels of IgG total anti-histone activity ($P < 0{\cdot}001$) were detected in the eluates than in the corresponding unfractionated sera both from SLE patients (both AHA-positive and -negative) and from healthy volunteers.

*Inhibition of IgG binding to histones by other serum components*

Since the above results indicated the presence of inhibitory factors in both SLE patients and normal sera, we studied the binding of a fixed amount of IgG in the presence of sequential dilutions of non-IgG fractions (effluent of a protein G-affinity column) from individual sera. Twelve IgG fractions, purified from eight SLE sera (four positive and four negative in the routine AHA assay) and four normal human sera, were studied adding non-IgG fractions obtained from their own sera. Results showed that when increasing amounts of the non-IgG fractions were added, IgG binding decreased markedly, in a dose-dependent manner, to levels similar to those obtained with the unfractionated sera.

Therefore, some serum component(s), present both in SLE sera and NHS, was either binding to histones or to IgG anti-histone antibodies and thereby blocking their reactivity. IgM antibodies were a likely candidate able to act both ways. Indeed, IgM anti-histone antibodies are common, and IgM has also been reported to inhibit several IgG autoantibodies (both natural and pathogenic) by an idiotype/anti-idiotype interaction [19–21]. So we tested whether IgM was involved in the inhibition of IgG binding to histones. After SLE sera had been passed through a protein G-agarose column, IgM was removed from the effluents by affinity chromatography in an anti-human IgM column. We then assessed its inhibitory effect on the anti-histone activity of IgG fractions. The experiments were performed by adding IgM-free protein G effluents (< 0·5% IgM) to autologous IgG fractions and comparing their effects with that of the whole effluents. As shown in Fig. 2, the IgM-free effluents inhibited IgG binding to histones in a way similar to those containing IgM. Same results were obtained when IgM was removed from normal control effluents.

Moreover, we tested for IgM antibody activity in total serum and IgG-free serum in order to exclude further a competition for histones in the ELISA test between IgM and IgG antibodies. IgM antibody binding was not significantly different in both groups of samples, neither was there any relationship between IgM antibody levels and the blocking effect (not shown).

*Histone-binding serum components are responsible for inhibition of IgG anti-histone activity*

In order to investigate further which other serum components were able to induce this effect, we passed the effluent of a protein G-agarose column (IgG-free serum) from two SLE and two healthy control sera through a histone-affinity column. Two of the eluates, treated with 5% β-mercaptoethanol, run in a 10% SDS–PAGE column and silver stained, are shown in Fig. 3.

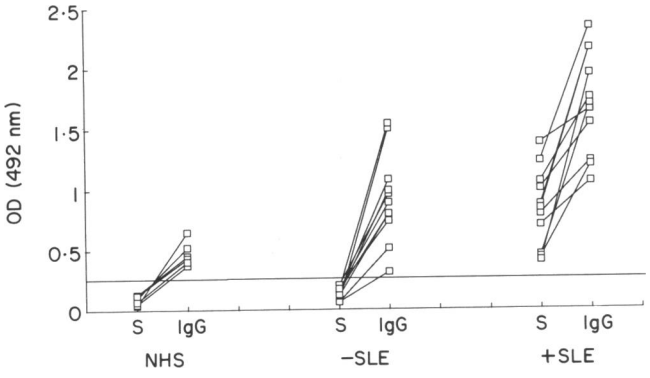


**Fig. 1.** Reactivity to histones of sera (S) and their IgGs (IgG) isolated on a protein G-agarose column. Both, sera and protein G eluates, were tested at the same dilution (1:100). NHS, Normal human sera ($n = 6$); −SLE, anti-histone antibody (AHA)-negative systemic lupus erythematosus (SLE) sera ($n = 11$); +SLE, AHA-positive SLE sera ($n = 11$). The horizontal line is 3 s.d. above the mean of NHS binding. $P < 0{\cdot}001$ in all instances when whole serum and isolated IgG from each individual case were compared.

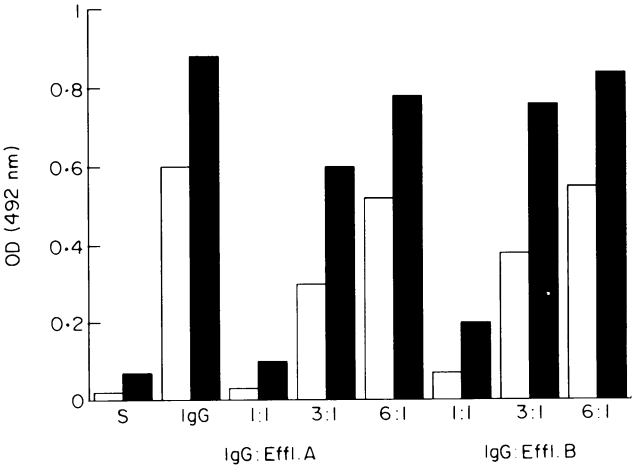


**Fig. 2.** Anti-histone activity of serum (S) and IgG fraction (both at 1:100 serum dilution) from an anti-histone antibody (AHA)-negative systemic lupus erythematosus (SLE) patient (no. 3) and a NHS, in the presence of increasing dilutions of autologous protein G effluent (Effl.A) or IgM-free protein G effluent (Effl.B). $P < 0{\cdot}01$ in all instances when IgG and IgG + Effl (A or B) were compared. □, NHS; ■, SLE.

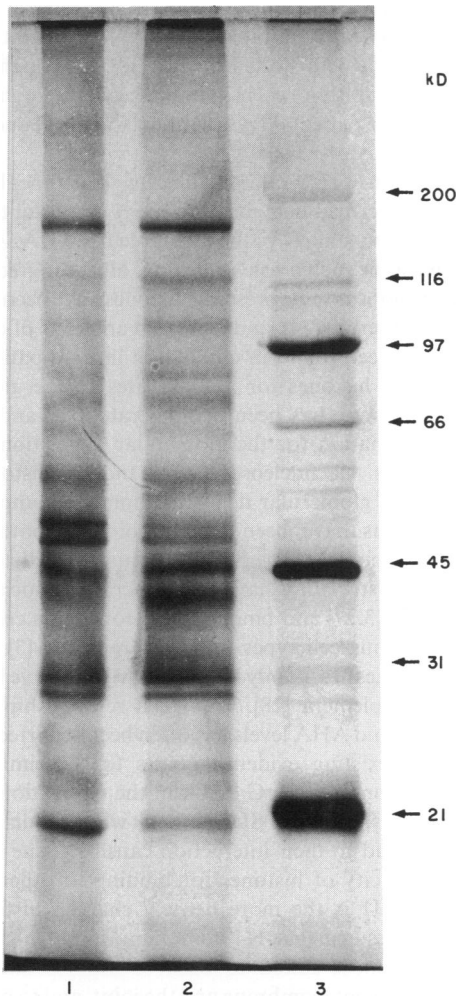


**Fig. 3.** Silver stained SDS–10% PAGE of histone-binding serum components (HBSC), under reducing conditions (5% β-mercaptoethanol). These HBSC were eluted from a histone–Sepharose column after protein G effluent of a systemic lupus erythematosus (SLE) patient (lane 1) or a control (lane 2) serum had been passed through. Lane 3, molecular weights protein standard, broad range.

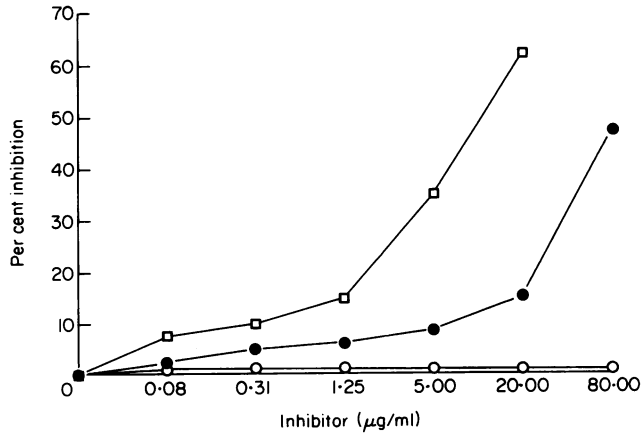


**Fig. 4.** Histone binding inhibition of isolated IgG, from systemic lupus erythematosus (SLE) patient serum no. 22, when increasing concentrations of either IgG-free serum (autologous protein G-effluent, ●), histone-binding serum components (HBSC, □) (autologous HBSC), or non-HBSC (autologous non-HBSC, ○) were added.

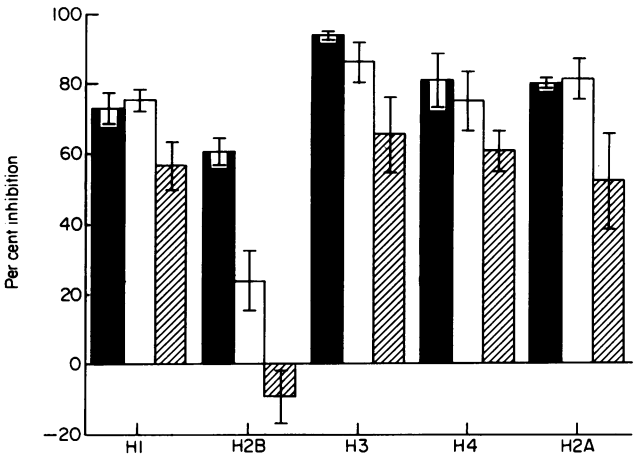


**Fig. 5.** Inhibition of antibody binding to purified histones, when comparing whole serum and autologous isolated IgG. Mean ± s.e.m. of per cent inhibition in each group was represented. Differences between serum and isolated IgG binding were significant for all antibody specificities ($P < 0.001$) except for anti-H2B in +SLE ($P > 0.05$). ■, NHS; □, −SLE; ■, +SLE.

Many proteins, of a wide range of molecular weight, were retained in the histone column. It is noted that there were only quantitative, but no qualitative, differences between SLE and control histone-binding serum proteins.

We next studied if inhibition was due to the HBSC or to any other serum factor. IgG fractions derived from sera of two SLE patients or two controls were incubated with total histones in the presence of HBSC. As shown in Fig. 4, the affinity-purified HBSC inhibited IgG anti-histone activity in a dose-dependent form, whereas the non-histone-binding fraction had no effect.

*Inhibition of antibody binding to individual histones*

In SLE patients, antibodies have been seen to react with all five histone classes. In order to see whether inhibition by HBSC was affecting all of them equally, we studied the binding of purified IgG from SLE patients and from normal sera to the different histone fractions. When HBSC had been removed, IgG antibodies were binding to all five histone classes, both in patients and in normal controls. Inhibition, when serum was tested and compared with IgG fraction results, was significant ($P < 0.001$) for all antibody specificities except for anti-H2B in anti-histone-positive SLE sera. Both in anti-histone-negative SLE and in normal sera, inhibition of binding to H2B was significantly lower than to any other histone class ($P < 0.01$) (Fig. 5).

Eight out of the 11 anti-histone-positive SLE sera were positive for anti-H1 when tested unfractionated. When only these patients' sera were considered, inhibition for H1 was equally significant (inhibition, mean ± s.e.m., 50·1 ± 6·45; $P < 0.001$).

*Anti-histone antibody avidity*

We studied the effect of varying ionic strength on IgG antibody binding to histones by carrying out the ELISA tests in the

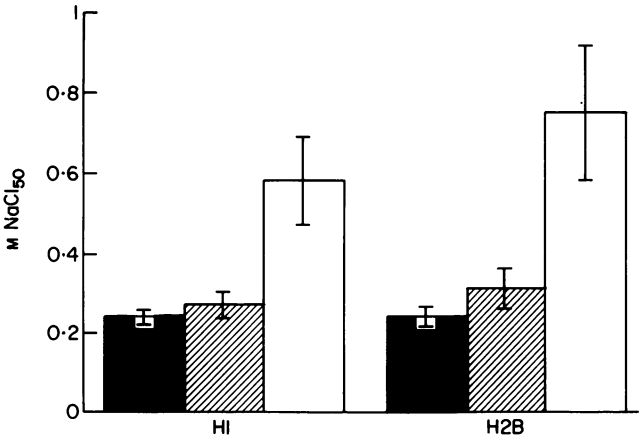


**Fig. 6.** Estimation of anti-H1 and anti-H2B antibody avidity in isolated IgG. Results are expressed as mean ± s.e.m. of M $NaCl_{50}$ (molarity of NaCl needed to decrease maximum antibody binding by 50%) in six control sera (NHS, ■), 11 anti-histone antibody (AHA)-negative sera (−SLE, ■), and 11 AHA-positive SLE sera (+SLE, □). $P < 0{\cdot}03$ when either anti-H1 or anti-H2B from +SLE were compared with either −SLE or NHS.

presence of different salt (NaCl) concentrations. M $NaCl_{50}$, defined as the NaCl molarity necessary to decrease antibody binding by 50%, was determined in IgG fractions from control and SLE serum samples. IgG antibodies from normal sera displayed a low avidity (M $NaCl_{50}$ $< 0{\cdot}3$) for all five histone classes. We considered binding avidity as high when M $NaCl_{50}$ was above mean +3 s.d. of normal sera for the specific histone. IgG antibody avidity in SLE patients was found high only to H1 and H2B (in 40% and 54% sera, respectively), mainly in sera positive for AHA when tested for total histones (Fig. 6). All these 11 sera had high-avidity antibodies for histone H2B (Fig. 6).

## DISCUSSION

In this study we have shown the presence of 'hidden' IgG anti-histone reactivity both in SLE and in normal human sera, the IgG–histone interaction being inhibited by HBSC.

Both normal human and SLE patients' sera were shown to have inhibitory HBSC, and disparity between positive and negative AHA tests were not dependent on differences in the blocking capacity but on IgG antibody levels. Although we do not know every component in the HBSC fraction, many plasma proteins do bind to histones [11–14,22,23]. By producing MoAbs to histone-binding proteins, we have identified, as two of these components, fibrinogen (data not shown), and a 55-kD protein forming complexes with IgG in some SLE sera [22]. Also $\alpha_2$-macroglobulin and C-reactive protein are well known histone-binding serum proteins [11,12]. In adition many intracellular proteins that have been shown to bind to histones might be present in serum when cellular death is taking place (i.e. nucleolin, actin, myosin, etc.) [13,14,23].

In AHA-positive SLE sera inhibition of IgG antibody binding was smaller for most histone fractions, and notably no inhibition could be seen for IgG anti-H2B binding. As one possible explanation was that only low-avidity binding was blocked, we undertook the study of antibody binding to the isolated histones at different ionic strengths. We observed that interaction of normal serum IgG with all five histone fractions was of low avidity, whereas interaction of IgG from most AHA-positive SLE sera with both H1 and H2B had high avidity. Nevertheless, inhibition of H1 binding by serum components was high both for patients' IgG (with high avidity) and normal serum IgG (low avidity).

The inhibition of IgG antibody binding to histones by HBSC that we have described here may have very important implications in relation to a likely pathogenic role for AHA. Although direct evidence for such a role is lacking, all the ingredients for pathogenicity appear to be present. Histones have been reported to be present in serum [24], and increased amounts of oligonucleosomes have been found in circulation in SLE patients [25]. High affinity of histones for anionic sites of the glomerular basement membrane has been demonstrated [2], and pointed out as an explanation for the glomerular deposition of anti-DNA antibodies, via nucleosomes [4]. Indeed, histones have been detected in glomerular deposited immune complexes [2]. Cationic antigens have been seen to bind to both hyaline cartilage and synovial tissue in mice joints [26]. Histones and nucleosome-like structures have been described associated with cell membranes [3,27] and binding of histones to a cell-surface protein on various cell types has been reported [3]. Histone reacting antibodies are mainly IgG and IgM, and have been seen to activate complement [28]. Finally, a relationship between disease activity and AHA levels has often been reported [1,6,29]. Therefore, all existing evidence points to the simultaneous presence of histones and IgG AHA in the circulation in some circumstances (especially in SLE patients when cellular death is taking place), and to their interaction causing tissue injury by the special capacity of histones for binding to other charged molecules. As H1 is the more densely charged histone, it is reasonable to assume that H1 containing immune complexes should be the most suited to bind to anionic sites in the glomerular basement membrane and the joint, and to play a role in initiating tissue injury.

In this context the presence in serum of histone-binding components could have a protective effect *in vivo* by interfering with histone binding to anionic surfaces and with histone–antibody interaction in a way similar to what we have observed *in vitro*. An intriguing aspect is whether interaction of some acute phase proteins with histones could be of any special significance in this respect. Notably, C-reactive protein (CRP), that may increase its plasma concentration as much as 1000-fold in response to acute tissue damage and various inflammatory stimuli, has been found to bind to chromatin with high affinity [30]. This binding promotes digestion of chromatin by complement and nucleases, suggesting that CRP may be important in the clearance of nuclear material [31]. H1 histone has the strongest interaction with CRP, and its presence is required for CRP binding to chromatin [12]. If histone-formed immune complexes were to play a pathogenic role, any histone-binding protein, and certainly CRP, could contribute to inhibit interaction of antibodies to histones, and of histones with glomerular basement membranes, cell surfaces or joint structures. Indeed, CRP is present in increased levels in synovial fluids in inflammatory arthritides [32], and it has been seen bound to the synovial membrane in patients with rheumatoid arthritis (RA) [33]. Lack of CRP plasma level increase observed during active SLE [34] could on the other hand facilitate disease progression.

The accumulated evidence indicates, therefore, that HBSC

inhibitory activity is at least clearly interfering with AHA measurements, even with high-affinity anti-H1 antibodies. This might explain why when correlation between AHA specificity (studied in whole serum) and disease has been investigated, levels of antibodies to core histones, but not anti-H1 antibodies, have been seen to reflect disease activity [35,36]. Antibodies to H2B histones have been shown to correlate best with the renal histologic and the clinical activity of the disease [35]. Lack of correlation between anti-H1 antibodies and clinical activity could just be due to the observed inhibition of anti-H1 antibodies in AHA tests performed in whole serum, and indicate the need for IgG purification when low-affinity IgG antibodies to any histone fraction, or high-affinity IgG antibodies, are to be accurately measured. But these observations also point to high-affinity anti-H2B, and not high-affinity anti-H1 antibodies, actually playing a pathogenic role. We favour the hypothesis that the inhibition of high affinity anti-H1 binding to H1 due to histone-binding serum components, reported here, might also take place *in vivo* and prevent H1/anti-H1 immune complex formation and its binding to anionic sites in the glomerular basement membrane and the joint.

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