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Rabbit anti-HMG-17 Antibodies Recognize Similar Epitopes on the HMG-17 Molecule as Lupus Autoantibodies. Relation with Histone H1 Defined Epitopes

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Abstract: HMG-17 is a nucleosomal protein which is an immune target of autoantibodies in systemic lupus erythematosus (SLE) and other autoimmune diseases. Autoantibody production in SLE is believed to result from autoantigen specific immune stimulation and subsequently, it is expected that antigenic determinants recognized by SLE autoantibodies and induced antibodies by immunization are quite similar. To examine this issue, rabbits were immunized with purified HMG-17. The produced antiserum showed cross reactivity on blots and in inhibition ELISA with histone H1, even after its affinity purification with immobilized HMG-17. Finally, purification of the antiserum over H1 absorbed on nitrocellulose membrane produced specific anti-HMG-17 antibodies in the supernatant and anti-HMG-17/H1 antibodies that were bound to H1. SLE sera positive for HMG-17 had also cross reactivity with H1, and following the same procedure as before we received HMG-17 specific SLE autoantibodies and anti-HMG-17/H1 autoantibodies. Using the multipin epitope mapping technology, 19 overlapping 15-mer HMG-17 peptides and six 15-peptides, corresponding to known epitopes of histone H1, were synthesized. Four major epitopes were identified on the HMG-17 molecule, reactive with induced anti-HMG-17 antibodies, and these were the same as major autoepitopes in SLE. The sequence 25–51 of HMG-17, part of its DNA-binding domain, was recognized by the anti-HMG-17/H1 antibodies that were bound to H1. These antibodies recognized also defined epitopes of H1. Our results show that SLE autoantibodies can be directed against the same or similar epitopes as do IgGs evoked during the active immunization of animals, and provide additional evidence that autosensitization with an autoantigen might be operative. The possibility that the same or similar epitopes are found on different molecules (in this study HMG-17 and H1) supports the fact that there are rules by which nature selects the most dominant immunodeterminant to a given protein, which often represents functional or structural sites in the autoantigen.

Keywords: HMG-17; histone H1; epitope mapping; rabbit antibodies; SLE autoantibodies; common epitopes

Abbreviations: Standard abbreviations are as defined in the Editorial *J. Pept. Sci.* 1999; **5**: 465–471. ANA, antinuclear antibodies; BCIP, bromochloroindolyl-phosphate; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence; HMG, high mobility group; HRP, horse radish peroxidase; IgG, immunoglobulin G; JRA, juvenile rheumatoid arthritis; MCTD, mixed connective tissue disease; NBT, nitroblue tetrazolium; OD, optical density; PBS, phosphate buffer saline; pSS, primary Sjogren's syndrome; RA, rheumatoid arthritis; RT, room temperature; SLE, systemic lupus erythematosus; TBS-T, Tris-buffer saline-Tween 20.

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INTRODUCTION

The high mobility group (HMG) proteins are evolutionary conserved, abundant and ubiquitous non-histone chromosomal proteins found in higher eukaryotes [1,2]. HMG-17 and its close homologue HMG-14 are representative of a family within this group [3] and are considered as the only specific nucleosomal binding proteins [2]. HMG-17 proteins have a sequence of 89 amino acids (MW ~9250), highly conserved in all eukaryotes studied [1,4]. Two molecules of HMG-17 (or HMG-14) bind to two sites on the nucleosome core [5–8], through their central, basic, nucleosome-binding domain that extends over 17–47 amino acid residues [8]. They serve as architectural or remodelling proteins that unfold the higher order chromatin structure, thereby enhancing various DNA-dependent activities such as transcription [9–13] and replication [14].

The HMG-17 containing nucleosomes are clustered into distinct domains in the chromatin fibre, and the presence of the protein reduces the repressive effect of the chromatin [15]. The association of HMG-17 with chromatin is dynamic rather than static and, in the absence of transcription, HMG-17 is released from chromatin and accumulates in inter-chromatin granule clusters [16]. Since HMG-17 binding to chromatin is related to chromatin structure, and subsequently chromatin function and gene activity, further studies on the mechanism of these interactions and the factors involved are essential, using specialized tools and methods, e.g. specific antibodies.

Moreover, HMG-17 is an immune target of autoantibodies. Previous reports have demonstrated an autoantibody response to HMG-17 in the sera of patients with systemic lupus erythematosus (SLE) [17,18], mixed connective tissue disease (MCTD) [17], ANA-positive pauciarticular juvenile rheumatoid arthritis (JRA) [19], but not in rheumatoid arthritis (RA) [17,18], primary Sjogren's syndrome (pSS) [18] and scleroderma [20]. Epitope mapping of the HMG-17 autoantigen revealed a proline and lysine rich octapeptide, PKPEPKPK (amino acids 34–41), as the major epitope, recognized by more than 70% of the HMG-17 positive JRA sera tested [21].

On the other hand, the antigenic properties of H1 variants (H1, H5, $H1^0$) have been studied by several groups [22,23] and most of the antigenic activity of the H1 molecule has been identified in the *C*-terminus and globular domains of the proteins, while less activity was detected in their respective *N*-terminal domains.

Epitopes localized at the basic *C*-terminus of the H1/H5 proteins were recognized by autoantibodies from SLE and drug induced lupus (DIL) patients [24–26]. Stemmer *et al.* agreed that linear epitopes on the H1 molecule are located at the *C*-terminus domain and, moreover, reported that they were recognized by rabbit induced antisera against H1 and H5 as well as by anti-histone H1 autoantibodies from patients with SLE, pSS and RA [27]. It was also reported that, anti-chromatin antibodies, induced in rabbits, recognize on the histone H1 molecule two major epitopes, similar or closely related to autoepitopes in SLE [28].

Autoantibodies in the serum of patients with SLE have specificity mainly against nucleic acids or nucleic acid binding proteins [29]. The nucleosome, in particular, has been proven to be a major immunogen of lupus [30]. These SLE autoantibodies, it is believed, are produced by an autoantigen specific-immune stimulation [31]. If an autoantibody production results from an antigen driven mechanism, then major autoepitopes on a given autoantigen could be considered to be major antigenic determinants for the antibodies induced in animals by immunization with that autoantigen.

We studied the previous hypothesis using the HMG-17 protein as antigen and we question whether linear epitopes to rabbit anti-HMG-17 antibodies share the same sequence on the HMG-17 molecule as do SLE anti-HMG-17 autoantibodies. In the present report, we attempt to map the linear B-cell epitopes within HMG-17, using rabbit anti-HMG-17 antibodies, and sera from patients with SLE, positive for anti-HMG-17 reactivity. Moreover, based on the observed cross reactivity with H1 of rabbit, as well as human antibodies, we question if HMG-17 and H1 share common or similar epitopes.

MATERIALS AND METHODS

Materials

BSA, Tween 20, *p*-nitrophenyl phosphate, BCIP, NBT and glutaraldehyde were purchased from Sigma (München, Germany). Freund's complete adjuvant and Freund's incomplete adjuvant were products of Gibco (Middlessex, UK). SDS and gelatin were products of Serva (Heidelberg, Germany). Protein A–alkaline phosphatase conjugate, biotinylated-goat antirabbit IgG, avidin-alkaline phosphatase

conjugate were purchased from Sigma (St Louis, USA). Diethanolamine was a product of Pierce (Oud-Beirland, Netherlands). Histones H1, H2A, H2B from calf thymus were obtained from Boehringer Mannheim. Affigel 10 matrice was a product of Bio-Rad (CA, USA). Nitrocellulose membrane, 0.2 μm, was obtained from Schleicher and Schuell (Germany). 96-well polystyrene plates were from Nunk (Roskilde, Denmark). Peptide synthesis was performed using the Multipin Peptide Synthesis kit (including the *N*-α-Fmoc-protected-amino acids) purchased from Chiron Mimotopes Peptide Systems (Victoria, Australia). All other chemicals, unless indicated, were obtained from Merck (Darmstadt, Germany).

Patients

Serum samples were obtained from four SLE patients, whose sera reacted strongly with HMG-17. These sera had been characterized previously [18]. Four sera of normal human donors had been pooled and used as control. All sera were supplied by the Internal Medicine Department of the University Hospital of Ioannina.

Rabbit Antibodies

The purified HMG-17 protein was used as the antigen for the immunization of two rabbits (one male, one female). Immunization was performed by emulsification of 200 μg HMG-17 in 2.5 ml Freund's complete adjuvant and injection was done at multiple intramuscular, intradermal and subcutaneous sites on days 1, 7 and 14. The final boost was administered on day 21 by an intravenous injection of 200 μg antigen dissolved in 0.5 ml sterilized physiological serum. The rabbits were bled at weekly intervals starting 1 week after the final boost. The highest titre of antiserum produced was from the male rabbit 2 weeks after the final boost.

Anti-HMG-17 rabbit antiserum was purified by affinity chromatography over HMG-17-Affigel 10 matrices, prepared according to the manufacturer's instructions (Bio-Rad, CA, USA). Fractions containing the antibody were pooled, dialysed against PBS-0.02% NaN_3, for 48 h, and stored at −20 °C. The final concentration of the pooled eluted fractions was 0.09 mg protein/ml.

Further purification of the eluted anti-HMG-17 rabbit antibodies was performed by the following procedure: Histone H1 was immobilized onto nitrocellulose membranes. Free sites were blocked by incubation with 0.5% gelatin in PBS-T (blocking solution) for 2 h at RT. Then 300 μl rabbit anti-HMG-17 antiserum or affinity purified anti-HMG-17 in blocking solution was added and incubated for 12 h at RT. The supernatant contained antibodies specific only for the HMG-17 protein and are referred to thereafter as specific anti-HMG-17 antibodies. Bound antibodies were eluted by incubation with 1 ml of 200 mM glycine, 500 mM NaCl (pH 2.2) and brought to neutrality. These antibodies recognized both histone H1 and HMG-17 protein and are referred to as anti-HMG-17/H1 antibodies.

Purification of SLE Autoantibodies

Sera of SLE patients were treated and purified with the procedure described before for rabbit anti-HMG-17 antiserum. Finally, we obtained autoantibodies specific only for the HMG-17 protein and are referred to thereafter as specific anti-HMG-17 autoantibodies, and autoantibodies that recognized both histone H1 and HMG-17 proteins (H1-bound) and were named anti-HMG-17/H1 autoantibodies.

Preparation of Subcellular Fractions

The isolation of calf thymus nuclei was performed using the procedure described by Aoronson and Blobel [32]. Crude mitochondrial fractions were prepared from calf thymus postnuclear supernatant, by centrifuging at 10 000 × **g** for 10 min, at 4 °C, and crude microsomes, by pelleting the membranes of a post-mitochondrial fraction at 110 000 × **g** for 60 min.

Immunoblotting

Proteins from subcellular fractions were separated by 15% SDS/PAGE as described by Laemmli [33] and electrotransferred onto activated nitrocellulose membrane. Alternatively, porcine thymus HMG-17 protein, calf thymus H1 and BSA diluted in 10 mM sodium acetate, pH 4.5, were spotted onto activated nitrocellulose membranes using the proper apparatus (Bio-Dot, Apparatus, Bio-Rad, CA, USA). Proteins were fixed onto the membrane with glutaraldehyde according to Karey and Sirbasku [34]. The membrane was washed with TBS-T, and finally blocked with 0.1% gelatin in TBS-T for 12 h at RT. Then, membranes were incubated with antiserum or affinity purified antibodies, at proper dilutions, in blocking solution for 2 h at RT. Bound rabbit antibodies were visualized by incubation with protein A-alkaline phosphatase conjugate, diluted

1 : 1000 in blocking solution for 1.5 h at RT, and subsequent reaction with the substrate BCIP/NBT in 10% diethanolamine buffer. When human antibodies were used, the membranes were incubated with anti-human IgG conjugated to HRP, as the second antibody, diluted in the blocking buffer (1 : 5000), and detected by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Amersham, Life Science).

Inhibition ELISA

Inhibition ELISA was performed as previously described [18] using HMG-17, H1, H2A and H2B as inhibitors.

Peptide Synthesis

Epitope mapping was performed using a Multipin Peptide Synthesis kit from Chiron Mimotopes (Australia). Nineteen pentadecapeptides overlapping by 11 amino acids, and covering the entire sequence of porcine HMG-17 [4] were synthesized in duplicate according to the multipin peptide synthesis of Geysen *et al.* [35], on prederivatized polyethylene gears attached to inert polyethylene stems. The following peptides were also synthesized in duplicate: the peptide corresponding to sequence 27–41 of the human HMG-14, four peptides corresponding to sequences 132–146, 147–161, 171–185, 205–219 of the human H1b variant, the peptide 113–127 of the human $H1^0$ and the synthetic peptide KTPKKAKKPKTPKKA.

The applied protocol was based on the principles of the solid phase synthesis, using the Fmoc-protecting group strategy [36]. The *N*-α-Fmoc-amino acid esters (60 mM) were activated with 1-hydroxybenzotriazole (HOBt, 72 mM) [37], using highly purified *N*, *N*-dimethylformamide (DMF) as solvent. α-Amino groups were deprotected by 20% v/v piperidine in DMF. *N*-termini of the peptides synthesized were acetylated using a mixture of DMF : acetic anhydride : triethylamine (5 : 2 : 1 v/v/v). Side chain deprotection was accomplished by treating the pins with a mixture of trifluoroacetic acid : ethanedithiol : anisole, 95 : 2.5 : 2.5 (v/v/v). In each synthesis cycle, a positive control peptide was used, sequence VRLRWNPADYGGIKKIRL (63–80) of the subunit of the acetylcholine receptor (AChR) recognized by an anti-AChR monoclonal antibody, while as a negative control peptide the sequence VRLRWAPAAYGGIKKIRL [38] was synthesized.

ELISA

Peptides coupled to polyethylene pins were tested for antibody binding by ELISA in 96-well polystyrene microtitre plates (all steps were carried out at room temperature, unless indicated). The pins were incubated with 200 µl of blocking buffer (PBS, pH 7.3, containing 0.1% Tween 20 and 2% BSA), for 1 h, to reduce non-specific binding. Then, the pins were incubated overnight, at 4 °C, with 175 µl of the appropriate dilution in blocking buffer, of the primary antibodies under investigation. After washing (four times) with 0.5% PBS-Tween 20, for 10 min, with agitation, each pin was incubated with 175 µl of donkey anti-rabbit IgG-HRP (1 : 2500 dilution in blocking buffer), or anti-human IgG-HRP (Fc specific), diluted 1 : 5000 in blocking buffer, for 1 h. The pins were then washed, as previously, and incubated with 150 µl/well of substrate solution (o-phenylenediamine 0.5 mg/ml in citric buffer, pH 5.0) in the dark. Then the pins were removed from the substrate solution and the absorbance of the produced colour was measured at 490 nm with an ELISA microplate reader. To test the cross-reactivity with other serum compounds, the ELISA testing was also performed using instead of the primary antibodies, normal rabbit serum and normal human sera, diluted 1 : 500, and the monoclonal antibody that recognized the non-related to HMG-17 positive control peptide.

After completion of the assay, the pins were sonicated for 15 min in a water bath with 0.1 M sodium dihydrogen phosphate, 1% SDS, and 0.1% 2-mercaptoethanol at 60 °C to remove antibodies. The pins were subsequently washed twice in hot water (60 °C) and immersed in methanol (60 °C) for 2 min. The pins were allowed to air-dry for a minimum of 20 min and then were ready for reuse or storage.

Inhibition Studies

Primary antibodies (rabbit or human) were preincubated with 200 µg/ml HMG-17 or H1, for 1.5 h, at RT, and then used in the former ELISA system.

RESULTS

Purification and Specificity of Rabbit Antibodies

As previously reported HMG-17 protein is poorly antigenic [39]. We support this evidence since we failed to raise antibodies against HMG-17 with other protocols [40]. The maximum response of the animal was observed 2 weeks after the last boosting.

The specificity of the antiserum was tested first by immunoblotting. Subcellular fractions from calf thymus (nuclei, mitochondria, microsomes and soluble fraction) as well as whole cells extract (CHO) were fractionated by SDS-PAGE, transferred to nitrocellulose membranes treated with glutaraldehyde. Then the membranes were probed with anti-HMG-17 antiserum, as described in Materials and Methods. Rabbit anti-HMG-17 antiserum recognized on blots, as was expected, the HMG-17 protein in nuclear and whole cell extracts as well as the pure protein. Meanwhile, the antiserum recognized also a double protein zone corresponding to the MW of histone H1 in the lanes corresponding to whole cells and nuclei. No other protein band was recognized on the cellular or subcellular fractions tested (Figure 1A). Further evidence that the anti-HMG-17 antiserum had cross reactivity with H1 came by dot-blotting onto nitrocellulose membranes HMG-17, H1 and BSA and probing the membranes with the antiserum. Again recognition of both HMG-17 and H1 occurred on blots (Figure 1B).

To test whether the cross reactivity of the anti-HMG-17 antiserum with histone H1 existed in the liquid phase as it did in the solid phase, an inhibition ELISA was performed. The antiserum was incubated with H1 before it was added to microplates to react with immobilized HMG-17 (heterologous inhibition). The reaction was inhibited by 35% with the highest antigen concentration (not shown). No inhibition was observed using the core histones H2A and H2B as inhibitors. Since the antiserum had cross reactivity with H1, both on blots and in liquid phase, we proceeded with its affinity purification.

The antiserum was purified over an affinity column with immobilized HMG-17. The affinity purified antibodies were checked again for their specificity with dot-blotting (Figure 1B). Unexpectedly, the cross reactivity with histone H1 still remained (Figure 1B). For this reason histone H1 was absorbed on nitrocellulose and the membrane was then incubated with affinity purified antiserum as described in Materials and Methods. Two populations of antibodies were prepared in that way. First, H1 unbound (or H1 purified) antibodies and, second, H1 bound antibodies. These two antibody populations were tested for their specificity on dot-blots. The first antibody population (H1 unbound) contained antibodies that recognize on blots only the HMG-17 protein (anti-HMG-17 specific IgGs) (Figure 1B). On the other hand, the second antibody population (bound on H1 antibodies), recognize both H1 and HMG-17 (anti-HMG-17/H1 IgGs) (Figure 1B). If rabbit antiserum was purified directly over H1 the same results were observed for bound on H1 and unbound antibodies (not shown). Preincubation of anti-HMG-17 specific antibodies with H1, before they were added to react with immobilized

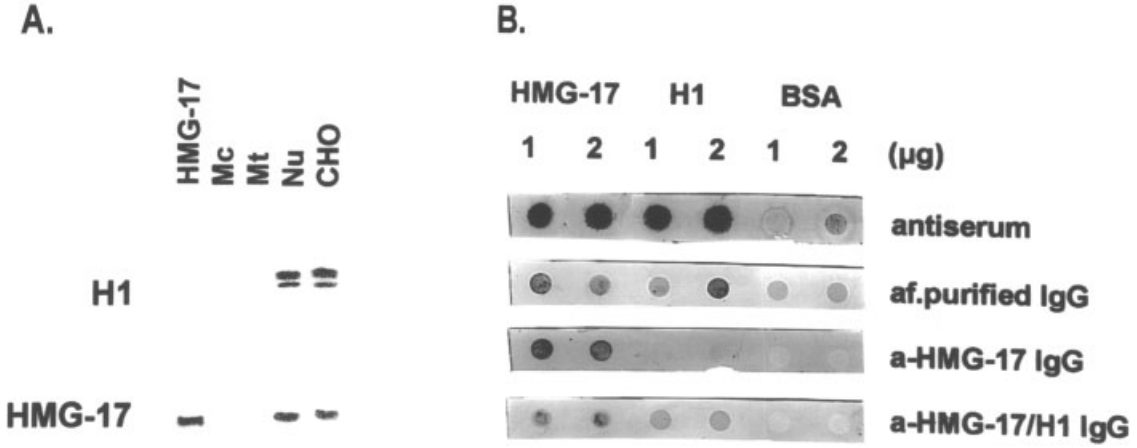

Figure 1 Specificity of anti-HMG-17 antibodies. (A) Pig thymus HMG-17 (2 µg), calf thymus subcellular fractions (Nu nuclei; Mt mitochondria; Mc microsomes; 60 µg each) and whole cell extract (CHO Chinese hamster ovary cells; 60 µg) were subjected to SDS-PAGE and transferred to nitrocellulose. After blocking the membranes were probed with rabbit anti-HMG-17 antiserum and visualized by incubation with protein A-alkaline phosphatase conjugate and reaction with BCIP/NBT. (B) Pig thymus HMG-17 protein, calf thymus H1 and BSA (1 and 2 µg) were spotted on nitrocellulose membranes. The strips were blocked and probed with rabbit anti-HMG-17 antiserum (antiserum), affinity purified rabbit anti-HMG-17 antibodies (af. purified IgG), rabbit anti-HMG-17 specific antibodies (anti-HMG-17 IgG), and rabbit anti-HMG-17/H1 antibodies (anti-HMG-17/H1 IgG) and visualized as in (A).

HMG-17 in microplates (heterologous inhibition), showed no inhibition (not shown). These results indicated that the anti-HMG-17 specific antibodies recognized only the HMG-17 protein. They were also indicative of the fact that HMG-17 protein and histone H1 share common epitopes, since immunization of rabbits with HMG-17 raised anti-HMG-17 antibodies that recognize also histone H1.

Purification of SLE Sera

Having in mind the previous observation that rabbit anti-HMG-17 antiserum, using intact HMG-17 as antigen, showed cross reactivity with H1, we performed dot-blot to test whether sera of patients with SLE positive for HMG-17 also reacted with H1. We found that sera of SLE patients, characterized before for their anti-HMG-17 reactivity [18], and having a highly positive reaction with HMG-17, recognized on blots both HMG-17 and H1 (not shown). Surprisingly, affinity purification of the sera, did not abolish the H1 reactivity of the resulting purified autoantibodies, although the reaction with HMG-17 was stronger (as with rabbit anti-HMG-17 antiserum). If the purified anti-HMG-17 autoantibodies were further purified over H1, then the anti-HMG-17 autoantibodies were divided in two sub-populations: H1 unbound autoantibodies that recognize specifically HMG-17 protein and H1 bound autoantibodies that recognize both HMG-17/H1 proteins (see Materials and Methods). These SLE autoantibodies, four HMG-17 positive affinity purified SLE sera and the eight autoantibody sub-populations obtained from these sera after purification over H1 (four anti-HMG-17 specific and four anti-HMG-17/H1 antibody populations), were used to perform the epitope mapping of the HMG-17 protein.

HMG-17, HMG-14 and H1 Synthetic Peptides

For epitope mapping of the HMG-17 protein, we synthesized, in duplicate, 19 15-mer peptides, overlapping by 11 residues, covering the entire sequence of porcine HMG-17, as previously determined [4]. Porcine HMG-17 has only one conservative substitution compared with the human protein. At position 64 an aspartic acid residue is substituted with glutamic acid (64 Asp/Glu). The following peptides were also synthesized: four peptides corresponding to sequences 132–146, 147–161, 171–185, 205–219 of the human H1b variant, the peptide 113–127 of the human $H1^0$ and the synthetic peptide KTPKKAKKPKTPKKA, which is part of the sequence $(KTPKKAKKP)_2$, that has been shown to bind DNA [40]. We also synthesized the peptide corresponding to sequence 27–41 of the human HMG-14 which is part of the DNA-binding domain 13–43 of the protein [1] and also contains the *C*-terminus of the HMG-14 peptide 13–35, which was shown to be immunogenic [42]. This particular HMG-14 peptide is homologous to HMG-17 only at the motif PKKA. In Figure 2 the amino acid sequences of the HMG-17 protein, as well as the peptide sequences corresponding to H1 and HMG-14 peptides, are represented by the single letter notation. The H1 peptides selected for synthesis have been proven to be major epitopes of H1 in previous studies [27] and have features similar to the central, basic domain of the HMG-17. These features are sequence homology to HMG-17, high lysine content and having in common with HMG-17 the tetrapeptide motif PKKA.

All former pin bound peptides were tested against purified IgG from rabbit anti-HMG-17 antibodies and anti-HMG-17 autoantibodies of SLE patients.

Epitope Mapping of HMG-17 Performed with Rabbit Anti-HMG-17 IgGs

The following rabbit antibodies were applied in the epitope mapping system: (i) polyclonal rabbit anti-HMG-17 antibody which was affinity purified from the rabbit antiserum raised against intact HMG-17 (it had both anti-HMG-17 and anti-H1 reactivity), (ii) purified rabbit anti-HMG-17 specific antibody and (iii) rabbit anti-HMG-17/H1 antibody, both (ii) and (iii) purified over H1 from (i) (see Materials and Methods).

The rabbit anti-HMG-17 antibody (i) recognized the 15-peptides corresponding to two amino acid sequences on the HMG-17 molecule, namely the regions 1–15 and 17–63. These clusters became more determined if mapping was performed with anti-HMG-17 specific antibodies (ii). The anti-HMG-17 specific antibody recognized the peptides corresponding to amino acid sequences 1–15, 17–39 and 41–63, respectively, (Figure 3). On the other hand, mapping with rabbit anti-HMG-17/H1 antibodies revealed antigenicity of the peptides that corresponded to the region 25–51 of HMG-17 (Figure 4). These data showed that rabbit anti-HMG-17 antibodies recognize on the HMG-17 molecule four epitopic sequences, namely the regions 1–15, 17–39, 25–51 and 41–63 (Figure 5). These sequences lie at the *N*-terminal and the central, basic, DNA binding domain of HMG-17 while the *C*-terminus was not significantly recognized by the anti-HMG-17 antibodies.

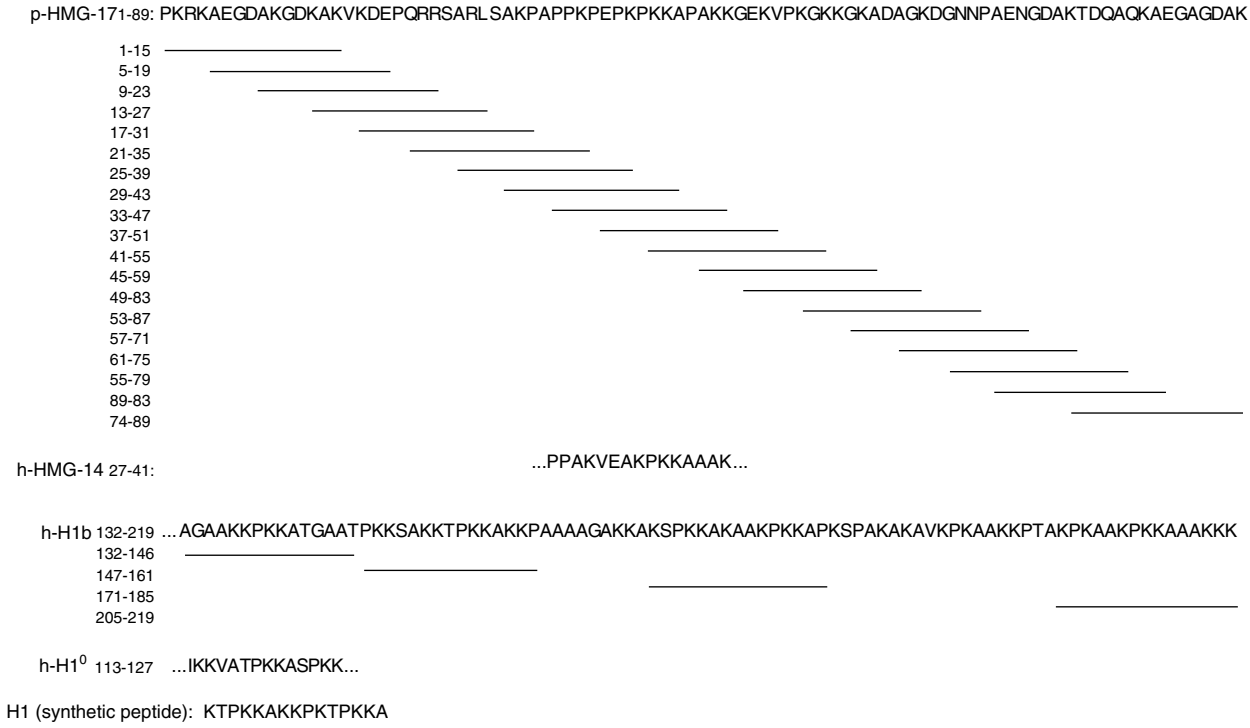

Figure 2 List of peptides used for the epitope definition of the HMG-17 protein.

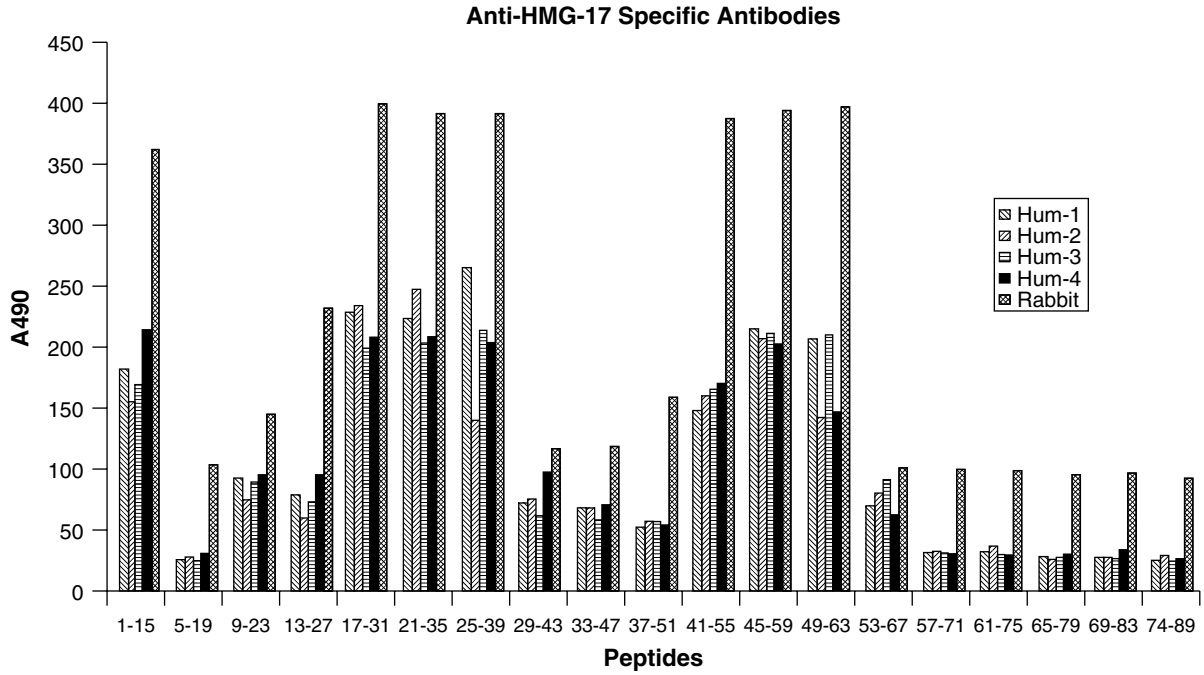

Figure 3 Epitope mapping of HMG-17 performed with rabbit anti-HMG-17 specific antibodies and SLE anti-HMG-17 specific auto-antibodies (H1 unbound) (data from a representative experiment).

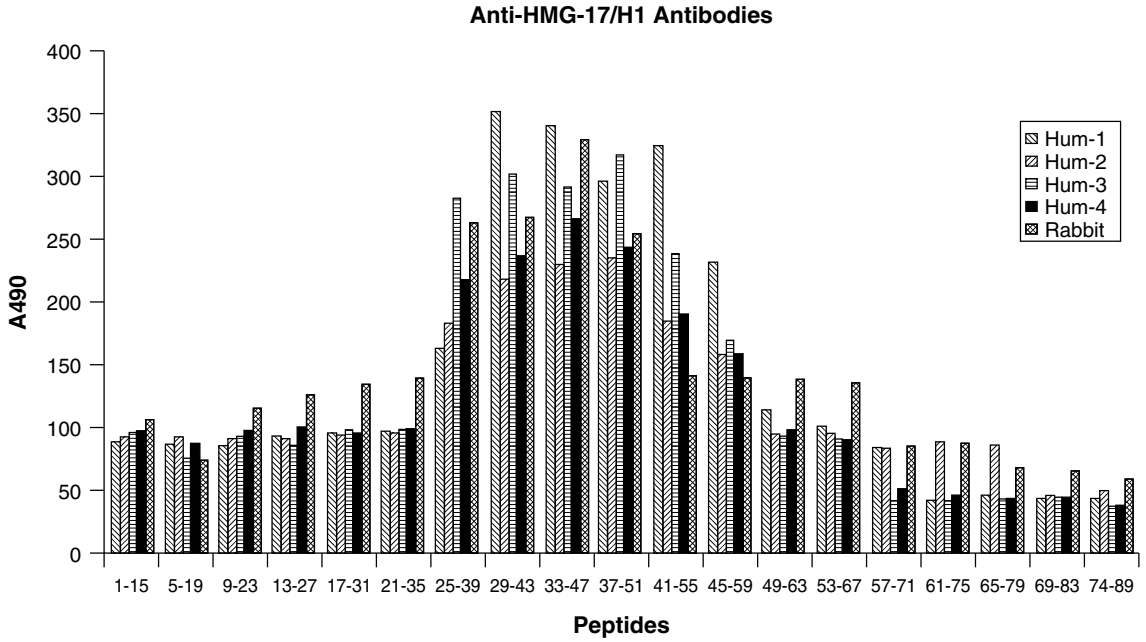

Figure 4 Epitope mapping of HMG-17 performed with rabbit anti-HMG-17/H1 antibodies and SLE anti-HMG-17/H1 autoantibodies (H1 bound) (data from a representative experiment).

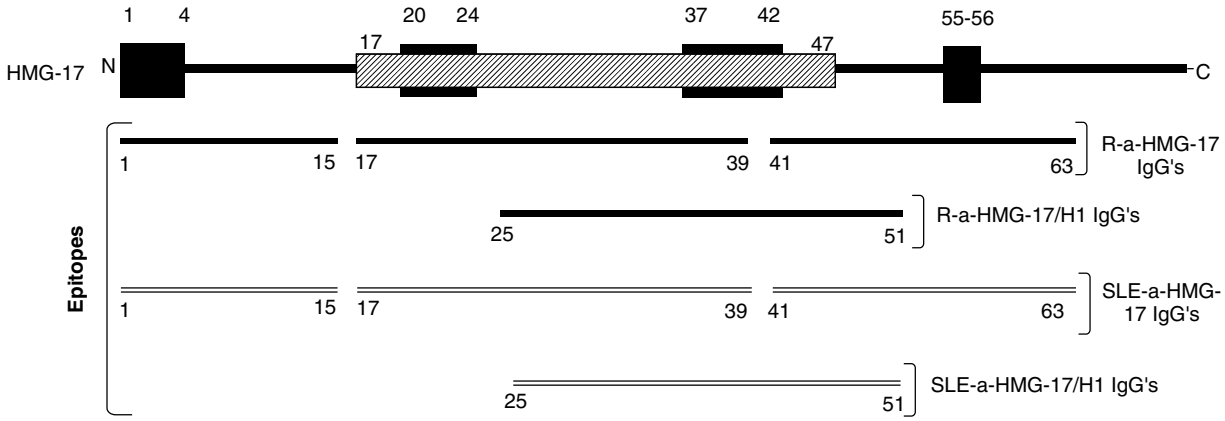

Figure 5 General presentation of the HMG-17 epitope mapping results obtained with polyclonal antibodies raised in rabbits against intact HMG-17 (▬) and with SLE anti-HMG-17 autoantibodies (═) as measured in ELISA with overlapping synthetic peptides. In the sequence of HMG-17, the nucleosomal binding domain (residues 17–47) is shown as a shaded box and the predicted strongly hydrophilic regions on the protein [44] are presented as black boxes.

On the other hand, the peptides corresponding to sequences 147–161, 171–185 and 205–219 of the H1b molecule, the peptide 113–127 of the $H1^0$ and the synthetic peptide KTPKKAKKPKTPKKA were recognized by the rabbit anti-HMG-17 antiserum and by the anti-HMG-17/H1 antibodies but not the rabbit anti-HMG-17 specific antibody. The H1b peptide 131–145 and the HMG-14 peptide 27–41 were not recognized by any of the former rabbit antibodies, indicating that it is not only the motif PKKA responsible for the antibody recognition, but probably the adjacent amino acids contribute to the antigenicity as well. The cut-off value of positive reaction for rabbit anti-HMG-17 antibodies was two times higher than the mean background value in each experiment. The OD range of positive reaction for H1 peptides was 0.18–0.25. Experiments were repeated twice with identical results.

The normal rabbit serum showed no significant non-specific binding to all tested peptides. The

monoclonal antibody against the VRLRWNPADYG-GIKKIRL peptide (not related to HMG-17 or H1), did not show any significant non-specific binding to the HMG-17 or H1 peptides either.

Epitope Mapping of HMG-17 Performed with SLE Anti-HMG-17 Autoantibodies

When the SLE autoantibodies were used for the epitope mapping of the HMG-17 a similar epitopic structure as with rabbit antibodies was obtained. All four SLE affinity purified sera recognized the peptides that corresponded to amino acid sequences 1–15 and 13–63 on the HMG-17 protein. The anti-HMG-17 specific autoantibodies (not bound to H1) recognized the peptides that corresponded to regions 1–15, 17–39 and 41–63 on the protein, respectively (Figure 3). Three of four anti-HMG-17 autoantibodies with anti-HMG-17/H1 activity (bound to H1) recognized the peptides corresponding to the amino acid sequence 25–59 of the HMG-17 molecule, while the fourth recognized the peptides that corresponded to sequence 29–51.

These data show again that SLE autoantibodies recognize on the HMG-17 protein four antigenic determinants, namely the regions 1–15, 17–39, 41–63 and 25–59 (except in one case where the recognized sequence was 29–51).

The H1b peptides 147–161, 171–185 and 205–219, the $H1^0$ peptide 113–127 and the peptide KTPKKAKKPKTPKKA were recognized by the SLE purified sera and by the H1 bound anti-HMG-17/H1 autoantibodies. On the other hand, the H1b peptide 131–145 and the HMG-14 peptide 27–41 were not recognized by any of the anti-HMG-17 autoantibodies tested. Normal human sera did not recognize any of the pin bound peptides. The cut-off value of the positive reaction for SLE autoantibodies was two times higher than the mean background value in each experiment. Experiments were repeated twice with identical results.

Inhibition Studies

When rabbit and SLE anti-HMG-17 specific antibodies were preincubated with HMG-17, the recognition of the corresponding pin bound peptides was abolished, but when they were treated with H1 the recognition was similar to before. On the other hand, when rabbit and SLE anti-HMG-17/H1 antibodies were pretreated with HMG-17 or H1 the corresponding HMG-17 pin bound peptides as well as the H1 corresponding pin bound peptides were not further recognized.

DISCUSSION

In this work anti-HMG-17 rabbit antibodies were raised, characterized and purified. We emphasize the fact that the produced rabbit antiserum, using the HMG-17 whole molecule as antigen, had cross reactivity on blots, as well as in the liquid phase, with HMG-17, as expected, and with histone H1. Surprisingly, this cross reactivity could not abolished by affinity purification of the antiserum on a column containing immobilized HMG-17.

The anti-HMG-17 autoantibodies from SLE patients had also cross reactivity with H1, as did the rabbit anti-HMG-17 antibodies produced by immunization, even after purification on an affinity column with immobilized HMG-17. Purification of the rabbit, as well as the SLE anti-HMG-17 antibodies, over H1, resulted in anti-HMG-17 specific rabbit antibodies and autoantibodies (H1 not bound anti-HMG-17 antibodies) and anti-HMG-17/H1 rabbit antibodies and autoantibodies (H1 bound anti-HMG-17 antibodies). These data indicate first the existence of major antigenic determinants in HMG-17 molecule and, second, the presence of common epitope(s) between HMG-17 and H1 in SLE autoantibodies, as well as, in rabbit induced antibodies.

Comparison of the sequences of the two proteins, HMG-17 and H1, revealed that they have an homologous region consisting of four amino acid residues (PKKA tetrapeptide). This sequence lies at the sites 40–43 on HMG-17 protein, at the DNA-binding domain of the protein and shows a predicted increased antigenicity. The PKKA sequence is repeated four times in the *C*-terminus region of the H1 molecule [42] at positions 119–122, 125–128, 153–156 and 172–175. This particular tetrapeptide is also part of the sequence of the synthetic peptide $(KTPKKAKKP)_2$, based on H1 sequence, that affects the DNA condensation on chromatin [41]. These observations are consistent with the fact that the H1-cross reactivity of the anti-HMG-17 antibodies was eliminated through purification over H1.

Nineteen 15-mer peptides overlapping by 11 residues and spanning the entire length of the HMG-17 molecule were used as antigenic substrates in ELISAs. Moreover, the HMG-14 peptide 27–41, five peptides corresponding to sequences 132–146, 147–161, 171–185 and 205–219 of the major human H1b variant, the $H1^0$ 113–127 peptide and the peptide KTPKKAKKPKTPKKA, were also used as antigenic substrates in the same ELISAs. The H1 peptides were chosen because they were recognized

as antigenic determinants by rabbit as well as SLE autoantibodies in previous studies [27] and they also had common features with the DNA-binding domain of the HMG-17 protein. The HMG-14 peptide 27–41 was chosen in our study for the following reasons: first, it contains the motif PKKA, but it differs in the other residues from the HMG-17 sequence (it has four lysine residues instead of seven or eight in the positive PKKA containing peptides), and second, it contains the *C*-terminus half of the peptide 13–35 which was used as antigen in a previous study [41], as part of the DNA binding region of the HMG-14 protein.

Four major epitopes were identified on the HMG-17 molecule in our study, spanning the sequences 1–15, 17–39, 25–51 and 41–63. Three of them, namely the sequences 1–15, 17–39 and 41–63 contain epitopes specific for the HMG-17 protein, since they were recognized by the specific anti-HMG-17 rabbit antibodies as well as the SLE autoantibodies (the former IgGs do not bind to H1). The fourth epitopic sequence of HMG-17, 25–51, has similarities with the H1 defined epitopes, since it is recognized by the rabbit and human anti-HMG-17/H1 antibodies (anti-HMG-17 antibodies that recognize also H1). This sequence lies within the highly conserved region comprising the nucleosomal binding domain of the HMG-17 (residues 17–47) [8] and has partial homology with histone H1. It contains the octapeptide sequence PKPEPKPK, which has been identified as constituting a highly antigenic epitope, recognized by the majority of HMG-17 positive sera from patients with pauciarticular-onset JRA [21].

The anti-HMG-17/H1 antibodies (rabbit and human) recognized also the peptides 147–161, 171–185, 205–219 of H1b, the $H1^0$ 113–127 peptide and the synthetic peptide KTPKKAKKPKTPKKA. The *C*-terminus peptide of H1b, 204–218 was previously characterized as a major epitope of the H1 molecule and was recognized by antibodies from SLE, RA and pSS patients as well as by rabbit antibodies [27]. The peptides 147–161 and 171–185 of H1b were also recognized as was the $H1^0$ peptide 113–127. These peptides contain sequence homologies to peptide 204–218 and have also similar features to the epitopic region 29–51 of the HMG-17 molecule. The H1 peptides share with that particular sequence of HMG-17 the high lysine content (7–8 residues per peptide) and they also have in common the tetrapeptide PKKA. The H1b sequence 144–159 has eight lysine residues and the motif PKKA in common with HMG-17 peptides 8–11, the $H1^0$ peptide 113–127 has two PKKA motifs as does the H1 synthetic peptide.

Computer algorithms for antigenicity prediction utilizing the amino acid sequence of HMG-17 [44], showed the existence of four strongly hydrophilic clusters on the protein, namely the sequences 1–4, 20–24, 37–42 and 55–56. This structural property has been proposed to be an important feature of antibody binding epitopes [45]. These highly hydrophilic sequences lie within the regions that were recognized as antigenic determinants in this study.

It was reported earlier that chromatin induced antibodies were directed against the same or similar epitopes on histone H1 molecule as did lupus autoantibodies [28]. It was also reported that specific natural anti-alliinase (a protein found in garlic cloves) antibodies presented in human serum had the same or overlapping epitopes with IgG evoked during immunization in rabbits [46].

In this report we demonstrate the existence of four epitopes on the HMG-17 molecule recognized by both antibodies elicited in rabbits and SLE autoantibodies. More interestingly, we demonstrated a major epitope of HMG-17, lying at position 25–51, which was recognized by anti-HMG-17/H1 antibodies from both species rabbit and human. These particulate anti-HMG-17/H1 antibodies (rabbit and human) recognized also the H1 defined epitopes which have sequence homology with the HMG-17 major epitope.

Histone H1 binding to chromatin has been shown to repress the gene activity [22]. On the other hand, binding of HMG-17 to H1 depleted nucleosomes unfolds the higher order chromatin structure, reducing consequently the repressive effects of chromatin [17]. Thus, it would be valuable for studies on the sequence of events and factors that result in active chromatin, an antibody that binds both proteins, HMG-17 and H1, particularly, since it recognizes an epitope at the DNA binding domain of HMG-17 and epitopes at the *C*-terminus of H1, which are known to be involved in interactions with other proteins.

The possibility that the same or similar epitopes are found on different molecules (here HMG-17 and H1) supports the hypothesis that autoantibodies in SLE are produced through an antigen driven mechanism, where 'molecular mimicry' plays a dominant role. The results of this study indicate that there are certain rules by which nature chooses the most dominant immunodeterminant to a given protein antigen (or autoantigen) and that specialized sequences on the antigen seems to be one of these.

The nucleosomal protein HMG-17 is one of the growing number of nucleic acid binding proteins known to react with antibodies in sera of patients with autoimmune diseases. Although the mechanisms of induction of anti-chromatin autoantibodies are poorly understood, autoimmune responses to these macromolecular complexes display certain common features. The autoantibody response is generally polyclonal with production of antibodies to multiple linear and conformational B-cell epitopes, representing often functional active sites in the autoantigen. Identification of B-cell epitopes of autoantigens is generally important, since it may provide useful information for the mechanism triggering autoantibody production, as well as on the functional and structural features of the antigenic determinants that are targeted. In addition, determination of the major epitopes of an antigen may considerably facilitate immunoassays with increased sensitivity of detection of autoantibodies and thus improve the diagnosis of autoimmune diseases.

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