Characterization of a monoclonal antibody reacting with histone H3

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Received 15 January 1985

A hybridoma cell line, 1GB3, has been obtained from a fusion between SP/O-Ag 14 myeloma cells and lymphocytes from BALB/c mice immunized with rat liver nuclear proteins. This hybridoma secreted a monoclonal antibody of the IgG2b class which reacted specifically with histone H3 in enzyme-linked immunosorbent assay (ELISA) as well as in immunoblotting and immunodot assays. Stringent test conditions were necessary to eliminate the presence of nonspecific or contaminating reactions with other histones than H3. The monoclonal antibody appears to recognize an epitope situated in the N-terminal residues 20–50 of histone H3; it recognizes this epitope in the octamer aggregate of core histones but not in the core particle.

Monoclonal antibody *Histone H3* *ELISA* *Immunodot assay*

1. INTRODUCTION

Immunochemical approaches have been widely applied to study the organization and structure of chromatin. In most of these studies antisera raised against histones, either free or complexed to nucleic acids, have been used (reviews [1,2]). However, this has limited the application of these studies, since histones within chromatin may be subjected to various chemical modifications, may have altered conformation in comparison to free histones and/or may be sterically inaccessible. On the other hand, antisera elicited by immunizations with chromatin did not react with pure histones, with the exception of H1. Therefore, histones in chromatin have been considered as non-immunogenic [3].

The informational value of immunochemical data could be enhanced if antibodies of defined specificity were available. Attempts have been made, therefore, to localize the antigenic areas within the primary structure of histones which are recognized by specific antibodies [4–10]. Furthermore, peptides obtained by enzymatic or chemical cleavage, as well as synthetic peptides, have been used to isolate the correspondent antibody population or to elicit specific antibodies [6,7,10–12].

The hybridoma technique for the production of monoclonal antibodies [13] offers an alternative approach for obtaining antibodies against one defined epitope. In addition to the advantages of specificity and availability of antibodies, it further permits the application of immunogens in the form of native complexes, allowing immunological responses to develop against specific molecular conformations of complex particles such as nucleosomes in chromatin. Recently, we applied the hybridoma technique to elicit monoclonal antibodies against nuclear proteins using a mildly prepared fraction from rat liver nuclear extract as immunogen. One of the clones isolated during this study produces a monoclonal antibody which recognizes an epitope on histone H3. Here, the characteristics of this monoclonal antibody are described.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. MATERIALS AND METHODS

2.1. *Preparation of antigens*

Rat liver nuclear extract was prepared by sonication at pH 8.0 and fractionated on a 15–30% sucrose gradient according to Louis and Sekeris [14]. The fractions containing the 30–50 S RNP monoparticles were pooled and submitted to high speed centrifugation (48000 rpm, 20 h at 4°C in a Ti 50 Beckman rotor). The pellet was taken up in 0.16 M NaCl, 3 mM KCl dissolved in 10 mM phosphate-buffered saline (pH 7.6) (or PBS).

Chicken erythrocyte chromatin and core particles were prepared as previously described [15]. H3-H4 complexes were a gift from P. Sautière (Lille, France). Chicken erythrocyte and calf thymus histones H2B, H2A, H3 and H4 were extracted and purified according to the acid method of Johns [16]. Some experiments were carried out with calf thymus H2B purchased from Boehringer, Mannheim (batch no.1253206) and from Sigma, St Louis. The mixture of the 4 histones was done according to the conditions previously described [17]. Core histone octamers were prepared by mixing equal volumes of equimolar aqueous solutions of the 4 core histones according to [18].

The fragment 27–128 of H3 was a gift from L. Böhm (Cape Town, South Africa). The synthetic peptide 1–21 of H3 was prepared by the solid-phase method of Baramy and Merrifield [19]. The properties of the 130–135 C-terminal hexapeptide of H3 and the procedure for coupling it to ovalbumin have been described previously [11].

2.2. *Immunization and fusion*

Three female BALB/c mice were immunized with 30–50 S RNP monoparticles, prepared as described above, emulsified with an equal volume of complete Freund's adjuvant. Each mouse received 45 μg of protein in each footpad. On day 12 after immunization 9×10^7 lymphocytes teased out from both inguinal lymph nodes of each animal were fused with 9×10^6 SP2/O-Ag 14 myeloma cells [20]. Fusion was performed at 37°C by adding 1 ml of 50% PEG 1500. After fusion the cells were suspended in HAT-RPMI-medium supplemented with 20% fetal calf serum and plated out at densities 1×10^6 and 1×10^5 cells/ml.

The cells were cultured in the same medium on the following days and transferred into HT-RPMI-medium after 2 weeks.

Supernatants were assayed by an enzyme-linked immunosorbent assay (ELISA). Positive hybridomas were subcloned twice by limiting dilution on mouse peritoneal macrophages feeder layers. The further characterization of the monoclonal antibodies was performed with cell culture supernatants.

2.3. *ELISA*

For screening the hybridomas for antibody secretion the following ELISA procedure was used: polystyrene (Dynatech) or polycarbonate (Nunc) microtiter plates were incubated at 4°C for 2 h with excess RNP particles (10 μg/well) diluted in PBS. Unspecific protein binding was prevented by blocking available sites with 1% horse serum in PBS. Antibodies were then added and allowed to react at 4°C for at least 2 h. Plates were thoroughly washed with PBS + 0.06% Tween 20 and incubated for 2 h at 25°C with alkaline phosphatase-conjugated goat anti-mouse IgG, diluted 1:500 in PBS. After a final rinse, *p*-nitrophenyl phosphate [Sigma (1 mg/ml in 0.1 M diethanolamine buffer (pH 9.8)] was added and the reaction was monitored at 405 nm with a Titertek Multiscan MC photometer (Flow Laboratories).

ELISA used to measure the antibody binding to histones and chromatin subunits was carried out as described by Laskov et al. [17]. Antigen dilutions used for coating microtiter plates were prepared in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T) in the case of chromatin or core particles, and in 0.05 M sodium carbonate buffer (pH 9.6) in the case of histones.

2.4. *Dot-immunobinding assay*

The procedure used was similar to the assay described by Hawkes et al. [21]. Aliquots of 0.5 μl of histone fractions dissolved in PBS-T were applied to nitrocellulose sheets already printed with a 3 × 3 mm grid (Millipore Corp., ref. HAWG). After successive washings of the filters for 5 min in PBS-T and blocking for 1 h at room temperature with PBS-T containing 1% BSA, dilutions of the antibody were incubated for 2 h at room temperature. Nitrocellulose sheets were then washed 3 times for 5 min with PBS-T and incubated for 2 h with antiglobulin peroxidase con-

jugates (peroxidase-linked goat anti-mouse Ig or peroxidase-linked goat anti-rabbit Ig supplied by Institut Pasteur Production no.75031 and 75011, respectively). A final extensive washing was performed in PBS-T. Then *o*-dianisidine (2%)–H_2O_2 (0.06%) in 10 mM Tris-HCl buffer (pH 7.4) was used as substrate for peroxidase reaction. When the stain was clearly visible, the reaction was stopped by washing the sheets in distilled water.

2.5. *Immunoblotting*

Electrophoresis was performed in 15% SDS-polyacrylamide gel according to Laemmli [22]. Proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schüll; pore size, 0.45 μm) as outlined by Towbin et al. [23]. The filters were first incubated with PBS-T containing 1% BSA to block remaining protein binding sites as described before for the dot-immunobinding assay. After 3 washings in PBS-T, the nitrocellulose sheets were independently incubated with various specific antisera or with monoclonal antibodies diluted in PBS-T. The reaction mixtures were gently shaken during 3 h at room temperature. Following extensive rinsing with PBS-T, ^{125}I-labelled protein A (≃30 mCi/mg, Amersham, IM144) diluted in PBS-T to a final concentration of 5×10^5 cpm/ml was added. After 1 h incubation at room temperature, the sheets were washed to remove unbound protein A; the blots were air-dried and exposed for autoradiography at −70°C.

3. RESULTS

3.1. *Isolation of the hybridoma 1GB3*

The hybridoma clone 1GB3 was one of several clones derived from a fusion of lymph node lymphocytes from BALB/c mice, immunized with a rat liver nuclear fraction containing mostly 30–50 S RNP monoparticles, and the myeloma cell line SP2/O-Ag 14. Clones were screened for antibody production by ELISA using the same antigens. The positive clones were further assayed by indirect immunofluorescence microscopy. Attention was focused on clone 1GB3, since its supernatant decorated not only nuclei of HeLa and PtK2 cells, but also their metaphase chromosomes. After 1GB3 was subcloned twice by limiting dilution, it was shown that the subclones solely secreted antibodies of the IgG2b subclass, as assayed by immunodiffusion against subclass-specific anti-mouse sera. This IgG2b antibody will be further referred to as antibody 1GB3 (subclone 14-34). Its light chain was found to belong to the kappa-class by a commercial ELISA-kit for antibody subclass determination (not shown).

3.2. *Identification of the antigen reacting with antibody 1GB3*

To identify the nuclear component stained in immunofluorescence microscopy by the monoclonal antibody 1GB3, rat liver nuclei were thoroughly extracted by sonication in 140 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris buffer (pH 8.0) and the extract was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting. Antibody 1GB3 was found to react with a polypeptide in the region of M_r = 15000, indicating that it may be specific for one of the histones.

When analysed by immunoblotting on nitrocellulose, the antibody preparation diluted 1/10 was found to reveal one band corresponding to the position of H3 (fig.1). When used at a dilution of 1/2 the same antibody preparation also stained a broad region corresponding to the other histones. The specificity of antibody 1GB3 was further established by a series of ELISA experiments using purified histones as antigens. In the concentration range of 0–400 ng/ml of histone antigen, the antibody reacted specifically only with histone H3 (fig.2A). However, at higher antigen concentration, some reactivity with the other histones (H2A, H2B, H4) was also observed (fig.2B). In particular, commercial preparations of H2B (Boehringer) were found to react distinctly with the monoclonal antibody. The origin of this apparent cross-reaction was further investigated by an immunodot experiment, using serial dilutions of the antigens. As shown in fig.3, the reaction of antibody 1GB3 with a commercial H2B preparation (Boehringer) appears to be caused by a contamination with about 1% of histone H3. Histones are notoriously difficult to purify and such a level of contamination is not unusual. In view of the sensitivity of ELISA and immunodot assay, it is essential to work at relatively low antigen concentrations when studying the specificity of histone-antibody reactions [10,24].

The fine specificity of antibody 1GB3 for

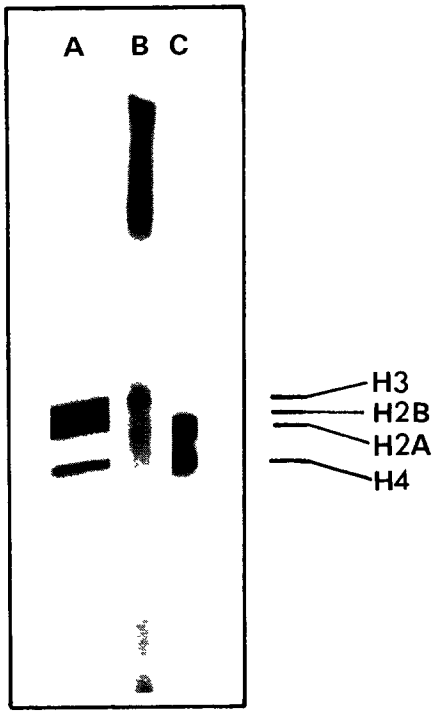

Fig.1. Analysis of antibody 1GB3 binding with calf thymus core histones by immunoblotting. Acid-extracted core histones were separated on 15% SDS-polyacrylamide gel and electrophoretically transferred on nitrocellulose paper. The antibody was incubated for 2 h after blocking the sheets with 1% BSA. After washing, ^{125}I-labelled protein A (5×10^5 cpm/ml) was added for 1 h. After a final rinsing, the blots were air-dried and exposed for autoradiography at −70°C. A, Control strip stained with Coomassie blue; B, blot incubated with antibody 1GB3 diluted 1:10; C, blot incubated with a mixture of anti-chicken erythrocyte H2A and anti-chicken erythrocyte H4 rabbit sera used as controls (diluted 1:500). Each band corresponds to about 1 μg histone.

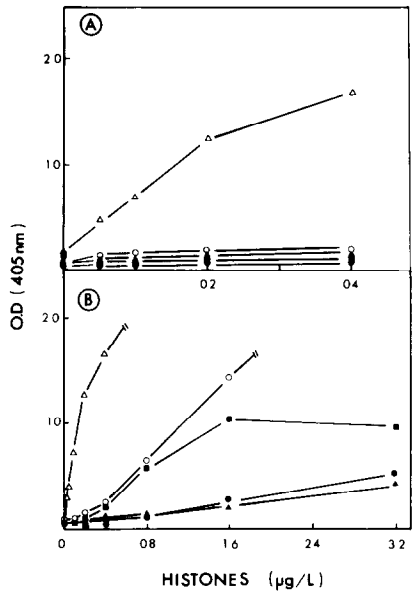

Fig.2. Binding in ELISA of antibody 1GB3 with different core histones used in the normal concentration range 0–0.4 μg/ml (A) and in large excess (B). Hybridoma culture supernatant diluted 1:2 was tested with H3 (Δ—Δ), H2A (●—●), H2B (▲—▲), H2B from Boehringer (○—○) and H4 (■—■).

histone H3 was further studied by ELISA using H3 histones of different origin, histone complexes and various fragments of histone H3. The results presented in fig.4A indicate that the monoclonal antibody recognizes H3 when complexed with H4 or when present in a mixture with the other 3 core histones or in the histone octamer, but that it does not react with H3 present in core particles. No reaction was observed when chromatin was used in the assay instead of core particles (not shown). From the reactivity pattern shown with the various peptides of H3 (fig.4B) it seems that the antibody recognizes an epitope in the region corresponding to residues 20–50 of histone H3.

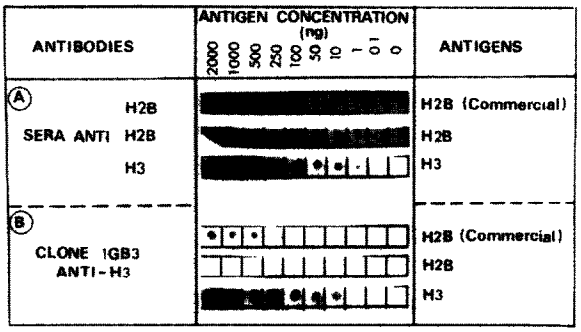

Fig.3. Analysis of the reaction between the antibody 1GB3 and histones H3 and H2B in a dot immunoassay on nitrocellulose paper. Hybridoma supernatant diluted 1:5 and control anti H2B and anti H3 sera diluted 1:1000 were allowed to react with serial dilutions of histone H2B (purified in our laboratory and purchased from Boehringer) and with histone H3. Antibody binding was revealed after incubation with peroxidase-linked goat anti-rabbit Ig (A) or peroxidase-linked goat anti-mouse Ig (B).

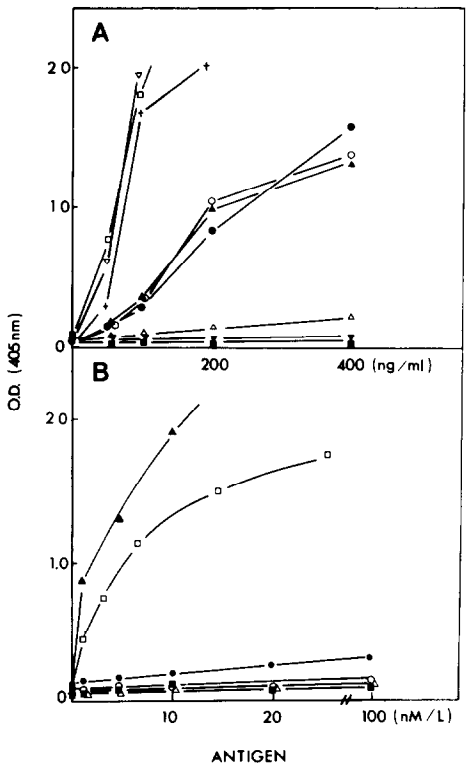

Fig.4. Binding in ELISA of antibody 1GB3 (supernatant diluted 1:2) to H3, different histone complexes containing H3 and various H3 fragments. (A) Antigens tested were: H3 extracted from calf thymus (○—○), chicken erythrocytes (▲—▲), barley (△—△), maize (■—■), calf thymus H3 dimer (●—●), calf thymus [H3-H4] complexes (+ — +), mixture of the 4 core histones H2A + H2B + H3 + H4 from calf thymus (□—□), calf thymus octamer (tested in 2 M NaCl, pH 7.5) (▽—▽), chicken erythrocyte core particles (▼—▼). Antigen concentrations are expressed in ng H3/ml contained in the various preparations used. (B) The antigens were: calf thymus [H3-H4] complexes (□—□), peptide fragments of H3 corresponding to residues 1–50 (▲—▲), 27–128 (●—●), 1–21 (■—■) and 130–135 conjugated to ovalbumin by glutaraldehyde (carrier/peptide ratio, 1:23) (△—△). The peptide 1–24 of H2B (○—○) was used as control.

4. DISCUSSION

The elicitation of a monoclonal antibody against histone H3 was quite an unexpected finding, since only traces of histones (detectable in SDS gels only by the silver stain) were present in the crude 30–50 S RNP fractions used for injection. It is thus interesting that such a small amount was enough to elicit an immune response. The most probable structural organization of the histones present in the RNP monoparticle fractions is a nucleosomal high order repeat structure of 33 S [25], although complexes between histones and hnRNA fragments cannot be excluded. The chromatin subunits might be products of endogenous nucleolytic digestion and, therefore, easily extractable. Note that until now the only anti-histone monoclonal antibodies that have been obtained by immunization of mice are H5 antibodies [26,27].

Initial attempts to determine which histone was recognized by the monoclonal antibody 1GB3 were complicated by the fact that when the hybridoma supernatant and the antigens were used in excess, several histones appeared to be recognized in different types of immunoassay. Nonspecific reactions are common with undiluted hybridoma supernatants, but in addition 'purified' preparations of histones often contain significant amounts of contaminating histones. As shown in fig.3, the histone H2B prepared in our laboratory was of much higher purity than the commercial H2B preparation. It is essential, therefore, not to use excessive antigen concentrations for establishing which histone is specifically recognized by a monoclonal antibody. In ELISA and immunodot assays the histone concentration had to be below 400 and 100 ng/ml, respectively, to ensure reliable results.

Fine specificity studies with H3 peptides indicated that monoclonal antibody 1GB3 recognizes a region in the vicinity of residues 20–50 of the H3 molecule (fig.4B). From the results obtained with the histone complexes (fig.4A) it appears that the region 20–50 of histone H3 is accessible in histone tetramer and octamer aggregates but not in the core particle where the histones are surrounded by DNA. The location of this region at the surface of the histone H3-H4 tetramer agrees with the results of Michalski-Scrive et al. [28] demonstrating the accessibility of residue 41 at the surface of the tetramer. Furthermore, the binding of this region to DNA agrees with the data of Kato and Iwai [29] showing that residues 1–40 are bound to DNA. The monoclonal antibody 1GB3, therefore, may turn out to be a useful probe for studying DNA-H3 interactions and core histone aggregation.

ACKNOWLEDGEMENTS

The authors are grateful to Dr P. Sautière (Lille) for a gift of H3-H4 tetramers, Dr L. Bohm (Cape Town) for the H3 cleavage peptide 27–128, Dr J.P. Briand (Strasbourg) for synthesizing the 1–21 peptide of H3, Dr C. Gigot for a gift of maize and barley H3, and Drs A. Mazen and G. de Murcia for preparations of core particles and chromatin. Thanks are due to C. Balz (Heidelberg) for excellent technical assistance. This work was supported by grants of the Deutsche Forschungsgemeinschaft to F.A.B. (Grant Ba 477/7). E.J.-W. was a recipient of a short term fellowship from the European Molecular Biology Organization.

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