PURIFICATION OF ANTI-HISTONE-H1 ANTIBODIES AND THEIR USE IN MEASURING HISTONE DETERMINANTS IN CHROMATIN BY RADIOIMMUNOADSORBANCE*

Michael Bustin** and Hanna Kupfer

Department of Chemical Immunology
Weizmann Institute of Science, Rehovot, ISRAEL

Received December 1,1975

Summary

Anti-histone H1 antibodies belonging to the IgG class were purified by affinity chromatography on H1-Sepharose columns. Such antibodies can be labeled with ^{125}I and used to measure the availability of H1 determinants in chromatin.

Current studies on the structure of chromatin suggest that the histones and DNA are arranged in repeating subunits roughly spherical in shape, which in the electron microscope have the appearance of "beads on a string" (1). Kornberg has suggested that each subunit contains a histone octamer composed of histones H2A, H2B, H3 and H4 (2). Histone H1 may be bridging between adjacent subunit (3). Investigations on the arrangement of histones in chromatin are hampered by a lack of methods for studying these proteins while they are still in the nucleoprotein complex. Thus it is still not known whether all the chromatin subunits are identical in histone content and arrangement (4).

Immunochemical techniques can provide information on the arrangement of histones in chromatin. We have previously shown that antisera specific to the various histone fractions specifically bind to chromatin (5,6). The advantage of these techniques is that the state of a particular histone can be probed without altering the "native" chromatin structure. Previously, we have used the complement fixation technique to measure chromatin-antibody reactions (5,6,8). This technique is sensitive, but yields only semiquantitative data and requires stringent conditions (pH, ion concentrations) thereby minimizing the experimental parameters which can be varied. Therefore, we have explored the use of alternate immunochemical techniques to investigate the state of chromatin-bound histones. In the present report we describe the isolation of antihistone H1 antibody, which belongs to the IgG class and its subsequent radioiodination.

---

* Supported by the ISRAEL Commission for Basic Research

** Present address: National Institutes of Health, Bldg 6, Room B1-26, Bethesda, Maryland for correspondence and reprint requests.

Radioactive antibody can then be used to obtain quantitative data on the exposure of histone antigenic determinants in chromatin.

MATERIALS AND METHODS

Methods to obtain "Triton nuclear chromatin", histone H1 and rabbit anti-H1 sera have been described before (5,6). The Ig fraction from the anti-H1 serum was obtained by either precipitation with 40% (w/v) ammonium sulfate at 4° or by chromatography on DEAE-cellulose (DE-52 Whatman) equilibrated and eluted with 0.02 M sodium phosphate pH 6.5 buffer. Pure anti-H1 antibodies were obtained by affinity chromatography as follows: 60 mg H1 dissolved in 4.5 ml HCl were dialysed overnight at 4° against 0.1 M $NaHCO_3$. The histone was added to 5.0 gm of CNBr activated Sepharose (with 0.5 gm solid CNBr-ref 9) and the mixture was shaken overnight at 4°. The reaction mixture was poured into a 0.5 x 5 cm column and washed with 1 mM HCl and with PBS (0.12 M NaCl, 0.02 M sodium phosphate pH 6.8) until the A_{230} of the eluate was less than 0.02. 45% of the histone was covalently bound to the column. 20 ml anti-H1 serum was added to the H1-Sepharose column. The column was washed with 500 ml PBS until the A_{230} of the eluate was less than 0.02. Bound antibodies were eluted with 0.1 M NH_4OH and immediately dialysed against PBS. Anti-H1 and Ig obtained from non-immunized rabbits by $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography were iodinated with ^{125}I using the lactoperoxidase method (10). The iodinated protein was separated from free iodine by filtration on Sephadex G-25 equilibrated and eluted with PBS containing 0.1% gelatin.

Immunological measurements. Quantitative microcomplement fixation (7) were performed as described before (5,6). In the radioimmunoadsorbance procedure ^{125}I gamma globulins were added to chromatin and carrier "artificial chromatin" (see results) suspended in 5 mM Tris, pH 8.0. The reaction mixture was incubated with shaking for 1 hr at 37° and for at least 6 hrs at 4°. Chromatin-bound antibodies were separated from nonbound antibodies by making the solution 0.12 M in NaCl centrifugation and washing the resulting precipitate 3X with PBS. The pellet was suspended in 0.1 M NaOH and counted. To increase reproducibility we performed the reaction in small tubes (i.e. Eppendorf type 3810).

RESULTS

Purification and characterization of antibodies. The data presented in Fig. 1A indicates that the immunoglobulin fractions obtained by ammonium sulfate precipitation and by DEAE-cellulose chromatography contain anti-H1 activity albeit somewhat less than that of the original serum. Fig 1B indicates that all the anti-H1 activity present in the serum was lost upon passage through the H1-Sepharose column and that this antibody activity could be eluted from the column with 0.1 M NH_4OH. When 20 ml anti-H1 serum was applied to the column the A_{280} recovered from the H1-Sepharose column was equivalent to 6.5 mg IgG. Assuming that the globulin concentration in rabbit serum is about 8.0 mg/ml we calculate that the column bound 4% of the Ig present in the sera. The results shown in Fig. 2 indicate that the antibody activity obtained from the column belong to the IgG fraction. Fig. 2A shows that the fractions obtained by $(NH_4)_2SO_4$ precipitation and by DEAE-cellulose chromatography contain several components while the material eluted from the H1-Sepharose column precipitated as a single band. Fig. 2B suggests that this band belongs to the IgG fraction. The observation is verified by the immunoelectrophoresis pattern shown in Fig. 2C.

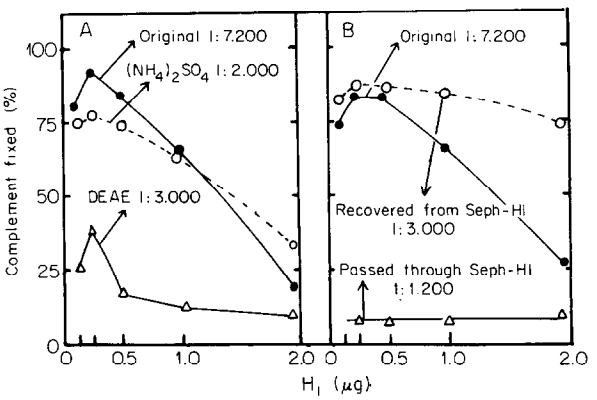

FIG. 1. Complement fixing activity of anti-Hl serum and immunoglobulin fractions purified by various techniques. The volume of the globulin fractions were the same as that of the serum from which they were derived. Sera dilution noted are the final dilutions present in the reaction mixtures.

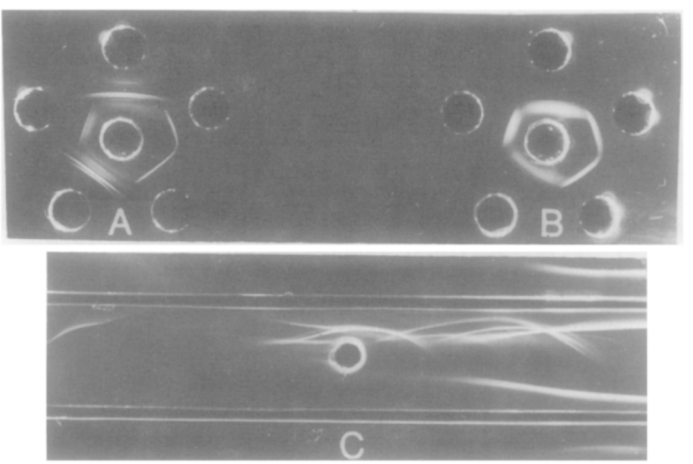

FIG. 2. Immunodiffusion and immunoelectrophoretic patterns of anti-Hl antibody. A, center well goat anti-rabbit serum, from top well clockwise: Ig obtained by $(NH_4)_2SO_4$; anti-Hl purified by affinity chromatography, 0.5 mg/ml; same, 2.0 mg/ml; normal rabbit serum; Ig obtained by DEAE-cellulose chromatography. B, same as A except center well contained goat anti-rabbit IgG. C, upper and lower well: anti-Hl purified by affinity chromatography, center well; normal rabbit serum. Top trough: goat anti-rabbit serum. Bottom trough: goat anti-rabbit IgG.

The anti-Hl antibody and our IgG fraction obtained from nonimmunized rabbits were labeled with ^{125}I using lactoperoxidase; protein-bound ^{125}I was separated from free ^{125}I by gel filtration. The proteins were iodinated with comparable efficiency. The specific activity of the anti-Hl antibody was 2.95×10^5 cpm/μg and that of non-immune Ig 5.3×10^5 cpm/μg. In the experiments described below

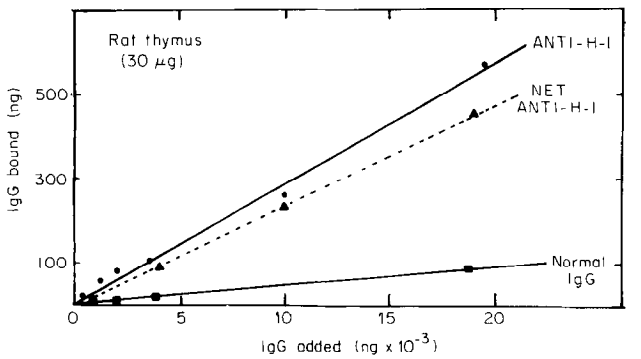

FIG. 3. Specific binding of ^{125}I anti-HI antibody to rat thymus chromatin. Each tube contained 30 µg chromatin, a variable amount of IgG (stock 1 x 10^5 cpm per 1 µg per 5 µl, PBS, 0.1% gelatin) in 0.25 ml 5 mM Tris pH 8.0 buffer, 0.5 mg/ml bovine serum albumin, 40 µg/ml "artificial chromatin" (see text).

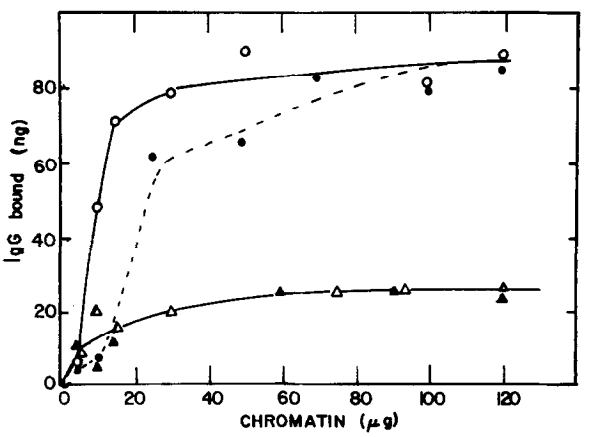

FIG. 4. Reaction of rat thymus and rat brain chromatin with ^{125}I-anti-HI antibody and with ^{125}I-normal rabbit IgG. Conditions as in Fig. 4 except that each tube contained 1000 ng antibody or IgG. Open symbols, adsorbed on rat thymus chromatin, closed symbols, adsorbed on rat brain chromatin. O, ● anti-HI antibody; Δ, ▲ IgG from normal rabbit serum.

---

we have used several ^{125}I preparations, some with specific activities 10 times higher. 98% of the counts were precipitable with 5% trichloroacetic acid, 88% with 40% $(NH_4)_2SO_4$ indicating that the counts were indeed incorporated in the IgG.

Binding of ^{125}I-IgG to chromatin. In these experiments it is mandatory to add carrier proteins to eliminate non-specific binding of ^{125}I-IgG to the reaction tubes. In the absence of such carriers, between 30% - 50% of the counts bind to

the tubes. In the presence of either 0.5 mg/ml RNA, 0.5 mg/ml gelatin or 0.5 mg/ml bovine serum albumin the percent radioactivity bound to the tubes was 40%, 15% and 3% respectively. The non-specific binding was reduced to less that 1% if in addition to 0.5 mg/ml bovine serum albumin the reactions contained 40 µg/ml of "artificial chromatin" composed of DNA and COP-1 in the ratio of 1:3 w/w (COP-1 is a cationic synthetic polypeptide with a molecular weight of 23,000, ref 11).

The results presented in Fig. 3 in which variable amounts of antibodies were added to a constant amount of chromatin indicate that ^{125}I-anti-H1 antibodies specifically bind to chromatin. Under the conditions studied about 2.5% of the IgG added bound to chromatin. There was a direct correlation between the amount of antibody added and the amount of the IgG bound. In the example shown in the figure, at the highest level of antibody added (20,000 ng), 30 µg of rat thymus chromatin, containing 3×10^{-10} moles of H1, specifically bound 450 ng antibody i.e., 4×10^{-12} moles of IgG. Fig 4 shows the result of an experiment in which, a constant amount of ^{125}I-IgG was added to various amounts of chromatin derived from rat thymus and rat brain. The ability of rat thymus chromatin to bind ^{125}I-IgG was compared to the ability of rat brain chromatin to bind these IgG. While the binding curves are very similar it seems that rat-thymus chromatin was somewhat more efficient in binding these antibodies. In both cases a maximum of 85-90 ng of antibody bound to the chromatin. Under the conditions studied about 35 µg of rat thymus chromatin were sufficient to bind this amount of antibody while 35 µg of rat-brain chromatin bound only 70 ng of antibody. More than 80 µg rat-brain chromatin were necessary to bind 80-85 ng antibodies.

DISCUSSION

In the experiments reported we explore, for the first time, the use of purified anti-H1 antbodies as reagents to probe the state of histones in chromatin. The results presented indicated that the immunological activity in anti-H1 sera resides in the IgG fraction. Active antibodies can be obtained by affinity chromatography on H1-Sepharose columns. Iodination of these antibodies affords a sensitive way to measure the amount of antibodies bound to chromatin. This technique requires less stringent experimental conditions than the previously used techniques which employed the complement fixation technique (5,6). The amount of ^{125}I antibody bound to chromatin is directly proportional to the amount of antibody added. All the anti-H1 activity which can bind to chromatin, present in a given amount of purified antibody, can be adsorbed by increasing the amount of chromatin used as immunoadsorbant (fig. 4). The radioactivity bound to chromatin is 3-8% of the total radioactivity added. The low reactivity of anti-H1 antibodies with chromatin-bound H1 could result from the following reasons: a, only part of the H1 determinants are exposed in chromatin; b, anti-

bodies were inactivated during elution from the H1-Sepharose column; c, antibodies were inactivated during the iodination procedure or as a result from radiation damage during storage (12,13) and d, the material eluted from the H1-Sepharose column was not exclusively anti-H1 antibody. Indeed, even though by immunochemical criteria the material eluted from the column was IgG (see fig.2) examination by polyacrylamide gels in the presence of SDS revealed a few minor additional bands.

The ^{125}I-IgG specifically bind to chromatin derived from rat thymus and rat brain. Small differences between the availability of H1 determinants present in the chromatins derived from these tissues were observed. Since the antibodies were used were elicited against calf thymus chromatin only the binding of cross reacting antibodies is measured. Previously we have shown (8) that in the rat thymus the H1 histone antigenic determinants which are exposed and available to interact with antibody are determinants which are shared among the H1 subfractions present in a tissue.

The novel approach described may prove useful in detecting and quantitating differences in the arrangement in chromatin of any chromosomal protein towards which antibodies can be prepared. Changes in the availability of antigenic determinants may reflect structural changes within the genome.

REFERENCES

1. Olins, A. L. and Olins, D. E. (1974) Science 183, 330-332.
2. Kornberg, R. D. (1974) Science 184, 868-871.
3. Baldwin, J. P., Boseley, P. G., Bradbury, E. M. and Ibel, K. (1975) Nature 253, 245-249.
4. Felsenfeld, G. (1975) Nature 257, 177-178.
5. Bustin, M. (1973) Nature New Biol. 245, 207-209.
6. Goldblatt, D. and Bustin, M. (1975) Biochemistry 14, 1683-1685.
7. Wasserman, E. and Levine, L. (1960) J. Immunol. 87, 290-295.
8. Zick, Y., Goldblatt, D. and Bustin, M. (1975) Biochem. Biophys. Res. Commun. 65, 637-643.
9. Axen, R., Porath, J. and Ernbach, S. (1967) Nature 214, 1302-1304.
10. Thorell, J. I. and Johansson, B. G. (1971) Biochim. Biophys. Acta 251, 363-369.
11. Webb, C., Teitelbaum, D., Arnon, R. and Sela, M. (1973) Eur. J. Immunol. 3, 279-286.
12. Yalow, R. S. and Benson, S. A. (1968) in Radioisotopes in Medicine: In Vitro Studies. (Hayes, R. L., Goswitz, F. A. and Murphy, B. P., eds) U.S. Atomic Energy Commission, 17-41.
13. Bogdanove, E. M. and Strash, A. M. (1975) Nature 257, 426-427.