Rabbit Antibodies to Histone Fractions As Specific Reagents for Preparative and Comparative Studies*

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SUMMARY

Antibodies specific for each of the F1, F2a1, F2a2, and F2b calf thymus histone fractions were induced by immunization of rabbits with varying histone-nucleic acid complexes. At appropriate dilution, each serum reacted in quantitative complement fixation with only one homologous fraction. With very much higher serum concentrations, cross-reactivities with other fractions occurred and were estimated to be approximately 5% in all but one case, in which 20% cross-reactivity was observed. Antibodies induced by F3 histones were less specific than the other four types of sera. The more specific sera were effective as reagents for the identification of individual histone fractions obtained by preparative electrophoresis, column chromatography, and dissociation of histones from nucleoprotein by increasing salt concentrations. With each of the anti-F2a1, anti-F2a2, and anti-F2b sera, comparisons were made of the corresponding fractions from human, calf, chicken, frog, and lobster tissues. With any one fraction and the corresponding antisera, little or no difference was seen in the reactivity of the protein isolated from any of these species.

In previous studies of experimentally induced antibodies to calf thymus histones, we have immunized rabbits with covalently linked histone-serum albumin complexes (1) or with noncovalent poly-L-lysine-phosphorylated bovine serum albumin complexes (2). The resulting antibodies reacted primarily with the lysine-rich histones, but also cross-reacted with other histone fractions in complement fixation and complement fixation inhibition reactions.

Riimke and Sluyser (3) have briefly reported the induction of rabbit antibodies which could distinguish between different histone classes by precipitation in gels. Another brief report (4) has claimed that insect larval histones may be distinguished from those of certain mammalian species. In a recent paper (5), we have used column chromatography to separate histone classes and electrophoresis to show that antibodies to calf thymus histones immunologically cross-reacted with other histones from human, calf, chicken, frog, and lobster origins.

EXPERIMENTAL PROCEDURE

Calf thymus was trimmed after collection and used immediately or stored at -20°C until used. Chicken blood was collected in Alsever’s solution. The erythrocytes were lysed by freezing and thawing, followed by the addition of saponin to a final concentration of 0.1%. Nuclei were collected by centrifugation and were washed with 0.43% NaCl solution-Versene buffer (0.075 M NaCl, 0.024 M EDTA, pH 8). Frog liver nuclei and lobster hepatopancreas nuclei were prepared by the method of Chaouev, Moulé, and Rouiller (5). Human spleen was obtained as a fresh surgical specimen. Nucleoprotein was prepared from the thymus and spleen and from isolated nuclei by the method of Zhuiba and Doty (6). Histones were prepared from nucleoprotein by the method of Johns (7). The arginine-rich F2a fraction was further separated into F2a1 and F2a2 components by the method of Phillips and Johns (8).

For column chromatography, Cm cellulose1 (Schleicher and Schuell Company, Keene, New Hampshire) was washed with 0.1 M HCl, 0.1 M NaOH, and water, equilibrated with glycine-HCl buffer (0.1 M, pH 4.2), and packed in a column (0.9 X 45 cm). A sample of 150 mg of whole histone in 3 ml of glycine-HCl buffer, pH 4.2, with 0.01 M dithiothreitol, was applied to the column, which was then washed with the glycine buffer. After an initial peak appeared, the following solutions were applied in stepwise additions: (a) 0.45 M NaCl in 0.1 M glycine-HCl buffer, pH 4.2 (70 ml); (b) 0.1 M glycine-HCl, pH 4.2 (30 ml); (c) 0.1 M glycine-HCl, pH 2.4 (120 ml); (d) 0.01 M HCl (80 ml); and (e) 0.04 M HCl (120 ml). Fractions of 2.0 ml were collected.

Analytical polyacrylamide gel electrophoresis was performed as described by Reisfeld, Lewis, and Williams (9) with 7.5% gels in tetramethylethylenediamine-acetate buffer, pH 4.3, and 6 M urea. Samples of 10 to 100 μg of protein in 5 μl urea were layered on gels (5 X 45 mm) and a voltage of 800 volts was applied for 90 min. Gels were stained with Amido black and destained with 7% acetic acid in 40% ethanol.

For elution of antigens, larger polyacrylamide gels (12 X 45 mm) were layered with 1 mg of a given histone fraction, and electrophoresis was carried out for 3 hours. In parallel, one such gel was stained for protein and a second gel was cut into 12 to 16 cross sections. Each section was minced, suspended in 4.0 ml

1 The abbreviation used is: Cm-, carboxymethyl-.
of 0.15 M NaCl, and shaken for 2 to 4 hours; the gel fragments were removed by centrifugation.

Histones were dissociated from nucleoprotein by increasing salt concentrations as described by Ohlenbusch et al. (10). Calf thymus DNA was purchased from Worthington, yeast RNA from Sigma, and Pronase from Calbiochem.

Protein concentrations were determined by absorbance at 275 nm, by a microbiuret assay (11) or by a turbidimetric assay in which 1.0 ml of 36% trichloracetic acid was added to 1.0 ml of samples of 10 to 100 μg of protein or of histone standards; turbidity was measured at 310 nm. Carbohydrate was assayed by the Molisch (12), cysteine-sulfuric acid (13), and orcinol (13) reactions. DNA was assayed by absorbance at 260 nm and by a diphenylamine assay (14). Hexosamine was determined by a modified Elson-Morgan reaction (15). The amino acid composition of Fraction C obtained from Cm-cellulose chromatography was kindly determined by L. J. Cuprak, by use of a Beckman-Spinco amino acid analyzer.

Complexes of yeast RNA with F2a1, F2a2, F2b, and F3 histones were formed by the addition of 200 μg of RNA to 600 μg of each histone fraction. The F2b histone used for immunization was Fraction C obtained by Cm-cellulose chromatography, as described below. Each of the complexes was injected into a corresponding group of three New Zealand white rabbits. Two doses, each containing 600 μg of histone, were given intradermally and subcutaneously in complete Freund's adjuvant on the 1st, 8th, and 15th days of immunization; an intravenous booster was given a week later, and serum was obtained 7 days after this. A similar course of immunization was repeated after a 1- to 2-month interval.

Three rabbits were immunized with complexes of whole histone and native DNA, which were formed by the addition of 20 ml of histone (1 mg per ml) to 0.75 ml of DNA (0.8 mg per ml). The fibrous complexes were homogenized at high speed in a Virtis 23 homogenizer. The immunization schedule was similar to that described above, except that a 4-month interval was allowed between the first and second courses.

Quantitative microcomplement fixation reactions were performed according to the method of Wasserman and Levine (16), in a total volume of 1.4 ml per reaction mixture, and with a buffer of 0.14 M NaCl, 0.01 M Tris, pH 7.4, 5 × 10⁻⁴ M Mg++, 1.5 × 10⁻⁴ M Ca++, and 0.1% bovine serum albumin.

RESULTS

All animals in the four groups of rabbits immunized with F2a1-RNA, F2a2-RNA, F2b-RNA, or F3-RNA complexes produced complement fixing antihistone antibodies. The serum of highest titer was selected from each group for detailed study. Of three other rabbits, which received whole histone-DNA complexes, one produced antihistone antibodies. None of the sera reacted with free RNA or DNA.

The complement fixation reactions of each of these sera with the histone fractions are shown in Fig. 1. At the serum dilutions used, each of the anti-F2a1, anti-F2a2, and anti-F2b antisera reacted specifically with the corresponding fraction used for immunization. The antiserum induced by whole histone-DNA complexes showed a fourth specificity, reacting with only the F1 histone. The less specific serum induced by F3 histone-RNA complexes reacted with the F2a1, F2a2, and F3 proteins.

With much higher serum concentrations, some cross-reactivity between fractions was observed. The percentage of cross-reactivity was calculated by comparison of the serum concentrations required to give a peak of 50% complement fixation with homologous and cross-reacting histone fractions. With the sera other than anti-F3 serum, reactivity of nonhomologous fractions was less than 10% in all but one case (Tables I and III to V); the F3 histone showed a 20% cross-reaction with anti-F2a2 serum. With the anti-F3 serum, F2a1 and F2a2 histones showed 35 and 65% cross-reactivity, respectively.

Normal rabbit serum, diluted 1:50, did not react in complement fixation with any of the histones. On the other hand,
double diffusion precipitation in gel could not be used in these studies because, at the higher concentrations of reagents required, there was nonspecific precipitation of histones with normal serum proteins.

The histones contained less than 0.3% carbohydrate as estimated by the Molisch and cysteine-sulfuric acid reactions. In samples of 1.0 mg of protein there was no RNA or DNA detectable by the orcinol or diphenylamine reactions. No hexosamine was found in 200 μg of protein in an assay capable of detecting 2 μg of glucosamine. In all cases, antigenic reactivity was abolished by Pronase digestion, which was carried to the extent that 50 to 75% of the protein became soluble in 18% trichloracetic acid, while none of the intact protein was soluble in this acid concentration.

To determine whether the antigens were single proteins, each was examined in analytical polyacrylamide gel electrophoresis, and was isolated from small scale preparative electrophoresis gels. The F2a1 and F2a2, as prepared by differential extraction and precipitation, each consisted of a major band but contained a very small amount of more slowly moving material, which was also present in the whole F2a (Fig. 2, A, B, C). In preparative electrophoresis, 1 mg of the F2a2 histone was applied to a larger gel (12 × 40 mm). The minor band was more prominent when this gel was stained, but still only a fraction of the total protein. Protein was eluted, in 60 to 80% yield, from serial sections of a similar parallel gel. All of the antigen was present in the major band. The isolated reactive protein was reexamined in analytical electrophoresis, and 40 μg migrated as a sharp single band (Fig. 2D). The F2a1 and F2b antigens were similarly characterized as single proteins. The F1 histones formed several bands in analytical gels. These merged in the larger scale gels, and reactive antigen was eluted from the entire merged region.

Use of Sera as Reagents for Detecting Specific Histone Fractions during Isolation Procedures—Whole histone preparations were separated by Cm-cellulose chromatography with a slight modification of the procedure of Johns et al. (17), in which a step of elution with 0.1 M glycine-HCl buffer, pH 2.4 was added (Fig. 3). Protein in each peak was pooled and assayed in complement fixation reactions with each serum (Table II). The anti-F1 serum reacted with only the early Peak B, although, for unknown reasons, this required a higher serum concentration than that required for reaction with the acid-extracted F1 histone. The reactive column Peak B corresponds to the lysine-rich histones (17). The anti-F2a1 serum reacted only with Peak D. Both Peaks D and E reacted with anti-F2a2 serum, but the former was more effective. The anti-F3 serum reacted mainly with

![Fig. 2. Analytical polyacrylamide gel electrophoresis of calf thymus histone fractions (A) F2a2; (B) whole F2a; and (C) F2a1, all prepared by differential extraction and precipitation (7, 8). The F2a2 histone was applied to a larger gel, eluted after electrophoresis, and the antigenically reactive eluate fraction was reexamined by analytical gel electrophoresis (D).](http://www.jbc.org/)

![Fig. 3. Carboxymethylecellulose chromatography of calf thymus histones. A sample of 150 mg was applied to the column (0.9 × 45 cm). Peak A was washed through with starting buffer (0.1 M glycine, pH 4.2). At points marked by base-line arrows, eluting solutions were applied as follows: a, 0.45 M NaCl in 0.1 M glycine, pH 4.2 (70 ml); b, starting buffer (80 ml); c, 0.1 M glycine, pH 2.4 (120 ml); d, 0.01 N HCl (80 ml); and e, 0.04 N HCl (120 ml). Fractions of 2.0 ml were collected.](http://www.jbc.org/)

| Antiserum       | Complement fixation with fractions |
|-----------------|-----------------------------------|
|                 | A  | B  | C  | D  | E  |
| Anti-histone-DNA (anti-F1) (1:300) | 0  | 45 | 0  | 0  | 0  |
| Anti-F2a1 (1:1500)               | 0  | 0  | 85 | 0  | 0  |
| Anti-F2a2 (1:300)                | 0  | 0  | 75 | 25 | 0  |
| Anti-F2b (1:750)                 | 0  | 25 | 0  | 0  | 0  |
| Anti-Fl (1:200)                  | 0  | 25 | 0  | 0  | 65 |
Peaks D and E, but to a small extent with Fraction B as well. The acrylamide gel electrophoresis patterns of these fractions are shown in Fig. 4. Only Fraction C migrated as a single protein. It was characterized as F2b histone by amino acid analysis, and was used, in complexes with RNA, for the induction of the anti-F2b antiserum. The resulting serum reacted specifically with this Cm-cellulose column fraction (Table II), and also reacted specifically with the F2b fraction prepared by differential extraction and precipitation, as described above (Fig. 1).

To test the sera as reagents for assaying histones in mixtures subjected to electrophoresis, whole F2a histone was applied to a preparative gel. Protein was eluted from sections and assayed by complement fixation with anti-F2a1 and anti-F2a2 sera. As shown in Fig. 5, the corresponding sera identified the positions of the two distinct components of F2a histone. Purified F2a1 and F2a2 preparations were used as standards for quantitation.

The sera have been similarly useful for detecting specific fractions in mixtures of protein dissociated from nucleoprotein by increasing salt concentration. For example, the anti-F2a2 serum did not react with proteins dissociated from nucleoprotein by 0.45 or 0.8 M NaCl but did react with those dissociated by 1.0 M NaCl or by higher salt concentrations. The antiserum specific for F1 histone did detect this fraction in the protein dissociated by 0.8 M NaCl; as with the Cm-cellulose column Fraction B, the reaction with this salt-dissociated fraction required a higher serum concentration than did the reaction of acid-extracted F1 histone.

Comparative Immunochemical Studies of Histone Fractions—Histone fractions were prepared from calf thymus, human spleen, chicken erythrocytes, frog liver, and lobster hepatopancreas by differential extraction and precipitation (7, 8). The fractions were applied to and eluted from preparative gels as described above, and the fraction with the peak amount of reactive eluted protein was identified for each gel. In all cases, protein from a single section (1 to 2 mm) of the gel was used for comparative study. Fig. 6 shows the complement fixation reactions of the anti-F2a1, anti-F2a2, and anti-F2b antisera with the corresponding histone fractions from several species. The percentage of cross-reactivity was calculated as described above. In contrast...
Cross-reactions and the chicken F2a1, slightly lower complement fixation was seen than with other corresponding samples (Tables III to V).

Cross-reactions of histone fractions with antiserum to calf thymus F2a1 histone

| Antigen   | Serum dilution required for 50% maximum complement fixation | Cross-reactivity |
|-----------|-------------------------------------------------------------|------------------|
| Calf F2a1 | 5500                                                        | 100              |
| Human F2a1| 6500                                                        | 120              |
| Chicken F2a1 | 4000                                                    | 75               |
| Frog F2a1  | 5000                                                        | 90               |
| Lobster F2a1 | 5500                                                    | 100              |
| Calf F1    | <200                                                       | <4               |
| Calf F2a2  | <200                                                       | <4               |
| Calf F2b   | 200                                                         | 4                |
| Calf F3    | <200                                                       | <4               |

Cross-reactions of histone fractions with antiserum to calf thymus F2a2 histone

| Antigen   | Serum dilution required for 90% maximum complement fixation | Cross-reactivity |
|-----------|-------------------------------------------------------------|------------------|
| Calf F2a2 | 800                                                         | 100              |
| Human F2a2| 850                                                         | 105              |
| Chicken F2a2 | 800                                                      | 100              |
| Frog F2a2  | 850                                                         | 105              |
| Lobster F2a2 | 650                                                     | 80               |
| Calf F1    | <50                                                         | <7               |
| Calf F2a1  | 80                                                          | 10               |
| Calf F2b   | 50                                                          | 7                |
| Calf F3    | 170                                                         | 20               |

Cross-reactions of histone fractions with antiserum to calf thymus F2b histone

| Antigen   | Serum dilution required for 90% maximum complement fixation | Cross-reactivity |
|-----------|-------------------------------------------------------------|------------------|
| Calf F2b  | 900                                                         | 100              |
| Human F2b | 850                                                         | 95               |
| Chicken F2b | 850                                                      | 95               |
| Frog F2b  | 900                                                         | 100              |
| Lobster F2b | 925                                                     | 100              |
| Calf F1   | 65                                                          | 7                |
| Calf F2a1 | 55                                                          | 6                |
| Calf F2a2 | 65                                                          | 7                |
| Calf F3   | 50                                                          | 6                |

Discussions

The findings described in this article indicate that antibodies may be used as reagents for studying and differentiating specific histone proteins. Few previous studies of histone immunoochemistry have been reported. Rümke and Sluyser (3) described the immunization of rabbits with histone components extracted from heated calf thymus, resulting in the production of some antisera with a specificity for the lysine-rich histones, and others which reacted with arginine-rich histones. In a recent report (18), Sluyser, Rümke, and Hekman have indicated that lysine-rich histones from different rat tissues reacted identically with each other and with the F1 histone of calf thymus when tested by precipitation in gel (at pH 6.6) with antibodies to the lysine-rich histones of rat liver.

In another study (4), rabbits were immunized with insect larval histones, and the resulting sera precipitated with the insect preparation but not with calf thymus histone; individual fractions were not examined.

We have not succeeded in immunizing rabbits with free histones, but have done so with varying complexes of histones and acidic carriers. This approach was suggested by the earlier finding that some human sera from patients with systemic lupus erythematosus reacted more effectively with histone-DNA or histone-RNA complexes than with free DNA or histone (19). As complexes made with a 3:1 excess of histone were maximally effective with these sera, we used similar complexes for immunization.

We have found the use of the microcomplement fixation technique advantageous for studying these antigens, as low concentrations of the basic proteins can be used, and nonspecific interactions with normal serum proteins can thus be avoided. In addition, microcomplement fixation has been shown to be more sensitive to differences in antigenic structure than are either precipitation or larger scale complement fixation (20).

The F2a1, F2a2, and F2b antigens were characterized by their behavior in differential extraction and precipitation, Cm-cellulose column chromatography, their susceptibility to proteolytic hydrolysis, the absence of significant amounts of carbohydrate or nucleic acid in the preparations, and by their behavior in electrophoresis; antigens which were recovered from sections of an electrophoresis gel migrated as single proteins on analytical gel electrophoresis. While the anti-F1 serum reacted with only this class of protein, it has been shown that the F1 histone includes at least four subcomponents (21); further comparison of the reactivities of these subcomponents is required before the serum can be used in comparisons of proteins from different species. The basis for lack of specificity of the anti-F3 serum has not yet been resolved. The material used for immunization was not a single protein, and the cross-reactions may represent contamination. On the other hand, with each serum there was a slight degree of cross-reactivity between the fractions, reflecting some similarities in structure; this is in agreement with cross-inhibition studies previously reported with other kinds of anti histone sera (1, 2). It is possible that common determinants were more effectively expressed on immunization with the F3 histone-RNA complexes than was the case with other fractions.

Individual histone fractions have not been found to have unique activities by which they can be identified separately. These antisera should therefore be of value as specific reagents in a variety of situations, as they were in identifying the components...
in fractions isolated by chromatography, electrophoresis, and in salt dissociation of histones from nucleoprotein.

Another example of the usefulness of the sera is seen in the comparative studies. Little or no difference was seen in histone fractions from sources as diverse as human spleen and lobster hepatopancreas, in contrast to the findings with most other kinds of proteins. Differences in complement fixation reactivity which were larger than these have been measured by this technique when growth hormone proteins of related primates were examined (22), corresponding to a much narrower range of species. The remarkable immunochemical similarities of the histones are in agreement with the similarities seen by amino acid analysis (23, 24), and, in the case of the F2a1 histone, by amino acid sequence determination of calf thymus and pea seedling proteins (25). The slight differences in the reactions of lobster F2a2 and chicken F2a1, in their corresponding groups, may reflect possible side chain modifications which can occur in these proteins (26). In the search for evolutionary changes in histones, earlier forms of plants and animals will be of interest, and the immunochemical assay should serve as a convenient screen to determine which samples will be of particular interest for amino acid sequence analysis.

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