Immunological Specificities of Lysine-rich Histones from Tumors*

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SUMMARY

The specificity of F1 histones from tumors was investigated with the following immunological probes: antisera elicited by each of the F1 subfractions obtained from rat thymus; antisera elicited by purified unfractionated rat hepatoma F1 histones; and antisera elicited by the COOH-terminal (N1) fragment of rat hepatoma F1 histones. The results indicate that F1 histones derived from tumors retain to a large extent the species and tissue specificity of the normal tissues. The species and tissue specificity was also observed with N1 fragments derived from the tumor F1 histones, suggesting that the COOH-terminal portions contain regions which vary from one molecular type to another.

Comparison of the chromatographic elution profiles of F1 histone from rat liver and rat hepatoma revealed that one of the peaks in rat liver (Peak 4a) was either absent or present in very low amounts in rat hepatoma. With the exception of Peak 2, the respective peaks of rat liver and rat hepatoma were immunologically closely related.

The lysine-rich histone class (F1) exhibits a higher specificity and multiplicity than the other histone classes. Preparations of F1 histones contain a number of closely related lysine-rich proteins, the type and relative concentrations of which depend on the source from which the histone is derived (1-7). Immunological procedures have been shown to be very useful for comparing F1 histones from different sources (8, 9). The finding that it is possible to distinguish immunologically between the various F1 subtypes in a certain tissue, by using antisera against any one of these subtypes, provides a highly sensitive method for detecting possible minute changes in histone structure (9, 10). We have therefore used immunological measurements to investigate whether the neoplastic condition involves changes in F1 histones. The following questions were asked: (a) are the F1 histones derived from tumors identical to those derived from normal tissues; (b) are there similarities between the F1 histones; (c) are the differences between the F1 histones located in a particular region on the polypeptide chain. For this study we employed antisera against each of the F1 subfractions from rat thymus, antisera against complete F1 histone from rat hepatoma, and antisera against the COOH-terminal fragment (N1) of rat hepatoma F1 histone.

MATERIALS AND METHODS

Materials—Guanidinium chloride (ultrapure grade) was purchased from Mann, Amberlite IRC-50 (Bio-Rex-70; 10 meq per g dry weight, 200 to 400 mesh) from Bio-Rad, and yeast RNA from Sigma. Carboxymethylcellulose (CM Nr-132) was obtained from Schleicher and Schuell, Dassel, Germany.

Complete F1 Histone—The histone was isolated as described before (11) by extraction with 5% perchloric acid, precipitation with 10% trichloroacetic acid, and chromatography on columns of carboxymethylcellulose. After removal of non-histone from the column by elution with borate buffer (pH 9) and with 0.4 M NaCl in the borate buffer, the F1 histone was obtained by elution with 1.5 M NaCl in the borate buffer. After the F1 histone was removed, some material was eluted from the column with an excess volume of 1.5 M NaCl in the borate buffer. This material, which had a lower A280:A260 than the F1 histone, was discarded.

F1 Subfractions—Crude F1 histones were obtained by initial extraction with 0.14 M NaCl, 0.05 M NaHCO3, followed by extraction of the washed nucleoprotein with 5% trichloroacetic acid (2, 7, 9), or by treatment of frozen tissue (solid CO2) with 0.14 M NaCl (pH 4) and extraction with 3.5% trichloroacetic acid (see Reference 12, final extraction procedure). Fractionation of the histone was carried out by chromatography on Amberlite IRC 50 with a shallow guanidinium chloride gradient (2, 10). The concentration of guanidinium chloride was measured with a conductometer. The elution of protein was monitored by the absorbance at 230 nm.

N1 and N2 Fragment—The NH2-terminal fragment (N2) and the COOH-terminal fragments (N1) of the F1 histones were obtained by N-bromosuccinimide treatment followed by gel filtration, as described before (4, 6).

Immunological Procedures—Rabbits were immunized with complexes of yeast RNA and histone samples as described previously (5). Quantitative microcomplement fixation reactions were performed according to the method of Wasserman and Levine (13) in a total volume of 1.4 Ml as described by Stollar and Ward (14). Immunological distance was calculated as described elsewhere (10, 15). The serum dilutions noted are the final dilution in the incubation mixtures.

Analytical Procedure—Protein concentration was determined by turbidimetry (16). The protein or peptide samples to be tested were dissolved in 0.5 ml of distilled water. Trichloroacetic acid (0.5 ml; 2.2 M) was added with rapid stirring, and the absorbance of the mixture read at 400 nm after standing for exactly 10 min.
Solutions containing 100 µg per ml of either F1, N1, and N2 had absorbances of 1.1, 0.33, and 0.12, respectively. The purity of the histone samples was checked by electrophoresis on polyacrylamide gels (17) and by amino acid analysis (18).

Tumors—Rat hepatoma BY 484 and rat hepatoma BY 252 were induced in male rats of the R-strain Amsterdam with dimethylaminoazobenzene, and serially transplanted. Rat hepatoma BY 484 was a rapidly growing loosely knit tumor. Hepatoma BY 252 was a solid tumor which grew more slowly. Rat mammary carcinoma was induced in Sprague-Dawley rats with dimethylbenzanthracene, and serially transplanted. The mouse mammary tumors originally arose spontaneously in mice of the inbred strain C57 Black and were serially transplanted. The human mammary tumor was cytologically a slowly growing cytosarcoma and adenocarcinoma phyloides (a malignant form of fibrosarcoma).

RESULTS

Species Specificity of Tumor F1 Histones—Previous studies have shown that antisera against F1 subtypes can detect differences between F1 histones which are not as easily detected using antisera against complete F1 (10). We therefore used these antisera to compare the F1 histones from mammary tumors obtained from different species. At the same time, we screened the F1 histones from rat liver and rat liver tumor.

As shown in Fig. 1, the antisera against rat thymus Peaks 2, 3, 4, and 5 reacted to various degrees with tumor F1 histones, depending on the source of the tumors. Antiserum against rat thymus Peak 2 reacted very strongly with the F1 histones derived from mammary tumors of various species (Fig. 1A). Mammary tumor F1 histones from human, mouse, and rat showed maximal complement fixations of 80 to 90%, whereas rat liver F1 and rat hepatoma F1 reacted more weakly (maximal complement fixations of 64% and 53%, respectively). Antisera against Peaks 3, 4, and 5 reacted significantly better with histones derived from the homologous species. Actually from Fig. 1, B, C, and D it can be seen that histones derived from heterologous species did not fix any complement when reacted with antisera to the latter rat thymus peaks. No significant differences between liver and hepatoma derived F1 histones could be detected.

The structure of the F1 molecule raises the possibility that sequence differences may be grouped in particular regions of the polypeptide chain (19, 20). It seemed therefore worthwhile to compare histone fragments. From Fig. 2 it is obvious that the COOH-terminal portion N1 (which contains about 66% of the molecule) retained, and in some cases, emphasized the specificity exhibited by the whole molecule. Thus, Anti-peak 2 reacted well with N1 fragments of mammary tumor F1 regardless of the species from which they were derived. Of interest was the finding that N1 fragments from rat hepatoma exhibited significantly less complement fixation that N1 fragments from rat liver, when tested with antisera against rat thymus Peak 4 (Fig. 2C) or Peak 5 (Fig. 2D). N1 fragment from regenerating rat liver gave intermediate values (Fig. 2D).

To see whether the NH2-terminal fragment (N2) also reacted specifically, we tested the ability of this fragment to lower the reaction between various antisera and antigens. We found that 3 µg of N2 derived from rat hepatoma F1 histone slightly inhibited the reaction with the homologous antisera (from 85% to 70% maximal complement fixation) but not that with antisera against rat thymus Peak 4.

Because the above mentioned tests were done with cross-reacting (anti-rat thymus F1 sera) it seemed reasonable to investigate the possibility that F1 histones from tumors contain

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**Fig. 1 (left).** Complement fixation of F1 histones with antisera against rat thymus Peaks 2 to 5. F1 histones obtained from: A, rat liver; •, rat hepatoma BY 484; O, rat hepatoma BY 252; □, mouse mammary tumor; ●, human mammary tumor; Δ, rat mammary tumor. Sera dilutions: anti-rat thymus Peak 2, 1:2400; anti-rat thymus Peak 3, 1:3600; anti-rat thymus Peak 4, 1:4800; anti-rat thymus Peak 5, 1:6000.

**Fig. 2 (center).** Complement fixation of N1 fragments with antisera against rat thymus Peaks 2 to 5. N1 fragments obtained from: A, rat liver; C, regenerating rat liver; •, rat hepatoma BY 484; Δ, rat mammary tumor; □, mouse mammary tumor.

**Fig. 3 (right).** A, complement fixation of antiserum against rat hepatoma F1 (serum dilution 1:2400) with F1 histones. B, complement fixation of antiserum against rat hepatoma N1 (serum dilution 1:12,000) with N1 fragments. Antigens obtained from: A, rat liver; O, regenerating rat liver; •, rat hepatoma BY 484; Δ, rat mammary tumor; □, mouse mammary tumor; ●, human mammary tumor.
FIG. 4. Chromatography of F1 histones on Amberlite IRC-50. F1 histones obtained from rat thymus (A) and rat liver (B) by extraction with 0.14 m NaCl, 0.05 m NaHSO4. F1 histones obtained from rat liver (C) and rat hepatoma BY 484 (D) by treatment of frozen tissue (solid CO2) with 0.14 m NaCl. The peaks are numbered by Arabic numerals according to their immunological relatedness. The rat thymus peaks served as the reference peaks. The Roman numbers in parentheses refer to the nomenclature given previously (2, 6, 10) which was based on the order of elution.

Tissue Specificity of Tumor F1 Histones—The data presented so far indicate that in addition to species specificity the F1 histones exhibit a certain degree of tissue specificity. Thus, from Fig. 1 to 3 it can be seen that there are differences between the F1 histones derived from rat liver tumors and those derived from rat mammary tumors. Anti-peak 2 reacts better with the F1 and N1 fragments of the mammary histones than with those of the hepatoma. Anti-rat thymus Peak 4 recognizes better the rat hepatoma than the rat mammary histones. Antisera to either hepatoma F1 or N1 also reacted better with hepatoma derived histones than those derived from mammary tumors. Again, the N1 fragments retained and re-emphasized the differences seen when intact F1 were compared. The most striking example was observed with anti-rat thymus Peak 3 sera (compare Fig. 1B to 2B) were the slight differences between intact F1 of hepatoma and mammary tumors were magnified when the N1 fragments were compared.

Subfraction Specificity—To obtain more information on possible alteration of F1 histones during oncogenicity, it is necessary to compare purified F1 subfractions. To this end, F1 histones derived from rat thymus, rat liver, and rat liver tumor (hepatoma BY 484) were subjected to chromatography on Amberlite IRC-50 eluted with shallow guanidinium chloride gradients. The elution profiles presented in Fig. 4 indicate that the F1 histones from the different rat tissues give characteristic, distinct elution patterns. In this study the peaks were numbered by Arabic numerals according to their immunological relatedness. The rat thymus F1 fractions against which the sera were eluted, served as the reference peaks. The Roman numbers in parentheses refer to the nomenclature given previously (2, 6, 10) which was based on the order of elution.

It can be seen that rat thymus Peak 1 was not detected in the rat liver tissues. The amount of Peak 2 seemed to be variable and to be dependent on the method of extraction, since preparations obtained from both normal liver and liver tumor by the solid CO2 extraction procedure (12) contained less Peak 2 than preparations obtained from saline washed nucleoprotein (compare Fig. 4, C and D with A and B). That Peak 2 was indeed present in Fig. 4, C and D, is indicated by the immunological data presented below.

The most striking chromatographic difference between the three types of F1 histones was found in Fraction 4. While rat thymus F1 displayed one apparently homogeneous Peak 4, rat liver Peak 4 contained two ill resolved components (designated 4A and 4B). Rat liver hepatoma contained one component which chromatographically corresponded to Fraction 4B.

None of the rat liver or hepatoma peaks reacted well with antisera to rat thymus Peak 1. Fig. 5 shows the complement fixation curves obtained when antisera against each of the rat thymus Peaks 2 to 5 were tested with each of the rat liver and rat hepatoma peaks. The data show that each peak of rat thymus cross-reacted most strongly with the respective peak of rat liver or rat hepatoma. The only clear difference between rat liver and rat hepatoma was seen with antisera against rat thymus Peak 2. This serum reacted significantly more strongly with rat liver Peak 2 (Fig. 5A) than with rat hepatoma Peak 2 (Fig. 5E). This difference could not be attributed to contamination of rat liver Peak 2 with Peak 3, since anti-rat thymus Peak 2 did not react with rat liver Peak 3 (Fig. 5A). Contamination of rat hepatoma Peak 2 with Peak 3 could not also be the cause of difference, since there was a vertical shift in the complement fixation curve (compare Fig. 5, E with A), and furthermore, antisera against rat thymus Peak 3 did not react more strongly with rat hepatoma Peak 2 than with rat liver Peak 2 (compare Fig. 5, F with B). The differences between rat liver and rat hepatoma could be detected also with antisera to hepatoma F1 which reacted better with rat hepatoma Peak 2 than with rat liver Peak 2 (Fig. 6). Both the antisera against...
FIG. 5. Complement fixation of antisera against rat thymus Peak 2 (serum dilution, 1:4800), Peak 3 (serum dilution, 1:3000), Peak 4 (serum dilution, 1:2700), and Peak 5 (serum dilution, 1:8000) with rat liver peaks and rat hepatoma peaks. ▼, ○, △, and □, rat liver Peaks 2, 3, 4, and 5, respectively. ▼, ■, △, and ○, rat hepatoma Peaks 2, 3, 4, and 5, respectively.

FIG. 6. Complement fixation of (A and B) antiserum against rat hepatoma F₁ (serum dilution 1:2400) and (C and D) antiserum against rat hepatoma N₁ (serum dilution 1:12,000) with rat liver peaks and rat hepatoma peaks. ▼, ○, △, and □, rat liver Peaks 2, 3, 4, and 5, respectively. ▼, ■, △, and ○, rat hepatoma Peaks 2, 3, 4, and 5, respectively.

rat hepatoma F₁ and that against rat hepatoma N₁ reacted more strongly with Peak 3 of rat liver and rat hepatoma than with any of the other peaks. This may be due to the fact that Peak 3 is the major component of rat hepatoma, so that a major fraction of the antibodies would be directed against this component.

DISCUSSION

Antisera against F₁ subfractions are more specific reagents for studying differences between histones than antisera against unfractionated F₁ histones. The extended structure of the F₁ histone molecules makes it seem likely that most of the antibodies are elicited by sequential determinants (21). Since the amino acid composition of the various F₁ histones is practically indistinguishable, it stands to reason that in a mixture of several histones or histone fragments (N₁ and N₅) the cross-reacting determinants tend to dominate. Our studies (8–10) showed that antisera against unfractionated F₁ histones from different species cross-react to such a degree that they cannot be used effectively to study histone specificity. In contrast, antisera against individual subfractions are so specific that they react well only with test antigens that closely resemble the subfraction used as the immunogen.¹ In comparative immunological investigations of histones several types of sera have to be used, since differences between histones not detectable by one antiserum may be detected by another. Cross-reacting antisera were mainly used in the present study (for example, antisera against rat thymus peaks to compare rat liver and rat hepatoma fractions) and therefore the reactions were based on determinants which were present both in the immunogen and in the test antigen. Obviously, immunogen-specific determinants do not participate in such reactions, and therefore any differences observed between test antisera reflect minimal differences between F₁ subfractions. The advantage of using cross-reacting sera is that when differences are seen they are more reliable, since interference by tissue-specific contaminants (which may elicit tissue-specific non-histone antibodies) is eliminated.

Species and Tissue Specificity—The results obtained indicate that F₁ histones derived from tumors retain to a large extent the characteristics of the respective normal tissues. Experiments in which F₁ histones from mammary tumors of various species (rat, mouse, and human) were compared revealed that these histones have species-specific features. For instance, each of the antisera against rat thymus Peaks 3 and 4 clearly distinguished between the F₁ histones derived from the rat tumor and those from other species. Experiments in which tumors in the same species were compared (rat liver tumor with rat mammary tumor) revealed that the F₁ histones of these tumors exhibited tissue specificity. This was shown clearly with antisera against rat thymus Peak 4, which reacted much more strongly with F₁ histones from rat hepatomas BY 484 and BY 262, than with those from rat mammary tumor. Rat thymus Peak 2, however, when tested with F₁ histones from various tumors reacted better with mammary tumors, irrespective of the species, than with the F₁ histones from liver tissues. Perhaps it is relevant that in two mammary tissues studied so far by chromatography, i.e. rabbit (12) and mouse (22), the early eluting peaks constitute a greater proportion of the F₁ group than in the liver.

The tissue- and species-specific differences seen with the intact F₁ histones, were also observed with the respective N₁ fragments. Moreover in some cases, comparison of N₁ fragments revealed differences which were not observed when the intact molecule was examined. Structural studies have revealed that the distribution of amino acid residues along the F₁ molecule

¹ The reaction between antisera to rat thymus subfractions and purified fractions obtained from rabbit tissues was so weak that meaningful studies of tissue specificity in rabbits could not be done (M. Bustin, unpublished observations).
is asymmetric, and that sequence variations between F1 subtypes occur in the first 72 residues (5). Our studies indicate that to some extent these differences are also serologically observable. Furthermore, the present immunological studies suggest that the COOH-terminal portions also contain regions which vary from one molecular species to another. Pertinent to this point is the finding that the N1 region of Peak 3 of rat liver differs in amino acid composition from that of the other peaks (6).

Comparisons of Subfractions—Studies of the individual F1 subfractions have yielded data concerning the relationships between subfractions from different tissues. In a previous study (10) in which the rat thymus and rat liver fractions were compared, the chromatographic peaks were numbered according to their order of elution from Amberlite IRC-50. It was noted then that certain rat thymus peaks immunologically closely resembled certain rat liver peaks, although chromatographically their order of elution differed. Since the present studies, using fractions from rat thymus, rat liver, and rat hepatoma confirm this result, we propose to revise the nomenclature of rat liver and rat hepatoma peaks to conform to their immunological relatedness, taking the rat thymus peaks as the standard reference (Fig. 4).

It could be argued that Peak 1 of rat thymus is a contaminant or a degradation product peculiar to rat thymus. The fact that this peak has been detected in three independent studies (2, 9, 23) argues against this. Other arguments supporting the notion that the chromatographic peaks are not artifacts have been put forward by Cole and his associates (4, 5, 10, 24). A peak equivalent to rat thymus Peak 1 was not detected in rat liver and hepatoma. Indeed none of the liver or hepatoma peaks reacted well with antisera to rat thymus Peak 1. As measured in the previous study (10), the closest fraction in the liver was Peak 2 with immunological distance of over 70.

Peak 2 was present in minimal amounts in the liver tissues. The amount of this fraction was so small that slight variations due to different extraction procedures appeared to mask its presence. However, the presence of this fraction was easily verified immunologically, using antisera against rat thymus Peak 2, since these sera distinguish between rat liver Peak 2 and Peak 3. The amounts of Peaks 3 and 5 appeared not to vary appreciably in rat thymus, rat liver, and rat hepatoma. The major differences between these tissues seemed to be in Peak 4. The amount of this peak in rat liver was very high, compared to that found in rat thymus. Peak 4 of rat liver was composed of two closely related components, one of which was absent or present in very low amounts in rat hepatoma (Fig. 4).

Tumor Specificity—The present results, and studies presented by Kinkade (25) indicate that the F1 histones from tumors have tumor-specific features superimposed on the general features of the respective normal tissue. Apparently, the tumor specificity can both be quantitative or qualitative. We have found that one of the F1 subfractions of rat liver (Peak 4a) is either absent in rat hepatoma BY 498 or else present in significantly diminished quantity. Similar results have been reported by Kinkade (25) who found that certain F1 subfractions differed qualitatively in Novikoff ascites hepatoma compared to liver, and in lymphosarcoma compared to spleen. The immunological data we present indicate that also certain qualitative differences may exist. Thus, Peak 2 of rat hepatoma reacts much weaker with anti-rat thymus Peak 2 than does Peak 2 of rat liver. Furthermore, antisera against rat hepatoma F1, and especially those against rat hepatoma N1, react more strongly with the homologous antigens than with F1 or N1, respectively, from rat liver. The general picture which emerges from these studies, therefore, is that changes occur in F1 histones from tumors depending on the type of tumor. It should be pointed out that this may not be the case with all tumors, since Hohman et al. (22) did not find differences in chromatographic elution profile between F1 histones from mouse mammary tumors and normal mammary gland tissue. However, in such cases, the application of immunological techniques may detect differences which are not observed by chromatographic analysis alone.

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