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Biochemical Characterization of the Antimicrobial Histone Released by Deoxyribonuclease and Lactic Acid* (33873)

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In 1965 Miller and Watson (1) proposed a model which may be determinative in the host–parasite interaction. It was shown that intact nuclei or deoxyribonucleohistone complexes (DNH) manifested little or no bactericidal activity, but the addition of deoxyribonuclease (DNase) liberated cationic bactericidal histones. The DNase contained in the lysosomes of the inflammatory leukocytes or paradoxically, the DNase produced by various pathogenic bacteria (2), may effectively liberate this bactericidal histone in an inflammatory area. Also, since lactic acid is produced by the glycolytic metabolism of the inflammatory cells (3), this lactic acid may also effectively liberate a bactericidal histone from the nucleus. The present paper describes the characterization of the type of histones released from DNH by treatment with DNase or lactic acid.

Materials and Methods. DNase I was obtained from Worthington Biochemical Corporation, Freehold, N.J. and DNH was isolated from calf thymus according to the method of Crampton *et al.* (4). The histones were extracted from the DNH by diluting it with a more concentrated acid solution until the desired acid concentration was achieved. The acidic solution was then stirred at 4° for 1 hr and centrifuged at 2000*g* for 10 min. The supernatant fluid was then dialyzed for 24 hr, pervaporated and lyophilized. Three acids were used for the extractions: 0.2 *N* HCl was used for the extraction of the "reference" total histones (5); 5% trichloracetic acid (TCA) was used to extract the "reference" lysine-rich histone (6), and 5% lactic acid (LA) was used experimentally to determine what type of histone might be released.

To release the histones, DNase at a concentration of 10 μg/ml was used to degrade the DNH during a 1-hr incubation at 37°. The undegraded DNH was precipitated out at 4° by making the solutions 0.85% with NaCl. The supernatant fraction from a 2000*g*, 10-min centrifugation was then dialyzed to remove the soluble nucleotides and then lyophilized. The precipitated DNH was then re-extracted with 5% TCA to remove the lysine-rich histones. DNase was omitted from the control tube.

For amino acid analysis, the proteins were hydrolyzed for 24 hr in sealed evacuated then lyophilized. The precipitated DNH was removed, *in vacuo*, in a desiccator over a desiccant and NaOH pellets. A Beckman model 120 B amino acid analyzer was then used for the analyses.

The electrophoretic separations were made in the E-C vertical acrylamide gel electrophoresis cell. A 5% concentration of acrylamide gel was prepared with 0.1 *M* acetate buffer at pH 4.2. Twenty μl of a 5% solution

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TABLE I. Amino Acid Composition of Calf Thymus Histones Extracted by Different Procedures.

	Method of extraction				
	1	2	3	4	5
Amino acid[a]	HCl	TCA	LA	DNase	DNase–TCA
Lysine	14.6	29.2	22.0	20.9	26.7
Histidine	2.1	0.1	1.2	0.4	0.2
Arginine	10.5	2.5	6.4	3.0	2.7
Aspartic acid	3.8	1.3	2.8	2.8	2.1
Threonine	5.2	4.9	5.6	5.7	5.9
Serine	4.3	3.2	3.4	5.6	4.5
Glutamic acid	4.7	1.3	1.3	5.0	0.9
Proline	4.9	10.9	8.0	10.6	9.4
Glycine	9.0	6.0	8.6	17.3	8.3
Alanine	14.5	27.4	20.2	19.3	26.2
Valine	7.2	4.5	5.9	4.1	5.1
Methionine	0.8	0.2	0.8	0.3	0.2
Isoleucine	4.7	0.9	3.0	1.1	1.3
Leucine	8.8	4.1	7.0	3.4	5.4
Tyrosine	2.8	0.7	2.3	0.5	0.8
Phenylalanine	2.1	0.6	1.6	0.5	0.7

[a] All values are expressed as percentages of total moles of amino acids recovered in the particular amino acid.

of each histone preparation was added to each slot. The electrophoretic run was made at a constant 200 mA for 4–8 hr. An amido black 10B dye was used to stain the protein bands.

The antibacterial assay was done by the turbidimetric method (1).

Results. Table I shows the amino acid composition of the various isolated histones. Column 1 is the amino acid composition of total histones extracted with 0.2 *N* HCl. The reference lysine-rich histone extracted by 5% TCA (6) is shown in column 2. The histones extracted by lactic acid (column 3) and deoxyribonuclease (column 4) are both more lysine-rich than the total histones but less so than the reference TCA-extracted lysine-rich histone.

A control for the DNase-liberated histone is found in column 5. It is the histone extracted from the DNase-treated DNH and subsequently treated with TCA. The lysine-rich histone from this sequential treatment is almost identical to the lysine-rich histone extracted from a native DNH with TCA. The significance of the difference in the amino acid composition of the various histone fractions is not known, since all fractions are shown in the following schematic electropherogram to be heterogeneous.

The electropherogram in Fig. 1 compares the various histones extracted by HCl, TCA, LA, DNase, and DNase–TCA. The total histones (slot A) extracted with hydrochloric acid have many different bands appearing. The reference lysine-rich histone (slot E) extracted with TCA shows a heavier concentration of the faster migrating bands of the total histone fraction. But the lysine-rich his-

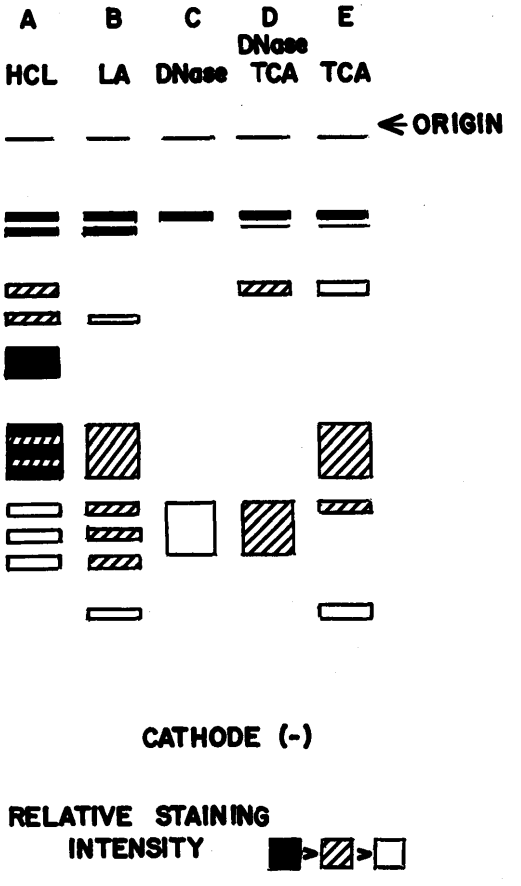

FIG. 1. Electrophoretic patterns of histones obtained by different extraction procedures. Each slot contains 20 μl of 5% histone extracted with: (A), 0.2 *N* HCl; (B), 5% lactic acid; (C), DNase; (D) DNase followed by 5% TCA; (E), 5% TCA. A 0.1 *M* acetate buffer, pH 4.2, was used.

tones extracted with lactic acid (slot B) reveal an even higher proportion of the more rapidly migrating proteins. The DNase and DNase–TCA extracted lysine-rich histones have an interesting electrophoretic mobility. As mentioned previously, the control lysine-rich histone extracted from the DNase-treated DNH with TCA is seen, on the basis of amino acid composition, to be almost identical to the lysine-rich histone extracted from native DNH with TCA. In contrast, as seen in the electropherogram, the fast-moving heavily stained protein band is displaced more toward the cathode in the TCA–DNase lysine-rich histone (slot D). Also, the two additional cationic protein bands of the reference TCA lysine-rich histone (slot E) are missing. The DNase-liberated lysine-rich histone (slot C) has only the fast-moving protein band, but even this is present in lower concentration on a dry weight basis. It is very likely that the displaced protein bands of the DNase and the DNase–TCA lysine-rich histones are the result of cathepsin degradation as also suggested by Reid and Cole (7).

Next, the same histone solutions which had been used for amino acid analysis and electrophoresis were utilized in an examination of the antibacterial potency. The results are shown in Fig. 2: The DNase-liberated histones manifest less activity than do the other histone preparations on a weight basis. This confirms the results of the electrophoresis which showed that on a weight basis, less protein was present than in the other histone preparations.

Discussion. These experiments demonstrated that DNase and lactic acid liberated a histone possessing antibacterial properties from nucleohistone. This histone was shown to be lysine-rich by amino acid analysis.

An explanation for the selective release of a lysine-rich histone by the enzyme DNase is not known, but two possibilities seem to exist. First, Johnson *et al.* (8) showed that the type of histone released from nuclear preparations was pH-dependent with the smaller molecular weight materials more easily extracted. Murray (9) demonstrated that

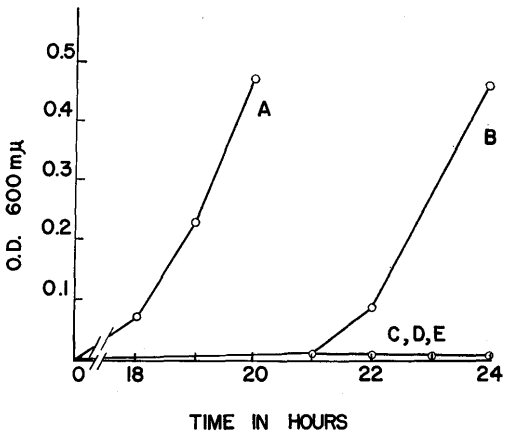

FIG. 2. Antibacterial activity of calf thymus histones isolated by different procedures. (A) refers to the medium control. The other letters identify the method of extracting histones from DNH: (B) DNase; (C),-lactic acid; (D) TCA; (E) hydrochloric acid. All histone preparations were at a final concentration of 10 μg/ml. The test organism was *Staphylococcus aureus* at an inoculum concentration of 10^2 into 10 ml of beef heart medium.

a lysine-rich histone fraction is released at weaker acid concentrations. His explanation is that the total number of basic acid residues is the important factor in the electrostatic linkage of histone to DNA. The first histone to be dissociated would be the lysine-rich histone fraction because it contains fewer electrostatic linkages. Although the release of a lysine-rich histone from DNH by DNase does not involve a pH elution process, this explanation might be plausible if one assumes that a certain length of DNA backbone is required for an effective ionic linkage to histone. Where the DNA has been cleaved by DNase, there is a spontaneous dissociation of the lysine-rich histone first because there are fewer electrostatic linkages to be broken.

Another plausible explanation would appear to be specificity of DNase cleavage with a resultant selective removal of histones. For example, Zamenhof and Chargaff (10) found nondialyzable oligonucleotides in DNase I resistant cores which contained a higher adenine to guanine and thymine to cytosine ratio than the original substrate. Interestingly, the lysine-rich histone appears to have a preferential linkage with DNA that is

relatively richer in guanine and cytosine.

It was of interest, as shown earlier (1), that these experiments confirmed the antibacterial properties of these lysine-rich histone preparations. It has been shown that some lysine-rich histone preparations have very little antibacterial activity (11) while others have suggested that it is very active (12, 13). An excellent analogy of this type of confusion is found in the inhibitor effect of histone on DNA-dependent RNA synthesis in *in vitro* assays (14, 15). Allfrey and Mirsky (14) concluded that the inhibitory activities cannot be attributed solely to the amount of lysine or arginine residues, but that many other factors may "play a role in determining the net inhibitor effect."

In conclusion, we wish to restate the possible significance of our hypothesis. In an inflammatory area, the terminal cells of the neutrophil type undergo dissolution (16) with the possible release of histones from the nucleus through the action of DNases or lactic acid. The former could be those from the polymorphonuclear leukocyte (PMN) lysosomes or could be produced by pathogenic microorganisms (2, 13). The lactic acid would be produced by the PMNs through their glycolytic metabolism (3). We do not propose this as an alternative to the important role of phagocytosis in host–parasite relationships; rather, we suggest that it exemplifies a means by which the host has adapted to cope with the parasite in an extracellular situation. Indeed, an extracellular mechanism is observed in skin lesions induced with *Bacillus anthracis* in resistant animals (17).

Summary. Both DNase and lactic acid are able to liberate histones with antimicrobial activity from an inactive deoxyribonucleohistone complex. The type of histone was shown in both cases to be of a lysine-rich variety by amino acid analyses and acrylamide gel electrophoresis. Since both DNase and lactic acid would be expected to be present in an inflammatory foci, the significance of this model is emphasized.

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