THE INHIBITION OF TWO ANTIBACTERIAL BASIC PROTEINS BY NUCLEIC ACIDS

WALTER L. BLOOM, MELISSA G. WINTERS, AND DENNIS W. WATSON

Departments of Biochemistry and Medicine, Emory University School of Medicine, Emory University, Georgia, the Research Division of Lawson VA Hospital, Chamblee, Georgia, and the Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minnesota

Received for publication March 19, 1951

Previous studies of certain aspects of natural resistance in experimental animals to infection by Bacillus anthracis suggested that during the course of the infection, antibacterial substances appear that alter the body fluids and create an environment unsuitable for the growth of this parasite. It was inferred that the antibacterial property might be derived from the polymorphonuclear leucocyte since in anthrax infection the major pathologic difference in the response of the susceptible, naturally resistant and immune host appeared to be the quantity of the polymorphonuclear leucocytes present in the local lesion (Cromarite, Bloom, and Watson, 1947). These observations led to the isolation and partial purification of an antibacterial protein which was shown to contain a high percentage of lysine (Bloom, Watson, Cromartie, and Freed, 1947). This type of compound was subsequently shown to have antibacterial activity against Micrococcus pyogenes var. aureus, beta-hemolytic streptococci, and Escherichia coli as well as Bacillus anthracis. Evidence was presented by Bloom and Blake (1947) that indicated that the basic protein combined with some component of the bacterial cell. Ribonucleic acid was shown to combine with the bacterial protein complex. However, if ribonucleic acid was first added to the basic protein, the antibacterial effects on the cell were inhibited. This observation indicated the necessity of studying the quantitative aspects of the basic protein-nucleic acid combination using the antibacterial property of the protein as the indicator.

Protamine, another basic protein, was used as a corollary antibacterial substance to determine whether the reaction was specific for the epsilon amino group of lysine or characteristic of proteins having a high content of other dibasic amino acids such as arginine. As has been shown by McClean (1930, 1931), protamine inhibited the growth of Salmonella typhosa and vaccinia virus. Reiner, deBeer, and Green (1942) showed that the respiration of Trypanosoma equiperdum was partially inhibited by protamine. Miller, Abrams, Dorfman, and Klein (1942) observed definite inhibition of respiration in a series of gram-positive and gram-negative bacteria. A similar observation was made by Negroni and Fischer (1944).

1 Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.
This communication presents quantitative data on the inhibition of the antibacterial properties of basic proteins by ribonucleic acid (RNA) and deoxy-ribonucleic acid (DRNA).

METHODS AND MATERIALS

The tissue protein used in these experiments was prepared from beef thymus by a modification of the method previously described (Bloom, Watson, Cromartie, and Freed, 1947). The dried preparations of tissue protein and protamine were weighed and dissolved in distilled water, and the pH was adjusted to 7.5 with 0.1 N NaOH. The DRNA was prepared by the method of Mirsky and Pollister (1942) with protein extraction of the nucleoprotein as reported by McCarty (1945 to 1946). The DRNA contained 13.4 per cent nitrogen and 8.0 per cent phosphorus. This material, when dissolved in distilled water, was very viscous. The RNA contained 13.6 per cent nitrogen and 8.5 per cent phosphorus. These solutions were made in distilled water, and the pH was adjusted to 7.5 with 0.1 N NaOH.

The amount of antibacterial proteins to be used in each experiment was determined by ascertaining the smallest concentration which would produce complete inhibition of oxygen uptake of the bacterium studied. This might be called a minimal antibacterial amount of the basic protein. For these experiments, two minimal antibacterial amounts of the basic protein were used in each flask. Five hundred µg of thymus protein per ml and 250 µg of protamine per ml were the amounts of each protein found to constitute two minimal antibacterial amounts of protein. The quantities of nucleic acid used in each experiment were constant in the series and ranged from 50 to 500 µg per ml. Two methods for the study of nucleic acid combination with the basic polypeptides were employed. In the first method, the nucleic acid was added to the culture medium and bacteria in the main well of the flask, and the antibacterial fraction was placed in the side arm. In the second method, the nucleic acid was mixed with the basic polypeptide in the side arm, and the bacteria and medium were placed in the main well of the flask. The volumes in each flask were kept constant in all experiments. The contents of the side arm of the Warburg flask in each instance was 1 ml. Saline in the side arm (0.85 per cent) served as the control.

The strain of Bacillus subtilis employed was isolated in this laboratory and maintained by frequent transfer in nutrient broth. The Warburg studies were conducted as previously described (Bloom, Watson, Cromartie, and Freed, 1947). Reproducibility of results was confirmed by repeating each Warburg experiment on three different occasions.

RESULTS

The data are presented in figures 1 to 4. Two types of experiments are presented in each figure. The nucleic acid was combined directly with the antibacterial fraction in the first experiment, and the combination was then tested

\* Kindly supplied by Dr. A. Lee Caldwell, Head, Nutrition and Vitamin Research Department, Eli Lilly Research Laboratory, Indianapolis, Indiana.
Figure 1. Quantitative titration of ribonucleic acid inhibition of the antibacterial effect of protamine on B. subtilis.

Description. Readings of oxygen uptake were taken at 10 minute intervals. Figures at the right of each curve refer to µg of nucleic acid added. In the first experiment the nucleic acid was added to the main well of the flask, and in the second experiment the nucleic acid was mixed with the basic protein in the side arm of the flask.

Figure 2. Quantitative titration of desoxyribonucleic acid inhibition of the antibacterial effect of protamine on B. subtilis.

Description. Readings of oxygen uptake were taken at 10 minute intervals. Figures at the right of each curve refer to µg of nucleic acid added. In the first experiment the nucleic acid was added to the main well of the flask, and in the second experiment the nucleic acid was mixed with the basic protein in the side arm of the flask.
Figure 3. Quantitative titration of ribonucleic acid inhibition of the antibacterial effect of thymus basic protein on B. subtilis.

Description. Readings of oxygen uptake were taken at 10 minute intervals. Figures at the right of each curve refer to μg of nucleic acid added. In the first experiment the nucleic acid was added to the main well of the flask, and in the second experiment the nucleic acid was mixed with the basic protein in the side arm of the flask.

Figure 4. Quantitative titration of desoxyribonucleic acid inhibition of the antibacterial effect of thymus basic protein on B. subtilis.

Description. Readings of oxygen uptake were taken at 10 minute intervals. Figures at the right of each curve refer to μg of nucleic acid added. In the first experiment the nucleic acid was added to the main well of the flask, and in the second experiment the nucleic acid was mixed with the basic protein in the side arm of the flask.
for its inhibition of oxygen uptake on *B. subtilis* after a control growth period of thirty minutes. In the second experiment, the nucleic acid was added directly to the bacteria in medium, and after the control growth period of thirty minutes, the antibacterial fraction was added from the side arm. This made it possible for the antibacterial polypeptide to combine with the nucleic acid, the bacterial cell, or both. In figure 1, it may be seen that a larger amount of RNA is needed to neutralize the protamine effect when the RNA is added to the culture medium. The same phenomenon was observed when DRNA and protamine were studied, in figure 1. Figures 1 and 2 show that the quantities of nucleic acid necessary to inhibit the antibacterial effect of protamine were of the same magnitude, 350 μg per ml for both RNA and DRNA.

In figures 3 and 4, the neutralization of the inhibitory effect of the thymus protein by both DNA and RNA may be observed. In contrast with the protamine results, when equal quantities of RNA and DRNA neutralized the antibacterial activity, the thymus protein required 500 μg of RNA and only 300 μg of DRNA. The greater affinity of DRNA for the thymus protein is further shown in figure 4 where the quantities of DRNA necessary to inhibit the thymus fraction are no different when combined directly in the side arm or added to the bacteria and media.

**DISCUSSION**

The quantitative nature of the neutralization of the antibacterial action of a thymus basic protein and protamine has been demonstrated. This study has been related to the observation that nucleic acid in adequate amounts blocks the basic protein inhibition of oxygen uptake of *B. subtilis*. RNA has the same affinity for both protamine and the thymus protein. DNA apparently has a greater affinity for the thymus fraction than protamine. Since both DNA and the thymus fraction are constituents of mammalian nucleoprotein, it is not surprising to find a strong affinity between these two compounds. Previous studies by Lewis, Bloom, and Smart (1949) have shown a quantitative difference in the amounts of RNA and DRNA necessary to form protein nucleates. Protamine is also a nuclear component, and one might expect to find a similar relationship between DNA and protamine, but this was not observed in this experiment. It is known that the thymus basic protein is high in lysine content (Bloom, Watson, Cromartie, and Freed, 1947), and the protamine is high in arginine content (Kossel, 1928). Whether the noted differences in the affinity of these proteins for nucleic acids are the result of these amino acid differences, cannot be determined until a synthetic polylysine (Stahmann, Graf, Patterson, Walker, and Watson, 1951) and a synthetic polyarginine are tested in an experiment of this type.

Titration data by Fletcher, Gulland, and Jordan (1944) and Gulland and Jordan (1948) on nucleic acids indicate that fewer phosphoryl groups are present in a mol of RNA than in a mol of DRNA. If the neutralization of basic proteins depended on combination of phosphoryl groups of nucleic acid with free amino groups of the protein, it might be expected that larger amounts of RNA would
be used. The nucleic acid linkage with the thymus protein could be explained on this basis. However, the quantities of RNA and DRNA required to block the protamine effect are the same and cannot be explained by a simple salt linkage. It has been shown that small amounts of nucleic acid may combine with the basic fractions, leaving free groups available to cause inhibition of oxygen uptake of the cell. When larger amounts of nucleic acid are added, all of the free groups of the antibacterial fraction are combined, and no effect on the cell is noted.

With the exception of the DRNA-thymus combination, more nucleic acid is necessary when added to the media than when combined directly with the basic proteins. This suggests that either the nucleic acids are being removed by the bacteria or that the basic protein, in the presence of bacteria and nucleic acid, combines with both.

The experiments have clarified the problem of the effects of RNA on the antibacterial action of basic proteins. Previously Weissman and Graf (1947) had shown that RNA could be combined with the basic polypeptide without altering the antibacterial effects of the protein. It is now clear that if sufficient RNA is added, the inhibition of oxygen uptake by thymus polypeptide is prevented.

Unequivocal evidence has not been presented here to prove that the inhibitory basic proteins act by combining with the phosphoryl groups of the nucleic acids in the bacterial cell. Recent studies of Stacey (1947) on the nature of the bacterial surface of the gram-positive bacteria have described the outer surface as being composed of layers consisting of magnesium ribonucleate-protein complexes. These nucleoproteins along with prosthetic groups and coenzymes are thought to form the enzyme systems responsible for cell function. The combination of basic proteins with the nucleic acids of such vital complexes could explain the mechanism by which these basic proteins exert their antibacterial effects as manifested by the inhibition of aerobic respiration.

**SUMMARY**

The quantitative nature of desoxyribonucleic and ribonucleic acid neutralization of the antibacterial action of thymus basic protein and protamine has been demonstrated. DRNA is more effective than RNA in inhibiting the thymus protein. In contrast no quantitative difference in inhibition was noted when these two nucleic acids were added to protamine. The findings suggest that the combinations of these fractions cannot be the result of a simple salt linkage, but depend upon some chemical difference in the structure of these two basic proteins.

These data suggest that the mechanism of antibacterial action of basic proteins depends upon their capacity to combine with nucleic acid complexes essential in the processes of aerobic respiration.

**REFERENCES**

Bloom, W. L., and Blake, F. G. 1948 Studies on an antibacterial polypeptide extracted from normal tissues. J. Infectious Diseases, 83, 118–123.


WEISSMAN, W., AND GRAF, L. H. 1947 Studies on infection with Bacillus anthracis. VII. A comparison of the antibacterial effects of calf thymus histone and a quaternary ammonium cationic detergent on B. anthracis. J. Infectious Diseases, 80, 145–153.