DNA Antibody in the Serum of Patients in Shock

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THIS REPORT describes the finding of antibodies to deoxyribonucleic acid (DNA) and its chemical subunits in the serum of patients in shock. DNA, the giant molecule in the cell nucleus, controls the process by which amino acids are aligned in proper sequence during protein synthesis.[7, 8, 20, 31] The integrity of DNA and its intermediary messenger, ribonucleic acid (RNA), are essential for continued cellular function and survival.[25]

The frequent failure of intensive therapy in severe shock, despite the application of advanced technics, emphasizes the continuing need for understanding the basic cellular changes that occur with shock. Little attention has been given to potential alterations in the subcellular mechanisms for protein synthesis during shock.[18] Of particular interest are several recent reports indicating the development of altered, but as yet undefined, immunochemical responses in patients in shock.[23, 24, 29] The possibility that immunochemical responses can disrupt the cellular coding mechanism of DNA is intriguing. These considerations and the existence of technics for detecting antibodies to DNA, prompted the analysis of the sera of patients in shock and the identification of circulating antibodies to DNA. The prognostic applications correlated with observations in 55 patients are presented.

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Material

All available patients with a clinical diagnosis of shock within the period of this study were utilized. The age of these 55 patients ranged from nine to 78 years; 30 were men and 25 were women. The clinical severity of shock was assessed by grading the signs and symptoms from transient to severe, according to the well established methods of Beecher and others.[3] While measurements of cardiac output were frequently unavailable for precise identification of the hemodynamic abnormality, an etiologic classification of cardiogenic, hypovolemic, neurogenic and septic shock was determined in these patients.

Fifteen patients had transient, mild shock of limited duration and responded readily to therapy. Of the remaining 40 patients, 16 had moderate and 24 patients had severe shock. The etiology of shock compared with the degree of severity is presented in Table 1.

Five of the 24 patients with severe shock survived. These five had either hypovolemic (three), cardiogenic (one) or septic shock (one). The remainder in severe shock died; 13 after a protracted course.

Methods

At least 10 ml. of blood were obtained from each patient, and kept at room tem-

TABLE 1. *A Comparison of the Etiology and Clinical Severity of Shock in 55 Patients*

Type Shock	Mild	Moderate	Severe
Cardiogenic	1	3	7
Hypovolemic*	12	11	8
Neurogenic	1	0	2
Septic	1	2	7
Total	15	16	24

* Includes hemorrhage.

perature until clot retraction occurred. The serum was separated by centrifugation, then frozen at −20° C and used for all subsequent procedures. No loss of antibody activity occurred with thawing or refreezing. In each case a sample was obtained during, or within 24 hours of the shock episode. Repeated sampling varied according to the patient's subsequent course.

Antibodies to DNA were demonstrated by a method of passive hemagglutination. Serum samples were heat-inactivated at 65° C. for 30 minutes and absorbed with human "O positive" red cells to inactivate nonspecific agglutinins and remove anti-erythrocyte antibodies. A double dilution of serum from 1:2 to 1:1024 was prepared after adding 0.5 ml. of serum to the first of 10 agglutination tubes containing 0.5 ml. of phosphate-buffered saline (pH 7.30). Erythrocytes coated with DNA constitutes a very sensitive reagent for detecting antibodies to DNA. To prepare a mixture of DNA and erytrocytes, 1.0 mg. of highly polymerized DNA (Sigma) was added to a 2.5% suspension of washed human "O positive" cells in 10 ml. of buffered saline. To couple DNA to the red cells, 0.5 ml. of a 1:16 dilution of bis-diazo-benzidine (0.5 ml. in 7.5 ml. of buffered saline) was added.[15] After washing once with 0.01 N rabbit serum to prevent spontaneous agglutination of the modified red cells, the cells were resuspended in 10 ml. of buffered saline. One-tenth milliliter of red cells coupled to DNA, acting as the test antigen, was then added to each tube of diluted serum. The reaction mixtures were incubated in a water bath at 37° C for 30 minutes, then centrifuged (Clay-Adams serofuge), and read for agglutination by observing the pattern of clumping in each tube. The titer of antibodies in the serum is expressed as the reciprocal of the highest dilution giving definite agglutination.

Erythrocytes must be freshly prepared, presenting the possibility of day-to-day variation. Multiple controls are used to insure that any positive results are not due to an error of technic.

Those subunits of the DNA molecule capable of combining specifically with antibody and inhibiting precipitation of the complete DNA molecule were determined at the nucleotide and nucleoside levels by inhibition tests. Antibody specificity to the nucleotides of thymine, adenine, guanine, cytosine and uracil, which replaces thymine in the RNA molecule, were tested for inhibition by adding 10 micrograms of the nucleotide monophosphate (Sigma) to separate preparations of diluted serum prior to the addition of DNA-coupled erythrocytes. Both ribonucleotides and deoxyribonucleotides were tested, and no difference was observed. This indicated the presence of antibody to RNA as well, in these patients, which can be further demonstrated by hemagglutination with RNA-coupled erythrocytes.

Inhibition of agglutination was compared to the DNA agglutination readings. A two-tube difference in the agglutination results was considered a valid determination of nucleotide specificity and not the result of variations in the procedure.

Specificity may also be determined by multiple absorptions of the serum with nucleotide-coupled erythrocytes prior to testing with DNA. Hemagglutination tests using red blood cells directly coupled to nucleotides have also proved helpful in determining nucleotide specificity. The inhibition method has the advantage of al-

lowing some quantitation of the amount of antibody in one milliliter of serum.

In our experience, normal serum did not contain DNA antibody although it is known to be present in one to three per cent of the population in large series.[32] A high percentage of patients with lupus erythematosus are known to have DNA antibody.[14, 27] DNA antibody has been found in smaller proportions of patients with connective tissue and other diseases.[2, 4] Such serum, particularly in lupus erythematosus, is valuable as a standard control for the hemagglutination test. Obviously, false positive results could be obtained depending upon the immunologic background of the patient.

Results

DNA antibody was found in the serum of all 40 patients in moderate and severe shock. No antibody was present in the serum of 15 patients with transient shock of mild severity.

Initial antibody titers ranged from 4 to 256. Low titers were found, even in patients in severe shock, during the hypotensive episode or within 24 hours thereafter. The mean initial titer was 16. Titers continued to rise to levels as high as 2,048 within an average of four days of the onset of shock, but in some cases not until 30 days. The mean maximum titer was 256.

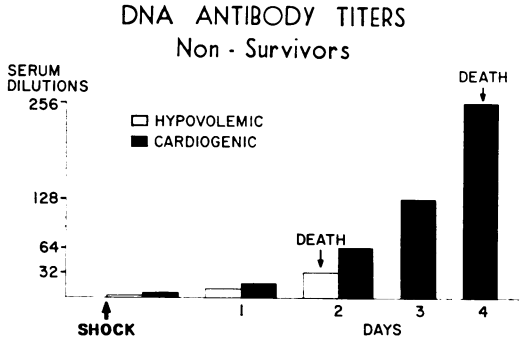

FIG. 1. A progressive rise in DNA antibody titers from relatively low values detected within hours of the onset of shock in one patient with hypovolemic shock and another, with cardiogenic shock, was a common observation.

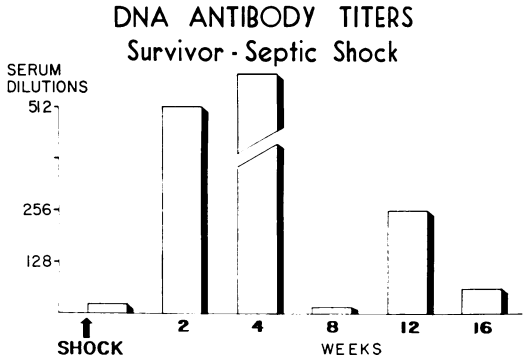

FIG. 2. A survivor of severe gram negative sepsis developed an antibody titer of 2048 within 4 weeks after the shock episode. A detectable titer of DNA antibody was still present four months later.

The titer of DNA antibody in an initial determination is of no practical importance other than signalling severe stress. While patients with severe and protracted shock tend to develop an increasing titer of antibody, Figures 1 and 2 illustrate a frequent finding that more antibody may be found in survivors of a severe shock episode than in patients succumbing to the stress of shock. A fall in subsequent titers was observed in three patients in severe shock, two of whom died. No increase in titers occurred in five patients with moderate shock.

No correlations could be made with age, sex or the rapidity of onset of the shock episode, and no relationship existed between antibody titer and the hemodynamic abnormality or the etiology of shock.

When it was observed that the antibody specificity was directed not only to the DNA molecule, but also to the nucleotides, distinct patterns emerged. Such patterns are not related to the etiology of shock, but are related to the eventual outcome. Thirty of 40 patients with DNA antibodies had sufficient titers (eight or more) to determine nucleotide specificities. Seventeen of these patients survived: five after a severe clinical course, the others after a mod-

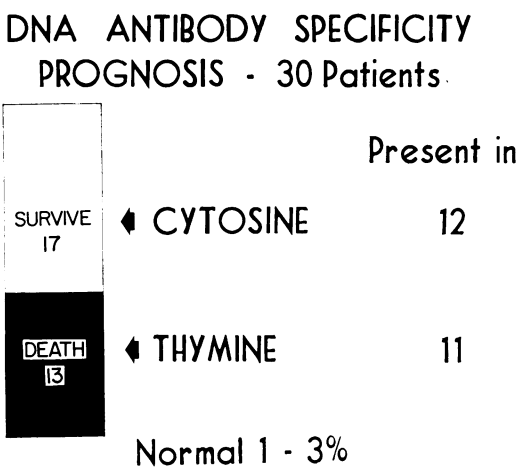

FIG. 3. The presence of cytosine and thymine-specific antibodies in the serum of patients in shock is related to the eventual outcome. DNA antibodies are uniformly present in severe shock, but only occur in a small percentage of the normal population.

erate shock episode. Thirteen patients died after a severe and protracted course of shock.

Thymine-specific antibodies, or combinations of thymine and other nucleotide specificities, were demonstrated in 11 of 13 deaths. Two patients had specificities directed to both adenine and uracil. No survivor had this combination.

Cytosine and guanine-specific antibodies alone, or combined with uracil, were present in 10 of 17 survivors. Five of these patients had severe shock. One survivor had antibody specificity directed to both adenine and guanine. The remainder had low titers of the following antibody combinations: thymine, cytosine and uracil (four survivors); thymine, cytosine and adenine (two survivors). Antibodies to thymine, cytosine and uracil was detected in the lupus erythematosus control serum. Two survivors with similar combinations had rheumatoid arthritis.

Our early experience uniformly associated the presence of thymine-specific antibody with a fatal outcome. Later, survivors with low thymine antibody were observed. Each survivor had a low titer of thymine-specific antibody (eight or less), hypovolemic shock due to fluid loss or hemorrhage, and responded rapidly to therapy. There is a statistical, as well as clinical, relationship between the presence or absence of thymine-specific antibodies and survival, at a probability level of less than 0.02 using Chi-square or exact probability tests (Fig. 3).

Discussion

The great majority of proteins are enzymes, directing the myriad chemical reactions within each cell. Their continued formation is essential for cell survival. Many observations of enzyme depletion and the consequent alterations in cell metabolism during shock have been reported.[5, 6, 9, 11, 17] Considerable attention has been directed to critical depletions of energy sources such as ATP and phosphocreatine.[16, 17, 28] Alterations in the mechanism for protein synthesis during shock have been inferred from such biochmical observations but direct evidence has not been reported.[18, 19, 26]

Since Watson and Crick proposed the basic molecular structure for DNA fifteen years ago, a vast amount of information concerning the nature of protein synthesis has accumulated.[31] Crick established that the chemical subunits of DNA (adenine thymine, cytosine and guanine) act as code letters which provide information used by the cell to construct proteins from 20 amino acids.[8] Gamow theorized that a sequence of three nucleotides, a code word, was necessary to direct each amino acid into a protein chain.[12]

Since DNA is not directly involved in protein synthesis, the nucleotide sequence must be transcribed by the synthesis of RNA as an intermediate messenger.[13] RNA contains the sugar ribose instead of deoxyribose, and the base uracil instead of thymine. The sequence of bases in messenger RNA specifies the amino acid sequence of the protein to be constructed at the ribo-

some, the cellular site of protein synthesis.[20] Nirenberg provided a convenient method for studying in vitro protein synthesis in cell-free extracts of Escherichia coli.[21] This permitted the determination of the sequence of bases which code for each amino acid.[22]

While this study does not present evidence demonstrating altered DNA function, the finding of antibodies in shock with activity directed to the individual nucleotides comprising the DNA molecule suggests further investigation to determine their potential effects. Preliminary studies of how these antibodies affect protein synthesis in cell-free extracts of E. coli have been undertaken in our surgical laboratories. These studies with purified antibody to thymine, adenine, cytosine, and uracil suggest that nucleotide-specific antibody prevents synthetic messenger RNA from directing the incorporation of amino acid into protein. Nucleotide-specific antibodies may have a similar effect or may prevent the formation of messenger RNA *in vivo*. The consequent effects may be similar to observations of the cessation of RNA production in tissue cultures after the introduction of drugs active at nucleotide levels, which resulted in cell autolysis.[25]

The origin or mode of production of DNA antibody is unknown. After separating serum fractions by column chromatography, using G-200 Sephadex (Pharmacia), the hemagglutination activity was found to be predominantly associated with the IgM (19 S) globulin fraction. In this analysis high titer serum obtained four days after a shock episode was used. Some activity appeared in the IgG (7 S) globulin fraction. Hemorrhagic and septic shock preparations in primates, in our laboratories, have demonstrated the uniform appearance of this antibody within 60 minutes of the onset of shock. These findings of a high molecular weight antibody as an early response to a stimulus are in accord with recent immunologic observations.[1, 30] Whether anti-DNA activity in the serum of patients in shock represents a response to an antigenic stimulus, an alteration in circulating globulins, or a release of preformed antibody raises considerable questions. The role of lysosomes in this phenomenon also merits investigation.[10]

Antibody to DNA is present in the serum of all patients in shock except those with a transient and mild course. No practical benefit will accrue from the use of antibody determinations in the patient who readily responds to therapy. However, in those with severe or protracted shock who do not respond to blood and extra-cellular fluid volume replacement, vasoactive drugs or other modes of advanced therapy, DNA antibody and nucleotide specificity tests may offer prognostic help.

Three-fourths of the problem patients in this series had a titer of sufficint magnitude to permit the determination of nucleotide specificity. Patients with specificities directed to cytosine or guanine had a favorable outcome. If the nucleotide specificity is that of thymine, it has been our experience that two-thirds will die and one-third survive. These survivors had low titers with quite similar patterns of nucleotide specificity. They also had a uniformly rapid response to treatment, and in each instance it was instituted quickly. Each had shock due to hypovolemia. We suspected that two of these survivors with thymine antibody had a pre-existing titer as both had rheumatoid arthritis, and multiple determinations subsequent to the shock episode showed no change in the titer levels.

From a practical standpoint, these determinations require approximately four hours of laboratory time. They are highly sensitive, inexpensive, and not diffijcult to perform, once facility is gained in reading agglutinations. Qualitative slide tests using immunofluorescent and other technics are being developed for preliminary screening.

Repeated determinations for DNA antibody have demonstrated the continued

presence of agglutination at variable titers in survivors as long as eight months after the shock episode. Continued follow up of these patients is being undertaken.

The possibility that DNA antibodies can prevent the formation of messenger RNA or otherwise block the mechanism for protein synthesis during shock suggests the therapeutic use of nucleotides to inhibit these potential antibody effects. The use of nucleotides in the treatment of hemorrhagic and septic shock in primates is being investigated. Their clinical use, as an adjunct to intensive therapy in shock, is under consideration.

Summary

Antibodies to deoxyribonucleic acid (DNA) and its chemical subunits, or nucleotides, were found in the serum of patients in shock using hemagglutination tests. Deoxyribonucleic acid controls the process by which amino acids are aligned in proper sequence during protein synthesis. The integrity of this molecule as well as its messenger, ribonucleic acid (RNA) are essential for continued cellular function.

Antibody was present in 40 patients with moderate and severe shock but was absent in 15 patients with mild or transient shock. No relationship exists between the presence of antibody and the etiology of shock.

Nucleotide specificity tests offered prognostic help in patients with severe and protracted shock. Patients with specificities directed to cytosine and guanine tended to have a favorable outcome. Two-thirds of the patients with thymine-specific antibodies died (in 11 of 13 deaths); one-third who had low titers survived.

The possibility that DNA antibodies can prevent the formation of messenger RNA or otherwise block the mechanism for protein synthesis suggests another approach to therapy.

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Discussion

Dr. William D. Holden (Cleveland): Doctors Nick and Dodd have entered an unusually difficult but very likely rewarding field of endeavor. The cellular biology of lethal shock is an almost totally untrammeled area and yet it is only here within the cell that the ultimate answers to the pathophysiology and distorted metabolism of lethal shock will be found. The observations made by the authors suggest that in shock nuclear DNA or its component nucleotides antigenically induce the formation of specific antibodies that are carried predominately in the 19S fraction of globulin and to a lesser extent in the 7S fraction. The ultimate course of the patients with antibodies related to guanine and cytosine was favorable whereas those with thymine specificities had less satisfactory courses. It is difficult to explain these observations and it is obvious that very complex biological phenomena are taking place. The technic of inhibition of agglutination is excellent and was used for the nucleotide bases. I could find, however, in the manuscript no mention of DNA itself being used in the patient's serum to attempt to qualify the extent of antibody formation.

There is so much of importance in the concept of a state of autoimmunity induced by DNA in shock that it would be highly desirable to test the hypothesis with more than one technic. The use of radioactive nucleotides and equilibrium dialysis with globulin would demonstrate the presence or absence of actual binding. The fractionation of the 7S globulin into F_{ab} and F_c fractions would permit identification of antigenic binding if true autoimmunity exists. In experimental animals, passive transfer studies could throw some light upon these phenomena. Since endotoxin has become such an important consideration not only in septic shock but possibly in hypovolemic shock, the use of E. coli DNA in place of calf thymus DNA to establish the DNA-benzidine-erythrocyte antigen might be a profitable pursuit.

It would appear important before the concept of DNA autoimmunity is prematurely seized upon and especially before nucleotide therapy is proposed for patients in shock that every technic be employed to establish unequivocally the specific nature of this process and that we are not observing some other non-specific phenomenon that is influenced by the presence of DNA, its nucleotides, or endotoxin.

The work is exciting to say the least, and we should all be pleased to see one of the most complex biological problems surgeons must deal with being attacked in a sophisticated investigative fashion by a young surgeon. Thank you. (Applause)

Dr. Fraser N. Gurd (Montreal): Mr. President, I was grateful for the opportunity to see the manuscript in advance, and would certainly like to reiterate Dr. Holden's compliments to the authors.

The approach which Dr. Nick has taken represents a most interesting attempt to explain the wide dissemination of organ damage which we are learning to recognize as a syndrome which may follow severe shock.

Our own approach has led us to favor the view that the fundamental metabolic handicap in the intestinal mucosa, to which we have assigned a certain primacy, is in the area of energy metabolism. The production of ATP and oxidative phosphorylation represent the initial areas of depression so far as we have been able to show.

Nevertheless, the creation of a deficiency of essential specific factors by immunological interference could indeed halt a necessary step in biosynthesis. Dr. Nick's concept is particularly exciting, because deficiencies can be treated when the time and place for the specific replacement therapy can be recognized.

What detrimental emanations could flow forth from tissues in the process of necrobiotic disin-