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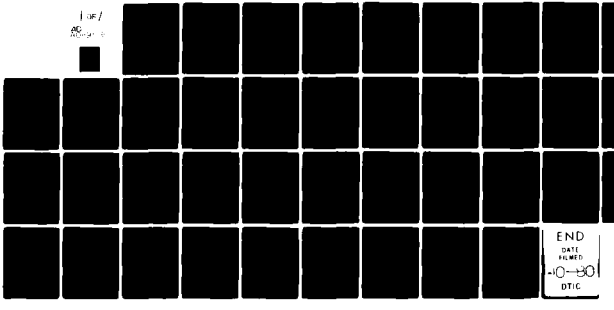

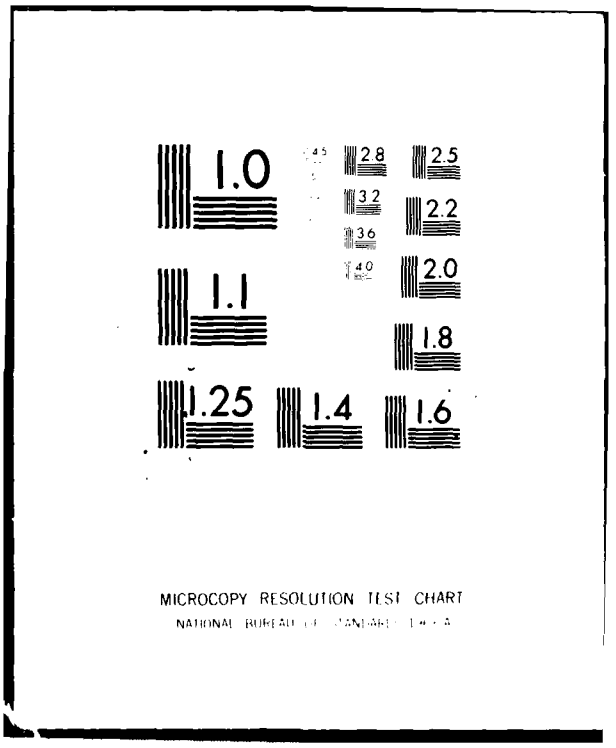
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REPORT #4 A060569

Host Defense Against Opportunist Microorganisms Following Trauma

(11)

ANNUAL SUMMARY REPORT

Ann B. Bjornson, Ph.D.
William A. Altemeier, M.D.
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JUNE, 1979

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Studies were performed to evaluate selected humoral components of host defense in 4 septic and 6 non-septic burned patients during 60 days postburn. The parameters measured in all patients were total hemolytic complement (CH_{50}), C3 conversion by inulin and cobra venom factor (CoVF), and immunochemical concentrations of C1q, C4, C2, C3, C5, factor B, properdin, and C3b inactivator (C3bINA). CH_{50} and C1q were decreased in the sera of the septic burned patients during the first 10 days postburn, but were restored to normal thereafter. — (cont'd.)

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CH_{50} and Clq were normal or elevated in the sera of the non-septic burned patients for the duration of the study. Concentrations of C4, C2, C3, and C5 in the sera of all of the patients were normal or elevated for the entire study period. C3 conversion by inulin and CoVF and the concentration of properdin were reduced in the sera of the septic and non-septic burned patients for the duration of the study, and concentrations of factor B and C3bINA were normal or elevated. When the non-septic burned patients were subdivided by the presence or absence of transient bacteremia, no significant difference in concentrations or functional activity of components of the classical or alternative complement pathways was demonstrated. The failure to demonstrate a decrease in multiple classical pathway components in the septic burned patients during the initial postburn period and classical pathway consumption during septic episodes was related to the administration of fresh frozen whole plasma.

Heat-labile and heat-stable serum opsonic activity, and agglutinin titers directed against the specific infecting bacterial strains were also measured in the 4 septic patients and in an additional group of 11 septic burned patients. Only two of the 15 patients had decreased heat-labile serum opsonic activity for their infecting bacterial strains, which occurred only during the initial postburn period. Heat-stable opsonins and agglutinin titers in the patients' sera were equivalent to those in normal human sera, except for the agglutinin titers to Streptococcus faecalis which were increased in the patients' sera in comparison to the normal sera. These results indicate that the multiple complement abnormalities which occur in septic burned patients do not predispose these patients to infection by decreasing serum opsonic activity. The results also indicate that heat-stable immune IgG antibodies are not produced during septicemia which facilitate opsonization of the infecting bacterial strains in the absence of an intact complement system.

Indirect evidence was provided to suggest that reduction in C3 conversion via the alternative complement pathway in burned patients is caused by an inhibitory serum factor with a molecular weight of less than 3,500 daltons. The inhibitory factor was shown to be distinct from ions required for formation of alternative pathway C3 convertases.

Inhibition of phagocytosis and intracellular killing of Staphylococcus aureus by normal human polymorphonuclear leukocytes was demonstrated in the presence of a physiologic concentration of burn serum. The inhibition of polymorphonuclear leukocyte bactericidal activity was not related to deficient opsonization of the bacteria by the burn serum. The factor in the burn serum which was responsible for the effect was shown to be distinct from the factor which inhibited C3 conversion via the alternative complement pathway. These preliminary results suggest that increased susceptibility to microbial infections in burned patients may be related to an inhibitory effect or one or more serum factors on polymorphonuclear leukocyte function.

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REPORT #4

Host Defense Against Opportunist Microorganisms Following Trauma

ANNUAL SUMMARY REPORT

Ann B. Bjornson, Ph.D.
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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

ACKNOWLEDGEMENT

The investigators express their gratitude to Dr. Clark D. West, Children's Hospital Research Foundation, Cincinnati, Ohio for the gift of reference sera and antisera to various complement proteins. We also wish to thank Ms. Mary Cannon for the excellent technical preparation of this report.

ABSTRACT

Studies were performed to evaluate selected humoral components of host defense in 4 septic and 6 non-septic burned patients during 60 days postburn. The parameters measured in all patients were total hemolytic complement (CH_{50}), C3 conversion by inulin and cobra venom factor (CoVF), and immunochemical concentrations of C1q, C4, C2, C3, C5, factor B, properdin, and C3b inactivator (C3bINA). CH_{50} and C1q were decreased in the sera of the septic burned patients during the first 10 days postburn, but were restored to normal thereafter. CH_{50} and C1q were normal or elevated in the sera of the non-septic burned patients for the duration of the study. Concentrations of C4, C2, C3, and C5 in the sera of all of the patients were normal or elevated for the entire study period. C3 conversion by inulin and CoVF and the concentration of properdin were reduced in the sera of the septic and non-septic burned patients for the duration of the study, and concentrations of factor B and C3bINA were normal or elevated. When the non-septic burned patients were subdivided by the presence or absence of transient bacteremia, no significant difference in concentrations or functional activity of components of the classical or alternative complement pathways was demonstrated. The failure to demonstrate a decrease in multiple classical pathway components in the septic burned patients during the initial postburn period and classical pathway consumption during septic episodes was related to the administration of fresh frozen whole plasma.

Heat-labile and heat-stable serum opsonic activity, and agglutinin titers directed against the specific infecting bacterial strains were also measured in the 4 septic patients and in an additional group of 11 septic burned patients. Only two of the 15 patients had decreased heat-labile serum opsonic activity for their infecting bacterial strains, which occurred only during the initial postburn period. Heat-stable opsonins and agglutinin titers in the patients' sera were equivalent to those in normal human sera, except for the agglutinin titers to Streptococcus faecalis which were increased in the patients' sera in comparison to the normal sera. These results indicate that the multiple complement abnormalities which occur in septic burned patients do not predispose these patients to infection by decreasing serum opsonic activity. The results also indicate that heat-stable immune IgG antibodies are not produced during septicemia which facilitate opsonization of the infecting bacterial strains in the absence of an intact complement system.

Indirect evidence was provided to suggest that reduction in C3 conversion via the alternative complement pathway in burned patients is caused by an inhibitory serum factor with a molecular weight of less than 3,500 daltons. The inhibitory factor was shown to be distinct from ions required for formation of alternative pathway C3 convertases.

Inhibition of phagocytosis and intracellular killing of Staphylococcus aureus by normal human polymorphonuclear leukocytes was demonstrated in the presence of a physiologic concentration of burn serum. The inhibition of polymorphonuclear leukocyte bactericidal activity was not related to deficient opsonization of the bacteria by the burn serum. The factor in the burn serum which was responsible for the effect was shown to be distinct from the factor which inhibited C3 conversion via the alternative complement pathway. These preliminary results suggest that increased susceptibility to microbial infections in burned patients may be related to an inhibitory effect of one or more serum factors on polymorphonuclear leukocyte function.

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I. INTRODUCTION

A primary cause of morbidity and mortality in military personnel who have sustained burns, gunshot and high explosive wounds, or crush injuries is microbial infection (1,2). The widespread prophylactic use of antibiotics has not only failed to decrease the incidence of infection, particularly in the burned patient, but has also contributed to the complexity of the problem through the development of infections caused by antibiotic resistant microorganisms. In our medical center, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans are the microorganisms which are most frequently associated with septic complications in thermally injured patients.

Management of surgical infections has classically involved antibiotic therapy and the meticulous care of the surgical wound. However, in addition to these therapeutic modalities, attention has been recently focused upon defining abnormalities of host defense mechanisms which may predispose the injured patient to microbial infection. Studies of immunological abnormalities in patients with severe thermal injury have shown that polymorphonuclear leukocyte bactericidal activity (3-5), leukocyte migration (6), phagocytic function of the reticuloendothelial system (7), serum opsonins (4,8-12), levels of immunoglobulins (10,13-18), the primary immune response (19-21), and classical (10,11,22,23) and alternative (9-11,23) complement pathway activity are reduced following burn trauma. In addition, sera from burned patients has been shown to contain an immunosuppressive peptide which depresses the mitogenic response of normal human peripheral leukocytes to phytohemagglutinin (24).

The investigation described in this report was undertaken to further evaluate the complement system, serum opsonins, and the immune response to bacterial infection in burned patients. An additional objective of the study was to determine the mechanism of the reduction in C3 conversion via the alternative complement pathway in burned patients.

II. BACKGROUND

A. Studies to determine the association between changes in humoral components of host defense and septicemia in burned patients

In our initial studies, reduction in the immunochemical concentrations and functional activities of components of the classical and alternative complement pathways was associated with septicemia in two burned patients (10). Studies on a group of five additional septic burned patients showed that reduction in the classical complement pathway occurred prior to and during septic episodes in three of the patients (11). In the other two patients, decreased classical pathway activity was demonstrated prior to the development of septicemia, but not consistently during septic episodes. Reduction in the functional activity of the alternative complement pathway was not found to be associated with septicemia in any of the five burned patients in this study group. Reduction in classical and alternative complement pathway activity did not reduce the ability of the patients' sera to promote phagocytosis and intracellular killing of the

specific bacteria causing infection by normal human peripheral polymorphonuclear leukocytes. In only one patient, consumption of components of the classical pathway occurring during septicemia decreased the opsonic capacity of the patient's sera for her infecting strain of E. coli; sera from the same patient which could not opsonize E. coli, opsonized her infecting strain of S. aureus normally.

In our subsequent investigation of seven septic burned patients and four non-septic burned patients, all of the septic patients had decreased classical pathway activity during their septic episodes (23). All of the patients who did not survive septicemia and who died of septic shock had consumption of the classical complement components (C1 to C5) during their septic episodes. Patients who survived septicemia had multiple patterns of classical complement pathway consumption during their septic episodes as follows: (a) consumption of C1 to C5, (b) consumption of only C1 and C4, (c) consumption of only C1, C4, and C2, and (d) consumption of C1, C4, C2, and C5, but not C3. In these patients, classical pathway activity was restored to normal within 20 days following the last positive blood culture. This observation is preliminary, however, since only two of the four surviving patients were studied long enough following septicemia to make this evaluation.

The alternative complement pathway was consumed in only one of the seven septic patients. C3b inactivator and factor B were markedly decreased in the sera of this patient for the entire study period. The results suggested that alternative pathway consumption occurring during septicemia in this patient resulted from the generation of C3b via consumption of the classical complement pathway. C3b together with factors B and $\overline{D}$, and properdin would lead to further depletion of C3 and C5 via the enzymes, C3bBb and $\overline{C3bBbP}$. This observation provides an explanation for the results of our earlier study, showing that both complement pathways were consumed in one septic burned patient, and only the classical pathway was consumed in the other septic patient (10).

The classical pathway appeared to be activated preferentially in all of the other burned patients during septic episodes. Although C3 and/or C5 conversion via the alternative complement pathway were often reduced during septic episodes, factor B was not decreased concurrently. Factor B was decreased in the sera of only three of the septic patients, and the reduction occurred only during the first 5 days post admission. Since it is well known that factor B is consumed during alternative pathway activation, the results suggested that the functional activity of the alternative pathway was decreased in the burned patients due to blockage of this pathway rather than to consumption of alternative pathway components.

Reduction in classical pathway activity was also demonstrated in all of the septic burned patients during the initial postburn period and during the time prior to when the first positive blood culture was obtained. Total hemolytic complement was always markedly reduced below the normal mean value for the non-septic patients, and early classical complement components were reduced as well. The reduction in classical pathway activity was not found to be the result of systemic infection, since blood cultures were consistently negative in the patients during this time. Reduction in

classical pathway activity was not demonstrated in the non-septic burned patients, suggesting that this humoral abnormality is predictive of septic episodes.

The lack of demonstration of classical complement pathway consumption during the study period in the non-septic burned patients could not be explained on the basis of the administration of blood products, since these patients as a group received less whole blood and single donor plasma than the septic burned patients. The administration of blood products also did not influence the outcome of septicemia, since the patient with the most prolonged septicemia received the largest amount of blood products per unit of time. In addition, the two other patients who died of septic shock received amounts of whole blood and single donor plasma per unit of time roughly equal to the non-septic patient who received the most blood products.

More abnormalities of C3 and C5 conversion via the alternative complement pathway were observed in the septic burned patients than in the non-septic burned patients. However, there was quite a discrepancy in the average burn ratio (% total injury/% third degree injury) between the two groups (55/43 for the septic patients and 49/19 for the non-septic patients). The increased number of abnormalities in the septic population is probably explained by this finding, since reduction in C3 conversion has been previously shown to be a function of increasing burn size (10,11). Values for concentrations and conversion of C3 and C5 were consistently higher when measured by hemolytic assays, in comparison to the results obtained from the immunochemical determinations. In addition, a marked variability in the results of the hemolytic assays was demonstrated. However, the most important observation which was derived from the C3 and C5 conversion data was that there appeared to be no correlation between these abnormalities and the occurrence, duration, or outcome of septicemia.

Consumption of components of the alternative and/or classical complement pathway did not decrease the ability of the patients' sera to promote phagocytosis and intracellular killing of their infecting bacterial strains by normal human polymorphonuclear leukocytes. Two possible explanations for this observation are as follows: (a) immune IgG antibodies may be produced during septicemia which effectively opsonize the infecting bacterial strains in the absence of complement; or (b) naturally occurring antibacterial antibodies may be present in the burn sera prior to the infection which together with minimal levels of alternative and/or classical complement pathway components effectively opsonize the bacterial strains. Data have been presented which show that gram-negative aerobic bacilli isolated from burned patients require immunoglobulin and utilize the alternative and/or classical complement pathways in normal sera during the opsonic process (25). This observation suggests that antibodies must be present in the burn sera prior to infection or be produced during colonization with the bacteria, if effective opsonization in the presence of minimal levels of complement components is to occur. In any event, the observation that the multiple complement abnormalities in the septic burned patients did not reduce the opsonic capacity of their sera for the bacteria causing infection suggests that current concepts regarding the role of immunoglobulin and

complement in opsonization of opportunist microorganisms require reevaluation.

B. Studies to determine the mechanism of the reduction in C3 conversion via the alternative complement pathway in burned patients

Our previous studies showed that conversion of C3 by inulin in sera from severely burned patients was reduced during the early acute burn phase and was normalized by the seventh week (9-11). The occurrence and duration of the reduction in C3 conversion were found to be directly related to the severity of the burn injury. Mixture of equal parts of burn sera with reduced C3 conversion and pooled normal human serum did not restore C3 conversion to normal. These preliminary results suggested that reduction in C3 conversion in the burn sera might be caused by a circulating inhibitor.

The hypothesis that reduction in C3 conversion in the burn sera was caused by an inhibitor was tested experimentally (Annual Summary Report, June 1977). Burn sera with normal or reduced C3 conversion were tested for their ability to inhibit C3 conversion by inulin in pooled normal human serum. None of the burn sera with normal C3 conversion inhibited C3 conversion to any extent when added in increasing concentrations to the normal serum. Several of the burn sera with reduced C3 conversion inhibited C3 conversion when added to the normal serum, whereas other burn sera with reduced C3 conversion had no inhibitory activity. When the data were subjected to statistical analysis, the differences which were observed between the burn sera with reduced C3 conversion and those with normal C3 conversion were not found to be significant. In addition, the values obtained when equal parts of burn sera and pooled normal serum were added together were not considerably lower than those obtained when a complement deficient normal serum was added to pooled normal serum.

The inability to demonstrate statistically significant differences between the inhibitory activities of burn sera with reduced versus normal C3 conversion might have been related to the concentration of the inhibitor in sera with reduced C3 conversion. If the inhibitor was present in minimal amounts in the sera, then fractionation of the sera and concentration of the fractions should presumably result in demonstration of inhibitory activity by one of the fractions. To test this hypothesis, pseudoglobulin and euglobulin fractions of burn sera with reduced C3 conversion were added in increasing concentrations to pooled normal human serum, and C3 conversion by inulin was measured and compared to C3 conversion in normal serum supplemented with pseudoglobulin or euglobulin fractionated from normal serum. Euglobulin fractions prepared from both burn sera and normal serum inhibited C3 conversion by inulin in normal serum, and the euglobulin fractionated from the burn sera appeared to be more inhibitory than the euglobulin fractionated from the normal serum. Since recognized regulatory proteins of the alternative complement pathway, C3b inactivator and β1H, are known to be euglobulins (26), the results described above provided preliminary support for the concept that reduction in C3 conversion in the burn sera might be caused by elevation of one or more of these proteins.

No correlation between reduction in C3 conversion by inulin and elevation of βlH and/or C3b inactivator in multiple burn sera was demonstrated (Annual Summary Report, June 1978). Euglobulin fractions of burn sera were found to inhibit C3 conversion when added in increasing concentrations to pooled normal serum; however, the inhibitory activity of the euglobulin fractions was found to be unrelated to the original C3 converting activity of the burn sera. These observations ruled out the possibility that reduction in C3 conversion was caused by elevation of regulatory proteins or that burn injury altered a normal euglobulin protein to a configuration with regulatory activity, thereby inhibiting C3 conversion.

In all of the studies described above, C3 conversion in the burn sera was measured by a single assay system and activating substance, inulin. The assay for measuring C3 conversion which utilizes reduction in the B antigenic determinant of C3 was shown to correlate well with a standard hemolytic assay for measuring C3 conversion which utilizes $EAC\overline{142}$ cells and purified classical complement components. Utilizing the B antigen reduction assay, results obtained when inulin was used as the activating substance correlated well with results obtained when cobra venom factor was used as the activating substance. In addition, the C3 conversion assays utilizing inulin or cobra venom factor as the activating substances were shown to measure alternative pathway activity. These observations strengthened the concept that an abnormality of the alternative complement pathway is caused by burn injury which blocks the pathway prior to the C3 conversion step.

III. EXPERIMENTAL APPROACH

Studies in the two areas of burn research which were presented in the preceding section of this report were to be continued during the project period. The first area was to include a continuation of studies to determine the integrity of the alternative and classical complement pathways in septic and non-septic burned patients, and the effects of septicemia on complement activity and opsonic function. In addition, comprehensive studies were to be initiated to determine if specific antibacterial antibodies were present in the sera of septic burned patients prior to infection or were produced during septicemia which could opsonize the infecting bacterial strains in the absence of complement. The second research area was to be focused on determining the mechanism of the reduction in C3 conversion via the alternative pathway in burned patients.

Ten to twenty burned patients, preferably of military age, were to be selected for the study. Sera were to be collected from the patients one time per week for 6 to 8 weeks postburn. The alternative complement pathway was to be assessed by measuring C3 conversion by inulin and cobra venom factor, and immunochemical levels of factor B, C3b inactivator, and properdin. The classical complement pathway was to be assessed by measuring total hemolytic complement and immunochemical levels of C1q, C4, C2, C3, and C5. Septicemia was to be documented in the burned patients by positive blood cultures and clinical findings. If a patient developed septicemia, blood was to be drawn one or two additional times per week until the

infection cleared. The hypothesis that multiple complement abnormalities in septic burned patients do not reduce the opsonic capacity of their sera for the bacteria causing infection was to be tested experimentally. The ability of the patients' sera to promote phagocytosis and intracellular killing of their infecting bacterial strains by normal human peripheral polymorphonuclear leukocytes was to be tested prior to and during septicemia, and, if the patients survived, after recovery. If more than one bacterial strain was isolated from a septic patient, each strain was to be tested separately as described above. To determine if specific antibacterial antibodies were present in the sera of the septic burned patients prior to infection or were produced during infection which opsonized the infecting bacterial strains in the absence of complement, heat-stable opsonins and specific agglutinating antibody titers directed against the infecting strains in the patients' sera were to be measured. The antibody measurements were to be performed on the seven septic patients which were identified and studied during the previous contract period (October 1, 1977 to September 30, 1978) and on all septic patients which were identified during the current project period. Pertinent clinical information was to be recorded on each burned patient by Ms. Geri Perkins, research nurse, on an appropriate flow sheet. The information was to include the antibiotic regimen, culture data, clinical signs of septicemia, administration of blood products, and surgical procedures. The clinical data were to be interpreted by Dr. S. Bjornson and Dr. Altemeier.

Experiments were to be performed to determine the mechanism of the reduction in C3 conversion via the alternative pathway in burned patients. Conversion of C3 was to be measured by reduction in the B antigenic determinant of C3 by radial immunodiffusion. Pooled normal human serum was to be fractionated by ion exchange chromatography and preparative isoelectric focusing, and fractions were to be added in increasing concentrations to burn sera in an attempt to restore C3 conversion by inulin to normal. Our initial experimental approach was to fractionate pooled normal human serum by column chromatography using TEAE-cellulose. Those fractions which restored C3 conversion in the burn sera were to be further purified by preparative isoelectric focusing. Purity of the protein or proteins which restored C3 conversion in the burn sera was to be determined by alkaline or acid polyacrylamide discontinuous electrophoresis and by immunoelectrophoretic analysis. Molecular weight determinations were to be performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

IV. PROGRESS REPORT

A. Studies to evaluate complement, opsonins, and the immune response to infection in burned patients

1. Results

In our previous studies, consumption of components of the classical complement pathway was demonstrated prior to and during septicemia in ten of 12 burned patients (11,23). In the other two patients, decreased classical pathway activity was demonstrated prior to the development of septicemia, but not consistently during septic episodes. Functional activity of the alternative complement pathway was reduced in all of the septic burned patients, and components of this pathway were consumed in one of the patients during septicemia. Despite these multiple complement abnormalities, no reduction in serum opsonic activity for the bacteria causing infection was demonstrated.

The primary objectives of the present investigation were as follows: (a) to further evaluate the integrity of the classical and alternative complement pathways in septic and non-septic burned patients, (b) to provide additional experimental evidence to confirm the observation that complement abnormalities in septic burned patients do not reduce the opsonic capacity of their sera for the bacteria causing infection, (c) to further determine the effects of the administration of blood products on the changes in humoral factors in septic and non-septic burned patients, and (d) to determine if antibacterial antibodies are present in the sera of septic burned patients prior to septicemia or are produced during septicemia which facilitate opsonization of the infecting bacterial strains in the absence of an intact complement system.

Ten patients with severe thermal injury were followed during 60 days postburn. Serum samples were drawn on all patients as soon after admission as possible and then at weekly intervals. In those patients who developed septicemia as documented by positive blood cultures and clinical findings, serum samples were obtained one additional time per week until blood cultures became negative. The clinical criteria used for the diagnosis of septicemia were (a) chills and fever, (b) tachycardia, (c) hypotension, and (d) disorientation.

Six of the ten burned patients had positive blood cultures during their clinical course. Clinical signs of septicemia were documented in four of these patients, when blood cultures were positive. The other two patients with positive blood cultures were considered to have transient bacteremia. Four of the ten patients had negative blood cultures. The burned patients were divided into three groups with the the four septic patients in group A, the two bacteremic patients in group B, and the four non-septic patients in group C. Clinical characteristics of the patients in each group are presented in Table 1. The temporal sequence of positive blood cultures in the septic and bacteremic patients is shown in Table 2.

Table 1. Clinical Characteristics of the Burned Patients

Group	Patient No.	Age	Sex[a]	Body Surface Injured (%)[b]	
				Total	Third Degree
A	1	4	M	38	36
	2	14	M	58	47
	3	11	M	85	75
	4	18	M	70	45
B	5	3	F	40	33
	6	11	F	40	35
C	7	9	M	70	0
	8	12	M	34	14
	9	14	M	32	24
	10	19	M	51	41

[a]M = male; F = female.

[b]All patients had flame burn injuries.

Table 2. Temporal Sequence of Positive Blood Cultures in the Burned Patients

Patient No.	Infecting Microorganisms	Positive Blood Cultures[a]
1	S. aureus	10
	P. aeruginosa	42,43
2	S. aureus	45,46,52
	C. albicans	45,46,52
3	S. aureus	6,32,34
	E. cloaceae	6,8,13
	P. aeruginosa	20,21,23,41
	C. albicans	30,31,34
4	S. aureus	18,19,21,34,48
	S. faecalis	21
	E. cloaceae	21
5	S. aureus	19
6	S. aureus	40

[a]Numbers indicate the number of days following the injury that positive blood cultures were obtained.

Every effort was made to include burned patients of military age in our study population. However, only two patients of military age (#4 and #10) were identified during the contract period who qualified for the study. Six other patients of military age were excluded from our study; two patients had liver disease secondary to excessive alcohol consumption, and the other four patients had burns involving less than 25% total body surface.

Functional activity of the classical complement pathway in the patients' sera was assessed by measuring total hemolytic complement (CH_{50}), a collective measurement of C1 to C9. Functional activity of the alternative complement pathway was assessed by measuring C3 conversion by inulin and cobra venom factor (CoVF), two recognized activating substances of the alternative complement pathway. Immunochemical serum concentrations of individual classical complement pathway components (C1q, C4, C2, C3, and C5) and alternative complement pathway components (factor B and properdin) were quantitated by radial immunodiffusion. The immunochemical serum concentration of the regulatory protease of the classical and alternative complement pathways, C3b inactivator (C3bINA), was also measured. Immunochemical concentrations and functional activity of the complement components in the patients' sera were compared to those in the sera of 20 age-matched normal controls. Statistical analysis on the data was performed by Student's t test.

CH_{50} and C1q were significantly decreased in the sera of the septic burned patients during the first ten days postburn in comparison to the controls ($p = < 0.05$) (Figure 1). After this time, CH_{50} and C1q in the sera of the septic patients were within the normal range. CH_{50} and C1q were normal or elevated in the sera of the non-septic and bacteremic burned patients for the duration of the study. Concentrations of C4, C2, C3, and C5 in the sera of all of the patients were normal or elevated for the entire study period.

Conversion of C3 by inulin and CoVF in the sera of the septic and non-septic burned patients was significantly decreased for the duration of the study in comparison to the controls ($p = <0.05$) (Figure 2). In the bacteremic patients, C3 conversion by CoVF was significantly decreased during this time ($p = <0.05$); however, C3 conversion by inulin was within the normal range. The concentration of properdin in all three groups of patients was significantly decreased during the entire study period ($p = <0.05$), and concentrations of factor B and C3bINA were normal or elevated.

The types and amounts of blood products administered to the burned patients are shown in Table 3. All of the patients received fresh frozen plasma and whole blood. As a group, the septic patients received more plasma and whole blood than the bacteremic and non-septic patients. All of the septic and bacteremic patients and one of the non-septic patients received packed cells. A retrospective analysis of the administration of blood products to burned patients at our medical center showed that the patients in this investigation, in contrast to all of the other burned patients studied during previous contract periods, received fresh frozen whole plasma rather than single donor plasma.

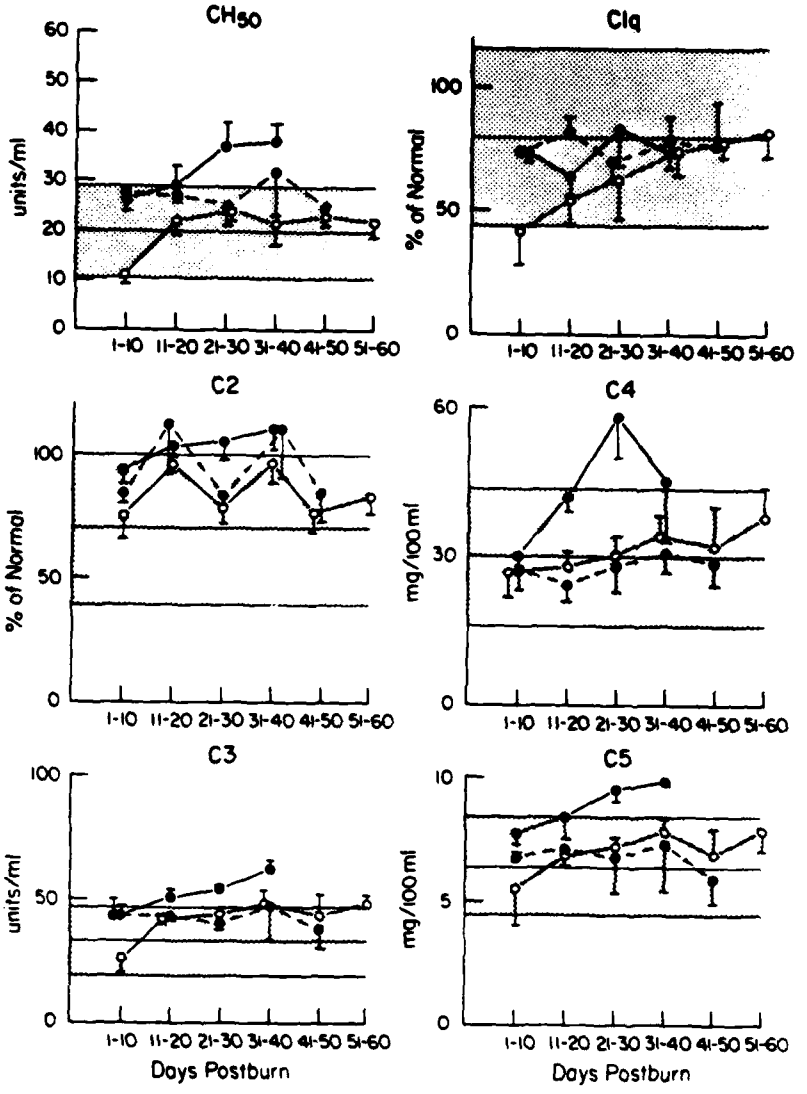

Figure 1. Immunochemical concentrations and functional activity of components of the classical complement pathway in the sera of septic (o——o), bacteremic (•----•), and non-septic (•——•) burned patients during 60 days postburn. The points represent mean values, and each vertical bar represents the standard error of the mean. The shaded areas represent the range of values in the sera of 20 age-matched normal controls (mean ± 2 S.D.).

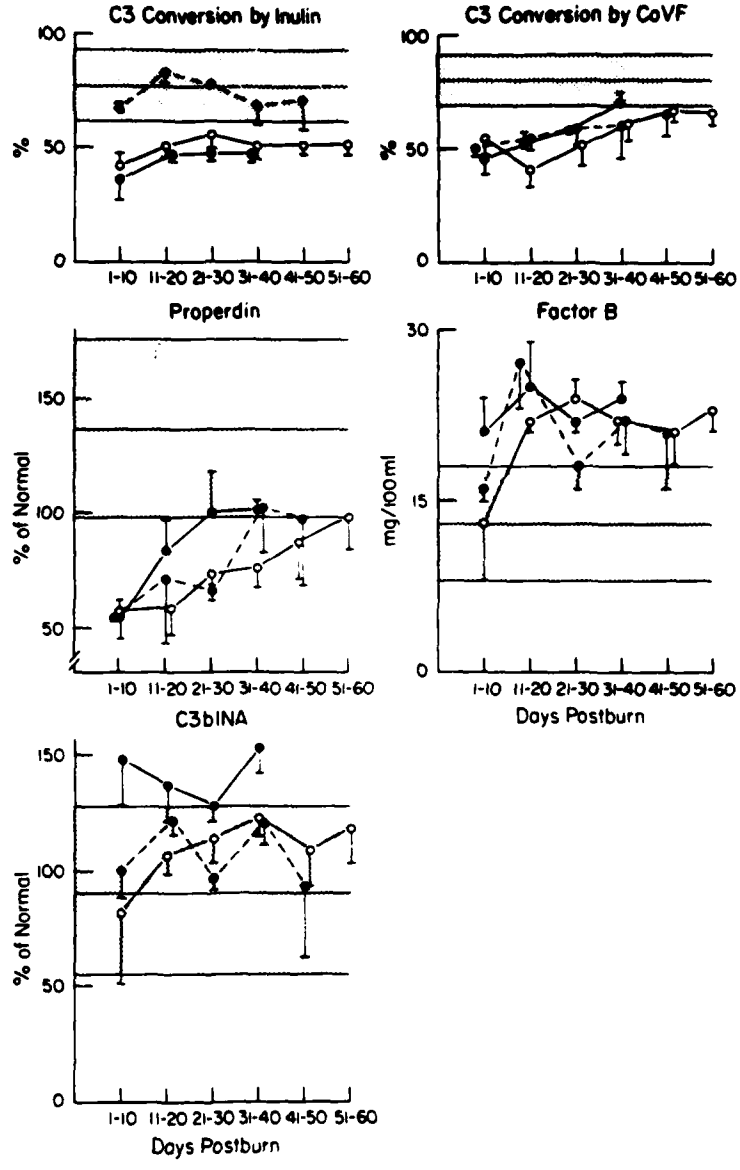

Figure 2. Immunochemical concentrations and functional activity of components of the alternative complement pathway in the sera of septic (o——o), bacteremic (•---•), and non-septic (•——•) burned patients during 60 days postburn. The points represent mean values, and each vertical bar represents the standard error of the mean. The shaded areas represent the range of values in the sera of 20 age-matched normal controls (mean ± 2 S.D.).

Table 3. Administration of Blood Products to the Burned Patients

Patient Group	Patient No.	Milliliters/24 Hours		
		Fresh Frozen Plasma	Whole Blood	Packed Cells
A	1	229	47	9
	2	247	171	15
	3	321	130	19
	4	37	241	33
B	5	143	15	41
	6	64	100	28
C	7	159	43	0
	8	13	45	0
	9	61	45	13
	10	15	80	0

Our next experiments were designed to determine if the initial decrease in classical complement pathway activity and the persistent functional abnormality of the alternative complement pathway decreased the opsonic activity of the septic patients' sera for the bacteria causing infection. An additional objective of this aspect of the investigation was to determine if heat-stable opsonins or agglutinating antibodies directed against the infecting strains were present in the patients' sera prior to septicemia or were produced during septicemia.

Preliminary experiments were performed to determine the minimal concentration of pooled normal human serum which promoted maximal phagocytosis and intracellular killing of each infecting bacterial strain by normal human polymorphonuclear leukocytes (PMNs). The bacterial strains were inoculated into brain heart infusion broth and incubated at 37°C for 18 hours. Aliquots of the cultures were frozen at -70°C. Prior to each experiment, a frozen culture was inoculated into brain heart infusion broth and incubated at 37°C for 18 hours. The bacteria were washed three times and resuspended to a final concentration of 1.0×10^7 cells/ml in Hank's Balanced Salt Solution containing 0.1% gelatin (HBG). This procedure was adopted to avoid multiple subculturing of the bacterial strains which might decrease virulence. The reaction mixtures consisted of 5.0×10^6 normal human PMNs, 1.0×10^6 bacteria, increasing concentrations of normal serum, and HBG in a final volume of 1 ml. HBG was substituted for the leukocytes or serum in the controls. The reaction mixtures and controls were rotated at 37°C, and aliquots were removed at 0 time and after 30, 60, and 120 minutes of incubation for enumeration of total surviving bacteria. Serum concentrations used in the assays ranged from 1% to 10%.

Once the minimal concentration of normal serum which promoted maximal phagocytosis and killing of each bacterial strain was determined, experiments to compare the opsonic activity of the patients' sera and the normal serum were performed. To measure heat-stable opsonic activity, patients' sera and normal serum were heated at 56°C for 30 minutes prior to testing. The reaction mixtures consisted of heated or unheated patients' sera or normal serum, leukocytes, and bacteria. HBG was substituted for the leukocytes or serum in the controls. The reaction mixtures and controls were rotated at 37°C, and total surviving bacteria were enumerated at 0 time and after 30, 60, and 120 minutes of incubation. The percent of heat-labile or heat-stable opsonins was calculated by the formula $\frac{a-b}{a} \times 100$, where a was the number of bacteria surviving at 0 time and b was the number of bacteria surviving after 30, 60, or 120 minutes of incubation. Maximal phagocytosis and killing of all of the strains was demonstrated after 60 minutes of incubation and, for this reason, the data which will be presented were calculated from the 60 minute results. Statistical analysis was performed by the Mann-Whitney test.

Four different methods were evaluated for preparation of the bacterial suspensions to be used for the measurement of agglutinating antibodies. The methods were as follows: (a) autoclave the bacteria at 121°C for 15 minutes at 20 pounds of pressure, wash and resuspend in physiologic saline, (b) incubate the bacteria in 0.5% phenol in physiologic saline for 12 hours at 20°C, wash with physiologic saline, resuspend in 86% ethanol and incubate at 37°C overnight, wash with physiologic saline, and resuspend in 20% glycerine containing 12% NaCl, (c) incubate the bacteria in 1.2% formalin at room temperature for 18 hours, wash with physiologic saline, and resuspend in 0.05 M phosphate buffered saline, pH 7.0, containing 0.1% formalin, and (d) incubate the bacteria at room temperature for 1 hour in physiologic saline containing 0.5% phenol, wash with physiologic saline, and resuspend in physiologic saline containing 0.5% phenol. For all four methods, the final concentration of bacteria in the suspensions was 3.0×10^8 cells/ml.

Each infecting bacterial strain was prepared by the four methods, and agglutinin titers were measured in 5 normal human sera utilizing tube agglutination and microtiter tests. The highest titers of agglutinins were demonstrated when all of the bacteria other than Streptococcus faecalis were prepared by autoclaving. The highest titers of agglutinins to S. faecalis were demonstrated when the bacteria were killed by overnight incubation in 1.2% formalin. Microtiter tests were found to be most suitable for the S. faecalis and Staphylococcus aureus strains, and tube agglutination tests were used for the other strains. Agglutinin titers in the patients' sera and normal sera were performed in triplicate. The data were expressed as geometric mean antibody titers, and statistical analysis was performed by Student's t test.

Heat-labile and heat-stable opsonic activity for the infecting bacterial strains and agglutinin titers directed against the same strains in the sera of the four septic burned patients are presented in Figure 3. Because of the similarity in the results, the data from the patients with the same species of infecting strain were grouped. Heat-labile serum opsonic activity was significantly decreased during the first ten days postburn in the two patients with septicemia caused by Pseudomonas aeruginosa ($p = <0.05$). After this time, heat-labile serum opsonins for the infecting P. aeruginosa strains were normal. Patients with septicemia caused by Enterobacter cloaceae, S. aureus, and S. faecalis had normal heat-labile serum opsonic activity for these strains during the entire study period. The only significant increase in heat-stable opsonins was demonstrated during the 41st to 50th post burn day period in the sera of patients with S. aureus septicemia ($p = <0.05$). Heat-stable opsonic activity for the other infecting bacterial strains in the patients' sera was equivalent to heat-stable opsonic activity for the strains in normal serum. In all patients, heat-stable opsonic activity was significantly decreased

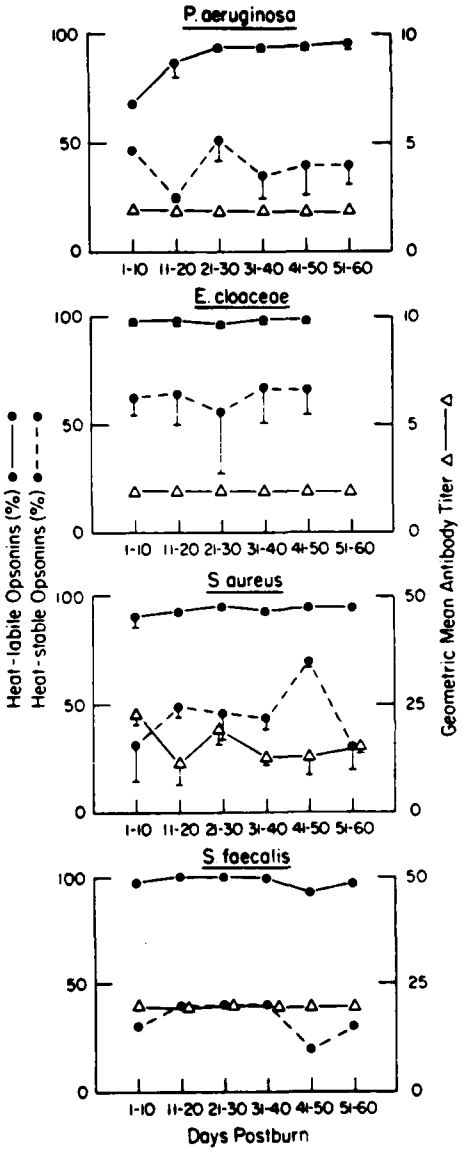

Figure 3. Heat-labile and heat-stable serum opsonins for the infecting bacterial strains, and agglutinin titers directed against the same strains in the sera of the four septic burned patients. The data from the patients with the same species of infecting strain were grouped as follows: (a) S. aureus (Patients 1-4), (b) E. cloaceae (Patients 3 and 4), and (c) P. aeruginosa (Patients 1 and 3). The data for S. faecalis from Patient 4 has been presented separately at the bottom of the figure. The points represent mean values, and each vertical bar represents the standard error of the mean.

in comparison to heat-labile opsonic activity for the duration of the study ($p = <0.05$). The agglutinin titers to S. faecalis in the sera of Patient 4 were significantly higher than the agglutinin titers in normal sera ($p = <0.05$). Agglutinin titers in the other patients' sera to their infecting strains were equivalent to the titers in normal sera. No rise in agglutinin titers in any of the patients' sera was observed.

We next attempted to confirm the results described above in an additional group of 11 septic burned patients. All of the patients had been studied during previous contract periods and had been shown to have decreased classical pathway activity prior to and during septic episodes and persistently decreased alternative pathway activity (10, 11, 23). Heat-labile and heat-stable opsonic activity for the infecting bacterial strains and agglutinin titers directed against the same strains were measured in the patients' sera as described above. Again because of the similarity in the results, the data from the patients with the same species of infecting strain were grouped.

During the entire study period, heat-labile and heat-stable opsonic activity in the patients' sera for the infecting strains of P. aeruginosa, Escherichia coli, S. aureus, and S. faecalis was equivalent to that in normal serum (Figure 4). Heat-stable serum opsonic activity for the infecting strains was significantly decreased in comparison to heat-labile serum opsonic activity ($p = <0.05$). Agglutinin titers directed against the S. faecalis strains in the patients' sera were significantly increased in comparison to the agglutinin titers for the strains in normal sera ($p = <0.05$). Agglutinin titers to all of the other infecting strains in the patients' sera were equivalent to the titers in normal sera, and no rise in agglutinin titers was demonstrated.

2. Discussion

The results of this investigation confirm our previous studies which showed that the functional activity of the alternative complement pathway and the concentration of properdin were persistently decreased during 8 weeks postburn in septic and non-septic burned patients (10, 11, 23). In addition, classical pathway activity was shown to be decreased during the initial postburn period only in patients who subsequently developed septicemia. However, in contrast to our previous results, the initial decrease in classical pathway activity in the septic burned patients was associated with a reduction only in the first component of complement, and classical pathway consumption was not demonstrated during septic episodes.

The failure to demonstrate a decrease in multiple classical pathway components during the initial postburn period and classical pathway consumption during septic episodes appears to be related to

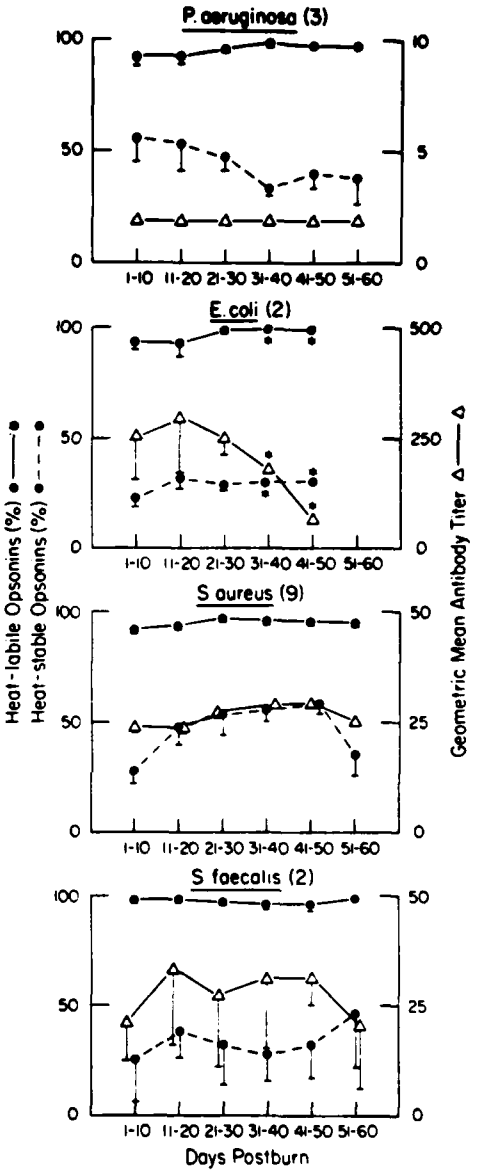

Figure 4. Heat-labile and heat-stable serum opsonins for the infecting bacterial strains, and agglutinin titers directed against the same strains in the sera of 11 septic burned patients. The data from the patients with the same species of infecting strain were grouped, and the number of patients in each group is shown in the parenthesis following the species designation. The points represent mean values, and each vertical bar represents the standard error of the mean. The asterisks indicate that the values were obtained from determinations on the sera from only one of the two study patients during the 31st to 50th postburn days.

the administration of blood products. Patients in this investigation received fresh frozen whole plasma rather than single donor plasma, which patients in all of our previous investigations had received. Fresh frozen whole plasma contains an intact complement system, and the infusion of this plasma into our patients probably replaced complement components which were consumed during septic episodes and during the initial postburn period. It is interesting that the functional abnormality of the alternative complement pathway and the reduction in properdin concentration persisted in our patients despite the administration of fresh frozen whole plasma. This observation provides support for the concept that the functional abnormality of the alternative complement pathway is not caused by a deficiency of plasma proteins, and that loss of properdin through the burn wound or increased catabolism of this protein perhaps occurs at a more rapid rate than previously realized.

Only two of 15 septic patients had decreased heat-labile serum opsonic activity for their infecting bacterial strains, which occurred only during the initial postburn period. The reduction in serum opsonic activity in these patients did not appear to be caused by decreased classical pathway activity, since other patients with decreased classical pathway activity had normal serum opsonic activity. These results confirm our previous observation that the multiple complement abnormalities which occur in septic burned patients prior to and during septic episodes do not decrease heat-labile serum opsonic activity for the bacteria causing infection (11,23). It should be emphasized, however, that the complement abnormalities may predispose burned patients to microbial infections by other as yet undefined mechanisms.

No rise in agglutinin titers directed against the infecting bacterial strains was demonstrated in the sera of the septic burned patients. A significant increase in heat-stable opsonic activity for S. aureus was observed in one group of four septic burned patients but not in another group of nine patients. Heat-stable opsonins and agglutinin titers in patients' sera were equivalent to those in normal sera, except for the agglutinin titers to S. faecalis which were increased in the patients' sera in comparison to the normal sera. These observations indicate that heat-stable immune IgG antibodies were not produced during septicemia which facilitated opsonization of the infecting bacterial strains in the absence of an intact complement system.

The observation that the titers of heat-stable opsonins and agglutinating antibodies directed against the infecting bacterial strains did not increase during septicemia in burned patients suggests that these patients may have a deficient humoral immune response to bacterial infection. In previous studies, the primary immune response to human erythrocytes in burned animals was shown to be depressed, if the erythrocytes were administered after but not prior to the burn injury (19). The in vitro primary immune response of mouse spleen cells to sheep erythrocytes has also been shown to be reduced following burn injury (20). Other investigators have failed to demonstrate a decrease

in the primary immune response to sheep erythrocytes (27,28), sheep erythrocytes coupled with TNP (28), or DNP-Ficoll (28) following burn injury. Kohn reported that the production of precipitating antibodies to Streptococcus pyogenes was decreased in experimentally infected burned animals (21). Others have demonstrated rising titers of staphylococcal antibodies in surviving burned patients (29) and humoral immune responsiveness to active immunization with bacterial vaccines (30,31).

Needless to say, this controversial area of burn research requires reevaluation. In particular, experiments to determine the temporal sequence of the humoral immune response in burned animals to experimental bacterial infection appear warranted. Studies of the humoral immune response in humans are complicated by the various therapeutic procedures, such as the administration of blood products, which preclude accurate interpretation of the results.

B. Studies to determine the mechanism of the reduction in C3 conversion via the alternative complement pathway in burned patients

1. Results

Our previous studies showed that conversion of C3 by inulin in sera from severely burned patients was reduced during the early acute burn phase and was generally normalized by the seventh postburn week (9-11, 23). The occurrence and duration of the reduction in C3 conversion were found to be directly related to the severity of the burn injury (10). Mixture of equal parts of burn sera with reduced C3 conversion and pooled normal human serum did not restore C3 conversion to normal (9,10). However, the C3 conversion values were not shown to be lower than the values obtained when equal parts of complement deficient control sera and pooled normal human serum were added together. These preliminary results suggested that reduction in C3 conversion in the burn sera might be caused by a deficiency of serum proteins, by an inhibitor of C3 conversion which had been diluted by mixture with normal serum, or by elevation of normal regulatory proteins of the complement system.

Subsequent studies failed to demonstrate a correlation between reduction in C3 conversion by inulin or CoVF and elevation of the regulatory proteins, β1H and C3bINA, in multiple burn sera (Annual Summary Report, June, 1978). This observation ruled out the possibility that reduction in C3 conversion was caused by elevation of these regulatory proteins. Data was presented to support the concept that reduction in C3 conversion in the burn sera represented a functional abnormality of the alternative complement pathway.

The primary objective of the present investigation was to determine if reduction in C3 conversion in the burn sera was caused by a deficiency of serum proteins or by an inhibitor of C3 conversion. The experimental approach which had been proposed was to supplement burn sera with pooled normal human serum and determine restoration of C3 conversion. Then purified fractions of the normal serum were to be prepared, and C3 conversion in burn sera supplemented with the fractions was to be measured. If reduction in C3 conversion in the burn sera was caused by a deficiency of a normal serum protein, then restoration of C3 conversion should be accomplished by supplementation of the sera with one of the purified fractions.

Our initial experiments were performed utilizing pooled serum samples from four burned patients, who had been identified during previous contract periods. The individual sera which were pooled on each patient had been dialyzed for 18 hours at 4°C against 0.01 M phosphate buffered saline, pH 7.4, containing 1.5×10^{-4} M $CaCl_2$ and 5.0×10^{-4} M $MgCl_2$. This procedure had been performed routinely to remove antibiotics and to standardize the concentration of calcium and magnesium ions in the sera.

C3 conversion by inulin was measured in each patient's pooled serum prior to and after supplementation with an equal part of pooled normal human serum. C3 conversion by inulin in three of the four pooled burn sera was markedly improved by supplementation with the normal serum (Table 4). However, mixture of the pooled serum from the fourth patient with the normal serum did not enhance C3 conversion.

This observation prompted us to consider the possibility that the pooled serum from Patient 4 had not been properly dialyzed prior to the reconstitution experiment. Patient 4's serum was therefore dialyzed a second time against fresh dialysis buffer, and C3 conversion by inulin was measured before and after the second dialysis. The C3 conversion values were found to be 0% and 51% respectively, indicating that a second dialysis of this patient's serum improved C3 conversion. This unexpected observation suggested that reduction in C3 conversion was not caused by a deficiency of serum proteins but by a factor which could be removed from the burn sera by extensive dialysis.

To test this hypothesis, pooled sera from the four burned patients were dialyzed for 18 hours at 4°C a second time against fresh dialysis buffer. To insure that the second dialysis per se was improving C3 conversion, the pooled sera were also incubated for 18 hours at 4°C without further dialysis. C3 conversion by inulin was measured in the pooled burn sera after the first dialysis with and without further incubation at 4°C and after the second dialysis.

A second dialysis of the pooled burn sera improved C3 conversion by inulin (Table 5). C3 conversion in the pooled sera which had been dialyzed once and then incubated for 18 hours at 4°C was equivalent to C3 conversion in the same sera which had not been incubated at 4°C. These results indicated that the factor causing reduction in C3 conversion in the burn sera could not be removed by merely incubating the sera at 4°C after the first dialysis. Two successive dialyses appeared to be required to reduce the concentration of the inhibitory factor in the burn sera.

Our next experiments were designed to determine the relative size of the factor causing reduction in C3 conversion in the burn sera. Sera from four burned patients, who were identified during the present contract period, were used for these experiments. Two of the burned patients (Patients #5 and #6) were included in section IV-A of this report (refer to Table 1 for the clinical characteristics of these patients). The third patient (#11) was an 11 year-old female with a 60% total body surface burn with 50% third degree injury, and the fourth patient (#12) was a 6 year-old male with a 75% total body surface burn with 70% third degree injury. The serum samples from each patient were frozen at -70°C without dialysis within two hours after collection.

Multiple serum samples were pooled on each patient, and each pooled serum was dialyzed for 18 hours at 4°C against 0.01 M phosphate buffered

Table 4. C3 Conversion by Inulin in Pooled Dialyzed Burn Sera Before and After Supplementation with Pooled Normal Human Serum

Patient No.	C3 Conversion by Inulin (%)[a]	
	Serum Alone	Serum + Normal Serum[b]
1	28	56
2	14	53
3	35	63
4	0	8
PNHS[c]	68	68

[a]The numbers represent mean values of two to five determinations.

[b]Burn serum was mixed with an equal part of pooled normal human serum prior to testing.

[c]PNHS = pooled normal human serum.

Table 5. C3 Conversion by Inulin in Pooled Burn Sera After Two Successive Dialyses

Patient No.	C3 Conversion (%)[a]		
	First Dialysis		Second Dialysis
	No Incubation	Incubation[b]	
1	14	12	47
2	3	0	46
3	45	45	79
4	0	0	51
PNHS[c]	70	72	78

[a]The numbers represent mean values of duplicate determinations.

[b]After the first dialysis, the burn sera were incubated for 18 hours at 4°C without further dialysis.

[c]PNHS = pooled normal human serum.

saline, pH 7.4, containing 1.5×10^{-4} M $CaCl_2$ and 5.0×10^{-4} M $MgCl_2$, using dialysis tubing with different pore sizes. The dialysis tubing had molecular weight cutoffs at 3,500 and 12,000 respectively. C3 conversion by inulin and CoVF in the pooled sera was measured before and after a single dialysis.

C3 conversion by inulin and CoVF in the burn sera was greatly enhanced by dialysis of the sera using either dialysis tubing (Table 6). In three of the four burn sera, C3 conversion was almost fully restored to normal by the dialysis procedure. C3 conversion in the serumof Patient 12 was the most markedly reduced prior to dialysis; although dialysis of this serumimproved C3 conversion, the values for C3 conversion after the dialysis procedure were still abnormally low. These results indicated that a single dialysis was sufficient to correct C3 conversion in the sera of certain burned patients but not in others. In addition, the results suggested that the molecular weight of the inhibitory factor in the burn sera which caused the reduction in C3 conversion was less than 3,500 daltons.

Since magnesium ions are known to be required for C3 conversion by inulin and CoVF, we next considered the possibility that an abnormal concentration of magnesium ions in the burn sera might have caused the reduction in C3 conversion. Dialysis of the sera would have standardized the concentration of magnesium ions, perhaps providing the ionic environment required for normal C3 conversion. To test this hypothesis, various chemical determinations were performed on the non-dialyzed sera from the four burned patients.

As shown in Table 7, magnesium, chloride, and potassium concentrations in the burn sera were within the normal range. Total protein concentrations were reduced in three of the four patients' sera, and sodium and calcium levels were decreased in all of the burn sera. These results indicated that reduction in C3 conversion in the burn sera was not caused by an abnormal concentration of magnesium ions.

2. Discussion

The results of this investigation support the concept that the functional abnormality of the alternative complement pathway in the sera of burned patients is caused by an inhibitor of C3 conversion. Evidence was provided to indicate that the inhibitor has a molecular weight of less than 3,500 daltons and is distinct from ions required for formation of alternative complement pathway C3 convertases.

The inhibitor of C3 conversion appears to be present in greater concentration in the sera of certain burned patients in comparison to others. The evidence for this conclusion comes from the observation that the inhibitor could be removed from the sera of certain burned patients by a single dialysis. In other patients, including those previously studied, a second dialysis of the sera was required to decrease the

Table 6. C3 Conversion by Inulin and Cobra Venom Factor in Pooled Burn Sera Prior to and After Dialysis

Patient No.	C3 Conversion (%)[a]					
	Before Dialysis		After Dialysis			
			3,500[b]		12,000[b]	
	Inulin	CoVF	Inulin	CoVF	Inulin	CoVF
5	34	28	65	64	67	63
6	26	31	84	69	86	71
11	31	39	61	69	58	70
12	8	16	33	57	46	47
PNHS[c]	72	92	76	86	79	85

[a]Numbers represent mean values of duplicate determinations.

[b]Molecular weight cutoffs of the dialysis tubing were 3,500 and 12,000.

[c]PNHS = pooled normal human serum.

Table 7. Chemical Determinations on the Burn Sera

Patient No.	Chloride mEq/l	Sodium mEq/l	Potassium mEq/l	Calcium mg/dl	Magnesium mg/dl	Total Protein g/100 ml
5	99	133	5.2	8.7	2.0	6.3
6	97	135	5.0	7.5	1.8	4.9
11	98	133	4.0	8.2	1.9	5.1
12	99	133	4.1	7.9	1.8	4.8
PNHS[a]	101	141	5.8	9.0	1.9	7.1
Normal Range	97-105	136-145	3.5-5.5	9.0-11.0	1.8-2.6	5.3-8.0

[a]PNHS = pooled normal human serum.

concentration of the inhibitor. These observations suggest that reduction in C3 conversion in certain burned patients may have been missed in our previous studies, in which only dialyzed sera were tested. This is perhaps most relevant to patients with less than 45% total body surface burn, who were previously shown to have no demonstrable reduction in C3 conversion (10). In addition, the use of dialyzed sera in our previous investigations precludes any conclusion regarding the temporal relationship between the reduction in C3 conversion and the onset of septicemia in burned patients.

Although the reduction in C3 conversion is not associated with a decrease in serum opsonic activity, it may predispose burned patients to microbial infection by another mechanism. Various investigators have demonstrated decreased PMN chemotaxis following burn injury (32-34). In one of these studies, no reduction in chemotaxis of normal PMNs was demonstrated in the presence of burn sera (33). However, Christou and Meakins recently reported that decreased PMN chemotaxis in surgical patients was associated with two serum inhibitors (35). These investigators postulated that the previous failure to demonstrate inhibitors of PMN chemotaxis in burn sera may have been related to the use of low serum concentrations in the assays. Perhaps our inhibitor of C3 conversion impedes the generation of chemotactic factors via the alternative complement pathway which are required for mobilization of leukocytes to sites of tissue injury in burned patients.

Increased susceptibility to microbial infections in burned patients has also been associated with a circulating immunosuppressive peptide (24). The peptide has been shown to suppress the mitogenic response of normal human peripheral leukocytes to phytohemagglutinin. The immunosuppressive peptide has also been demonstrated in the sera of patients following major operative trauma (36). The peptide has been recently isolated in purified form from the sera of these patients and has been shown to have a molecular weight of approximately 1,000 daltons (Dr. John A. Mannick, personal communication). Because of the similarity in the molecular weight of the immunosuppressive peptide and the inhibitor of C3 conversion, it is interesting to speculate that there may be a relationship between these two serum factors.

The inhibitory factor in the sera of burned patients was shown to affect C3 conversion by both inulin and CoVF. This observation suggests that the factor inhibits the formation of the alternative pathway convertase, C3bBb. Formation of this convertase requires magnesium ions, C3b or its analog (CoVF), and factors B and D (37,38). The burn serum factor may inhibit the biological activities of one of these essential complement components or the formation of the convertase. Further studies are required to chemically characterize the inhibitor and determine its mode of action.

C. Studies to determine the effects of burn sera on PMN bactericidal activity

1. Results

In our previous studies to determine the opsonic activity of septic burned patients' sera for their infecting bacterial strains, an in vitro bactericidal assay consisting of combinations of bacteria, serum, and normal human PMNs was utilized. Serum concentrations ranged from 1% to 10% and were based on the minimal concentration of pooled normal human serum which promoted optimal phagocytosis and killing of each bacterial strain. This experimental approach maximized the chance of demonstrating decreased opsonic activity in the burn sera; however, no reduction in opsonic activity was demonstrated. (Refer to Section IV-A of this report).

Before ruling out the possibility that burn sera had an inhibitory effect on PMN bactericidal activity, phagocytosis and intracellular killing of bacteria by normal human PMNs was measured in the presence of a physiologic concentration of burn serum or pooled normal human serum. The results were compared to those obtained from assays in which 10% of the sera was employed. Combinations of 5.0×10^6 normal human PMNs, 1.0×10^6 bacteria, 98% or 10% of the test serum, and HBG were added to plastic capped tubes in a final volume of 1 ml. HBG was substituted for the serum or leukocytes in the controls. The tubes were rotated at 37°C, and 100 µl aliquots were removed at 0 time and after 30, 60, and 120 minutes of incubation. The aliquots were diluted in distilled water to rupture the leukocytes, and total surviving bacteria were enumerated.

In these preliminary experiments, the pooled serum from Patient 5 and this patient's infecting S. aureus strain were used (Refer to Table 1 of Section IV-A for the clinical characteristics of this patient.) Prior to testing, the pooled burn serum and normal serum were dialyzed for 18 hours at 4°C against 0.01 M phosphate buffered saline, pH 7.4, containing 1.5×10^{-4} M $CaCl_2$ and 5.0×10^{-4} M $MgCl_2$. The dialysis procedure was performed to insure that antibiotics, which would result in killing of the bacteria in the absence of PMNs, were removed from the burn serum. The sera were dialyzed using 12,000 molecular weight retention tubing and were identical to the sera used in the C3 conversion experiments presented in Table 6 of Section IV-B of this report.

The dialyzed serum from Patient 5 promoted normal phagocytosis and killing of the S. aureus strain by the PMNs at a concentration of 10% (Figure 5). However, when the concentration of the serum in the bactericidal assays was increased to 98%, the patient's serum was unable to support phagocytosis and killing of the bacteria by the PMNs. Pooled normal human serum promoted over a one-log reduction in bacterial counts by the PMNs at concentrations of 10% and 98%. Killing of the bacteria was not demonstrated in the presence of patient's or normal serum in the absence of PMNs, or by PMNs in the absence of serum. These results indicated that the serum from

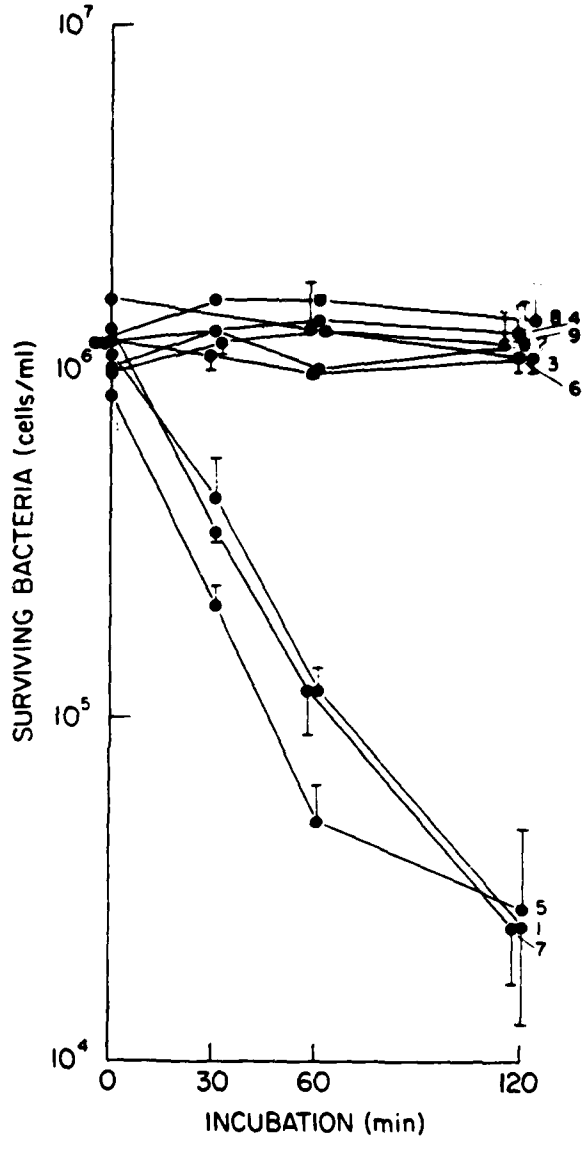

Figure 5. Phagocytosis and intracellular killing of S. aureus by normal human PMNs in the presence of 10% or 98% of pooled burn serum or normal serum. The reagents in addition to the bacteria and diluent which were added to the reaction mixtures were as follows: (1) patient's serum (10%), and leukocytes; (2) patient's serum (10%); (3) patient's serum (98%), and leukocytes; (4) patient's serum (98%); (5) pooled normal human serum (PNHS)(10%), and leukocytes; (6) PNHS (10%); (7) PNHS (98%), and leukocytes; (8) PNHS (98%); and (9) leukocytes. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

Patient 5 had an inhibitory effect on PMN bactericidal activity when tested at a physiologic concentration.

To determine if the inhibition of PMN bactericidal activity was related to deficient opsonization of the bacteria by the burn serum, the bacteria were preopsonized with burn serum or normal serum and tested for their ability to be phagocytosed and killed intracellularly by normal human PMNs. Washed bacteria (1.0×10^7) were incubated with 98% of the test serum or HBG at 37°C for 30 minutes. The bacteria were then washed once and resuspended in HBG. Preopsonized bacteria (1.0×10^6), 5.0×10^6 normal human PMNs, and HBG were added to plastic capped tubes in a final volume of 1 ml. The tubes were rotated at 37°C, and total surviving bacteria were enumerated at 0 time and after 60 and 120 minutes of incubation.

Bacteria preopsonized with Patient 5's pooled serum were phagocytosed and killed by the PMNs as efficiently as bacteria preopsonized with pooled normal human serum (Figure 6). Phagocytosis and killing of bacteria preopsonized with HBG without serum was not demonstrated. These results indicated that the inhibition of PMN bactericidal activity was not related to deficient opsonization of the bacteria by the burn serum.

2. Discussion

Decreased phagocytosis and/or intracellular killing of various bacterial strains by PMNs has been demonstrated following burn injury (4,5,39-41). In all of these studies, the bactericidal assays were performed utilizing the patients' PMNs and pooled normal human serum as the source of opsonins. It should be emphasized that the results of our investigation are unique in that they demonstrate an inhibitory effect of burn serum on the bactericidal activity of normal PMNs. The pooled serum from a bacteremic burned patient was shown to be unable to support phagocytosis and intracellular killing of this patient's infecting S. aureus strain by normal human PMNs when tested at a physiologic concentration.

The failure of the burn serum to support phagocytosis and intracellular killing of the S. aureus strain in the presence of a physiologic concentration of the burn serum was not caused by deficient opsonization of the bacteria by the burn serum. No reduction in phagocytosis and intracellular killing of the S. aureus strain by the PMNs was observed in the presence of 10% of the burn serum. In addition, bacteria preopsonized with the burn serum were phagocytosed and killed intracellularly by the PMNs as efficiently as bacteria preopsonized with pooled normal human serum. These observations suggest that the inhibition of PMN bactericidal activity was related to an effect of the burn serum on leukocyte function.

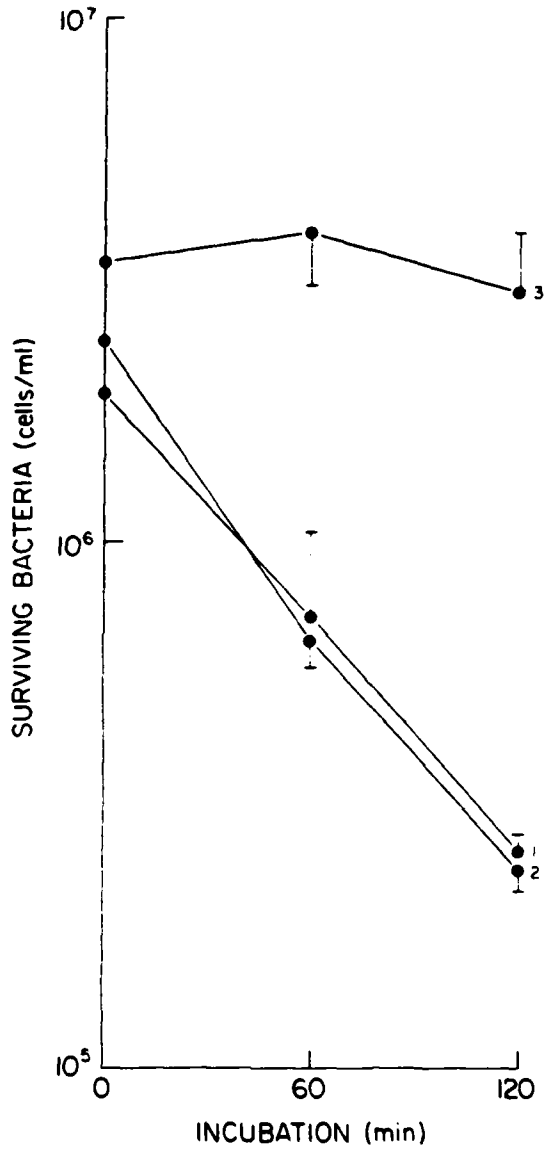

Figure 6. Phagocytosis and intracellular killing of S. aureus preopsonized with pooled burn serum or normal serum by normal human PMNs. All reaction mixtures consisted of preopsonized bacteria, leukocytes, and diluent. The reagents used to preopsonize the bacteria were as follows: (1) pooled normal human serum, (2) pooled burn serum, and (3) HBG without serum. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

The factor in the burn serum which was responsible for the inhibition of PMN bactericidal activity was shown to be distinct from the inhibitor of C3 conversion via the alternative complement pathway. Inhibition of PMN bactericidal activity was demonstrated in the presence of a physiologic concentration of dialyzed burn serum. The burn serum was dialyzed using 12,000 molecular weight retention tubing, suggesting that the serum factor which inhibited PMN bactericidal activity has a molecular weight of greater than 12,000 daltons. In contrast, the inhibitor of C3 conversion is dialyzable and has a molecular weight of less than 3,500 daltons.

The inhibition of PMN bactericidal activity may be related to an effect of one or more burn serum factors on phagocytosis or intracellular killing or both processes. Previous studies by Glaser et al. demonstrated that the alpha-globulin fraction of normal human plasma increased the susceptibility of mice to experimental infection with Streptococcus pyogenes, decreased the bactericidal activity of normal human whole blood against this bacterial strain, and inhibited the ingestion of Staphylococcus albus by mouse peritoneal leukocytes (42,43). When phagocytosis of the S. albus strain by the leukocytes was permitted to occur prior to the addition of alpha-globulin, intracellular killing of the bacteria was not inhibited. This observation suggested that the site of action of alpha-globulin was on the plasma membrane of the leukocyte. Serum concentrations of alpha-globulin have been shown to be elevated following burn injury (18). It is interesting to speculate that the inhibition of PMN bactericidal activity demonstrated in our studies is related to the concentration of alpha-globulin in the burn serum.

A cytotoxic effect of the burn serum on the leukocytes is another mechanism to explain the inhibition of PMN bactericidal activity. Previous studies have shown that a toxin prepared from burned mouse or human skin decreased the response of mice to experimental infection with P. aeruginosa by damaging the membranes of parenchymal cells (44). Perhaps this toxin also damages the membranes of PMNs.

Subsequent studies in my laboratory have confirmed the observations presented in this preliminary report. The pooled dialyzed sera from two additional burned patients have been shown to inhibit the bactericidal activity of normal human PMNs when tested at a physiologic concentration. Further studies are required to determine the occurrence and duration of the inhibitory effect of burn sera on PMN bactericidal activity and the relationship between this abnormality and the onset of septicemia.

V. CONCLUSIONS

A. Classical complement pathway activity was decreased in septic burned patients prior to but not during septic episodes. The initial decrease in classical pathway activity was associated with a reduction in the concentration of the first component of complement. Concentrations and activity of components of the classical complement pathway were normal or elevated in bacteremic and non-septic burned patients.

B. Reduction in the functional activity of the alternative complement pathway and decrease in the concentration of properdin were demonstrated in septic, non-septic, and bacteremic burned patients, indicating that these abnormalities were not caused by septicemia. In addition, a cause and effect relationship between the abnormalities was not demonstrated.

C. The failure to document consumption of complement during septic episodes in the burned patients in this investigation was related to the administration of fresh frozen whole plasma.

D. Reduction in heat-labile serum opsonic activity for the specific infecting bacterial strains was demonstrated in only 2 of 15 septic burned patients; the reduction in opsonic activity was only demonstrated during the initial postburn period. Heat-stable immune IgG antibodies were not produced during septicemia in the burned patients which facilitated opsonization of the infecting strains in the absence of an intact complement system. Heat-stable opsonins and agglutinin titers directed against the strains in the patients' sera were equivalent to those in normal human sera, except for the agglutinin titers to S. faecalis which were increased in the patients' sera in comparison to the normal sera.

E. The reduction in C3 conversion via the alternative complement pathway in the sera of burned patients was shown to be caused by an inhibitory serum factor with a molecular weight of less than 3,500 daltons. The inhibitory factor was found to be distinct from ions required for formation of alternative pathway C3 convertases.

F. Inhibition of phagocytosis and intracellular killing of S. aureus by normal human PMNs was demonstrated in the presence of a physiologic concentration of burn serum. The inhibition of PMN bactericidal activity was not related to deficient opsonization of the bacteria by the serum. The factor in the burn serum which was responsible for the effect was shown to be distinct from the factor which inhibited C3 conversion via the alternative complement pathway.

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