STAPHYLOCOCCAL ANTIBODIES IN BURNED PATIENTS

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RAISED titres of agglutinins to *Pseudomonas pyocyanea* have been found in the serum of patients whose burns were colonised by that organism ; the rise of titre was related to the area of the burn and the duration of infection (Fox and Lowbury, 1953). A renewed interest in antibacterial immunity of burned patients has been prompted recently by the recognised limitations of antibiotic prophylaxis and therapy, and also by claims that convalescent serum from burned patients may have a beneficial effect when infused into patients with severe burns (Feodorov and Skurkovitch, 1962 ; Rosenthal, Hartney and Spurrier, 1962 ; Dobrkovsky, Dolesalova and Pavkova, 1962). Animal experiments have suggested that gamma globulin or specific antisera to *Ps. pyocyanea* may possibly succeed where chemotherapy has failed either in prophylaxis or in therapy of burned patients (Rosenthal, Millican and Rust, 1957 ; Millican and Rust, 1960 ; Millican, Rosenthal, Rust and Janesky, 1963).

In the studies reported here we have assessed the level of two antibodies to *Staphylococcus aureus*, the anti-α-haemolysin and the antibody to an erythrocyte-coating polysaccharide originally described by Rountree and Barbour (1952), who considered that it might provide a better index of resistance to staphylococcal infection than anti-α-haemolysin. To throw light on some unexpected features in the pattern of antibody response, we have made limited studies of other antibodies in the serum of burned patients.

MATERIAL AND METHODS

Antibody to erythrocyte-coating polysaccharide (ECP)

Estimations of this antibody were made by a method based on that of Oeding (1957) ; sheep erythrocytes were sensitised with a polysaccharide extract from a strain of *Staph. aureus* and the sensitised cells were used for the detection of antibody in the serum of burned patients and of normal subjects.

Preparation of erythrocyte-coating polysaccharide.—Strains of *Staph. aureus* of phage types 29, 55, 6/47/75/75B, 3C, 83, 79, 71 and a non-typable strain were used. Cultures incubated overnight in nutrient broth (' Lemco ') containing 1 per cent sucrose were centrifuged at 3000 r.p.m. for 20 min. The supernatant was concentrated to one-tenth of its original volume by vacuum distillation and centrifuged. From this concentrate a polysaccharide extract was obtained (see Oeding, 1957) by precipitation with three volumes of 96 per cent ethanol at 4°, extraction with 90 per cent phenol in water, and removal of traces of protein from the phenol-insoluble residue by shaking with chloroform-butanol mixture (10 : 1) ; the extract was dialysed, concentrated by vacuum distillation, freeze dried and stored over calcium chloride in a vacuum desiccator. Further details of this material are described elsewhere (Jones, 1962). The largest yield was obtained from the strain of type 29 (5530), and this material was used in studies of antibody in human serum.

Production of immune serum.—Antisera were obtained by immunising rabbits with the 8 strains of *Staph. aureus* used for preparing the polysaccharide extracts. Suspensions were

prepared by washing the growth from overnight nutrient agar cultures in saline and suspending in 0·1 per cent formol saline to give a turbidity of $5{\cdot}4 \times 10^9$ organisms per ml. (by Brown's opacity scale); intravenous injections of 0·1, 0·2, 0·4, 0·4, 0·6, 0·8, 0·8, 1·0 and 1·0 ml. were given on days 1, 2, 3, 9, 10, 11, 17, 18 and 19. Blood was taken before the first injection and 10 days after the last injection. Serum was separated and inactivated at 56° for 30 min.

Preparation of sensitised sheep erythrocytes.—Sheep erythrocytes (Wellcome) were washed three times in physiological saline. Doubling dilutions to 1 in 256 of a solution of the polysaccharide (1 mg. per ml.) were made in physiological saline. To 1·0 ml. of each dilution 0·1 ml. of washed, packed erythrocytes was added. The mixture was incubated in a water bath at 37° for 2 hr. with frequent shaking. The cells were then washed four times with large volumes of saline, and suspended in saline at a dilution of 0·2 per cent.

Rabbit antisera were diluted 1 in 10 and absorbed with normal packed, washed sheep erythrocytes for 30 min. at room temperature. The smallest sensitising concentration of polysaccharide was estimated by adding 0·2 ml. of absorbed rabbit antiserum to 0·2 ml. amounts of sheep cells sensitised with serial dilutions of polysaccharide; controls using sensitised erythrocytes without antiserum and without polysaccharide were included in the test. The mixtures were shaken and left at room temperature overnight; they were then examined for haemagglutination with a hand lens under a bright lamp before and after shaking. Haemagglutination was found with dilutions of polysaccharide up to 1 in 32 when homologous serum was tested, and with 1 in 8 and 1 in 16 dilutions of polysaccharide in tests with heterologous serum; no agglutination was found in tests with serum from un-immunised rabbits.

For titrations of antipolysaccharide by the haemagglutination method, 0·1 ml. of packed sheep erythrocytes was sensitised with 1·0 ml. of polysaccharide solution (1 mg. per ml.).

Titration of antibody to erythrocyte-coating polysaccharide (anti-ECP) in serum from burned patients and normal subjects.—Serum from blood taken by venepuncture was obtained from patients with burns and from 9 normal subjects at the times stated below.

The serum was heated at 56° for 30 min. to inactivate complement; of this serum 0·5 ml. amounts were absorbed for 30 min. with 0·04 ml. of packed sheep erythrocytes at room temperature to remove antibodies against sheep erythrocytes. To 0·2 ml. amounts of a series of doubling dilutions of the absorbed serum (from undiluted to 1 in 128) were added 0·2 ml. amounts of a 0·2 per cent suspension of sensitised sheep erythrocytes. The tubes were well shaken and left at room temperature for 18 hr. Two controls were included: (1) 0·2 ml. sensitised erythrocytes with 0·2 ml. physiological saline, (2) 0·2 ml. amounts of sensitised erythrocytes with a range of doubling dilutions of rabbit antistaphylococcal serum of known titre.

Tests were read with a hand lens; the reciprocal of the last serum dilution showing definite agglutination was recorded as the titre.

Anti-α-haemolysin titrations

All sera which were tested for anti-ECP were also tested for anti-α-haemolysin by the method described by Lack and Wailling (1954). To 0·5 ml. amounts of serial dilutions of the serum inactivated by heating at 56° for 30 min. were added 0·5 ml. amounts of Wellcome staphylococcal α-haemolysin (2 units per ml., adjusted when necessary after titration with Wellcome anti-α-haemolysin). The tubes were shaken and left for 30 min. at room temperature. Then 0·1 ml. of 10 per cent fresh, defibrinated, thrice washed rabbit erythrocytes were added. The tests were read after incubation for 1 hr. at 37°. Serum and α-haemolysin controls were included with each batch of sera tested. The titre of anti-α-haemolysin (units per ml.) was recorded as twice the reciprocal of the highest serum dilution in the presence of which there was no haemolysis.

Titration of agglutinins to Ps. pyocyanea *and of ABO blood group agglutinins*

Serum from 4 burned patients was obtained on a number of occasions from the day of admission or shortly afterwards and examined for agglutinins to *Ps. pyocyanea* and for blood group agglutinins (ABO), as well as for staphylococcal anti-α-haemolysin and anti-ECP. Parallel titrations of *Ps. pyocyanea* agglutinins and of blood group agglutinins were also made on serum from 8 normal subjects.

Agglutinins to *Ps. pyocyanea* were titrated by a method similar to that of Fox and Lowbury (1953), using a thrice washed suspension of *Ps. pyocyanea* (in patients, the strain isolated from

the burn) diluted to a turbidity corresponding with Brown's opacity tube number 1 (approximately $5{\cdot}48 \times 10^8$ bacterial cells per ml.). Doubling dilutions of serum were incubated in Dreyer's tubes with equal volumes of bacterial suspension in a water bath at 37° for 18 hr. The tubes and saline controls were then examined for agglutination with a hand lens.

Blood group agglutinins were titrated as follows. Doubling dilutions of serum in saline were mixed in $3 \times \frac{1}{2}$-in. tubes with equal volumes of 2 per cent saline suspension of thrice-washed fresh human blood of appropriate blood group (for group O serum, erythrocytes from donors of groups A and B, for group A serum, erythrocytes of a group B donor, and for group B serum erythrocytes of a group A donor). Tubes were incubated at 37° for 1 hr. in a water bath and examined with a hand lens for agglutination ; sedimented cells were also examined under the low power of the microscope.

Culture of bacteria from burns

Bacteriological swabs were taken from all burns on admission, at changes of dressings, at operations, and daily if they were treated by the exposure method. The swabs were examined by methods described elsewhere (Lowbury, 1960).

RESULTS

Anti-ECP and anti-α-haemolysin titres in burned and unburned subjects

Sera from 120 patients with burns and from 9 unburned subjects (laboratory staff) were examined for titres of anti-ECP and anti-α-haemolysin. Samples of venous blood from most of the patients were obtained as soon as possible after burning and at approximately weekly intervals ; in some patients the first sample was obtained 1 week or more after the injury. From each of the normal subjects 5 samples were obtained for antibody titration at intervals of time ranging from 4 days to 92 days.

TABLE I.—*Staphylococcal Antibody Titres in Serum of Nine Normal Subjects*

Subject	Test	Titre of antibody on day 1	5	10	17	109
E. L.	anti-α-haemolysin	<2	<2	<2	<2	<2
	anti-ECP	1	2	1	1	1
R. J.	anti-α-haemolysin	<2	<2	<2	<2	<2
	anti-ECP	1	1	1	1	1
T. K.	anti-α-haemolysin	<2	<2	<2	<2	<2
	anti-ECP	2	4	4	8	2
J. B.	anti-α-haemolysin	<2	<2	<2	<2	<2
	anti-ECP	32	32	32	32	32
A. J.	anti-α-haemolysin	2	2	2	—	2
	anti-ECP	1	1	1	—	8
G. B.	anti-α-haemolysin	<2	<2	—	<2	<2
	anti-ECP	8	32	—	32	32
P. D.	anti-α-haemolysin	2	2	2	2	—
	anti-ECP	2	2	2	2	—
K. P.	anti-α-haemolysin	<2	<2	<2	<2	<2
	anti-ECP	8	8	8	8	8
B. C.	anti-α-haemolysin	<2	<2	<2	<2	<2
	anti-ECP	64	128	64	64	128

Table I shows the antibody titres of sera from unburned controls. Anti-α-haemolysin was persistently less than 2 units per ml. in 7 subjects, and in the

other 2 it was persistently at 2 units per ml. The titre of anti-ECP, on the other hand, varied in different individuals between 1 unit and 128 units per ml., but successive samples usually showed similar titres, and there was never more than a 4-fold difference in titre of sera obtained from the same individual at different times. One of the subjects (T. K.) who showed a transient rise of titre was suffering from an upper respiratory infection between days 1 and 10, and the highest titres were found in one (B. C.) who suffered from a chronic sinus infection.

Anti-ECP and anti-α-haemolysin titres of sera from 6 burned patients and the presence or absence of *Staph. aureus* in their burns are summarised in Figs. 1 and 2. Titres of each antibody rose and fell independently of each other and sometimes precipitately. The association with staphylococcal infection was not obvious; indeed there was sometimes a fall in titre of one or the other antibody in the presence of continuing staphylococcal infection of the burns. High titres of both antibodies, however, were found in patients with staphhylococcal infection, and the only patient in our series (Fig. 1c) with the minimal detectable level of each antibody in each sample of serum was one from whose burns *Staph. aureus* was not isolated.

The development of an antibody response to staphylococcal infection is shown by the data in Tables II and III which summarise results from all the patients examined. In Table II the proportion of anti-α-haemolysin titres above 2 units per ml. and of anti-ECP titres above 4 units per ml. were shown to be significantly greater in sera from patients whose burns were colonised with *Staph. aureus* than in sera from patients with no staphylococci in their burns.

TABLE II.—*Staphylococcal Antibody Titres of Serum and presence of* Staph. aureus *in Burns*

	Sera with titres of anti-α-haemolysin (units per ml.)					Sera with titres of anti-ECP				
Patients with	0–2	2	4	>4	%>2	0–1	2–4	8–16	>16	%>4
No *Staph. aureus* in burns	60	9	3	0	4·1*.	34	12	10	4	23†
Staph. aureus isolated from burns	142	21	27	21	22·5*.	77	28	48	47	47†

* $\chi^2 = 11{\cdot}2$, $P < 0{\cdot}01$. † $\chi^2 = 10{\cdot}2$, $P < 0{\cdot}02$.

Table III shows that anti-α-haemolysin titres above 2 units per ml. increased during the course of treatment and were most frequent in sera from burns of 4 weeks' duration or more; but there were fluctuations in the frequency of raised titres in sera of the more extensively burned patients (with more than 10 per cent of body surface burned), and these showed consistently fewer titres above 2 units per ml. than the sera of less extensively burned patients. Anti-ECP titres showed a different relationship with duration of infection and area of burn. The lowest proportion of titres above 4 units per ml. was found in serum from the less extensively burned patients, but in these there was little evidence of an increase in the proportion of raised titres after the second week; in sera from the more extensively burned patients the highest proportion of titres above 4 units per ml. was found in the third week, but the lowest proportion of raised titres in these patients was found in the 4th week when raised titres of anti-α-haemolysin were also least frequent.

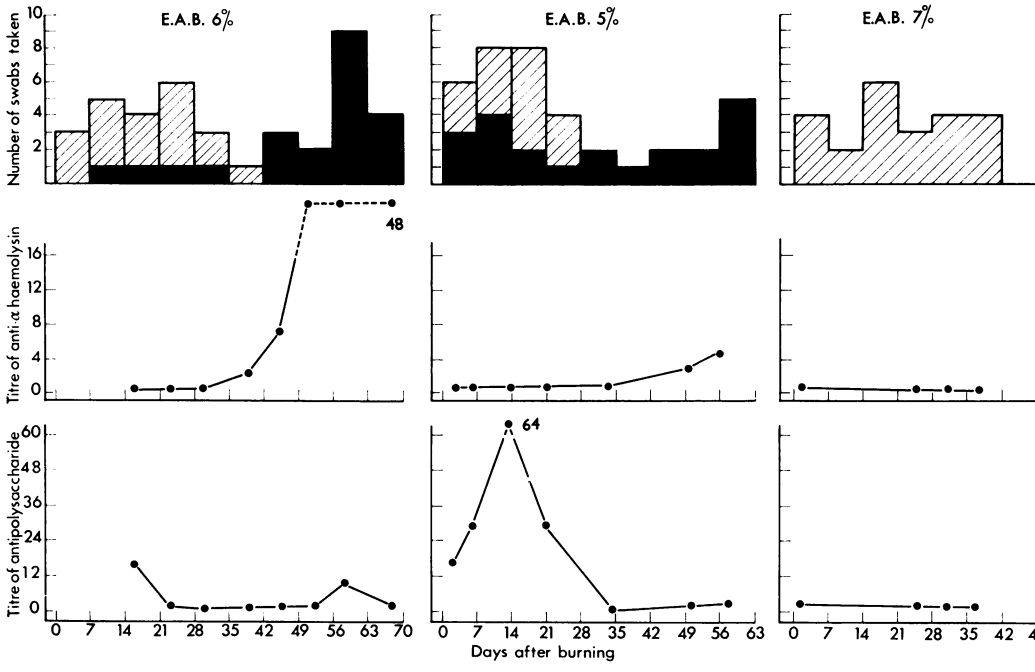

FIG. 1.

FIGS. 1 and 2.—Anti-α-haemolysin and anti-ECP titres of serum taken during the course of treatment in hospital of 6 patients with burns. The proportions of swabs from burns yielding *Staph. aureus* and the estimated area of burns (EAB) are shown at the top of each patient's record.

Staph. aureus absent.

Staph. aureus present.

TABLE III.—*Staphylococcal Antibody Titres, Area of Burns and Duration of Infection with* Staph. aureus

Duration of *Staph. aureus* in burns (weeks)	Anti-α-haemolysin (units per ml.)				Anti-ECP (units per ml.)			
	Burns 10% or less		Burns >10%		Burns 10% or less		Burns >10%	
	Titre >2	% >2	Titre >2	% >2	Titre >4	% >4	Titre >4	% >4
<1	3	15·0	1	6·2	3	15·0	11	44·0
1–2	2	16·7	4	14·2	5	35·7	12	44·0
2–3	4	19·0	2	11·1	6	31·6	22	68·7
3–4	3	21·4	1	9·0	3	21·4	3	30·0
4–6	6	40·0	2	13·3	5	29·4	6	46·1
>6	9	64·3	15	38·5	2	40·0	18	48·6

These data suggest a slow anti-α-haemolysin response and a much more rapid response of anti-ECP to staphylococcal infection; they also suggest that the build-up of anti-α-haemolysin occurs more readily in patients with small burns, while raised anti-ECP titres are on the whole commoner in patients with more extensive burns.

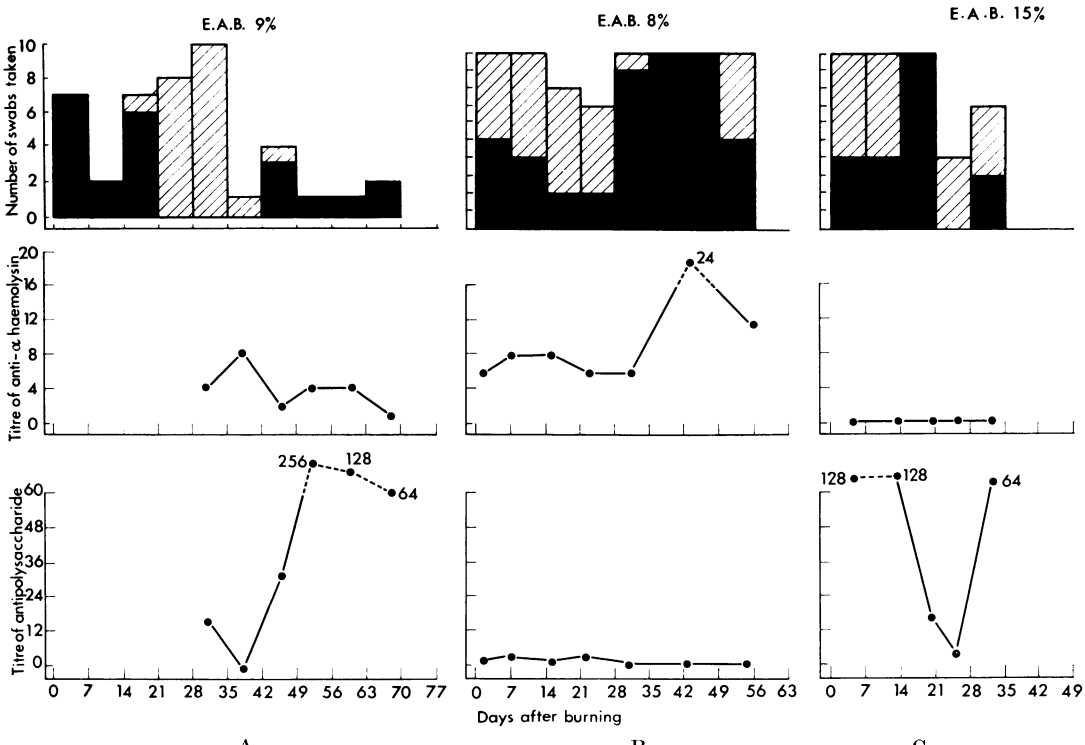

FIG. 2.

Antibody titres and mortality

A significantly higher proportion of serum samples with anti-α-haemolysin titres of less than 2 units per ml. and anti-ECP titres of less than 8 units per ml. was obtained from patients who died than from those who survived (Table IV).

One of the patients who died 9 days after injury had a septicaemia with *Staph. aureus* in blood cultures taken on the 7th day ; serum obtained on that

TABLE IV.—*Staphylococcal Antibody Titres and Mortality*

Subjects	Days since burning injury	Sera with anti-α-haemolysin titre (units)					Sera with anti-ECP titres (units)				
		<2	2	4	>4	%<2	<2	2–4	8–16	>16	%<8
Patients who died	1–7	14	0	0	0	100	4	6	3	1	71
	later	9	0	1	0	80	6	4	0	0	100
	Total	23	0	1	0	95·8*	10	10	3	1	83·4†
Patients who survived	—	120	21	25	21	64*	67	17	45	46	48†
Normals (nine subjects)	—	34	8	0	0	81	12	9	8	13	50

* $\chi^2 = 7{\cdot}98$, $P < 0{\cdot}02$. † $\chi^2 = 9{\cdot}03$, $P < 0{\cdot}02$.

day showed a titre of 16 units per ml. of anti-ECP and no detectable anti-α-haemolysin.

Changes in titre of other antibodies in burned patients

Because of the irregularity of anti-α-haemolysin and anti-ECP titres following burns, a series of tests was made on the level of other antibodies in serum specimens taken at intervals from burned patients. The antibodies chosen for these tests were agglutinins to *Ps. pyocyanea*, which had previously been shown to rise during infection of burns with *Ps. pyocyanea* (Fox and Lowbury, 1953), and ABO blood group agglutinins, which might be assumed to show little change in response to antigenic stimuli from infecting bacteria (though some increase in blood group agglutinins in response to bacterial antigens has been demonstrated; see Crawford, Cutbush, Falconer and Mollison, 1952). The titre of these antibodies in repeated samples of serum from 8 normal subjects was also estimated.

Table V shows the titre of agglutinins for *Ps. pyocyanea* and of ABO agglutinins in normal subjects. In 7 of these no change occurred in the titre of either antibody, but in 1 (J. B.) there was an increase in both antibodies.

TABLE V.—Pseudomonas pyocyanea *and Blood Group (ABO) Agglutinins in Serum of Normal Subjects*

Subject	Blood group (ABO)	Antigen used in test (cells)	Titre of antibody on day 1	11	36
E. L.	O	Group B blood	4	4	—
		Group A blood	64	64	—
		Ps. pyocyanea	80	80	—
R. J.	A	Group B blood	32	32	32
		Ps. pyocyanea	<40	<40	<40
A. K.	A	Group B blood	64	64	64
		Ps. pyocyanea	<40	<40	<40
C. C.	O	Group B blood	64	64	—
		Group A blood	128	128	—
		Ps. pyocyanea	<40	<40	—
J. B.*	O	Group B blood	16	32	32
		Group A blood	64	128	128
		Ps. pyocyanea	80	640	640
A. J.	O	Group B blood	64	64	64
		Group A blood	512	512	512
		Ps. pyocyanea	40	40	40
M. W.	O	Group B blood	8	8	8
		Group A blood	8	8	8
		Ps. pyocyanea	40	40	40
G. B.	O	Group B blood	64	64	64
		Group A blood	32	32	32
		Ps. pyocyanea	<40	<40	<40

* This subject had recently had an attack of viral pneumonia.

Fig. 3 shows the titre of 4 antibodies (staphylococcal anti-ECP and anti-α-haemolysin, *Ps. pyocyanea* agglutinins and ABO agglutinins) in 4 burned patients over a period of weeks during their treatment in hospital. They show variable and independent fluctuations in the level of each antibody. In spite of these fluctuations, the antibody titres reflected the bacterial colonisation of the burns. For example, the patient whose serology is summarised in Fig. 3 (A) had a heavy

growth of *Ps. pyocyanea* in his burns from the day of admission, but no staphylococci at any time during the period shown. On the other hand the burns of the patient whose serology is shown in Fig. 3 (D) were colonised with *Staph. aureus* from the day of admission, but no *Ps. pyocyanea* was found in them until 11 days after admission. The burns of the patients represented in Fig. 3 (B) and (C) were

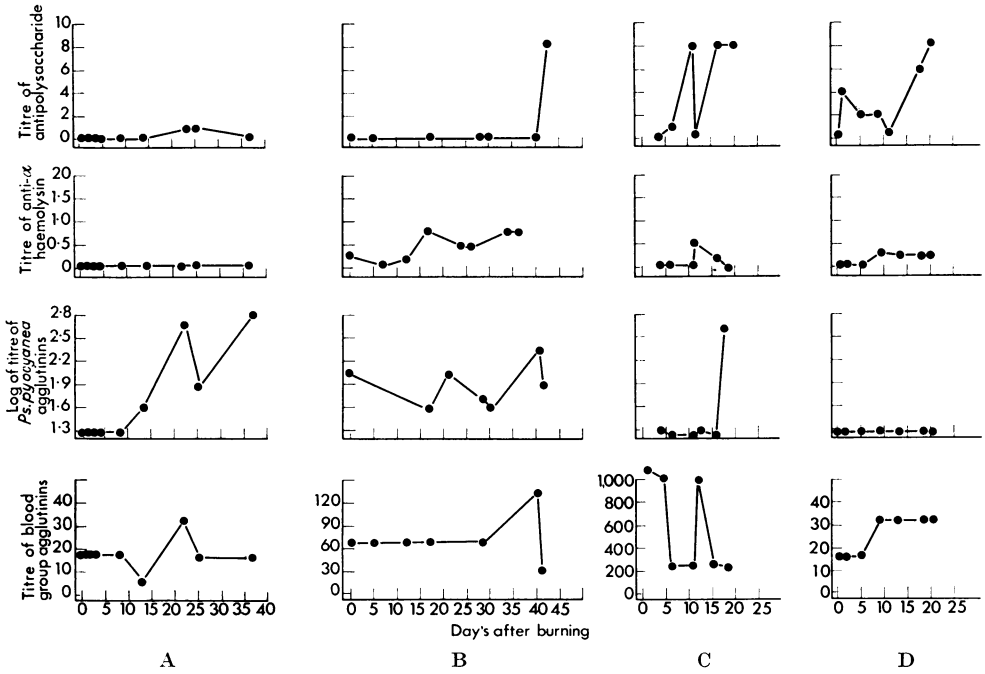

FIG. 3.—Titres of four antibodies (anti-α-haemolysin, anti-ECP, agglutinin to *Ps. pyocyanea* and ABO blood group agglutinins) in the serum of 4 patients during the course of treatment for burns.

colonised with *Staph. aureus* from the day of admission and from the 6th day respectively, and with *Ps. pyocyanea* from the 6th day in both cases.

DISCUSSION

Repeated examination of the serum of burned patients showed evidence of an antibody response to infection of the burns with *Staph. aureus* ; this was apparent in respect of both types of antibody examined, anti-α-haemolysin and antibody to an erythrocyte-coating polysaccharide (anti-ECP). The pattern of this response, however, was unexpected. Repeated samples of serum from normal subjects showed consistent titres of these antibodies—anti-α-haemolysin low when detectable, anti-ECP low in some and moderately raised in other subjects (*cf.* Rountree and Barbour, 1952). In burned patients, on the other hand, there were considerable fluctuations in the titre of both antibodies, which rose and fell independently of each other ; sometimes only one or the other antibody was detected and showed changes of titre. Similar fluctuations were found in the titre of agglutinins to *Ps. pyocyanea* and to the ABO blood group antigens.

Various factors might be involved in causing the irregularity of antibody levels in the serum of burned patients. The loss and replacement of plasma during the " shock " phase is probably not an important factor, as the fluctuations of titre occur over a long period of time after the injury. Loss and replacement of blood after operations is another unlikely factor ; some patients show marked fluctuations of antibody titre when there has been no skin grafting operation or blood transfusion. Moreover, fluid loss and fluid replacement would be expected to cause a rise or a fall of all antibodies rather than the independent fluctuations noted in our study ; for the same reason the altered nitrogen metabolism of burned patients is an improbable factor. Such independent fluctuations, however, might be due to a shifting balance between specific antibody formation in response to bacterial colonisation of the burns and specific absorption of these antibodies by the same bacteria and their toxins. This hypothesis could explain the paradoxical observation of lower anti-α-haemolysin levels in the serum of patients with more extensive burns (Table III), and also the greater irregularity of titre in these patients. The larger the burn, the larger the numbers of staphylococci present in them ; but beyond some point the increase in numbers of staphylococci will probably be associated with less increase of antibody formation than with capacity to absorb antibody from the circulation.

The variability of antibody levels in the serum from burned patients raises some important questions. If convalescent serum were to be accepted as a valuable component of treatment for severely burned patients, it would be important to confirm the presence in such serum of antibodies from which protective action is expected. The anti-α-haemolysin levels show that it might be easier to obtain serum with high titres of certain antibodies from patients with burns of less than 10 per cent than from patients with extensive burns, but this would not apply in the case of antibodies that behave like anti-ECP. In view of the independent fluctuation of antibodies it seems likely that few sera from donors would contain all the desired antibodies.

A significantly higher proportion of low anti-α-haemolysin and low anti-ECP titres was found in the 13 patients of our series colonised with *Staph. aureus* who died than in those who survived ; the titres in sera from normal subjects were closer to the levels found in survivors from burns. Although more than half of the serum samples from those who died were obtained during the first week after burning, none of the samples taken later than this showed anti-ECP titres of more than 4 units per ml., and only 2 of these samples (from 1 patient) showed anti-α-haemolysin titres above 2 units per ml. The preponderance of low titres in the patients who died may have been due to diminished antibody production or, alternatively, to the greater absorption by the burn flora of antibody from the circulation of more severely burned patients. In either case, the association between low titres and mortality suggests an enhanced hazard of infection in these patients. These findings support the rationale of prophylaxis against infection and therapy by convalescent serum or specific antibacterial serum.

Anti-ECP has been thought to have more importance as a factor in defence against staphylococcal infection than anti-α-haemolysin (Rountree and Barbour, 1952) ; this may be due to interference with a mechanism by which polysaccharides have been shown to protect staphylococci against phagocytic action (Morse, 1962). Preliminary studies (Jones, 1962) have shown opsonic action of heat-inactivated serum from rabbits immunised against *Staph. aureus*, but no association between

opsonic activity and anti-ECP titre. Further studies are planned on the bacteriotropic effects of unheated sera on leucocytes, and on antileucocidin titres of sera from burned patients.

SUMMARY

Antibody to erythrocyte-coating polysaccharide of *Staph. aureus* and staphylococcal anti-α-haemolysin were titrated in the serum of 120 patients with burns and of nine normal subjects.

The level of both antibodies was higher in patients whose burns became infected with *Staph. aureus* than in those who did not acquire this infection. By contrast with the findings in serum of normal subjects, there were considerable fluctuations in the antibody titre of successive samples of blood from the same burned patient ; similar fluctuations were found in the titre of agglutinins to *Ps. pyocyanea* and of ABO blood group agglutinins. The titre of each antibody varied independently. Anti-α-haemolysin appeared to respond slowly to the antigenic stimulus of staphylococcal infection, and the rise in titre was greater in patients with less extensive burns. The rise of anti-ECP titres in response to staphylococcal infection was apparently more prompt, and greater in the more extensively burned patients.

Low titres of anti-α-haemolysin and anti-ECP were significantly more frequent in the serum of patients who died than in those who survived.

Possible explanations for the variable titre of antibodies and the relevance of this phenomenon to immunity and to the possible prophylactic use of convalescent serum are discussed.

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