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Comparative Studies of Immunoglobulin Opsonins in Osteomyelitis and Other Established Infections

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Summary. The opsonic activity of serum and isolated immunoglobulin fractions has been studied in twenty patients with osteomyelitis, using a quantitative assay which measures phagocytosis and killing of bacteria by human polymorphonuclear leukocytes. Opsonic functions as well as agglutinating and complement-fixing antibodies were compared with normal human sera as well as with a control group of eighteen patients with miscellaneous established severe infections. A surprising lack of heat stable γG opsonic activity, particularly for various species of *Staphylococcus aureus*, was documented among many patients with chronic active osteomyelitis. A similar apparent deficiency or low level of heat stable γG opsonin was also demonstrated among some of the controls with severe soft tissue infections. These weak opsonins showed a marked contrast to the potent heat stable γG opsonin activity previously documented in sub-acute bacterial endocarditis. Heat stable γG opsonin proved to be present in effective concentration in Gram-negative infections of bone and in the control group. Evidence was obtained for competitive blocking of phagocytic mechanisms by relatively weak immune γG opsonins allowed to react with bacteria prior to contact with more potent γG opsonins subsequently added to the test system. High titres of agglutinating anti-bacterial antibodies contrasted with relatively low levels of complement-fixing antibodies in many patients with chronic osteomyelitis. Opsonic activities of both 7S and 19S fractions of heat inactivated sera were studied in sera from osteomyelitis patients and control infections. When phagocytosis-promoting properties were not detectable in such fractions, opsonic capacity could be effectively restored by adding fresh serum devoid of anti-bacterial antibodies but possessing complement activity. Opsonic capacity of serum fractions then depended on the marked facilitative effect of heat labile factors. Activity ratios were derived of opsonic activity to actual amount of immunoglobulin present acting in concert with a constant amount of complement.

INTRODUCTION

The opsonic properties of various immunoglobulin fractions as well as heat labile serum factors have recently been studied in a group of patients with sub-acute bacterial endocarditis (Laxdal, Messner, Williams and Quie, 1968; Messner, Laxdal, Quie and Williams, 1968a, 1968b; Quie, Messner and Williams, 1968). During this latter work it became apparent that γG-immunoglobulin was often associated with an active opsonic or phagocytic and killing capacity which in many instances did not require the addition of

heat labile serum components. Wishing to extend these observations on the interaction of various immune opsonins, we turned to what we presumed to be an additional rich clinical source of opsonin associated with another chronic human infection—purulent, draining osteomyelitis. To our surprise in some instances of staphylococcal osteomyelitis, patients showed an apparent low level of opsonic capacity in various isolated immunoglobulin G fractions. The finding of such weak opsonins stood in sharp contrast to the clinical picture of long standing chronic osseous suppuration. It also could be contrasted to the vigorous immunoglobulin opsonins previously encountered among sera from patients with subacute bacterial endocarditis (Messner *et al.*, 1968a, 1968b; Williams and Quie, 1968). In some patients with staphylococcal osteomyelitis, high titres of agglutinating antibody to the infecting organisms were accompanied by a glaring lack of both opsonic capacity of isolated immunoglobulin components and in many instances low titres or absence of demonstrable complement-fixing antibodies. This did not hold true, however, among patients with Gram-negative infections of bone. The immune antibody response in patients with chronic draining osteomyelitis seemed of considerable interest. Furthermore, these patients provided a unique opportunity to investigate what serological or immunological qualities might be responsible for the presence of relatively weak immunoglobulin opsonins despite months or even years of draining osteomyelitis. In the studies recorded here the opsonic properties and conventional antibody activities as measured by agglutination or complement fixation with infecting bacteria were analysed in patients with osteomyelitis and compared with those previously documented in subacute bacterial endocarditis (SBE). In addition, comparative studies were made using normal human sera as well as sera from a control group of patients with miscellaneous soft tissue, systemic urinary tract or pulmonary infections. However, viewed in direct comparison with opsonins present in the control groups of other soft tissue infections or in normal human subjects, it became apparent that even the relatively weak opsonins encountered among some osteomyelitis patients or in the control group were probably adequate and could function quite well in phagocytosis when acting in concert with heat labile complement components.

MATERIALS AND METHODS

Bacteria and immune human sera studied

Twenty patients with active osteomyelitis provided the primary clinical material for this work. In all instances the patients' own infecting bacterium was isolated either by culture at operation or from draining sinuses. The salient clinical and pertinent anti-bacterial antibody data of these twenty patients are shown in Table 1. Duration of active osteomyelitis ranged from 3 days to 30 years; however, most patients had chronic infections present for months or years. It is perhaps important to note that none of these patients was febrile, toxic or acutely ill with the exception of patient MIN (Table 1) who had acute active osteomyelitis.

Control sera were obtained from ten normal blood donors as well as from a group of eight patients with miscellaneous severe infections. This latter group included several patients with chronic cystic fibrosis and recurrent pulmonary infections (Table 2) chosen as appropriate controls because of their repeated infections wth *Staphylococcus aureus* or pseudomonas species. In addition, the opsonic capacities of serum dilutions or isolated immunoglobulin fractions from patients with subacute bacterial endocarditis (Laxdal *et al.*, 1968) were used in many comparative control studies.

TABLE 1

CLINICAL FEATURES AND ANTI-BACTERIAL ANTIBODY CHARACTERIZATION AMONG TWENTY PATIENTS WITH OSTEOMYELITIS STUDIED

Patient	Infecting organism	Duration of osteomyelitis	Agglutination			Complement Fixation		
			Whole serum	Controls		Whole serum	Controls	
Sex, age				Mean	Range		Mean	Range
YU, M, 45	*S. aureus*	24 months	6*	3*	2–4	3*	2*	0–4
GRI, M, 50	*S. aureus*	30 years	9	4	2–5	0	3	0–6
FUR, M, 84	*S. aureus*	16 years	8	3	1–4	0	3	0–5
DUR, M, 50	*S. aureus*	24 months	10	2	0–5	0	2	0–3
BEC, M, 22	*S. aureus*	30 months	7	3	1–5	2	2	0–3
BRE, M, 20	*S. aureus*	20 months	10	4	0–5	2	3	0–7
PER, M, 20	*S. aureus*	9 months	8	2	0–4	8	4	3–8
BUR, F, 45	*S. aureus*	11 years	10	4	3–5	0	2	0–4
MID, M, 62	*S. aureus*	25 months	7	5	4–6	0	2	0–3
MON, M, 21	*S. aureus*	1 month	7	3	0–4	0	1	0–4
PAR, M, 28	*S. aureus*	9 months	9	4	2–6	6	3	0–6
JUL, M, 4	*S. aureus*	6 weeks	5	2	1–5	0	2	0–4
MIN, F, 12	*S. aureus*	3 days	8	5	2–8	6	4	0–6
MOH, M, 32	*S. epidermidis*	24 years	6	4	2–6	0	2	0–4
KAM, M, 40	*Pseudomonas aeruginosa*	3 months	9	2	0–4	AC†	AC	AC
STA, M, 60	*Pseudomonas aeruginosa*	4 months	–	–	–	0	0	0
LAH, M, 50	*Pseudomonas aeruginosa*	24 months	7	3	2–4	0	1	0–2
SHI, M, 39	*E. coli*	14 months	10	6	5–8	0	0	0
GIL, F, 50	*Proteus mirabilis*	9 years	7	2	0–4	0	0	0
SCH, M, 5	*Serratia marcescens*	3 weeks	1	1	0–3	7	< 1	0–1

* Numbers refer to $\log_2$ dilution of titres; $\log_2$ dilution of 1, being 1 : 2. Mean agglut. titre among osteomyelitis group was 6·0 as compared with mean of 3·2 in controls; mean CF titre among osteomyelitis group was 1·2 as compared to 1·3 in controls.

† AC indicates anticomplementary effect of organism tested.

Serological testing

Agglutinating antibodies to the various infecting bacteria were measured as previously described (Laxdal *et al.*, 1968; Kinsella, 1917) using bacterial suspensions washed with distilled water and adjusted to optical density at 600–620 mμ in a Coleman Junior Photocolorimeter. Complement-fixing antibodies to the various bacteria were measured by a standard complement fixation test (Casals and Palacios, 1941; Levine, Cowan, Osler and Mayer, 1953) and included appropriate controls for antigen, anti-complementary effect of serum and buffers. In all serological procedures a group of ten normal human sera obtained from blood donors were always included as controls, and served as an index of the means and ranges of antibacterial antibody titres to the various strains of organisms tested (Tables 1 and 2).

Fractionation of immunoglobulins

γG was prepared from immune sera in most instances using diethylaminoethyl cellulose chromatography and buffers of pH 8·2, 0·02 M (Sober, Gutter, Wyckoff and Peterson, 1956; Fahey, McCoy and Goulian, 1958). In some instances zone electrophoresis of whole serum on Pevikon (Müller-Eberhard, 1960) was used to obtain γ-globulins. 7S and 19S fractions from 10 to 40 per cent sucrose density gradient separations were utilized in many phagocytic systems. All of the twenty sera from patients with osteomyelitis were separated into 7S and 19S fractions (Kunkel, 1960; Messner *et al.*, 1968a) and their respective

TABLE 2

CLINICAL FEATURES AND CHARACTERIZATION OF ANTI-BACTERIAL ANTIBODIES AMONG CONTROL GROUP OF EIGHTEEN PATIENTS WITH MISCELLANEOUS INFECTIONS

Patient sex, age	Infecting organism	Type of infection	Duration	Agglutination			Complement fixation		
				Whole serum	Controls		Whole serum	Controls	
					Mean	Range		Mean	Range
DUV, M, 48	*S. aureus*	Surgical wound	4 weeks	7*	4	2–6	5	4	3–6
SHE, M, 8	*S. aureus*	Recurrent boils, abscesses	5 years	7	4	3–5	5	4	0–6
MIN, F, 18	*S. aureus*	Recurrent skin abscesses	4 years	8	5	2–6	6	3	2–5
SEL, M, 10	*S. aureus*	Recurrent skin abscesses and boils	1 year	8	4	0–6	2	3	1–6
FLA, M, 48	*S. aureus*	Surgical wound	6 weeks	8	5	3–6	0	0	0
KOR F, 15	*S. aureus*	Recurrent pneumonias, bronchitis†	15 years	5	4	0–7	3	4	3–7
OLS, M, 14	*S. aureus, Pseudomonas aeruginosa*	Recurrent pneumonias, bronchitis†	14 years	10	4	3–6	5	3	3–5
				5	3	1–5	5	< 1	0–1
HOC, F, 38	*Pseudomonas aeruginosa*	Surgical wound	2 weeks	8	3	0–5	AC	AC	AC
HER, F, 70	*Pseudomonas aeruginosa*	Surgical wound	4 months	8	3	0–5	0	< 1	0–2
FLE, M, 45	*E. coli*	Surgical wound	3 weeks	9	4	2–7	7	2	0–6
HAR, F, 49	*E. coli*	Acute bacterial endocarditis	2 weeks	8	2	0–5	6	1	0–3
HUU, M, 5	*E. coli*	Urinary tract infection	2½ years	6	4	2–6	5	1	0–6
SEA, F, 4	*Klebsiella*	Urinary tract infection	2½ years	6	3	0–4	5	< 1	0–2
WEI, M, 30	*Serratia marcescens*	Infected decubitus ulcer	4 years	5	< 1	0–1	4	< 1	0–1
FRI, M, 45	*Serratia marcescens*	Infected venous cannula	2 weeks	6	< 1	0–1	5	< 1	0–1
ELE, M, 57	*S. aureus*	Sub-acute bacterial endocarditis	2 months	5	4	2–5	4	2	2–6
FOS, F, 62	*S. aureus*	Sub-acute bacterial endocarditis	2 months	7	2	0–4	6	3	0–6
SHR, M, 46	*S. aureus*	Sub-acute bacterial endocarditis	8 weeks	5	2	0–4	4	2	0–3

* Numbers refer to $\log_2$ dilution of titres; $\log_2$ dilution of 1 being 1 : 2. Mean agglutinin titre among miscellaneous infections group was 6·7 with mean central titre of 3·3. Mean CF titre among group was 4·5 as compared to 1·2 for controls.

† Patients with cystic fibrosis.

bacterial agglutinating and complement-fixing antibodies characterized. Similar gradient separations and titrations of antibacterial antibody activity were performed on all eighteen control patients with miscellaneous infections. An estimation of effective final concentrations of γG or γM was made in pooled 7S and 19S sucrose gradient fractions by quantification in Oudin tubes (Oudin, 1952; Wollheim and Williams, 1965) of fractions in parallel with whole serum. Since the actual concentrations produced by dilution during gradient separation were in some instances below the range of sensitivity of the Oudin tube method, a system of inhibition of agglutination of sensitized red cells was used for measurement of actual 19S γM in gradient fractions. The tanned cell method (Boyden, 1951) was used to coat human erythrocytes with human γM prepared as a pool of ten isolated Waldenstrom's macroglobulins. Inhibition of agglutination of these coated cells by a dilution of specific rabbit anti-γM antibody could detect γM in concentrations of 0·01 mg/ml.

Phagocytic system

Essentially the same technique as outlined previously (Maaløe, 1946; Hirsch and Strauss, 1964; Laxdal *et al.*, 1968) was used for the experiments reported here. This method is designed to quantify phagocytosis and killing of ingested bacteria by human polymorphonuclear and mononuclear cells, obtained by dextran sedimentation from blood of normal human donors.

Characterization of bacterial species

The staphylococci isolated from osteomyelitic lesions as well as from control patients were examined for phage type, Müller phenomenon, antibiotic sensitivity and general growth characteristics in order to obtain some profile of their bacteriological qualities.

RESULTS

SEROLOGICAL STUDIES AND MOLECULAR CLASS OF ANTI-BACTERIAL ANTIBODIES

In many of the sera from patients with active osteomyelitis, high titres of agglutinating antibodies to the individual specific organisms were present. It can been seen from Table 1 that in every instance agglutinating antibody titre was above the mean of control normal sera. Of interest was the virtual absence of detectable complement-fixing antibody against the same organisms in the face of extremely high agglutinating antibody activity (as in patients GRI, FUR, DUR, BUR, MOH, LAH, SHI and GIL, Table 1). Anti-complementary effects of sera alone could be detected only in one instance. In the sera from patients with osteomyelitis, the specificity of agglutinating antibodies for individual infecting

TABLE 3

COMPARATIVE AGGLUTINATING ANTIBODY TITRES OBTAINED WITH FOUR SERA FROM PATIENTS WITH OSTEOMYELITIS TESTED AGAINST A PANEL OF ORGANISMS DERIVED FROM OSTEOMYELITIC LESIONS OR BLOOD CULTURES

Serum tested	KAM (*Pseudomonas aeruginosa*)	GIL (*Proteus mirabilis*)	BUR (*S. aureus*)	FUR (*S. aureus*)	DUR (*S. aureus*)	NOR (*Enterococcus*)	FRI (*Serratia marcescens*)
KAM	9*	2*	4	1	4	4	1
GIL	4	8	4	2	4	5	0
BUR	0	2	10	2	4	4	0
FUR	0	0	4	8	2	4	0

*Numbers represent $\log_2$ dilution of agglutination titre; doubling dilutions were used and first dilution was 1 : 2.

TABLE 4

COMPARATIVE MOLECULAR DISTRIBUTION OF AGGLUTINATING AND COMPLEMENT FIXING ANTI-BACTERIAL ANTIBODIES IN OSTEOMYELITIS AND CONTROL INFECTIONS AS DETERMINED BY SUCROSE GRADIENT SEPARATIONS

Infecting organism	No. studied	Agglutination		Complement fixation	
		7S	19S	7S	19S
Osteomyelitis sera:					
S. aureus	13	5·5*	2·1*	<1*	<1*
S. epidermidis	1	4·0	4·0	0	0
Pseudomonas	3	5·5	2·5	0	0
E. coli	1	6·0	3·0	0	0
Proteus	1	4·0	2·0	0	0
S. marcescens	1	3·0	1·0	2·0	0
Control sera:					
S. eureus (soft tissue infection)	7	5·0	2·2	1·2	<1
S. aureus (Sub-acute bacterial endocarditis)	3	4·0	1·3	2·6	0
Pseudomonas	3	4·0	2·6	1·0	0
E. coli	3	5·0	3·0	3·5	0
Klebsiella	1	4·0	0	1·0	0
S. marcescens	2	4·0	1·0	0·5	0

* Numbers refer to average $\log_2$ dilution titres from sucrose gradient titrations.

organisms was shown by parallel agglutination reactions using the patients' serum, own organisms, and four other non-related bacterial species (Table 3). This was necessary because the extremely high titres of agglutinating antibodies as in patients DUR, BUR or KAM raised the question of the nature or specificity of these agglutination reactions. The antibody profiles of separated 7S and 19S fractions from the osteomyelitis and control (miscellaneous infections) groups are compared in Table 4. Similar profiles obtained on whole sera from these groups are shown in Tables 1 and 2.

In general, agglutinating antibody titres tended to be high in both groups but complement-fixing antibodies showed somewhat higher levels in the control group of miscellaneous infections than were seen in patients with osteomyelitis. Data on the relative molecular distributions of 7S and 19S anti-bacterial antibodies among the osteomyelitis and control subjects are summarized in Table 4. It was apparent that agglutinating as well as complement-fixing antibodies were predominant among 7S fractions. These comparative data established the basis for comparison of the two groups of patients. In addition, Oudin tube quantitative estimations of serum γG, γA and γM showed no significant differences between the two groups. Neither the osteomyelitis nor control groups showed positive tests for anti-γ-globulin factors (Messner *et al.*, 1968b).

OPSONIC PROPERTIES OF SEPARATED SERUM FRACTIONS

Effect of heat labile factors

Since we had expected to recover brisk opsonic activity in immunoglobulin fractions derived from sera of patients with chronic osteomyelitis, the relatively weak opsonic effect observed in many instances of staphylococcal osteomyelitis came as a distinct surprise. In six experiments, comparisons were made between native whole serum previously stored at $-70°$ and the same serum inactivated at 56° for 30 minutes. In every instance absent or only weak opsonic activity was noted after heat inactivation. Furthermore, isolated immunoglobulin G from many of these sera showed little or no demonstrable opsonic effect.

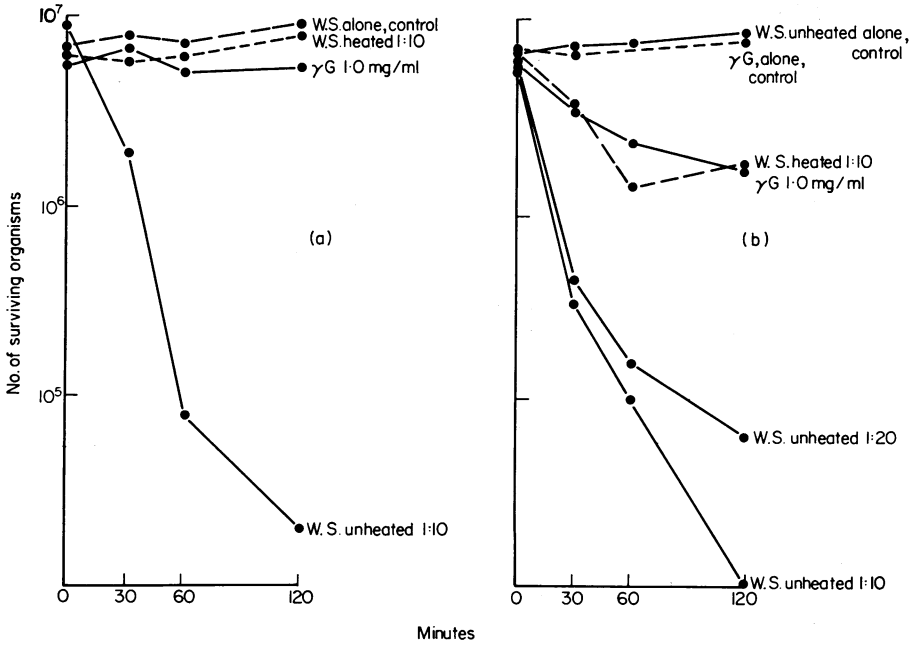

FIG. 1. Opsonic activity of whole serum and γG immunoglobulin fractions from two patients FUR (a) and DUR (b) with osteomyelitis due to *S. aureus* organisms. Unheated whole serum (W. S. unheated) showed rapid opsonic effect in both examples. In patient FUR whose opsonic functions are shown in (a), heated whole serum as well as isolated γG at 1·0 mg/ml showed no opsonic effect. In the patient DUR (b) both heat-inactivated whole serum and γG 1·0 mg/ml showed weak opsonic activity. Controls which included unheated serum or γG fractions without leukocytes produced no bacterial killing (W. S. unheated alone, control and γG, alone control).

Examples of these results are shown in Fig. 1. Of importance was the universally good opsonic effect noted with fresh unheated immune sera in all of these experiments. It appeared, therefore, that heat labile components in these sera were necessary to demonstrate efficient opsonic function and that the fresh whole serum from the osteomyelitic patients was effective in promoting phagocytosis.

Effect of γG fractions

Because an active opsonic effect could be demonstrated in fresh whole immune sera from patients with staphylococcal osteomyelitis but was markedly diminished by heating and because only weak or moderate specific opsonic activity was demonstrable in isolated immune γG from DEAE separations, a series of comparative experiments were next undertaken. Immune γG isolated from whole serum and adjusted to concentrations of 1·0, 0·5 and 0·1 mg/ml was used. In these comparisons, γG from the patients with active staphylococcal osteomyelitis was tested with their own infecting organisms and compared with several other DEAE-isolated γG preparations from patients with active subacute bacterial endocarditis due to a different strain of *S. aureus*. Representative results of such comparative experiments are shown in Fig. 2 (a), (b) and (d) in which different concentrations of γG were used. In the majority of such comparisons, the isolated γG of the patients with staphylococcal osteomyelitis was less potent against their own organisms than were various γG preparations from staphylococcal endocarditis sera tested at the same protein concentration.

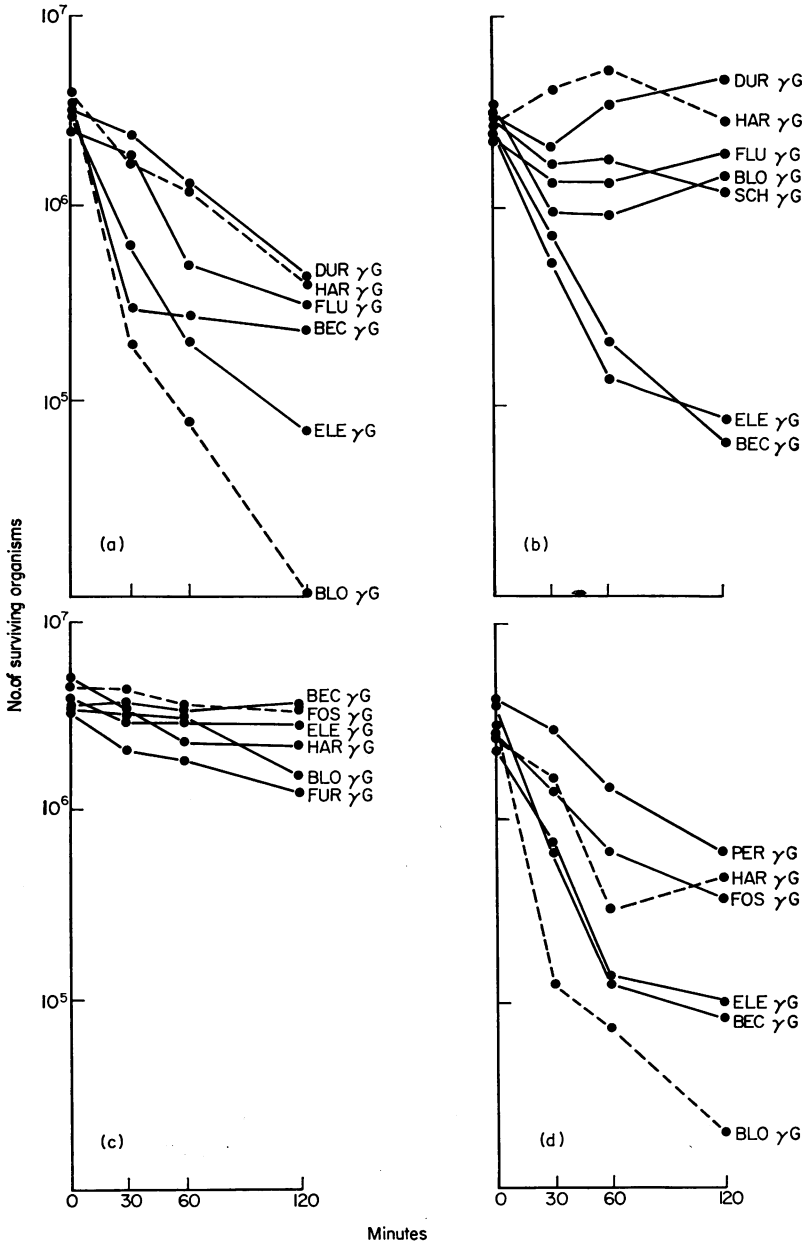

FIG. 2. (a) and (b) Comparative opsonic activity of γG preparations from patient DUR with *S. aureus* osteomyelitis tested with the organism from DUR. Parallel opsonic activities of individual γG from patients HAR, FLU, BEC, ELE and BLO—all with staphylococcal endocarditis—have been tested at 0·5 mg/ml (a) and 0·1 mg/ml (b). These experiments demonstrated the relatively weak opsonic activity of osteomyelitic γG DUR when tested with the DUR infecting organism. On the left (c) are shown a similar panel of immune γG opsonins from patients with bacterial endocarditis (BEC, FOS, ELE, HAR and BLO) tested in parallel with γG and *S. aureus* organism from osteomyelitis patient FUR (γG at 0·5 mg/ml). Of interest was the poor opsonic function noted with all preparations tested. In such instances, subsequent experiments indicated brisk reconstitution of opsonic activity when heat labile serum factors were added. On the right (d) are shown comparative opsonic studies using isolated γG from osteomyelitis patient PER (γG at 1·0 mg/ml) and the panel of immune γGs from five patients with bacterial endocarditis. Again relatively weak PER γG opsonic function is apparent with the patients' own organism.

TABLE 5

COMPARATIVE OPSONIC CAPACITIES OF γG FRACTIONS OF SERUM FROM PATIENTS WITH OSTEOMYELITIS AND SUB-ACUTE BACTERIAL ENDOCARDITIS STUDIED USING ORGANISMS (*S. aureus*) ISOLATED FROM THE PATIENTS WITH OSTEOMYELITIS

Patients with osteomyelitis studied	Own γG opsonin*	γG opsonins from SBE patients*				
		FOS	HAR	ELE	BEC	BLO
1. PER	47†	46	37	95	98	99
2. DUR	85	90	90	98	92	100
3. MIN	0	10	87	96	95	94
4. BEC	80	70	83	99	98	85
5. YUN	30	66	71	24	60	99
6. MON	30	50	54	70	77	60
7. FUR	65	34	60	27	10	58
8. MOH	0	25	30	30	30	22
9. JUL	0	0	0	0	0	0

* All γG opsonins used at 1·0 mg/ml in a phagocytic system employing osteomyelitis patient's organism and own opsonin in parallel with γG from SBE patients.

† Numbers refer to percentage of test bacteria phagocytosed and killed after 120 minutes.

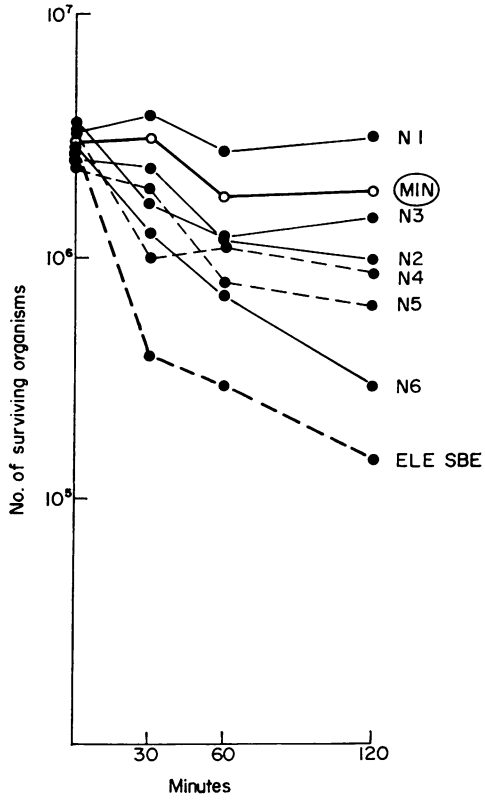

FIG. 3. Comparative opsonic function of isolated γG (at 1·0 mg/ml) from osteomyelitis patient MIN run in parallel with γG from six normal blood donors Nl–N6 and γG from patient ELE with bacterial endocarditis. The *S. aureus* organism isolated from the osteomyelitis patient MIN was used throughout. It can be seen from the phagocytic curves illustrated here that several of the normal donors' γG preparations were considerably more active in opsonic activity than the patients own γG. Isolated γG from bacterial endocarditis patient ELE, as in other experiments, showed the most rapid and efficient opsonic activity.

A summary of these comparative experiments is shown in Table 5. Of interest was the situation observed in two instances (MOH and JUL) where none of the γG preparations tested showed a strong opsonic effect. One example of extremely weak opsonic effect can be seen in Fig. 2(c). Using the same FUR fresh whole serum, however, excellent opsonic activity for FUR organism could be demonstrated (Fig. 1).

A series of comparative experiments were next undertaken in which opsonically weak γG from patients with staphylococcal osteomyelitis was compared with γG from six normal blood donors' sera, using the osteomyelitis patients' own infecting organism. Surprisingly, the normal donors' γG preparations clustered in disperse fashion near the activity of γG from the osteomyelitis subject. In some comparisons very little or no opsonic effect was noted with some normal γG preparations, whereas in other examples a normal blood donor γG at equivalent concentrations performed far better than the osteomyelitis patient's γG itself. These comparisons are clearly apparent in Fig. 3. Moreover, when isolated γG from patients with other types of soft tissue staphylococcal infections were tested in similar simultaneous comparative studies, it was evident that many were only weak or moderate opsonins often behaving in a similar fashion to those of the osteomyelitis group from which the test organisms were derived. It, thus, appeared that isolated γG from patients with SBE often contained extremely potent heat stable opsonin: γG from patients with staphylococcal osteomyelitis or other soft tissue infections was weak or intermediate, while normal γG displayed a diverse degree of opsonic capacity against the staphylococcal species tested.

Comparative opsonic studies were also done using isolated γG from the three patients with pseudomonas osteomyelitis (KAM, STA and LAH) and two other control patients with pseudomonas wound infections. In contrast to the situation recorded with staphylococcal osteomyelitis, no deficiency of osteomyelitic γG opsonin was noted in these cases. In addition, two patients with *Serratia marcescens* osteomyelitis were similarly compared with three other control patients showing *Serratia* infections. Again no deficiency of γG opsonin derived from these patients with osteomyelitis was noted. During these studies, marked individual specificity of opsonic γG for the infecting *Serratia* species was apparent (Fig. 4).

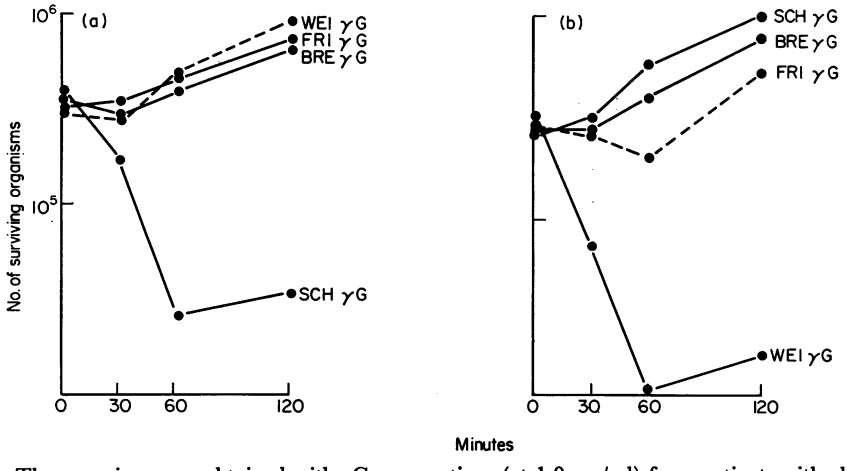

FIG. 4. The opsonic curves obtained with γG preparations (at 1·0 mg/ml) from patients with chronic *Serratia marcescens* infections are shown. Patient SCH (a) had chronic osteomyelitis whereas WEI (b) had a decubitus ulcer of 4 years duration. Marked individual specificity of immune γG opsonic function is shown. Patients FRI and BRE also had chronic *Serratia* infections. This group of isolated γG preparations had the most pronounced individually specific opsonic activity of all tested.

Competitive interactions of two distinct opsonins

Because γG opsonins from some patients with staphylococcal osteomyelitis appeared to be weak or relatively deficient when compared to those associated with intravascular systemic staphylococcal infections such as SBE or with osteomyelitis produced by the various gram negative bacteria studied, a series of experiments were next performed involving competitive interactions of two distinct γG opsonins for one test bacterial species. These experiments were designed to test primary avidity or, more precisely, blocking ability of specific immune opsonic γG acting in the presence of another γG antibody with primary reactivity for the same bacterial species. In several instances good evidence for

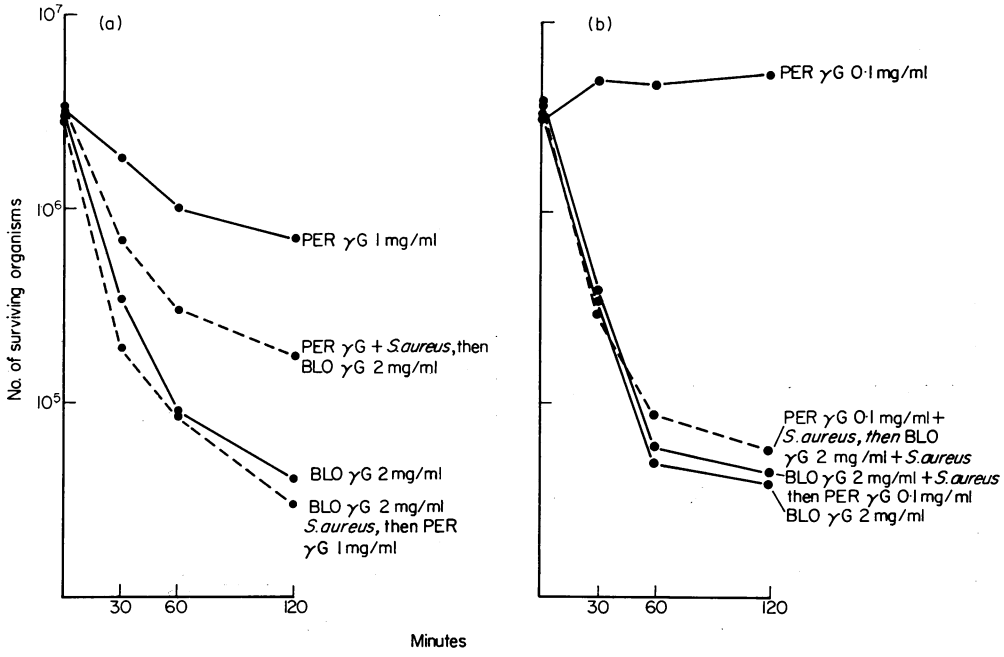

FIG. 5. Opsonic curves show competitive blocking action between a weak γG opsonin PER derived from a subject with osteomyelitis and a strong γG preparation BLO from a patient with staphylococcal endocarditis. PER *S. aureus* bacteria were used in the phagocytosis tests. In (a) it can be seen that when the weak PER γG is allowed first to react with PER bacteria and then opsonized by strong BLO γG, partial blocking or attentuation of the strong γG BLO effect is noted (PER γG+*S. aureus*, then BLO γG 2 mg/ml). When, however, BLO γG is allowed to react with PER bacteria first, no blocking by γG PER is apparent (lower two curves). In (b) the loss of the above described blocking effect is apparent as γG PER is diluted out to 0·1 mg/ml. Results intermediate to that shown in the middle curve on the left were noted with PER γG at 0·5 mg/ml. Several consistent similar experiments of this type confirmed the abilities of *initially reacting* opsonins to control subsequent opsonic activity.

blocking of rapid opsonization by weak but specific opsonin was obtained. A represeetative experiment is shown in Fig. 5. When the relatively weak opsonin PER γG was pre-incubated with PER *S. aureaus* and the mixture then allowed to react with a strong opsonic BLO γG, some impedance to BLO γG opsonic effect was demonstrable. Pre-incubation did not affect the opsonic function of BLO γG when the concentration of PER γG was reduced to 0·1 mg/ml. This type of interference with opsonic activity by pre-incubation with a relatively weak γG opsonin was readily demonstrable using three different systems and strains of *S. aureus* from patients with osteomyelitis.

Separation of opsonins by zone electrophoresis

A different approach towards better characterization of opsonic serum immunoglobulin fractions was next sought using separate or pooled fractions obtained after zone electrophoresis of heat-inactivated serum on Pevikon blocks. Six individual Pevikon block titrations of complement-fixing and bacterial agglutinating antibodies indicated relatively similar curves for both types of antibody; however, the opsonic activity in parallel experiments was largely confined to the slow γ-globulin segments (Fig. 6a). Attempts were next made to test for blocking effects or competitive opsonic functions by mixing fractions from

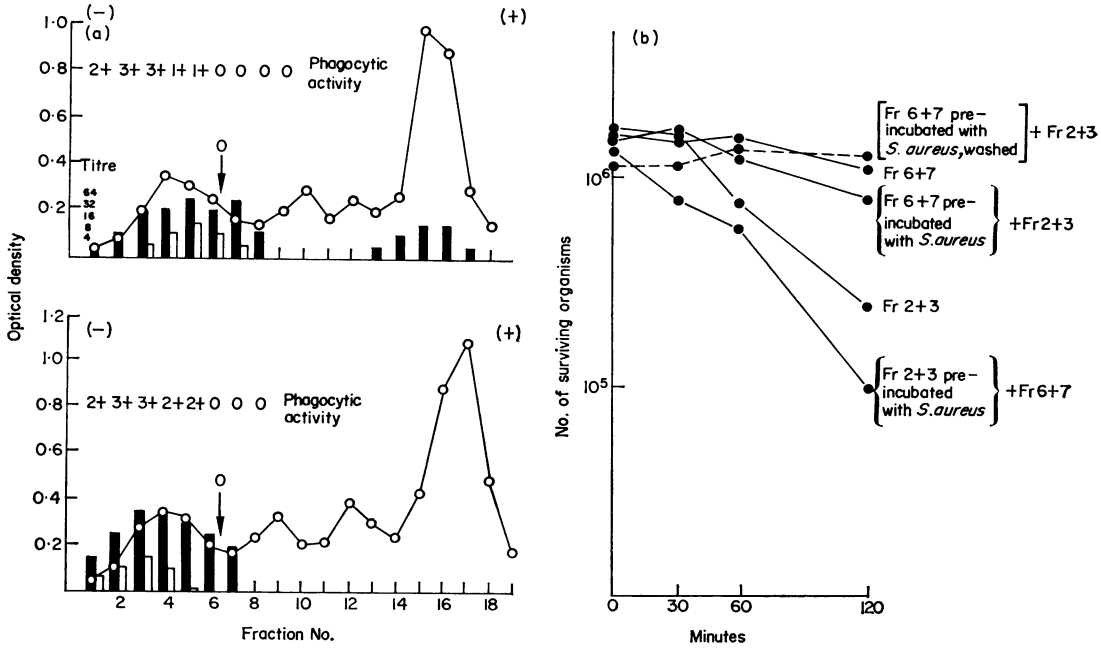

FIG. 6. Two heat-inactivated sera from patients with osteomyelitis were separated by Pevikon block zone electrophoresis and individual fractions tested for bacterial agglutinating activity (solid columns) complement fixation (open columns) and phagocytic or opsonic activity. In all instances opsonic activity was most prominent in electrophoretically slow Pevikon fractions. (a) BRE *S. aureus* (top) and KAM *Pseudomonas* (bottom); (b) BRE *S. aureus*.

Diminution of phagocytosis-promoting or opsonic activity in faster electrophoretic Pevikon block fractions prompted an attempt to demonstrate blocking of opsonic action by electrophoretically fast γ-globulin fractions. In one of four instances, such blocking could be demonstrated and is shown in (b). In this instance the opsonic activity present in Pevikon block Fractions 2+3 was largely blocked by pre-incubation with fractions 6+7. However, if the electrophoretically fast fractions 6+7 were added *after* opsonic fractions 2+3, no blocking occurred.

the same preparative zone electrophoresis separation. In one instance, of four tested the faster electrophoretic immunoglobulin fractions containing both agglutinating and complement-fixing antibody to the test organisms could be shown partially to block opsonic activity in the electrophoretically slow γ-globulin fractions (Fig. 6b). No such intrinsic blocking effect could, however, be shown in other similar experiments using serum and organisms from other patients with either staphylococcal or *Pseudomonas aeruginosa* osteomyelitis.

Opsonic capacities of 7S and 19S frations

Comparative phagocytosis promoting capacities of 7S and 19S fractions of osteomyelitis sera were obtained by measuring opsonic titres of pooled fractions from sucrose density gradient separations of sera previously inactivated at 56° for 30 minutes. Such comparisons were completed on fifteen serum samples from patients with osteomyelitis. In two instances a significant intrinsic opsonic effect was notable in such 19S fractions and in only seven cases was moderate to strong opsonic activity documented in 7S fractions as indicated by more than 50 per cent killing of the test organisms. The results of these comparative opsonic titres of 7S and 19S gradient fractions are shown in Table 6 where they are presented along with similar data on the separated fractions from seventeen patients with miscellaneous infections studied as controls. Table 6 shows that in many instances 7S fractions

TABLE 6

COMPARATIVE OPSONIC AND BACTERICIDAL CAPACITIES OF 7S AND 19S SUCROSE GRADIENT FRACTIONS OF SERUM* FROM FIFTEEN PATIENTS WITH OSTEOMYELITIS AND SEVENTEEN CONTROL SUBJECTS WITH MISCELLANEOUS INFECTIONS

Osteomyelitis				Control group			
Patient	Organism	7S	19S	Patient	Organism	7S	19S
FUR	*S. aureus*	0†	0†	DUV	*S. aureus*	0†	0†
DUR	*S. aureus*	0	0	MIN	*S. aureus*	0	0
BUR	*S. aureus*	0	0	SHE	*S. aureus*	29	0
YUN	*S. aureus*	0	0	FLA	*S. aureus*	20	0
MON	*S. aureus*	0	0	OLS	*S. aureus*	56	0
PER	*S. aureus*	59	0	SEL	*S. aureus*	59	0
JUL	*S. aureus*	60	0	KOR	*S. aureus*	90	0
MIN	*S. aureus*	70	0	ELE‡	*S. aareus*	98	0
PAR	*S. aureus*	80	27	FOS†	*S. aureus*	99	0
MOH	*S. epidermidis*	45	0	SHR‡	*S. aureus*	99	0
SCH	*Serratia marcescens*	99	25	HOC	*Pseudomonas aeruginosa*	0	0
KAM	*Pseudomonas aeruginosa*	80	84	HER	*Pseudomonas aeruginosa*	90	0
LAH	*Pseudomonas aeruginosa*	84	0	HUU	*Pseudomonas aeruginosa*	99	42
STA	*Pseudomonas aeruginosa*	92	66	FLE	*E. coli*	0	0
SHI	*E. coli*	0	0	HAR	*E. coli*	94	0
				WEI	*Serratia marcescens*	98	50
				FRI	*Serratia marcescens*	99	50

* All fractions studied after sucrose had been removed by dialysis against pH 7·4 0·1 M phosphate buffer.
† Numbers refer to percentage of bacteria phagocytized and killed 120 minutes after phagocytosis assay begun.
‡ These patients had staphylococcal endocarditis.

from heat inactivated sera showed little or no opsonic effect in both osteomyelitis and control infections due to *S. aureus*. The contrast noted with similar 7S fractions from patients with *S. aureus* endocarditis (ELE, SHR and FLU) can readily be noted. Moreover, the separated gradient fractions obtained from patients with gram negative infections of bone or soft tissue in general showed considerably better opsonic and bactericidal capacity than those due to *S. aureus*. It is important to mention that the protein concentrations in 7S fractions were generally about twelve times that of the 19S materials separated simultaneously, however, the relative dilutions by the various concentrations of sucrose used to construct the density gradients were approximately the same. This was further checked by immunoglobulin quantification of γG and γM on whole serum and the actual 7S and 19S sucrose gradient fractions used in phagocytosis assays. Average relative dilution of 7S γG calculated by this method was 1 : 28 and of 19S γM, 1 : 25. Thus, comparative opsonic

activity could be compared to quantitative estimations of γG or γM actually present in the fractions tested. This type of opsonic activity ratio to actual quantity of immunoglobulin present in separated gradient fractions provided only a general estimate since it could not be assumed that more than a minority of the immunoglobulin molecules within such fractions actually had specific opsonic antibody capacity (Messner *et al.*, 1968a).

The next line of approach to the study of specific opsonic activity utilized addition of heat labile serum fractions (HLF) (Laxdal *et al.*, 1968; Messner *et al.*, 1968b) to isolated 7S and 19S sucrose gradient fractions in the *in vitro* phagocytosis system. Two sources of heat labile factors were used, fresh pooled newborn infant serum and fresh pooled serum from adult blood donors. Both were absorbed twice or three times with test bacteria at 4° for 1 hour in the presence of 0·01 M EDTA. Calcium and magnesium ions were restored to absorbed serum by dialysis against Hanks's balanced salt solution as previously described (Williams *et al.*, 1968). Residual haemolytic complement in such absorbed sera was measured by lysis of amboceptor-sensitized sheep cells. β_{1C}-globulin was measured by radial diffusion using Hoechst diffusion plates and standard serum (Mancini, Carbonara and Heremans, 1965; Kohler and Müller-Eberhard, 1967). Effective haemolytic complement activity in such absorbed sera ranged from 22 to 30 $C'H_{50}$ units/ml.

The concentration of heat labile factors added to separated gradient fractions was kept constant and varying dilutions of 7S and 19S materials used in the phagocytosis assay. Whereas only a weak or no opsonic effect could be demonstrated by the use of 7S and 19S sucrose gradient fractions alone, striking recovery of opsonic activity in both 7S and 19S fractions was noted after addition of HLF. In several instances 7S and 19S fractions could be diluted to approximately equal end-point titres 1 : 20 or 1 : 30 and still showed reconstitution of opsonic activity in the *in vitro* phagocytosis assay system. Heat labile factors could restore or reconstitute efficient opsonic activity in 7S or 19S fractions considered deficient or relatively weak when tested alone.

DISCUSSION

The presence of high titres of anti-bacterial agglutinating antibodies in the face of relatively little complement-fixing antibody and in some instances rather weak γG opsonic capacity among some patients with osteomyelitis is of considerable interest. This clinical situation can be immediately contrasted with that of patients with subacute bacterial endocarditis where high agglutinin, moderate complement-fixing and high opsonic titres were found to be the general rule (Laxdal *et al.*, 1968). Initially, it appeared that a qualitative immune γG opsonin deficiency might be present in some patients with staphylococcal osteomyelitis. This could proceed from the relative anatomical isolation of the necrotic bone containing bacteria and a rather indolent antigenic stimulus. The extremely slow interchange by diffusion in bone of molecules of a variety of types as studied by Frost (Frost, Villaneuva and Roth, 1960; Frost, 1960) emphasizes the unusual physical properties of infected bone as an antigenic stimulus. However, the control studies of isolated γG fractions using both normal blood donors and γG from patients with other soft tissue infections indicated that immunoglobulin opsonic activity similar to that found in osteomyelitis was also present in a group of miscellaneous infections. Thus, no overall γG opsonic deficiency could be ascribed to osteomyelitis. The placid clinical vista in many patients with active draining osteomyelitis has been repeatedly emphasized in the clinical studies of this disorder (Brodie, 1845; Orr, 1927; Baer, 1931; Kelly, Martin and Coventry, 1965;

Clawson and Dunn, 1967). Systemic manifestations such as malaise, fever, or profound leukocytosis are often absent in chronic osteomyelitis. These clinical aspects and the very chronicity in many cases suggests a symbiotic relationship between the active draining osteomyelitic focus and the infecting organism, and may account for the discrepancy noted between serum levels of agglutinating and complement-fixing antibody.

The extremely high bacterial agglutinating titres in the face of low or undetectable antibodies which fixed complement among some patients with staphylococcal osteomyelitis suggested that an unusual qualitative difference in the anti-bacterial antibody might be present. Absence of ability to fix complement has been described with relationship to the γG_4 H-chain subgroup of molecules (Ishizaka, Ishizaka, Salmon and Fudenberg, 1967). Attempts using the sera studied here were made to relate the percentage of γG_4 molecules to the total mg/100 ml of γG-globulins. No direct correlation between relatively high γG_4 serum levels in some sera and low or absent complement-fixing anti-bacterial antibodies was noted.

A ready explanation for what appeared to be weak γG opsonins among some of the patients with staphylococcal osteomyelitis studied here is not at hand. No definable special characteristics were noted among the individual bacterial species studied. Phage typing of staphylococci, Müller phenomena, colony or growth characteristics or antibiotic sensitivity did not single out features linking these organisms to the osteomyelitic process. Moreover, direct comparison with γG opsonins from patients with staphylococcal endocarditis confirmed the impression of opsonic deficiency in many cases. Some insight into the kinetics of two competing γG opsonins was obtained by the mixing experiments illustrated in Fig. 5. It appeared that initial exposure to a weak γG opsonin could partially block the opsonic capacity of a stronger γG added subsequently. In addition, the Pevikon block experiments in which electrophoretically slow or fast γ-globulins were added to bacteria in various sequences were helpful in characterizing opsonic functions. The slow γ-globulin fractions contained the opsonic activity in all samples studied.

The low levels of demonstrable complement-fixing antibodies against infecting bacteria were observed in many whole sera or their 7S and 19S fractions (Tables 1, 2 and 3). However, as demonstrated by the experiments in which heat labile factors were added to gradient fractions, this appeared to be a situation in which extremely small amounts of these immune antibodies were capable of interacting with heat labile components to reconstitute brisk opsonic activity. The experiments reported here are quite similar in many respects to those of others in which 7S or γG immunoglobulins were capable of opsonizing by themselves but 19S or high molecular weight antibodies required addition of complement for efficient opsonic function (Gerlings-Petersen and Pondman, 1962, 1965). The precise logistics of how many molecules are required or the exact quantitative ratios of the efficiency of 7S and 19S antibodies as opsonins in the systems studied here are not yet known. Attempts to determine ratios of numbers of γG or γM antibody molecules actually active as opsonins (Robbins, Kenny and Suter, 1965) have thus far been unsuccessful because of difficulties in eluting opsonins directly from bacteria in the systems reported here.

Finally, it seems evident that effete or weak γG opsonins cannot be the prime cause of the chronicity or of all the characteristics of the osteomyelitis in our group of patients. For instance, patient BEC stepped on a land mine in Vietnam 30 months previously and had thousands of residual metal splinters in his tibia while patient FUR had cut his femur with a crosscut saw some 16 years before study. However, the finding of a muted opsonic

antibody response in this condition suggests that the hosts' immune response may be altered and is somehow not ideal. A surprising comparative aspect of this study indicated that in many of the control infections studied, relatively weak heat stable opsonins were present, and that heat labile serum factors—presumably complement—could provide the margin necessary for effective opsonic activity. It thus appeared that the activity of heat stable γG opsonins previously documented in SBE stood far above the rather moderate or weak heat stable opsonins of patients with osteomyelitis or various control infections. The final saving aspect in initiating opsonic activity in these latter infections appeared to be the remarkably efficient effect of heat labile serum factors—presumably involving the complement system.

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