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Serum Opsonin, Bacteria, and Polymorphonuclear Leukocyte Interactions in Subacute Bacterial Endocarditis

ANTI–γ–GLOBULIN FACTORS AND THEIR INTERACTION WITH SPECIFIC OPSONINS

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A B S T R A C T   The effect of anti–γ-globulin factors on 7S γ-globulin opsonins from patients with subacute bacterial endocarditis has been examined with a quantitative in vitro phagocytosis system. Human anti–γ-globulin factors from patients with subacute bacterial endocarditis and rheumatoid arthritis inhibited the opsonic action of 7S γ-globulin specifically bound to bacteria. A similar antiopsonic effect was obtained with rabbit antiserum to human γG globulin. The antiopsonic effect of anti–γ-globulin factors did not correlate with their ability to potentiate agglutination of bacteria by 7S antibody. Competition was demonstrated between the antiopsonic effect of anti–γ-globulin factors and the phagocytosis-promoting action of heat-labile serum factors containing hemolytically active complement.

INTRODUCTION

Characterization of antibacterial antibodies and immune opsonins in subacute bacterial endocarditis (SBE) (1) has revealed that in most patients 7S γ-globulin constitutes the major class of antibody directed at the infecting bacteria as measured by bacterial agglutination, complement fixation, and opsonizing parameters. These findings provided a basis for pertinent observations on the

physiological role of autoreactive factors. The in vitro interactions of anti–γ-globulin or rheumatoid factors (RF) with agglutinating or opsonizing antibodies in the sera from patients with SBE is the subject of this report. Anti–γ-globulin antibodies similar to rheumatoid factors were present in about one-half of the patients with SBE. These anti–γ-globulin factors disappeared after effective therapy (2) and were thus presumed to be a response related to the infection. In contrast to many of the diseases in which anti–γ-globulin factors are found (3–6), the bacterial agent initiating these changes in SBE can usually be isolated with standard culture techniques. This natural model, therefore, offers an unusual opportunity for study of interactions of anti–γ-globulin factors with specific antibodies to infecting bacteria.

An in vitro phagocytosis test system was chosen to examine the effect of RF on antibacterial antibody activity. The question was asked whether anti–γ-globulin factors in sera from patients with bacterial endocarditis (autologous) or derived from patients with rheumatoid arthritis (homologous) may affect the phagocytosis of bacteria by polymorphonuclear leukocytes.

METHODS

Serum and bacteria from 12 patients with SBE, previously studied for agglutinating, complement fixing, and opsonic antibodies (1), were utilized in these experiments.

In all instances the major opsonic activity contained in these sera was demonstrated in 7S fractions obtained by sucrose density gradient ultracentrifugation. Anti–γ-globulin factors were measured by the latex fixation test (7), and the agglutination of Rh positive cells (R^1R^1) coated with incomplete anti-Rh antibody Ripley (8). Standard methods were used for Gm (a), Gm (b), and Gm (f) typing (9–12). The anti-Rh sera as well as the agglutinators used in Gm typing were obtained from Hoechst Pharmaceuticals, Cincinatti, Ohio and others were generously provided by Drs. S. D. Litwin and H. G. Kunkel or Dr. A. G. Steinberg.

Various 7S and 19S immunoglobulin preparations isolated from serum by sucrose density gradient ultracentrifugation, gel filtration with Sephadex G-200, or (O-diethylaminoethyl) cellulose (DEAE-cellulose) column chromatography were utilized in phagocytosis experiments. Autologous 19S anti–γ-globulin factors were separated from sera of patients with SBE. After dialysis against 0.1 M acetate buffer pH 4.1 for 16 hr to dissociate any anti–γ-globulin–γG complexes (13), sucrose density gradient ultracentrifugation was performed with a 10–40% gradient of sucrose in 0.1 M acetate buffer pH 4.1. The 19S fractions were pooled and dialyzed for 4 hr against phosphate buffered saline pH 7.4 before use. Homologous anti–γ-globulin factors were obtained from sera of patients with classic rheumatoid arthritis. The 19S fractions from Sephadex G-200 gel filtration in 0.1 M acetate buffer pH 4.1 were pooled and dialyzed against buffered saline before use. These 19S fractions containing anti–γ-globulin activity were absorbed by incubation with test bacteria to remove directly reactive antibodies. In addition, similar preparations of two isolated Waldenström's γM proteins, H. J.[1] and G. S.,[2] with high titer of anti–γ-globulin activity were used as sources of RF in some experiments.

The phagocytosis system described by Hirsch and Strauss (14) adapted from Maaløe (15) was modified for experiments with RF. Tests were performed in 15 × 75-mm siliconized pyrex glass tubes capped with siliconized rubber stoppers. The final volume of the phagocytosis test system was 1 ml, which was prepared with 0.3 ml of polymorphonuclear leukocytes (PMN) suspension containing 1×10^7 PMN/ml, 0.1 ml of bacterial suspension of 5×10^7 bacteria/ml, and 0.6 ml of opsonin components and (or) dilutions of RF. Dilutions of all ingredients were made in a medium of Hanks's balanced salt solution with 0.1% gelatin (gel-Hanks' solution) (14). Final ratio of PMN to bacteria in the phagocytic mixture was approximately 1 : 1. Each test included two controls: (a) a suspension of serum or serum fraction and bacteria without PMN's to demonstrate antibiotic activity or any direct bactericidal effects of the serum; and (b) suspension of bacteria and PMN without serum or serum fractions to demonstrate necessity of opsonins for phagocytosis of individual bacterial species. The phagocytic mixtures were tumbled end over end at 37°C at 10

rpm on a Rotorack (Fischer Scientific Co., Pittsburgh, Pa.). We sampled the mixture for viable bacterial counts at 0, 30, 60, and 120 min by placing 0.001 ml into 1 ml of distilled water with a calibrated platinum loop.

Clumping of extracellular and intracellular staphylococci in the presence of serum did not seem to be a factor in reducing the total counts (16). The curve showing the drop in colony count during the 120 min most often had a smooth downwards slope. Moreover, examinations of stained smears from the phagocytic mixture revealed minimal clumping of extracellular organisms.

After 120-min incubation, phagocytic mixtures were centrifuged at 450 rpm for 5 min (International Centrifuge, model U. V., Head No. 219). After sampling we discarded the supernatant. The leukocyte pellet was washed in heparinized saline, resuspended to original volume, and 0.001 ml was placed in 1 ml of distilled water for leukocyte lysis. In some instances streptomycin in a final concentration of 400 μg/ml was added to the cell pellet, the samples were rotated at 37°C for an additional 15 min, and bacterial counts performed after rewashing with heparinized saline.

Pour plates were prepared with 0.1-ml aliquots of these dilutions in nutrient agar. Phenol-red agar base (Difco Laboratories, Detroit, Mich.), was used for streptococcal species and Penassay agar (Difco) for staphylococci. The plates were incubated for 24 hr at 37°C and bacterial colonies enumerated with a darkfield Quebec colony counter (American Optical Company, Buffalo, N. Y.). In certain experiments complexes of bacteria and serum components were prepared by incubation of 0.1 ml of bacteria suspension with 0.2 ml of each serum component for 30 min at 37°C. These bacteria were then washed with balanced salt solution and added to the phagocytic mixture.

The effect of RF on bacterial agglutination was studied by the addition of aliquots of RF preparations to doubling dilutions of 7S serum fractions known to possess bacterial agglutinating antibody. Bacteria and 7S serum fractions were preincubated at room temperature for 30 min before the addition of 0.05 ml of RF preparation. Tubes to which 0.05 ml of saline were added served as controls. In this manner isolated antibacterial agglutinating antibodies from patients with SBE as well as individuals with rheumatoid arthritis were studied after the addition of RF prepared from autologous (their own) serum and RF derived from other individual sera.

RESULTS

Interaction of anti–γ-globulin factors with specific antibacterial antibody. The interaction of RF and specific antibacterial antibody was examined in bacterial agglutination tests. It was felt that RF might act as a Coombs-type reagent and augment the agglutination of bacteria by isolated 7S fractions. Rheumatoid factors derived from the patients' own serum (autologous) or isolated from serum of patients with rheumatoid arthritis (ho-

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[1] Supplied by Dr. P. S. Schur.
[2] Supplied by Dr. P. Kohler.

TABLE I

Effect of Autologous* and Homologous* Anti–γ-Globulin Factors on the Agglutination of Bacteria by 7S γG
Isolated from Sera of Patients with Subacute Bacterial Endocarditis (SBE)

SBE serum from which γG was isolated	Latex titer of whole SBE serum	Latex titer of isolated RF	Increase in twofold agglutination titer with addition of isolated RF		
				Homologous	
			Autologous	CAR	SWE
SUN	10240	320	1	1	0
MIN	2560	40	4	0	0
MAY	640	80	1	3	3
GUL	640	40	0	0	0
WEA	640	640		0	0
SMI	40	80	0	1	1

* Autologous RF derived from the patient's own serum obtained during SBE; homologous RF refers to anti–γ-globulin factors isolated from patients with rheumatoid arthritis.

mologous) were absorbed with test bacteria before use. Although latex titers of the isolated RF preparations were lower than in whole sera, no further loss occurred during the absorption process.

Of the five autologous RF preparations derived from SBE sera, one, MIN, showed striking potentiation of the agglutination reaction, raising the titer four doubling dilutions. Two of the autologous SBE RF preparations demonstrated minimal potentiation, and two had no effect (Table I). By comparison, homologous RF obtained from patients with rheumatoid arthritis and used at equivalent reactivity showed greater augmentation in some cases, but less in others. These results were compared with similar experiments using rheumatoid factor preparations together with 7S antibacterial agglutinating antibody all derived from sera of patients with rheumatoid arthritis. In these instances, autologous RF obtained from serum of rheumatoid arthritis patients showed consistent maximum potentiation of bacterial agglutination when tested with the patients' own 7S antibody (Table II).

The effect of anti–γ-globulin factors on phagocytosis. Since engulfment and subsequent intracellular killing of organisms is an important mechanism in host response to infection, it seemed pertinent to study the effect of RF on phagocytic functions. Previous characterization of the opsonins in sera had revealed high titers of opsonic activity associated with 7S fractions in the 12 patients studied with SBE. Immune sera, there-

fore, were ideally suited for quantitative study of the effect of RF on opsonic activity.

Anti–γ-globulin factor preparations were absorbed with the test bacteria and adjusted to give a latex titer of 1:20 in the final phagocytic mixture. Control observations indicated that the bacterial species used in these experiments were not phagocytized without opsonin or in the presence of RF alone. Addition of 7S opsonin resulted in rapid uptake and killing of bacteria by the PMN's. The opsonic activity of these preparations was completely removed by absorption with the patient's infecting bacteria. Absorption with unrelated bacteria caused only slight reduction in

TABLE II

Effect of Anti–γ-Globulin Factors Isolated from Sera of
Patients with Rheumatoid Arthritis on Bacterial Agglutination Reactions Obtained with 7S γG Isolated
from Rheumatoid Arthritis Patients' Sera

RA serum from which 7S γG bacterial agglutinating antibody isolated by sucrose gradient ultracentrifugation	RF preparations obtained from serum of patients with rheumatoid arthritis used to potentiate bacterial agglutination reaction			
	CAR, 1:2560*	SWE, 1:5120	KIN, 1:320	FOS, 1:40
CAR	7‡	4	3	0
SWE	4	6	2	0
KIN	3	2	5	0
FOS	2	3	2	2
WIL	3	4	2	1
PET	5	4	3	1

* Indicates latex titer of RF preparation added.
‡ Numbers indicate increase in $\log_2$ dilution agglutination titer after addition of RF preparation.
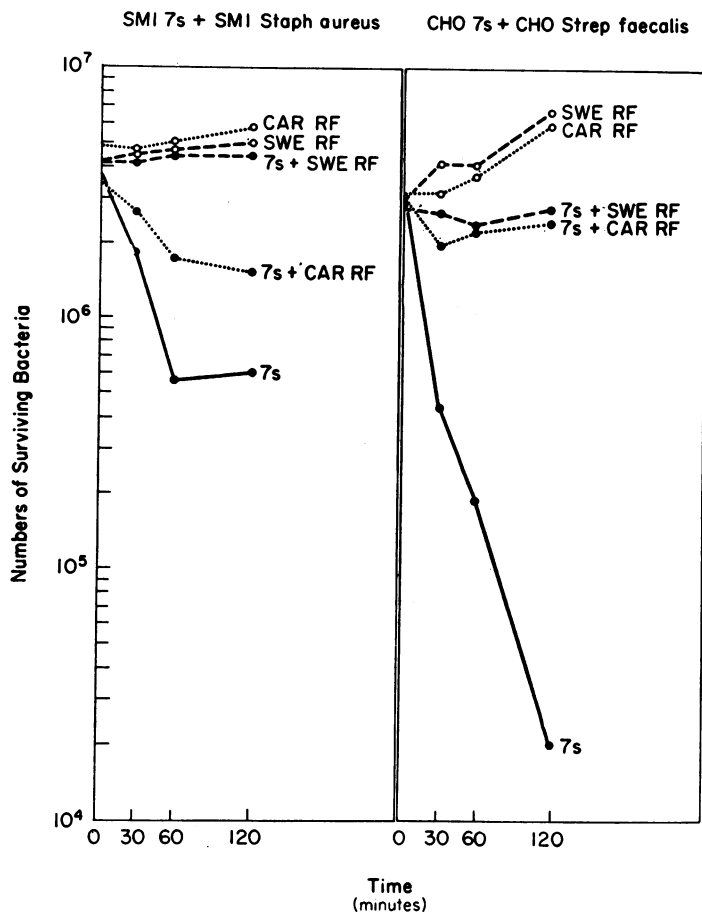
SMI 7s + SMI Staph aureus   CHO 7s + CHO Strep faecalis

FIGURE 1 Effect of homologous anti–γ-globulin rheumatoid factor preparations (RF) derived from patients with rheumatoid arthritis on opsonic activity of 7S antibody obtained from subacute bacterial endocarditis (SBE) patients. Bacteria were phagocytized and killed only in the presence of 7S antibody as can be noted in the curves labeled 7S. Addition of RF inhibited this opsonic action. Results using 7S opsonin from patient SMI and his own infecting bacteria (*staphylococcus aureus*) are indicated on the left; the results with 7S opsonin from patient CHO with SBE (*streptococcus faecalis*) are graphed on the right. No opsonic effect of RF preparations alone (CAR and SWE RF) was noted.

opsonic activity, which demonstrated specificity of the opsonin.

Striking inhibition of the opsonic activity of the 7S fractions was found with the addition of RF to the phagocytic system (Fig. 1). Anti–γ-globulin factor preparations inhibited the 7S opsonin in all five of the patients studied in this manner (Table III). The homologous RF, SWE, derived from serum of a patient with rheumatoid arthritis showed complete inhibition of opsonic activity of all 7S fractions examined. The inhibiting effect of homologous RF, CAR, also obtained from a rheumatoid arthritis serum, was not complete and varied in degree with 7S fractions from different SBE sera. The opsonic activity of γG fractions isolated by DEAE-cellulose chromatography as

TABLE III

Effect of Homologous Rheumatoid Factors Derived from Serum of Patients with Rheumatoid Arthritis on Opsonizing Capacity of 7S Serum Fractions Isolated from Patients with Subacute Bacterial Endocarditis

Patient	Organism	Per cent of bacteria phagocytized and killed in 120 min		
		7S	7S + CAR RF	7S + SWE RF
SMI	*Staphylococcus aureus**	84	67	0
THO	*Staphylococcus aureus*	65	24	0
WEA	*Staphylococcus epidermidis*	66	45	0
CHO	*Streptococcus faecalis*	99	30	0
WOL	*Streptococcus faecalis*	93		0

* Bacteria tested were not phagocytized in the absence of 7S opsonin.

1112   R. P. Messner, T. Laxdal, P. G. Quie, and R. C. Williams, Jr.

well as 7S fractions from sucrose density gradients was inhibited by homologous RF preparations, obtained from serum of patients with rheumatoid arthritis.

19 S fractions from five normal human sera isolated in an identical fashion with those containing RF were prepared and absorbed with test bacteria. No antiopsonic effect of such fractions was apparent using several test organisms. The possibility that 19S preparations containing RF activity affected phagocytic mechanisms on the basis of concomitant immunoconglutinin (17, 18) activity was considered. However, several preparations of isolated monoclonal γM with high titers of anti–γ-globulin and antiopsonic activity contained no detectable immunoconglutinin as assayed by the method of Coombs, Coombs, and Ingram (19) as modified by Bienenstock and Bloch (18).

Experiments were designed to test whether RF reacted with immune 7S opsonin before or after opsonin combined with bacteria. When 7S fractions obtained from SBE serum were preincubated with bacteria and the bacteria subsequently washed to remove the unbound antibody, there was no reduction of opsonic activity. In fact, the opsonic capacity of many 7S fractions was increased by this procedure. Control experiments in which 7S

opsonin was added to bacteria which had been washed after preincubation with RF revealed no reduction in opsonic activity ([SWE RF] + 7S, Fig. 2). Therefore, the antiopsonic activity of RF was not due to binding of RF itself to bacteria but required the previous binding of 7S opsonin. If RF derived from the immune SBE serum (autologous) or isolated from a serum of a rheumatoid arthritis patient (homologous) was added to bacteria which had been washed after incubation with opsonin, there was inhibition of opsonic activity similar to that observed when RF, bacteria, and opsonin had been incubated together.

The problems of interaction of bacteria, 7S opsonin, RF, and PMN were also studied in two additional ways. Bacteria were incubated with 7S opsonin, washed, and preparations of RF added. Subsequent washing of this mixture was followed by elution of bacteria-opsonin-RF pellets with acid glycine–saline buffer at pH 3.0. Material thus eluted from bacteria showed strongly positive reactions for RF by latex fixation reactions. Eluates from pellets of bacteria previously mixed with RF alone were negative.

In separate experiments, direct immunofluorescent staining of phagocytic mixtures of PMN, bacteria, 7S opsonin, and RF were performed with
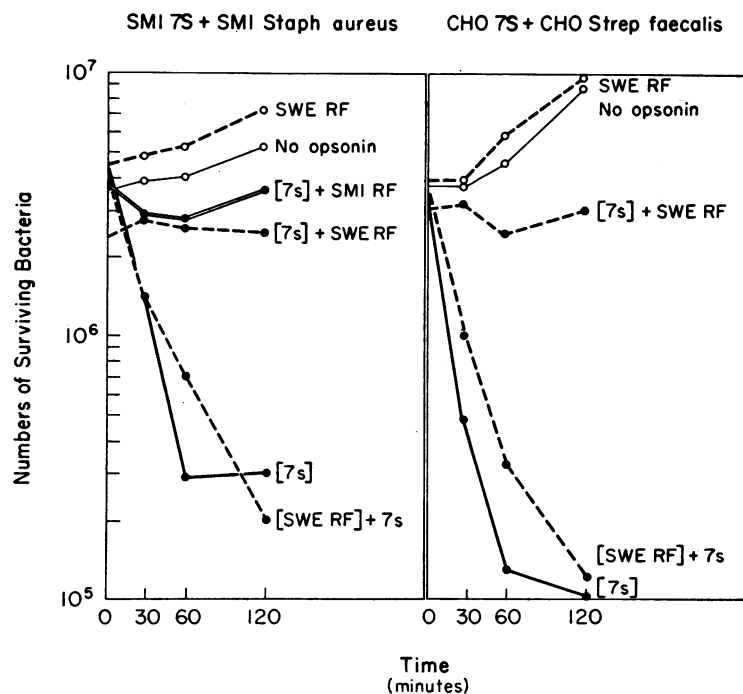

FIGURE 2 Effect of RF on phagocytosis of bacteria washed after preincubation with 7S γG opsonin. Bacteria were preincubated with the preparations shown in brackets. Autologous SMI RF was derived from the patient SMI with subacute bacterial endocarditis (SBE). Homologous SWE RF was isolated from serum of a rheumatoid arthritis patient. Addition of RF to bacteria washed after preincubation with 7S opsonin inhibited phagocytosis as shown by curves labeled [7S] + SMI RF and [7S] + SWE RF. Preincubation of bacteria with RF did not inhibit the action of 7S opsonin in either of the two representative experiments shown in curves labeled [SWE RF] + 7S. The experiment denoted to the left [7S] + SMI RF demonstrates antiopsonic effect of autologous RF derived from the SBE patient's own serum.

specific fluorescein-conjugated anti-γG and anti-γM antisera. Twice washed PMN alone stained brightly for both γM and γG. However, staining in mixtures of bacteria, 7S opsonin, and RF showed γG coating bacteria as well as strong anti-γM staining surrounding isolated bacteria. Conjugated antisera (previously absorbed with heat-killed bacteria) showed no staining of bacteria studied alone. It appeared that anti–γ-globulin factors could act on 7S opsonin bound to bacteria.

Isolated γG from five patients with SBE and two patients with rheumatoid arthritis used as sources of RF in the phagocytosis experiments were typed for Gm (a), (b), and (f). No distinct pattern of specificity was noted between the Gm type of the RF donors and the 7S opsonins which they inhibited.

It was considered that the formation of immune complexes might have a direct damaging effect on PMN and account for decreased phagocytic function. Therefore, in some experiments, the test bacteria were washed after incubation with 7S opsonin and RF before addition of PMN. The antiopsonic effect of the RF was unchanged, indicating that RF affected the opsonization of bacteria and not PMN function. Moreover, the effect of RF appeared to be on phagocytosis and not on the mechanism of intracellular killing. Quantitative bacterial counts on supernatant fractions and cell fractions separated by low-speed centrifugation indicated diminished phagocytosis in the presence of RF. There was no increase in numbers of leukocyte-associated bacteria in these preparations.

Comparison of agglutination potentiation and opsonin inhibition of anti–γ-globulin factors. The degree to which homologous RF preparations CAR and SWE obtained from patients with rheumatoid arthritis potentiated agglutination of bacteria by isolated 7S fractions did not correlate with inhibiting effect of the opsonic activity of the same 7S preparations. Agglutination of bacteria by immune SBE 7S antibody, CHO, was potentiated and opsonic activity inhibited by both homologous RF preparations (CAR and SWE, Table III). Agglutination of bacteria by 7S antibody, SMI, was weakly potentiated by CAR RF while SWE RF had no effect. On the other hand, SWE RF exerted a much stronger antiopsonic influence than CAR RF. Autologous RF SMI did

not potentiate agglutination by 7S antibody, SMI, but did inhibit its opsonic effect.

Effect of rabbit anti-human γG serum on phagocytosis. In attempts to elucidate the mechanisms of the antiopsonic effect of RF, specific anti-human γG rabbit antiserum was used in place of RF. These experiments were designed to determine whether other reagents with reactivity for γ-globulin would act in a similar fashion. The rabbit antiserum used showed specificity for H-chain determinants of γG and no cross-reactivity for γA or γM. Agglutinating titer of the rabbit anti-γG antiserum for cells coated with human incomplete Rh antibody Ripley was adjusted to equal that of the RF preparations previously used. When the rabbit antiserum was added either together with SMI 7S opsonin and bacteria, or after preincubation of the 7S opsonin and bacteria, inhibition of opsonic effect was noted (Fig. 3). The antiopsonic activity of the rabbit antiserum was equal to that of homologous SWE RF, derived from serum of a patient with rheumatoid arthritis.

Interaction of heat-labile factors and anti–γ-globulins in phagocytosis. Currently available data suggest that anti–γ-globulin factors may block the fixation of complement by 7S antibodies (20–

SMI 7S with SMI Staph aureus
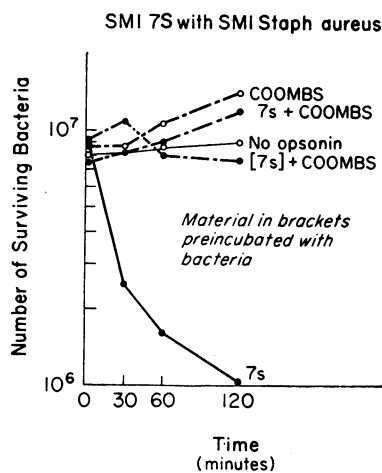

FIGURE 3 Rabbit anti-human–γG Coombs serum inhibited the opsonic activity of 7S serum fractions when added either together with the 7S opsonin and bacteria in curve indicated by 7S + Coombs or to bacteria preincubated with 7S opsonin ([7S] + Coombs). This result was similar to that observed with preparations of human anti–γ-globulin factors. Coombs serum alone, as indicated by the curve labeled Coombs, showed no inate opsonic effect.

22).[3] Since complement components may facilitate certain phagocytic systems (23–26), it was considered important to determine if complement and anti–γ-globulin factors acted competitively or in concert in the phagocytic system under study. Previous work (1) had demonstrated that serum containing only heat-labile opsonic factors (HLF), possessing hemolytic activity and presumably complement could be prepared by absorbing fresh serum with bacteria in the presence of ethylene-diaminetetraacetic acid and readjusting the concentration of calcium and magnesium. Serum prepared in this manner (HLF) was diluted 1:40 with gel-Hanks's medium and preincubated with opsonin and bacteria. The bacteria were washed and RF added. The antiopsonic effect of RF was markedly reduced by this process. Experiments were then performed in which titrations of these two reactants, HLF and RF, were used. When the dilution of HLF was held constant at 1:40 and a twofold greater concentration of RF was used, phagocytosis was diminished. If HLF was used in a dilution of 1:5 and the concentration of RF held constant, the antiopsonic effect of RF was obscured. When HLF was used diluted 1:60 and SWE RF used in a concentration to give a latex titer of 1:20 in the final mixture, directly competitive effects between HLF and RF became apparent (Fig. 4). If HLF was added to bacteria washed after incubation with 7S opsonin and RF, the antiopsonic effect of the RF predominated. However, if RF was added to bacteria washed after incubation with 7S opsonin and HLF, the opsonic effect of the HLF was dominant. If both the RF and HLF were added simultaneously to bacteria washed after incubation with 7S opsonin, the effect was intermediate (Fig. 4). Neither HLF in a dilution greater than 1:40 nor RF showed opsonic activity when added alone.

Effect of RF on the opsonic properties of whole serum. When whole sera from patients with SBE were employed as opsonin some similarities, but also interesting differences, were noted.

In previous experiments homologous RF SWE derived from a patient with rheumatoid arthritis had consistently exerted a strong antiopsonic effect on 7S opsonin from the serum SMI (Figs. 1 and

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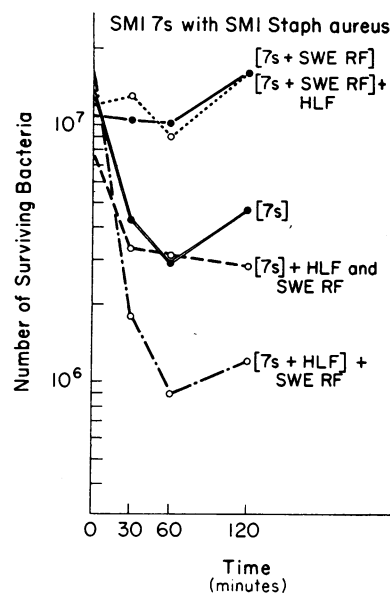[3] Messner, R. P., and R. C. Williams, Jr. 1967. Unpublished data.

FIGURE 4 Interaction of heat-labile factors and RF with 7S opsonin. Bacteria were washed after preincubation with the preparation shown in brackets. Heat-labile factors (*HLF*) and RF had opposite, competitive effects on immune 7S opsonic activity.

2, Table III). When added to heated SMI whole serum, however, no antiopsonic effect of RF was noted. When RF was added to bacteria washed after incubation with whole serum SMI, antiopsonic effect was again demonstrated (Fig. 5). These results obtained with whole serum SMI were reproducible on numerous occasions.

A much different result was noted with heat-inactivated whole serum, TOM. Preparations of RF enhanced phagocytosis when added to bacteria incubated with this whole serum. The addition of RF to bacteria washed after incubation with whole serum inhibited phagocytosis as it had previously with the isolated 7S opsonin. The results from one of two similar experiments with TOM whole serum are shown to the right of Fig. 5. The opsonic effect of TOM serum was also increased if the bacteria were washed after preincubation with the serum. In other experiments, it was noted that the HLF opsonic activity, with whole serum depleted of specific antibacterial antibody, was increased by washing opsonized bacteria before the addition of PMN.

To extend these observations, two other heat-inactivated whole sera from patients, SHR and BEC, with staphylococcal SBE were studied for
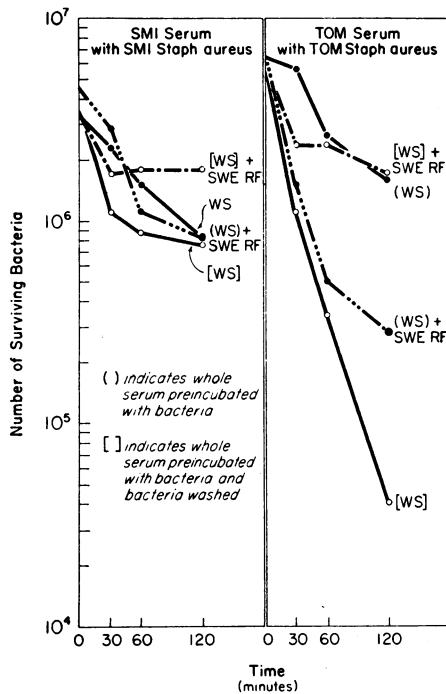
FIGURE 5 Effect of homologous RF obtained from patients with rheumatoid arthritis on opsonic properties of heat-inactivated immune whole serum from two patients with subacute bacerial endocarditis. On the left, opsonic curve of whole serum alone is indicated by curve labeled WS. Addition of RF to bacteria incubated with SMI serum had no effect (WS) + SWE RF but addition of RF (SWE) to bacteria, washed after preincubation with inactivated whole serum showed slight antiopsonic effect. On the right are shown results of addition of homologous RF SWE to various phagocytic systems with inactivated immune whole serum TOM. Basic opsonic curve is denoted by (WS) indicating whole serum was preincubated with bacteria. When whole serum was preincubated with test bacteria and homologous RF SWE added, apparent enhancement of phagocytosis occurred (WS) + SWE RF. In addition, basic whole serum opsonic activity was potentiated by preincubation of inactivated whole serum and washing as shown in curve labeled [WS]. In this latter instance, when RF was added, antiopsonic effect was again demonstrated ([WS] + SWE RF). Thus, this whole serum opsonin, obtained from heat inactivated serum, could be blocked by RF SWE after the bacteria were preincubated with whole serum and washed to remove excess serum components. In this respect the antiopsonic effect of RF resembled that seen with isolated 7S opsonins. (Figs. 1, 2).

their interaction with six different homologous RF preparations obtained from patients with rheumatoid arthritis. These SBE sera contained no intrinsic or autologous RF. In all instances, no direct antiopsonic effect was noted with whole undiluted

serum opsonin and RF. Slight potentiation, as with serum TOM above, occurred in two instances. Again as in the case of experiments with whole sera TOM and SMI when whole serum opsonin was added to bacteria, the mixture washed, and RF added, distinct antiopsonic effect could be demonstrated. In like manner, when whole serum opsonin was used in increasing dilutions and RF preparations added to phagocytic mixtures, moderate antiopsonic effects were noted in all of 12 experiments. Representative experiments with several dilutions of heat-inactivated whole serum opsonin from patient SHR are shown in Fig. 6. Antiopsonic effect of RF CAR can be seen at the left of Fig. 6 where heat-inactivated whole serum opsonin was applied to bacteria and then excess serum removed by washing before the addition of RF preparations. No antiopsonic effect was demonstrated when whole serum opsonin diluted 1:20, RF, and PMN were added simultaneously. However, further dilution of the heat-inactivated whole serum opsonin SHR to 1:30 (Fig. 6, right diagram) allowed two homologous RF preparations to exhibit moderate antiopsonic activity. Thus, RF seemed capable of blocking 7S or whole serum opsonin most effectively when acting in isolated or dilute systems.

A series of experiments using whole serum opsonins from patients SHR and BEC with staphylococcal endocarditis were then carried out to define the effect of anti–γ-globulin factors in the presence of whole fresh serum opsonin as compared to whole serum inactivated at 56°C before use. Such experiments were believed to approximate more closely the situation which might exist in vivo. These phagocytic assays indicated that six anti–γ-globulin factors isolated from sera of rheumatoid arthritis patients exerted no antiopsonic or potentiating effect in the presence of fresh noninactivated whole serum. However, as shown in Figs. 5 and 6, distinct antiopsonic effect of RF could be demonstrated with sufficiently dilute whole serum opsonins previously inactivated at 56°C; this was always more pronounced if excess serum components were removed by washing before the addition of anti–γ-globulin factor preparations. From these results it seemed clear that the antiopsonic effect of RF could not overcome positive opsonic factors present in fresh native immuneserum.
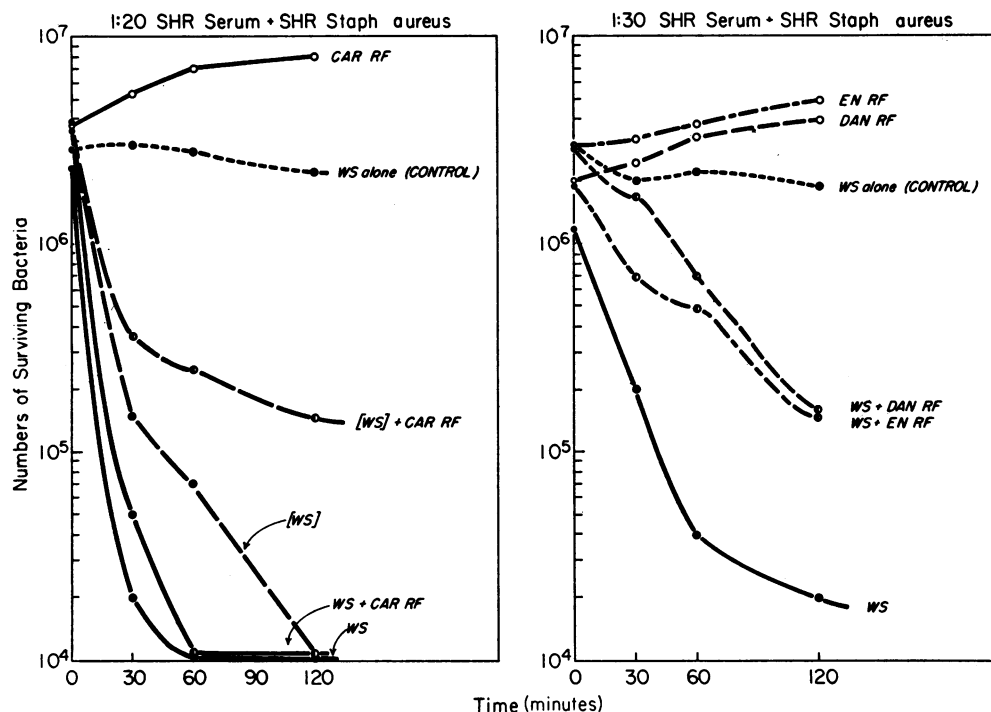
FIGURE 6 To the left is shown opsonic activity of whole serum opsonin SHR from patient with *Staphylococcus aureus* endocarditis. Serum was inactivated at 56°C before use and used at 1:20 dilution. Curve labeled WS indicates efficient opsonic activity of this immune serum. Addition of RF CAR (obtained from patient with rheumatoid arthritis) produced no demonstrable change in phagocytosis as seen in curve labeled WS + CAR RF. When inactivated whole serum opsonin was preincubated with bacteria and excess serum removed by washing, antiopsonic effect of CAR RF was noted ([WS] + CAR RF). The control curve for effect of washing opsonin treated bacteria is shown by line labeled [WS]. No opsonic or killing effect of whole serum alone or RF preparation CAR was present as can be noted in the two upper curves.

To the right are shown representative experiments using a higher dilution (1:30) of heat-inactivated whole serum opsonin from SBE patient SHR. In this instance two RF preparations obtained from sera of patients with rheumatoid arthritis, DAN RF and EN RF, showed moderate antiopsonic effect (WS + DAN RF and WS + EN RF). No antiopsonic effect of these RF preparations was demonstrated when added to fresh noninactivated whole serum opsonin. Anti–γ-globulin factors were not capable of blocking opsonic capacity of native fresh immune serum from patients with subacute bacterial endocarditis.

DISCUSSION

Anti–γ-globulin factors obtained from immune SBE serum (autologous) or isolated from unrelated patients with rheumatoid arthritis (homologous) were found to exhibit varying activity in augmenting bacterial agglutination by 7S antibacterial antibody. Thus, their action with this antigen-antibody system is analogous to that described with other systems (27, 28).

The inhibiting effect of homologous and autologous anti–γ-globulin factors on specific immune 7S opsonins from sera of patients with SBE was an unexpected finding. From the results of experiments with washed complexes of bacteria and 7S opsonin, it was evident that preparations containing RF exerted this effect on opsonin bound to bacteria rather than by combining with opsonin before its attachment. Specific rabbit anti-human γG antiserum demonstrated the same inhibiting effect on 7S opsonin. This supports the concept that the antiopsonic action of RF is related to its combination with γG opsonin. The paradox of antiopsonic activity of RF and concomitant potentiation of bacterial agglutination suggests that ob-

served antiphagocytic effect was not related to aggregation of bacteria. The antiphagocytic effect of RF appeared to be exerted against some inherent opsonic property of the γG molecule.

Several methods of treating γG are associated with reduction in opsonic activity. Rowley, Thöni, and Isliker (29) have shown that heat aggregation of γ-globulin at 63°C for 10 min destroys opsonic effect. In many systems, heat aggregation may produce some loss of specific antibody function. In the experiments reported here, opsonins specifically combined with bacteria were inhibited by RF. In addition, opsonins in unfractionated serum were inhibited by RF if bacteria were washed after incubation with serum. Thus, alterations or aggregates formed in the isolation of γG opsonin do not appear to explain the antiopsonic effect of the anti–γ-globulin preparations studied. Other methods of inactivating opsonin involve splitting of γG by papain or pepsin. Shands, Stalder, and Suter (30) and Rowley (29) have shown that enzyme split preparations of γG devoid of Fc have considerably diminished opsonic capacity. Human anti–γ-globulin factors as well as specific rabbit anti-γG antisera show primary reactivity for the Fc portion of the γG molecule (31). Antiopsonic effect of RF might involve interference with, or alteration of sites on the Fc fragment essential to opsonic activity. The combination of anti–γ-globulin antibodies with γG could thus be analogous to the condition in which the Fc fragment is removed by proteolytic digestion.

There is some additional indirect evidence to support the concept of a specific phagocytosis promoting site on the Fc portion of the γG opsonin, activated when opsonin combines with antigen. Rowley and Turner (32) have demonstrated that relatively few molecules of opsonin are necessary for the phagocytosis of one bacterium. This observation is more compatible with a specific phagocytosis-promoting mechanism than the concept of a buttering layer of opsonins. A conformational alteration of γG sufficient for the specific function of promoting phagocytosis may be formed during combination with bacterial antigen. Such changes are apparently responsible for reactivity of γG with some anti–γ-globulins (31, 33, 34).

Competition between RF and the complement system has been demonstrated with several antigen-antibody reactions (20–22, 35–37). The experiments reported here have shown that effects on phagocytosis may also be competitive. The initially reacting complement component (C'_{1q}) and RF are known to attach to closely adjacent but separate sites on the Fc fragment (38). Competition for binding sites might explain the observed antagonistic actions. However, the rate-limiting complement components most active in phagocytosis are not yet clear, and must be individually studied among distinct Gram-positive or Gram-negative bacterial species. The observed antagonistic effects of RF and heat-labile serum factors on phagocytosis may depend primarily on a balance of the antiopsonic effect of RF on γG opsonin and the positive opsonic effect of complement constituents fixed to the bacterial cell wall (39).

Parker and Schmid (40) demonstrated that heat-aggregated γG was phagocytized much more avidly in fresh serum containing RF than in fresh normal serum. The heat-aggregated γG used by these workers had probably lost any intrinsic phagocytosis-promoting properties native to specific antibody. If the effect of RF in the antibacterial antibody system is due to inhibition of phagocytosis-promoting properties of γG exposed by combination with bacteria, its action may be very different in these two situations. The observation that anti–γ-globulin factors can in some instances promote phagocytosis (40) suggests that they do not possess universal antiphagocytic properties.

Anti–γ-globulin factors were found to exert an antiopsonic effect when bacteria were preincubated with whole serum and the excess serum removed. These conditions may simulate those with isolated 7S opsonin. On the other hand, if phagocytosis was allowed to take place in the presence of undiluted heated whole serum, no consistent antiopsonic effect was noted, and indeed in occasional instances potentiation was seen. The significant finding that preparations of several anti–γ-globulin factors did not block phagocytosis in the presence of native fresh whole serum opsonin indicates that potent opsonic activity of such fresh immune sera prevails to insure phagocytosis. Whether the major positive opsonic activity is entirely due to heat-labile complement components capable of easily overcoming anti–γ-globulin factors is not yet clear. Direct competitive experiments with purified com-

plement components and RF must now be performed to answer this question.

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