Detection of staphylococcal antibodies in human sera

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Diagnostic detection of *Staphylococcus aureus* antibodies in patients suspected of having a staphylococcal infection has always been a problem. No satisfactory serologic test has yet been described, although there has been a renewed interest in the matter and new data have been provided. We are going to present studies on an indirect haemagglutination (IHA) technique for the detection of antibodies to teichoic acid. The results did not turn out as well as the first experiments had indicated. We therefore find it desirable to discuss why an anti-staphylococcal antibody rise during disease is so difficult to demonstrate.

Knowledge of the antigens, their immunogenicity and specificity is a necessary basis for the diagnostic detection of antibodies. The extracellular antigens of *S. aureus* are quite well known. Most of what we know of the cellular antigens, however, comes from research performed during the last 20 years (see Oeding, in press). Regarding the antibody response in man and the diagnostic detection of antibodies, the cellular antigens may at present be of primary interest. In the first part of our paper we will briefly review our present knowledge of the cellular antigens. The extracellular antigens and capsular antigens will not be discussed here.

Cellular Antigens

S. aureus produces many cellular and extracellular substances (fig. 1). These components possess a variety of functions in addition to their antigenic activity, making the pathogenesis, immune response and immunity unusually complex. The staphylococcal cell wall contains two covalently linked major substances: the peptidoglycan (PG) and the wall teichoic acid, which, in *S. aureus*, is of the ribitol type (RTA). The cytoplasmic membrane is composed of glycerol teichoic acid covalently linked to a glycolipid

is composed of glycerol teichoic acid covalently linked to a glycolipid moiety, lipoteichoic acid (LTA). The *S. aureus* cell wall contains the unique protein A (pA) in addition to small amounts of a number of carbohydrate and protein antigens.

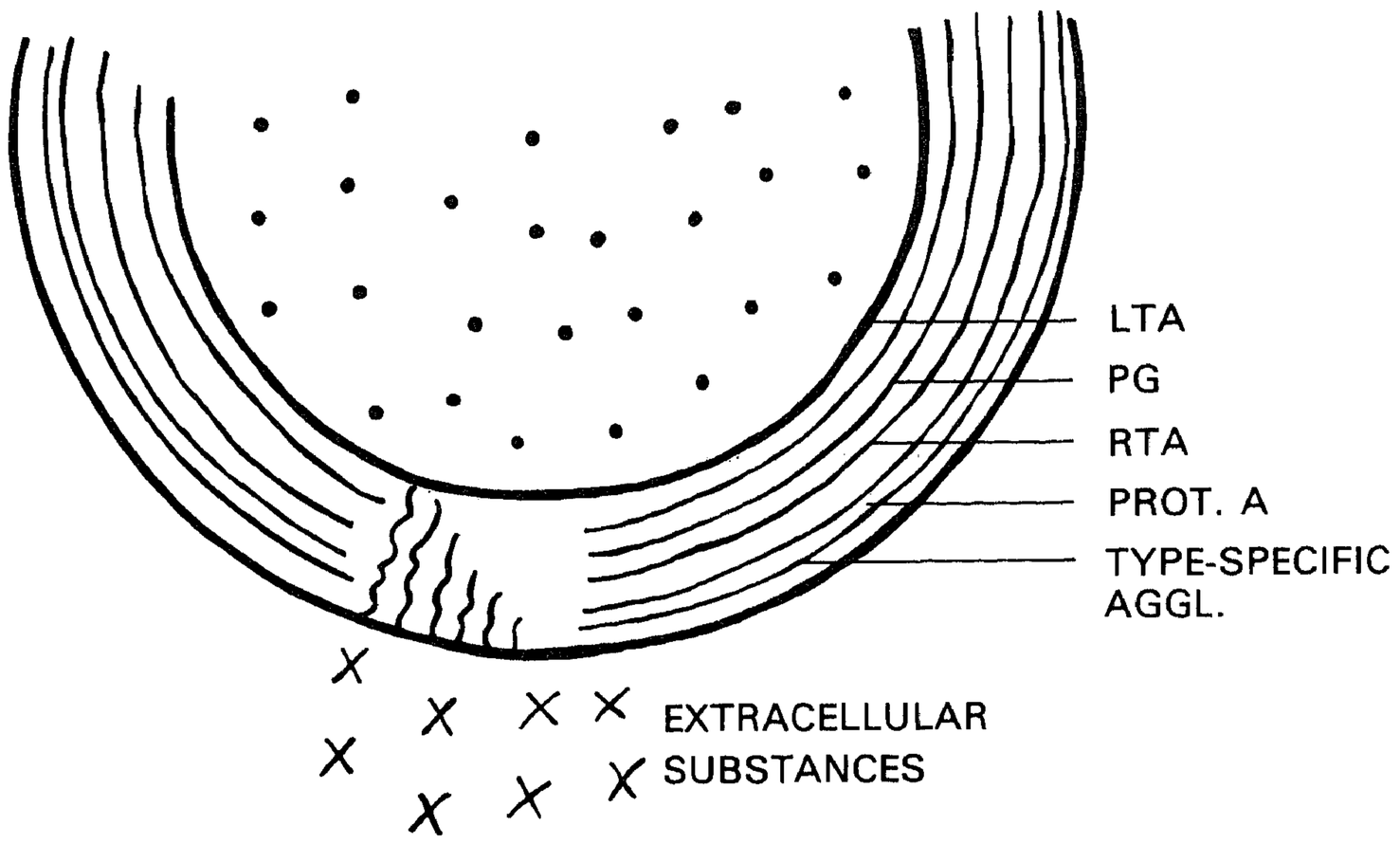

Fig. 1 Schematic representation of the *S. aureus* cell wall and cytoplasmic membrane.

The PG forms a tight structure around the entire wall. Immunological techniques combined with ultramicroscopic examinations of whole cells have shown that the components of the cell wall are intimately integrated. Antigenic determinants from all the components, including the LTA, may be exposed on the cell surface (indicated by wavy lines in fig. 1) and may thus be able to react with the homologous antibodies.

The PG is the only cell wall polymer common to gram-negative and gram-positive bacteria (table 1). PGs with a high content of glycine are typical of staphylococci. The peptide moiety shows considerable variation particularly in the interpeptide bridges which possess specific antigenic determinants. Many gram-positive bacteria, including all staphylococci, contain wall teichoic acids (WTAs) of varying types and quantities. Except for phage 187

strains, essentially all *S. aureus* strains contain a species-specific RTA with N-acetylglucosaminyl substituents, usually in the β-position. LTAs occur in all gram-positive bacteria. The common glycerol phosphate backbone has a broad antigenic specificity (Rantz antigen) whereas the sugars are species-or type-specific. pA and the minor carbohydrate and protein antigens are present only in *S. aureus*. pA is particularly known for its ability to combine non-specifically with the Fc part of IgG. However, it also has a species-specific Fab reactivity. The minor antigens are type-specific.

TABLE I

Occurrence and antigenic specificity of *S. aureus* cell wall and cytoplasmic membrane substances

Substance	Occurrence	Antigenic specificity
PG	Gram-pos. and gram-neg. bacteria	Species-specific (?) interpeptide bridge determinant(s)
WTA	Many gram-pos. bacteria, all staphylococci	Species-specific N-acetylglucosaminyl RTA
LTA	All gram-pos. bacteria	Heterogenetic (Rantz) glycerol phosphate. Sugar specificity
pA	*S. aureus*	Non-specific Fc-reactivity. Species-specific Fab-reactivity
Type-specific agglutinogens	*S. aureus*	Type-specificity

PG : peptidoglycan
WTA : wall teichoic acid
LTA : lipoteichoic acid
RTA : ribitol teichoic acid
pA : protein A

When whole *S. aureus* cells are injected into rabbits, potent antibodies are produced against PG, WTA and LTA. When the substances are given in an isolated form, they are non-immunogenic. Rabbit antisera against PG contain haemagglutinating antibodies of different specificities. Most antisera have more antibodies against the peptide moiety of PG than against the glycan moiety. Antibodies against the LTA glycerol phosphate backbone are formed regularly. In antisera against the *S. aureus* RTA, high levels of antibody against the immunodominant amino sugar are found. High levels of antibodies are also produced against the type-specific antigens, both when whole cells and when isolated antigens have been injected.

Staphylococcal Antibodies

It is well-known that most adult human sera possess a variety of anti-staphylococcal antibodies. This is due to the nasal carrier state coupled with repeated subclinical or minor infections. However, antibodies in human sera reacting with *S. aureus* may also be non-specific, due to exposure to other bacteria containing cross-reacting antigens (table I).

The anti-staphylolysin (ASta) test with α-haemolysin or more recently with γ-haemolysin was for many years used diagnostically to detect staphylococcal antibodies in human sera. Few laboratories. however, use the ASta test today because it is considered to be of little diagnostic help. Serious staphylococcal infections are often not recognized, and high antibody levels are found in non-staphylococcal diseases such as chronic polyarthritis and spondylitis ankylopoetica (Schaefer & Zeidler, 1970). When patient sera with high 'non-specific' ASta titres were treated with dextran sulphate-$CaCl_2$ or heparin-$MnCl_2$, we observed no reduction of the ASta titres. Therefore, the high ASta titres in these sera are not due to non-specific inhibitors of the β-lipoprotein (LDL) serum fraction. By fractionation of serum on a DEAE ion exchange column, the majority of the ASta activity was found in the IgG peak, and pepsin treatment showed that the antibodies bind to the haemolysin through their Fab portion. When the active IgG fractions were run through a pA Sephadex column, almost all ASta activity was retained and could subsequently be eluted. It was concluded that the high ASta levels found in rheumatic patients are due to an increase of specific staphylococcal IgG or cross-reacting auto-antibodies.

pA creates special problems for the diagnostic detection of *S. aureus* antibodies because of its reactivity with the Fc portion of immunoglobulins, particularly IgG. Therefore, cells or antigen preparations used for the detection of antibodies should not contain pA. A *S. aureus* strain not possessing pA, e.g. strain Wood 46, is preferable. If a strain containing pA (Lafferty strain) is used, pA must be completely removed from the extract on an immunosorbent column.

Specific agglutination of *S. aureus* cells in human serum is difficult, not to say impossible, to detect. If a strain not containing pA is used for the detection of staphylococcal antibodies, non-specific agglutination does not occur. But *S. aureus* contains such a variety of type-specific surface agglutinogens varying from one strain to another that this diagnostic procedure can be excluded. When the infecting strain is at hand, it will regularly contain pA which causes strong non-specific agglutination (Forsgren & Nordstrøm, 1974) masking specific agglutination. pA is not

found in coagulase negative staphylococci. In serious infections, such as septicaemia and endocarditis, caused by *S. epidermidis*, bacterial agglutination can therefore be used with advantage when the organism has been isolated and the aetiological relationship ought to be established. We have observed a great increase in agglutination titres during such infections.

Our knowledge of PG and LTA is still too limited for their use in the diagnostic detection of *S. aureus* antibodies. However, such antigens represent interesting possibilities. Today, the RTA is the only cell wall antigen which is suitable for use. This substance is present in almost all strains of *S. aureus*, it is easily purified and is strongly immunogenic in a complexed form. Although the specificity is good, it should be remembered that β-N-acetylglucosamine is an antigenic determinant of many substances.

Several authors have during recent years reported on the use of crude or purified RTA preparations for the detection of antibodies in the sera from patients with different types of staphylococcal infection (Crowder & White, 1972; Nagel *et al.*, 1975; Parker & Fossieck, 1976; Flandrois *et al.*, 1979). Double diffusion in agar and countercurrent immunoelectrophoresis (CIE) have been used. Even by the insensitive double diffusion test, antibodies were often detected in control sera. Generally, precipitation tests are not very suitable for measuring antibodies, and precipitins appear late. Indirect haemagglutination (IHA) tests are more sensitive and become positive sooner.

Indirect haemagglutination tests

The IHA test was used for the detection of anti-teichoic acid antibodies in patients' sera. The tests performed in Bergen were compared with CIE (performed in Lyon). In both tests, purified β-glucosamine RTA from *S. aureus* strain Wood 46 was used as an antigen. Finally, the tests were compared in Bergen with a standard α-ASta test.

Sensitization of erythrocytes with RTA is not possible using the conventional methods (Oeding, 1965; Yoshida & Ekstedt, 1968; Grov, 1969). Therefore, sheep erythrocytes were senstized with β-glucosaminyl RTA using chromium chloride ($CrCl_3 \cdot 6H_2O$) as the coupling agent (Baker *et al.*, 1969; Brock & Reiter, 1971; Oeding *et al.*, in press). A 0.1 mg per ml solution of the RTA prepared according to Haukenes (1962) was used for sensitization. Serial dilutions of human sera were made in 0.5 per cent normal rabbit serum in PBS, starting with a 1:8 dilution. Thereafter 0.5 per cent sensitized erythrocytes were added. Readings were made after 2 h at room temperature. A rabbit antiserum produced against whole Wood 46 cells (Oeding *et al.*, 1964), and a high-titred human osteomyelitis serum,

served as controls. The agglutination patterns were easy to read, and the reproducibility of the test was good. We did not find it necessary to remove heterophil antibodies by absorption of the sera with non-sensitized chromium chloride coupled erythrocytes (Oeding *et al.*, in press).

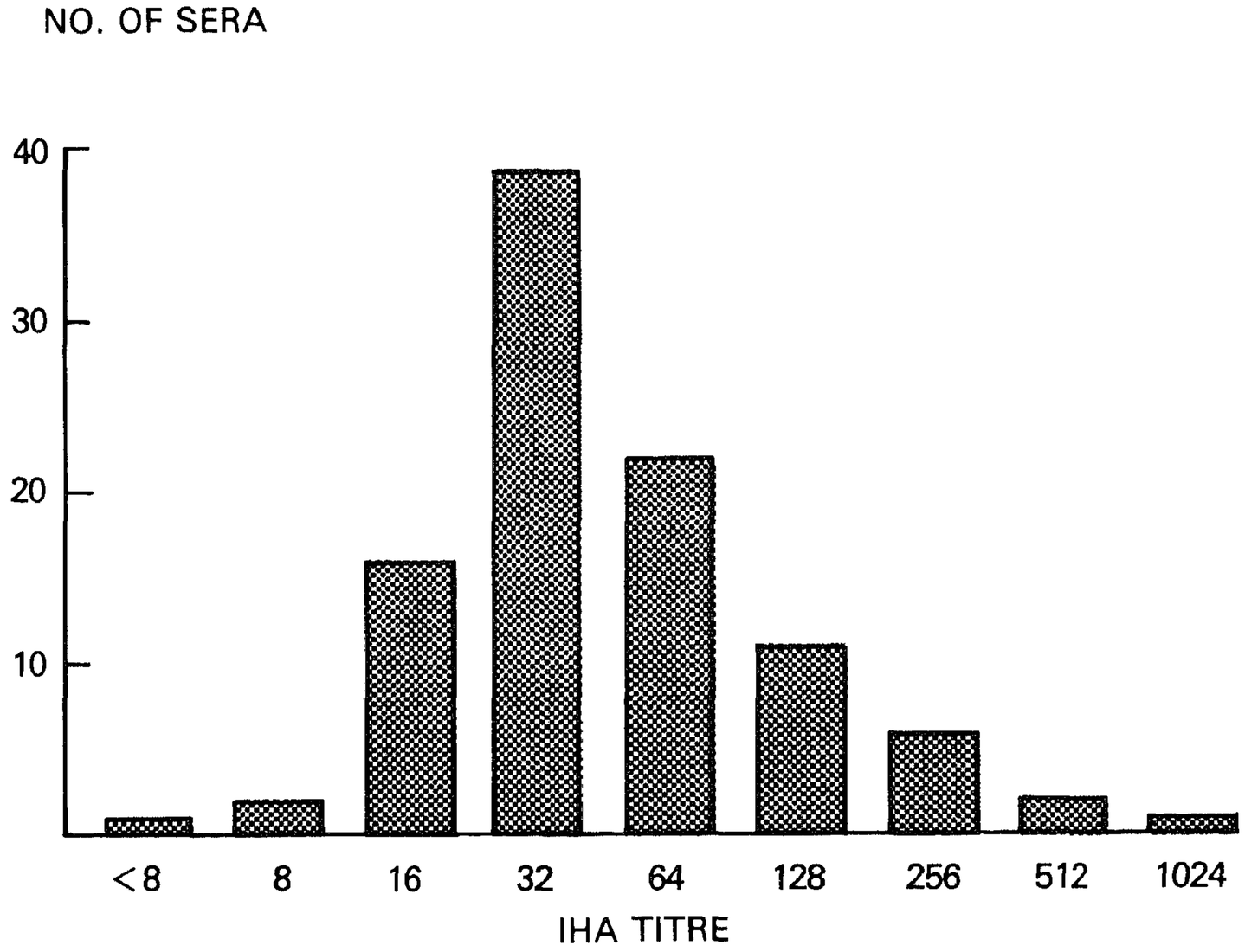

Fig. 2 Distribution diagram of 100 sera from blood donors in IHA using Wood 46 RTA as antigen.

CIE was performed by the procedures described by Flandrois *et al.* (1979). The ASta test was performed according to standard procedures using α-toxin produced from *S. aureus* Wood 46. The IHA test as well as the ASta test were performed in microtitre plates.

One hundred sera from blood donors in Bergen served as control material for the IHA test. The distribution curve for the IHA titre values was approximately normal (fig. 2), although it was somewhat skewed to the right hand side and had heavy tails. These abnormalities might have been avoided with closer dilution intervals and a larger number of sera. Twenty per cent of the sera had titre values $\geqslant 128$.

The IHA test was compared to the CIE test. Eighty sera from patients hospitalized in Bergen and Lyon, with different types of staphylococcal infections or other diseases, were tested (fig. 3). A statistically significant correlation was found between the two tests both by the Spearman Rank correlation test (0.69) and by the Pearson correlation test. This could be expected since the same antibody is detected. Provided that the techniques were adequate, one explanation of the aberrant values observed may be that different Ig classes are detected. The heavy lines (fig. 3) indicate the border values for the two tests. IHA values $\geqslant 128$ and CIE values $\geqslant 8$ (Flandrois *et al.*, 1979) have been estimated as elevated.

CIE TITRE

	<8	8	16	32	64	128	256	512	1024	2048	4096
128											1
64								1			
32					1		2	2	2	1	
16					1	3	6	4	1		
8				5	3	5	5	1			
4		1		2	4	3	1				
2		1		3	4		1				
1			2		3	1					
<1		1	4	1	1	2	1				

IHA TITRE

Fig. 3 Comparison of IHA and CIE using Wood 46 RTA as antigen. Numbers show distribution of 80 sera from patients having *S. aureus* infections and patients not known to have such infections.

No clear correlation was observed between the IHA test and the ASta test by examination of control sera (blood donors, and patients not known to have staphylococcal infection) and sera from hospital patients in Bergen and Lyon with serious staphylococcal infections. In the sera from staphylococcal patients (fig. 4), no strong correlation was found based on a

Spearman Rank correlation (0.28). The low correlation was expected since antibodies of different specificities are measured (Flandrois *et al.*, 1979). These antibodies apparently do not occur in parallel in the sera. Based on an asymptotic standard error we find a t-value = 2.44, which shows that the correlation is significantly different from zero ($p < 0.01$).

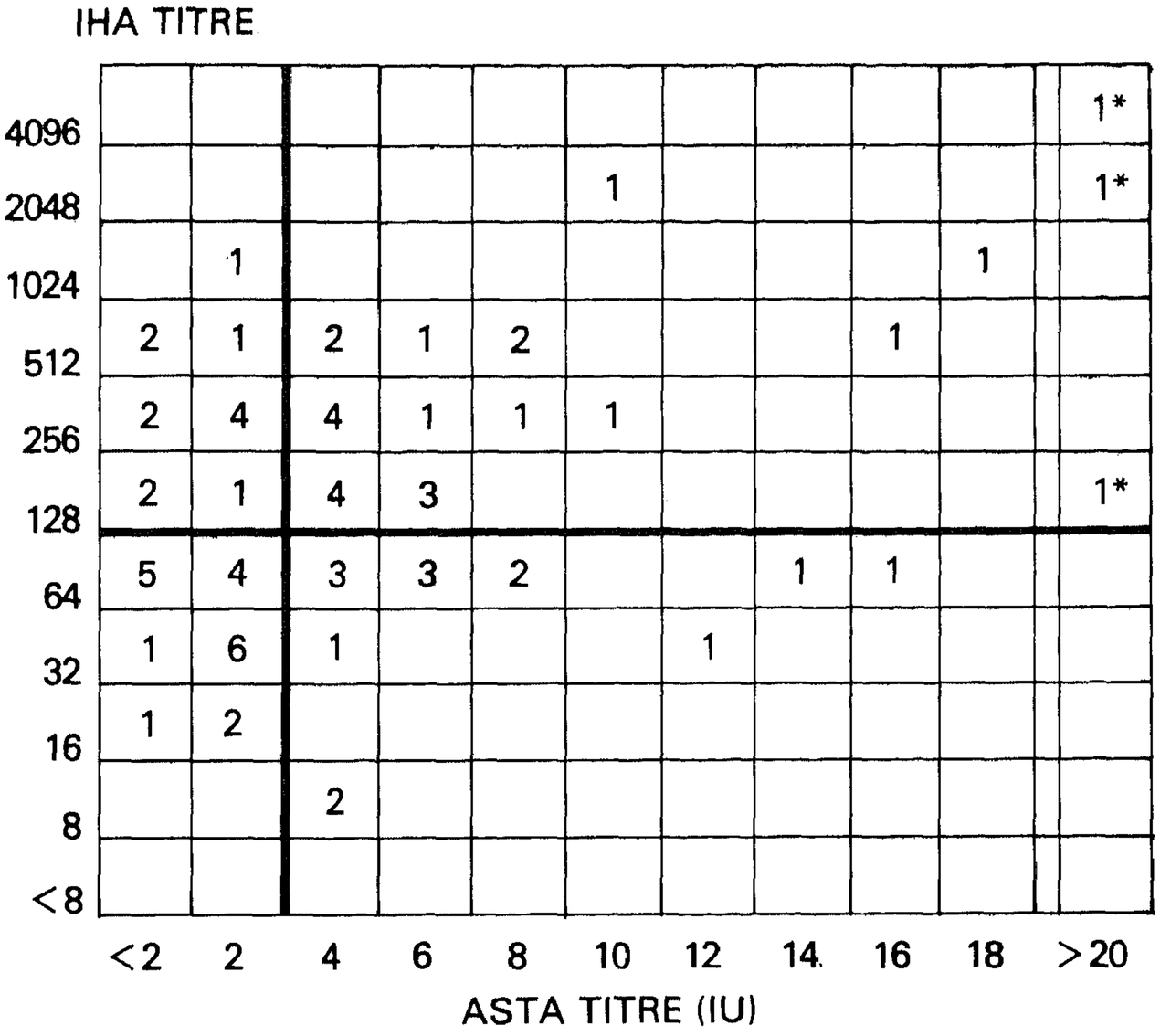

Fig. 4 Comparison of IHA and ASta test using respectively Wood 46 RTA and α-toxin as antigens. Numbers show distribution of 71 sera from 43 patients having serious, bacteriologically verified *S. aureus* infections.

* ASta titres 26, 40, 40.

Only 38 sera out of 71 (53.5 per cent) from the patients with serious, bacteriologically verified staphylococcal infections had IHA titres $\geqslant 128$, and 27 had titres $\geqslant 256$ (fig. 4). Raised ASta titres ($\geqslant 4$) were found in an equal number of sera (39). Both tests showed elevated titres in 25 sera, the IHA test alone was increased in 13 sera and the ASta test alone in 14 sera. Combined, the two tests showed elevated titres in 52 out of the 71 sera. In a

few cases, high titres were recorded by one test, while the titres were not elevated or were only slightly elevated in the other test. The finding that the results of the ASta test were as good as those of the IHA test was surprising in view of the generally low opinion of the ASta test.

Three out of the 4 patients (5 sera) with endocarditis had high IHA titres ($\geqslant 512$) (table II). In contrast, only 2 out of the 6 patients (11 sera) with deep abscesses or pneumonia had increased titres. Elevated titres were found in a somewhat higher proportion of osteomyelitis sera compared to septicaemia sera. In superficial staphylococcal infection (not included in the material) IHA titres $\geqslant 128$ were only exceptionally found.

TABLE II

IHA titres related to staphylococcal disease

IHA titres	Endo-carditis	Septi-caemia	Osteo-myelitis	Deep abscess, pneumonia
Number of sera	5	26	29	11
4096	1			
2048		1	1	
1024	1	1		
512	1	4	4	
256		4	7	2
128	1	2	7	1
64		6	5	8
$\leqslant 32$	1	8	5	

A group of 125 sera selected from patients with symptoms from the joints and muscles in addition to other patients with increased ASta values, were examined by the IHA test. The patients were not known to have staphylococcal infection. Forty-eight per cent of all sera had IHA titres $\geqslant 128$ and 22 per cent had titres $\geqslant 256$. Of the 61 sera from rheumatic patients, 27 had elevated IHA titres (table III) while 38 had elevated ASta titres. Twenty sera had elevated titres in both tests. In patients with verified

or suspected *Streptococcus pyogenes* infections and in patients with different types of skin diseases, the IHA titres were surprisingly often elevated. In *S. pyogenes* infections the elevated IHA titres may be due to the presence of N-acetylglucosamine in the C antigen. In rheumatic diseases and skin diseases the elevated IHA (and ASta) titres may be explained by a non-specific stimulation of staphylococcal antibody production or by cross-reacting auto-antibodies. Although the IHA titres were generally higher in serious staphylococcal infections (fig. 4), the elevated titres seen in patients with rheumatic diseases, skin diseases and streptococcal diseases create a serious problem in the evaluation of the serological results.

TABLE III

IHA titres in patients not known to have staphylococcal infection

IHA titres	Rheumatic diseases	*S. pyogenes* infection	Skin diseases
	Number of sera		
	61	15	19
⩾128	27	11	11
⩾256	15	2	8

DISCUSSION

The anti-RTA tests have been reported to be of value in the diagnosis of endocarditis, and for the distinction of complicated from uncomplicated bacteraemia (Crowder & White, 1972; Jackson *et al.,* 1978; Lé & Lewin, 1978). In deep staphylococcal infection, including osteomyelitis, the titre values are frequently not elevated (Lé & Lewin, 1978; Editorial, 1979). Altogether, the anti-RTA tests seem to be of limited value in the diagnosis of serious staphylococcal infections. This conclusion can be drawn from the present results with the IHA anti-RTA test as well as from other recent evaluations (Editorial, 1979; Martin *et al.,* 1979; Wheat *et al.,* 1979). The rise in antibody titre is usually not high, and it occurs irregularly and unpredictably. Only rarely are the titres so high that a single result can be considered as conclusive evidence of a staphylococcal infection. Often the

titres are not significantly higher than those found in many healthy individuals. A four-fold rise in titre is found only exceptionally. Typical titre curves like those seen in many other infections are not observed. Less rigorous requirements should not be put to anti-staphylococcal antibody tests than to other serological tests. Otherwise the results may be subjected to arbitrary evaluation.

In the present situation we must realize that a single test will probably not detect more than 50 per cent of all serious staphylococcal infections. The individual results depend on earlier experience with staphylococci in addition to the booster effect of the various antigens in the new infecting strain. The diagnostic yield can be improved by combining tests for the detection of different antibodies. This has been shown by the combined use of an anti-RTA test and the ASta test (present report; Flandrois *et al.*, 1979), and by combining the ASta test with a heat stable nuclease test (Taylor *et al.*, 1976) or an anti-leucocidin test (Taylor *et al.*, 1975). A battery of tests including all the important *S. aureus* antigens might detect strong antibody response against at least one antigen. Such a program is, however, not feasible for routine use. Another possibility is to use crude extracts of *S. aureus* cells containing a mixture of antigens and to detect the corresponding antibodies by for example RIA (Schopfer *et al.*, 1979; Wheat *et al.*, 1979). An objection to this approach is that the antigens are not defined and the antibodies detected are not identified. It is possible that antigens which have still not been tested for the diagnostic detection of antibodies in human sera can prove useful, e.g. species-specific antigenic determinants of PG and LTA.

Efforts to increase the sensitivity, e.g. by employing RIA or ELISA (Mackowiak & Smith, 1978) do not seem to solve the problem. The only result is higher antibody titres both from healthy individuals and from patients, i.e. a dislocation of the level. The problem is not the detection of staphylococcal antibodies but rather the demonstration of an antibody rise during disease. Therefore, *the fundamental problem is why a serious staphylococcal infection rarely results in a strong and regular booster effect* whereas staphylococcal carriage and minor infections give a seemingly normal antibody response.

S. aureus is a very special organism containing a variety of antigenic substances of which the presence of pA may be of particular importance. Furthermore, staphylococcal infections have a varied picture, the strong tendency to the formation of abscesses being of particular interest in relation to antibody formation. The irregular detection of increased antibody levels by infection is probably due both to properties of the staphylococcal cell and to what is happening in the host.

Staphylococci within an abscess are not likely to penetrate the fibrous wall. This may also be true for fragments of dead bacteria and extracellular antigenic material. In this case, antigenic material does not reach the antibody forming centres, and a booster effect does not occur. If some antigenic material does escape, the question is in which form it is presented to the macrophages. Uncomplexed excreted antigens, like the RTA coupled to a small peptide moiety, are not immunogenic. This is known from animal experiments. Furthermore, antigens present in the form of complexes may not be of the same nature as the material processed by the macrophages after phagocytosis of whole bacteria.

pA has been shown to be antiphagocytic by competing with polymorphonuclear leukocytes for the Fc sites of IgG opsonin (Dossett *et al.*, 1969). Moreover, pA in the cell wall may mask important antigenic sites or react with non-specific IgG, thus creating steric hindrance to the attachment of specific opsonins. If this happens, the stimulation of the antibody-producing cells may be reduced. *S. aureus* strains containing little or no pA should consequently be expected not to exert such an effect, and increased antibody levels should be found in the serum after infection. The capacity of small numbers of *S. aureus* cells to survive long periods within leukocytes may also be important. Furthermore, pA has been shown to be an unspecific mitogen for human B lymphocytes (Forsgren *et al.*, 1976). pA may thus block the stimulation of B lymphocytes carrying specific staphylococcal IgG on their surface and thus prevent the production of specific antibodies.

When a deep staphylococcal infection occurs in a patient who already possesses clones of B lymphocytes producing antibodies against all major *S. aureus* antigens, free antigen may combine specifically with antibodies and form circulating antigen-antibody complexes. The amount of free antibody will consequently be reduced and an antibody increase resulting from the infection may not be detected. To explain the absence of a booster response by infection, it has been postulated that antibody synthesis is already proceeding at maximal rates and cannot be further reinforced (Rogers & Melly, 1965; Ekstedt, 1965). This does not seem likely since a stimulated B cell is capable of producing an appreciable quantity of antibody. In addition, this theory does not explain why high levels of antibodies are detected in one osteomyelitis patient but not in another patient with the same disease. One might speculate on the possibility that one major immunogen like the RTA stimulates a strong homologous booster response and simultaneously hinders the production of antibodies against other antigens. Although this may be theoretically unlikely, the observation of elevated titres against one antigen but not against another in the same

patient remains to be explained. It is unlikely that a booster response might be inhibited by large doses of bacteria or free antigen, since very large doses are probably required for such an effect. Individual host differences may play a role, but in a situation like this the properties of the infecting strain should perhaps attract most attention.

Although most *S. aureus* strains contain the same major antigenic substances *in vitro,* the quantities of the various antigens no doubt vary considerably from one strain to another. This has been shown for pA, the quantity of which may have a decisive influence on the stimulation of antibody formation against other staphylococcal antigens. It has also been demonstrated that some strains contain much RTA whereas others contain little of this antigen. This may explain why an anti-RTA antibody rise is not observed in some patients with serious staphylococcal infections. It is also known that other antigens, such as the type-specific agglutinogens, vary both qualitatively and quantitatively from one strain to another (Oeding, in press). However, the production of antigens may be different *in vivo* than in culture.

Conclusions

In conclusion, in our opinion none of the existing tests for detection of anti-staphylococcal antibodies contributes appreciably to the diagnosis of suspected staphylococcal infections. The controversy between the universal presence of staphylococcal antibodies in healthy individuals and the failing booster effect by later serious infection is the major problem which has to be settled before any definite improvement in diagnostic staphylococcal serology can be expected. One approach is to study the properties of the infecting strain. We are at present preserving *S. aureus* strains isolated from patients with deep-seated staphylococcal infections in addition to serum samples drawn from the same patients. We plan to analyse the antigens of each strain qualitatively and quantitatively and to relate the findings to the corresponding antibodies found in the same patients.

In the introduction of this paper it was pointed out that an impressive body of knowledge has been obtained since 1960 on the cellular antigens of staphylococci. We want to conclude by expressing the hope that the coming years will bring about similar progress in the research on staphylococcal antibody formation.

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