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PNEUMOCOCCAL ANTIBODIES IN IgA OF SERUM AND EXTERNAL SECRETIONS

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SUMMARY

An indirect immuno-fluorescent method is described for the detection of group and type specific antibodies against pneumococci. The localization of these antibodies in the various immunoglobulin classes can be established by this method. Difficulties may be encountered, due to masking of sites on the antigen by antibodies, present in excess in a particular class of immunoglobulin. Relatively high titres of group specific and type specific antibodies against pneumococcus types, 3, 6, 9, 14 and 24 could be demonstrated in colostrum IgA. Occasionally these antibodies were also found in serum IgA. Group specific antibodies were detected in low titres in IgA of saliva and nasal secretions. The findings are discussed in relation to the concept of the secretory IgA system.

INTRODUCTION

The presence of specific antibodies in external secretions is of importance in the development and maintenance of immunity. This has recently been established for a number of virus infections of the respiratory tract (Smith *et al.*, 1966; Tremonti, Lin & Jackson, 1968; Kasel *et al.*, 1969). In some cases it was found that the immunity showed no correlation with the serum antibody titres but did show a correlation with antibody activity in the external secretions (Smith *et al.*, 1966; Tremonti *et al.*, 1968).

The antibodies found in secretions after local challenge with virus preparations are mainly IgA immunoglobulins (Smith, Bellanti & Chanock, 1967; Ogra & Karzon, 1969; Kasel *et al.*, 1969).

Such a correlation between immunity and the presence of antibodies in external secretions has not previously been investigated in regard to bacteria. A number of documented cases have, however, been reported of healthy persons with no secretory IgA (Rockey *et al.*, 1964), whereas others with the same abnormality exhibited frequent and severe bacterial infections.

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In many cases the infection is localized in the respiratory tract, with pneumococci and *Haemophilus influenzae* as the principal pathogens. In previous studies (Stoop, 1965) on young children with the clinical picture of immunological deficiency, in whom other causes of frequent severe infections of the respiratory tract had been excluded, the immunological reactivity was investigated. Some patients had low serum immunoglobulin levels and low serum titres of specific antibodies against pneumococci and *Haemophilus influenzae*. Others had normal serum immunoglobulin levels and high serum titres of specific antibodies against these bacteria. In regard to the latter group of patients, it was clearly of importance that not only the serum but also the external secretions should be tested for immunoglobulins and antibodies. For this purpose it is not sufficient for type specific antibodies against pneumococci and *Haemophilus influenzae* to be determined. The localization of these antibodies in the various immunoglobulin classes must also be studied. To this end we developed an indirect fluorescent antibody technique, which has so far been confined to the determination and classification of pneumococcal antibodies in serum and external secretions from normal adults. A description of the technique and the first results obtained are presented in this paper.

MATERIALS AND METHODS

Antigen

Fresh pneumococcal suspensions of various types in phosphate-buffered saline, pH 7·2 (PBS), served as antigens. The densities of the suspensions were adjusted nephelometrically to 10^8 cells/ml.

Sera and external secretions

Sera were separated from the clot by centrifugation a few hours after venipuncture and stored at −20°C. Parotid saliva was obtained by means of a parotid cup (Curby, 1953). Nasal secretions were obtained by rinsing both nasal cavities with 50 ml saline and homogenizing ultrasonically. Both saliva and nasal secretions were concentrated twenty to fifty times by ultrafiltration before use. Colostrum was obtained by suction 6 hr after birth or as soon as it was produced.

Conjugates

Commercial fluorescein isothiocyanate (FITC) conjugated antiglobulin antisera were used (equine anti-human globulin and equine anti-guinea-pig were obtained from Roboz Surgical Instr. Inc., Washington; sheep anti-rabbit globulin was purchased from Sylvana Comp., U.S.). Specific anti-human immunoglobulin conjugates were prepared by ourselves. Anti-IgG fluorescent antiserum was prepared in a goat by immunization with purified normal IgG. After precipitation with $(NH_4)_2SO_4$ and fractionation on a Sephadex A50 column the serum was conjugated with FITC and then purified on G25 and A50 Sephadex columns. This procedure was followed by absorption with human red cell ghosts and rat liver powder. The F/P ratio of the conjugate was about 4. Anti-IgM and anti-IgA sera were prepared in rabbits by immunization according to Goudie, Horne & Wilkinson (1966) with immunoprecipitation lines of IgM and IgA respectively. The anti-IgM serum was absorbed with a serum lacking IgM. To obtain specificity the anti-IgA serum was absorbed with IgG and transferrin. Conjugation of both antisera and further absorption procedures were the

same as for the anti-IgG serum, resulting in conjugates with F/P ratios of 2·4 and 4 respectively. All antiglobulin sera were tested for specificity by immunoelectrophoresis and immunodiffusion and after conjugation by immunofluorescence on bone marrow smears from myeloma patients.

Optical apparatus

A Reichert Biozet microscope with a three-lens UV condenser and Binolux lamphouse was used. It was equipped with an HBO 200 high pressure mercury vapor lamp, a BG 12 (E3) exciter filter and an Sp3 barrier filter.

Fluorescent antibody method

A standard loop of the antigen was applied to a premarked area on a slide, resulting in 80–150 organisms per high power field. The antigen was fixed by warming. One drop of the material to be tested (serum, colostrum, etc.) was applied to the antigen. After 30 min at 37°C the slides were rinsed in PBS for 30 min. After drying, one drop of a given dilution of the conjugate was applied. The slides were kept for 30 min at 37°C and rinsed again with PBS for 30 min. They were mounted in a mixture of glycerol 90% and PBS 10% and read with an oil immersion system. Preliminary experiments showed, that titres of antibodies should be determined by reading the number (classification into three categories: <20, 20–50 and >50/field) and brightness ($-$, $+$, $+\pm$, $++$, $++\pm$, $+++$) of fluorescent organisms at various dilutions of the material tested according to a scheme developed for this purpose. The evaluation proved to be reproducible by having the slides judged by two persons unaware of each other's results.

Specificity and controls

The specificity of the method was proved by determining pneumococcal antibodies in specific pathogen free (SPF) guinea-pigs before and after immunization. Pre-immunization sera gave negative results; immune sera contained antibodies against the type of pneumococcus used for immunization but also, though at lower titre, against other types of pneumococci (group specific antibodies).

We tried to achieve type specificity of the test by absorption of sera with packed cells of a rare pneumococcal type: two parts of serum (dilution) were incubated for 30 min at 37°C with one part of washed packed cells of type 24 pneumococcus. The cells were then removed by centrifugation. Antibody determinations in sera of rabbits immunized with type 6 pneumococci showed that titres against heterologous types after absorption were at least eight times lower. No change in titre was observed with the homologous type 6. Preliminary experiments with human sera showed that a similar reduction of antibody titre against most types tested could be obtained by absorption with type 24 packed cells. In view of the much lower titres (2–64) found in humans and the frequent absence of antibody titres after absorption we conclude there must be type specificity of the antibody titres of absorbed human sera.

The negative controls of the test system included tests with all conjugates and antigen without serum, external secretions or protein fractions, the same but with albumin, and tests with isolated immunoglobulins and nonmatching anti-immunoglobulin conjugates. The antibody character of the immunoglobulins reacting with the antigen was proved by using papain-produced fragments of IgG isolated from the serum of a patient with relatively

high titres of anti-pneumococcal antibodies. The Fc fraction gave negative reactions when tested with anti-IgG conjugate whereas the Fab fraction, as well as complete IgG, gave positive reactions in the same system. Further controls used are given in Table 1.

TABLE 1. Control-experiments in immunofluorescent determination of pneumococci antibodies

Antigen	First incubation	Blocking	Conjugate	Result
Pneum.	Rabbit anti-pneum. serum	Pos. human serum	Anti-human glob.	neg.
Pneum.	Pos. human serum	Anti-human glob.	Anti-human glob.	neg.
Pneum.	Pos. human serum	Anti-IgG	Anti-IgG	neg.
Pneum. 24	Pos. human serum abs. type 24	—	Anti-human glob.	neg.
Pneum. 3	Pos. human serum abs. type 24	—	Anti-human glob.	pos.

Quantitative immunoglobulin determination

The immunoglobulin levels of sera and external secretions were determined by the radial diffusion method of Mancini, as described earlier (van Munster en Stoelinga, 1965; Zegers *et al.*, 1968).

RESULTS

All normal human sera were found to contain anti-pneumococcal antibodies. The antibody titres of human sera, when tested with an anti-human immunoglobulin conjugate, generally varied from 2 to 64 and never exceeded 1:256. After absorption the type specific antibody titres tended to be rather low (titre of 0–8), with some exceptions.

With an anti-IgG conjugate, approximately the same titres were obtained as with an anti-immunoglobulin conjugate (Table 2). As a rule, no antibodies could be detected in human

TABLE 2. Pneumococcal immunofluorescent (I.F.) antibody titres of a pooled human serum tested with four conjugates

Conjugate	Absorption	Titres against type		
		3	19	24
Anti-human globulins	—	64	128	32
	Type 24	16	8	0
Anti-IgG	—	64	64	16
	Type 24	8	0	0
Anti-IgA	—	0	0	0
	Type 24	0	ND	0
Anti-IgM	—	0	0	0
	Type 24	ND	ND	ND

ND: not done.

sera when anti-IgA or anti-IgM conjugates were used: only twelve out of fifty-four sera contained IgA antibodies, mostly group specific, in low titres, and one out of five sera contained IgM antibodies. The titres of one of these sera are given in Table 3.

In preliminary tests with an anti-IgA conjugate and concentrated saliva specimens or nasal

TABLE 3. Pneumococcal I.F. antibody titres of a human serum tested with four conjugates

Conjugate	Absorption	Titres against type		
		3	6	24
Anti-human globulins	—	128	128	32
	Type 24	64	32	0
Anti-IgG	—	64	64	64
	Type 24	32	16	0
Anti-IgA	—	16	16	0
	Type 24	4	2	0
Anti-IgM	—	2	0	0
	Type 24	0	0	0

TABLE 4. Group specific pneumococcal antibody titres of some colostra and matched sera, tested with anti-IgA conjugate

Patient	Material	IgA content (mg/100 ml)	Titres against type			
			3	6	14	24
SIL	Serum	198	0	0	0	0
	Colostrum	2196*	⩾32	⩾32	16	16
CAS	Serum	166	⩾2	0	0	0
	Colostrum	2322	⩾32	16	16	32
OOS	Serum	187	0	0	0	0
	Colostrum	1602	⩾64	16	32	8
PIT	Serum	209	16	16	0	0
	Colostrum	1422	320	320	⩾64	20

* Colostrum IgA contents were determined using a standard curve for serum IgA.

secretions, IgA pneumococcal antibodies could only be demonstrated sporadically. Since low IgA contents, particularly of the nasal secretions (23–30 mg/100 ml), were a probable cause of these negative results, a series of tests with human colostrum was instituted; in view of the high IgA content of colostrum, positive results in IgA pneumococcal antibody determinations could be expected by using this material.

Group specific and type specific pneumococcal antibodies were always demonstrable in colostra. In none of the colostra tested could IgG antibodies against pneumococci be detected. IgM antibodies were found only sporadically and at low titres. Table 4 lists the IgA anti-pneumococcal titres and IgA levels of colostra and the corresponding sera of a

number of patients. It is obvious from these data that a discrepancy exists between the anti-pneumococcal titre of colostra and sera on the one hand and the corresponding IgA levels on the other. For instance the difference of serum and colostral IgA of patient 'Pit' differ by a factor 7 and IgA antibodies differ by a factor of 20. Since the IgA levels of colostra were determined by the radial diffusion method, using a standard curve for serum IgA, the values should be interpreted with caution. In spite of this possible source of error, other explanations for this discrepancy were considered (see Discussion). One concerns the possibility of false negative or low IgA antibody titres in the sera as a consequence of masking of the antigen for IgA antibodies by excess IgG antibodies in the sera. This phenomenon had been described earlier for IgM antibodies reactive with *N. gonorrhoeae* (Cohen, Norins & Julian, 1967). In order to establish the extent to which a masking phenomenon might play a

TABLE 5. IgA pneumococcal I.F. antibody titres of a human colostrum specimen. Influence on titres by the addition of serum with IgG pneumococcal antibodies

Colostrum pat. D diluted with	Titres against type		
	3	6	24
Saline	80	80	20
Serum	20	10	0

confusing role in serum IgA antibody determinations, we prepared mixtures of colostrum with a high IgA antibody titre and serum with a high IgG antibody titre. The results of these tests were reproducible and fairly uniform. Table 5 shows that before addition of serum the IgA pneumococcal antibody titres of colostrum are higher than after addition of serum, indicating the masking of bacterial antigenic sites for IgA antibodies by the excess of IgG antibodies.

No IgA antibodies could be detected in the nasal secretions and saliva of five patients with high IgA antibody titres in the colostrum. The data on IgA levels and IgA pneumococcal antibodies of these three external secretions (Table 6) suggest that the negative results of antibody determination in saliva and nasal secretions, in contrast to the positive tests with colostrum, might be explained by the difference in IgA content. This was substantiated by a second series of experiments on nasal secretions obtained from sixteen other persons. In five of these, group specific IgA pneumococcal antibodies were found, although again at low titres. Once more there seemed to be a relationship between antibody titre and IgA level: antibody activity was never found in nasal secretions with an IgA level of 7 mg/100 ml or lower while two nasal secretions with relatively high IgA contents (23 and 30 mg/100 ml) were positive.

DISCUSSION

The results of the present study are interesting from several points of view. Practically all human sera contained, apart from group specific antibodies, antibodies to one or more types

TABLE 6. Group specific IgA antipneumococcal antibody titres and IgA levels in serum and three external secretions of five patients

Patient	Serum				Colostrum				Saliva				Nasal secretion			
	IgA	penumoc. type			IgA	pneumoc. type			IgA	pneumoc. type			IgA	pneumoc. type		
	(mg/100 ml)	3	6	24	(mg/100 ml)	3	6	24	(mg/100 ml)	3	6	24	(mg/100 ml)	3	6	24
DUA	199	0	0	0	2154	160	160	20	12	0	0	0	10	0	0	0
GOU	228	16	16	0	3720	64	128	16	20	0	0	0	6	0	0	1
OST	320	4	4	0	2581	32	64	32	63	—	—	—	7	0	0	0
PIT	209	16	16	0	1422	320	320	20	7	0	0	0	5	0	0	0
STO	167	4	4	0	2083	256	512	16	14	0	0	0	10	0	0	0

of pneumococci. The same results were obtained in rabbits and guinea-pigs. That the antibodies in question were actually the product of immunization was proved by the results of immunization experiments in SPF guinea-pigs. The study of immunity against pneumococci in man is rendered difficult by the presence of group specific antibodies, this immunity being correlated with type specific and not with group specific antibodies (MacLeod *et al.*, 1945).

The determination of type specific antibodies is frequently difficult because of technical problems. The indirect immunofluorescence method described in the present article not only allows the determination of type specific antibodies but also renders it possible in principle to determine what immunoglobulin classes the antibodies belong to.

There is, however, a restriction to the latter possibility. In most sera, no antibodies were found in the IgA or IgM fraction. In samples of colostrum (with a relatively high IgA level and low IgM and IgG levels), on the other hand, it was mostly antibodies localized in the IgA that were found. The results of experiments in which serum and colostrum were mixed clearly show that a masking phenomenon occurs. This implies that specific IgA antibodies, to a titre which in colostrum is sufficient for a positive fluorescence test with anti-IgA, cannot be demonstrated on the bacteria in the presence of an excess of similar IgG antibodies. When it is desired to determine the precise titres of pneumococcal antibodies in IgA and IgM in the serum, the masking effect of IgG will have to be eliminated, for instance by the use of anti-IgG immuno-absorbents.

The high antibody titres against pneumococci in colostrum IgA constituted an interesting finding. Infections with these micro-organisms are typically localized in the respiratory tract. The presence of IgA antibodies in colostrum might be attributed to local synthesis of anti-pneumococcal antibodies in the mamma or to secretion of serum antibodies into the colostrum (Heremans, 1968). From the ratio of the serum and colostrum antibody titres in relation to the IgA levels, the conclusion might be drawn that active local synthesis of anti-pneumococcal antibodies occurs in the mamma. This would agree with the concept of the secretory IgA system (Tomasi & Bienenstock, 1968). Since local antigenic stimulation by pneumococci can be considered highly improbable, local immunization by circulating pneumococcal polysaccharides or migration of antibody-producing cells into the mammary gland has to be considered. It must, however, be borne in mind, that, owing to the masking effect of IgG, the titres of anti-pneumococcal antibody found in the serum IgA are relatively too low. As mentioned under 'Results', the same phenomenon was observed in the determination of IgM antibodies specific for *N. gonorrhoeae* (Cohen *et al.*, 1967). However, this is not a generally valid rule, because while our work was in progress Tourville, Bienenstock & Tomasi (1968) published data concerning the determination of antibodies against *E. coli* in serum, saliva and urine. The immunofluorescence method used is essentially the same as the one we have developed. Tourville *et al.* found a 'prozone' effect for the IgM due to 'competitive inhibition between IgA and IgM antibodies', but they encountered no difficulties in demonstrating IgA or IgM antibodies in the serum, in addition to antibodies of the IgG category. It seems likely to us that these discrepancies are due to differences in the nature and immunogenicity of the respective antigens. However this may be, from the relatively high titres in colostral IgA which we found, one cannot simply deduce that local synthesis of anti-pneumococcal antibodies takes place in the mammary gland. The question of the extent to which transportation of serum IgA antibodies into the external secretion occurs is difficult to answer on the basis of the present material. To demonstrate, as Tourville *et al.* have done, that the secretory IgA antibodies contain 'transport piece' constitutes no proof in this

respect. Adinolfi *et al.* (1966) published the interesting finding that colostral IgA contained antibodies against *E. coli* WF 96 whereas in the serum the antibodies were localized in the IgM. This is suggestive of local synthesis. A similar finding was previously published by Hodes *et al.* (1964) for poliomyelitis IgA antibodies.

Our findings concerning the presence of IgA anti-pneumoccocal antibodies in colostrum, saliva and nasal secretions confirm and extend the data reported by Quie, Messner & Williams (1968) on the presence of anti-pneumococcal antibodies in 11S colostral IgA against types 14 and 6. The significance of these antibodies in external secretions with regard to a possible role in specific immunity remains to be established. The preliminary data on the antibody content of nasal secretions presented in this paper will therefore have to be confirmed and extended. Since significant results could not be obtained due to low antibody levels, experiments are in progress to overcome these difficulties.

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