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BACTERIAL BLOCKING ACTIVITY OF SPECIFIC IgG IN CHRONIC *PSEUDOMONAS AERUGINOSA* INFECTION

R. M. GUTTMAN AND B. A. WAISBREN

Burn Research and Clinical Systems Laboratory, St Mary's Hospital, Milwaukee, Wisconsin, U.S.A.

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SUMMARY

A factor has been demonstrated in the serum of some patients with chronic Gram-negative infections which specifically blocks bactericidal activity against the infecting organism. Sera with this factor, that had been obtained from patients suffering from chronic *Pseudomonas aeruginosa* infections, were fractionated by Sephadex G-200 and DEAE–Sephadex column chromatography. Blocking activity was detected in the Sephadex G-200 '7S' peak and eluted from the DEAE column along with the serum IgG. Immunoelectrophoresis studies of this material along with pure IgG showed that the bactericidal blocking factor in these patients was IgG. The blocking factor was specific in its ability to protect bacteria, and could be absorbed from the serum by the particular bacterial strain isolated from the patient. Possible clinical importance of blocking activity by IgG is suggested by the persistent nature of the *Ps. aeruginosa* infections in these patients.

INTRODUCTION

Specific bactericidal blocking activity in the serum associated with chronic Gram-negative infections was reported by Waisbren & Brown in 1966. They demonstrated that the blocking factor was heat-stable and specific for the particular bacteria isolated from the patient. It was suggested that the blocking factor might be some type of antibody, but they were unable to find any correlation between the serum titres of the factor and agglutinating, or passive haemagglutinating, antibodies.

Louria *et al.* (1972) described a factor in the serum of many patients with mucocutaneous or systemic candidiasis which interfered with the *Candida* clumping activity of normal serum. This factor was present at titres less than 20 and could not be removed by dialysis or by heating the serum to 60°C for 1 hr.

Taylor (1972) demonstrated bactericidal blocking activity in the serum of patients suffering from chronic urinary tract infections caused by *Escherichia coli*. He identified the block-

Correspondence: Dr R. M. Guttman, Burn Research and Clinical Systems Laboratory, St Mary's Hospital, Milwaukee, Wisconsin 53211, U.S.A.

ing factor as a specific IgG and suggested that it competed with the normally bactericidal IgM for sites on the bacteria. The present study extends these observations to *Pseudomonas aeruginosa* infections, and suggests that 7S antibodies that block specific serum bactericidal activity may be associated with persistent infections by various species of Gram-negative organisms.

MATERIALS AND METHODS

Serum

The patients' sera were obtained periodically and stored at −70°C. Except where noted, patients' sera were inactivated by heating to 56°C for 30 min immediately prior to use. Normal human serum, having good bactericidal activity, was obtained from a single donor, stored at −70°C, and used within 2 weeks.

Bacteria

P. aeruginosa were isolated from the sputum and urine, respectively, from a patient (H.T.) who suffered from a chronic intractable infection of the lungs, and from a patient (S.M.) who had a chronic infection of the urinary tract. Growth was removed from 18-hr trypticase soy agar cultures, transferred to trypticase soy broth containing 15% glycerine, and frozen at −70°C until needed. Bacteria for assay of the bactericidal activity of patient's serum were obtained by inoculating a trypticase soy agar plate with one of the above broth–glycerine suspensions and, after 18 hr of incubation at 37°C, transferring the growth to 10 ml of sterile saline. Enough of this heavy suspension was added to another 10 ml of saline to produce a uniform bacterial suspension with a percentage transmission of 50–55 at 550 nm. A 10^{-3} dilution of this second suspension contained approximately 10^6 bacteria per millilitre and was used for the bactericidal assay.

Test system

The test system for detection of bactericidal blocking activity consisted of a set of three sterile 12 × 75 mm plastic test tubes (Falcon number 2054) maintained in an ice bath. One tube contained 0·9 ml of normal human serum and 0·9 ml of phosphate-buffered saline, pH 7·2; the second contained 0·9 ml of the patient's serum and 0·9 ml of phosphate-buffered saline; and the third tube had 0·9 ml of normal human serum and 0·9 ml of patient's serum. To each of these tubes was added approximately $2{\cdot}0 \times 10^5$ bacteria in 0·2 ml of normal saline. The tubes were thoroughly mixed and then incubated for 2 hr, with no additional stirring, in a 37°C water bath. After 2 hr they were rapidly chilled to 0°C in an ice bath to retard further cell division. Plate counts were done in triplicate using 0·2-ml samples of the initial bacterial suspension, and on each test mixture at the end of the 2-hr incubation period.

The results were expressed as the percentage of the original inoculum remaining viable after 2 hr: (2-hr mean plate count × 100)/(mean plate count of bacterial suspension) = percentage survival.

In experiments studying the specificity of the bactericidal activity, *P. aeruginosa* strains from burn patients and urinary tract infection sources were substituted into the above procedure, in place of the patient's own bacterial strain.

The *P. aeruginosa* were immunotyped by slide agglutination (Fisher, Devlin & Grabasik,

1969), using monospecific antisera supplied by Dr Myron Fisher (Parke–Davis Company, Detroit, Michigan).

Soluble bacterial antigen preparations for immunodiffusion were prepared by transferring the 18-hr bacterial growth from ten trypticase soy agar plates to 50 ml of sterile saline containing 5% formalin. After 24 hr the bacteria were washed with three changes of saline, counted in a Petroff–Hauser chamber, and adjusted to 10^9 cells/ml. The suspensions were placed in an ice bath and each sonicated for 15 min at 380 watts/in^2, using a Biosonic III immersion probe sonicator.

Absorption of bactericidal blocking factor by whole bacteria

Bacteria isolated from the patient and other sources were grown on trypticase soy agar plates for 18 hr and then transferred to 5% formalinized saline. After washing three times with normal saline, 10^9 bacteria were mixed with 1·0 ml of heat-inactivated patient's serum. After incubation at 5°C for 24 hr with occasional mixing, the bacteria were removed by centrifugation at 1200 *g* for 15 min. The absorbed serum was assayed for bactericidal blocking activity along with unabsorbed samples of the same serum.

Gel diffusion

Immunodiffusion was carried out with 1·0% agar (Ionagar number 2, Consolidated Laboratories, Incorporated, Chicago, Illinois) in 0·85% saline. After 48 hr of diffusion the gel was sequentially washed in saline and distilled water, dried, and stained with Buffalo Black or Ponceau S stain.

Immunoelectrophoresis

Immunoelectrophoresis of serum fractions was done on 25 × 75 mm microscope slides overlaid with 2·0 ml of 1% Ionagar in 0·075 M barbital–acetate buffer, pH 8·6. A constant current of 5 mA/slide was maintained for 1·5 hr at 20°C. After the electrophoresis period, the troughs were filled with the appropriate reagents and incubated at 20°C for 24 hr. The slides were then washed, dried, and stained as with gel diffusion. Purified IgG, goat antisera to human whole serum, IgG, and IgM were obtained from Hyland Laboratories (Costa Mesa, California). Goat anti-whole human serum was diluted 1:5 with saline before use.

Sephadex gel chromatography

Patients' sera were chromatographed on Sephadex G-200 (Pharmacia, Upsala, Sweden) using Tris–NaCl buffer (pH 8·0) by the method of Killander (1963). Ten to 15 ml of sera were concentrated to 2–5 ml by negative pressure dialysis using a Schleichre & Schuell (Keene, New Hampshire) collodion bag apparatus, and applied to a 2·5 × 100·0 cm Sephadex G-200 column. The flow rate was maintained at 10·0 ml/hr and 5·0-ml fractions were collected. The relative protein concentration was determined by measuring the absorbancy of each fraction at 280 nm on a Beckman model DU spectrophotometer. Pooled or unpooled fractions were concentrated by negative pressure dialysis before testing for blocking activity.

Ion exchange chromatography

Ion exchange chromatography was done on a 2·5 × 25 cm column of DEAE–Sephadex (A-50, Pharmacia, Upsala, Sweden) equilibrated with 0·1 M Tris-HCl buffer (pH 8·3) at 20°C (Gelotte, Flodin & Killander, 1962). The material to be fractionated was dialysed

against the starting buffer and applied to the top of the DEAE–Sephadex bed. The column was sequentially washed through with 0·1, 0·2, 0·3 and 0·4 M Tris–HCl buffer (pH 8·3) at a flow rate of 10 ml/hr. Five-millilitre fractions were collected and their absorbancy at 280 nm was determined. Fractions from each peak were pooled, concentrated, and tested for bactericidal blocking activity.

Haemagglutination titres

Haemagglutination titres of the patients' sera were determined by the microtitre method of Gaines & Landy (1955). Aliquots of fresh human type O erythrocytes were individually sensitized with the first seven *Pseudomonas* immunotype antigens (0·1 mg/ml, supplied by Dr M. Fisher, Parke–Davis, Detroit, Michigan).

Immunoglobulin levels

IgG, IgM, and IgA concentrations in the patients' sera were determined using commercial radial immunodiffusion plates (Quanti-plate, Kallestad Labs, Minneapolis).

RESULTS

When strain H.T. was incubated in medium containing equal amounts of normal human serum and phosphate-buffered saline only 0·6 ± 2·3 per cent of the bacteria survived (Table 1). In the patient's own unheated serum and phosphate-buffered saline mixture there was a

TABLE 1. Bactericidal blocking activity of serum from patients H.T. and S.M.

Patient	Mean percentage of bacteria surviving			
	Normal human serum + phosphate-buffered saline	Fresh patient's serum + phosphate-buffered saline	Normal human serum + fresh patient's serum	Normal human serum + heated patient's serum*
H.T.	0·67 ± 2·36	123·00 ± 13·05†	130·87 ± 19·12†	146·77 ± 17·11†
Number of duplicate assays	5	3	5	7
S.M.	1·21 ± 2·36	135·30 ± 65·92†	178·98 ± 69·97†	104·50 ± 25·50†
Number of duplicate assays	5	3	4	2

* Heated to 56°C for 30 min.

† Significantly higher than the corresponding normal human serum and phosphate-buffered saline value ($P<0·01$).

significant ($P<0·01$) increase in the percentage of bacterial survival to 123·0 ± 13·0. When equal amounts of normal human serum and patient's serum were mixed and used as the test mixture, the patient's serum was able to block the bactericidal activity of the normal human serum. Fresh and heated patient serum resulted in 130·8 ± 19·1 and 146·7 ± 17·1 per cent of the bacteria surviving respectively. Both these means were significantly higher ($P<0·01$) than the value observed for normal human serum alone. However, they were not significantly different from each other or from the percentage at survival observed for patient serum alone.

TABLE 2. Blocking activity of H.T. serum for other immunotypes of *P. aeruginosa*

P. aeruginosa strain	Fisher immunotype	Percentage of bacteria surviving					
		Experiment I			Experiment II		
		Normal human serum + phosphate-buffered saline	Fresh patient's serum + phosphate-buffered saline	Normal human serum + patient's serum*	Normal human serum + phosphate-buffered saline	Fresh patient's serum + phosphate-buffered saline	Normal human serum + patient's serum*
H.T.	1	2	147	107 (105)	0	47	40 (40)
478	1	36	90	62 (26)			
97	1				3	72	22 (19)
864	1				3	10	9 (6)
939	2	4	6	7 (3)			
595	2	26	41	38 (12)	2	9	8 (6)
871	3				0	24	2 (2)
876	3	9	43	21 (11)			
550	4	76	60	65 (11)			
615	5	4	5	3 (−1)	0	9	0 (0)
463	6	< 0·1	< 0·1	< 0·1 (0)	0	2	0 (0)
840	7				1	0	0 (−1)
587	3, 7	25	64	42 (17)	28	31	33 (5)
46	8				10	32	17 (7)
70	8	56	67	53 (3)			

* The figure in parentheses show the net change in percentage survival from control mixture = percentage survivors (normal human serum, patient's serum) minus percentage survivors (normal human serum, phosphate-buffered saline).

TABLE 3. Absorption of blocking factor from patient H.T. serum using formalinized bacteria*

P. aeruginosa strain used for absorption	Immunotype	Percentage survivors of H.T. strain
Unabsorbed	—	61
H.T.	1	2
478	1	24
595	2	17
587	3, 7	31
550	4	32
615	5	35
463	6	52
70	8	< 0·1

* Test mixture included absorbed or unabsorbed patient's serum, normal human serum, and patient's own bacterial strain.

TABLE 4. Bactericidal blocking activity by pooled Sephadex G-200 fractions

Test mixture	Percentage survivors	
	H.T.	S.M.
Normal human serum, phosphate-buffered saline	13	< 0·1
Normal human serum, patient whole serum	228	14·8
Normal human serum, 19S pool*	71	0·1
Normal human serum, 7S pool*	237	17·7
Normal human serum, 4·5S pool*	3	< 0·1

* Each pool was concentrated to the original sample volume by negative pressure dialysis before being assayed.

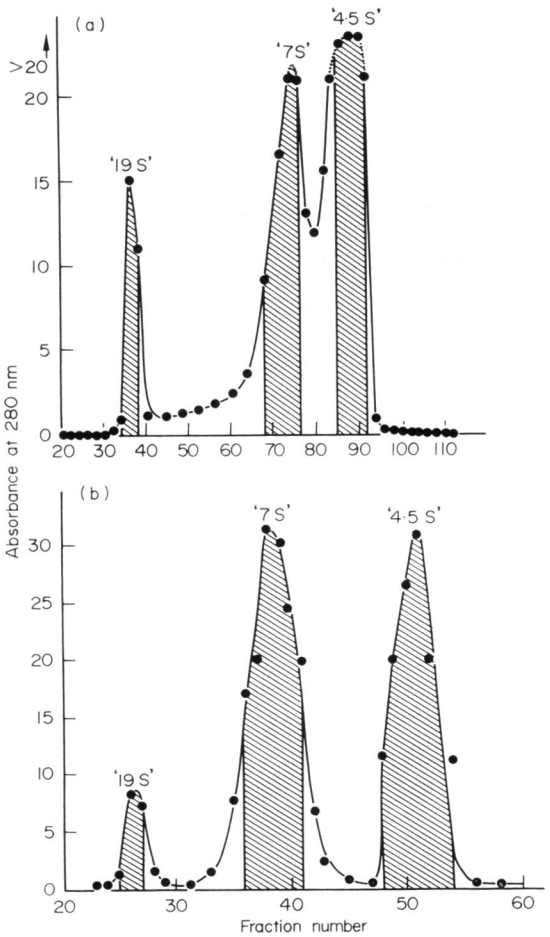

FIG. 1. Sephadex G-200 column protein elution pattern of serum from (a) patient H.T. and (b) patient S.M. Shaded areas under each peak denote pooled fraction. The dotted line shows the part of peaks which were approximated since absorbance was too high to be determined directly. Both sera were diluted in Tris–NaCl buffer, pH 8·0.

Similar results were obtained with the *P. aeruginosa* and serum of patient S.M. The percentages of bacterial survival were $1{\cdot}2 \pm 2{\cdot}3$ in the normal human serum–phosphate-buffered saline combination; $135{\cdot}3 \pm 65{\cdot}9$ ($P < 0{\cdot}01$) in patient's serum–phosphate-buffered saline combination; $178{\cdot}9 \pm 69{\cdot}9$ ($P < 0{\cdot}01$) in the fresh patient's serum–normal human serum mixture; and $104{\cdot}5 \pm 25{\cdot}5$ ($P < 0{\cdot}01$) in heated patient's serum–normal human serum. Again, there was no difference between the blocking ability of fresh and heated patient's serum.

This blocking activity has been detected in the serum of these patients repeatedly for 3 years during which time their infections were never completely eradicated.

The passive haemagglutination titres to the seven Fisher *P. aeruginosa* immunotype antigens were within normal control ranges for both patients. The IgG levels of both patients, however, were elevated. H.T. had an IgG concentration of 2020 mg% and that of

TABLE 5. Bactericidal blocking activity by pooled DEAE fractions

Test mixture	Percentage survivors	
	H.T.	S.M.
Normal human serum, phosphate-buffered saline	0·1	< 0·1
Normal human serum, patient's serum	163·0	111·0
Normal human serum, 0·1 M pool*	51·0	33·0
Normal human serum, 0·2 M pool*	0·1	< 0·1

* Each pool was concentrated to the original sample volume by negative pressure dialysis before being assayed.

S.M. was 1850 mg% (normal range 600–1200 mg%). IgG and IgM levels were normal. Immunodiffusion with sonicated preparations of the H.T. strain of *P. aeruginosa* demonstrated one precipitin line against H.T. serum. Normal human serum produced two precipitin lines against the same antigen preparation, one of which fused with the line formed by the patient's serum.

In order to determine the specificity of the bactericidal blocking activity, duplicate samples of patient H.T. sera were assayed with a panel of bacteria representing each of the Fisher *P. aeruginosa* immunotypes. Table 2 shows the results of two separate experiments using H.T. serum obtained at different bleedings. The increases in the percentage of bacterial survival due to blocking by patient's serum are in parentheses. When the patient's own strain of *P. aeruginosa* was used, blocking by patient's serum resulted in an increased bacterial survival of 105% in one experiment and 40% in the other.

The blocking activity appeared to be specific for the patient's own strain. When other *P. aeruginosa* strains were substituted for that of the patient, the change in the percentage of survival due to blocking activity ranged from -1 to 26, and from -1 to 19 in the two experiments. Some strains having the same immunotype as the patient's own were protected better than those of other immunotypes. However, the best protection was for the particular strain isolated from the patient.

Essentially all the bactericidal blocking activity could be absorbed from the patient's serum by the homologous bacteria (Table 3). Other *P. aeruginosa* strains reduced the block-

ing ability of the H.T. serum by various amounts, but in most cases were not as effective as he patient's own strain.

Sephadex G-200 fractionation of 10 ml of either patient's serum yielded the typical three protein peak pattern (Fig. 1). After being pooled and concentrated, the material under each peak was analysed by immunodiffusion and electrophoresis with specific anti-IgG and anti-IgM antisera, along with purified IgG. IgM was found in the first peak, and IgG was identified in the second. Neither immunoglobulin was detected in the third peak. Blocking activity

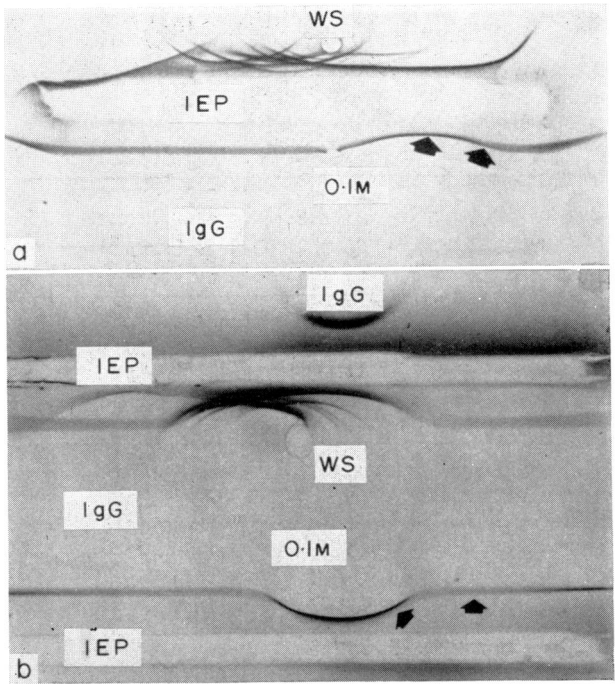

FIG. 2. Immunoelectrophoresis of (a) H.T. and (b) S.M. serum fractions eluted from a DEAE column. The pooled and concentrated sephadex G-200 '7S' peaks were equilibrated to 0·1 M Tris–HCl buffer, pH 8·3, and then fractionated on a DEAE–Sephadex, A-50 column. W.S. = whole patient's serum, 0·1 M concentrated pool eluted by 0·1 M Tris–HCl buffer, pH 8·3. IEP = Hyland goat anti-whole human serum. IgG = immunophoretically pure human IgG obtained from Hyland Laboratories. Arrows indicate the line of identity between patient's serum fraction and IgG standard. The positive pole was to the left.

determinations on these concentrates showed most of the activity to be in the 7S pool (Table 4). The fractions from another Sephadex separation of H.T. serum were concentrated and tested individually for blocking activity. In this case, the blocking activity curve was found to centre directly under the 7S peak.

DEAE–ion exchange chromatography of the 7S material yielded prominent protein peaks in the 0·1 M and 0·2 M buffer fractions only. Assay of each pooled and concentrated peak demonstrated bactericidal blocking activity in the 0·1 M peaks only (Table 5). Immunoelectrophoresis of material from each of these 0·1 M peaks demonstrated a single arc with an

electrophoretic mobility similar to IgG. This arc was positively identified as IgG by virtue of the fact that it fused with the precipitin line formed by a known IgG standard (Fig. 2).

The 0·1 M peaks were also analysed by Ouchterlony immunodiffusion using monospecific goat antisera to human gamma, alpha, and mu immunoglobulin heavy chains. Again, only IgG was detected in the final blocking factor preparation. Controls included purified IgG, IgA, IgM, and whole serum.

DISCUSSION

Lack of overt serum bactericidal activity may be caused by an absence of bactericidal antibody, a serum-resistant bacterium, or the presence of a bactericidal blocking factor in the patient's serum. The observation that serum from other individuals was bactericidal for these patient's bacteria eliminated the possibility that these organisms were serum-resistant. The fact that the patients' sera were able to interfere with the bactericidal activity of normal human serum demonstrated that the defect was due to the presence of a blocking factor rather than a serum deficiency (Waisbren & Brown, 1966).

It has been reported that most of the bactericidal activity of normal serum towards Gram-negative organisms is due to the IgM class of antibodies (Michael & Rosen, 1963; Chernokhvostova *et al.*, 1968; Wiederman *et al.*, 1970). The present findings suggest that some patients with chronic Gram-negative infections have developed a population of antibodies which effectively inhibits and/or replaces the normally bactericidal IgM population. The result is a net increase in the number of viable bacteria when they are incubated *in vitro* with the patients' serum. The final blocking antibody preparation was immunoelectrophoretically pure and was identified as IgG.

IgG may be produced in other cases but only manifests itself as a blocking antibody when the structure of the infecting organism is unfavourable for IgG-mediated complement activation. The density and distribution of antigen receptor sites on the surface of the infection bacteria may play a role in the effect of the antibacterial IgG. It has been shown that single molecules of IgM can initiate complement-dependent bacteriolysis, while complement activation by IgG requires the close proximity of at least two molecules bound to the cell surface (Borsos & Rapp, 1965; Cohen & Becker, 1968). If IgG competitively inhibited the binding of IgM on the surface of a bacterium whose receptor sites were too far apart for IgG activation of complement, the cell would be effectively protected from lysis.

Likewise, the response of the patient may determine whether the blocking antibody is observed. The two precipitin lines formed by immunodiffusion of normal human serum against the H.T. bacterial preparations, as opposed to only one formed by H.T. sera, suggests that H.T.'s immune system did not recognize all the possible antigens on the H.T. *P. aeruginosa* strain.

The antigenic specificity which was blocked by patient H.T. serum did not appear to be identical to that recognized by the rabbit immunotyping antisera. Bactericidal protection was not equally afforded to all, the *P. aeruginosa* strain having the same immunotype as strain H.T. Likewise, the ability of various *P. aeruginosa* strains to absorb blocking activity from the patient's serum was not related to immunotype either.

The similarity in the bacteria isolated from the same patient at different times, with respect to immunotypes and bactericidal protection, suggests a long-term infection with the same

organism. In these patients the blocking antibody may play a selection role, giving this particular pathogen an advantage over other organisms.

It is impossible to make a cause and effect determination regarding the presence of the blocking IgG and the chronic nature of the patient's infection. The chronic infection may be a direct result of early production of the blocking IgG. On the other hand, the IgG antibody may have been produced only as a result of continued immunological stimulation by an organism which was not effectively dealt with, and eliminated by the patient's defences. However, it is clear that this phenomenon, which has now been associated with numerous Gram-negative organisms (Waisbren & Brown, 1966; Gower *et al.*, 1972) will cause decreased bactericidal activity against specific pathogenic strains of bacteria causing chronic infections.

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