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Assessment of biological activity of immunoglobulin preparations by using opsonized micro-organisms to stimulate neutrophil chemiluminescence

C. S. MUNRO, P. J. STANLEY & P. J. COLE *Host Defence Unit, Department of Medicine, Cardiothoracic Institute, Brompton Hospital, London, UK*

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SUMMARY

We have used the ability of opsonised bacteria to stimulate luminol enhanced chemiluminescence of human neutrophils to examine the opsonic capabilities of normal and hypogammaglobulininaemic sera for four common bacterial pathogens. Preparations of human immunoglobulin modified for i.v. use have then been compared with unmodified Cohn Fraction II for their effectiveness in improving opsonization when added to antibody deficient sera *in vitro*. Hypogammaglobulinaemic sera exhibited impaired opsonisation of *Haemophilus influenzae*, and severely antibody deficient sera also opsonized *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* poorly. The opsonization of these organisms was improved by Cohn Fraction II, and by pH 4 and β-propionolactone treated immunoglobulins, in descending order of effectiveness. Pepsin digested immunoglobulin was inactive, and in some cases impaired opsonic capacity. The opsonisation of *Staphylococcus aureus* by hypogammaglobulinaemic sera was near normal, and was not improved by any immunoglobulin. This technique, which assesses biological activity of immunoglobulin, is useful in comparing preparations, and may help to establish appropriate dosage and frequency for intravenous immunoglobulin replacement therapy.

Keywords hypogammaglobulinaemia immunoglobulin opsonization *Haemophilus influenzae* chemiluminescence

INTRODUCTION

Intravenous (i.v.) replacement therapy for hypogammaglobulinaemia has been shown to be have advantages over i.m. treatment (So *et al.*, 1984) and numerous preparations of human immunoglobulin for i.v. use are being developed. There are real hazards of such treatment, from transmissible agents (Lever *et al.*, 1984), from the presence of kinins (Alving *et al.*, 1980) and from spontaneous complement activation by aggregates (Baradun *et al.*, 1962). Immunoglobulin preparations are therefore pre-treated to render them safe for i.v. administration, but such pre-treatment can affect their properties (Skvaril, Roth-Wicky & Baradun, 1980; Romer *et al.*, 1982a). To assess whether these modified preparations retain biological activity, functional assays of immunoglobulin are required. We have adapted the technique of Williams *et al.* (1980) by using bacteria opsonised by normal and hypogammaglobulinaemic sera to stimulate luminol-dependent

Correspondence: Dr C. S. Munro, Host Defence Unit, Cardiothoracic Institute, Brompton Hospital, Fulham Road, London SW3 6HP, UK.

neutrophil chemiluminescence. The relative potency of various immunoglobulin preparations has been assessed by their ability to improve the impaired opsonic activity of hypogammaglobulinaemic sera.

MATERIALS AND METHODS

Targets. The bacteria used were single clinically isolated strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*; a serum resistant strain of *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Oxford strain). These organisms were grown in broth overnight, then pelleted and washed three times in PBS. The final suspension was adjusted with a nephelometer to a standard concentration for each organism of approximately 10^9 organisms per ml.

Neutrophils. These were obtained from healthy donors by adding 20 ml of blood to 4 ml of 6% Dextran and 200 iu heparin. After sedimentation at room temperature for one hour, the buffy coat was layered onto Ficoll-Paque (Pharmacia) separation medium and centrifuged at 500 *g* for 45 min at 18°C. Residual erythrocytes were lysed by resuspension of the pellet in 0·8% ammonium chloride buffer, pH 7·2, and the cells washed twice (100 *g* for 10 min at 4°C) in colourless medium 199 (Flow Labs). The final suspension contained 10^6 neutrophils per ml.

Sera. Sera were obtained from ten healthy subjects. A pool formed from these sera was used as standard throughout. Sera were also obtained from five patients with hypogammaglobulinaemia, of whom four were already receiving replacement therapy but had immunoglobulin levels below the normal range of our laboratory. Using radial immunodiffusion (Mancini, Carbonara & Hereman, 1965), we measured total IgG, IgA, and IgM with standard polyclonal antisera, and IgG subclasses with specific monoclonal antisera (Seward Laboratory). Details of the patients are given in the table.

Immunoglobulin preparations. Cohn fraction II was provided by the Blood Products Laboratory, Elstree, UK. The i.v. immunoglobulin preparations tested were Sandoglobulin (pH 4 treated; Sandoz Products Ltd.); Intraglobin (β-propionolactone treated; Biotest Folex Ltd.) and Gammavenin (pepsin cleaved; Behring-Werke GmbH).

Opsonization. All opsonizations were performed in triplicate. 100 μl of the target suspension was incubated for 30 min at 37°C in 1 ml of PBS containing 10% normal or hypogammaglobulinaemic serum. Immunoglobulin preparations, if added, were to a final concentration of 10 mg/ml serum. The opsonized targets were centrifuged at 3,000 r/min for 10 min at 4°C. Immediately prior to the chemiluminescence assay, they were resuspended in 900 μl of pre-warmed (37°C) colourless Hanks' solution (GIBCO), buffered to pH 7·4 by 10 mM HEPES, which contained 10^{-5} M luminol, diluted from a stock solution of 10^{-2} M luminol (Sigma) in dimethylsulphoxide.

Chemiluminescence. Five hundred microlitres of the neutrophil suspension, warmed to 37°C, was added to the suspension of target particles in luminol medium. The final ratio of target particles to neutrophils was 50–140:1, depending on the target. Chemiluminescence was measured with an LKB 1250 luminometer at 15 and 30 min after addition of the cells. Results are expressed as a percentage of CL produced when the particle was opsonized by pooled normal serum, and are the mean of the three readings.

Statistical analysis. These were by Student's *t*-test. A P value of less than 0·05 was considered significant.

RESULTS

All sera opsonized all targets better than either normal serum inactivated by heat (56°C for 30 min) or saline alone. Fig. 1 shows the individual and mean values of CL produced by opsonisation using normal and hypogammaglobulinaemic sera (at peak: 15 min for pseudomonas and 30 min for other targets). With normal sera the range of opsonic activity was wide for streptococcus and pseudomonas, and overlapped that of the antibody deficient sera. For staphylococcus the normal range was narrower, but hypogammaglobulinaemic sera showed only slightly depressed opsonisa-

Table 1.

Patient	Age/sex	Immunoglobulin (g/l) Total IgG	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM
AS	33 M	1·52	0·67	0·45	0·05	0·12	ND	ND
PW*	60 M	1·88	1·12	0·45	0·04	0·30	0·21	ND
MH	76 F	2·79	1·36	1·40	0·09	0·24	ND	0·15
JL	37 F	3·66	1·41	1·29	0·06	0·19	0·03	0·05
RP	34 M	3·76	2·32	1·25	0·61	0·19	0·31	0·33

ND = not detected. * No recent immunoglobulin treatment.

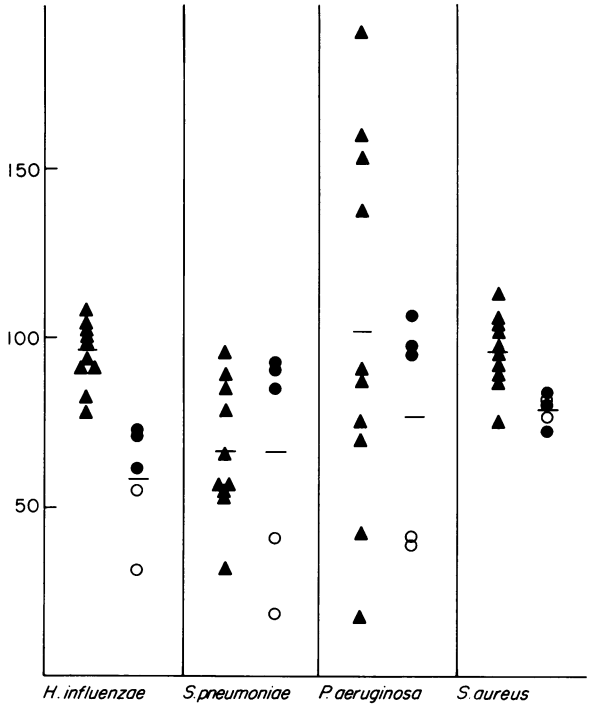

Fig. 1. Chemiluminescence (% of pooled serum standard) stimulated by bacteria opsonized by 10 normal (▲) and five hypogammaglobulinaemic sera (●). (○) indicate sera with IgG < 2·0 g/l.

tion within this range. In contrast the opsonization of *H. influenzae* by hypogammaglobulinaemic sera was significantly ($P < 0·001$) impaired, and there was no overlap with the normal range. The same result was obtained using two other strains of *H. influenzae* (data not shown).

In the absence of serum no immunoglobulin preparation produced CL greater than the saline control after 30 min. The results of adding the preparations to hypogammaglobulinaemic sera are shown in Fig. 2. Hypogammaglobulinaemic serum had shown the clearest defect with *H. influenzae*. When Cohn fraction II was added there was in every case an improvement in the opsonic properties for this target, and the mean value was considerably greater than with untreated sera ($P < 0·001$). Significant benefit was also seen with pH 4 treated immunoglobulin ($P < 0·01$). β-propionolactone treated immunoglobulin improved opsonization, though the effect was less marked ($P < 0·05$).

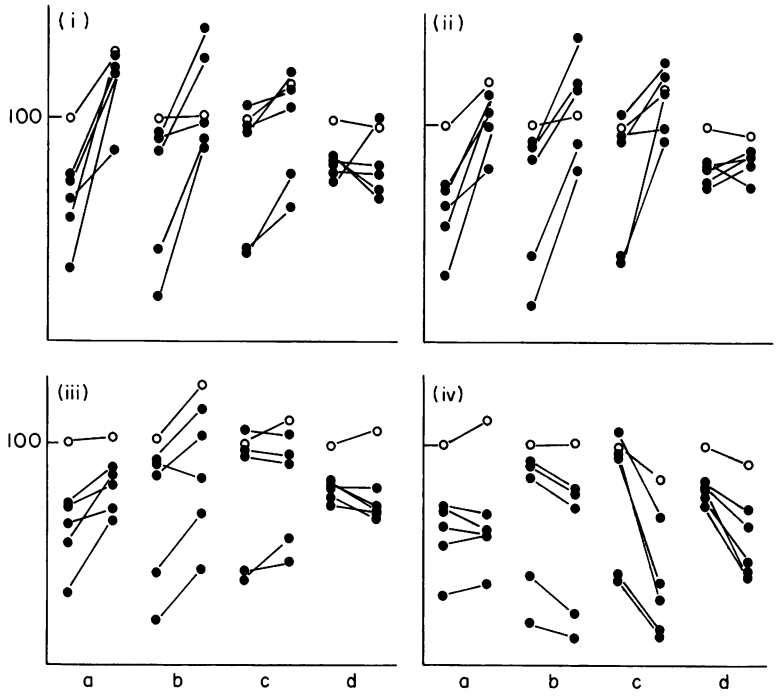

Fig. 2. Chemiluminescence as % of standard (vertical axis) before and after adding immunoglobulin preparations at 10 mg/ml serum to hypogammaglobulinaemic (●——●) and pooled normal (○——○) sera. (i) Cohn Fraction; (ii) pH 4 treated immunoglobulin; (iii) β-propionolactone treated immunoglobulin; (iv) pepsin digested immunoglobulin. The target bacteria were (a) *H. influenzae*, (b) *S. pneumoniae*, (c) *P. aeruginosa* and (d) *S. aureus*.

These effects on CL were not due to changes in pH, and were maintained when the stabilizing sugars in the preparations were removed by dialysis. The effect of increasing the concentration of immunoglobulin added to one hypogammaglobulinaemic serum is shown in Fig. 3: the stimulation of opsonic activity was dose dependent. Addition of the pepsin cleaved product to antibody deficient sera did not affect opsonization of *H. influenzae*.

The pattern of improvement found with *H. influenzae* was repeated with streptococcus and pseudomonas, except that the β-propionolactone treated immunoglobulin showed no consistent effect with the latter. None of the preparations consistently improved the opsonization of *S. aureus*, and for targets other than haemophilus the pepsin cleaved product actually diminished the CL obtained. Addition of the various immunoglobulins to pooled normal serum resulted in similar, though generally less marked effects on the opsonization of the four targets.

DISCUSSION

Although the conventional i.m. replacement therapy for chronic hypogammaglobulinaemic states is generally well tolerated and clinically effective, the volume of injection required to give large doses by this route is uncomfortable. Intravenous administration of immunoglobulin allows larger and therefore less frequent doses to be given with less discomfort. Preparations intended for i.v. use are processed by a variety of means, which involve chemical or enzymatic modification of the immunoglobulin molecule. As well as reducing spontaneous complement activation, these processes may affect other properties of the molecule such as Staphylococcal protein A binding (Skvaril *et al.*, 1980; Romer *et al.*, 1982b). Immunodiffusion assay (Romer *et al.*, 1982a; Stanley &

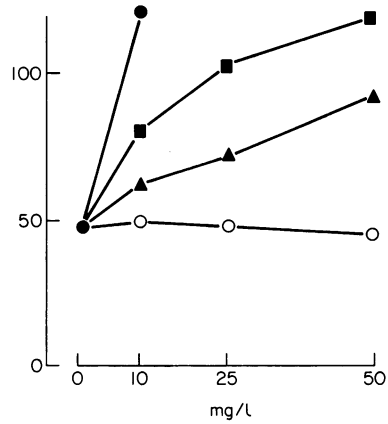

Fig. 3. Chemiluminescence as % of standard (vertical axis) produced by *H. influenzae* opsonised by one hypogammaglobulinaemic serum, showing the effect of adding Cohn Fraction II at 10 mg/ml (●), and increasing concentrations of pH 4 treated (■), β-propionolactone treated (▲), and pepsin digested (○) immunoglobulin preparations.

Cole, 1983) suggests that β-propionolactone treated immunoglobulin is deficient in IgG subclass 3. However, alteration in these *in vitro* measurements need not imply impaired biological function (Stephan & Dichtelmuller, 1983). Valid assays of these preparations ought therefore to relate to the *in vivo* function of immunoglobulin. Phagocytosis and killing by neutrophils following opsonization of *S. aureus* (van Furth & Leijh, 1981), or other bacteria (Hill, Augustine & Shigeoka, 1984) by immunoglobulin preparations have been used for this purpose, but involve cumbersome bacteriological quantification.

The present study was undertaken to provide a simple and rapid means of comparison between immunoglobulin preparations. Luminol-dependent neutrophil chemiluminescence of neutrophils can be stimulated by opsonised micro-organisms (Williams *et al.*, 1980), and gives a rapid result. The production of reactive oxygen metabolites detected by this technique relies on interaction of appropriate membrane receptors with immunoglobulin Fc segments, activated C3b, or bacterial components such as cell wall polysaccharides. Complement activation by the bacteria occurs by both classical (immunoglobulin-dependent) and alternative pathways, and the relative importance of these in opsonization may depend on the concentration of serum used (Tofte *et al.*, 1980).

Under the conditions of the study, the opsonization of *S. aureus* was not dependent on the immunoglobulin content. For *S. pneumoniae* and *P. aeruginosa*, there was a scattered normal range perhaps because of differing individual experience of capsular antigens. Opsonization comparable with normal by some of the hypogammaglobulinaemic sera may be due to replacement therapy. With *H. influenzae*, however, a clear difference between the normal and all hypogammaglobulinaemic sera was seen, and conversely the improvement following addition of immunoglobulin was most marked. Non-capsulated *H. influenzae* is a frequent isolate in respiratory infections, and membrane antigens common to all strains (Loeb & Smith, 1980), some of which are also shared with other gram negative bacteria (Burns & May, 1967) may explain the narrow normal range of opsonization. More widespread opsonic defects might be found with agammaglobulinaemic sera: the two sera with IgG $< 2{\cdot}0$ g/l and undetectable IgM consistently exhibited low opsonic activity for all targets except staphylococcus.

The consistent effects of added immunoglobulin on the opsonization of three of the targets permit general conclusions about the relative potency of the preparations. It appears that treatment of Cohn Fraction II with pH 4 (and a mild exposure to pepsin) slightly abrogates its opsonic capacity, and supra-physiological concentrations are required to match the effect of Cohn Fraction II *in vitro*. β-propionylation results in a greater reduction of effectiveness, but opsonisation by hypogammaglobulinaemic serum is still improved. Previous comparisons between these prep-

arations have found comparable differences in subclass content (Romer *et al.*, 1982a), and ability to bind staphylococcal protein A (Skarvil *et al.*, 1980). Immunoglobulin cleaved by pepsin into Fc and $F(ab)_2$ fragments does not improve opsonization, and actually impairs it for some targets. $F(ab)_2$ fragments of rabbit immunoglobulin have been reported to activate complement by the alternative pathway (Seiler *et al.*, 1980) but we found no evidence of this phenomenon. The reduction of CL seen may have resulted from masking of bacterial antigens by $F(ab)_2$ fragments.

We conclude that the use of luminol-dependent neutrophil chemiluminescence stimulated by opsonized bacteria, in particular *H. influenzae*, provides a convenient means of determining whether immunoglobulin preparations have been functionally damaged by the methods used to improve their tolerability in i.v. use. The optimal choice of preparation for clinical use must depend also on other variables, such as safety (absence of adverse reactions and freedom from transmissible agents), and the biological half-life of the modified immunoglobulin. The use of the present assay will make it possible to observe and follow the *in vivo* effect of i.v. immunoglobulin treatment, and may be helpful in determining the optimum dosage schedule for antibody replacement therapy by this route.

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