A Factor in Human Gamma Globulin Preparations Active Against *Pseudomonas aeruginosa* Infections. (22904)

SANFORD M. ROSENTHAL, R. CARL MILLICAN AND JOHN RUST
(Introduced by DeWitt Stetten, Jr.)
National Institute of Arthritis and Metabolic Diseases, N.I.H., Public Health Service, Bethesda, Md.

Pseudomonas aeruginosa exhibits low virulence for mice, producing death only when injected intraperitoneally in large numbers (10^8 to 10^9 organisms). An endotoxin probably plays a part in these deaths, which occur chiefly within 12 to 24 hours.

The susceptibility of mice to Pseudomonas can be enhanced by pretreatment with cortisone, so that smaller inocula can be used(1). Under these conditions a more delayed mortality is produced, affording a more suitable technique for *in vivo* studies of antibacterial agents. By this procedure it has been possible to demonstrate a potent therapeutic effect from normal human γ globulin preparations, while human serum albumin is devoid of this principle.

Methods. Female albino mice of the NIH general purpose strain, weighing 17 to 22 g were used. Two strains of *Ps. aeruginosa,* isolated from patients with burns(2), were employed. They have been classified by Dr. Elizabeth Verder of the National Microbiological Institute as serological types 10: 1, 2. Lyophilized milk suspensions of the bacteria were inoculated into meat extract broth and

TABLE I. Results in Mice Pretreated with Cortisone. γ-globulin, albumin and plasma were all from human sources. One inj. only, within 3 hr after infection, unless otherwise specified. Organisms intraperitoneally.

Therapy	No. of organisms	No. of mice	% mortality — Days after infection — 1	2	3	4	5	6	7
A. Controls, untreated	8×10^5	110	13	50	63	71	72	76	84
B. 16% γ-globulin									
.2 ml i.v. × 2	8×10^5	25	4	4	4	8	16	20	24
.2 ml i.v.	"	29	3	10	10	24	24	31	31
.1	"	45	2	9	13	13	16	18	20
.05	"	15	0	7	20	27	27	33	40
.02	"	29	0	7	10	14	14	14	14
.005	"	14	0	21	28	50	50	50	57
.002	"	23	0	22	30	35	39	48	57
.0005	"	15	0	13	33	33	47	53	67
C. Controls, untreated	8×10^6	20	30	70	75	75	80	80	80
γ-globulin, .1 ml i.v.	"	14	0	0	0	7	14	36	36
" .007	"	9	0	11	22	22	33	33	45
" .002	"	10	0	30	40	40	60	60	60
D. Plasma, .3 ml i.v. or i.m.	8×10^5	26	12	19	31	35	39	46	50
Plasma, heated, 30 min. i.v. or i.m.									
56° — .3 ml	8×10^5	26	4	15	23	23	23	27	31
65° — .3	"	25	8	28	32	36	44	48	52
85° — .3	"	12	25	58	67	75	75	83	83
E. 16% serum albumin i.v.									
.2 ml i.v. × 2	8×10^5	15	13	53	60	60	73	73	73
.2 ml i.v.	"	10	10	70	70	80	90	100	100
F. 16% γ-globulin									
.2-.4 ml i.v.	0	10	0	10	10	10	10	20	20
Plasma, .3 ml i.v.	0	9	0	0	0	0	0	0	0
Cortisone only	0	28	0	4	7	7	7	7	7
Organisms only	8×10^5	155	0	0	0	0	0	0	1

i.p. = intraperitoneally; i.m. = intramuscularly; i.v. = intravenously.

incubated for 5 hours at 37°. After centrifugation the organisms were suspended in 0.9% NaCl to an optical density of 0.40 to 0.45 (at 650 mμ) representing an approximate concentration of 1.7×10^9 organisms per ml. This suspension was further diluted with saline 10^2 to 10^3 fold. Mice received intraperitoneally 0.5 ml of the diluted saline suspension (approximate infective dose, 8×10^6 to 8×10^5 organisms). *Cortisone pretreatment* of mice consisted of 5 intramuscular injections (0.1 ml) of 1.25 mg each of cortisone acetate at daily intervals beginning 4 days prior to infection, the last dose being given on the morning of inoculation. Under these conditions 80 to 100% of the animals died within 10 days, the majority of deaths occurring between the second and fifth days. Control animals receiving cortisone alone or bacteria alone have only occasionally died(1).

*Human γ globulin** was injected intravenously, intramuscularly or intraperitoneally within 3 hours after bacterial inoculation, in a volume of 0.1 to 0.2 ml; only one dose was given, unless otherwise specified. Dilutions of the original γ globulin, containing 16% protein, were made in 0.9% NaCl. Normal human serum albumin, 25%, was similarly diluted.

Results. Cortisone-treated mice. Very small amounts of γ globulin afforded a high degree of protection. Thus, a single intravenous injection representing 0.002 ml of the original 16% solution, or 320 μg of protein, protected approximately half the animals, while 0.02 to 0.2 ml prevented death in 70 to 86% of them (Table I, B; Fig. 1A).

While a high degree of protection was afforded by 0.02 ml, complete protection was not always attained by increasing the dosage of γ globulin 10 to 20 fold, in single or multiple injections. Some deaths from large doses may be due to extraneous toxicity of the cortisone plus globulin (Table I, F), since blood cultures were not taken on all fatalities. It

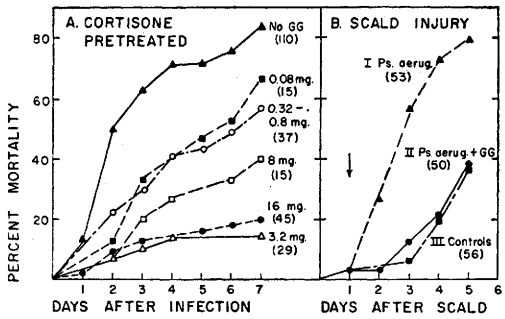

FIG. 1. Protective action of γ-globulin (GG) on Pseudomonas infections. A. Mice pretreated with 5 intramusc. injections of 1.25 mg of cortisone; organisms intraper. on fifth day, followed within 3 hr by varying amounts of GG intrav. as indicated. B. Susceptibility of thermally injured mice to Pseudomonas infections, and response to GG. Infection intraper. in Groups I and II (arrow), the day after injury; 16 mg GG intramusc. in Group II one hr after inoculation (8×10^5 organisms). Figures in parentheses = No. of mice.

should also be pointed out that heterologous γ globulin rapidly disappears from the circulation of the rabbit after a period of 4 or 5 days, due to antibodies formed by the host against the foreign protein. Therefore, with the use of a heterologous protein, the protective substances may be antagonized after the first few days. The *protective action* is manifest against many lethal doses of organisms, since increasing the inoculum 10 fold (8×10^6 organisms) did not greatly alter the results (Table I, C).

Human citrated plasma pooled from 3 donors, in dosage of 0.3 ml, intravenously or intramuscularly, gave a degree of protection in cortisone-treated mice consistent with its γ globulin content; experiments are in progress to determine the relative titre of plasma, and whether its activity is confined to the γ globulin fraction. A sample of chromatographically purified γ globulin from fresh human serum(3)† gave a degree of protection similar to that of commercially prepared material. Preliminary studies with normal mouse plasma (NIH strain) revealed little or no protective action in doses of 0.3 ml intravenously. To test the heat stability of the factor, samples of human plasma were heated for 30 minutes at 56°, 65° and 85°. The

* γ globulin preparation was kindly supplied by American Red Cross. This preparation is marketed as poliomyelitis immune serum globulin (human). Electrophoretically it is 99.1% γ globulins and 0.9% β globulins.

† Kindly supplied by Dr. Herbert A. Sober of National Cancer Institute.

TABLE II. Effect of γ-Globulin on Pseudomonas Infections in Normal Mice (No Cortisone Administered). γ-globulin inj. i.p. 3-6 hr before bacterial inoculation, to allow time for absorption.

Therapy	No. of organisms	No. of mice	% mortality — Days after infection						
			1	2	3	4	5	6	7
Controls, untreated	8×10^7	20	80	83	83	83	83	83	83
	1.6×10^7	20	5	5	10	10	10	10	10
	8×10^6	40	0	0	0	0	0	0	0
γ-globulin, 16% i.p. 3-6 hr before inoculation									
.1 ml	8×10^7	20	33	38	38	38	38	38	40
.1 ml	1.6×10^7	20	0	0	0	0	0	0	0

sample heated at 56° was centrifuged at 3000 r.p.m. to remove precipitated proteins, and the supernatant was injected intravenously or intramuscularly. Samples heated at 65° became gelatinous, while those at 85° developed a heavy precipitate; these were injected (without centrifugation) intraperitoneally or intramuscularly. Under these conditions activity was retained in the samples heated at 56° and 65°, and was lost at 85° (Table I, D). Experiments with normal human serum albumin injected intravenously in doses comparable to the highest amount of γ globulin employed, showed it to be devoid of protective action (Table I, E).

Normal mice. A few experiments were carried out on the antagonism in mice without cortisone pretreatment. An inoculum of 8×10^7 organisms intraperitoneally was required to kill normal mice. 0.1 ml of γ globulin injected intraperitoneally 3-6 hours before bacterial inoculation reduced the mortality from 83% in the untreated group to 40% (Table II).

Experiments with thermal trauma. Systemic infection with *Ps. aeruginosa* and other organisms normally of low virulence is a frequent sequel to extensive burns and other trauma. In mice subjected to a standardized trauma, in which acute death from shock was prevented by therapy with isotonic saline solutions, a high percentage of animals succumb within 2 to 3 weeks(4). In the present experiments a somewhat less severe degree of trauma was employed (unshaved etherized mice, dipped to the axilla for 6 seconds in water at 70°) in an attempt to retard the incidence of delayed deaths. Two ml of 0.9% NaCl was administered immediately after the trauma to prevent death from shock. The following day the mice were divided into 3 groups. Group I received intraperitoneally 0.5 ml of *Ps. aeruginosa* (8×10^5 organisms); Group II received organisms intraperitoneally and 0.1 ml of γ globulin intramuscularly within an hour following infection, and Group III served as a control group (injury only). In this way it was possible to test susceptibility of thermally injured mice to *Pseudomonas* infections, as well as the effect of γ globulin therapy upon infection.

Most of the traumatized mice injected with an inoculum of *Pseudomonas,* non-fatal to normal animals, succumbed within the first 4 days, while the mortality curve of those receiving γ globulin was much lower, and similar to that of the control group (Fig. 1B). Many of the animals in Groups II and III died at a later interval, and the cause(s) of death require elucidation. It remains to be determined whether more prolonged treatment with a homologous antibody will influence these later deaths.

The results with *Pseudomonas* infections in burns are similar to those obtained with the use of cortisone, and suggest that in extensive trauma the susceptibility to infections of this type may be related to excessive secretion of steroid hormones by the animals, as a consequence of the trauma. The above experiments also suggest that γ globulin therapy may be of benefit in *Pseudomonas* infections subsequent to trauma.

Discussion. Further work is in progress to supplement the present incomplete report. The nature of the protective mechanism, and

its application to other bacterial infections, must await future experimentation. Landy and Pillemer have shown protection against *Pseudomonas* and other Gram-negative bacterial infections by prior injection into mice of lipopolysaccharides that increase the properdin titre of blood serum(5). However, properdin is reported to be in Fraction III (Cohn) of the plasma proteins(5), while γ globulin is obtained from Fraction II. Likewise, heating serum for 30 minutes at 56° destroys properdin titre(5), while in our experiments with plasma no loss of activity occurred.

Summary. A factor in the γ globulin fraction of human plasma has been shown to prevent death in mice from *Ps. aeruginosa* infections, when injected subsequent to the inoculation. Protection was demonstrated in normal mice, and in mice rendered more susceptible to this infection by pretreatment with cortisone, or by subjecting them to thermal trauma. Human serum albumin was without activity.

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1. Millican, R. C., Rust, J., Verder, E., and Rosenthal, S. M., *Antibiotics Annual,* 1956-57.

2. Markley, K., Gurmendi, G., Morichavez, P., and Bazan, A., *Ann. Surg.*

3. Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A., *J. Am. Chem. Soc.,* 1956, v78, 756.

4. Landy, M., and Pillemer, L., *J. Exp. Méd.,* 1956, v104, 383.

5. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science,* 1954, v120, 279.

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Tissue Heparin and Mast Cells in Rats and Rabbits.* (22905)

W. MARX, L. MARX, P. RUCKER, L. RUGGERI AND L. FREEMAN
Department of Biochemistry and Nutrition, School of Medicine, University of S. California, Los Angeles.

It is generally assumed that heparin is synthesized and stored in the mast cells, and that variations of mast cell numbers and of heparin contents are parallel, when different tissues are compared(1,2,3). Recently species differences in susceptibility to atherosclerosis were correlated with differences in mast cell numbers; the greater resistance to this disease of certain species such as, *e.g.*, the rat, was attributed to heparin being available in relatively greater quantities, since this anticoagulant was shown to accelerate removal of certain plasma lipids, via the clearing factor(4). To test this hypothesis, tissue heparin concentrations and mast cell numbers were compared in species resistant and susceptible to atheromatosis, *i.e.*, the rat and rabbit.

Material and methods. Twenty adult male rats of the University of S. California strain, fed Purina Chow, and 12 adult male albino rabbits obtained from a commercial breeder, were used without further treatment. The animals were anesthetized with nembutal, and liver, intestine, kidneys, lungs, spleen and thymus were excised and weighed. From each of these organs, a small slice was removed for histological examination; samples of 1-5 g were then taken for heparin extraction, weighed and stored in the frozen state. Heparin was extracted, defatted, deproteinized by tryptic digestion and partially purified according to a method adapted to relatively small samples, and relatively low heparin concentrations, as reported recently (5). The heparin content of the extracts obtained was measured in line with a semimicro

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