Increase in Bactericidal Activity as a Result of Injection of Zymosan. (22600)

BENJAMIN BLATTBERG. (Introduced by M. N. Levy.)
General Medical Research Laboratory, Veterans Administration Hospital, Albany, N. Y.

In an attempt to isolate one of the components of complement, Pillemer *et al.*(1) separated a protein from normal serum which may have significance in natural immunity. The protein, called properdin, acts only in the presence of complement and Mg^{++} and is capable of destroying bacteria, of neutralizing viruses, and of lysing certain red cells(1,2).

Zymosan,* the insoluble carbohydrate residue from yeast cells, combines with properdin at 17°C in the presence of fresh serum to form a complex which can be removed by centrifugation. Serum deficient in properdin after zymosan treatment resembles normal serum except that the activity against bacteria, viruses, and red cells is greatly diminished or entirely absent(1).

In vivo experiments in mice have demonstrated the ability of zymosan to cause marked changes in susceptibility to experimental *Escherichia coli* infection(2). The ability of zymosan as well as other polysaccharides such as dextran, levan, and glucan to react with properdin(2) suggested the possibility that specific configurations or spatial arrangements are involved and that the injection of one of these polysaccharides into a normal animal would stimulate antibody production to these specific configurations.

Pillemer *et al.*(1) have shown that properdin has a bactericidal action against *E. coli* B. This paper concerns itself with an attempt to increase the cidal activity of rabbit and guinea pig serum for *E. coli* B by means of injections of zymosan incorporated in Freund adjuvants (3).

Methods. Zymosan was suspended in 100 volumes of saline and placed in a boiling water bath for one hour(4). After centrifugation at 3500 rpm for 45 minutes at 0°C, the zymosan was resuspended in a 3.5% solution of fresh egg-white and incubated for 2 hours at 37°C. This mixture was shaken occasionally during incubation and was then taken up in Freund adjuvants(3). As zymosan is a carbohydrate, there is the possibility that it may not be antigenic of itself but may act as a hapten. Therefore, fresh egg-white was added to the zymosan because it has been suggested that antigenic proteins may complex with carbohydrates and impart their antigenic property to the complex(5).

Six guinea pigs and 6 rabbits, all young adult males, were used. Each animal received a subcutaneous injection into the back of the neck, weekly, over a period of 3 weeks. A fourth injection was given 17 days after the third. Each guinea pig received a total of approximately 12 mg of zymosan and each rabbit approximately 24 mg. The animals were bled from the heart before injection and at intervals thereafter. Clotted blood was allowed to remain at room temperature for 2 hours. The serum was then separated in a refrigerated centrifuge and stored in sealed vials in a dry ice chest. The *E. coli* B culture was maintained on nutrient agar slants and transfers made to complete liquid medium(6). After the second serial transfer in this medium, an 18-hour culture was used for inoculum. After 4 or 5 such serial transfers, a new culture was started from the agar slant.

Bactericidal tests were set up in the following manner: Nine ml amounts of complete medium were sterilized in tubes calibrated at 10 ml. Each tube was inoculated with 1 ml containing approximately 1,000 cells of an 18-hour *E. coli* B culture. Sera were added to each tube in the amounts indicated. Mg^{++} was supplied by the addition of $MgCl_2 \cdot 6H_2O$ to the inoculum so as to give a final concentration of 0.07 mg of Mg^{++} ml in the test(7). The volume was made up to the 10 ml mark with distilled water to compensate for loss during sterilization, and after the contents were well mixed, 1 ml amounts were distributed into small

* Standard Brands, Inc.

sterile tubes. Incubation was at 37°C. At hourly intervals, one tube of each serum concentration was well shaken in a rack to avoid foaming, and pour plates of the entire contents were made in complete agar(6). Plates were incubated for 24 hours and colony counts made.

For skin testing, zymosan was prepared in a boiling water bath as previously described, but was centrifuged lightly at 500 rpm for one minute. The very opalescent supernatant was removed and 0.1 ml injected intracutaneously. Boiled as well as formalin treated cells of *E. coli* B were also used in skin tests, 0.1 ml amounts containing 2 x 10^7 and 2 x 10^5 bacteria being injected intracutaneously.

Antigens for agglutination tests were the same boiled and formalinized *E. coli* B suspensions as used for skin tests, but in a concentration of 1 x 10^8 cells ml. Incubation temperatures of 37°, 46°, and 56°C for 18 hours were all tried.

As not all preparations of zymosan are active, the zymosan used in this experiment was tested for its ability to absorb properdin from normal sera without affecting the hemolytic activity of the serum(4). Rabbit sera were absorbed twice with 5 mg of zymosan/ml at 17°C for one hour each time. The sera were then tested in a standard hemolytic system and found to have retained more than 80% of their original hemolytic activity. Bactericidal activity was found to be greatly diminished, indicating an active zymosan preparation(4).

Results. In order to eliminate as far as possible the variables which arise in experiments employing small numbers of bacteria and small amounts of serum, it was found necessary: (a) to report only those experiments in which the inoculum was within the arbitrarily chosen limits of 95 to 105 bacteria/ml; (b) to test in the same experiment, all sera which were to be compared; (c) to repeat the testing of these sera 3 or 4 times at intervals of approximately a week. Table I represents the results of 4 experiments with 0.75% and 1.0% concentrations of normal rabbit serum pool, of rabbit Pool G bled 7 days after the last injection and of Pool I bled 30 days after the last injection. Table II represents 4 experiments with 1.75% and 2.0% concentrations normal guinea pig pool, of guinea pig Pool B bled 11 days after the last injection and of Pool C bled 25 days after the last injection. The hourly average values in Tables I and II clearly indicate a sustained increase in bactericidal activity resulting from the zymosan injections. *Skin test*: Sixteen days after the third injection, the animals were skin tested with the zymosan suspension. There was no reaction at 8 hours, but by 24 hours there was marked reddening. The skin was raised and hard, the central areas were blanched, and later became necrotic. The rabbits showed more severe reactions than did the guinea pigs. Normal animals, skin tested at the same time, showed a slight pink area at 24 hours which had completely faded by 48 hours. Thirty-two days after the last injection (subsequent to the final bleeding), the animals were skin tested with both boiled and

TABLE I. Bactericidal Activity of Normal and Immunized Rabbit Sera as Determined by Plate Count.

Hr of incubation	Viable cells/ml at end of each incubation period					
	Pool N		Pool G		Pool I	
	Final conc. of serum					
	.75%	1.0%	.75%	1.0%	.75%	1.0%
1	77	79	76	40	5	2
	69	66	63	45	11	3
	92	88	53	46	2	0
	69	63	49	30	6	0
Avg	77	74	60	40	6	1
2	34	43	6	0	0	0
	32	29	12	2	0	0
	58	31	0	6	0	0
	45	33	6	2	0	0
Avg	42	34	6	3	0	0
3	118	65	4	0	0	0
	96	70	20	0	0	0
	260	82	2	4	0	0
	105	46	1	0	0	0
Avg	145	66	7	1	0	0
4	TM*	240	42	4	0	0
		200	94	0	0	0
		250	8	30	0	0
		TM*	20	4	0	0
Avg			41	10	0	0

Pool N: Normal serum pool.
Pool G: Serum pool 7 days after last inj.
Pool I: " " 30 " " " " ".
* TM = Too many bacteria to count.

TABLE II. Bactericidal Activity of Normal and Immunized Guinea Pig Sera as Determined by Plate Count.

Hr of incubation	Viable cells/ml at end of each incubation period					
	Pool N		Pool B		Pool C	
	Final conc. of serum					
	1.75%	2.0%	1.75%	2.0%	1.75%	2.0%
1	90	92	84	66	85	84
	95	90	75	94	75	74
	89	85	83	82	65	57
	79	67	78	73	78	68
Avg	88	84	80	79	76	71
2	104	124	15	2	4	0
	130	90	12	11	3	0
	139	75	4	5	1	0
	140	85	10	0	4	2
Avg	128	94	10	5	3	1
3	280	360	5	1	0	0
	410	210	4	6	0	0
	360	140	0	4	0	0
	440	170	2	0	0	0
Avg	376	220	4	3	0	0
4	TM*	TM*	14	0	0	0
			0	2	0	0
			0	4	0	0
			5	0	0	0
Avg			5	2	0	0

Pool N: Normal serum pool.
Pool B: Serum pool 11 days after last inj.
Pool C: " " 25 " " " " ".

* TM = Too many bacteria to count.

formalinized suspensions of *E. coli* B cells. The experimental animals failed to show appreciable differences from the normals.

Agglutination tests with *coli* antigens and rabbit and guinea pig sera failed to show consistent differences between normal and injected animals.

Rabbit Pool I, bled 30 days after the last injection, was absorbed with 5 mg of zymosan/ml of serum at 17°C for one hour. After centrifugation at 3500 rpm for 45 minutes at 0°C, this serum lost none of its hemolytic activity but showed a marked decrease in bactericidal potency. A second absorption of the same serum resulted in some 25% loss of hemolytic activity and a further loss in cidal activity (Table III).

Discussion. The increase in bactericidal titer, the positive Arthus phenomenon, and the ability to absorb bactericidal activity with zymosan, all suggest that antibodies were produced as a result of immunization with zymosan. Thus the possibility exists that antibody against zymosan possesses receptor sites capable of reacting with a specific polysaccharide configuration; a configuration which may be present in *E. coli* B, viruses, and red cells. The concept of the antigenicity of zymosan may be carried a step further to help explain a number of experiments(8-12) which have recently appeared in the literature. Workers have injected substances such as levan(8), lipopolysaccharides(9), typhoid vaccine(10), and zymosan(10-12) into mice and found a rapid fall in properdin level followed in 3 to 6 days by a rise to a level greater than normal. After about 10 to 14 days, the properdin level returned to normal. This fall and rise in properdin was paralleled by the degree of susceptibility of the mice to infection(8-12). Thus it would seem that there may be groupings on levan, lipopolysaccharides, typhoid vaccine, zymosan, *E. coli* B, viruses and perhaps other substances which can combine with receptor sites on properdin. The fact that the properdin level falls rapidly on injection of the above substances coincides with what would be expected if one injected antigen into an immune animal; namely, a rapid fall in antibody followed by a subsequent rise and then a downward levelling off. The response of properdin in the serum of a normal animal to injections of levan, zymosan, etc., is very much like the antibody response of the immune animal to specific antigen.

Landsteiner(5) in his discussion of natural antibodies concluded that normal serum contains substances which are formed indepen-

TABLE III. Bactericidal Activity* of Pool I Rabbit Serum after Absorption with Zymosan as Determined by Plate Count.

Hr of incubation	Absorbed once		Absorbed twice	
	.75%	1.0%	.75%	1.0%
1	91	85	88	87
2	97	40	172	183
3	300	68	TM†	TM†
4	TM†	TM†	TM†	TM†

After first absorption serum lost no hemolytic activity.
After second absorption serum lost 25% of hemolytic activity.

* Due to lack of serum, figures represent avg of only 2 duplicate experiments.
† TM = Too many bacteria to count.

dently of external antigenic stimuli, have a low order of specificity and resemble the antibodies produced by immunization. It is proposed that the properdin fraction of serum contains these normal substances which destroy bacteria, neutralize viruses, and destroy red cells, and that the injection of zymosan stimulates the production of antibodies with receptor configurations similar to those found in the properdin of normal serum. Such a proposal would readily explain the sustained increase in bactericidal activity of the serum due to the presence of small amounts of zymosan, also the ability of zymosan to absorb bactericidal activity due to immunization as well as to properdin.

Kabat and Berg(13) in demonstrating the antigenicity of dextran in man, suggested that "In all probability, other polysaccharides composed of only a single sugar, levans, mannans, etc., will also prove to be antigenic in man —". Zymosan belongs to this group of polysaccharides and it may be possible, by injecting zymosan, to increase the "natural antibodies" in the human species.

Summary. 1. Bactericidal activity against *Escherichia coli* B was increased in rabbits and guinea pigs as a result of injections of zymosan mixed with egg-white and incorporated in Freund adjuvants. 2. These rabbits and guinea pigs responded to intracutaneous injection of zymosan with the Arthus phenomenon. 3. Zymosan absorbed the bactericidal activity without affecting the hemolytic activity of the sera. 4. The possible antigenicity of zymosan is discussed.

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Turbidimetric Determination of Proteins with Sulfosalicylic and Trichloracetic Acids. (22601)

RICHARD J. HENRY, CHARLES SOBEL, AND MILTON SEGALOVE.
Bio-Science Laboratories, Los Angeles, Calif.

Folin and Denis(1), using a method proposed earlier for the nephelometric determination of protein in milk and digestion mixtures, introduced the turbidimetric determination of protein in urine with sulfosalicylic acid (SA). Subsequently, this technic was extended to protein determination in spinal fluid by Denis and Ayer(2) and in serum or plasma by Looney and Walsh(3). Mestrazat(4) used trichloracetic acid (TCA) for the turbidimetric determination of protein in spinal fluid.

The biuret reaction has almost completely supplanted turbidimetry for the routine determination of protein in serum in the clinical laboratory, but because of its greater sensitivity the turbidimetric method is still widely used for urine and spinal fluid and most textbooks advise the use of SA.

Although some workers(3,5) have reported observing no difference in the turbidity pro-