Antigenicity of Zymosan. (23399)

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Pillemer *et al.*(1) have demonstrated that zymosan, the soluble carbohydrate residue from yeast cells, combines with the properdin of fresh serum at 17°C to form an insoluble complex thereby removing the capacity of the normal serum to destroy bacteria, neutralize viruses, and lyse red cells.

Previously(2), it was demonstrated that the serum of rabbits and guinea pigs which had been injected with zymosan incorporated in adjuvants showed an increased bactericidal activity for *Escherichia coli* B. Skin testing of these animals with zymosan resulted in an Arthus reaction thereby suggesting the possible antigenicity of zymosan.

Additional experiments have been carried out in rabbits injected with zymosan plus adjuvants. It has been demonstrated by a quantitative agglutination technic that heat stable antibodies to zymosan are formed and that a concomitant increase in bactericidal activity for *E. coli* B can be absorbed by *E. coli* B cells.

Methods. Zymosan,* suspended in saline instead of egg-white, was prepared for injection by incorporation in Freund adjuvants as previously described(2). The presence of egg-white was found to be unnecessary.

Young adult male rabbits were used. Each of 6 rabbits received a subcutaneous injection of 8 mg of zymosan in adjuvants† followed by a 16 mg injection one week later. Five rabbits received no injections and were regarded as normal controls. Three rabbits received 2 injections of adjuvants without zymosan to test the effect of adjuvants alone. Two additional groups of 3 rabbits each were injected with zymosan alone, one group receiving 2 I.V. injections a week for 3 weeks and the other group 2 intracutaneous injections a week for 3 weeks. Each rabbit received a total of 12 mg of zymosan. All animals were bled from the heart before the injections began and periodically up to 100 days after the last injection. Sera from the rabbits within each group were pooled at each bleeding and placed in sealed tubes in a dry ice chest.

Bactericidal tests were carried out with *E. coli* B as previously described(2). Antigen for quantitative agglutination test was prepared by thoroughly grinding 0.1 g of zymosan in an agate mortar and slowly suspending it, with grinding, in 20 ml of sterile saline. The suspension was heated in a boiling water bath for 1½ hours. After centrifugation for 30 minutes at 1770 g, the precipitate was resuspended in 20 ml of sterile saline according to the method of Pillemer *et al.*(3). This suspension was stored in the refrigerator and usually used within the next 2 days. To 1 ml of serum to be tested, 1 ml of zymosan suspension (5 mg) was added. The mixture was shaken and placed in a 16.5°C water bath for 2 hours; during this time the tubes were well mixed every 10-15 minutes. The flocculant precipitate was centrifuged at 1550 g for 20 minutes and washed twice with 2 ml of ice-cold saline as described by Kabat and Mayer (4). Five mg of zymosan was found to be sufficient as further addition of zymosan to the supernatant failed to precipitate additional antibody.

All centrifugations and washings were conducted at 2-5°C in a refrigerated room. The washed precipitate was suspended in 4 ml of 0.25 N acetic acid and the supernatant clarified by centrifugation. The supernatant was read in the Beckman DU spectrophotometer at 277 mμ(5), with 0.25 N acetic acid in the reference cell. A zymosan blank was carried through the procedure without added serum. Optical densities of supernatants were corrected for the optical density of the blank which never read more than .005. The results were calculated in μg N/ml from E =

* Standard Brands, Inc.

† Adjuvants are Bayol F (Esso Standard Co.), Falba (Pfaltz and Bauer, Inc.), and killed tubercle bacilli strain H37Ra.

$\frac{O.D.}{\mu g\ N \text{ in sample}}$ where E, the extinction coefficient, is 0.0099 as determined by McDuffie and Kabat(5) for rabbit antibodies.

E. coli B was grown on nutrient agar, thoroughly washed with distilled water, centrifuged, suspended in 0.4% formalin and incubated until no longer viable as determined by plate count. Before use, the formalin was removed by centrifugation and the cells suspended in saline to a concentration of 2.2 x 10^9/ml. For absorption, 1 ml of cell suspension was added to 1 ml of serum and incubated at 16.5°C for 2 hours with shaking at 10-15 minute intervals. The suspensions were centrifuged in the refrigerated room and the supernatants were tested for remaining bactericidal and hemolytic activity.

Results. Fig. 1 indicates the rise and fall of the rabbit antizymosan serum titer. Zymosan plus adjuvants caused a delayed but sharp rise followed by a slow decline. The intravenously injected rabbits seemed to have been little affected by the injections. The rabbits given zymosan intracutaneously showed a definite though small increase in serum titer during the first 4 weeks. The titer of rabbits injected with adjuvants alone rose to about twice its original level and remained there throughout the experiment. Each point in Fig. 1 is the average result of 3 or more determinations performed on separate days. Four sera were titrated 7 times each and the standard deviation was calculated from $S = \sqrt{\frac{\Sigma x^2}{n-1}}$ with the following results in μg N/ml: 231.6, S = 2.43; 237.8, S = 3.20; 81.9, S = 3.45; 20.2, S = .70.

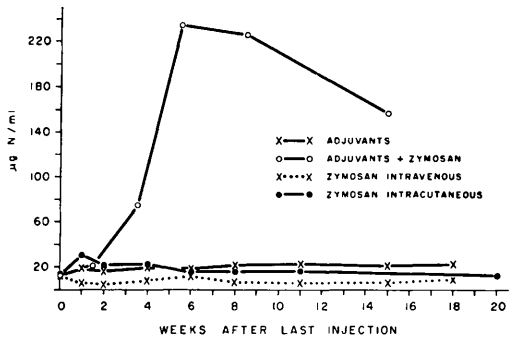

FIG. 1. Comparison of amt of nitrogen precipitated by zymosan from sera of rabbits immunized according to the various regimens.

Antibody nitrogen determinations made on immune sera, heated to 56°C for 30 minutes, were not found to be significantly different from the determinations made with unheated sera. It is therefore concluded that the antibodies are heat stable.

The bactericidal activity of the sera of rabbits bled 3½, 5½, and 8½ weeks after immunization with zymosan plus adjuvants was compared with the activity of sera of normal rabbits bled at the same time. The sera were absorbed with *E. coli* B at 16.5°C for 2 hours and then again tested for bactericidal activity. Table I records the results on the 5½ week sera. The 3½ and 8½ week sera gave similar results. The immune sera had greater bactericidal activity than normal sera, and after absorption with *E. coli* B neither exhibited bactericidal activity. Wardlaw and Pillemer(6) have demonstrated that lysis of bacteria requires all 4 components of com-

TABLE I. Bactericidal Activity. Viable counts of *E. coli* B/ml after incubation in sera of rabbits immunized according to the various regimens.

Sera	Final % conc. in test	Hr incubation 1	2	3	4
Adjuvants + zymosan	.75	3	0	0	0
" " " "absorbed"*	1	118	340	TM†	TM†
Normal	1	79	47	225	"
" "absorbed"*	1	101	350	TM	"
Adjuvants	1	86	45	180	"
Zymosan intrav.	1	71	44	200	"
" intracut.	1	49	32	140	"

* Sera were absorbed with 2.2 × 10^9 *E. coli* B/ml.
† TM = Too many colonies to count.
Plate count of *E. coli* B at start of exp. 100 ± 5/ml.
Each plate count is the avg of 3 exp. set up on different days.

plement. Therefore, after absorption with *E. coli* B the sera were titrated for hemolytic activity in a standard test with sensitized sheep cells. Less than 15% of their original activity was lost so that the decrease in bactericidal activity could not be attributed to a loss of complement. Table I also records the bactericidal activity of normal sera, of the sera of rabbits injected with adjuvants alone, and with zymosan alone injected intravenously or intracutaneously. These sera were obtained 2 weeks after the last injection. It can be seen that there is no evidence of increased bactericidal activity nor was any increase found in later bleedings. Each viable count in Table I represents the average of 3 experiments.

Discussion. Previously it was demonstrated that animals injected with zymosan incorporated in adjuvants would respond to an intracutaneous injection of zymosan with an Arthus phenomenon(2). Further evidence of the antigenicity of zymosan is demonstrated by the rise and fall of nitrogen content of zymosan antibodies in sera of rabbits immunized with zymosan plus adjuvants. These antibodies were shown to be heat stable.

The small increase in antibody nitrogen evident at the first week and continuing at a reduced level for the next 3-4 weeks in sera of rabbits injected intracutaneously with zymosan might indicate that, had much larger amounts of zymosan been injected, it is possible that high titered sera could have been produced without the aid of adjuvants. The intravenously injected rabbits too may not have received sufficient antigen.

The small increase in precipitable nitrogen in the sera of rabbits injected with adjuvants alone would indicate a substance in the adjuvants to which the rabbits responded and which was precipitated by the zymosan. Further investigation(7) revealed that rabbits injected with Bayol F or Falba, either alone or in combination, failed to give increased precipitates with zymosan. However, the killed tubercle bacilli, either alone or in combination with Bayol F or Falba, was found to be the cause of the increased titer represented in Fig. 1. It may be that the tubercle bacilli contain an antigenic grouping in common with zymosan.

Further it should be noted that rabbits immunized with zymosan plus adjuvants plus egg-white(2) when tested by quantitative agglutination with zymosan gave results of the same order as those given by rabbits without the addition of egg-white(7). Since a high titer of anti-egg-white antibodies was produced in these rabbits, and since the zymosan antizymosan titer was not increased, it would seem that in this case at least, zymosan did not precipitate the nonspecific gamma globulin.

It has been demonstrated that either zymosan or *E. coli* B is capable of absorbing bactericidal activity of normal as well as of zymosan immune sera(2). In addition, injections of zymosan were capable of increasing bactericidal activity of rabbit sera for *E. coli* B. It would seem, therefore, that *E. coli* B and zymosan may be related antigenically through spatial arrangements or specific configurations.

It is likely that zymosan contains a number of antigens due to the fact that it is a relatively crude commercial preparation derived from the cell walls of yeast. It is suggested that the increased bactericidal activity to *E. coli B* is a response to one of the antigenic groupings on the zymosan molecule, and that the amount of bactericidal antibody is a small part of the total antibody precipitated from the immune sera by zymosan.

Further work is planned to define more clearly the relationship between bactericidal and anti-zymosan properties of the sera of rabbits injected with zymosan.

Summary. Heat stable antibodies to zymosan were produced in rabbits and measured by quantitative agglutination. The increased bactericidal activity for *Escherichia coli* B resulting from injections of zymosan could be absorbed by *E. coli* B. Some aspects of the antigenicity of zymosan are discussed.

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Effect of Partial Hepatectomy on Liver and Kidney Coenzyme A Content in the Rat.* (23400)

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The rat liver regenerates rapidly after surgical extirpation of as much as two-thirds of the liver mass. During the regenerative process protein content and organ weight are restored to the original levels within a period of from 14 to 21 days after partial hepatectomy(1,2). Protein synthesis proceeds most rapidly in the first 4 to 8 days and affords the opportunity for study of protein anabolic processes in liver. The investigation described below was undertaken in the effort to evaluate the role of coenzyme A in this process. Acetyl coenzyme A is important in systems of carbohydrate and fat metabolism through mechanisms of utilization of 2 carbon fragments(3,4.5,6,7). It is to be presumed that similar mechanisms are involved in systems of protein metabolism. In this investigation the concentrations of coenzyme A in liver and kidney of rats were studied during the period of increased protein synthesis induced by subtotal hepatectomy.

Procedure. Male Sprague-Dawley rats weighing 95 to 135 g were divided into 4 groups. A subtotal hepatectomy was performed on 44 rats in Group I by excision of median and left lateral liver lobes *in toto* leaving the posterior and caudate lobes intact (major hepatectomy). The amount of liver removed was approximately 66% of total wet liver weight, a value corroborated by performing the identical operation on 10 additional animals which were immediately sacrificed and the excised and remaining liver mass determined. Rats in this group were sacrificed at intervals up to 14 days following major hepatectomy. Group II consisted of 10 rats which were subjected to excision of the left lobule of the median lobe of the liver (minor hepatectomy). Minor hepatectomy thus performed removed approximately 8.5% of total liver, or about 1/8th as much liver tissue as was removed at major hepatectomy (Group I). Animals in Group II were sacrificed 24 hours post-operatively and served as a control in evaluation of surgical stress and trauma to the liver on hepatic coenzyme A concentrations. Group III included 10 rats which were subjected to major hepatectomy and sacrificed 24 hours later, in order to determine the effect of early liver regeneration on kidney coenzyme A concentrations. Group IV consisted of 10 normal rats which were sacrificed and kidney coenzyme A concentrations determined. This group thus served as a control for Group III.

All animals were fed a stock diet (Purina Laboratory Chow) *ad libitum.* The daily food consumed by each rat in Group I was measured for one week prior to surgery and throughout the post-operative period daily intake of calcium pantothenate was estimated from amount of food consumed. Hepatectomized rats in Groups I, II and III received 15,000 units of procaine penicillin, intramuscularly, immediately following surgery. Body weights were measured at time of hepatectomy and at sacrifice. At the conclusion of the experimental periods, animals were decapitated. The remaining hepatic tissue and

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