A Heat-Labile Zymosan Agglutinating Factor Observed in Rabbits after Intravenous Injections of Quartz.* (24519)

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Zymosan, an insoluble carbohydrate derived from yeast(1) has remarkable immunological properties: it combines with properdin(2), with bovine conglutinin(3) and with anti-zymosan antibodies(4). Zymosan, therefore, is capable of binding different, although perhaps not entirely unrelated serum proteins. In investigations on possible role of immunological reactions in pathogenesis of silicosis (5,6), it was observed that sera of rabbits which had received several intravenous injections of quartz agglutinated zymosan, even at comparatively high dilutions. Obviously in serum of these rabbits a zymosan agglutinating factor had appeared; this factor will be referred to as quartz-factor (Q.F.), although no claim is made that a direct causal relationship exists between its appearance and administration of quartz. As this factor did not appear to be identifiable with any proteins already known to combine with zymosan, it was studied in detail and comparison was made between its reaction with zymosan and those of properdin and bovine conglutinin.

Materials and methods. Quartz dust (99% pure quartz, particle size 1-3 μ) was suspended in saline, autoclaved and injected intravenously in 10 hybrid rabbits of either sex. Twenty mg of quartz, suspended in 2 ml of saline, were injected twice weekly during 8 weeks, each rabbit receiving a total of 320 mg of quartz. One week after last injection, the rabbits were bled and their sera tested for ability to agglutinate zymosan. Ten control rabbits were treated with the same amounts of sterile saline. Fresh bovine sera containing natural conglutinin were obtained directly at the slaughter house. *Sera* were examined either immediately after clotting at room temperature or after a period (up to 4-5 months) of preservation in frozen state at –20°C, and thawing immediately before use.

For agglutination 2-fold serial dilutions of each serum were made at pH 7.4 barbital buffer containing Ca^{++} and Mg^{++}(7) starting from 1:8. 0.5 mg of zymosan, finely† suspended in 0.1 ml pH 7.4 barbital buffer, were added to 1 ml of each serum dilution and the mixture incubated overnight at different temperatures, *viz*, 2°C, 17°C and 37°C; for every experiment each reagent was brought to the desired temperature before mixing. Three different zymosans were tested: Zymosan G-6, (obtained from Dr. M. E. Scevola of Ist. Sieroterapico Milanese) had been perepared by pyridine hydrolysis(8) and was composed of comparatively large particles (4 μ average diameter); according to its behaviour with the properdin system it could be classified in category B of Pillemer *et al.*(7) inasmuch as it combined with properdin to give a suitable RP, but did not give a good R_3; Zymosan D i.s.m., obtained from the same source, had been prepared by tryptic hydrolysis had definitely smaller particles (2 μ average diameter), and was classified in category A of Pillemer *et al.*, as it made a suitable RP and R_3; particle size and immunological properties of the third sample, Zymosan 6B 14 (obtained from Dr. M. Landy, National Cancer Inst., Bethesda), were similar to those of Zymosan D i.s.m. All 3 zymosans were agglutinated by Q.F. and conglutinin, however Zymosan G-6 gave better results presumably because its definitely coarser particles required less agglutinating factor to give visible clumping: Zymosan G-6 was therefore used throughout. *Agglutination* was observed after gently inverting the tubes thrice: presence of large macroscopic clumping was classified as a +++ reaction, while agglutination giving finer agglomerates, but visible by eye, was estimated as ++; a reaction was considered

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† Shaking 1 hour with rapid vibrator (Microid Flask shaker, Griffin, London) gave homogeneous zymosan suspensions.

+ when only microscopic observation showed that most particles were agglutinated. The influence on agglutination of *high ionic strength or absence of divalent cations* was studied by preparing sera in 2-fold serial dilutions with ionic strength = 0.69 veronal-NaCl buffer as used by Pillemer *et al.* to elute properdin from PZ(7) or with 0.01 M trisodium-ethylenediaminotetra-acetate (Na_3-EDTA) dissolved in saline. In some experiments sera were deprived of divalent cations by treatment with ion-exchange resin(9) and dilutions then performed in 0.15 M NaCl. Zymosan agglutinating activity of *sera treated with typhoid endotoxin or with gastric mucin* was tested by adding 0.25 mg of purified typhoid endotoxin‡ or 0.5 mg of gastric mucin§ to 1 ml of 2-fold serial dilutions (starting from 1:8) of serum, incubating 30′ at 17°C and finally adding 0.5 mg zymosan to each test tube. Agglutinating activity of *sera deprived of C'4* by treatment with 0.125 N NH_4OH(10) or with 6.0×10^{-3} M hydrazine (11) was also tested. To investigate the possibility that agglutination of zymosan by Q.F. could be due to interaction between some normal serum component absorbed by zymosan and the agglutinating factor of rabbit sera, agglutinability of *zymosan pre-treated with normal serum* was studied by performing 2-fold serial dilutions with pH 7.4 barbital buffer(7) of fresh normal rabbit serum starting from 1:2; to 1 ml of each dilution. 0.5 mg of zymosan were then added, the mixture incubated 1 hour at 17°C and frequently mixed; the tubes were then centrifuged and zymosan particles washed 3 times with barbital buffer. Finally, 1 ml of 1:32 dilution of Q.F. containing rabbit serum previously shown to agglutinate zymosan strongly, was added to the sedimented zymosan particles in each test tube; particles were resuspended and mixtures incubated overnight. Pretreatment of zymosan with fresh normal serum was also made in the presence of 0.01 M EDTA, the subsequent steps performed as before. Finally all sera were tested for *conglutinin activity* on sheep red cells sensitized with bovine antibody and treated with horse complement, as described by Coombs and Coombs(12).

Results. After intravenous administration of quartz the sera of 8 out of 10 rabbits agglutinated zymosan at titres 1:128 or 1:256. Zymosan agglutinating activity could be removed by absorbing the sera once with zymosan. With some fresh sera of quartz-treated rabbits a prozone was observed, agglutination at 1:8 being absent or definitely weaker than at higher dilutions. Sera of control rabbits did not agglutinate zymosan, while 2 out of 45 untreated farm-bred rabbits had sera agglutinating zymosan to dilutions of 1:64 and 1:128 respectively; investigation showed that these sera contained zymosan agglutinating factor which behaved in all respects (see below) as the factor induced by quartz.

The incidence of zymosan agglutinating activity amongst untreated hybrid rabbits of 4 different stocks bred in different laboratories was then made; while 2 stocks had 0% positive animals, the other 2 had 41% and 50% respectively; in total the sera of 16 out 97 rabbits agglutinated zymosan at titres varying between 1:32 and 1:128. This observation prompted the possibility that latent or active infection might determine appearance of Q.F. or similar factors in "untreated" rabbits. This was suggested by the fact that, in weekly examinations, the titres of one stock with high incidence of zymosan agglutinating activity declined in some animals while agglutinating activity appeared in others in which it had previously been absent. In an effort to determine the nature of the infectious agent (or agents), 5 untreated rabbits whose sera showed higher zymosan agglutinating activity were sacrificed; necrospy showed in one case a fibrino-purulent pleuro-pericarditis due, as ascertained bacteriologically, to *Pasteurella multocida*. The possibility that the "spontaneous" appearance of zymosan agglutinating activity is due to gram-negative bacteria appears attractive in view of some characteristics of Q.F. (see below) and is presently being investigated; it is apparent that conditions other than intravenous administration

‡ Kindly prepared by Dr. A. Di Nardo, Inst. of Microbiol., Univ. Milan, from *S. typhi* 0901.

§ Crude hog gastric mucin (Armour) 2 mg of which were shown previously to absorb >90% properdin from 1 ml human serum.

of quartz may induce its production.

Normal bovine sera also agglutinated zymosan at high dilutions. This was due to a factor different from that induced in rabbits by quartz *i.e.*, normally occurring bovine conglutinin(2). The simpler way to show that bovine conglutinin and zymosan agglutinating factor induced in rabbits by quartz have different properties is to inactivate the sera; bovine conglutinin is unaffected by heating for 30′ at 56°C, and inactivated bovine serum agglutinates zymosan as well as before inactivation(11). On the contrary Q.F. sera lose entirely their ability to agglutinate zymosan after heat inactivation.

That this is not due to simple loss of active complement was supported by the fact that addition of fresh rabbit or guinea-pig serum to heated Q.F. serum, did not restore ability to agglutinate zymosan. The effect of heating at 56°C on Q.F. is reminiscent of the total loss of properdin activity from sera heated to the same temperature.

The possibility was then considered that Q.F. might react with a number of substrates already known to combine with properdin; it was found that typhoid endotoxin and hog gastric mucin abolished agglutination of zymosan by Q.F. while not affecting that due to bovine conglutinin.

The next step was to consider the possibility that properdin could interfere with combination of Q.F. with zymosan; pretreatment of zymosan with fresh normal rabbit serum in dilutions up to 1:4 (and with some sera up to 1:8) made it inagglutinable by Q.F., while no inhibition was observed by pre-treating zymosan with fresh rabbit sera in presence of Na_3EDTA or with sera deprived of C′4 by ammonia or hydrazine.

These results show that something exists in normal fresh rabbit sera which reacts with zymosan, thus rendering it inagglutinable by Q.F.; this substance may well be properdin. It appears probable that the prozone sometimes observed with fresh Q.F. containing sera is due to a like interaction; treatment of the agglutinating sera with ammonia or hydrazine, while not affecting the limit titre of agglutination of zymosan, caused prozone to disappear.

The failure of Q.F. to agglutinate zymosan after pretreating the latter with fresh normal rabbit serum is important, in that it indicates that Q.F. combines with zymosan directly and that agglutination is not the consequence of a reaction between Q.F. and some normal serum component absorbed by zymosan; should this be the case, zymosan pretreated with normal rabbit serum should be agglutinated at least as well as the untreated one.

Possible similarities or differences between reactions with zymosan, of properdin on one side and Q.F. and conglutinin were investigated. It was ascertained that, while properdin does not combine with zymosan at temperature lower than 5°C(12), Q.F. and bovine conglutinin agglutinate zymosan equally well at 2°C and at 17°C; at 37°C agglutination by Q.F. was slightly improved while that by bovine conglutinin was at least 2 titres less than at 17°C.

At ionic strength = 0.69 bovine conglutinin does not agglutinate zymosan and preformed agglutinates are promptly reversed indicating that conglutinin is being eluted from zymosan much in the same way as is properdin(7); the behaviour of Q.F. at ionic strength = 0.69 is, however, different inasmuch as it normally agglutinates zymosan and is not eluted from it.

Presence of divalent cations was necessary for the combination with zymosan of both Q.F. and bovine conglutinin, as it is for properdin(12); neither Q.F. nor bovine conglutinin, agglutinated zymosan in the presence of 0.01 M Na_3EDTA or after treatment of sera with cation-exchange resin. The zymosan agglutinating activity was restored almost to original extent by performing dilutions of resin-treated sera in Mg^{++} and Ca^{++} containing barbital buffer(7) instead of simple saline.

The above results show therefore that Q.F. behaves differently from properdin and conglutinin. This was further borne out by investigation of conglutinin activity. As expected, all bovine sera showed strong (up to dilution 1:1024) conglutination of sheep red cells treated after Method I of Coombs and Coombs(13), no such activity was found in fresh and inactivated Q.F. sera. This differentiates Q.F. not only from bovine congluti-

TABLE I. Comparison of Some Properties of Properdin (Rabbit or Human), Quartz Factor (Rabbit) and Conglutinin (Bovine).

	Properdin	Quartz factor	Conglutinin
Agglutination follows reaction with zymosan	No	Yes	Yes
Combines with zymosan at 2°C	"	"	"
At ionic strength = 0.69	"	"	No
In presence of 0.01 M EDTA	"	No	"
After heating serum at 56°C for 30′	"	"	Yes
After absorption with *Salmonella typhi* endotoxin	"	"	"
After absorption with hog gastric mucin	"	"	"
In absence of C'4	"	Yes	"
Conglutinates sensitized sheep red cells treated with horse complement	"	No	"

nin, but also from immuno-conglutinin, obtained in rabbits by intravenous injections of particulate substances such as killed gram-negative bacteria or kaolin particles covered with horse complement. Like natural bovine conglutinin, rabbit immunoconglutinins are resistant to heating at 56°C for 30′, and agglutinate zymosan as well; their possible presence(13) in "normal" rabbits may explain our observation of a zymosan agglutinating activity which resisted heating at 56°C for 30′ in 2 out of 97 untreated rabbits. In another case the titre of the agglutination was reduced by heating from 1:128 to 1:16; this suggests the possibility that in some instances Q.F. may coexist with immunoconglutinin, to which it is obviously similar. Q.F. appears entirely unrelated with Cx-reactive protein; purified Cx-reactive protein (obtained through the courtesy of Dr. H. F. Wood, Irvington House, N. Y.) did not agglutinate zymosan either alone or after addition of fresh normal rabbit serum.

Discussion. That Q. F. needs divalent cations to combine with zymosan suggests that Q.F. does not behave as an antibody, at least not as a classical antibody. The evidence summarized in Table I, shows rather that Q.F. behaves in some respects like properdin and in other respects like a conglutinin; it might be asserted that Q.F. appears to be an intermediate between properdin and conglutinin.

Unlike properdin, however, it is not present in detectable amounts in blood of every rabbit. Not much is known concerning the mechanism of production of Q.F.: it is not known what conditions, other than intravenous administration of quartz, can determine its appearance, nor whether quartz acts directly or through some intermediate process, such as superimposed infections or cellular damages connected with widespread lesions of liver and spleen produced after intravenous injections of quartz(14).

Summary. After intravenous injections of fine quartz dust, a serum factor appeared in 8 out of 10 rabbits which agglutinated zymosan to a dilution of 1:256. A similar factor was also noted in occasional uninjected rabbits. This factor differed in certain respects from classical antibodies and appeared to be different from any other hitherto described, to react with zymosan. The conditions of its reaction with zymosan have been studied; they are in some respects similar to those of properdin, and similar to those of conglutinin. In certain respects, however, the factor induced by quartz behaves differently from both properdin and conglutinin. It is not believed that this factor is specifically induced by quartz but rather due to a non-specific immune response.

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Incorporation *in vivo* of P^{32} from Condensed Phosphates. (24520)

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The reported symptomatic benefit obtained in treatment of tumor metastasis to bone with P^{32} phosphate(1,2,3) has suggested the use of other forms of phosphate. In particular, condensed phosphates such as trimetaphosphate and polymetaphosphate (previously misnamed hexametaphosphate) appeared worthy of investigation since they have been hydrolyzed by mammalian tissues(4,5). Additional favorable features of these substances are their strong binding to proteins(6,7), and relatively high molecular weights(8). Both characteristics would tend to increase body retention, thus prolonging their action. This, in addition to ease with which these condensed phosphates can be prepared from other phosphates, has prompted the present study of localization of P^{32} from these higher forms of phosphates.

Methods. Radioactive trimetaphosphate was prepared in platinum crucibles from $NaH_2P^{32}O_4 \cdot H_2O$ by the method of Jones(9). Larger quantities of a non-radioactive preparation were obtained simultaneously for analytical purposes. The final preparation was an amber-colored clear glass, not precipitable in solution with $AgNO_3$ indicating an absence of phosphate. The pH of 1% solutions was 5.5. Phosphorus content was 28%. Polymetaphosphate also was prepared from $NaH_2P^{32}O_4 \cdot H_2O$ by the method of Jones(9). This material was a colorless transparent glass which dissolved slowly in water. In solution, it did not precipitate with $AgNO_3$. One % solutions had a pH of 5.8. Phosphorus content was 26%. Both substances probably do not represent pure compounds but contain small amounts of other condensation products. According to Jones(9), 92-95% of pure metaphosphates may be prepared by these procedures. Injections of $NaH_2P^{32}O_4$, radioactive trimetaphosphate or polymetaphosphate, were given intraperitoneally to 25 g male mice for 4 successive days, and the mice were sacrificed 24 hours after last injection. When larger bone specimens were required, 5 kg male rabbits were used. No toxic reactions or discomfort were observable. Preliminary experiments established intravenous and intraperitoneal routes as being equivalent and markedly superior to oral administration. That condensed phosphates are poorly absorbed by the intestine has been noted by other workers(10). Amount of radioactivity given was approximately 3×10^5 counts/minute for the 3 substances. Depending upon the experiment, representative samples of pooled organs of 2-5 animals were obtained, wet-ashed with HNO_3 and H_2O_2 and aliquots plated on stainless steel planchets. Radioactivity was counted with an end window counter coupled with an automatic sample changer attachment. All counts were corrected for decay. No correction for self absorption was necessary for the mass of sample taken. Standard deviation for the counts measured is $\pm$ 2 cts/min. To evaluate biological retention of these substances, radioactivity of approximately 4×10^5