# A METHOD FOR THE PREPARATION OF COMPLEMENT-FIXING ANTIGENS IN A STUDY OF EXPERIMENTAL TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS)

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Other investigators (Bengtson, 1944, 1945; Topping and Shepard, 1945) have recently reported the successful preparation of complement-fixing antigens of *Rickettsia orientalis*, grown in the yolk sac of fertile hens' eggs, that were suitable for use in the diagnosis of tsutsugamushi disease (scrub typhus fever). These findings have been confirmed by other workers as well as by ourselves (Cox *et al.*, 1945). However, the methods of preparation described in these reports are not too readily adaptable to large-scale production. Moreover, those authors have stated that their preparations contained only specially selected yolk sacs that showed large numbers of rickettsiae by microscopic examination (Topping, 1945).

In view of the results hitherto obtained it seemed advisable to attempt to develop a method that would be applicable to fairly large-scale production and that would utilize the yolk sacs from all living eggs harvested daily for the preparation of antigens of at least a moderately potent titer. In other words, yolk sacs showing only moderate or even poor rickettsial growths would be utilized, and the processing procedure should be of such a nature as to conserve and concentrate all antigenic substances present.

This paper describes a method of preparing complement-fixing antigens that seems to meet the foregoing requirements.

## MATERIALS AND METHODS

*Rickettsial strain.* The Karp strain of *Rickettsia orientalis*, obtained through the courtesy of Dr. Norman H. Topping of the National Institute of Health and with the permission of the United States of America Typhus Commission, was used throughout the experiments.

*Source of infectious material.* With a few exceptions, all antigens were prepared from infected yolk sac membranes of fertile hens' eggs inoculated as described by Cox (1938), and were processed from daily harvests containing from 300 to 900 eggs a harvest. A few antigens were prepared from other portions of the fertile eggs, such as the yolk fluid and the chorio-allantoic membrane, but, as will be shown later, such preparations were not nearly so active as those prepared from yolk sac membranes. Twelve to thirty-six yolk sacs were examined microscopically from each lot of eggs on the eighth, ninth, tenth, or eleventh day after inoculation, and the results of these

samplings were considered as representative of the whole lot and determined the time of harvesting of all remaining, living eggs.

The majority of the antigen preparations were prepared from yolk sacs that showed an average of at least "one plus" rickettsial growth. Such a numerical evaluation was determined in the following manner: Smears showing rickettsiae varying in numbers from 1 up to 6 or 8 per oil immersion field were termed "positive"; smears showing from 6 to 8 up to a countable number of rickettsiae per field were termed "one plus"; and smears showing uncountable numbers of rickettsiae in nearly every field were termed "two plus." It must be emphasized that in no instance could the *average count* of the yolk sac harvests concerned in this report be considered as "two plus" growth.

*Preparation of antigens.* A general outline of the method used for preparing the antigens is as follows:

(1) Infected yolk sacs (either freshly harvested or those kept frozen *in toto* in the $CO_2$ icebox at $-70$ C and later thawed in a water bath at about 37 C) were weighed and homogenized in a Waring blender to a 50 per cent tissue suspension with the addition of an equal volume of distilled water containing phenol to 0.8 per cent concentration.

(2) The resulting 50 per cent tissue suspension was held for at least 48 hours in the cold room at 4 C.

(3) The inactivated suspension was dispensed into freezing bottles of the blood plasma type, shell-frozen in a dry ice alcohol mixture, and completely dried by evacuation from the frozen state.

(4) The shell of dried tissue was well broken up in the bottle by means of a spatula and the material transferred to an Erlenmeyer flask.

(5) One volume of diethyl ether (based on the original amount of 50 per cent tissue suspension employed) was added to the flask, and the contents were well mixed. The flask was tightly stoppered and allowed to stand at 4 C for 3 to 4 hours with occasional shaking.

(6) The ether was separated from the tissue mass by means of a Buchner funnel fitted with two layers of coarse (crepe) filter paper. Small particles of tissue adherent to the walls of the extraction flask were collected by rinsing the flask with small portions of fresh ether and adding the washings to the mass in the funnel. After being freed of all excess ether by suction, the tissue was carefully scraped from the funnel in small thin flakes and replaced in the extraction flask.

(7) Step no. 5 was repeated except that 1½ to 2 volumes of fresh ether were used. The extraction with ether was again carried out for 3 to 4 hours or even overnight at 4 C with occasional shaking.

(8) Step no. 6 was repeated, and the dry, pulverized material was then transferred to thick-walled, pyrex glass bottles suitable for the subsequent removal of the remaining ether by high vacuum. A simple apparatus consisting of a cenco-megavac pump and an ether trap immersed in a dry ice alcohol bath served to remove traces of residual ether by allowing the material to remain under high vacuum for 6 to 18 hours at room temperature.

(9) The dried powder was weighed and placed in a bottle containing a few sterile glass beads. A quantity of saline (containing phenol to 0.3 per cent con-

centration), sufficient to resuspend the material to a volume equal to that of the original 50 per cent tissue suspension, was added. The suspension was stored at 4 C for 3 to 5 days with occasional shaking each day.

(10) The suspension was centrifuged at 3,000 rpm for 30 minutes at room temperature in an International, size I centrifuge equipped with an angle head rotor. The sediment was discarded. The resulting supernatant fluid constituted the antigen.

Various modifications were made in this procedure in an attempt to secure antigens of higher complement-fixing titers, and these will be noted later in describing each antigenic preparation.

Each preparation was evaluated by the results obtained in complement-fixing tests in which the antigens were titrated in the presence of a known scrub-typhus-immune guinea pig serum.

*Complement-fixation test.* For the complement-fixation tests a modified Kolmer and Boerner (1941) technique was employed using 2 units of amboceptor, 2 exact units of complement, and a 2 per cent suspension of washed sheep cells.

In the titration of the antigen, 0.25 ml of diluted, inactivated serum from guinea pigs recovered from tsutsugamushi disease were added to 0.25-ml amounts of antigen that had been serially diluted 2- fold. Two exact units of complement in a volume of 0.5 ml were then added to each tube and the tubes held in the cold room at 4 C overnight. The following morning the hemolytic system (consisting of 2 units of hemolysin in a volume of 0.25 ml plus 0.25 ml of a 2 per cent suspension of washed sheep cells) was added, and the tubes were kept at 37 C until the control tubes cleared. Readings were then made immediately.

In the determination of the titer of tsutsugamushi disease antiserum, 0.25 ml of antigen containing approximately 3 antigenic units were added to 0.25-ml amounts of inactivated serum that had been serially diluted 2- fold. Two exact units of complement (determined in the presence of antigen) in a volume of 0.5 ml were then added to each tube and the tubes held in the cold room at 4 C overnight. The method of adding the hemolytic system and of incubating the tubes at 37 C before the reading was the same as that used in determining the antigenic titer.

The pool of tsutsugamushi-disease-immune serum, against which all the antigen preparations were tested, was obtained from a group of convalescent guinea pigs that had been inoculated with infected mouse liver suspensions of the Karp strain. This serum gave a complement-fixing titer of "4 plus" in a dilution of 1:128 and in all antigenic titration tests was used in a dilution of 1:20.

## EXPERIMENTAL

*The effect of prolonged contact with phenol upon the crude tissue suspension.* Living yolk sacs were harvested from over 900 eggs on the tenth day after inoculation and stored frozen *in toto* at − 70 C in a $CO_2$ box for 3 to 6 weeks. The yolk sacs were then processed as shown above except that step no. 2[1] was modified as follows: Phenol (0.4 per cent concentration) was allowed to act on

[1] These numbers refer to the steps involved in the processing procedure outlined under the section *Preparation of antigens*.

one portion of the crude tissue suspension for 2 days, on a second for 12 days, and on a third for 33 days before continuing with the refining process.

The complement-fixation results obtained with these antigens (table 1) indicate that 0.4 per cent phenol, in contact with the crude tissue suspension for as long as 33 days, exerts no appreciable deleterious effect upon the final product.

*Comparative antigenicity of various portions of the infected fertile egg.* The yolk sacs, chorio-allantoic membranes, and yolk fluid were harvested separately from 8 eggs that were still living on the tenth day after inoculation.[2] The various

TABLE 1

*Effect of prolonged contact with phenol upon crude tissue suspension*

| ANTIGEN SERIES | PROCEDURE NOTES | YOLK SAC CONCENTRATION OF FINAL PRODUCT | ANTIGEN DILUTION TESTED | | | | | | ANTIGEN CONTROLS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 | 4 | 8 | 16 | 32 | 64 | 2 | 4 | 8 |
| K-11-Aa*.. | 2 days in phenol | 25 per cent | 4 | 4 | 4 | 1 | | | | | |
| K-11-B.... | 12 days in phenol | 50 per cent | 4 | 4 | 4 | 4 | 2 | | | | |
| K-11-C.... | 33 days in phenol | 50 per cent | 4 | 4 | 4 | 4 | 2 | | | | |

* This antigen was resuspended to a volume equivalent to a 25 per cent yolk sac suspension for other purposes of study. From data presented later (series K-16, table 6) it is reasonable to assume that if the concentration of this antigen had been 50 per cent instead of 25 per cent the complement fixation results would have been identical to those of antigens K-11-B and K-11-C.

TABLE 2

*Comparative antigenicity of various portions of the infected fertile egg*

| ANTIGEN | EGG MATERIAL | ANTIGEN DILUTIONS TESTED | | | | | | ANTIGEN CONTROLS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 8 | 16 | 32 | 64 | 2 | 4 | 8 |
| K - 12 - A.... | Yolk sac membrane | 4 | 4 | 4 | 4 | 4 | 1 | 4 | 1 | |
| K - 12 - B.... | Chorio-allantoic membrane | 4 | 4 | 1 | | | | 4 | | |
| K - 12 - C.... | Yolk fluid | 4 | 1 | | | | | | | |

portions were then processed as outlined above except for steps no. 1 and no. 2. No. 1: The membranes and fluid were prepared as 10 per cent suspensions by weight in 0.4 per cent phenolized saline. No. 2: The suspensions were all stored at 4 C for 40 days before processing. After being processed each preparation was resuspended to a volume calculated to be equivalent to that of an original 50 per cent tissue suspension.

The data in table 2 show that when these various preparations were titrated

[2] The embryos were likewise harvested separately but an accident prevented completion of the antigen. This experiment is being repeated.

as antigens the yolk sac proved to be at least 8 times more active than the chorioallantoic membrane or yolk fluid.

*The effect of inactivating the rickettsiae with ether or phenol and preserving the antigens with formalin or phenol.* Yolk sac membranes were harvested from 310 living eggs on the tenth day after inoculation and were held frozen *in toto* at −70 C for 2 to 3 days. The yolk sac suspensions were then processed as outlined above except for steps no. 1 and no. 10. No. 1: One half of the 50 per cent tissue suspension was inactivated by the addition of 0.4 per cent phenol, and the other half was inactivated by the addition of 10 per cent ether by volume. No. 10: Both preparations, after ether extraction, were resuspended in saline containing 5 per cent ether. To one half of each preparation phenol was added to 0.1 per cent concentration, but to the other half of each preparation was added 0.1 per cent formalin.

TABLE 3

*The effect of inactivating the rickettsiae with ether or phenol and preserving the antigens with formalin or phenol*

| ANTIGEN | PROCEDURE NOTES | RESULTS SOON AFTER PREPARATION | | | | | | RESULTS AFTER 6 WEEKS' STORAGE AT 4 C | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 8 | 16 | 32 | 64 | 2 | 4 | 8 | 16 | 32 | 64 |
| K-16-B.... | Ether-killed, formalin-preserved | 4 | 4 | 4 | 4 | 1 | | 4 | 4 | 1 | | | |
| K-16-C.... | Ether-killed, phenol-preserved | 4 | 4 | 4 | 4 | 1 | | 4 | 4 | 4 | 3 | | |
| K-16-J.... | Phenol-killed, phenol-preserved | 4 | 4 | 4 | 4 | 2 | | 4 | 4 | 4 | 3–4 | | |
| K-16-K.... | Phenol-killed, formalin-preserved | 4 | 4 | 4 | 4 | 3 | | 4 | 4 | 2 | | | |
| K-11-C.... | (Used as a control) | 4 | 4 | 4 | 4 | 2 | | 4 | 4 | 4 | 3–4 | | |

Table 3 shows the results obtained when the various preparations were titrated as antigens soon after their preparation and again after 6 weeks' storage at 4 C.

The data show that no appreciable differences could be discerned in the antigens when they were tested soon after their preparation. When tested after 6 weeks' storage, however, all showed a decrease in titer. The preparations preserved with phenol showed the least drop in titer—approximately 2-fold—but those preserved with formalin showed the greatest loss in activity—approximately 4-fold.

The preparations inactivated with phenol gave slightly higher titers than those inactivated with ether, but the differences were not great enough to be considered significant.

*The antigenicity of yolk sac membranes harvested from dead embryos.* Yolk sac membranes were harvested from embryos that had died at various intervals after inoculation. The yolk sac suspensions were processed as outlined except for steps no. 1 and no. 2. No 1: The yolk sac membranes were homogenized to a 50

per cent suspension in 0.85 per cent salt solution containing 0.25 per cent phenol. No. 2: The suspensions were held at 4 C for 10 to 15 days before processing.

The data in table 4 indicate that yolk sacs harvested from embryos dead on the eighth to tenth day after inoculation demonstrate some activity when tested as antigens. However, their activity was so poor when compared with that of

TABLE 4

*The antigenicity of yolk sac membranes harvested from dead embryos*

| ANTIGEN | PROCEDURE NOTES | ANTIGEN DILUTIONS TESTED | | | | ANTIGEN CONTROLS | | |
|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 8 | 16 | 2 | 4 | 8 |
| DD-4... | Dead 4 days after inoculation | | | | | | | |
| DD-5... | Dead 5 days after inoculation | | | | | | | |
| DD-6... | Dead 6 days after inoculation | | | | | | | |
| DD-7... | Dead 7 days after inoculation | | | | | | | |
| DD-8... | Dead 8 days after inoculation | 3 | | | | | | |
| DD-9... | Dead 9 days after inoculation | 4 | 1 | | | | | |
| DD-10.. | Dead 10 days after inoculation | 4 | 3 | | | | | |

TABLE 5

*The effect of various lipid solvents upon antigenicity*

| ANTIGEN | SOLVENT | EXTRACTION TEMPERATURE | ANTIGEN DILUTIONS TESTED | | | | | | ANTIGEN CONTROLS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 | 4 | 8 | 16 | 32 | 64 | 2 | 4 | 8 |
| K-15-A.... | Ether | 4 C | 4 | 4 | 4 | 4 | 4 | | | | |
| K-15-B.... | Alcohol | 4 C | 4 | 4 | 4 | | | | | | |
| K-15-C.... | Acetone | 4 C | 4 | 4 | 4 | 4 | 4 | 1 | 1 | | |
| K-15-D.... | Toluene | 26 C | 4 | 4 | 4 | 4 | 4 | | | | |
| K-15-E.... | Chloroform | 26 C | 4 | 4 | 4 | 4 | 3 | | | | |
| K-15-F.... | Benzene | 26 C | 4 | 4 | 4 | 4 | 3 | | | | |
| K-15-G.... | Ethyl acetate | 26 C | 4 | 4 | 4 | 4 | 3 | | | | |

TABLE 6

*The quantitative nature of the method for preparation of antigens*

| ANTIGEN | PER CENT OF SUSPENSION IN TERMS OF ORIGINAL TISSUE | ANTIGEN DILUTIONS TESTED | | | | | | ANTIGEN CONTROLS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 8 | 16 | 32 | 64 | 2 | 4 | 8 |
| K-16-E..... | 10 | 4 | 4 | 1 | | | | | | |
| K-16-F..... | 25 | 4 | 4 | 4 | 1 | | | | | |
| K-16-G..... | 50 | 4 | 4 | 4 | 4 | 1 | | | | |

the antigens prepared from living eggs that it seemed advisable to discard all dead embryos.

*The effect of various lipid solvents upon antigenicity.* Yolk sac membranes were harvested from 300 living embryos on the tenth day after inoculation and held frozen *in toto* at − 70 C for 10 to 30 days. The frozen and dried material was divided into 7 equal portions and these were processed as outlined except for

steps no. 5 and no. 7 wherein various other fat solvents were employed in the same volume as ether.

The data in table 5 show that, with the single exception of alcohol, all the fat solvents gave antigen preparations as potent as that obtained by using ether.

TABLE 7

*Specificity tests of antigen K-11-C*

Antigen titration in the presence of various antirickettsial immune serums

| SERUM NO. | CONVALESCENT GUINEA PIG SERUM | HOMOLOGOUS TITER OF IMMUNE SERUM | IMMUNE SERUM DILUTION | ANTIGEN DILUTIONS TESTED | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 2 | 4 | 8 | 16 | 32 | 64 |
| K-3..... | Tsutsugamushi disease (Karp) | 1:256 | 1:20 | 4 | 4 | 4 | 4 | 1 | |
| Ep. typhus no. 21..... | Epidemic typhus (Breinl) | 1:128 | 1:10 | 2 | 1 | | | | |
| End. typhus no. 2-10..... | Endemic typhus (Wilmington) | 1:512 | 1:10 | 2 | 1 | | | | |
| RMSF no. 3... | Rocky Mt. spotted fever (McCullough) | 1:512 | 1:10 | 1 | | | | | |
| Q9M no. 1..... | American Q fever (Nine Mile) | 1:128 | 1:10 | | | | | | |

TABLE 8

*Specificity tests of antigen K-11-C*

Immune serum titrations in the presence of antigen K-11-C diluted 1:6 (3 antigenic units)

| SERUM NO. | CONVALESCENT GUINEA PIG SERUM | SERUM DILUTIONS TESTED | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| K-3..... | Tsutsugamushi disease (Karp) | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 1 |
| Ep. typhus no. 21..... | Epidemic typhus (Breinl) | | | | | | | | |
| End. typhus no. 2-10..... | Endemic typhus (Wilmington) | 1 | | | | | | | |
| RMSF no. 3... | Rocky Mt. spotted fever (McCullough) | | | | | | | | |
| Q9M no. 1..... | American Q fever (Nine Mile) | | | | | | | | |

*Quantitative nature of the method for preparation of antigens.* Yolk sac membranes were harvested from 310 living embryos on the tenth day after inoculation and held frozen *in toto* at − 70 C for 2 to 3 days. The yolk sac suspension was processed as outlined except for step no. 10. No. 10: The dried and extracted material was divided into 3 portions and these were resuspended to volumes equivalent to 10, 25, and 50 per cent tissue suspensions.

The results in table 6 show that these antigens gave complement-fixing titers quantitatively directly proportional to the concentration of tissue used in their preparation.

*Specificity of antigen.* Approximately 1,200 ml of antigen K-11-C were prepared by the outline described above and this preparation was set aside as a reserve stock antigen. Specificity tests of this preparation were carried out against immune serums obtained from guinea pigs convalescent from epidemic (louse-borne) typhus, endemic (murine) typhus, Rocky Mountain spotted fever, American Q fever, and tsutsugamushi disease (scrub typhus), Karp strain.

Table 7 demonstrates the specificity of this antigen when it was titrated in the presence of various antirickettsial immune serums, and table 8 shows the results obtained when various antirickettsial immune serums were titrated in the presence of tsutsugamushi disease antigen K-11-C (Karp).

## *Comment*

Various preparations similar to those noted in this report are being tested as vaccines to determine their immunizing capacities in experimental animals and man.

In studies to be reported later it has been determined that this method is readily applicable to the preparation of other rickettsial diagnostic antigens such as epidemic (louse-borne) typhus, endemic (murine) typhus, Rocky Mountain spotted fever, and American Q (Nine Mile) fever.

## SUMMARY AND CONCLUSIONS

(1) A method is described for the preparation of complement-fixing antigens in a study of experimental tsutsugamushi disease (scrub typhus).

(2) The freezing and drying procedure employed in this method seems to possess several advantages over other methods used in the preparation of rickettsial complement-fixing antigens processed entirely in the fluid state:

(a) It is not necessary to use specially selected yolk sacs (based on microscopic examination) to prepare an antigen of practical value.

(b) Various lipid solvents, either miscible or immiscible with water, may be used partially or completely to extract the lipids from the yolk sac tissues without showing any appreciable adverse effect on the antigenic preparation.

(c) It is possible to work with yolk sac suspensions equivalent to 50 per cent tissue content and thus conserve and concentrate the antigenic components without resorting to further physical or chemical procedures.

(3) Complement-fixing antigens of *Rickettsia orientalis* prepared by this method do not show cross fixation with immune serums of epidemic (louse-borne) typhus, endemic (murine) typhus, Rocky Mountain spotted fever or American Q (Nine Mile) fever.

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