# PRIMARY SERUM TOXICITY AS DEMONSTRATED BY THE CHICKEN EMBRYO

By ERNST WITEBSKY, M.D., AND ERWIN NETER, M.D.

*(From the Laboratories of The Mount Sinai Hospital, New York)*

(Received for publication, January 3, 1935)

The origin of normal antibodies for bacteria and their toxins is still a moot question. There is, however, no doubt that antibody functions of normal sera against such antigens as blood group properties or Forssman antigen are due to constitutional, genetic factors. Normal sera containing Forssman antibodies react with cells and tissues which possess Forssman antigen; that is to say, such sera can display "primary toxicity."

Szepsenwol and Witebsky (1, 2) have recently shown that from the beginning of its development the chicken embryo, as well as its vascular network, contains Forssman antigen. Addition of normal human serum, rabbit serum or Forssman antisera to the chicken embryo causes a peculiar phenomenon. The vascular network shrinks, the embryo turns around and sinks into the yolk. One can often see the heart beating within the sunken embryo, until it finally dies[1] (Baumann and Witebsky (3, 4)). Duck embryos, lacking Forssman antigen, however, do not show the vascular phenomenon after the addition of normal human or rabbit serum. The experiments to be reported in this paper deal with the analysis and the mechanism of this phenomenon.[2]

[1] For the sake of brevity, this sequence of events occurring in the chicken embryo as a result of the several experimental procedures to be described, will be referred to as the "vascular phenomenon."

[2] In 1928 Sherwood (5) used chicken embryo hearts for experiments on anaphylaxis. The change in frequency and regularity of the heart beat was employed as the test. He succeeded in producing a sensitization of the heart by means of passive transfer of chicken antiserum (rabbit antiserum had no effect).

### *Method*

The following method (Kaufman (6), Szepsenwol (7)) is used to remove the embryos from the eggshell: Fertilized chicken eggs are kept for 66 to 72 hours in the incubator at about 40–42°C. Then the eggshell is cleaned and washed with alcohol. The eggshell is opened, preferably at the small pole, about a third to a half of the shell is carefully removed and the egg poured into a sterile beaker. The beaker is then covered with a sterile watch glass and kept in the incubator at 37–38°C. In the experiments to be cited the sera were added to the chicken embryo drop by drop.

### *Experiments on Primary Toxicity of Rabbit Type Sera toward the Chicken Embryo*

*1. Effectiveness of Active, Inactivated and Reactivated Normal Sera.*— Normal rabbit type sera lose their ability to produce the vascular phenomenon in the chicken embryo after being heated at 56°C. for ½ hour. Hence the first question which arose was whether or not inactivated normal human serum can be reactivated by addition of complement (fresh guinea pig serum). Table I illustrates a typical experiment designed to elucidate this question (Table I).

As is seen, normal human serum inactivated at 56°C. cannot be reactivated in its potency to induce the vascular phenomenon by addition of fresh guinea pig serum.

In order to study the relationship between the destruction of complement and the potency of the phenomenon-producing factor ("factor toxic" to the embryo) in normal rabbit type sera, the following experiment was performed.

Normal human serum was divided into three equal portions. Two of these were placed in a water bath for 20 minutes at a temperature of 49°C. and 51°C. respectively. The complement content of each specimen was then determined as follows:

Decreasing amounts of each of the above heated sera as well as active serum were made up to 0.2 cc. volume with physiological saline. To each of these there was added 0.4 cc. of strongly sensitized sheep cells. The mixtures were placed in the incubator at 37°C. Hemolysis was noted after 30 minutes (Table II).

As is seen from Table II, the complement of human serum was slightly inactivated at 49°C., but completely destroyed at 51°C. These sera were then tested for their effect on the chicken embryo. For this purpose decreasing amounts of the above sera were made up

TABLE I

*Effect of Normal Active, Inactivated and Reactivated Human Serum on the Chicken Embryo*

| Amount of serum | Reaction of the chicken embryo after | |
|---|---|---|
| | 3 hrs. | 8 hrs. |
| *cc.* | | |
| 0.5 active human | ++++ | ++++ |
| 0.25 " " | ++ | ++++ |
| 0.15 " " | − | − |
| 0.5 inactivated* human | − | − |
| 0.5 " " + 0.2 cc. fresh guinea pig serum | − | − |

− represents no reaction; + to ++++ represent different stages in the development of the phenomenon varying from the beginning contraction of the vascular net (+) to final contraction associated with sinking of the embryo into the depth of the yolk and its death (++++).

* Inactivation in water bath at 56°C. for ½ hour.

TABLE II

*Influence of Heat on Complement as Determined by Hemolysis of Sensitized Sheep Cells Produced by Normal Human Serum*

| Amount of human serum | Extent of hemolysis | | |
|---|---|---|---|
| | Active serum | Serum incubated at | |
| | | 49°C. | 51°C. |
| *cc.* | | | |
| 0.1 | C. | C. | 0 |
| 0.05 | C. | M. | 0 |
| 0.025 | M. | M. | 0 |
| 0.0125 | M. | Tr. | 0 |
| 0.00625 | Tr. | Tr. | 0 |
| 0.003125 | Tr. | 0 | 0 |
| 0.0 | 0 | 0 | 0 |

C., complete hemolysis; M., moderate hemolysis; Tr., trace of hemolysis; 0, no hemolysis.

to 1.0 cc. by the addition of physiological saline and then added to the chicken embryo preparation. The results are given in Table III.

Table III shows that at the end of 2 hours serum heated at 49°C.

was less effective against the chicken embryo than the unheated serum. At the end of 8 hours, however, it appeared to be just as potent as the unheated serum. On the other hand, serum heated at 51°C. completely lost its effect. Repeated experiments invariably disclosed this parallelism between the complement content of the serum and its ability to produce the vascular phenomenon in the chicken embryo.

It has been shown (Table I) that the addition of guinea pig serum (complement) to human serum inactivated at 56°C. fails to restore its potency to produce the vascular phenomenon. Since it is known that antibodies of normal serum are relatively thermolabile it seemed of interest to determine whether the addition of complement to serum in-

TABLE III

*Effect of Heating on Phenomenon-Producing Potency of Normal Human Serum*

| Amount of serum | Reaction of chicken embryo after | |
|---|---|---|
| | 2 hrs. | 8 hrs. |
| *cc.* | | |
| 0.5 active | ++++ | ++++ |
| 0.25 " | +++ | ++++ |
| 0.15 " | − | − |
| 0.5 heated at 49°C. | ++ | ++++ |
| 0.25 " " 49°C. | + | ++++ |
| 0.15 " " 49°C. | − | − |
| 0.5 " " 51°C. | − | − |

activated at lower temperature could restore this potency. However, on repeated attempts it was found impossible to reactivate human serum which was inactivated at 51°C. even by addition of large quantities of fresh guinea pig serum (0.1 to 0.5 cc.). Nor was such reactivation accomplished by the use of many variations in the method of experiment; *e.g.*, instead of mixing inactivated serum and guinea pig serum before its addition to the chicken embryo, we added first the inactivated serum and several hours later the guinea pig serum.

In this connection it seemed of special interest to study umbilical cord serum inasmuch as this serum is apparently characterized by a poverty of normal antibodies. It was, therefore, surprising to find that two samples of undiluted umbilical cord serum produced a typical vascular phenomenon even though sheep cell hemolysins could not be

demonstrated *in vitro*. The effectiveness of umbilical cord serum which does not contain demonstrable sheep cell hemolysins seems to argue against the identity of the latter with the vascular phenomenon-producing factor. The half diluted umbilical cord serum, however, was without effect. On the other hand, despite demonstrable complement content, such half diluted serum did not reactivate inactivated normal human serum.

*2. The Specific Inhibitory Effect of Guinea Pig Type Sera.*—The vascular phenomenon could be obtained by means of sera of animals belonging to the rabbit type. Up to now, normal sera of man, rabbit,

TABLE IV

*Inhibitory Effect of Sera of the Guinea Pig Type on Active Human Serum*

| Amount of serum | Reaction of chicken embryo after 8 hrs. |
|---|---|
| *cc.* *cc.* | |
| 0.5 active human + 0.5 physiological saline | ++++ |
| 0.5 " rat + 0.5 " " | ++++ |
| 0.5 " human + 0.5 inactivated chicken | − |
| 0.5 " " + 0.5 " guinea pig | − |
| 0.5 " " + 0.5 " human | ++++ |
| 0.5 " " + 0.5 " rat | ++++ |

rat and pigeon[3] were found effective, while those of guinea pig,[4] horse and chicken were ineffective.

Sera of animals of the guinea pig type contain dissolved Forssman antigen. For this reason, it seemed of interest to determine whether sera of the guinea pig type would alter the potency of rabbit type sera in respect to their ability to produce the vascular phenomenon. In order to determine this, the following experiment was carried out.

0.5 cc. of active human serum was mixed with 0.5 cc. of inactivated serum from chicken, rat, guinea pig and man respectively. These mixtures were left at room temperature for 10 minutes and then added to chicken embryos. The reaction resulting after 8 hours is noted in Table IV.

[3] The pigeon has been described as lacking in complement, and for this reason the mechanism of its effectiveness should be investigated further.

[4] Guinea pig sera are encountered which contain sheep cell hemolysins; it would be interesting to study the effectiveness of such a serum against the chicken embryo.

As is seen from Table IV, inactivated chicken and guinea pig sera, containing Forssman antigen, inhibit the potency of normal human serum, but inactivated rat and human sera, lacking Forssman antigen, do not display any inhibitory effect.

### *Experiments on the Toxicity of Forssman Antiserum for the Chicken Embryo*

*1. Effectiveness of Inactivated and Reactivated Forssman Antiserum.*— In contrast to normal sera of the rabbit type, inactivated Forssman antiserum can be made effective against the chicken embryo by the addition of fresh guinea pig serum. Each of these is ineffective by itself. One may infer that when the vascular phenomenon is produced by the addition of antisera it is necessary for antibody and complement to be present. The following experiment illustrates the quantitative relationship between both factors.

TABLE V

*Effect of Forssman Antisera on the Chicken Embryo*

| Inactivated sheep cell antiserum | | Guinea pig serum | Reaction of chicken embryo after 15 hrs. |
|---|---|---|---|
| Amount | Dilution | | |
| *cc.* | | *cc.* | |
| 1.0 | 1:10 | 0.2 | ++++ |
| 1.0 | 1:30 | 0.2 | ++++ |
| 1.0 | 1:90 | 0.2 | − |
| 1.0 | 1:10 | 0.1 | − |
| 1.0 | 1:30 | 0.1 | − |
| 1.0 | 1:10 | 0.05 | − |

Decreasing amounts of fresh guinea pig serum were made to 0.2 cc. with physiological saline and mixed with 1.0 cc. of various dilutions of an inactivated sheep cell antiserum. Then the mixtures were added to 3 day old chicken embryos and the reaction resulting after 15 hours noted in Table V.

As is seen from Table V, relatively large amounts of fresh guinea pig serum (complement) were necessary in order to reactivate inactivated Forssman antiserum against the chicken embryo. Sometimes smaller amounts (0.1 cc.) of fresh guinea pig serum can reactivate it however. The Forssman antiserum in a dilution up to 1:30 to-

gether with 0.2 cc. fresh guinea pig serum is capable of producing the vascular phenomenon; but not every sheep cell antiserum is effective in so high a dilution. Thus, of 9 sheep cell antisera which reacted well *in vitro*, 4 were ineffective against the chicken embryo even in a dilution of 1:10. In higher concentrations (dilution 1:2 to 1:5) they produced the phenomenon. On the other hand, one sheep cell antiserum was able to induce the vascular phenomenon even in a dilution of 1:200. As a rule antiserum and complement were mixed in a test tube before they were added to the embryo, but there was no difference in the reaction of the embryo after separate addition of both components provided the interval was less than 12 hours.

TABLE VI

*Reactivation of Inactivated Forssman Antiserum by Guinea Pig and Chicken Serum*

| Sheep cell antiserum | | Complement | Reaction of chicken embryo after 15 hrs. |
|---|---|---|---|
| Amount | Dilution | | |
| *cc.* | | *cc.* | |
| 1.2 | 1:10 | 0.2 guinea pig serum | ++++ |
| 1.2 | 1:10 | 0.3 chicken serum | – |
| 1.2 | 1:10 | 0.1 " " | – |
| 1.2 | 1:10 | 0.05 " " | – |
| 1.2 | 1:10 | 0.3 saline | – |

The remarkable difference between Forssman antiserum and normal sera of the rabbit type in that the former can be reactivated in its potency toward the chicken embryo by the addition of complement, suggests the possibility of differentiating in this way between normal antibodies of the blood serum and those produced by immunization or diseases.

It is known that bird serum does not complement amboceptors of mammalian sera. Therefore, it seemed of interest to determine whether or not it would be possible for normal chicken serum to be used as complement in the reactivation of inactivated Forssman antisera in their effect on the chicken embryo. For this purpose the following experiment was set up.

Decreasing amounts of chicken serum and guinea pig serum respectively were made up to 0.3 cc. with physiological saline and each mixed with 1.2 cc. of an inactivated sheep cell antiserum in a dilution of 1:10. The mixtures were then added to 3 day old chicken embryos. The results are given in Table VI.

Table VI shows that chicken serum does not restore the inactivated Forssman antiserum to phenomenon-producing potency. Thus, chicken serum does not function as complement in this preparation.

*2. The Specific Inhibitory Influence of Guinea Pig Type Sera on Forssman Antisera.*—Since chicken serum contains dissolved Forssman antigen capable of fixing the corresponding antibodies, we examined the influence of chicken serum on reactivated Forssman antiserum in

TABLE VII

*Inhibitory Effect of Chicken Serum on Reactivated Forssman Antiserum*

| Sheep cell antiserum, dilution 1:2 | Guinea pig serum | Saline | Chicken serum | Reaction of the chicken embryo after | |
|---|---|---|---|---|---|
| | | | | 3 hrs. | 12 hrs. |
| *cc.* | *cc.* | *cc.* | *cc.* | | |
| 0.4 | 0.2 | 0.5 | 0.0 | ++++ | ++++ |
| 0.4 | 0.2 | 0.0 | 0.5 | − | − |

order to determine whether an inhibitory effect could be demonstrated. The experiment was carried out in the following way.

To 0.6 cc. of an equal mixture of sheep cell antiserum, fresh guinea pig serum and saline respectively were added (*a*) 0.5 cc. chicken serum, and (*b*) 0.5 cc. saline. These mixtures were left at room temperature for 10 minutes and then dropped on 3 day old chicken embryos. The reaction resulting after 3 and 12 hours respectively is noted in Table VII.

As is seen from Table VII chicken serum prevents a reactivated Forssman antiserum from producing the vascular phenomenon.

## DISCUSSION

One of the characteristic qualities of the Forssman antigen is its widespread presence in the living world. It is found on the one hand in animals which have no zoological relationship at all, guinea pig,

horse, dog, cat, chicken (animals of the so called guinea pig type). On the other hand, closely related animals may differ in their content of Forssman antigen. Serum of such animals whose organs lack Forssman antigen contains antibodies against this antigen: rabbit, man, cow, duck, hog (animals of the so called rabbit type). In this characteristic the distribution resembles that of the blood group properties of man. In the serum there are always present antibodies against such group qualities as are absent in the individual himself.

The vascular phenomenon of the chicken embryo is produced by Forssman antiserum and by normal sera of the rabbit type. Sera of the guinea pig type are ineffective. Normal rabbit type sera lose their effectiveness after being heated at 51°C. The disappearance of the vascular phenomenon-producing potency parallels the loss of complement. Up to now, it has not been possible to reactivate heat-inactivated normal serum by the addition of complement, in contrast to Forssman antisera which can be easily reactivated by the addition of fresh guinea pig serum. This difference between normal and immune serum discloses a similarity to those antibody functions which play an important rôle in phagocytosis.

The vascular phenomenon as such is to be considered as a sequela of the reaction between the Forssman antigen of the chicken embryo on the one hand and the corresponding antibodies of the added serum on the other. This statement is supported by the following facts.

1. The vascular phenomenon of the chicken embryo is produced only by sera containing Forssman antibodies.

2. Duck embryos, which lack Forssman antigen, do not give the vascular phenomenon.

3. Forssman antisera lose their effectiveness toward the chicken embryo after being absorbed with sheep cells. However, attempts to fix the phenomenon-producing factors of normal sera by absorption with sheep cells, have as yet been inconclusive.

4. Guinea pig type sera containing dissolved Forssman antigen inhibit normal rabbit type sera as well as Forssman antisera in their effectiveness toward the chicken embryo.

These findings parallel the so called inverted anaphylactic shock of guinea pigs, which is characterized by the fact that the antibodies of

the serum are the actuating factor, while the antigen is present in the cells and tissues of the reacting organism itself.[5]

The significance of complement for anaphylactic reactions has been discussed for many years. Dale's (8) demonstration of anaphylactic reactions on the isolated uterus suggests that complement is unnecessary for anaphylaxis. Hyde (9), however, failed to induce the inverted anaphylactic shock in guinea pigs which did not possess complement in their blood. On the other hand, Doerr and Pick (10) were able to produce a typical anaphylactic shock in the adult chicken by the intravenous injection of an inactivated Forssman antiserum without addition of complement, an observation which we can confirm. However, for the production of the vascular phenomenon with the chicken embryo, complement is absolutely necessary, as has been shown above. The necessity of adding complement to inactivated Forssman antiserum suggests that either the embryo itself does not contain sufficient complement or that its complement will not function with mammalian antibodies.

It is to be borne in mind that the Forssman antigen is not a uniform substance, but consists of various components with different corresponding antibodies. This fact may perhaps help to explain the observation that Forssman antisera are encountered with sheep cell hemolysin titer 1000 times higher than that of a normal human serum, yet with a potency to produce the vascular phenomenon that may be almost the same as that of the normal serum. In this connection it may be mentioned that umbilical cord serum produced the vascular phenomenon in the chicken embryo, in spite of the fact that demonstrable sheep cell antibodies were not present. As yet it is impossible to state whether the difference between the Forssman antibodies of the agglutinin and hemolysin type and those responsible for the production of the vascular phenomenon are due to differences in the titer of partial antibodies or to qualitatively different antibody functions.

[5] Regarding nature and mechanism of the inverted anaphylactic shock, *cf.* Doerr, R., in Bethe, A., von Bergmann, G., Embden, G., and Ellinger, A., Handbuch der normalen und pathologischen Physiologie, Berlin, Julius Springer, 1929, **13,** 757. Forssman, J., in Kolle, W., and von Wasserman, A., Handbuch der pathogenen Mikroorganismen, Jena, Gustav Fischer, 3rd edition, (Kolle, W., Kraus, R., and Uhlenhuth, P.), 1928, **3,** Liefg. 23, 469.

SUMMARY

1. The 3 day old chicken embryo removed from its shell is a suitable test object for the demonstration of primary serum toxicity. Addition of normal rabbit type sera as well as Forssman antiserum causes the vascular network to contract and the embryo sinks in the yolk and dies.

2. Only sera of animals of the so called rabbit type produce this phenomenon. Sera of the guinea pig type are ineffective.

3. Heating to 51°C. destroys the complement content of normal human serum as also its effectiveness to produce the vascular phenomenon.

4. Up to the present it has not been possible to reactivate heat-inactivated normal serum by the addition of complement, while inactivated Forssman antiserum can be easily reactivated.

5. The vascular phenomenon of the chicken embryo is produced not only by the addition of a mixture of Forssman antiserum and complement but also by separate addition of both components.

6. Guinea pig type sera, containing dissolved Forssman antigen, are not only ineffective but actually exert an inhibitory influence on effective rabbit type sera as well as on Forssman antiserum.

BIBLIOGRAPHY

1. Witebsky, E., and Szepsenwol, J., *Compt. rend. Soc. biol.*, 1934, **115**, 291.
2. Szepsenwol, J., and Witebsky, E., *Compt. rend. Soc. biol.*, 1934, **115**, 1019.
3. Baumann, A., and Witebsky, E., *Compt. rend. Soc. biol.*, 1934, **116**, 10.
4. Baumann, A., and Witebsky, E., *Ann. Inst. Pasteur*, 1934, **53**, 282.
5. Sherwood, N. P., *J. Immunol.*, 1928, **15**, 65.
6. Kaufman, L., *Compt. rend. Assn. anat. Amsterdam*, 1930, **25**, 183.
7. Szepsenwol, J., *Arch. anat. hist. et embryol.*, 1933, **17**, 307.
8. Dale, H. H., *Lancet*, 1929, **1**, 1179, 1233, 1285.
9. Hyde, R. R., *Am. J. Hyg.*, 1932, **15**, 824.
10. Doerr, R., and Pick, R., *Z. Immunitätsforsch.*, 1913, **19**, 251.