THE CONGLUTINATION PHENOMENON

IX. THE PRODUCTION OF IMMUNO-CONGLUTININ IN RABBITS

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(With 9 Figures in the Text)

CONTENTS

INTRODUCTION

Before discussing immuno-conglutinin it is perhaps pertinent to remind readers of the definition given to the word conglutinin by Bordet & Streng (1909). Conglutinin was the name given to the substance in bovine serum, which was protein in nature and resistant to heating for half an hour at 56° C. and which reacted with an antigen-antibody complex, after the complex had adsorbed complement, bringing about a marked clumping or conglutination of the reactants. Conglutination is best observed using red cells as the antigen, an antibody which does not produce agglutination by itself and a complement which is not haemolytic at the strength used.

Streng (1930) carried out some experiments by which he hoped to show unequivocally that there was such a thing as an anti-complement, i.e. an antibody capable of reacting specifically with the complement of a serum while not reacting with the other proteins of the serum. By inoculating rabbits with the complement of the guinea-pig or horse adsorbed on sensitized bacteria he succeeded in stimulating the production of immune bodies which had the power of flocculating unrelated sensitized bacteria or red cells which had adsorbed a complement also unrelated to that used for the inoculations. These immune bodies, reacting characteristically with adsorbed complement, appeared to behave as true anti-complements; not being simply antibodies to the serum proteins concerned. As the properties and mode of action of these immune bodies or anti-complements were apparently analogous to naturally occurring conglutinin in bovine serum Streng gave them the name of immuno-conglutinins.

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https://doi.org/10.1017/S0022172400036792 Published online by Cambridge University Press

Thus, following Streng, immuno-conglutinins may be defined as immune bodies produced in the serum of an animal, following certain inoculations, which have the property of reacting with complement adsorbed on an antigen-antibody complex causing a marked clumping or flocculation of the reactants. Since Streng's original work in 1930 and the monograph by Wartiovaara (1932) no further work on this subject has been published to the knowledge of the present authors.

Quite a number of reasons have strengthened the desirability for renewing afresh and continuing the investigations on immuno-conglutinins. It seemed likely that a study of the *in vitro* properties of immuno-conglutinins would throw light on the whole phenomenon of conglutination. As the work progressed it became evident that the study of the conditions under which immuno-conglutinins were produced *in vivo*, was of peculiar interest since it was found that they could be invoked by inoculations of bacteria, unsensitized and without previous treatment with complement.*

Thus immuno-conglutinins have to be considered in association with the changes in the serum proteins consequent upon immunization or infective processes although they are unrelated in the specific sense to the bacterium or antigen immunizing the host. The circumstances of their stimulation are similar in some respects to those associated with the development of C-reactive protein.

Again it is possible that the development of immuno-conglutinin may subsequently be shown to be one of the best examples of a process of auto-immunization. This concept, however, will be discussed later.

In this paper experiments are reported on methods for stimulating the production of immuno-conglutinins in rabbits. Different inocula have been studied, as also has the influence of the route of the inoculations. The *in vitro* properties of sera containing immuno-conglutinins and the mode of their interaction with adsorbed complement have also been studied. Finally the possible significance of immuno-conglutinins to the host-animal is discussed.

MATERIALS AND METHODS

Animals in which immuno-conglutinins were stimulated

Fully grown rabbits weighing between 2 and 3 kg. and of either sex were used in these experiments. They were of mixed laboratory strains.

Inocula

Bacterial suspensions

The stock bacterial suspensions from which the inocula were prepared were obtained as follows:

Salmonella pullorum. The organisms were harvested in saline from a 48 hr. growth on nutrient agar. They were then killed by adding 0·5 % formol saline, then washed in 0·5 % formal saline and finally resuspended in this medium so that the suspension, when diluted 1 in 10, matched in opacity Brown tube no. 8.

* To differentiate immuno-conglutinins produced in this latter way from those produced by inoculating complement of another animal species adsorbed on some complex, the names immuno-conglutinin (auto-stimulation) and immuno-conglutinin (hetero-stimulation) respectively are suggested.

Proteus OX 19. Obtained from the National Collection of Type Cultures. The suspension was prepared in the same way as was the suspension of *S. pullorum*. The stock suspension when diluted 1 in 10 matched the opacity of Brown tube no. 9.

Corynebacterium hofmanni. This was harvested after 48 hr. growth on horse blood agar plates. The saline suspension was killed by heating at 58° C. for 90 min. The washed saline suspension when diluted 1 in 10 matched Brown tube no. 6. Methiolate, in a final dilution of 1 in 10,000, was used as the preservative.

Staphylococcus aureus. The suspension obtained after 36 hr. growth in liquid digest broth was deposited by centrifugation, washed in saline and heated at 80° C. for 15 min. The killed suspension when diluted 1 in 10 matched Brown tube no. 6. Methiolate was used as the preservative.

All the suspensions were kept at +4° C. The preservatives were removed before inoculation by washing the organisms in saline.

Soluble antigens

Soluble extract of S. pullorum. A 48 hr. growth of the organism on nutrient agar was harvested in sterile saline, and the organisms deposited by centrifugation. The deposit was resuspended in 10 times its volume of distilled water and twice frozen and re-thawed. The disintegration of the bacterial bodies was completed in a Mickle sonic vibrator.

The mixture was then diluted and centrifuged, and the clear supernatant fluid passed through a Seitz E.K. pad. A Kjeldahl estimation showed a protein concentration of 0·56 %. This soluble extract and the standard suspension of formolized organisms had similar end-points when titrated for antigenic capacity by a complement-fixation test, against a rabbit anti-pullorum serum.

Egg albumen. This was prepared by Hopkin's method described by Cole (1933). A 4 % protein solution was used for the injections.

Casein. This was a commercial sample further purified by the method of Lea & Hannan (1950). A 3·28 % solution was used for inoculation.

Bence-Jones protein. A 1·5 % solution was used for inoculation.

Horse dandruff protein. This was prepared by the method described by Squire (1950). The protein concentration was 0·65 %.

Adsorbed complement on sensitized bacterial or other suspensions. The preparation of these suspensions is described under the individual experiments.

Inoculations and bleedings

Inoculations were given on day 0, 3, 6 and 9 with doses of $\frac{1}{2}$, 1, $1\frac{1}{2}$, and $1\frac{1}{2}$ ml. for each occasion respectively. The animals were bled to obtain small amounts of serum usually on the 5th, 13th, 17th, 19th and 28th day after the day of the first inoculation. The sera were separated and stored at −20° C. until tested.

Methods used to examine sera for conglutinin or immuno-conglutinin

To demonstrate the conglutination of sheep red cells by the conglutinin or immuno-conglutinin in a heated serum, it is necessary to add, besides the sheep cells and the serum under investigation, an antibody to sheep cells and the

following complement components—C'1, C'2 and conglutinating C'4. Other complement components may also be necessary, although we have no certain evidence on this point (Coombs, Blomfield & Roberts, 1950).

Horse complement with its various components is well suited for this purpose for, besides containing a suitable C'1 and C'2, it contains also a conglutinating C'4. If horse complement is used, a convenient antibody to sensitize the sheep cells is that which occurs naturally in bovine serum. This is a Forssman antibody which, on combining with sheep cells, produces only extremely weak agglutinating activity, probably because of the situation of the Forssman antigen sites on the surface of the sheep cells. This failure to produce agglutination to any marked degree makes this antibody very well suited for conglutination studies because the sensitized sheep cells in the absence of conglutinating complement and conglutinin are scarcely clumped. A rabbit Forssman antibody may also be used instead of the bovine antibody and is of especial value when certain other conglutinating complements are being studied.

In the present experiments the following systems have been used to detect conglutinin or immuno-conglutinin activity in sera.

Method I

1 vol. of varying dilutions of the serum to be tested, previously heated at 56° C. for half an hour.

1 vol. 0·4 % sheep cell suspension previously sensitized with bovine antibody and subsequently washed free of the other constituents of heated bovine serum.

1 vol. horse complement diluted 1/10, a dilution previously shown to contain an adequate amount of conglutinating C'4.

A control test is also set up replacing the dilution of horse complement by a similar dilution of heated horse serum. The absence of clumping of the cells in these control tests assures that the clumping observed in the experimental test is a manifestation of conglutination.

Method II a

1 vol. of varying dilutions of the heat inactivated serum to be tested. The serum previously absorbed with sheep cells to remove sheep cell antibodies.

1 vol. of saline.

1 vol. 0·4 % suspension of alexinated* sheep cells.

Method II b

As method II*a*, except that the volume of saline is replaced by a volume of inactivated horse serum diluted 1/10.

Methods II*a* and II*b* are controlled for clumping action other than conglutination by replacing the alexinated cells by a suspension of antibody sensitized cells which have previously been exposed to inactivated horse serum instead of horse complement.

* The term alexinated cells is used in this paper to denote cells sensitized with antibody and which have also adsorbed complement.

That the clumping activity measured is, in fact, an expression of conglutinin or immuno-conglutinin may be further demonstrated by the fact that the responsible substance in the serum can be absorbed with alexinated cells but not by sensitized cells. Method II*b*, allowing as it does, the serum to react in the presence of inactivated horse serum, supplies evidence that the phenomenon is not a manifestation of an antiglobulin reaction.

The advantage of methods II*a* and II*b* over method I is that they allow of an examination of undiluted serum for conglutinating activity which by method I may quite often be masked by an anti-complementary action of the concentrated serum on horse complement. Method I is easiest of execution.

Preparation of a suspension of sheep cells sensitized with bovine antibody

2 ml. 4% suspension of sheep cells with 2 ml. inactivated bovine serum diluted 1 in 2 is incubated at 37° C. for 15 min. The cells are deposited by centrifugation, washed once in saline, and finally saline added to 20 ml. to give a 0·4% sensitized cell suspension.

Preparation of alexinated cell suspension

A mixture is made of the following: 2·5 ml. 4% suspension sensitized cells; 2·5 ml. undiluted horse complement, 2·5 ml. undiluted inactivated horse serum which is added to insure ample C′4; 17·5 ml. saline.

The mixture is incubated at 37° C. for 20 min. The cells are then deposited by centrifugation, washed once in saline, and finally saline added to 25 ml. to give a 0·4% alexinated sheep cell suspension.

EXPERIMENTAL STUDY

First series of experiments: production of immuno-conglutinin by hetero-stimulation

In the first attempts to produce immuno-conglutinins in rabbits, the animals were inoculated with complement adsorbed on sensitized bacteria or with fresh unheated serum adsorbed on kaolin with the object of presenting the animal with what might be called the surface configuration of complement.

Unless otherwise stated, the development of the conglutinating activity in the rabbits' serum was tested for by method I.

A. *Inoculum: the complement of the horse or pig adsorbed on Salmonellae sensitized with rabbit antibody*

In these experiments rabbit antibody was used to avoid introducing into the inoculum an antibody globulin which would be antigenic in the rabbit.

Preparation of the alexinated bacterial suspension

The stock *S. pullorum* suspension was washed, resuspended in saline and mixed with a heat-inactivated, rabbit antiserum to *S. pullorum*. After half an hour at 37° C. the bacteria were deposited by centrifugation, the supernatant fluid discarded and the bacteria resuspended in saline.

An equal volume of undiluted serum was than added as complement. After half an hour at 37° C. the bacteria were again deposited by centrifugation, washed once in saline, and finally resuspended in saline to the original volume ready for inoculation.

(1) *Complement of the horse: intravenous route for inoculation.* The level of immuno-conglutinins produced in the three rabbits R 2209, 2210 and 2211 is shown in part A, Fig. 1. The interpretation of the prozone of inhibition of conglutination is discussed later.

(2) *Complement of the horse: intravenous route for inoculation.* Expt. 1 was repeated on another three rabbits—R 2217, 2218 and 2219—with the difference that the alexinated sensitized bacteria were washed three times in saline, instead of only once, before being inoculated. This was to insure that there was no free horse serum, other than that adsorbed as complement and perhaps as antibody on the sensitized bacteria, in the inoculum. It may be seen (part B, Fig. 1) that this extra washing of the alexinated bacteria did not reduce the extent of the prozone of inhibition of conglutination.

(3) *Complement of the horse: subcutaneous route for inoculation.* In this experiment also the alexinated bacteria were washed three times in saline before inoculation. The immuno-conglutinin level produced in the sera of rabbits R 2605, 2606 and 2607 is shown in part C, Fig. 1.

(4) *Complement of the pig: intravenous route for inoculation.* In this experiment the alexinated bacteria were washed only once before inoculation. The level of immuno-conglutinin produced in rabbits R 2213, 2214 and 2215 is shown in part D, Fig. 1.

Comments on the results of experiments A. 1–4

The inoculation of rabbits with complement adsorbed on sensitized *S. pullorum* always resulted in the production of immuno-conglutinins.

Using method I with horse complement for the detection of conglutinin or immuno-conglutinin, none of the pre-inoculation sera, at a dilution of 1 in 5 or greater, showed any conglutinating activity.

Sera with a higher demonstrable titre of immuno-conglutinin were produced when horse rather than pig complement was used in the preparation of the inoculum. However, the prozone of inhibition of conglutination was also greater in these circumstances. Although the nature of this prozone of inhibition is discussed later, it may be noted that very thorough washing of the alexinated bacteria before inoculation did not prevent the manifestation of the prozone. The washing had as its purpose the removal of horse serum protein other than that adsorbed as complement, but the washing would not remove any adsorbed naturally occurring horse *S. pullorum* antibody which might have been present in the complement serum.

Finally, it appeared that inoculation by the intravenous route was better than the subcutaneous route for the stimulation of immuno-conglutinin.

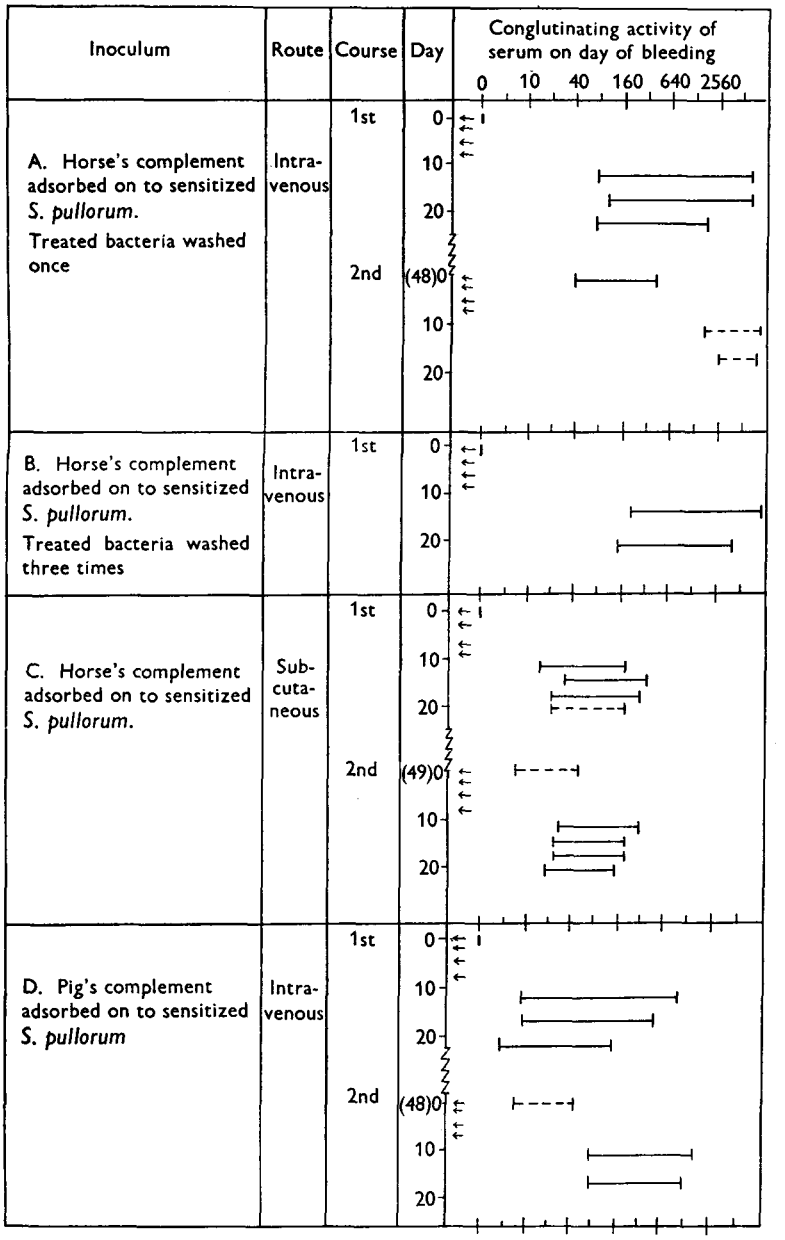

Fig. 1. Immuno-conglutinin response of rabbits inoculated with complement adsorbed on sensitized *S. pullorum*. Key to Figs. 1–5 and 8: a broken line indicates that the conglutination demonstrated was weak. The results shown are a mean of those found on testing individual sera from the two or three rabbits used for each experiment.

B. *Inoculum: unheated and heated serum of the horse or guinea-pig adsorbed nonspecifically on kaolin*

Experiments were performed to see if immuno-conglutinin could be produced in rabbits by intravenous inoculations of kaolin particles coated with fresh horse serum. Such a possibility was suggested by the fact that the naturally occurring

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conglutinin in heat-inactivated bovine serum can be specifically absorbed by kaolin particles previously coated with fresh horse serum, but not by particles previously coated with heat inactivated horse serum.

Preparation of inoculum

To the amount of serum to be used for the inoculation—0·5, 1·0, 1·5 and 1·5 ml. was added 0·25, 0·5, 0·5 and 0·75 g. respectively of sterile kaolin powder. The suspension was very lightly centrifuged to deposit the larger particles. The undeposited particles were left in contact with the serum for 10 min. at 37° C., after which they were deposited by centrifugation, washed twice in distilled water and finally resuspended in saline to the original volume for the inoculation.

The sera in this series of experiments (B 1–5) were examined with horse complement for immuno-conglutinin content using method I.

(1) *Kaolin particles coated with the unheated serum of the horse: intravenous route for inoculation.* Two rabbits, R 1245 and 1334, were subjected to a course of inoculations prepared as above. The results of the examination of their sera for immuno-conglutinin activity are shown in part A, Fig. 2.

(2) *Kaolin particles coated with the heated serum of the horse: intravenous route for inoculation.* The horse serum used for this experiment was heated at 56° C. for half an hour before being used to coat the kaolin particles. Part B, Fig. 2, shows the results obtained with the two rabbits R 1250 and 1327. Though very low titred immuno-conglutinins were produced in these rabbits, the picture was quite different from that obtained in Expt. B 1.

(3) *Kaolin particles coated with the unheated serum of the guinea-pig: intravenous route for inoculation.* Rabbits R 1473 and 1474, part C, Fig. 2.

(4) *Kaolin particles coated with the heated serum of the guinea-pig: intravenous route for inoculation.* Rabbits R 1475 and 1476, part D, Fig. 2.

(5) *Fresh unheated and heated serum of the horse.* The results obtained using these inocula are given here as they act in certain respects as controls to the above experiments (B 1–4), although they belong more properly to a later section (Fig. 3).

It can be seen that whether the horse serum had been heated or not made little difference to the level of immuno-conglutinin developed in the serum.

Comments on the results of Expts. B 1–5

The difference in the immuno-conglutinin response in rabbits between those, on the one hand, receiving inoculations of kaolin coated with fresh unheated serum and those, on the other hand, receiving inoculations either of kaolin coated with heated serum or of fresh or heated horse serum in solution was quite evident. In the former procedure the picture was very similar to that observed after inoculation of alexinated sensitized bacteria. The small but definite amount of immuno-conglutinin produced under the latter conditions will be discussed after consideration of the experiments in *Series* 2 *and* 3.

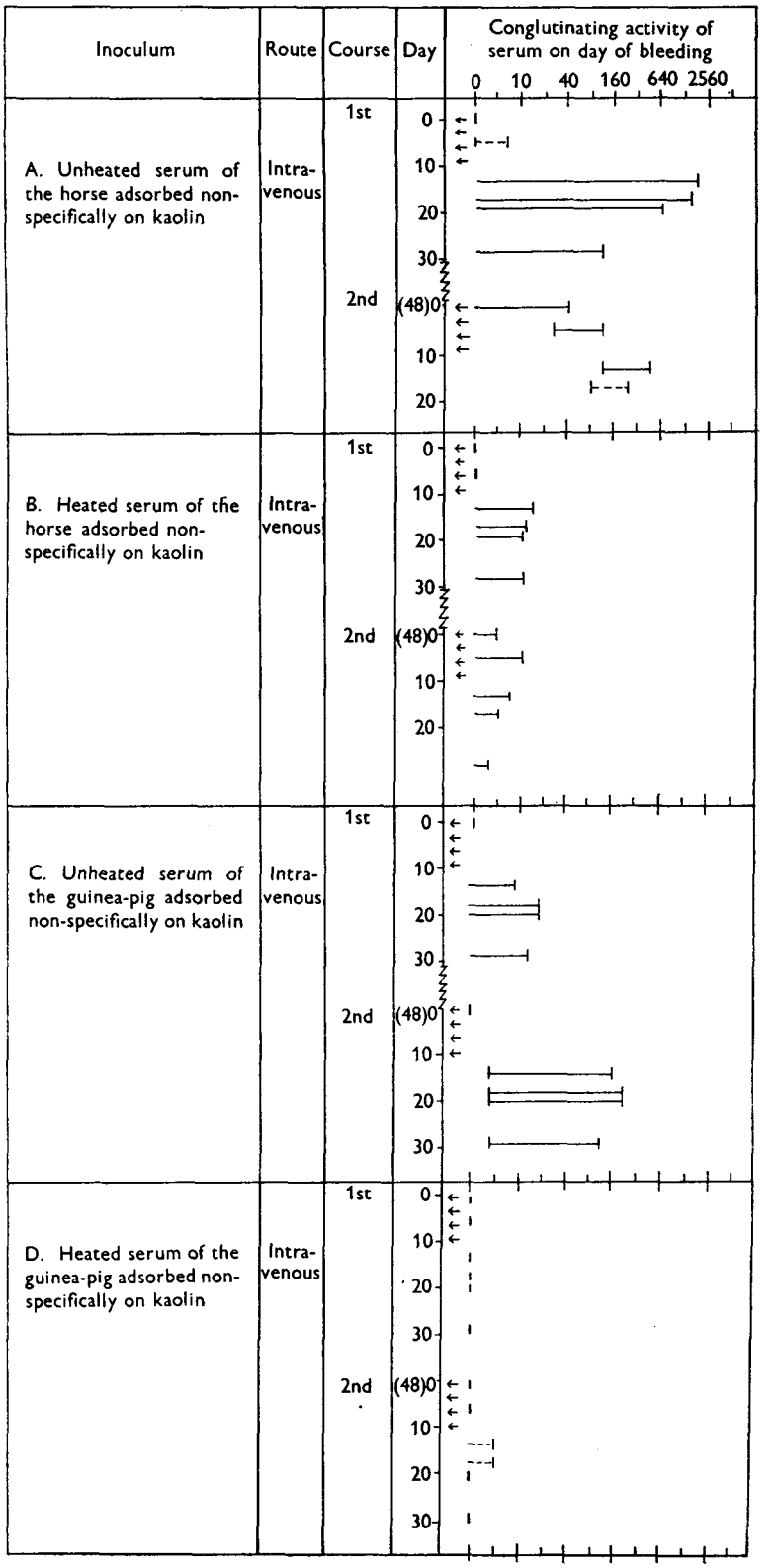

Fig. 2. Immuno-conglutinin response of rabbits inoculated with heated and unheated serum adsorbed on kaolin.

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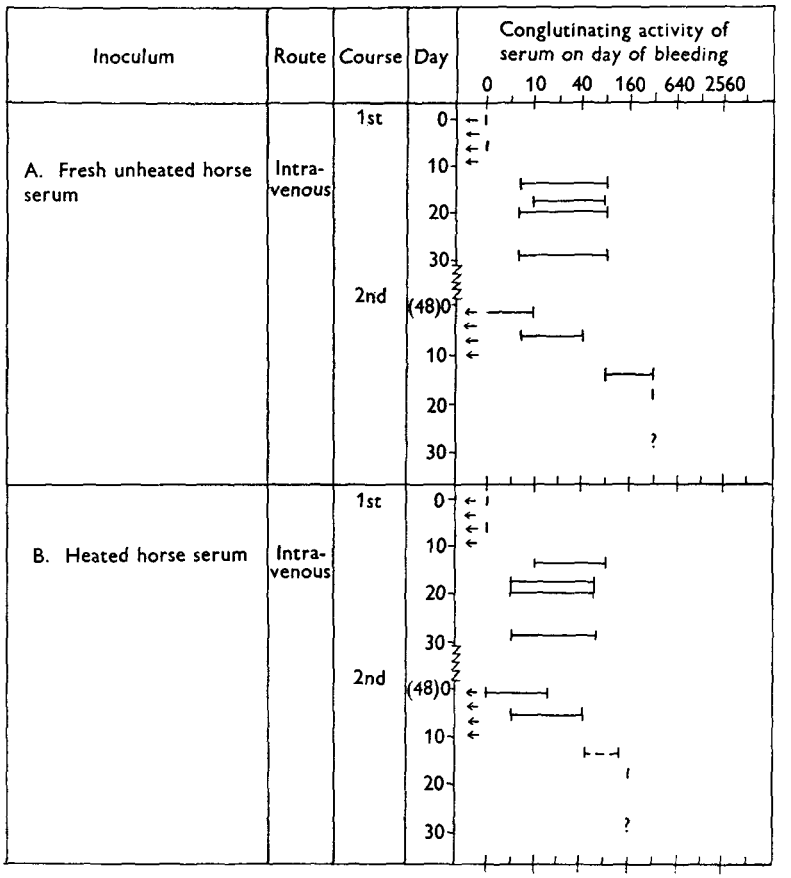

Fig. 3. Immuno-conglutinin response of rabbits inoculated with heated and unheated horse serum.

Analysis of the sera produced as described in A and B for their different serological properties

From theoretical and empirical considerations the injection of rabbits with sensitized bacteria treated with horse complement should result in the rabbit producing at least three reactive properties in its serum. These should be:

(*a*) Antibodies to the bacterial antigens.

(*b*) Antibodies to horse serum proteins. The antigenic stimulus coming from some of the proteins constituting complement and from any natural *S. pullorum* antibody globulin molecules in the horse complement which adsorbed on to the bacteria.

(*c*) Antibodies to the configuration which is characteristic of adsorbed complement of many, if not all, animal species—so-called immuno-conglutinins.

Likewise the injection of kaolin particles coated with fresh horse serum would also be expected to stimulate antibodies to certain of the horse serum proteins besides the immuno-conglutinin which might be developed.

To illustrate the different properties and the components responsible for them,

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in the sera which were investigated in this first series of experiments, the reactions of serum R 2209 were analysed.

It could be shown that this serum, produced in response to the inoculum of sensitized *S. pullorum* previously treated with horse complement, possessed the three properties (*a*), (*b*) and (*c*) mentioned above. The prozone of inhibition of conglutination shown with the low dilutions of the serum when it is tested by method I was due to horse serum protein antibodies reacting either with the proteins of horse complement directly or with non-complement horse protein with the subsequent fixation of the complement. Thus the complement is either prevented or deviated from taking part in the conglutination of the red cells. It was possible by experiments using selective absorptions to demonstrate that the three reactive properties (*a*), (*b*) and (*c*) were expressions of three separate components in the serum.

Experiment. The titre of immuno-conglutinin in the serum of rabbit R 2209 was determined by method I; the extent of the prozone of inhibition of conglutination being carefully noted. The antibody content in the anti-serum to *S. pullorum* was measured using a complement-fixation test with guinea-pig complement.

Samples of the inactivated serum were absorbed with killed *S. pullorum* organisms treated in the following ways:

(i) *S. pullorum* organisms without further treatment.

(ii) *S. pullorum* organisms previously sensitized with rabbit antibody.

(iii) *S. pullorum* organisms previously sensitized with rabbit antibody and exposed to NH_3-treated unheated horse serum. Such organisms, after being washed, would then have adsorbed on their surface certain proteins of horse serum constituting the C′1 and probably also some of the C′2 of complement, but would not have the characteristic configuration of adsorbed total complement since C′4 was lacking. Such cells do not conglutinate in the presence of immuno-conglutinin but are capable of adsorbing antibodies to certain proteins of horse serum.

(iv) *S. pullorum* organisms previously sensitized with rabbit antibody and exposed to the complement of horse serum. Such treated cells, after washing, present the configuration of adsorbed complement and react with and adsorb immuno-conglutinin.

(v) *Proteus* OX19 organisms without further treatment.

(vi) *Proteus* OX19 organisms previously sensitized by rabbit *Proteus* OX19 antibody and exposed to the complement of fresh horse serum as in (iv).

The results of testing the absorbed and unabsorbed samples of the serum are shown in Table 1. This table shows that each of the three properties studied could be influenced independently. The titre of immuno-conglutinin in the serum was unaffected by absorptions which removed either the bacterial antibodies or the antibodies to certain horse serum proteins which were responsible for the prozone of inhibition of conglutination. Immuno-conglutinin, like conglutinin, could be absorbed only by sensitized bacteria previously treated with whole fresh complement. The immuno-conglutinin produced by the inoculum containing adsorbed pig complement was, in a like manner, adsorbed by sensitized bacteria previously treated with horse complement.

The inoculation of a rabbit with horse complement adsorbed on a sensitized cell produces, besides immuno-conglutinin, antibodies to other horse serum

proteins. Such sera would give an antiglobulin reaction (Coombs, Mourant & Race, 1945) with cells sensitized with horse antibody globulin. However, a fundamental difference between this latter reaction and that of conglutination using immuno-conglutinin is that to get clumping or agglutination by the antiglobulin reaction free unabsorbed horse globulin must be removed, whilst in the clumping or conglutination by complement and immuno-conglutinin free unabsorbed horse serum proteins do not interfere with the interaction. Thus if antiglobulin antibodies and immuno-conglutinin are present in the same serum, the antiglobulin antibody can be neutralized or absorbed without influencing the manifestation of the immuno-conglutinin content.

Table 1. *Analysis of the three different properties developed in a rabbit serum* (R 2209) *after inoculation of* Salmonella pullorum *organisms previously treated with horse complement and subsequently washed before inoculation*

Treatment of serum	Titre of serum showing: *S. pullorum* antibody	Immuno-conglutinin activity	Prozone of inhibition of immuno-conglutinin activity
Unabsorbed	320	5120	160
Absorbed with *S. pullorum* organisms	0	5120	160
Absorbed with *S. pullorum* organisms sensitized with rabbit antibody	40	5120	160
Absorbed with sensitized *S. pullorum* previously treated with NH_3-treated fresh horse serum and subsequently washed	20	5120	0
Absorbed with sensitized *S. pullorum* previously treated with horse complement and subsequently washed	20	40	0
Absorbed with *Proteus* OX 19 organism	320	5120	160
Absorbed with sensitized *Proteus* OX 19 previously treated with horse complement and subsequently washed	320	40	0

Nevertheless to have antibodies to the ordinary serum proteins also present in a serum specially prepared for studying the properties of immuno-conglutinins was not desirable. For this reason the further investigations were directed towards the production by auto-stimulation of sera containing immuno-conglutinin.

Second series of experiments: production of immuno-conglutinin by auto-stimulation using particulate inocula

Wartiovaara (1932) showed that the production of immuno-conglutinin could regularly be stimulated in rabbits by the intravenous inoculation of untreated killed bacteria and that their previous adsorption of complement was not essential for this purpose.

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In order to confirm his findings and, at the same time, to attempt to find the optimal method for the production by auto-stimulation of immuno-conglutinin the following experiments were performed.

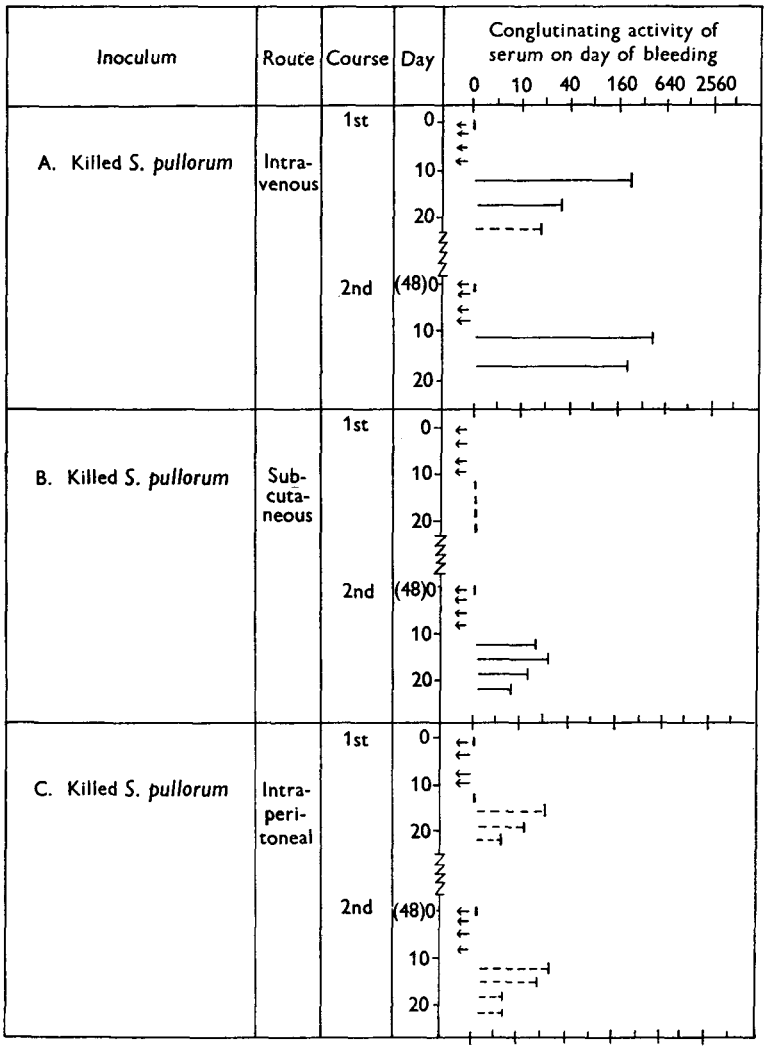

Fig. 4. Immuno-conglutinin response of rabbits inoculated with killed *S. pullorum*.

Rabbits were injected with killed suspensions of both Gram-positive and Gram-negative bacteria. The influence of the route by which the injection was given, was also studied.

(1) *Killed* Salmonella pullorum: *intravenous route for inoculation*

The serum titre of immuno-conglutinin produced in rabbits R 2204 and 2228 after a first and second course of injections is shown in part A, Fig. 4. A third rabbit, R 2229, died after the fourth injection.

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A definite and powerful immuno-conglutinin response always results when rabbits are inoculated in this way. The serum level may be maintained by repeated courses of inoculations, and the sera even after the seventh course of inoculations show no prozones of inhibition of conglutination.

(2) *Killed* Salmonella pullorum: *subcutaneous route for inoculation*

Rabbits R 2606, 2603 and 2604 (part B, Fig. 4).

After the first course of inoculations antibodies to *S. pullorum* were present in the sera to a high titre, yet no immuno-conglutinin could be detected by method I. After the second course, however, immuno-conglutinins appeared in the sera although the titre was not very high.

We noticed that the injections of the first course produced large and firm nodules in the subcutaneous tissue and it is probable that organizms immured within an abscess would not easily gain entrance into the general circulation. The second-course injections did not result in such nodules.

(3) *Killed* Salmonella pullorum: *intra-peritoneal route for inoculation*

Rabbits R 2608, 2609 and 2610 (part C, Fig. 4).

Rabbit R 2609 died after the fourth inoculation.

Again with this route for inoculation high titred antibodies to *S. pullorum* were produced, but the immuno-conglutinin response was very poor and irregular.

(4) *Killed* Proteus OX19: *intravenous route for inoculation*

Rabbits R 2601, 2220 and 2644 (part A, Fig. 5).

The immuno-conglutinin response was not uniformly good after the first course of injections. Rabbit R 2220 did not receive the third injection because at the time it appeared to be ill. The response after the second series of injections was much more uniform and satisfactory.

(5) *Killed* Staphylococcus aureus: *intravenous route for inoculation*

Rabbits R 2679, 2680 and 2650 (part B, Fig. 5).

The rabbits received a single course of inoculations. Immuno-conglutinins were stimulated but only to a low titre.

(6) *Killed* Corynebacterium hofmanni: *intravenous route for inoculation*

Rabbits R 2677 and 2678 (part C, Fig. 5).

Here again the rabbits were only given a single course of inoculations and the immuno-conglutinin response, although definite, was of low titre.

(7) *Killed* Salmonella pullorum: *single injection intravenously*

Rabbits R 2288 and 2289.

The sera of these two rabbits before injection showed neither conglutinating activity when tested by method I nor any antibodies to *S. pullorum* when tested by the usual conglutinating complement-absorption test using horse complement.

Examination of the sera, after the rabbits had received a single injection of 1·5 ml. suspension containing approximately 10^8 organisms, gave the results shown in Fig. 6.

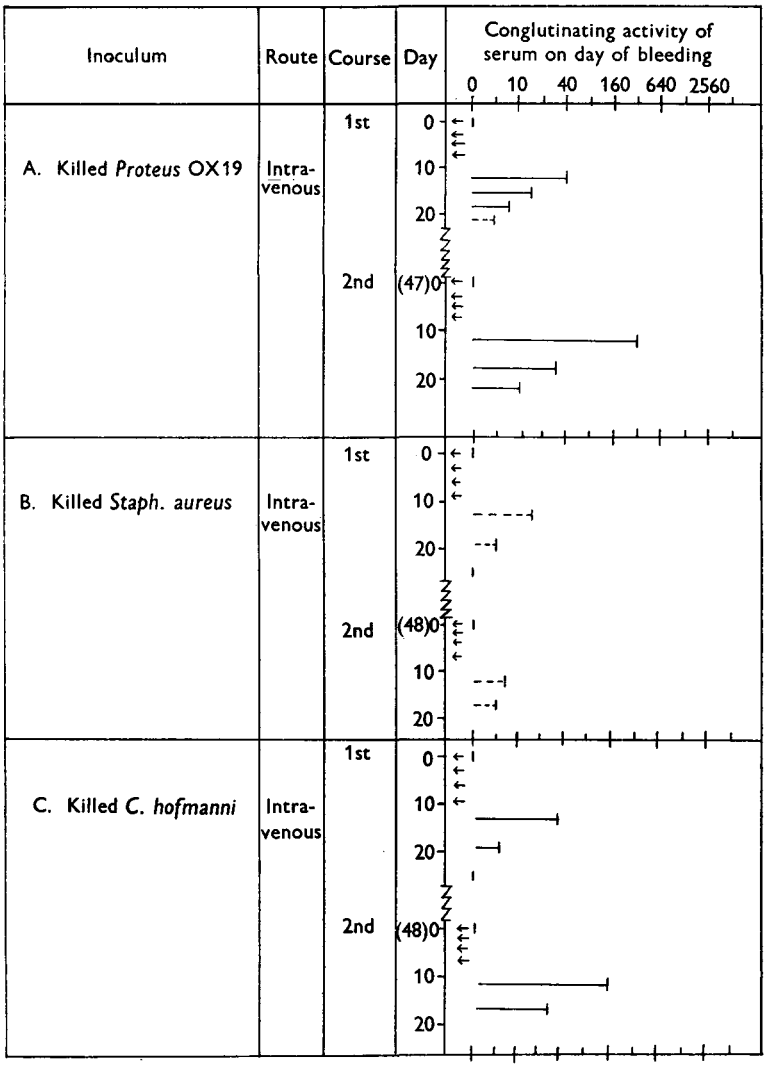

Fig. 5. Immuno-conglutinin response of rabbits inoculated with killed *Proteus* OX 19, *Staph. aureus* and *C. hofmanni*.

Although the methods of testing for the two properties being studied were different, it appeared that both antibody and immuno-conglutinin in the serum began to rise on the second or third day. The strength of the conglutination produced by the immuno-conglutinin was not great and the level in the serum fell after the sixth day despite there being no fall in the antibody level.

The fact that the conglutinating complement-absorption test failed to demonstrate any antibodies to *S. pullorum* in the sera before the animals were inoculated is no proof that none was present for certain factors in concentrated serum are able

to mask the demonstration by a complement-fixation test of small amounts of antibody (see Blomfield, Coombs & Hole, 1950).

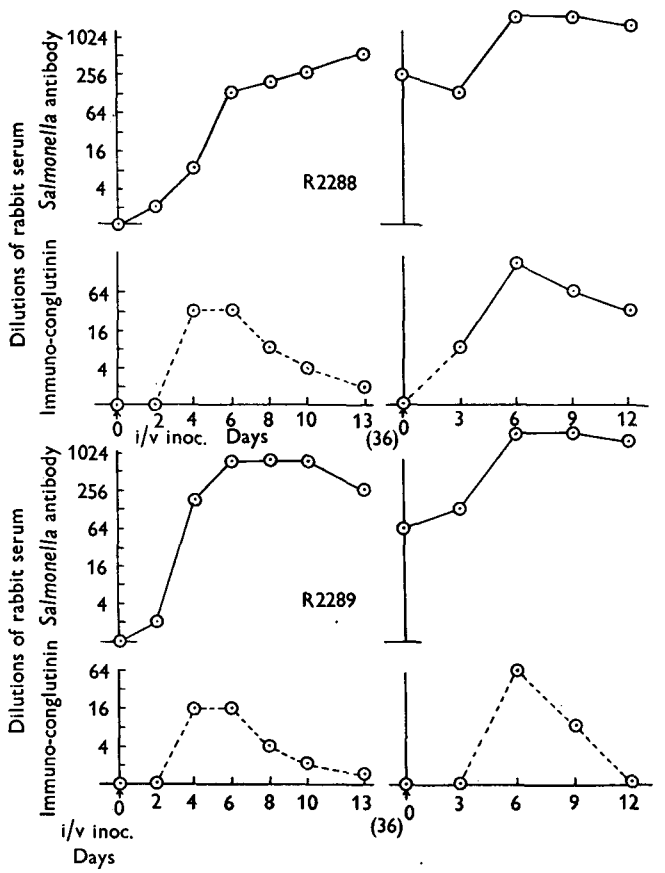

Fig. 6. The variations in immuno-conglutinin and anti-*S. pullorum* antibody titre after a single intravenous inoculation of *S. pullorum*. A broken line indicates that conglutination was only partial.

Conclusions from experiments in Series 2

Immuno-conglutinin appears in the serum of rabbits receiving injections of killed bacteria. These experiments confirm the earlier work of Wartiovaara (1932).

Comparing the reactions to *S. pullorum*, *Proteus* OX 19, *Staph. aureus* and *C. hofmanni*, it seems that Gram-negative bacteria are much more potent in stimulating the rise of immuno-conglutinin. There was no doubt that the injections of the Gram-negative organisms distressed the animals much more than did the Gram-positive organisms.

The route by which the injection is administered greatly influences the immuno-conglutinin response. The best response after a single course of inoculations was obtained using the intravenous route for injection. The intraperitoneal route only produced poorly demonstrable and low titred immuno-conglutinin, while with the subcutaneous route no detectable immuno-conglutinin could be shown in the

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serum. After a second course, however, rabbits injected by all three routes developed immuno-conglutinin in their sera; but here again, those animals injected by the intravenous route showed the property to the most marked degree.

The development of immuno-conglutinin in serum as a consequence of the injection of killed bacteria is of great interest, for this property of the serum, which in so many respects behaves as an antibody, is in no way specifically related in the serological sense to the antigens of the inoculum. This can be shown by the fact that repeated absorptions of the heated serum with similar bacteria to those used in the inoculum leaves the immuno-conglutinin unaltered. However, these bacteria or quite unrelated bacteria, once sensitized with their homologous antibodies and treated with complement, especially that of the horse or guinea-pig, will absorb the immuno-conglutinin from the heated serum.

Third series of experiments: production of immuno-conglutinin by auto-stimulation using soluble inocula

The object of this series of experiments was to see if soluble antigens are able to supply the stimulus necessary for the production of immuno-conglutinin. The soluble antigens chosen were egg albumen, casein, horse dandruff protein, Bence-Jones protein and a soluble extract of *S. pullorum*. The injections were administered intravenously and the time intervals between the injections and the bleedings were as in previous experiments.

When the sera were tested by method I for any developed immuno-conglutinin the results were nearly all negative or certainly equivocal. In experiments in Series 1 and 2 the sera were not examined more concentrated than a 1 in 5 dilution. It was clear, however, that the sera in this third series of experiments would have to be examined undiluted. It was soon realized that method I had certain disadvantages when used for testing undiluted serum because the possibility existed of factors in the rabbit serum interfering with the adsorption of horse complement. Therefore method II*b* was used since it appeared to have no drawbacks as long as the heated rabbit sera were first absorbed with sheep red cells and a control test using inactivated horse serum instead of horse complement was also set up.

Using this latter method not only did some of the post-inoculation sera show unequivocally immuno-conglutinin activity, but there was also definite evidence of conglutinin or immuno-conglutinin activity in some of the pre-injection sera. In the light of this the sera of 39 adult and apparently normal rabbits were also examined by method II*b* for conglutinin or immuno-conglutinin activity.

Conglutinin or immuno-conglutinin level in the sera of thirty-nine adult, apparently normal rabbits

The result of the examination by method II*b* of the sera of thirty-nine adult and apparently normal rabbits is given in the form of a frequency distribution diagram (Fig. 7). This shows that the majority of apparently normal rabbits possess a property in their sera which will conglutinate sensitized cells which have adsorbed a conglutinating complement. Whether one regards this substance, which is

present only in very low titre, as conglutinin or immuno-conglutinin is either a moot point at the present state of our knowledge or simply a matter of words.

This apparently normal level of conglutinin is not demonstrable if tested for by method I.

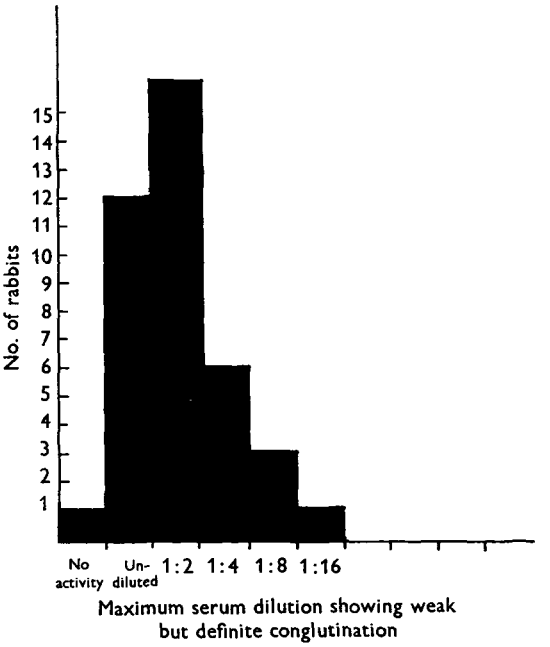

Fig. 7. Distribution of conglutinating activity in the sera of thirty-nine adult normal rabbits.

Injection of soluble antigens: intravenous route for inoculation

The conglutinin or immuno-conglutinin in the pre- and post-inoculation sera of rabbits injected with the various soluble antigens is shown in Fig. 8. All animals produced antibodies corresponding to the antigens injected, and these were demonstrated by ordinary interfacial precipitation tests.

The rabbits receiving injections of casein and the soluble extract of *S. pullorum* produced definite, although low-titred, immuno-conglutinin, while with the other antigens the responses were equivocal as the levels in these sera were well within the range shown by the sera of the thirty-nine apparently normal uninjected rabbits, but there did seem to be an indication of a rise in the serum level after the inoculation.

The significance of these findings must await further work. Factors other than the physical dispersion of the antigens, such as the multiplicity of antigens stimulating the animal, or the toxicity of the antigens, may also be involved.

The results using heated or unheated horse serum as the inoculum may conveniently be considered in this section (see B. 5). Compared with the other soluble antigens, horse serum, whether heated or not, appears to be a moderately good stimulant to immuno-conglutinin production. The inevitably high titre of

anti-serum antibodies in these immune sera made it very difficult to draw definite conclusions about the content of immuno-conglutinins.

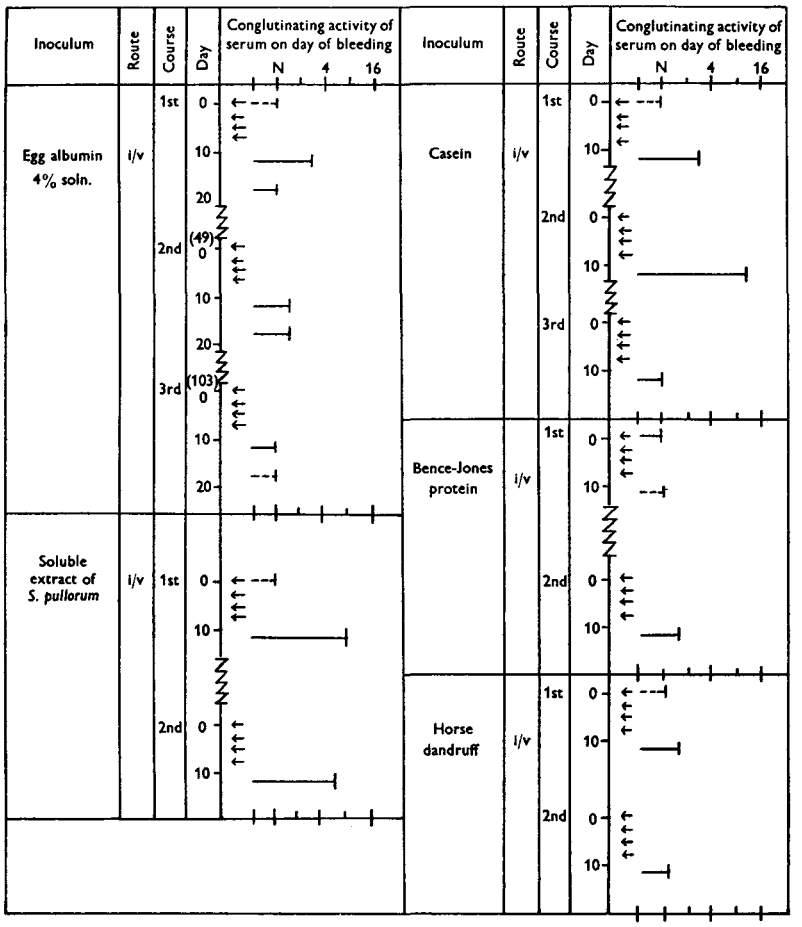

Fig. 8. Immuno-conglutinin response of rabbits inoculated with soluble antigens. Three rabbits were inoculated with egg albumin and two with each of the other antigens.

A note on the in vitro *serological properties of rabbit immuno-conglutinin*

In our work on immuno-conglutinin to date we have observed no difference in the *in vitro* serological properties of rabbit immuno-conglutinin and the naturally occurring conglutinin found in bovine serum. Certain differences, however, might be expected as a reflexion of the species nature of the molecules concerned.

They are both globulins and stable to 56° C. for half an hour. Bovine conglutinin is destroyed by heating between 62° and 64° C. for half an hour, whereas rabbit immuno-conglutinin is much more stable under these conditions.

Rabbit immuno-conglutinin and bovine conglutinin (Coombs *et al.* 1950) react with antigen-antibody complexes to produce conglutination only after C′1, C′2 and C′4 components of complement have all been adsorbed and at no stage before. If the antigen consists of red cells the C′4 must be of the conglutinating type

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otherwise, if of the haemolytic type, lysis may be produced before the cells can conglutinate.

To absorb rabbit immuno-conglutinin or bovine-conglutinin from a heated serum the same conditions are necessary. Suitable adsorbants are either fully sensitized bacteria which have been treated with excess of complement and subsequently washed or inert small particles such as kaolin previously treated with fresh serum such as that of the horse or guinea-pig and subsequently washed.

These remarks apply to immuno-conglutinins produced either by hetero- or auto-stimulation.

DISCUSSION

No work on immuno-conglutinin has been reported, to the knowledge of the authors, since that carried out by Wartiovaara (1932) at Prof. O. Streng's laboratory in Finland. This was a very extensive study, covering work over a period of 4 years. It seems surprising that the biological implications of this phenomenon have not been further investigated. The findings of our preliminary experiments amply confirm the work of Wartiovaara, and give a basis from which a further study can be planned.

Streng (1930), reasoning that a true anti-complement would behave serologically like conglutinin, originally stimulated the production of immuno-conglutinin in rabbits by inoculating sensitized bacteria which had adsorbed guinea-pig complement. For immuno-conglutinin sera produced in this way we suggest the name *immuno-conglutinin sera* (*hetero-stimulation*).

Wartiovaara found that immuno-conglutinins were most easily produced by Streng's technique, but that a great many antigens which had not been treated with complement *in vitro* were nevertheless effective under certain circumstances in stimulating their production to a moderate degree. Amongst these antigens were substances as different as bacteria, red blood cells, and heated and unheated sera. We suggest that immuno-conglutinin sera produced under these circumstances, to an inoculum quite unrelated in specificity to complement, might be called *immuno-conglutinin sera* (*auto-stimulation*).

In the present study immuno-conglutinin sera have been produced by both hetero- and auto-stimulation. The ease with which immuno-conglutinin can be produced by the former method was evident. The production of high-titred sera by the inoculation of fresh unheated horse serum adsorbed on kaolin is of interest as it illustrates that the antigen-antibody complex on which serum complement is adsorbed may play no part in conglutination other than that of an adsorbent for complement. Although very powerful immuno-conglutinin sera were produced by these methods of hetero-stimulation, investigations on the *in vitro* serological behaviour of the sera were complicated by the reaction of antibodies to the serum proteins of the inoculum which were simultaneously produced in the sera.

In our experiments on the production of immuno-conglutinins by auto-stimulation it was found that the intravenous inoculations of large doses of killed bacteria regularly produced powerful immuno-conglutinin sera. The Gram-negative bacteria, besides being very toxic for the rabbits, were more effective than the Gram-

positive bacteria in stimulating immuno-conglutinin production. The soluble antigens studied were found either to be poorly effective or apparently completely ineffective. The difference in response, well exemplified by the inoculation of *S. pullorum* antigens, dependent upon whether the antigen is in a soluble or particulate state, may well be concerned with the different processes of the body for dealing with soluble and particulate foreign substances. The influence of toxicity also needs to be studied.

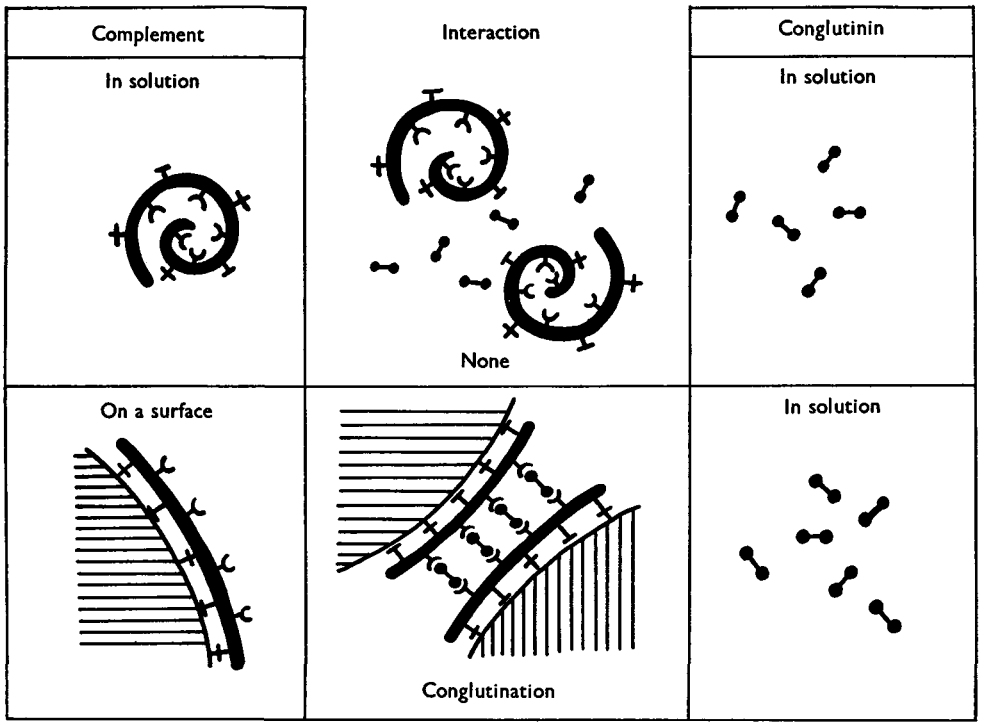

Fig. 9. Diagram of a possible method of interaction between complement and conglutinin.

A close comparison has been made of the conglutinating activity of the sera produced by the different methods of both hetero- and auto-stimulation. Although the methods vary in the ease with which they stimulate the production of immuno-conglutinin, it has not been possible to detect any difference in the reactivity of the immuno-conglutinins so produced. The striking similarity in their properties argues an ultimate common origin, even if the stimuli applied from without are different.

Coombs (1947) advanced a possible theory for the interaction of naturally occurring bovine conglutinin and complement. This postulated that conglutinin reacts with certain groupings of the complement proteins which are only exposed when complement is 'unfolded' on a surface (see Fig. 9). This hypothetical explanation also holds for the reactivity of immuno-conglutinin. There is suggestive

evidence that complement adsorbed either on an immune aggregate or on a surface such as that which kaolin presents, assumes a configuration which it does not possess in solution. This configuration would appear to be a common characteristic of adsorbed complement regardless of the species of the animal from which the serum comes, an organ specificity as opposed to a species specificity.

It has been shown that when a rabbit is inoculated with sensitized bacteria which have adsorbed a complement from another animal species, it responds in two ways to the adsorbed complement. It produces antibodies whose specificity is directed against the serum proteins of the species of animal which supplied the complement and it also produces immuno-conglutinin, the specificity of which is directed against the configuration characteristic of adsorbed complement.

If a rabbit's own complement is adsorbed *in vivo* by an immune aggregate and displays this characteristic configuration of adsorbed complement there would seem to be no reason why it should not be antigenic for the rabbit, notwithstanding the fact that the rabbits own proteins constitute the building stones for this configuration. Thus immuno-conglutinin produced by auto-stimulation, where no foreign complement is included in the inoculum, may represent a physiological example of auto-immunization.

The question must be considered of the relationship of immuno-conglutinin to other apparently non-specific consequences of immunization. Since immuno-conglutinins appear in the globulin fraction of the serum and are in all probability ordinary antibodies they must contribute to the rise of apparently non-specific globulin which has often been observed following a process of immunization. The intense stimulation which is necessary for the experimental production of immuno-conglutinins (by auto-stimulation) might easily find a parallel in naturally occurring acute infections, and therefore an attempt was made to find out if there was any relationship between the acute-phase or C-reactive protein and immuno-conglutinin. It could be shown that the two substances are not identical. Acute phase protein appeared in the serum of rabbits given a single intravenous inoculation of a suspension of killed *Proteus* OX 19 as early as 12 hr. after the injection, whereas immuno-conglutinin did not appear till 3 days later. When the injections were made subcutaneously the Cx-reactive protein appeared, but there was never any demonstrable immuno-conglutinin. Immuno-conglutinin is completely precipitated by 50 % saturation with ammonium sulphate, while Cx-reactive protein is precipitated only between 50 and 70 % saturation.

Having found a very low titre of conglutinin or immuno-conglutinin activity in the sera of certain apparently normal healthy rabbits, the sera of other animals species are being examined preparatory to an attempt to produce immuno-conglutinins in these other animal species.

The present distinction between naturally occurring conglutinin in a serum such as that of cattle and immuno-conglutinin such as that produced under certain conditions in the rabbit may well disappear as the whole problem is further examined. Finally, a study must be made of the role which conglutinin or immuno-conglutinin plays in the immunity of the animal.

SUMMARY

1. An investigation has been carried out into the conditions necessary for the production of immuno-conglutinin in rabbits.

2. The inoculation of complement adsorbed on sensitized cells stimulates the production of immuno-conglutinin. We have called this procedure *hetero-stimulation*.

3. Immuno-conglutinin also results from the inoculation of untreated but killed bacteria. This procedure for its production we call *auto-stimulation*. Gram-negative bacteria appear to be more effective stimulants than Gram-positive bacteria. Soluble antigens did not appear to be as effective as bacterial suspensions.

4. The necessity for clarifying the part played by immuno-conglutinin in *in vivo* immune processes has been stressed.

The authors would like to thank the following for gifts of experimental materials: Mr H. I. Field, strains of *S. pullorum*; Dr J. Boissard, strain of *C. hofmanni*; Dr C. H. Lea and Dr R. S. Hannan, purified casein; Miss Dunkerley, Bence Jones protein. Cx polysaccharide for testing acute phase protein in rabbit serum was made available by the kindness of Dr McCarty of the Hospital of the Rockefeller Institute for Medical Research, New York; and much of the work on this aspect was done in consultation with Dr H. C. Anderson, to whom we are most grateful.

REFERENCES

Blomfield, A. M., Coombs, R. R. A. & Hole, N. H. (1950). The conglutination phenomenon. VI. An experimental investigation of the factors determining the adsorption of complement by an antigen-antiserum mixture. *J. Hyg., Camb.*, **48**, 73.

Bordet, J. & Streng, O. (1909). Les phénomènes d'absorption et la conglutinine du sérum de bœuf. *Zbl. Bakt.* **49**, 260.

Cole, S. W. (1933). *Practical Physiological Chemistry*, 9th ed., p. 93. Cambridge: W. Heffer and Sons Ltd.

Coombs, R. R. A. (1947). The conglutination and sensitization reactions. Dissertation to the University of Cambridge for the degree of Ph.D. (p. 77).

Coombs, R. R. A., Blomfield, A. M. & Roberts, G. F. (1950). The conglutination phenomenon. VII. A study of the interaction of complement components and conglutinin in the process of conglutination. *J. Hyg., Camb.*, **48**, 484.

Coombs, R. R. A., Mourant, A. E. & Race, R. R. (1945). A new test for the detection of weak and 'incomplete' Rh agglutinins. *Brit. J. exp. Path.* **26**, 255.

Lea, C. H. & Hannan, R. S. (1950). Studies of the reaction between proteins and reducing sugars in the 'dry' state. II. Further observations of the formation of the casein-glucose complex. III. Nature of the protein groups reacting. *Biochim. biophys. Acta*, **4**, 518; **5**, 433.

Squire, J. R. (1950). The relationship between horse dandruff and horse serum antigens in asthma. *Clin. Sci.* **9**, 127.

Streng, O. (1930). Immunokonglutinin-Antikomplement. *Acta path. microbiol. scand.* **20**, 411.

Wartiovaara, T. W. (1932). Über die Entwicklung der konglutinierenden Eigenschaft bei der Immunisierung. *Acta Soc. Med. Duodecim*, Ser. A, **14**, Fasc. 3.

(*MS. received for publication* 3. v. 53)