THE CONGLUTINATION PHENOMENON

X. CONGLUTININ AND IMMUNO-CONGLUTININ IN GUINEA-PIGS, PIGS, DONKEYS, RATS AND MICE

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(With 8 Figures in the Text)

CONTENTS

INTRODUCTION

In a previous paper (Coombs & Coombs, 1953) experiments were reported which confirmed the observations of earlier workers on the production of immuno-conglutinin in rabbits (Streng, 1930; Wartiovaara, 1932). Conglutinin was found to be present in very small amounts in the sera of normal uninoculated rabbits and the production of immuno-conglutinin was induced by injecting them with complement from a different animal species which had been adsorbed either specifically on to sensitized bacteria or non-specifically on to kaolin; the term hetero-stimulation was suggested for this procedure. The immuno-conglutinin response was not found to be dependent on the inoculation of complement from a foreign species for it was also elicited when certain bacterial suspensions, untreated with complement, were injected. To differentiate immuno-conglutinin produced in this latter way, the name immuno-conglutinin (auto-stimulation) was suggested. The hypothesis was advanced that an animal's own complement, fixed *in vivo* on to the specific bacterial antigen-antibody aggregate, was the direct stimulus for the production of immuno-conglutinin (auto-stimulation). The response was, therefore, regarded as a physiological example of auto-immunization.

This paper reports a continuation of this work. Normal adult guinea-pigs, pigs, donkeys, rats and mice were first examined for the occurrence of conglutinin in

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their serum. In each species an attempt was then made to stimulate the production of immuno-conglutinin (auto-stimulation) by the intravenous injection of Gram-negative bacteria. In rats and mice the attempt was successful but in the other three species there was no evidence of immuno-conglutinin production. Further experiments were carried out in guinea-pigs in an attempt to elucidate the apparent failure of this species to produce immuno-conglutinin by auto-stimulation.

MATERIALS AND METHODS

Animals

Fully grown guinea-pigs of mixed laboratory strains and weighing between 500 and 600 g. were used. They were bled by cardiac puncture and were injected intravenously into an ear vein as described by Blomfield, Herbertson & Coombs (1952). The pigs were pedigree Large Whites which were 3 months old when first bled for pre-inoculation samples of serum. Veins of the ear were used for injection, and the animals were bled either from an ear vein, when small amounts of serum were required, or from the subclavian vein. The final bleeding after the second course of injections was taken at slaughter when the pigs had reached bacon weight. The donkeys used were adult. The rats and mice were adults of the Wistar and Glaxo strains respectively. They were injected intravenously into one of the tail veins and bled by cardiac puncture.

Inocula

The preparation of the stock bacterial suspensions of *Salmonella pullorum*, *Proteus* OX 19 and *Staphylococcus aureus* has been described in a previous paper (Coombs & Coombs, 1953). The preparation of a suspension of sensitized bacteria which had adsorbed complement was also described in that paper; the procedure was similar in the present experiment except that, when guinea-pigs were being injected, a guinea-pig anti-proteus serum was used to sensitize the proteus organisms.

Inoculations and bleeding

After preliminary bleeding, the animals were injected on days 0, 3, 5 and 8. For guinea-pigs the washed bacterial suspensions were used at a dilution equivalent to 1:2 of the stock suspension and the following amounts were injected $-0{\cdot}5$, 1·0, 1·5 and 1·5 ml. Pigs were injected with a bacterial suspension equivalent to the strength of the stock suspension and the amounts injected were 1, 1·5, 1·5 and 3 ml. The donkeys also received the equivalent of stock suspension in washed organisms and the doses were 1, 2, 4 and 5 ml. The rats and mice were injected with washed suspensions at $\frac{1}{2}$ and $\frac{1}{4}$ strength respectively and in amounts of 0·25, 0·5, 0·5 and 1·0 ml. Post-inoculation bleedings were made on the 12th day unless otherwise stated. The sera were separated and stored at $-20°$ C. till tested. Before testing they were heated at 56° C. for half and hour and absorbed with packed sheep cells.

Method of testing for conglutinating activity

Method 1 as described by Coombs & Coombs, (1953) in which sensitized sheep cells and a dilution of fresh horse serum were added consecutively to the test serum,

was found to give equivocal results with guinea-pig serum and to fail to detect any conglutinating activity in pig, rat or mouse serum. Because in this method the horse complement is exposed, before or during adsorption, to any anti-complementary action of the serum being tested, method 2*b* as described by Coombs & Coombs (1953) was used routinely since in this method the complement was adsorbed on to the cells in the absence of test serum. The indicator system in method 2*b* was sheep cells sensitized with the antibody from normal bovine serum and then exposed to a dilution of horse complement. After 20 min. at 37° C. the unabsorbed horse serum was removed after centrifugation and the alexinated cells were washed and resuspended in saline. Heated horse serum diluted 1:10 was added to serial dilutions of the serum under investigation, and finally a suspension of alexinated cells was added to each tube. Cells for the control tests were prepared in exactly the same way except that they were exposed to horse serum which had been heated at 56° C. for half an hour instead of to fresh horse serum.

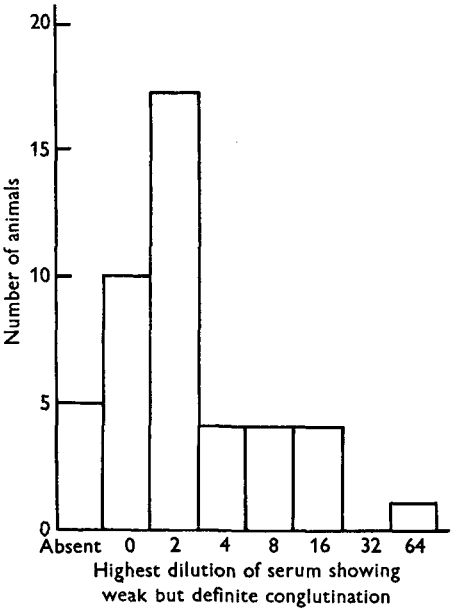

Fig. 1. Distribution of conglutinating activity in the serum of forty-five guinea-pigs.

EXPERIMENTAL STUDY

(1) NORMAL CONGLUTININ LEVELS AND THE EFFECT ON THEM OF INTRAVENOUS INJECTIONS OF A BACTERIAL SUSPENSION

(*a*) *Guinea-pigs*

(i) *Conglutinin in the serum of normal guinea-pigs*

Streng (1909) reported a low-titred conglutinating activity in the serum of normal guinea-pigs. The sera from forty-five of the laboratory stock of guinea-pigs whose individuals varied in age from young adults of 4 months to old breeding does aged 2–3 years were tested in the present series. The conglutinating activity of these sera is shown in Fig. 1. A serum dilution was considered positive for

conglutinin if it could bring about weak but definite conglutination of the cells of the indicator system. The range of values found for young animals did not differ in any marked degree from the values found for older animals.

(ii) *Intravenous injection of bacterial suspensions*

Six guinea-pigs were injected intravenously with a suspension of *Staph. aureus*, and twelve were similarly injected with a suspension of *Proteus* OX 19. The Gram-negative organisms were not nearly so toxic for guinea-pigs as they had been for rabbits. The animals were given two courses of injections and were bled on the 12th day after the beginning of each course. Examination of the sera failed to reveal any increase in conglutinating activity that could be attributed to the effect of the injections; the post-inoculation conglutinin titres of both first and second course sera fluctuated above and below those found in the pre-inoculation bleedings.

Definite production by auto-stimulation of immuno-conglutinin in guinea-pigs has not been possible, although a technique was used which gave good results in the rabbit, namely the intravenous injection of Gram-negative organisms such as *Proteus* OX 19. Gram-positive organisms also failed to produce immuno-conglutinin in guinea-pigs.

(*b*) *Pigs*

(i) *Conglutinin in the serum of normal pigs*

There are two properties of pig serum which can interfere with the demonstration of its conglutinating activity. The sera are mostly very anti-complementary to horse complement and contain antibodies to sheep erythrocytes. The anti-complementary action may be masked and a pro-complementary effect be apparent because of the high titre of anti-sheep cell antibody, which appears to be a Forssman antibody since it can be absorbed from the serum by minced guinea-pig kidney and its agglutinating power for sheep erythrocytes is weak. However, this antibody can hardly be called a haemolysin since it fails to adsorb guinea-pig complement when combined with sheep erythrocytes. The presence of the antibody when combined with sheep cells can be shown either by an antiglobulin reaction, when the washed sensitized cells are agglutinated by the addition of a rabbit anti-pig globulin antiserum, or by a direct conglutination reaction in which the sensitized cells adsorb horse complement and can then be conglutinated by the addition of heated bovine serum from which the anti-sheep cell antibodies have been removed by absorption.

To overcome anti-complementary action and any confusion there might be between agglutination and conglutination, alexinated sheep cells were used as an indicator system of conglutinating activity and the heated pig sera were tested after absorption with packed sheep cells.

The results found when normal pig sera were tested under these conditions are shown in Fig. 2. The bloods were obtained from forty-eight pigs which had been sent to a bacon factory for slaughter. The pigs were all of a similar age but came from different farms and small holdings.

(ii) *Intravenous injection of bacteria*

Three pigs were injected intravenously with a suspension of *S. pullorum*.

The results found on testing the inactivated absorbed sera are shown in Fig. 3. Any increase in conglutinating titre in post-inoculation sera was not great, and its significance cannot be assessed without further knowledge of the normal fluctuations of serum conglutinin in pigs.

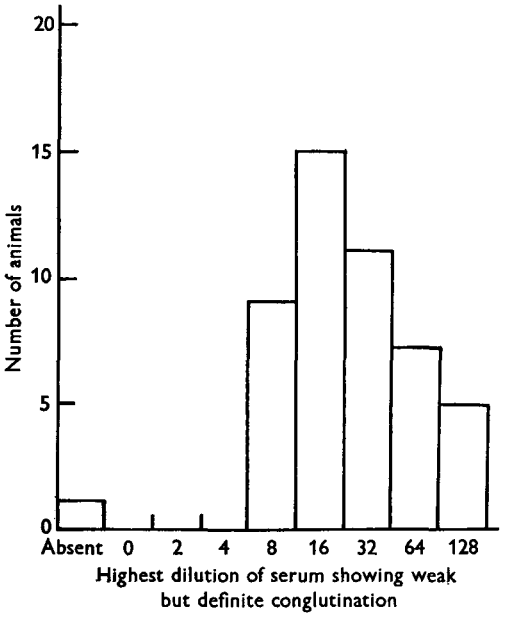

Fig. 2

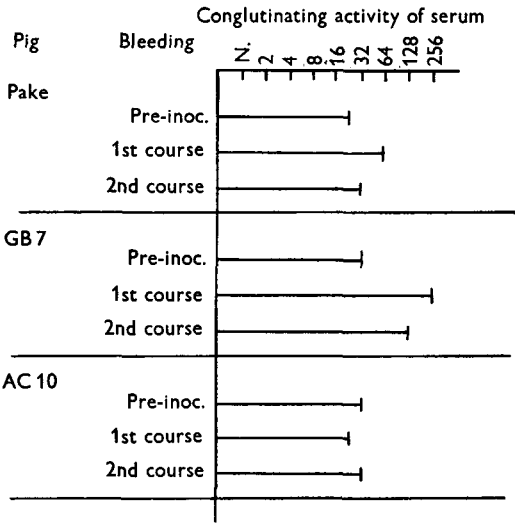

Fig. 3

Fig. 2. Distribution of conglutinating activity in the serum of forty-eight normal pigs.
Fig. 3. Conglutinating activity in the serum of pigs inoculated intravenously with bacterial suspensions.

(c) *Donkeys and horses*

(i) *Conglutinin in the serum of normal horses*

Sera from twelve normal horses when examined for their content of conglutinin yielded negative results.

(ii) *Injection of a suspension of formolized* Salmonella pullorum

Five donkeys were used for these experiments. Two were injected by the subcutaneous route and three by the intravenous route. Serial bleedings were made throughout each course of inoculation and the sera obtained were tested for conglutinating activity. Intravenous injections of *S. pullorum* were toxic to donkeys and one donkey died as a result of this toxicity. This toxic property made it impossible to use a dosage for the donkeys which was comparable, on a weight basis, with that received by the rabbits.

No conglutinating activity could be demonstrated in the post-inoculation sera. Injections were continued in one donkey until it had received five courses intravenously. The results were still negative.

(*d*) *Rats*

(i) *Conglutinin in the serum of normal rats*

Heated rat serum has been found to be very anti-complementary to horse complement. For this reason no conglutinating activity could be detected in rat serum unless alexinated cells were used for the indicator system. The results found by testing the serum of twenty-five normal rats with alexinated cells and inactivated horse serum are shown in Fig. 4. The sera of many rats showed no conglutinating activity and a large majority showed a titre of 1:2 or less.

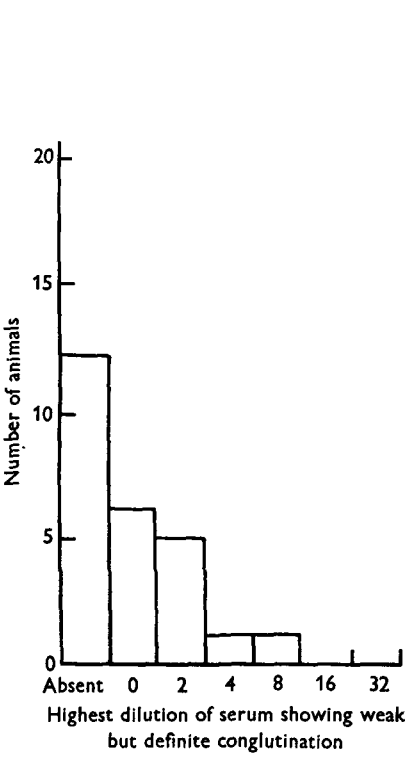

Fig. 4

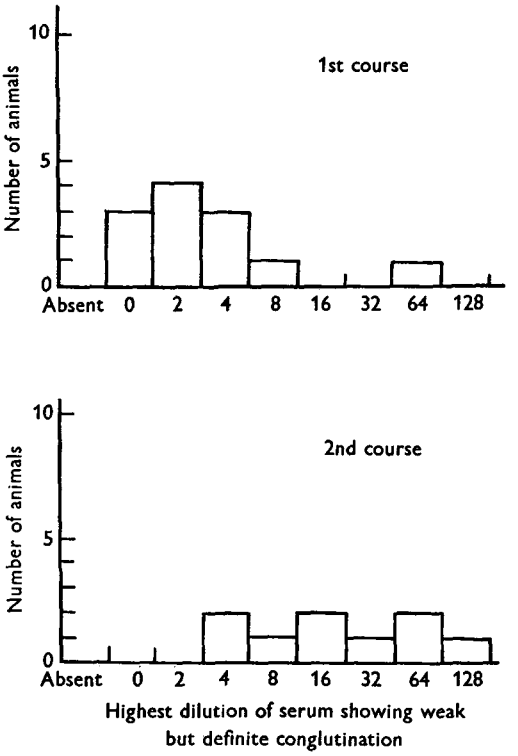

Fig. 5

Fig. 4. Distribution of conglutinating activity in the serum of twenty-five normal rats.
Fig. 5. Distribution of conglutinating activity in the post-inoculation serum of rats.

(ii) *Intravenous injection of formolized* Salmonella pullorum

The results of testing the post-inoculation sera of the first and second courses are shown in Fig. 5. The intravenous injection of these organisms stimulated the production of immuno-conglutinin in rats.

(*e*) *Mice*

(i) *Conglutinin in the serum of normal mice*

Like rat serum, the serum of mice is markedly anti-complementary to horse complement. The serum of normal mice must, therefore, be tested for its conglutinating activity with sensitized cells which have already adsorbed complement

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and firmer aggregates occur on conglutination if inactivated horse serum at a dilution of 1:10 is also included with the indicator system as in method 2*b*. The result of testing the serum of fourteen normal mice is shown in Fig. 6. The sera of many mice lacked conglutinating activity but a low titre was present in some serum samples.

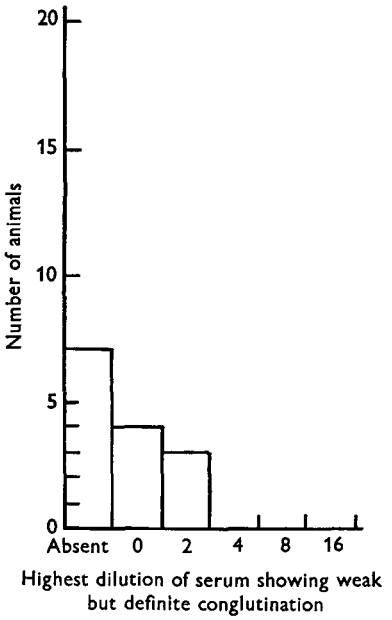

Fig. 6

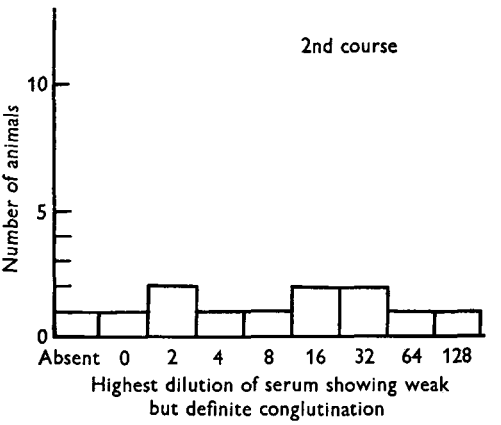

Fig. 7

Fig. 6. Distribution of conglutinating activity in the serum of fourteen normal mice.
Fig. 7. Distribution of conglutinating activity in post-inoculation serum of mice.

(ii) *Intravenous injection of* Salmonella pullorum *(formolized suspension)*

Only a small amount of serum was obtained from each mouse bled after the first course of inoculations. Two pools of sera were made, M3 which was the pooled sera of six mice and M4 which was the pooled sera of five mice. After the second course of inoculations the serum of each mouse was tested separately. The conglutinating titres of the pools of the first-course sera were M3 1:16, and M4 1:32, and the second-course sera had titres ranging from 1:120 downwards (see Fig. 7).

These results are similar to those found in rats, in that intravenous injection of mice with a suspension of Gram-negative bacteria stimulates the production of immuno-conglutinin in their serum.

(2) AN ATTEMPT TO ELUCIDATE THE REASONS FOR THE FAILURE OF GUINEA-PIGS TO PRODUCE IMMUNO-CONGLUTININ (AUTO-STIMULATION)

The hypothesis has been advanced in a previous paper (Coombs & Coombs, 1953) that the animal's own complement, fixed *in vivo* on to injected bacteria, might be the direct antigenic stimulant for the production of immuno-conglutinin. The failure

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of immuno-conglutinin (auto-stimulation) production in guinea-pigs, when considered in the light of this hypothesis, could be due to any of at least three causes. First, the complement configuration, that is the grouping common to adsorbed complement of many different animal species, might not be antigenic in guinea-pigs. Secondly, a guinea-pig's own complement might not be fixed *in vivo*. Thirdly, the guinea-pig's own complement, fixed *in vivo*, might not be antigenic. These three possibilities were investigated experimentally.

(*a*) *Intravenous injection of sensitized bacteria that had adsorbed either horse or rabbit complement*

In these experiments, the capacity of guinea pigs to respond to the configuration of adsorbed complement from another animal species was tested. The complements chosen were those of the horse and the rabbit. They were freed of antibodies to *Proteus* OX 19 by absorbing them with these organisms at 0° C. for 15 min. The complement activity of the sera after this treatment was apparently unaltered. The complements were then adsorbed on to formolized *Proteus* organisms which had been sensitized with guinea-pig antibody.

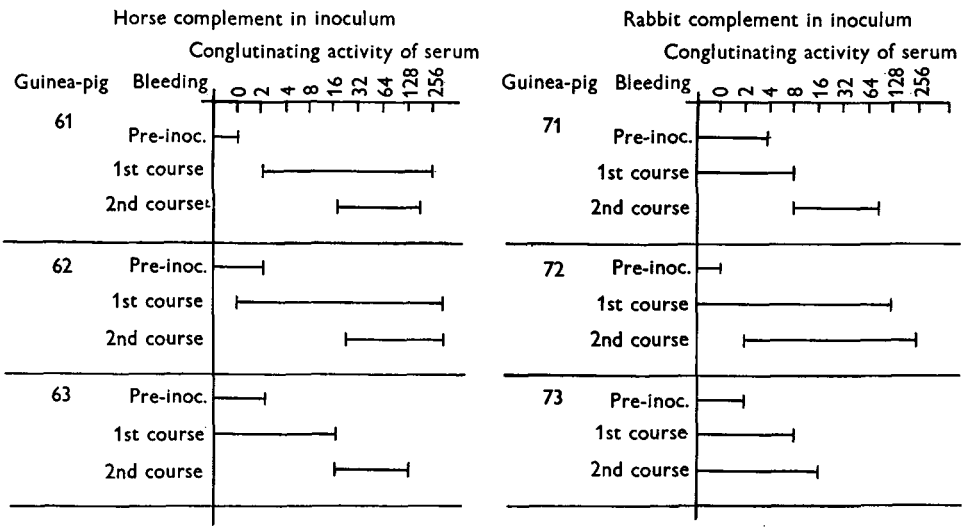

Fig. 8. Conglutinating activity in the serum of guinea-pigs inoculated with a foreign complement adsorbed on to sensitized proteus.

Three guinea pigs were injected intravenously with adsorbed horse complement and three with adsorbed rabbit complement. The animals were given two courses of injections. The results found on testing the pre-inoculation sera and those drawn on the 12th day after the beginning of each course are shown in Fig. 8. The figure shows that immuno-conglutinin was produced by both groups of guinea-pigs. Guinea-pigs injected with adsorbed horse complement produced high-titred conglutinating sera after the first course of injections. Adsorbed rabbit complement, however, did not stimulate such high titres of immuno-conglutinin. Antibodies

were also produced in both groups of animals which reacted with horse serum proteins and these interfered with the demonstration of conglutinating activity by immuno-conglutinins by producing a prozone of inhibition.

The conglutinating activity of these sera could be absorbed out with sensitized *S. pullorum* organisms which had adsorbed horse complement. Salmonellae either in the native state or sensitized with horse or rabbit antibody did not absorb out any of the conglutinating activity. This is evidence of the true conglutinin-like nature of these immune substances.

This experiment demonstrates that the complement configuration, when made up of serum constituents of another animal, can be antigenic in the guinea-pig.

(*b*) *The intravenous injection of bacteria, sensitized with guinea-pig or with rabbit antibody and treated with guinea-pig complement* in vitro

The original experiments of Streng (1930) had demonstrated that guinea-pig complement adsorbed *in vitro* on sensitized bacteria was very effective in stimulating immuno-conglutinin production in rabbits (hetero-stimulation). This is evidence that guinea-pig complement after being adsorbed *in vitro* takes up the active configuration that will stimulate certain animal species to produce immuno-conglutinin. Experiments were therefore designed to discover whether *in vitro* adsorption of the guinea-pig complement before injection would be able to influence the production of immuno-conglutinin in guinea-pigs.

In the same series of experiments, the influence of the antibody sensitizing the injected bacteria was investigated. Experiments with mallein and *S. pullorum* antisera had demonstrated that haemolytic guinea-pig complement was not very readily absorbed by guinea-pig antibody combined with the homologous antigen although, by contrast, rabbit antibody readily absorbed guinea-pig complement (Blomfield, Coombs & Hole, 1949). The bacteria for injection in this experiment were, therefore, sensitized either with guinea-pig or with rabbit antibody before exposure to guinea-pig complement *in vitro*. Complement was adsorbed by both sensitized suspensions. Organisms sensitized with rabbit antibody were also injected without having been treated with guinea-pig complement. Groups of three guinea-pigs were used for each of the three inocula. Two standard courses were given and each animal was bled on the 12th day of each course.

None of the animals in this experiment produced any demonstrable immuno-conglutinin. These experiments therefore led to the conclusion that neither the complement of the guinea-pig adsorbed *in vitro* nor that adsorbed *in vivo* was able to stimulate the guinea-pig to the production of immuno-conglutinin.

DISCUSSION

The occurrence of conglutinin in the sera of normal animals has been surveyed by Streng (1909), who reported that conglutinin was to be found in the greatest concentration in the sera of ruminants; guinea-pig sera possessed a weak but definite conglutinin whereas the sera of pigs, man, horses, rabbits and poultry gave equivocal results. As a preliminary to experimental work on the production of

immuno-conglutinins we needed to know the conglutinin level in the serum of each experimental animal before inoculation and also how representative this might be of the species as a whole. The results with guinea-pigs serum confirmed the findings of Streng. Pig serum also contained a conglutinin which could only be demonstrated by using an indicator system which suffered a minimum of interference from other serum factors. The sera of rats, mice and rabbits rarely contained conglutinin and conglutinin could never be demonstrated in the sera of horses.

The significance of the actual figures of conglutinin levels recorded only applies to the particular laboratory stock that was tested. On theoretical grounds such factors as the flora of the digestive tract or the activity of any pathogens which might be endemic in the animal rooms or introduced with feeding-stuffs might affect the conglutinating activity of the animals' sera. The figures given, therefore, serve only as a base-line for the particular stocks of animals that were used in these experiments.

The production of immuno-conglutinins could be stimulated with ease in rabbits by the intravenous injection of a suspension of killed Gram-negative bacteria (Coombs & Coombs, 1953). The experiments recorded here have shown that similar injections will also stimulate the production of immuno-conglutinins in rats and mice. Rabbits, therefore, are not unique in the animal kingdom in their response of immuno-conglutinin production by auto-stimulation.

Some species of animals, however, appear to be resistant to this particular stimulation. Guinea-pigs, pigs and horses inoculated intravenously with suspensions of Gram-negative organisms failed to respond by a rise in the conglutinating activity of their sera. In the guinea-pig and pig this failure may possibly be correlated with the normal presence of conglutinating activity in the serum, but horses do not appear to have conglutinin in their serum.

Human sera are now being examined to find whether conglutinin occurs normally and whether there is evidence that immuno-conglutinin production can be stimulated.

A further study will be carried out, on a comparative basis, of *in vivo* complement fixation in animals since the mechanism may be different in different species.

SUMMARY

1. Immuno-conglutinin production by auto-stimulation was attempted in guinea-pigs, pigs, donkeys, rats and mice by the intravenous injection of a suspension of Gram-negative bacteria. The attempt was successful in rats and mice.

2. Experiments were carried out to elucidate the lack of response in guinea-pigs.

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