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Antibody Responses to Bacteria

THE EFFECT OF THE IMMUNOGLOBULIN TYPE OF ANTI-BACTERIAL ANTIBODY ON IMMUNOCONGLUTININ STIMULATION IN RABBITS AND GUINEA-PIGS

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Summary. Three manifestations of the antibody response of rabbits and guinea-pigs to injections of killed bacteria have been examined, namely, complement-fixing and agglutinating anti-bacterial antibodies and immunoconglutinins (I-Ks) directed against fixed complement. Animals given intramuscular injections of the bacteria in Freund's complete adjuvant before a course of intravenous injections were compared with those receiving the latter course only.

The prior immunization with organisms in adjuvant increased the I-K responses in rabbits. This was correlated with an increase in that part of the complement-fixing, anti-bacterial antibody contained in the IgG fraction. The animals receiving the course of intravenous injections only, produced more agglutinating anti-bacterial antibody (which was shown to be predominantly IgM in type) and less I-K.

In guinea-pigs there was little difference in the I-K response between animals receiving the two injection schedules. The prior immunization with organisms in adjuvant did produce higher levels of anti-bacterial antibody, but in this species the increase was mainly in the IgM fraction.

It is suggested that in both species the presence of complement-fixing anti-bacterial antibody of IgG immunoglobulin type is necessary for the stimulation of I-K production and that high levels of IgM antibody may have an inhibitory effect.

High titres of I-K can be produced regularly by injecting bacteria intravenously into rabbits with predominantly IgG anti-bacterial antibodies. Where these do not occur naturally they can be produced in reponse to two intramuscular injections of bacteria in adjuvant. In guinea-pigs it was difficult to produce predominantly IgG anti-bacterial antibody and high titres of I-K were difficult to achieve.

INTRODUCTION

Wartiovaara (1932) first demonstrated the stimulation, by injections of bacteria into rabbits, of substances causing agglutination of alexinated cells. Coombs (1947) suggested that these substances were autoantibodies produced against complement components which exposed new antigenic determinants by becoming fixed to antibody combined with bacteria *in vivo*. The evidence for this hypothesis is now considerable (Lachmann, 1967).

The majority of the early work was done in rabbits and mice, although most mammals studied, including man, exhibited some immunoconglutinin (I-K) activity (Coombs,

Coombs and Ingram, 1961; Bienenstock and Bloch, 1966). Guinea-pigs were shown to be poor producers of I-K by Lachmann and Coombs (1965), this despite the high circulating levels of haemolytic complement present in the species.

Coombs and Coombs (1953) found that particulate antigens, such as bacteria (alive or dead) circulating in the blood produced the highest levels of I-K although there was considerable species variation among bacteria in this respect. Higher titres of I-K were obtained with repeated courses of injections (Lachmann and Coombs, 1965) and Ingram (1962b) found that 'immune' rabbits also produced more I-K.

The experiments reported were undertaken to examine the effect of the immune status of the animal on the I-K response to bacteria, and are the first part of a study of the role of the immunoconglutinin–complement system in health and disease. This system also represents a possible way of examining the antibody response to bacteria with respect to *in vivo* complement fixation.

MATERIALS AND METHODS

Animals

Young adult New Zealand white rabbits were used, weighing 3·5–4·5 kg at the beginning of the experiments. The guinea-pigs were adult outbred animals of about 250 g.

Bacteria

Escherichia coli, *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus megaterium* were grown on nutrient agar at 37° for about 30 hours and harvested into saline. The Gram-negative organisms were killed with 1 per cent formalin and the Gram-positive bacteria by heating at 100°. *Brucella abortus* was obtained from the Central Veterinary Laboratory, Weybridge. After checking for lack of viability the bacteria were washed in sterile saline and an aliquot counted by haemocytometer, or measured by opacity. Stock suspensions of about 10^{11} organisms/ml were stored at 4°, plated for sterility and diluted in saline before injection.

Adjuvant

Freund's complete adjuvant was made up by grinding together 8·5 ml Bayol '85' (Esso), 1·5 ml Arlacel A (Honeywill and Stein) and 20 mg dried human tubercle bacilli. Equal volumes of bacterial suspension and adjuvant were emulsified together, and injected into the thigh muscles.

Ground-nut protein

This was obtained from Dr Philip, Unilever Ltd.

Intravenous injection course

A dose of 0·5 ml of the bacterial suspension was injected into the ear vein in rabbits or foot vein in guinea-pigs at intervals of 2 days. Each course consisted of four such injections.

Serum sampling

Rabbit sera were collected 19, 9 and 0 days before the first intravenous injection, and 6, 12, 19 and 26 days after it. Guinea-pigs were bled before inoculation and on the 7th,

9th, 13th and 20th days after the first intravenous injection. The sera were inactivated at 56° for 30 minutes, absorbed at room temperature with ½ volume of washed, packed, sheep erythrocytes and stored at −20° so that all samples from each course could be tested together.

Immunoconglutinin levels

These were determined by the conglutination of indicator sheep erythrocytes alexinated with whole rabbit complement at 10° (EAC'_{Rab}) (Lachmann and Liske, 1966). The pools of complement used were kept at −196°. Guinea-pig sera were titrated against sheep cells alexinated with equine complement using the conditions described by Lachmann (1962) (EAC'_{Eq}). In both these methods the tubes were read by re-suspension, the end-point being the last tube showing a clear background. Sera were diluted two-fold and controls set up to ensure that the activity was directed against fixed complement.

Anti-bacterial antibody levels

Complement fixation tests were carried out on the sera, using bacterial suspensions as antigens. Rabbit complement pools were absorbed with packed bacteria at 1° (in the case of *Staph. aureus*, in the presence of 0·01 M EDTA, adding Ca^{++} and Mg^{++} later), and used at 1·5 haemolytic doses per tube. For titrating rabbit sera optimal antigen concentrations were found to be as follows. *S. typhimurium* and *B. megaterium*, 2×10^9 cells/ml; *Staph. aureus*, 8×10^9 cells/ml; *E. coli* 1×10^9 cells/ml. Equal volumes of all the reagents were used. Complement-fixing titres in the guinea-pig sera were estimated by using 1·5 50 per cent haemolytic doses of a guinea-pig complement pool which did not require absorptions. *Staph. aureus* and *S. typhimurium* were used at 1×10^9 cells/ml and *E. coli* at 5×10^8 cells/ml. A microtitration system giving three-fold dilutions of the sera was employed.

Agglutinating titres of the sera for the bacterial suspensions were also estimated. These were carried out in agglutination plates and were read, after overnight settling, at 4°.

Sephadex separation

Some of the sera were separated on Sephadex G-200 (Pharmacia) columns in 0·1 M Tris–HCl buffer, pH 7·2, with 0·5 NaCl and 1/1000 sodium azide. The peaks were pooled and concentrated by salt precipitation, to the original volume of the serum applied.

2-*Mercaptoethanol treatment*

Sera were treated with 0·1 M 2-mercaptoethanol for 2 hours at 37°, the reagent being dialysed away in the presence of saline. This was found to leave most of the complement-fixing ability of the IgG antibody.

RESULTS

RABBITS

The effect of prior immunization with bacteria in adjuvant on the immunoconglutinin response to a course of intravenous injections

Rabbits were given two injections of bacteria in Freund's complete adjuvant 19 and 14 days before a course of four intravenous injections. These are referred to as the test rabbits. They are compared with similar animals receiving intravenous injections only, which are referred to as the control rabbits. At all injections 5×10^9 bacteria were given. Results

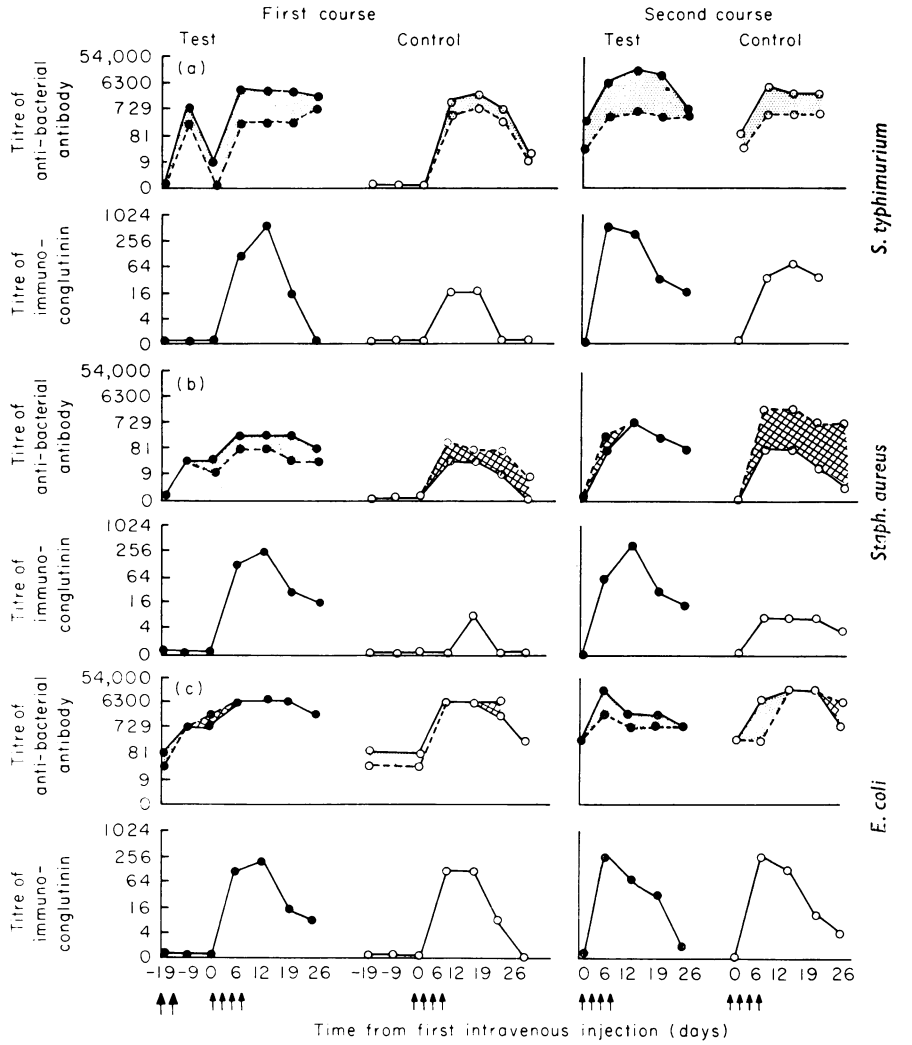

FIG. 1. Anti-bacterial antibody and immunoconglutinin levels in rabbits, produced by different injection schedules. The test rabbit received two intramuscular injections of bacteria in adjuvant (large arrows) before the first intravenous course. The control rabbit received the intravenous injection courses only (small arrows). Each inoculum contained 5×10^9 bacteria. Anti-bacterial antibody: continuous line, complement fixation; broken line, agglutination. Stippling indicates higher complement fixation than agglutination titres and cross-hatching the reverse. These are typical examples of the results obtained with a number of rabbits, i.e. *S. typhimurium* (a): three test rabbits and six controls; *Staph. aureus* (b): three test rabbits and nine controls; *E. coli* (c): two test rabbits and fourteen controls.

for three bacterial species are shown in Fig. 1 and for *B. megaterium* and *Br. abortus* in Tables 1 and 2. The I-K titres shown were measured on EAC'_{Rab}. Parallel titrations on EAC'_{Eq} were carried out and yielded similar results although the end-points were generally slightly lower.

The test rabbits showed a notable increase in the immunoconglutinin response to four of these species of bacteria. With *E. coli*, no difference was observed compared with the

control rabbits which themselves showed high values. The results shown in Fig. 1 are typical of those obtained with many rabbits. Thus eight test rabbits, three given *Staph. aureus*, three given *S. typhimurium* and two given *E. coli*, all produced high I-K levels. Fourteen control rabbits receiving *E. coli* showed similar high I-K titres whereas nine control rabbits

TABLE 1

I-K AND ANTI-BACTERIAL ANTIBODY TITRES PRODUCED IN RABBITS INJECTED WITH *Bacillus megaterium*

Titre	Days from first intravenous injection		
	−19	0	6
Immunoconglutinin			
Test	0	0	512
One previous intravenous course	0	0	64
Control	0	0	32
Complement-fixing (rabbit complement)			
Test	27	243	18000
One previous intravenous course		2100	6300
Control	27	27	2100
Agglutinating			
Test	9	81	243
One previous intravenous course		243	729
Control	27	27	243

The test rabbit was immunized, intramuscularly, with bacteria in adjuvant as well as by the intravenous route. The injection schedule was as shown in Fig. 1. Each inoculum contained 5×10^9 bacteria.

TABLE 2

I-K AND ANTI-BACTERIAL ANTIBODY TITRES IN RABBITS INJECTED WITH *Brucella abortus*

Titre	Days from first intravenous injection				
	−19	0	6	12	20
Immunoconglutinin					
Test	4	8	512	64	32
Control	8	4	16	32	8
Complement-fixing (rabbit complement)					
Test	0	243	2100	2100	729
Control	0	0	243	243	81
Agglutinating					
Test	0	729	2100	2100	729
Control	0	0	2100	2100	729
Complement fixing (guinea-pig complement)					
Test	0	243	2100	2100	729
Control	0	0	2100	2100	729

The test rabbit was immunized, intramuscularly, with bacteria in adjuvant as well as by the intravenous route. The injection schedule was as shown in Fig. 1. Each inoculum contained 5×10^9 bacteria.

given *Staph. aureus* produced low titres and six control rabbits given *S. typhimurium* produced intermediate I-K levels. The maximum titres achieved were of the same order with all five species of bacteria. A rabbit which had had one intravenous course of *B. megaterium*, 2 months previously, showed I-K levels intermediate between the test and control rabbits. (Table 1).

The I-K response using larger doses of bacteria

Ingram (1962a) reported that increased antigen dose gave greater I-K responses. The experiments above were, therefore, repeated using *Staph. aureus* and *S. typhimurium* at 10^{10} and 2×10^{10} bacteria per injection, the schedule otherwise being the same. Table 3 illustrates the height of the I-K response in these animals.

The larger doses did increase the I-K levels in the controls, particularly when *S. typhimurium* was the stimulating bacterium. On the other hand, the titres of I-K in the test rabbits showed little change with the increased dosage. In only one of the rabbits, that given 10^{10} *Staph. aureus* per injection, was I-K production stimulated by the injections in adjuvant alone and this animal had a low level of I-K present before the experiment began. This rabbit also produced very high titres of I-K in the first course.

TABLE 3

I-K TITRES IN RABBITS INJECTED WITH INCREASING DOSES OF BACTERIA

Bacteria	Dose		Days from first intravenous injection					
			First course			Second course		
			0	6	12	0	6	12
Staphylococcus aureus	10^{10} per injection	Test	4	1024	2056	16	1024	512
		Control	0	64	64	8	128	256
		Control	0	24	128	0	512	128
	2×10^{10} per injection	Test	0	128	1024	16	2056	1024
		Control	0	16	16	0	96	64
		Control	0	0	0			
Salmonella typhimurium	10^{10} per injection	Test	0	256	256	0	1024	128
		Control	0	32	128	0	512	256
		Control	0	64	64	0	256	256
	2×10^{10} per injection	Test	0	1024				
		Control	0	64	128			
		Control	0	128	64			

The test rabbit was immunized, intramuscularly, with bacteria in adjuvant as well as by the intravenous route. The injection schedule is shown in Fig. 1.

I-K production after a second course of intravenous injections

The rabbits were given a second course of intravenous injections after a 2- or 6-month interval (Fig. 1 and Table 3). The sera from the control rabbits generally showed higher titres of I-K than in the first course, in some cases approaching those of the test animals, especially if the first course response had been a good one. The test rabbits produced similar levels in both courses. The two rabbits injected with *E. coli* again reacted identically. The other noticeable effect was that most of the test rabbits showed maximum titres on the 6th day in the second course rather than on the 12th day as in the first. The control rabbits were variable in this respect, those producing high levels of I-K tending to the earlier maximum.

The effect of pre-immunization with an unrelated antigen in adjuvant on the I-K response

Rabbits given ground-nut protein, with or without adjuvant, before the usual intravenous course of *Staph. aureus* responded with I-K levels similar to the control animals (Table 4). That is, the adjuvant itself had no effect on the subsequent I-K production.

TABLE 4

I-K AND ANTI-BACTERIAL ANTIBODY TITRES IN RABBITS PRE-IMMUNIZED WITH GROUND-NUT PROTEIN FOLLOWED BY AN INTRAVENOUS COURSE OF *Staphylcoccus aureus*

Titre	Days from first intravenous injection				
	−19	0	6	12	20
Immunoconglutinin					
Test	4	4	16	24	16
Control	2	2	16	16	16
Complement-fixing (rabbit complement)					
Test	0	0	27	81	54
Control	0	0	18	54	9
Agglutinating					
Test	18	18	729	2100	729
Control	27	27	243	729	243

The test rabbit received ground-nut protein in adjuvant, intramuscularly, whereas the control received the protein in saline. The injection schedule was as shown in Fig. 1. Each inoculum contained 5×10^9 bacteria.

TABLE 5

THE I-K RESPONSE IN RABBITS AND GUINEA-PIGS IN THE PRESENCE OF HIGH TITRES OF AGGLUTINATING ANTI-BACTERIAL ANTIBODY

RABBIT—*Staphylococcus aureus*. Third intravenous course (5×10^9 per injection)

Titre	Days from first intravenous injection			
	0	6	12	20
Immunoconglutinin				
Test	32	2056	256	128
Control	0	32	16	16
Complement-fixing (rabbit complement)				
Test	81	1400	729	729
Control	81	4200	2100	729
Agglutinating				
Test	81	2100	2100	2100
Control	81	162000	54000	18000

GUINEA-PIG—*Salmonella typhimurium*. First intravenous course. Prior immunization in adjuvant

Titre	Days from first intravenous injection				
	−14	0	9	12	20
Immunoconglutinin					
Test	2	16	32	24	8
Control	0	0	32	16	
Complement-fixing (guinea-pig complement)					
Test	0	27	729	2100	2100
Control	0	0	27	81	
Agglutinating					
Test	0	160	18000	18000	6300
Control	0	0	81	243	

The test animals were immunized, intramuscularly, with bacteria in adjuvant, as well as by the intravenous route.

The anti-bacterial antibody response

Anti-bacterial antibody was measured in all the rabbit sera by complement fixation with rabbit complement and by agglutination. The results are shown in Fig. 1 and Tables 2, 5 and 6.

Prior immunization with bacteria in adjuvant generally increased the levels of complement fixing antibody compared with the controls. On the other hand, the agglutination titres were higher in the the controls. This difference is seen very well in those rabbits given *Br. abortus* (Table 2). The antibody responses in the two animals injected with *E. coli* were similar, as were the I-K responses. Thus a reasonable correlation was seen between the levels of complement fixing antibodies and the I-K response. Those rabbits receiving increased doses of bacteria also showed this correlation (Table 6). Similar results were obtained when the sera were tested with an 'O' suspension of *S. typhimurium* or *E. coli*.

The correlation did not always hold, however. In Fig. 1, the rabbits given *S. typhimurium*

TABLE 6

THE SENSITIVITY OF THE RABBIT ANTI-BACTERIAL ANTIBODIES TO 2-MERCAPTOETHANOL (2ME)

Bacteria	Dose		Anti-bacterial antibody				I-K
			Untreated		Treated with 2ME		
			CFT	Agglutination	CFT	Agglutination	
Staphylococcus aureus	1×10^{10} cells per injection	Test	243	54	243	18	1024
		Control	54	54	0	0	64
		Control	27	27	0	0	24
	2×10^{10} cells per injection	Test	243	81	81	27	128
		Control	9	81	0	0	16
		Control	3	81	0	0	0
Salmonella typhimurium	1×10^{10} cells per injection	Test	1400	243	480	81	256
		Control	729	729	54	54	32
		Control	729	729	81	27	64
	2×10^{10} cells per injection	Test	2100	729	2100	243	1024
		Control	729	2100	27	27	64
		Control	2100	6300	81	81	128
Staphylococcus aureus	Third course, 6th day	Test	1400	2100	1400	243	2056
		Control	4200	162000	81	2100	32

The test rabbits were immunized, intramuscularly, with bacteria in adjuvant, as well as by the intravenous route. Except where indicated the sera were taken on the 6th day in the first course.

may be seen to have similar complement-fixing antibody levels, even though the I-K titres are so different. Here, the higher agglutinating antibody titres in the control seemed to be associated with diminished I-K production. This effect was observed in other rabbits, for example, a control rabbit given a third course of *Staph. aureus* (Table 5). The test rabbit may be seen to have produced less complement-fixing antibody than the control but sera from the latter also had extremely high agglutination titres which were again associated with a low I-K response.

Some of the sera were also titrated for their ability to fix guinea-pig complement and

this is shown in Table 2 for the rabbits stimulated with *Br. abortus*. The guinea-pig complement fixation titres were seen to parallel the agglutination titres and did not, therefore, show any correlation with I-K levels.

The immunoglobulin type of the anti-bacterial antibodies

Sera from the height of the response to *S. typhimurium* (5×10^9 cells per injection, Fig. 1) in the test and control rabbits were fractionated on Sephadex G-200. The success of the procedure was checked by immunoelectrophoretic analysis, showing the exclusion peak to contain the IgM and the second peak the IgG immunoglobulins. The results of titrations of pools from this separation for complement fixing and agglutination antibodies

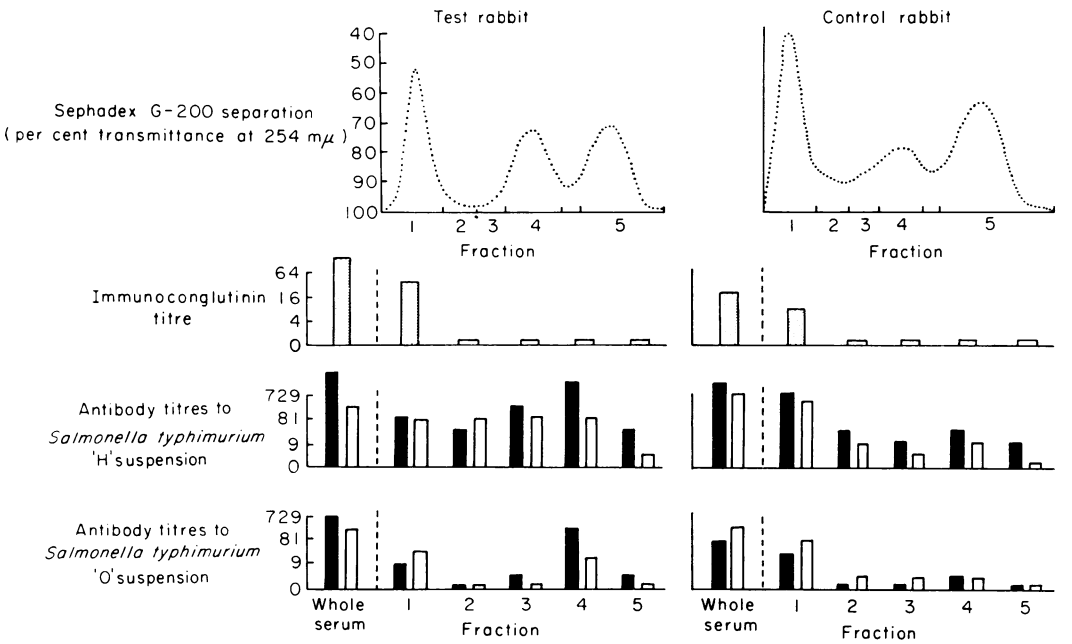

FIG. 2. Sephadex G-200 separation of two rabbit sera taken on the 6th day after stimulation with *Salmonella typhimurium*. The test rabbit received two intramuscular injections in adjuvant before the intravenous course. The control rabbit received the intravenous injections only. Anti-bacterial antibody: black columns, complement fixation; white columns, agglutination.

are shown in Fig. 2. Whilst 90 per cent of the antibody in the control rabbit was found in the IgM peak, 85 per cent of that in the test animal appeared in the second IgG containing peak.

The second peak also had higher titres of complement-fixing than agglutinating antibodies. It may be seen that the distribution of antibodies to an 'O' suspension of bacteria followed the same pattern. Immunoglobulin of both types reacted with this antigen. It is possible that when the anti-bacterial antibodies were measured by complement fixation anti-O antibodies were detected on the formolized bacteria. This may account for the high complement-fixing titres obtained with the 'H' suspension (Fig. 2).

Titration of pools obtained from a similar fractionation of sera from rabbits given 10^{10} *Staph. aureus* per injection gave similar results. The activity in the exclusion peak was 2-mercaptoethanol-sensitive, whereas that in the second peak was not. The results show,

therefore, that IgG antibody levels are higher in the test rabbits and that this is associated with complement-fixing rather than agglutinating activity in the whole serum, and with increased I-K production.

These findings were confirmed by testing sera taken on the 6th day after the first intravenous injection for sensitivity to 2-mercaptoethanol (Table 6). Test rabbits showed higher

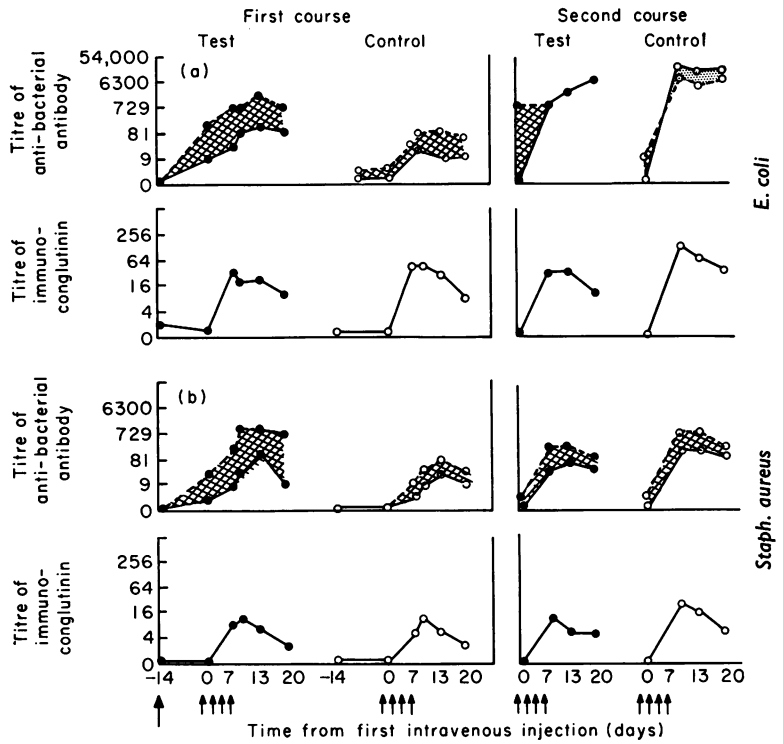

FIG. 3. Anti-bacterial antibody and immunoconglutinin levels in guinea-pigs, produced by different injection schedules. The test guinea-pigs received two intramuscular injections of bacteria in adjuvant (large arrows) before the first intravenous course. The control guinea-pigs received the intravenous injection courses only (small arrows). Each inoculum contained 1×10^{10} bacteria. Anti-bacterial antibody: continuous line, complement fixation; broken line, agglutination. Stippling indicates higher complement fixation than agglutination titres and cross-hatching the reverse. The results are typical of a number of animals tested with each bacterium, i.e. *E. coli* (a): two test guinea-pigs and ten controls; *Staph. aureus* (b): five test guinea-pigs and five controls.

titres of 2-mercaptoethanol-resistant antibody (IgG) than the controls. This was particularly marked in the rabbits given *Staph. aureus*. Although appreciable levels of IgG antibody were present in the *S. typhimurium* control rabbit sera, the titres were also much lower than in the corresponding test animals. Table 6 also shows that the very high level of agglutinating antibody associated with a low I-K response in a rabbit given a third course of *Staph. aureus* is in fact largely IgM in nature.

GUINEA-PIGS

The effect on the I-K response of an injection of bacteria in adjuvant before the intravenous course

Guinea-pigs received one dose of bacteria in adjuvant 14 days before the intravenous injections. These animals did not produce higher levels of I-K than the controls given the

intravenous course only. In the case of *E. coli* they were lower and in that of *Staph. aureus*, about the same (Fig. 3). The *S. typhimurium* test guinea-pig produced some I-K before the intravenous course but its I-K response was no higher than that of the control (Table 5). A second course of intravenous injections stimulated increased titres of I-K and in both cases the controls produced more than the test animals (Fig. 3).

A guinea-pig given ground-nut protein in adjuvant before an intravenous course of *E. coli* produced I-K and antibody titres practically identical with those of a control animal receiving the bacteria only.

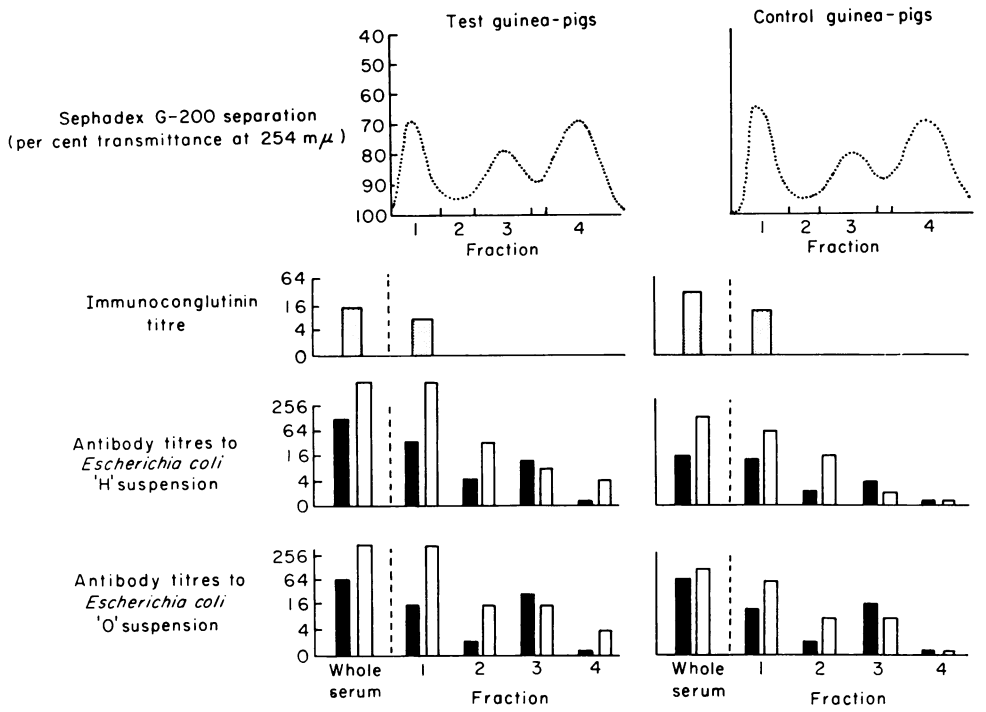

FIG. 4. Sephadex G-200 separation of two guinea-pig serum pools taken on the 9th day after stimulation with *Escherichia coli*. Serum from two animals was used for each pool. The test guinea-pigs received one intramuscular injection in adjuvant before the intravenous course. The control guinea-pigs received intravenous injections only. Anti-bacterial antibody: black columns, complement fixation; white columns, agglutination.

The anti-bacterial antibody response

Anti-bacterial antibody levels were higher in the test guinea-pigs even though the I-K titres were not (Fig. 3). In both test and control guinea-pigs, higher titres of antibody were detected by agglutination than by complement fixation. However, the difference between the levels measured by the two methods was much greater in the test than in the control animals.

Sera taken on the 9th day from the two test guinea-pigs given *E. coli* were pooled and fractionated on Sephadex G-200. They were compared with a similar fractionation of sera from the controls (Fig. 4). The I-K was seen to fall entirely in the exclusion peak and was

also 2-mercaptoethanol sensitive. Antibody levels were higher in the test than in the control guinea-pigs and this increase was seen in both the IgM and IgG fractions. The IgG peak did show an increase in complement fixation over agglutination titre in the test guinea-pigs but this effect was less than that seen in the rabbit. Serum from the test guinea-pig given S. *typhimurium* was also fractionated and again showed an increase in both types of antibody. Thus the same effect was seen in guinea-pigs as in rabbits in that high levels of IgM antibody were associated with a lower I-K response but in these animals, prior immunization with organisms in adjuvant increased the IgM as well as the IgG antibody levels.

DISCUSSION

The early work on the autostimulation of immunoconglutinins showed that antigens were extremely variable in their stimulating abilities. Thus, soluble proteins were largely ineffective and certain bacteria most active in this respect (Coombs and Coombs, 1953; Coombs *et al.*, 1961; Ingram, 1962a, b). The I-K responses of the control rabbits in the experiments described here are in agreement with those found by these workers, who also gave multiple intravenous injections of bacteria. They showed that bacteria of the family Enterobacteriaceae were most effective in stimulating I-K production. Gram-positive species (with the exception of *Listeria monocytogenes*) and other Gram-negative bacteria were less efficient, *Staph. aureus* being one of the least efficient species. The larger doses and different species of bacteria used may account for the higher titres of guinea-pig I-K achieved in these experiments when compared with previously reported work. As has been found in other species, the I-K produced by guinea-pigs was of the IgM immunoglobulin type.

The intramuscular injection of bacteria with Freund's complete adjuvant into rabbits produced no detectable I-K titre. This process did, however, raise the anti-bacterial antibody titres. On the subsequent administration of the usual intravenous course all these animals produced high levels of immunoconglutinin, irrespective of the species of bacteria used. This effect was particularly noticeable with *Staph. aureus*, previously a poor antigen, which under these conditions proved as good an antigen as the other bacteria. After a second course of intravenous injections the effect, although less marked, was still apparent. Rabbits given a different antigen in adjuvant before the intravenous injections were not affected in this way, showing the stimulus in adjuvant to be a specific one.

It has been demonstrated that antibodies from different species of animal vary in their capacity to fix different species of complement (Blomfield, Coombs and Hole, 1949). Consequently, the measurement of complement-fixing, anti-bacterial antibodies in the experiments described was carried out with complement from the same species. However, when guinea-pig complement was used to test the complement-fixing ability of some of the rabbit antibodies, higher titres were found in the sera containing predominantly IgM antibodies than was the case with rabbit complement, whereas similar titres were given by IgG containing sera with both complements.

When the anti-bacterial antibody levels were examined in rabbits, a correlation was observed between the I-K titres and the level of complement-fixing antibody (as measured by rabbit complement). This antibody was shown to be mainly of the IgG immunoglobulin type and was particularly stimulated by the prior immunization with organisms in adjuvant. IgM anti-bacterial antibody was primarily associated with the agglutinating

activity of the serum and higher titres were found in the control animals. Ingram (1962b) also found that his 'immune' rabbits generally produced more antibody as detected by complement fixation with guinea-pig complement, as well as producing more I-K. The presence of complement-fixing antibody of IgG type seems, therefore, to be a prerequisite for the stimulation of an I-K response. Thus the rabbits given *E. coli* had naturally occurring antibodies of this type and might be expected to have experienced similar organisms before, i.e. both groups were showing a secondary type of antibody response which would account for the high I-K titres found in the control. In the sera from rabbits given other organisms by intravenous injection only, agglutinating antibodies (IgM) predominated in the case of *Staph. aureus* whereas against *S. typhimurium*, a higher proportion was IgG. This may be due to an adjuvant effect of the endotoxin in the latter organism and/or differences in previous experience by the rabbits and may explain why such Gram-negative bacteria were shown in the past to be more efficient in stimulating I-Ks than *Staph. aureus*.

Prior immunization of guinea-pigs by injections of bacteria in adjuvant was effective in increasing the anti-bacterial antibody response to intravenous injections, when compared to the controls. In this species, however, the I-K response was, if anything, lower in the test animals. Both IgM and IgG antibody levels were raised in the test guinea-pigs and this is compatible with the hypothesis that IgM anti-bacterial antibody is in some way inhibitory to I-K production. This supposition was supported by finding a similar situation in some rabbits, where the presence of high levels of agglutinating antibody, shown to be of IgM type, were associated with lower I-K titres than in other rabbits with similar complement-fixing antibody responses.

It is not readily apparent how such an inhibition might occur. IgM antibodies may bring about a rapid clearance of bacteria, possibly as a result of *in vivo* agglutination. Moreover, such aggregates might be cleared from the blood in sites that are not congenial to formation of antibody to any fixed complement. The work described here also suggests that, with this system, IgM antibodies may be relatively poor at fixing complement from the same species when compared with antibodies of IgG type. This might enhance an effect of the rate or site of clearance on I-K production. Experiments are being continued to examine these possibilities.

The stimulation of high IgM anti-bacterial antibody titres in guinea-pigs by these injection schedules is noteworthy, as the response to bacteria injected intramuscularly in adjuvant in rabbits is predominantly of IgG type. This may perhaps partially explain the low I-K titres detected after injection of bacteria into guinea-pigs. In this species, the presence of IgG, anti-bacterial antibody of γ_1, non-complement-fixing type may also play a part in the poor I-K response. Another point of interest is the production in both rabbits and guinea-pigs of antibodies of both immunoglobulin types to the 'O' antigens of *S. typhimurium* and *E. coli*. This is in contrast to the findings of Lospalluto, Miller, Dorward and Fink (1962) in man, and Bauer, Mathies and Stavitsky (1963) in rabbits, where IgM antibodies to this antigen predominated. However, these workers did not employ Freund's complete adjuvant and the use of this in the experiments described may account for the stimulation of IgG antibodies to the somatic antigens. Altemeier, Robbins and Smith (1966) have also demonstrated IgG anti-O antibody production in rabbits using a more sensitive detection system.

A regime with the first course consisting of two intravenous injections of organisms in Freund's adjuvant and the second course consisting of four intravenous injections of the

bacterial suspension alone provides a technique for regularly producing high titres of I-K in rabbits. The method is also relatively safe—only one rabbit died during these experiments, possibly as a consequence of this injection schedule. Intravenous injections must still be employed, probably because I-K is an IgM antibody, but the bacteria are introduced into an environment which contains IgG, complement-fixing antibodies to them. This situation appears to be optimal for the production of autostimulated immunoconglutinins.

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