THE RELATIONSHIP BETWEEN THE ACUTE PHASE RESPONSE AND ANTIBODY PRODUCTION IN THE RABBIT

II. The Stimulation of Cx-Reactive Protein Response by Certain Adjuvants and the Relation of This Response to the Enhancement of Antibody Formation

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In the preceding paper it has been shown that a correlation exists between the production of Cx-reactive protein by rabbits in response to the administration of the two antigens, human C-reactive protein and human gamma globulin, and the subsequent production of precipitating antibody in high titer (1, 2). This finding suggested that it would be of interest to investigate the effects of an adjuvant and its individual components in stimulating the production of Cx-reactive protein. Since the addition of adjuvants to poor antigens is widely used to increase the titers of antibody produced against such antigens, it was of interest to determine whether an adjuvant alone, without any antigen incorporated in the saline phase, would be capable of stimulating the production of Cx-reactive protein.

There has been one report of the production by rabbits of an acute phase substance which probably represents Cx-reactive protein in response to the injection of non-antigenic substances. Hedlund, who described the non-specific capsular swelling of certain strains of Type XVI pneumococci in the presence of acute phase rabbit serum, noted the capsular swelling substance in the sera of rabbits which had been injected intramuscularly with a colloidal sulfur preparation, and with salts of manganese, gold, and copper (3). In addition to Hedlund's findings employing non-specific agents for the production of the capsular swelling substance, it has been found in unpublished experiments in this laboratory that the production of Cx-reactive protein can be stimulated by the administration of such diverse agents as molar NaCl given intramuscularly, 5 per cent yeast nucleic acid given subcutaneously, and by the intravenous injection of Todd-Hewitt broth, 1 per cent solution of trypan blue, and by as little as 1 cc. of India ink.

The adjuvant most commonly employed consists of paraffin oil and a saline solution of antigen, mixed with the aid of emulsifying agent to yield a stable water-in-oil emulsion. It has been used for three purposes in immunological work (4, 5). First, to promote the formation of antibody in high titer, second, to maintain production of the antibody over a long period of time without repeated administration of the antigen, and third, to confer the delayed or tuberculin type of sensitivity to the incorporated antigen when heat-killed tubercle bacilli are included in the emulsion.

The first property of adjuvant, that of increasing antibody titers, is the one of importance for the present study. It was found that the adjuvant used, as

well as certain of its individual components, is highly active in stimulating the production of Cx-reactive protein in rabbits. In view of this finding and the results presented in the preceding paper, further experiments were carried out to determine the effect of the adjuvant on the production of antibody. In these experiments the antigen and the adjuvant were administered separately by different routes; therefore, the results were due to some effect of the adjuvant other than creation of a local depot of antigen.

Materials and Methods

Two types of experiments were carried out in the course of this study. In the first set of experiments the adjuvant and its individual components, aquaphor, heavy grade mineral oil, and heat-killed tubercle bacilli in mineral oil were administered subcutaneously to groups of rabbits. The sera of these rabbits were tested with a sensitive guinea pig antiserum to Cx-reactive protein before the injection of the materials and on each of the 7 days following injection. In the second set of experiments two 1 cc. portions of adjuvant were administered subcutaneously to rabbits on the 1st day of each course of intravenously administered antigen, human C-reactive protein, or human gamma globulin. The control animals received only the intravenous antigen.

Experimental Animals.—Hair brown rabbits weighing between 2500 and 3500 gm. were used in all experiments.

Adjuvant.—Sterile adjuvant incorporating heat-killed Jamaica strain tubercle bacilli and adjuvant identical in composition except for the omission of the mycobacteria was used. This adjuvant, a stable water-in-oil emulsion obtained by the use of autoclaved aquaphor, contained two parts of heavy grade mineral oil and two parts of physiological saline to one part of aquaphor. All the emulsions used were smooth and were so thick in consistency that they could be ejected from the syringe through a 19 gauge needle only with difficulty. The emulsions withstood storage at 4°C. for 4 or 5 days without separation of the phases. When killed, dried tubercle bacilli were used, 0.5 mg. of organisms was incorporated into each 10 cc. of the adjuvant.

Aquaphor.—Equal parts of sterile physiological saline and of melted aquaphor were thoroughly blended with heating. While still soft two 2 cc. portions were injected subcutaneously into abdominal sites on 4 rabbits. Each animal received the amount of aquaphor contained in 10 cc. of adjuvant.

Mineral Oil.—Two 1 cc. portions of the heavy grade mineral oil used in making the adjuvant were injected subcutaneously into two separate abdominal sites of 4 rabbits. Each rabbit received the amount of mineral oil contained in 5 cc. of the adjuvant.

Tubercle Bacilli.—Four rabbits were given 0.2 cc. of a suspension of heat-killed Jamaica strain tubercle bacilli in mineral oil subcutaneously. Each 0.2 cc. of the suspension contained 0.25 mg. of the killed organisms, one-half the weight contained in 10 cc. of complete adjuvant.

Immunization Procedures.—Two 1 cc. portions of adjuvant were administered subcutaneously to rabbits on the 1st day of each course of intravenously administered antigen. Control animals received only the intravenous antigen. In three groups of animals three successive courses of human C-protein were given to provide the antigenic stimulus. In the case of gamma globulin, one group of animals received two courses and another three courses. A course of antigen consisted of the intravenous administration of three or four successive daily injections, each series followed by a 5 day rest period. Control bleedings and bleedings from each animal on each day of the experiment were tested with guinea pig antiserum by the capillary tube precipitin technique for the presence of Cx-reactive protein. Serum from bleedings taken

from each animal on the last day of each rest period was tested for antibody by the capillary precipitin technique.

Only roughly quantitative tests were done for Cx-reactive protein. The column of antigen-antibody precipitate obtained in each instance was read in terms of pluses, each plus representing approximately 1 mm. of precipitate. The method for quantitative determination of C-reactive protein in human serum has not been successfully adapted for use in quantitative determination of Cx-reactive protein in rabbit serum because of the failure of even the purest preparations of Cx carbohydrate to precipitate completely the Cx-reactive protein. Complete precipitation is probably prevented by the presence of inhibitory C carbohydrate in preparations of the Cx carbohydrate (2, 6).

Preparation of C-reactive protein for use as antigen and *preparation of the Cx carbohydrate* were carried out as in the preceding study.

Human Gamma Globulin.—Human gamma globulin, Fraction II, and Fraction II, 1-2 were employed.[1]

RESULTS

Findings with the Complete Adjuvant.—Fifty rabbits given adjuvant without incorporated antigen responded with the production of Cx-reactive protein. Subcutaneous injection of 1 cc. of the adjuvant into either two or three sites gave rise to the production of large amounts of the acute phase protein 24 hours after the injection. The response was of the same order of magnitude and of approximately the same duration regardless of whether or not tubercle bacilli were incorporated in the adjuvant. The level of Cx-reactive protein in the serum of rabbits stimulated with adjuvant alone is as great as that obtained in response to dermal infection with a virulent Type I pneumococcus and persists longer, since the animals usually succumb to the pneumococcal infection within 2 days.

It was noted that the administration of two separate 1 cc. injections of adjuvant within 2 days after the disappearance of the Cx-protein response to a previous injection of the material was again followed by the presence of large amounts of this protein in the serum for from 3 to 5 days.

Results Using Aquaphor, Mineral Oil, and Heat-Killed Tubercle Bacilli.—In an attempt to determine which component of the adjuvant was responsible for the stimulation of Cx-protein production, the ointment base aquaphor, mineral oil, and heat-killed tubercle bacilli in mineral oil were given separately to groups of 4 rabbits. The results obtained with these agents are presented in Table I.

It was found that the aquaphor blended with saline is only slightly less effective than the complete adjuvant in that its stimulation of the Cx-protein response persists from 3 to 4 days rather than from 3 to 6 . The administration of the amount of mineral oil contained in 5 cc. of the adjuvant stimulated the production of Cx-reactive protein for only 2 days and the amount in the serum

[1] The gamma globulin, fraction II, was obtained from the American Red Cross through the kindness of E. R. Squibb & Sons, New Brunswick, New Jersey. The gamma globulin, fraction II, 1-2, was prepared in the laboratory of Dr. E. J. Cohn, Harvard Medical School.

was very much less than that found in the sera of animals given the complete adjuvant. The weight of heat-killed Jamaica strain tubercle bacilli equivalent to that contained in 5 cc. of adjuvant did not, when suspended in 0.2 cc. of mineral oil, stimulate the injected rabbits to produce the Cx-reactive protein.

From these experiments it was evident that the constituent of the adjuvant which was most effective in stimulating rabbits to produce the Cx-reactive protein was the ointment base, aquaphor. This substance, an ointment base which acts as an emulsifying agent, is solid at room temperature. It was noted during the course of the experiments that those animals in which the adjuvant remained as a firm nodule beneath the skin at the injection site were the

TABLE I

Effect of Individual Components of the Adjuvant on the Production of Cx-Reactive Protein by Rabbits

Preparation injected	No. of rabbits injected	No. of rabbits which produced Cx-reactive protein	Maximum level of Cx-reactive protein precipitin titer		No. of days Cx-reactive protein was detectable	
Aquaphor blended with saline	4	4	Rabbit 1	++++++	Rabbit 1	4
			2	++++	2	4
			3	++++	3	3
			4	++++	4	3
Mineral oil	4	3		++		2
Jamaica strain tubercle bacilli 0.25 mg. in 0.2 cc. mineral oil	4	0				

ones which produced the largest amounts of Cx-reactive protein over the longest period of time. Intramuscular injections of the adjuvant were not carried out in the course of these experiments.

Findings with Subcutaneous Adjuvant and Intravenous Antigen, C-Reactive Protein.—In one experiment employing subcutaneous adjuvant and intravenously administered C-reactive protein, each animal of a group of 8 rabbits was given two separate 1 cc. subcutaneous injections of the adjuvant with incorporated heat-killed tubercle bacilli on the 1st day of the first course of intravenously administered antigen. On the 1st day of each of two succeeding courses of antigen two 1 cc. injections of adjuvant without incorporated tubercle bacilli were given to each animal. Each course of antigen consisted of three successive intravenous injections of 0.15 mg. of lipid-free C-reactive protein. All 8 animals responded to each course of adjuvant and antigen with

the production of large amounts of the Cx-reactive protein. As determined in the preceding study, only 1 out of 3 rabbits would have been expected to produce a significant amount of Cx-reactive protein in response to the intravenously administered C-reactive protein alone. All 8 rabbits which received subcutaneous adjuvant and intravenous C-reactive protein developed precipitating antibody to the C-reactive protein. The amount of precipitate obtained with an acute phase human serum known to contain a high titer of C-reactive protein ranged from 3+ to 8+, each + representing approximately 1 mm. of antigen-antibody precipitate. Almost no cross-reactivity with normal human serum was noted. A tabulation of the precipitin titers attained by

TABLE II

The Effect of Subcutaneously Administered Adjuvant on the Production of Antibody to Human C-Reactive Protein Given Intravenously

	Precipitin titers with acute phase human serum							
Rabbit No.	1	2	3	4	5	6	7	8
Rabbits given adjuvant subcutaneously on the 1st day of each of three courses of intravenous C-reactive protein	++++ ++++	++++ ++++	+++ +++	+++ +++	++++	++++	++++	+++
Rabbit No.	9	10	11	12	13	14	15	
Rabbits given three courses of C-reactive protein intravenously. No adjuvant given	++++ ++++	++++	++++	++	+	−	−	

these 8 rabbits is shown in Table II in comparison with the titers attained by 7 rabbits which received three courses of the same amount of intravenously administered C-reactive protein without subcutaneous adjuvant. Only 3 of the 7 control animals produced levels of antibody comparable to those of the animals which received adjuvant in addition to antigen.

In another experiment 3 animals of a group of 4 given subcutaneous adjuvant on the 1st day of each of three courses of three daily successive injections of 0.1 mg. of C-reactive protein developed precipitating antibody levels of 3+, 3+, and 4+ respectively. The 4th animal, the serum of which gave only a + precipitin reaction with acute phase human serum at the end of the experiment failed to produce appreciable amounts of the Cx-reactive protein in response to the adjuvant and antigen.

In a third experiment employing subcutaneous adjuvant and intravenous C-reactive protein one of the 3 adjuvant-treated animals died. The sera of

the surviving 2 gave 3+ and 4+ precipitin reactions respectively with acute phase human serum. These animals received 0.25 mg. of C-reactive protein in each intravenous injection of the antigen. 1 of 3 control animals which received the antigen courses without subcutaneous adjuvant developed a 5+ precipitin titer of antibody to C-reactive protein. However, this animal produced large amounts of Cx-reactive protein in response to the intravenously administered antigen.

TABLE III

Comparative Antibody Responses of Rabbits Given Human γ Globulin Fraction II, 1–2 Intravenously with and without Adjuvant Given Subcutaneously

(Two courses of 4 injections of 10 mg. gamma globulin)

Animals given intravenous gamma globulin alone							Animals given intravenous gamma globulin and subcutaneous adjuvant						
5 days after first antigen injections							5 days after first antigen injections Two 1 cc. subcutaneous injections of adjuvant						
Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*							Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*						
Rabbit No.	10	5	2.5	1.0	0.1	0.01	Rabbit No.	10	5	2.5	1.0	0.1	0.01
1	—	—	—	+	+	±	5	±	+	+±	++	+±	+
2	—	—	—	tr	tr	tr	6	tr	tr	±	±	+	±
3	—	—	—	±	±	tr	7	±	+	+±	+++	+++	++
4	—	—	—	tr	tr	tr	8	tr	±	+	+	++	+
5 days after second antigen injections							5 days after second antigen injections Two 1 cc. subcutaneous injections of adjuvant						
Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*							Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*						
Rabbit No.	10	5	2.5	1.0	0.1	0.01	Rabbit No.	10	5	2.5	1.0	0.1	0.01
1	+±	++	+±	+±	+	tr	5	+++	++++	+++ +++	+++ +++	+++	+
2	+	+	+	+	+	tr	6	++	++	++±	+++	+	±
3	++	++	+++	++	±	±	7	+++	+++++	+++ +++	+++ +++	+++	+
4	+	+	+	+	+	+	8	+++	++++	+++++	+++++	+++	±

Results Employing Subcutaneous Adjuvant and Intravenous Gamma Globulin. —Two experiments employing subcutaneous adjuvant and intravenous human gamma globulin were carried out. In the first experiment, the results of which are presented in Table III, subcutaneous adjuvant was given to four rabbits on the 1st day of each of two courses of antigen.

4 control animals which were given no adjuvant received the intravenous gamma globulin, 10 mg. of fraction II, 1–2 in each injection. It was found that the subcutaneous administration of adjuvant of the beginning of both courses of antigen resulted in enhanced antibody production in comparison with the control animals which received intravenous gamma globulin alone. The serum

of the animal in the adjuvant-treated group, rabbit 6 in Table III, which had the least antibody at the end of the experiment gave approximately the same precipitin reactions as did the control animal, rabbit 3 in Table III, which gave the greatest response. It is apparent from inspection of Table III that the rabbits which received adjuvant and intravenous antigen had as much anti-

TABLE IV

Comparative Antibody Responses of Rabbits Given Human Gamma Globulin Fraction II Intravenously with and without Adjuvant Given Subcutaneously

(Three courses of 4 injections of 2.5 mg. gamma globulin)

Animals given intravenously gamma globulin alone 5 days after first antigen injections Precipitin reactions with dilutions of antigen γ globulin, *mg./cc.*							Animals given intravenously gamma globulin and subcutaneous adjuvant 5 days after first antigen injections Two 1 cc. subcutaneous injections of adjuvant Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*						
Rabbit No.	10	5	2.5	1.0	0.1	0.01	Rabbit No.	10	5	2.5	1.0	0.1	0.01
1	—	—	tr	tr	—	—	4	—	—	±	±	±	—
2	—	—	—	—	—	—	5	±	+	+	+±	±	tr
3	—	—	—	—	—	—	6	—	—	—	—	—	±
5 days after second antigen injections Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*							5 days after second antigen injections Two 1 cc. subcutaneous injections of adjuvant Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*						
Rabbit No.	10	5	2.5	1.0	0.1	0.01	Rabbit No.	10	5	2.5	1.0	0.1	0.01
1	—	—	tr	tr	tr	—	4	++	++++	+++ +++	++++	+	—
2	—	—	—	—	tr	—	5	+	++	++++	+++	+±	±
3	—	—	—	±	tr	—	6	+++	++	++++	++++	+	tr
5 days after third antigen injections Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*							5 days after third antigen injections Two 1 cc. subcutaneous injections of adjuvant Precipitin reactions with dilutions of antigen gamma globulin, *mg./cc.*						
Rabbit No.	10	5	2.5	1.0	0.1	0.01	Rabbit No.	10	5	2.5	1.0	0.1	0.01
1	—	—	—	—	—	—	4	+++++	+++++	+++ +++	++++	+	tr
2	—	—	—	tr	tr	—	5	+++	++++	+++++	++++	+	tr
3	—	—	—	tr	tr	tr	6	+++++	+++++	+++ +++	+++++	+	±

body at the end of their first rest period as did the animals given antigen alone at the end of their second rest period.

In the second experiment using subcutaneous adjuvant and intravenous human gamma globulin three courses of antigen were given. Only 2.5 mg. of human gamma globulin, total fraction II, were given in each injection of antigen. This was ¼ the amount of antigen used in the preceding experiment. The results of this experiment are shown in Table IV.

In this experiment the contrast between the antibody responses of the

adjuvant-treated animals and their controls is even more marked than it was in the preceding experiment in which four times as much antigen was given in each injection.

Daily bleedings on the animals in all experiments employing subcutaneous adjuvant and intravenous antigen were tested for the presence of Cx-reactive protein. All the animals responded with the production of their acute phase protein in good amount for a minimum of 4 days after each injection of adjuvant.

DISCUSSION

In the experiments described in this study, rabbits were stimulated to produce Cx-reactive protein in response to injections of a complete adjuvant as well as to its individual components, aquaphor and mineral oil. The consistency with which the animals produced Cx-reactive protein in response to the adjuvant was far greater than in the instance of the response to only moderately good antigens such as human C-reactive protein and human gamma globulin which, as shown in the preceding paper, stimulated good Cx-reactive protein responses only in those animals which produced good titers of precipitating antibody. Adjuvant is composed of non-antigenic substances and, when injected into the animal, incites the foreign body type of inflammatory response (4). Aside from the established fact that adjuvant serves as a depot in which incorporated antigen is present for an extended period of time, little is known about its mechanism of action in enhancing antibody production. Whether or not the depot for antigen provided by adjuvant is its important contribution to enhancing the titer of antibody to incorporated antigens is not known. There is, however, little doubt that the antigen depot provided by adjuvant is important in maintaining antibody titers over extended periods of time.

Aside from demonstrating that rabbits respond to the injection of adjuvant with the production of acute phase Cx-reactive protein, the experiments undertaken in this study showed that enhanced antibody titers could be obtained by giving adjuvant subcutaneously and antigen intravenously. This fact indicated that adjuvant of itself, independent of the presence of antigen in the saline phase, stimulates the mechanism of the production of antibody to the intravenously administered antigens, human C-reactive protein and human gamma globulin. The nature of this stimulatory action of adjuvant is unknown, but every rabbit in which adjuvant was effective in stimulating production of antibody also developed large amounts of the Cx-reactive protein. In these rabbits, as in those described in the preceding paper, a correlation was found between the production by the rabbits of Cx-reactive protein and the subsequent development of high titers of antibody to the two antigens, human C-reactive protein and human gamma globulin.

The production of the Cx-reactive protein by rabbits is a non-specific response in that it can be induced by a variety of agents, both antigenic and non-antigenic. All these agents, however, have one property in common, the ability to cause inflammation. From the large amount of available data bearing on the human C-reactive protein and the rabbit Cx-reactive protein it is evident that these substances are sensitive indicators of inflammation. Whether they form a direct link in the sequence of biochemical events leading to the production of antibody, or whether they are non-specific indicators of an inflammatory response which may independently result in the production of antibody, is not yet known.

SUMMARY

The ability of an adjuvant and its individual constituents to induce the production of Cx-reactive protein in rabbits has been studied. It was found that the adjuvant stimulated rabbits to produce large amounts of the acute phase protein for 3 to 6 days. Melted aquaphor blended with saline stimulated the production of Cx-reactive protein for 3 or 4 days. Mineral oil was less effective in stimulating the production of the protein than either adjuvant or aquaphor. Heat-killed Jamaica strain tubercle bacilli suspended in mineral oil did not induce the Cx-protein response.

The ability of subcutaneously administered adjuvant without antigen incorporated in the saline phase to potentiate the antibody response of rabbits to the intravenously administered antigens, C-reactive protein and human gamma globulin, was investigated. It was found that the adjuvant-treated animals produced more precipitating antibody to the two intravenously administered antigens than did the control animals given intravenous antigen alone.

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