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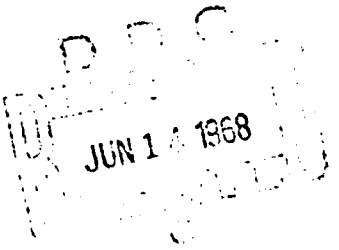

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PRELIMINARY TRIALS TO ATTEMPT TO DETERMINE THE MODE OF ACTION OF ADJUVANT AND IMMUNITY-STIMULATING SUBSTANCES

PRELIMINARY TRIALS TO ATTEMPT TO DETERMINE THE MODE OF ACTION OF ADJUVANT AND IMMUNITY-STIMULATING SUBSTANCES

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It is well known that certain substances injected into human beings or animals, either simultaneously or in a mixture with an antigen, can act on the development of the immunity produced by this antigen.

In 1925, G. Ramon (1) demonstrated the principle of adjuvant and immunity-stimulating substances, showing that an increase of immunity can be induced by adding to the antigen, prior to its injection, nonspecific substances such as tapioca. Because of it, augmentation of the rate of antibodies is produced by means of the local inflammation developed by the adjuvant substance at the point of injection of the antigen.

Other substances, such as the antibiotics (penicillin, streptomycin, etc.) have no influence on the appearance and development of the specific antitoxin produced by diphtheria, tetanus, and staphylococcus toxoids (2).

On the other hand, we know that injection of the tetanus toxoid-antitoxin mixture with a great preponderance of antitoxin does not confer immunity (3) and that human gamma globulin of placental origin injected in mixture with staphylococcus toxoid interferes with the production of the specific antitoxin (4); these latter two examples constitute very particular cases.

Finally, various authors have asked themselves what

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influence a hormone such as cortisone, whose action is above all anti-inflammatory, could exert in the production of antibodies.

In the case of animals, cortisone would be capable of inhibiting the formation of antibodies [Germuth and Ottinger (5)]; in the case of rabbits previously immunized, it would cause a fall in the proportion of antibodies [Fischel and colleagues (6)]. In the case of man, it would not entail notable changes in the production of diphtheria antitoxin [Hevens and Schaffer (7)] and in that of pneumococcus antibodies, [Miwick (8)] where it would have rather a tendency to favor the development.

Faced with these results, which are truly quite inconsistent, it has seemed interesting to us to study the influence of cortisone (9) on the production of staphylococcus antitoxin, produced in the rabbit, by injections of specific toxoid. We have established in such a case that cortisone inhibits partly the development of staphylococcus beta antitoxin and especially that of alpha antitoxin, which could be caused - - we suggest - - by the anti-inflammatory action of this hormone.

These results have led us to study the repercussions that another type of anti-inflammatory agent: proteolytic enzymes, could have on the development of immunity when introduced into the organism of a subject in the course of immunization.

We have chosen alpha-chymotrypsin as proteolytic enzyme and staphylococcus toxoid as antigen, because of the ease of experimentation and the simplicity with which we can evaluate the proportion of specific antitoxin in the serum of the immunized subjects.

In a first series of experiments, we have used staphylococcus toxoid alone, and in a second and third, staphylococcus toxoid alone or mixed with an adjuvant and immunity-stimulating substance.

FIRST SERIES OF EXPERIMENTS

Twenty-four rabbits, whose serum contained no trace of staphylococcic antitoxin of natural origin, were divided into four series of six animals each (10).

The animals of series I, II, and III received, at four day intervals, three subcutaneous injections respectively of 1, 2, and 4 milliliters of a toxoid titrating 8 antigen units

to the milliliter.

In addition, in the course of immunization, the animals of series II and III each received 12 intraperitoneal injections of alpha-chymotrypsin: 1 milligram per kilogram for series II, 5 milligrams per kilogram for series III.

The animals of series IV received three subcutaneous injections of 1, 2, and 4 milliliters of staphylococcus toxoid supplemented extemporaneously with an amount of alpha-chymotrypsin so that, at the time of each injection of toxoid, the animals received 25 milligrams per kilogram of alpha-chymotrypsin.

The animals were bled seven days after the last injection of toxoid and the staphylococcus antitoxin titrated, by the hemolytic method, in the mixtures of the serums of each series and in each serum separately.

The results of these titrations are recorded in table I.

TABLE I

SERIES	NUMBER OF THE RABBITS	ANTITOXIN TITER (in units)
Series I Toxoid alone	7	+0.2 - 0.5
	13	+3 - 5
	29	+2 - 3
	31	+7 -10
	37	+10 -15
	mixed serums	+5 -7
Series II Subcutaneous toxoid; intraperitoneal alpha-chymotrypsin (1 mg/kg).	17	+ 2 - 3
	45	+ 7 - 10
	69	+10 - 15
	27	+ 7 - 10
	41	+ 7 - 10
	57	+15 - 20
	mixed serums	+ 7 - 10
Series III Subcutaneous toxoid; intraperitoneal alpha-chymotrypsin (5 mg/kg).	47	+ 1 - 2
	61	+15 - 20
	59	+ 1 - 2
	mixed serums	+7
Series IV Toxoid + alpha-chymotrypsin (mixed).	75	+ 5 - 7
	23	+ 2 - 3
	43	+ 0.2 - 0.5
	34	+ 3 - 5
	mixed serums	+3

Contrary to cortisone, although alpha-chymotrypsin also possesses anti-inflammatory properties, when administered intraperitoneally, 12 times to the animal in the course of immunization in the amount of 1 or of 5 milligrams per kilogram, it in no way damages the development of antistaphylococcic immunity, the titers of specific antitoxin obtained being practically the same.

On the other hand, immunity is, in this experiment, a little weaker in the case of animals which received subcutaneously the mixture of toxoid and alpha-chymotrypsin. This can be very simply due to the often considerable differences of reactivity of the experimental animals.

These results have been moreover confirmed in another experiment based on this one and of which we will not give details here.

SECOND SERIES OF EXPERIMENTS

Twenty-five rabbits, not possessing any natural antistaphylococcic immunity were divided into five series of five animals each.

They were immunized according to the record below, receiving each, at four day intervals, three injections of staphylococcus toxoid No. 4325, titrating 12 antigen units to the milliliter.

SERIES I

1st injection: 2 ml of a mixture of 6 ml of toxoid + 6 ml of water for injection.
2nd injection: 3 ml of a mixture of 12 ml of toxoid + 6 ml of water for injection.
3rd injection: 4 ml of a mixture of 18 ml of toxoid + 6 ml of water for injection.

SERIES II

1st injection: 2 ml of a mixture of 6 ml of toxoid + 0.2 ml of 3 percent saponin (11) solution + 5.8 ml of water for injection.
2nd injection: 3 ml of a mixture of 12 ml of toxoid + 0.4 ml of 3 percent saponin solution + 5.6 ml of water for injection.
3rd injection: 4 ml of a mixture of 18 ml of toxoid + 0.8 ml of 3 percent saponin solution + 5.2 ml of water for injection.

SERIES III

1st injection: 2 ml of a mixture of 6 ml of toxoid + 0.2 ml of a 3 percent saponin solution + 50 mg of alpha-chymotrypsin (12) dissolved in a volume of 5.8 ml.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 0.4 ml of a 3 percent saponin solution + 50 mg of alpha-chymotrypsin dissolved in a volume of 5.6 ml.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 0.8 ml of a 3 percent saponin solution + 50 mg of alpha-chymotrypsin dissolved in a volume of 5.2 ml.

SERIES IV

1st injection: 2 ml of a mixture of 6 ml of toxoid + 0.2 ml of a 3 percent saponin solution, heated before addition to the toxoid for five hours at 37° with 50 mg of alpha-chymotrypsin in a volume of 0.8 ml, this latter being neutralized with 1 million units of iniprol in a volume of 5 ml.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 0.4 ml of a 3 percent saponin solution, heated before addition to the toxoid for five hours at 37° with 50 mg of alpha-chymotrypsin in a volume of 0.6 ml, this latter being neutralized with 1 million units of iniprol in a volume of 5 ml.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 0.8 ml of a 3 percent saponin solution, heated before addition to the toxoid for five hours at 37° with 50 mg of alpha-chymotrypsin in a volume of 0.2 ml, this latter being neutralized with 1 million units of iniprol in a volume of 5 ml.

SERIES V

1st injection: 2 ml of a mixture of 6 ml of toxoid + 50 mg of alpha-chymotrypsin, in a volume of 1 ml, neutralized after being heated for five hours at 37° with 1 million units of iniprol in a volume of 5 ml.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 50 mg of alpha-chymotrypsin, in a volume of 1 ml, neutralized after being heated for five hours at 37° with 1 million units of iniprol in a volume of 5 ml.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 50 mg of alpha-chymotrypsin, in a volume of 1 ml, neutralized after being heated for five hours at 37° with 1 million units of iniprol in a volume of 5 ml.

The following reactions were recorded.
Series I : no reaction
Series II : marked reactions
Series III: reactions still more marked than in Series II
Series IV : reactions identical with those of Series II

Series V : no reaction

All the animals were bled seven days after the last injection of antigen and the staphylococcic antitoxin titrated, by the hemolytic method, in the mixtures of the serums of each series and in each series separately.

The recorded results appear in table II.

TABLE II

SERIES	NUMBER OF THE RABBITS	ANTITOXIN TITER (in units)
Series I Toxoid alone	1	+ 7 - 10
	3	+ 0.5 - 1
	35	+ 1 - 3
	49	+ 7 - 10
	73	+ 3 - 5
	mixed serums	+ 3 - 5
Series II Toxoid + saponin . . .	39	+ 7 - 10
	13	+ 10 - 15
	87	+ 7 - 10
	53	+ 1 - 3
	17	15
	mixed serums	+ 7 - 10
Series III Toxoid + saponin + alpha-chymotrypsin (in mixture)	77	+ 3 - 5
	21	+ 7 - 10
	51	+ 20 - 30
	69	+ 5 - 7
	27	+ 20 - 30
	mixed serums	+ 15 - 20
SERIES IV Toxoid + saponin treated "in vitro" with alpha-chymotrypsin and neutralized then with iniprol	79	+ 1 - 3
	65	+ 3 - 5
	71	+ 5 - 7
	67	7
	mixed serums	5
SERIES V Toxoid + alpha-chymotrypsin neutralized with iniprol (in mixture)	43	+ 7 - 10
	5	+ 7 - 10
	23	+ 3 - 5
	33	+ 3 - 5
	47	+ 1 - 3
	mixed serums	+ 5 - 7

Examination of this table permits certain statements:

- - As it usually did, saponin performed well here in its role of adjuvant and immunity-stimulating substance (Series II).

- - The addition of alpha-chymotrypsin to the saponin further increased the role of adjuvant and immunity-stimulating substance performed by saponin (Series III). It is to be

noted in this series, where the animals possess the highest titers of antitoxin, that the local reactions at the point of injection are the most marked.

- - Addition to the toxoid either of saponin treated in vitro with alpha-chymotrypsin, then neutralized afterwards with iniprol (Series IV), or of alpha-chymotrypsin neutralized with iniprol (Series V) does not appreciably increase the rate of immunity.

***

These results have led us to carry out a third series of experiments, in order to verify if it is accurate :

1. that alpha-chymotrypsin added to the specific antigen has only a weak action on the development of immunity (1st series of experiments, Series No. IV; 2nd series of experiments, Series No. IV and V, with several variants, since in this case the alpha-chymotrypsin had been neutralized with iniprol);

2. that, on the contrary, alpha-chymotrypsin mixed extemporaneously with saponin clearly increases the adjuvant and stimulating power of this latter (2nd series of experiments, Series III).

THIRD SERIES OF EXPERIMENTS

Twenty-five rabbits not possessing any natural antistaphylococcic immunity, were - - as in the preceding experiment - - divided into five series of five animals each.

They each received at four day intervals, three injections of staphylococcus toxoid No. 4340, titrating 8 antigen unitsto the milliliter.

Series I

1st injection: 2 ml of a mixture of 6 ml of toxoid + 6 ml of water for injection.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 6 ml of water for injection.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 6 ml of water for injection.

SERIES II

1st injection: 2 ml of a mixture of 6 ml of toxoid + 50 mg of alpha-chymotrypsin dissolved in a volume of 6 ml.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 100 mg of alpha-chymotrypsin dissolved in a volume of 6 ml.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 100 mg of alpha-chymotrypsin dissolved in a volume of 6 ml.

SERIES III

1st injection: 2 ml of a mixture of 6 ml of toxoid + 0.2 ml of 3 percent saponin solution + 5.8 ml of water for injection.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 0.4 ml of 3 percent saponin solution + 5.6 ml of water for injection.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 0.8 ml of 3 percent saponin solution + 5.2 ml of water for injection.

SERIES IV

1st injection: 2 ml of a mixture of 6 ml of toxoid + 0.2 ml of 3 percent saponin solution + 50 mg of alpha-chymotrypsin dissolved in a volume of 5.8 ml.

2nd injection: 3 ml of a mixture of 12 ml of toxoid + 0.4 ml of 3 percent saponin solution + 100 mg of alpha-chymotrypsin dissolved in a volume of 5.6 ml.

3rd injection: 4 ml of a mixture of 18 ml of toxoid + 0.8 ml of 3 percent saponin solution dissolved in a volume of 5.2 ml.

SERIES V

The animals of this series were immunized exactly like those of Series III. In addition, each received, in the course of immunization, intraperitoneally, 12 injections each of 2.5 mg of alpha-chymotrypsin.

The reactions recorded, at the point of injection of the antigen, were the following:

Series I: no reaction.
Series II: marked reactions.
Series III: marked reactions.
Series IV: the most marked reactions.
Series V: reactions clearly less marked than in Series II, III, and IV.

On the third day after the injections, there was an important decrease of the reactions for the 2nd, 3rd, and 4th series. There was practically no more reaction on this day for the 5th series. In Series II we observe several scabs.

All the animals were bled seven days after the last injection of antigen, and the staphylococcic antitoxin titrated,

by the hemolytic method, in the mixtures of serums of each series and in each serum separately.

Table III summarizes the results obtained.

TABLE III

SERIES	NUMBER OF THE RABBITS	ANTITOXIN TITER (in units)
Series I	11	+ 10 - 15
	17	+ 5 - 7
	39	+ 0.5- 1
Toxoid alone	59	+ 3 - 5
	21	+ 15 - 20
	mixed serums	+ 7 - 10
Series II	41	+ 0.2- 0.5
	55	+ 0.5- 1
	23	+ 0.2- 0.5
Toxoid + alpha-chymotrypsin	31	+ 1 - 2
(in mixture)	5	+ 3 - 5
	mixed serums	+ 2 - 3
Series III	53	+ 15 - 20
	19	+ 15 - 20
	47	+ 10 - 15
Toxoid + saponin	37	+ 7 - 10
	75	+ 20 - 30
	mixed serums	+ 15 - 20
Series IV	65	+ 3 - 5
Toxoid + saponin + alpha-	57	+ 7 - 10
chymotrypsin (in mixture)	25	+ 10 - 15
	7	+ 7 - 10
	mixed serums	+ 7 - 10
Series V	77	+ 7 - 10
Toxoid + saponin + intra-	2	+ 20 - 30
peritoneal alpha-chymo-	45	+ 15 - 20
trypsin	25	+ 15 - 20
	73	+ 10 - 15
	mixed serums	+ 15 - 20

We observe on examination of this table:

- - that alpha-chymotrypsin injected in mixture with anastaphylotoxin entails a lessening of specific immunity, whether this toxoid had been mixed with saponin (Series IV) or not (Series II);

- - that intraperitoneal injection of alpha-chymotrypsin does not damage development of immunity (Series V).

RESUME AND CONCLUSIONS

- - Alpha-chymotrypsin, administered intraperitoneally to experimental animals, does not damage development of anti-staphylococcic immunity (Experiment No. I, Series II and III) even when the toxoid is mixed with saponin (Experiment No. 3, Series V), although in this case it entails a very definite lessening of the local reaction at the point of injection of the antigen.

- - When we inject into experimental animals, the staphylococcus toxoid + alpha-chymotrypsin mixture, the immunity obtained is a little weaker (in Experiment No. 1, Series IV) and decidedly weaker (in Experiment No. 3, Series II) than in the control experiment, nevertheless, in the latter series of experiments, the reactions are definitely more marked than in the control experiments.

- - Injection of the toxoid + saponin mixture (Experiment No. 2, Series II; Experiment No. 3, Series III) gives, since saponin acts as an excellent adjuvant and immunity-stimulating substance, a better immunity than toxoid alone.

This immunity is in one case increased (Experiment No.2, Series III) and in the other case decreased (Experiment No. 3, Series IV) when we add alpha-chymotrypsin to the toxoid mixed with saponin, the mixtures thus obtained causing the most marked local reactions. On the other hand, the immunity obtained with toxoid mixed with saponin previously treated in vitro with alpha-chymotrypsin and subsequently neutralized with iniprol, (Experiment No. 2, Series IV), although the local reactions stated are as marked as in Series II (toxoid + saponin), is practically the same as that produced by the toxoid alone.

Likewise, the toxoid mixed with alpha-chymotrypsin neutralized with iniprol (Experiment No. 2, Series V), an antigen which entails no local reaction, gives a rate of immunity practically equal to that established in the control experiments.

From this experimentation, whose purpose it was to find precisely the mode of action of adjuvant and immunity-stimulating substances, and in particular the influence of the local reaction at the point of injection of the antigen by using either generally or locally an anti-inflammatory, alpha-chymotrypsin, it appears that the proportion of antitoxin obtained does not always seem relative to the local reaction involved at the point of injection of the antigen. We have moreover made the same statement in the course of previous research on saponin (13).

These tests, which employed staphylococcus toxoid as antigen, saponin as adjuvant immunity substance, and alpha-chymotrypsin as anti-inflammatory, merit being pursued using other antigens, for example: diphtheria toxoid and tetanus toxoid, other adjuvant immunity substances, and other anti-inflammatories administered not only generally or mixed with the antigen, but also in repeated local applications at the point of injection of the antigen and under varying conditions.

(Work of the National Institute of Health and of Medical Research).

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(10) See our preliminary communication: R. Richou and R. Thevenot, Comptes Rendus de l'Academie des Sciences, Vol. 255, 1962, page 2849.

(11) The saponin No. 1 used has acted, in some previous experiments as an excellent adjuvant and immunity-stimulating substance. R. Richou, R. Jensen and Cl. Belin, Revue d'Immunologie, Vol. 28, 1964, page 49.

(12) Dosage at which alpha-chymotrypsin does not entail mortality in the experimental animals. See in this connection, our preliminary note. Loc. cit.

(13) R. Richou, R. Jensen and Cl. Belin, Revue d'Immunologie, Vol. 28, 1964, page 53.