STUDIES ON THE RELATIONSHIP BETWEEN TOXIN-ANAPHYLAXIS AND ANTITOXIC IMMUNITY

I. QUANTITATIVE STUDIES ON THE TOXIN-ANAPHYLAXIS IN VITRO

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INTRODUCTION

Since the discovery of anaphylaxis by Richét in 1902, the relationship between allergy and immunity has been studied extensively by many investigators. The opinions of those investigators, however, are quite divergent. Such confusion seems to come from the idea that immunity is always established by antibody alone. But, it is not yet known whether or not the anti-infectious immunity is always established by the formation of antibody alone. Generally speaking, moreover, immunity and allergy are not always developed by the antigen antibody reaction of the same nature. Thus, in order to clarify the fundamental relationship between immunity and allergy, studies should be carried out on those cases in which both of them are caused by the same antigen antibody reaction.

As for antitoxic immunity, it has hitherto been believed that the immune-state is due to the presence of sufficient antitoxin in the circulating blood. In this connection, however, the antitoxin in the tissues is reported to play an important role in the antitoxic immunity against diphtherial toxin. While, the anaphylaxis to diphtherial toxin was observed by Lawrence & Pappenheimer (1948) and experimentally demonstrated in vitro by the present authors (1953). Accordingly, both the antitoxic immunity and the toxin anaphylaxis of diphtheria are caused by the same antigen antibody reaction. Thus, the studies on the relationship between the antitoxic immunity and the toxin-anaphylaxis of diphtheria would throw light on the fundamental relation between immunity and allergy.

For this purpose, quantitative studies on toxin-anaphylaxis are indispensable. Kabat (1942) tried to clarify the relation of the amount of antigen and antibody to the degree of anaphylaxis, however, the anaphylaxis in vivo is too complicated for the clarification of their relation.

In this paper attempts were made to determine quantitatively the intensity of toxin-anaphylaxis in vitro.

MATERIALS AND METHODS

Antigen used was purified diphtherial toxoid (purity: 0.00057 mg P-N/Lf). For the the passive sensitization of guinea pigs, pooled antitoxic serum (65
units per ml) obtained from hyperimmunized guinea pigs was used. Namely, guinea pigs were sensitized by the intravenous injection of the antitoxic serum.

For the determination of the antitoxic titer of each serum, Jensen's method was used. One M. R. D. of toxin used in this test was 0.00000038 ml and its 20LR/200 was 0.008 ml. The test was carried out taking the toxin level as LR/10. On the other hand, K1 of antitoxin was determined by Jerne's method. Namely, the following equation was applied:

\[ C_A = m s = 2 \left( \sqrt[2]{\frac{C_T}{T}} - 1 \right) \left( \sqrt[2]{\frac{C_T[T]}{T}} + \frac{1}{K_1} \right) \]  

\[ m: concentration of antitoxin in the serum, C_A: concentration of antitoxin in the toxin-antitoxin mixture, C_T: concentration of toxin in the mixture, T: concentration of free toxin necessary to cause the reaction, K_1: coefficient representing avidity of antitoxin \]

In other words, test antitoxic serum was put into twofold dilutions with buffer solution and each dilution was mixed with an equal amount of high level of toxin (C_T=100LR/1). Then, after 2 hours at 37°C, each mixture was diluted 100 times with buffer and 0.1 ml of the dilution was injected intracutaneously into a normal rabbit. The readings of the reaction responded permit an estimate of m as follows:

\[ m \cong 2 \left( \frac{C_T - 1}{s''} \right) \sqrt[2]{\frac{C_T[T]}{T}} \]

\[ s'': concentration of test serum in the final mixture \]

On the other hand, the diluted serum was mixed with low level of toxin (C_T=1LR/1) at 0°C, and immediately, 0.1 ml of the mixture was injected. An estimate of mK_1 may be obtained by the following equation:

\[ m K_1 \cong 2 \frac{\left( \sqrt[2]{\frac{C_T}{T}} - 1 \right)}{s'''} \]

\[ s''': concentration of the serum in the mixture \]

Then, K_1 was calculated from the results of these two calculations.

It seems that one of the difficulties in quantitative studies on anaphylaxis is to exclude the individual difference of experimental animals. In order to eliminate this difficulty, anaphylaxis in vitro with the aid of Schultz-Dale's apparatus was applied. Namely, the intestine of passively sensitized guinea pig was taken out 24 hours after sensitization and its anaphylactic contraction against various amounts of purified toxoid dropped into the bath was observed. On the other hand, the concentration of acetylcholine required to cause equivalent contraction in the tyrode bath was determined. As shown in Fig. 1, the specific contractions of intestinal strips of the same animal caused by 2.5 Lf, 5 Lf, and 10 Lf of purified toxoid were equivalent to the contractions caused by acetylcholine...
in the concentration of $0.4 \times 10^{-8}$, $0.8 \times 10^{-8}$, $1.4 \times 10^{-8}$ g/ml in the bath respectively. With this method, the intensity of anaphylactic reaction can be designated by the concentration of acetylcholine, and the individual difference in sensitivity to the effective substance which develops anaphylaxis can mostly be excluded. By the way, Nakamura et al\(^7\) reported that the effective substance in anaphylaxis is acetylcholine-like substance.

Moreover, by the application of this method, the Lf dose of antigen required to cause a certain specific reaction (for example, specific contraction corresponding to the one caused by acetylcholine of $10^{-8}$ g/ml) can be determined in each sensitized animal.

Thus, in this paper, such method was applied to investigate the relation of the intensity of anaphylaxis to the dose of antigen and antibody concerned.

**Experimental Results**

1. **Relation between the degree of specific contraction and the amount of antigen used**

As a basic experiment, the contraction of intestine sensitized against purified toxoid was proved in vitro to be the anaphylaxis developed by genuine toxoid. Namely, a guinea pigs was sensitized with antitoxic serum and 24 hours later, the intestine of the sensitized guinea pigs was taken out. Then, the doses of crude toxin and purified toxoid required to cause the same degree of specific contraction of the intestine were determined. As the result of this, the specific contractions of the intestine caused by the same Lf dose of two antigens, the crude toxin and purified toxoid, were equivalent to the control contraction of the
intestine caused by acetylcholine in the same concentration. Therefore, the anaphylactic reaction observed was regulated only by Lf of the antigen and it has nothing to do with the protein content of the antigen. This finding is considered to give a basis to the opinion that these contractions of intestine are the anaphylactic reaction caused by the genuine diphtherial toxin (Ishizaka & Ishizaka, 1953).

Various amounts of the antitoxic serum were injected intravenously into normal guinea pigs and 24 hours later, their intestines were taken out and used for the experiment. Namely, various doses of purified toxoid were added to the bath, in which a strip of an intestine was suspended and the degree of specific contraction appeared was determined by acetylcholine as described above. As shown in Table 1, in the guinea pig sensitized with 134 units of antitoxin, the specific contraction of its intestine caused by 15 Lf to 1.5 Lf of toxoid was comparable to the one caused by acetylcholine in the concentration: of $2.6 \times 10^{-8}$ g/ml to $0.8 \times 10^{-8}$ g/ml.

Table 1. Degree of the anaphylactic reactions caused by various amounts of toxoid

<table>
<thead>
<tr>
<th>Body-weight</th>
<th>Dosis of antitoxin</th>
<th>Circulating antitoxin titer after sensitization</th>
<th>Dosis of toxoid</th>
<th>Acetylcholine concentration ($10^{-8}$ g/ml)</th>
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<tbody>
<tr>
<td>g</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>Lf</td>
</tr>
<tr>
<td>430</td>
<td>134</td>
<td>6</td>
<td>2.5</td>
<td>15</td>
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<td>10</td>
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<td>5</td>
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<td>2.5</td>
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<td></td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>245</td>
<td>6.7</td>
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<td>60</td>
<td>0.4</td>
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<td></td>
<td></td>
<td></td>
<td>40</td>
<td>0.2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* Concentration of acetylcholine in the bath required to cause a comparable contraction to that by toxoid.

When a guinea pig was sensitized with such a small amount of antitoxic serum as 6.7 units, the specific contraction of the intestine was observed by adding 60 Lf of toxoid, while no contraction was observed by 20 Lf. In either case of the sensitization made with a large amount of antitoxin or that with a small amount, the more the amount of antigen used resulted in the production of so much stronger reaction. Namely, inhibition of the anaphylactic reaction due to antigen excess was never observed in this experiment.

2. Relation between the dosis of antitoxin used and the degree of sensitization

Guinea pigs were sensitized with varying doses of antitoxin injected intravenously and 24 hours or 48 hours later, intestines of these guinea pigs were taken out and used for the experiment. Prior to this, the animals were bled,
immediately after sensitization and again immediately before removing intestine for the determination of their respective antitoxic titers. Then, the dose of antigen (Lf) required for developing the specific contraction comparable in intensity to the one developed by acetylcholine in the concentration of 10^{-8} g/ml, was determined for the intestine of each animal. And the relation between the reciprocal of the Lf dose used and the dose of antitoxin used for the sensitization was investigated. The findings are shown in Fig 2. Namely, when the reciprocal of Lf used and the unit of antitoxin were transferred into log, a certain correlation was found between them (P<0.01). The correlation constant calculated was 0.907.

It is presumable, therefore, for the development of a contraction of certain intensity, the more the unit of antitoxin used in the sensitization, the amount of antigen required becomes the less. Accordingly, the reciprocal of Lf required to cause specific reaction comparable in intensity to that caused by acetylcholine in the concentration of 10^{-8} g/ml was used to represent the degree of sensitization.
In the next place, investigation was made on the relation between the degree of sensitization of the intestine and the units of circulating antitoxin in the blood taken immediately before removal of the intestine. As the result of this investigation, a certain correlation was found to exist between them (Fig. 3). The correlation constant was 0.906 ($P<0.01$).

Fig. 3
Relationship between the degree of sensitization and the circulating antitoxin titer.

\[
\log y - \log 0.257 = 1.17 (\log x - \log 0.871) \\
\rho = 0.906 \quad N = 17 \quad P < 0.01
\]

Namely, in the guinea-pigs which had been sensitized with the same antitoxic serum, the higher the circulating antitoxic titer, the less the amount of antigen required for the development of a contraction of certain intensity of the intestine.

On the other hand, it is believed that the anaphylactic reaction is caused by antigen-antibody reaction in the tissues. Accordingly, the tissue antibody related to the anaphylactic reaction should also be determined quantitatively. Besides, it is assumed that the decrease of circulating antitoxin during such a short period of 24 hours is due to the deposition of antitoxin in the tissues, the unit of tissue antitoxin was calculated from the difference in the unit of circulating
antitoxin between immediately after sensitization and immediately before removal of the intestine.

Next, the relationship between the tissue antitoxin and the degree of the sensitization was examined. As illustrated in Fig. 4, a certain correlation was found between them ($P<0.01$, $r=0.734$). In other words, the degree of sensitization seems to be dependent on the unit of antitoxin in the tissues. From these findings, it may safely be said that the inhibition of the reaction due to excess antigen or excess antibody, which is observed in the other serological reactions, does not occur in anaphylactic reaction.

**Fig. 4**
Relationship between the degree of sensitization and the unit of antitoxin in the tissues.

\[
\log y - \log 0.28 = 0.926 (\log x - \log 5.28)
\]
\[
r=0.734, \ N=13, \ P<0.01
\]

3. **Effect of precipitating and non-precipitating antitoxin in passive sensitization**

In the next place, an examination was done on the problem whether or not the effectiveness of antitoxin in sensitizing animals is dependent on the properties of antitoxin used. Two kinds of homologous antitoxic sera ($A_1$ and $A_2$) with different avidity were used for sensitization in order to examine the relation between sensitizing dose of antitoxin and the degree of sensitization resulted.
Intestines of sensitized guinea pigs were removed 24 hours after sensitization and the degree of sensitization was determined for each animal. In Fig 5, P₁ represents the relation between the unit of antitoxin used and the degree of sensitization in the guinea pigs sensitized with the antitoxic serum A₁ (65 units/ml, K₁=0.36). On the other hand, P₂ represents the same relation in the guinea pigs sensitized with the antitoxic serum A₂ (65 units/ml, K₂=0.51). The antitoxin titer of these sera was the same as determined by Jensen's method the concentration of antitoxin (CA) contained in these sera, however, seems to be different. As the result of calculation made by the equation (1), it was demonstrated that the ratio of CA between the two sera was as follows:

\[
\frac{A₁}{A₂} = \frac{2.14}{1.74}
\]
In other words, the absolute amount of antitoxin contained in 10 units of $A_2$ equals to that contained in 8.36 units of $A_1$. Thus, the relations between the absolute amount of antitoxin and the degree of sensitization in each of $A_1$ and $A_2$ are illustrated as $P_1$ and $P_2'$ respectively. Therefore, it may safely be said that this relation is scarcely influenced by such a small difference in $K_1$ as described above, but that the degree of sensitization is dependent on the absolute amount of antitoxin used for sensitization. So far as the experiments carried out within 24 hours after sensitization are concerned, the relation between the absolute amount of circulating antitoxin and the degree of sensitization is scarcely influenced by such a small difference in $K_1$ as shown in Fig 6, in which $P_1$ and $P_2$ represent the relation between the circulating antitoxin unit and the degree of sensitization in guinea pigs sensitized with $A_1$ and $A_2$ respectively. $P_1$ and $P_2'$ represent the relation between the absolute amount of antitoxin in the serum and the degree of sensitization in each case.

**Fig. 6**

Sensitizing capacity of antitoxins with different avidity (2)

<table>
<thead>
<tr>
<th>Unit of circulating antitoxin per ml</th>
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</thead>
<tbody>
<tr>
<td>$P_1$: $y - \log 0.257 = 1.17$ (log $x - \log 0.871$)</td>
</tr>
<tr>
<td>$N = 17$, $K_1 = 0.358$, $r = 0.906$</td>
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<tr>
<td>$P_2$: $y - \log 0.156 = 1.575$ (log $x - \log 0.68$)</td>
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<tr>
<td>$N = 15$, $K_1 = 0.506$, $r = 0.95$</td>
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Further, so-called non-precipitating antitoxin was obtained after serial absorption of the antitoxic serum with purified toxoid. Namely, 4.5 Lf of purified toxoid was added to per ml of antitoxic serum (A₂). After precipitation, the precipitate was removed. Again, the same amount of toxoid was added. After repeating the same procedure for 3 to 4 times, antitoxic sera A₃' (33 units/ml) and A₃'' (12.5 units/ml) were obtained. Serological properties of the two sera are illustrated in Table 2. Namely, A₃'' seems to contain only non-precipitating antitoxin. By the way, the K₁ of A₁' and A₂'' are similar to the K₁ of A₂. Thus, guinea pigs were sensitized with these antitoxic sera and 24 hours later, their intestines were removed. Purified toxoid was added to the bath in which a strip of the intestine was suspended, however, the specific contraction of the strip was not observed (Table 2). Then, the intestines of guinea pigs 48 to 72 hours after sensitization were used, but the specific contraction was not observed inspite of the decrease in the circulating antitoxin. In short, the non-precipitating antitoxin contained in the homologous antitoxic serum was incapable of sensitizing the intestine of guinea pigs.

DISCUSSION

Quantitative studies on anaphylaxis have been carried out by many investigators. For example, Goodner & Horsfall (1937) studied on the passive sensitivity to pneumococcal capsular polysaccharide and reported that the fatal anaphylactic sensitivity to this antigen can be caused by such a proportion of antigen to antibody as the amount of carbohydrate is slightly in excess of the amount necessary to satisfy the available antibody and if the amount of antigen is excessively large, the result will be negative. On the other hand, Kabat (1944) reported that the best anaphylactic response (to ovalbumin) occurs in the region of large antigen excess and that the more antigen is necessary to cause severe anaphylaxis when too much amount of antibody is used for sensitization. These results will make us assume that there is the inhibition of reaction due to antigen excess or antibody excess in anaphylaxis as it is observed in precipitation. However, the mechanism of anaphylaxis or allergy seems to be more complicated than the mechanism of precipitation. Firstly, anaphylaxis is caused by antigen-antibody reaction in the tissues and the circulating antibody seems to take no part in anaphylaxis but sometimes it even inhibits the occurrence of anaphylaxis (Ishizaka 1953). Secondly, it is doubtful whether the degree of allergic reaction is only dependent on the degree of antigen-antibody reaction occurring in the tissues. In other words, the stability of autonomous nervous system seems to have a great importance in determining the intensity of allergic reaction (Nakamura et al 1954). In addition the individual difference in the stability of autonomous nervous system among experimental animals appears to be considerable. Accordingly, it would be dangerous to form an inference of the fundamental relation between the degree of allergic reaction and the amount of antigen.
or antibody concerned from the findings obtained in anaphylactic shock. In the present study, therefore, in vitro anaphylaxis was applied. Namely, the contraction of a certain intensity of the sensitized intestine was designated in the term of acetylcholine concentration for the quantitative investigation of anaphylaxis. As the results of this investigation, the inhibition of anaphylactic reactions due to antigen excess or antibody excess was not observed. Moreover, a certain correlation was assumed between the amount of antibody in the tissues and the amount of antigen required to cause the contraction of a certain intensity.

On the other hand, a number of investigators have studied the effectiveness of antibody in passive sensitization. Especially, Kabat & Benacerraf (1949) demonstrated that guinea pigs can be sensitized to fatal anaphylaxis with non-precipitating rabbit anti-ovalbumin and with precipitating anti-ovalbumin of approximately the same quantity. Later, however, an equivalent does of non-precipitable antibody was found to be unable to induce Arthus reaction comparable in sensitivity to those produced by the antibody in whole serum (Benacerraf & Kabat). According to Pappenheimer et al (1952), human non-precipitating diphtherial antitoxin as well as precipitating antitoxin is capable of sensitizing guinea pigs to fatal anaphylactic shock. On the other hand, non-precipitating antitoxin is capable of sensitizing human skin to toxin or toxoid, but precipitating antitoxin is incapable of sensitizing normal skin. Thus, in the present study

### Table 2. Sensitizing capacity of nonprecipitating antitoxin.

<table>
<thead>
<tr>
<th>Antitoxic serum</th>
<th>Antitoxin units</th>
<th>Floculation test (time for precipitation)</th>
<th>Ring test</th>
<th>$K_1$</th>
<th>Sensitizing antitoxin (unit)</th>
<th>Circulating antitoxin (unit)</th>
<th>Toxoid (2f)</th>
</tr>
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<tr>
<td>$A_2$</td>
<td>67</td>
<td>min</td>
<td>5</td>
<td>0.51</td>
<td>4.5</td>
<td>0.4</td>
<td>10</td>
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<td></td>
<td>8.2</td>
<td>0.6</td>
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<td>13.5</td>
<td>1.3</td>
<td>4</td>
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<td></td>
<td>21</td>
<td>2</td>
<td>1</td>
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<tr>
<td>$A_2'$</td>
<td>33</td>
<td>&gt;240</td>
<td>+</td>
<td>0.51</td>
<td>14.7</td>
<td>2</td>
<td>&gt;70</td>
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<td>27.5</td>
<td>1****</td>
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<tr>
<td>$A_2''$</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>0.53</td>
<td>14</td>
<td>1.2</td>
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<td>18.5</td>
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<td>14.2</td>
<td>1***</td>
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</table>

* immediately before the removal of intestine
** required to cause a specific contraction comparable to the one by acetylcholine in the concentration of $10^{-8} \text{g/ml}$
*** intestine removed 48 hours after sensitization
**** intestine removed 72 hours after sensitization
the sensitizing activity of homologous precipitating and non-precipitating antitoxin was examined. Non-precipitating antitoxin contained in the homologous antitoxic serum was observed to be incapable of sensitizing guinea pig intestine. So far as the present experiments are concerned, the avidity of antitoxin has no relation to its sensitizing activity. At any rate, it should be noted that guinea pig serum is easily denatured by heating (Cohn & Pappenheimer 194914, Ishizaka & Ishizaka 195415), so that, it is uncertain whether the non-precipitating antitoxin used for the experiment was the same as that in the original serum. Thus, as control experiment, antitoxic serum of guinea pig heated at 56°C for 30 minutes was also used for sensitization. The heated serum was not precipitated with toxoid, however, it was capable of sensitizing guinea pig intestine to a degree comparable to the original serum. In view of these findings, the non-precipitating antitoxin contained in the homologous antitoxic serum seems to be incapable of sensitizing guinea pig intestine.

As described above, many factors seem to affect the sensitizing activity of antibody for which further investigations are required.

**SUMMARY**

1) Inhibition due to antigen excess or antibody excess was not observed in the anaphylactic contraction of guinea pig intestine.

2) The more the amount of antitoxin existed in the tissues, the less amount of toxoid is required to cause the anaphylactic contraction of the same intensity. Moreover, a certain correlation is assumed between the amount of antitoxin in the tissues and the reciprocal of the dosis of toxoid required to cause the anaphylactic contraction.

3) Non-precipitating antitoxin of guinea pig is incapable of sensitizing guinea pig intestine.

The authors wish to express their gratitude for cordial guidance of Prof. K. Nakamura of the National Institute of Health.

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