Studies on the Immunological Reactions of a Snake Venom by the Crossing Paper Electrophoresis

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It is well known[1] that the snake venom contains many active substances including enzymes. In regard to the one Japanese viper, Agkistrodon halys (Pallas), almost the same results were obtained[2], although the toxicity is not so violent. Owing to these results, it can be inferred that the venom would contain many antigens against its antiserum. In the following report their immunological reactions studied by the "crossing paper electrophoresis" are reported.

MATERIALS AND METHODS

Snake venom. Solutions of the venom of Japanese viper: 0.1g of dried venom was dissolved in 1 ml of water. The protein content of the solution was 8 per cent. It was diluted, if neccessary.

Horse antisera. Horse antisera against the viper venom were authorized commercial preparations. They were already purified but were different in their content in fractions, according to the manufacturer. One contained chiefly γ-fraction and the other β-fraction. Their protein concentration were about 12 per cent. In some cases mixtures of antisera were also used, as the studies reported here were concerned chiefly with the distribution of antigens in the fractions of the viper venom.

"Crossing paper electrophoresis". Procedures of the crossing paper electrophoresis were already described elsewhere[3][4][5]. It consists in making two substances encounter each other on a filter paper, during electrophoresis. Apparatuses used were of the horizontal type or of the hanging paper type. A paper sheet of 30×30 cm can be placed in them. Available area of the paper was 22×22 cm. Barbiturate buffer solution of pH 8.6 and ionic strength 0.05 was used. The electrophoreses were carried out at room temperature and 50–100 v, for 10–24 hours.

One dimensional crossing paper electrophoresis was carried out in the same way as the usual paper electrophoresis, except that two reactants were applied on two lines, in order to be crossed with each other, owing to the difference in their electrophoretic mobility.

Two-dimensional procedure was carried out as described already[6]: In the first electrophoresis, the snake venom was applied on a line and separated, then the antiserum was applied on the other line which was drawn perpendicularly to the first one and the second electrophoresis was carried out in the direction perpendicular to the first run. By this pair of successive runs of electrophoresis, the "crossing diagram of the snake venom" could be obtained. This "crossing diagram" shows the distribution of antigens (components of the venom) in the fractions of the venom. If the order of the application of the snake venom and of the antiserum, hence the order of the two runs of electrophoreses, was reversed, the "crossing diagrams of anti-venom" were obtained. The first electrophoresis was carried out usually at 100–200 v. for about 10 hours and the second one at about 50 v. for 12–24 hours.

The zones of proteins were stained by bromophenol blue according to Durrun[7].

RESULTS

A. Electrophoretic patterns of viper venom and its antisera.

As shown in Fig. 1, about 7 components could be separated in the venom of Agkistrodon halys (Pallas), by the paper electrophoresis. The number of components was from batch to batch somewhat different. But usually 7 were common and named provisionally, Fraction 1, 2, 3, 4, 5, 6, and 7, in the order of electrophoretic mobility. Estimated by the density of the staining, the most abundant component is No. 4, then No. 5. The quantities of faster components than No. 4 are relatively

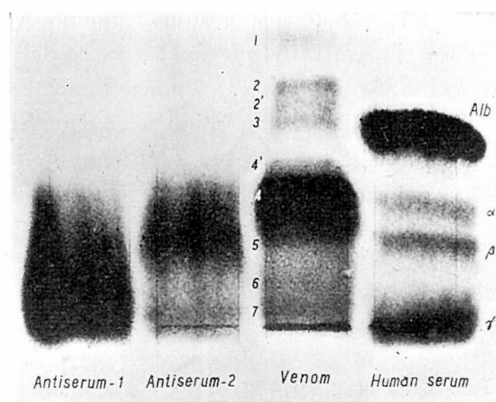

Fig. 1. Electrophoretic pattern of the venom of the Japanese viper, Agkistrodon halys (Pallas) and those of purified antisera.

1. Normal human serum. 0.02 ml/3 cm were applied on the original line. 2. Viper venom. 0.02 ml /3 cm of a 10 per cent solution of the viper venom were applied. (Fractions were numbered.) 3. and 4. 0.02 ml/3 cm of purified antisera were applied. Antiserum-1: Preparation of Biseibutu-kenkyusyo, Corp. Antiserum-2: Preparation of Takeda-yakuhin-kogyo Co.
Electrophoresis at 160 v., for 9 hours. Veronal buffer of pH 8.6 and ionic strength 0.05. Filter paper, Toyo No. 52. Stained with bromophenol blue.

little.

The electrophoretic patterns of the used antisera were also shown in the figure. They were already purified. The one (prepared by the Biseibutsu-kenkyusho Corp.) contained almost exclusively γ-globulin, whereas the other (prepared by Takeda-Yakuhin-Kogyo Co.) contained chiefly β-globulin.

B. Reactions of the viper venom with its antiserum by the one-dimensional paper electrophoresis.

Fig. 2 shows the reaction of the viper venom with its antiserum by the one-dimensional crossing paper electrophoresis. The antiserum was originally applied on the line MN and the viper venom on the line XY. In the upper case shown in Fig. 2, the β-globulin migrated from the line MN toward the anode and the γ-globulin toward the cathode. At the end of the electrophoresis, Fraction 4 of the venom just crossed over with the β-globulin of the antiserum. As can be seen from the figure, four sharply defined lines of precipitate of antigen-antibody reactions appeared in the zone of γ-globulin. Thus it is clear, that they come from the antibodies contained in the latter. In the lower case the fractions of the antiserum ran after those of the snake venom, whereas some of the latter migrated opposite to the former. Here appeared each one line of precipitate in the zone of β-and of γ-globulin. The distribution of the antigens, which took part in the formation of the lines of precipitate, can be roughly inferred from the inclination of the lines as follows: The angles between the lines and the direction of the electrical field depend upon the direction and velocity of each reacting antibody and antigen. If the reacting pair migrated toward each other with equal velocity, the line would be perpendicular to the direction of the electrical field; if the reacting antigen had a velocity

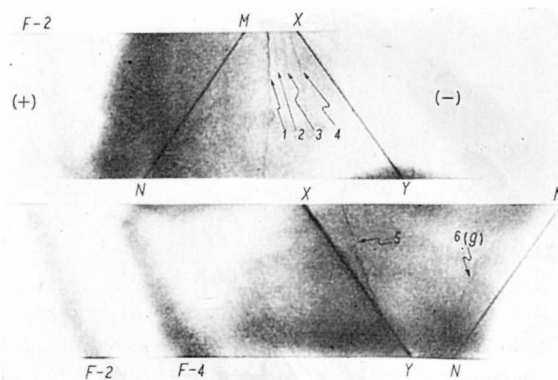

Fig. 2. One-dimensional crossing electrophoresis of the viper venom and its purified horse antiserum.

0.007 ml/cm of the purified antiserum were applied on the line MN, and 0.002 ml/cm of a 10 per cent venom solution was applied on the line XY. Electrophoresis at 55 v. and 2.1 mA, for 15 hours. Other conditions were the same as in Fig. 1. Explanation see text.

greater than that of antibody, the line of precipitate would be inclined in the direction of antibody, and vice versa.

But the distribution of the antigens contained in the fraction of the viper venom and that of antibodies contained in the antiserum can not be definitely determined by this method. This is only possible by the two-dimensional crossing paper electrophoresis.

C. "Crossing diagrams" of the Japanese viper venom against its antiserum

As discussed already by Nakamura et al.[6], the advantage of the procedure of crossing electrophoresis over other methods in immunological studies lies in the fact that it can be carried out two-dimensionally to obtain the "crossing diagrams". According to the distribution of the curves of precipitate, two sorts of "crossing diagrams" can be obtained.

(i) "Crossing diagram of anti-viper venom" shows the distribution of the antibodies contained in the fractions of antiserum. One example of this sort of crossing diagrams obtained is shown in Fig. 3. As can be seen from the figure, after the antiserum was separated by the first electrophoresis, the viper venom was applied perpendicularly to the line of the former. Thus the fractions of the venom, which

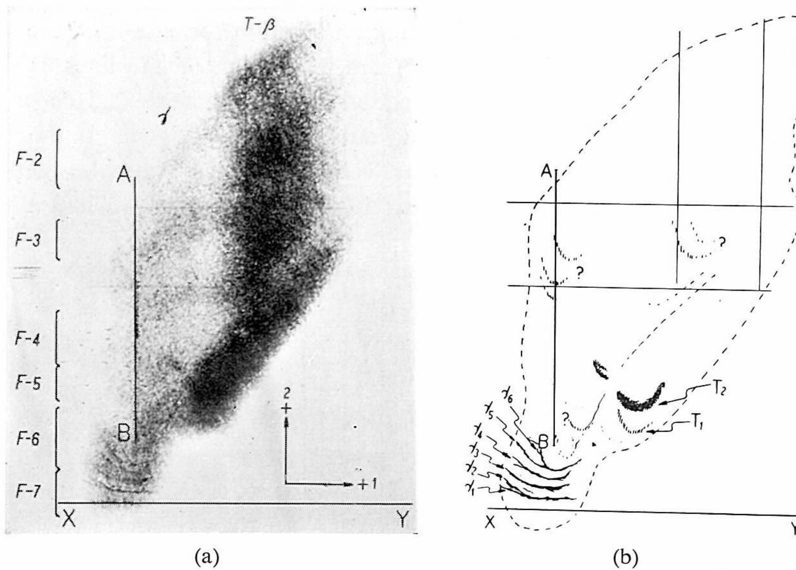

Fig. 3. An example of the crossing diagrams of the anti-viper venom. (Fig. 3 b, a sketch to illustrate peaks in Fig. 3 a)

1st electrophoresis: After 0.015 ml/cm of a mixture of antisera (antiserum-1: antiserum-2 of Fig. 1 = 1:5) were applied on the line AB, the first electrophoresis was carried out in the direction 1, at 100 v., for 15 hours. 2nd electrophoresis: After 0.000125 ml/cm of a 10 per cent solution of the viper venom were applied on the line XY, the second electrophoresis was carried out at 60 v. for 25 hours. Other conditions were the same as in Fig. 1.

were separated by the second electrophoresis migrated one after the other through the zones of γ-and β-globulins to form curves of precipitate.

(ii) "Crossing diagram of the viper venom" shows the distribution of the antigens contained in the fractions of the applied viper venom. Fig. 4 shows one example. As has been described above, first only the venom was applied on the line XY and the first electrophoresis was carried out in the direction 1, and then the antiserum was applied on the line AB and the second electrophoresis was carried out in the direction 2. Under the experimental conditions (especially the relative concentrations of the applied venom and the antiserum were important), two peaks appeared in the β-globulin, corresponding to Fraction 1 and 2 of the venom proteins.

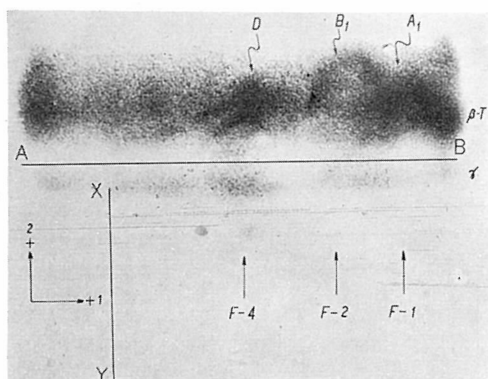

Fig. 4. An example of the crossing diagrams of the venom of the Japanese viper (1). 1st electrophoresis was carried out in the direction 1, at 100 v. for 10 hours, after 0.003 ml/cm of a 10 per cent venom solution were applied on the line XY. 2nd electrophoresis was carried out in the direction 2 at 50 v. for 15 hours, after 0.0075 ml/cm of an antiserum were applied on the line AB. Other conditions were the same as in Fig. 1.

In the regions of β-globulin corresponding to Fractions 4 and 5, several sharply defined lines (or peaks) of precipitate can be seen. These peaks appeared more clearly in other examples by changing the relative concentrations of the venom and the antiserum. Fig. 5 shows one of these cases. In this case, the peaks of the crossing diagram appeared in the regions of viper venom, Fractions 4, 5, 6, and 7, whereas the peaks corresponding to Fractions 1, 2, and 3 did not appear. This comes perhaps from the reason that the quantities of these fractions were excessive to the corresponding antibodies and hence the peaks of precipitate were redissolved in the venom fractions and carried away with them. This would apply as well to the fact that in the case shown in Fig. 4, no peak corresponding to the Fraction 3 of the venom appeared.

In Fig. 5 b is shown a sketch of Fig. 5 a, where not clearly defined peaks were also drawn. Thus some of the peaks shown in the sketch, especially those marked

Fig. 5. An example of the crossing diagrams of the venom of the Japanese viper (2). (Fig. 5 b, a sketch to illustrate peaks in Fig. 5 a)

1st electrophoresis: After 0.005 ml/cm of the venom solution were applied on the line XY, the 1st electrophoresis was carried out in the direction 1, at 170 v. for 8.5 hours. 2nd electrophoresis: 0.008 ml/cm of an antiserum were applied on the line AB and the second electrophoresis was carried out in the direction 2, at 55 v. for 15 hours. Other conditions were the same as in Fig. 1.

with ('), ('') or (''') are only probable. The sign given to each peak is arbitrary and bears no relation to those given in Fig. 2 and 3. It is intended to correspond the fraction number of the venom and the alphabetical signs of the peaks. But the correspondence is not always certain, as some peaks appear in the zone between the fractions or are so flat that they extend over several fractions. However, eight antigens can be observed in Fraction 4 of the venom, of which six have their corresponding antibodies in β-globulin of the antiserum, and two in γ-globulin; seven antigens in Fraction 5, of which one has its antibody in β-globulin and six, their antibodies in γ-globulin; three antigens in Fraction 6 and one antigen in Fraction 7, which have their antibodies in γ-globulin.

As discussed already by Nakamura et al.[4], one of the disadvantages of the crossing electrophoresis lies in the fact that two reactants of the same electrophoretic mobility can not be brought to encounter on the filter paper. In the case shown in Fig. 5 a, Fraction 5 is of almost the same mobility as β-globulin and Fraction 7 is slower than the latter. Therefore they cannot encounter with β-globulin by the procedure employed here. Fig. 6 shows one of the other examples of the "crossing diagrams of the viper venom", in which the order of the electrophoretic runs of the venom and the antiserum is the same as that in Fig. 5, but the relative position of them is reversed: After the venom was separated into fractions by the first electrophoresis, the antiserum was applied on the line drawn below the venom instead of a line above it, and the second electrophoresis was carried out. Thus β-globulin of the antiserum migrated over Fraction 7 and partly Fraction 6. As can be seen from the figure, there appeared at least two peaks in the zone of Fraction 7.

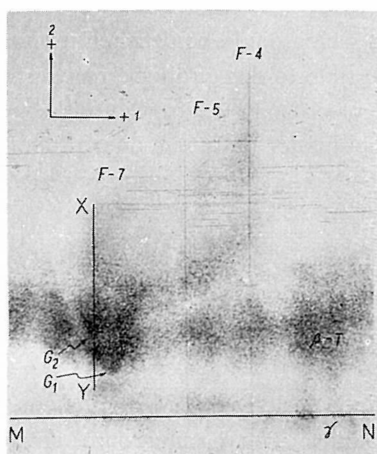

Fig. 6. An example of the crossing diagrams of the venom of the Japanese viper (3). 1st electrophoresis: in the direction 1, at 50 v. for 15 hours. A venom solution only was applied on the line XY; 0.005 ml/cm. 2nd electrophoresis: in the direction 2, at 25 v. for 24 hours. An antiserum was applied after the first electrophoresis; 0.006 ml/cm on the line AB. Other conditions were the same as in Fig. 1.

Any one line of precipitate in the "crossing diagram of the anti-venom" should appear also in the "crossing diagram of the venom". But it is not easy to establish the correspondence between the two lines of precipitate. Thus the signs of number given to the peaks in one type of crossing diagram were independent from those in the other. In this respect, the total number of antigens contained in the venom should be counted only in one type of crossing diagram. In this paper the "crossing diagram of the venom" was preferred as reference, owing to the higher resolution of the lines, and the results obtained in Fig. 4, 5, and 6 are summed up and tabulated in Table I. As can be seen from the table, the venom of the Japanese

Table I. Distribution of antigens observed in the fractions of Japanese viper venom.

Fraction of viper venom	Antigens reacting with antibodies contained in		Total Nos.
	β-globulin	γ-globulin	
1	A_1		1
2	B_1		1
3			0
4	D_1, D_1', D_1'', D_1''', D_2, D_2'	d_1, d_2	8
5	E_1	e_1, e_1', e_2, e_3, e_4, e_5	7
6		f_1, f_1', f_1''	3
7	G_1, G_2	g_1, (g)?	3
Total			23

Symbols for individual peaks are arbitrary and those dotted are not clearly discernible.

viper, Agkistrodon halys (Pallas), contained not a little number of (probably over 20) antigens, or substances which could be detected immunologically. As mentioned above, however, it is impossible to detect all the reactants contained in the reacting solutions by the procedure of crossing electrophoresis. Thus the number of antigens shown in the table might be smaller than true one, although some probable ones are also counted therein.

SUMMARY

Immunological reactions of the venom of the Japanese viper, Agkistrodon halys (Pallas) with its antiserum was studied by the technique of the crossing paper electrophoresis.

The viper venom contained 7 electrophoretic fractions, and the purified antisera contained β-and/or γ-globulins. The distribution of antigens in the fractions of the viper venom, and that of antibodies in the fractions of the antiserum were elucidated by the crossing diagrams of the viper venom against the antiserum, which could be obtained by the two-dimensional techniques.

It was concluded, that the venom of the Japanese viper contained over 20 antigens, namely substances which could be distinguished immunologically. Of them about 8 were distributed in the 4th fraction of the venom, and of the corresponding antibodies 6 in β- and 2 in γ-globulin of the antiserum. The 1st and the 2nd fractions contained one antigen each, corresponding to the antibodies in β-globulin. The 5th, the 6th, and the 7th fractions contained 7, 3, and 3 antigens respectively. Of them one in the 5th fraction and 2 in the 7th fraction had their corresponding antibodies in γ-globulin of the antiserum.

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