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Citation	Japanese Journal of Veterinary Research, 3(2), 82-89
Issue Date	1955-08-10
DOI	https://doi.org/10.14943/jjvr.3.2.82
Doc URL	https://hdl.handle.net/2115/1669
Type	departmental bulletin paper
File Information	KJ00002372930.pdf



STUDIES ON A PYROGENIC SUBSTANCE IN BLOOD PLASMA OF VIROSIS

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(Received for Publication, May 2, 1955)

Reports have been published about some kinds of pyrogenic substances, bacterial pyrogens from several species of bacteria and filamentous fungus, pyrexin isolated by MENKIN from exudates of acute sterile inflammations, pyrexin as termed by HATTA in exudates of human tubercular pleurisy, and pyrogenic substance found by YOSHIDA in exudates of human cancerous peritonitis etc.

There are, however, few reports available describing the pyrogenic substances in virosis, except that a few viruses have been shown to possess pyrogenic properties.

Accordingly, the authors' desire was aroused to learn about the mechanism of febrility in virosis and to know whether the pyrogenic substances exist in body fluid other than exudate. Therefore, experiments have been performed with blood of equine infectious anemia cases.

Equine infectious anemia is one of the most important diseases in the field of veterinary science because it is spread wide in the world, further because there is no conclusive factor in diagnosis and no sure medical treatment. Diseased animals often suffer high fever, but little is known about the pathogenesis of this fever fit as is also the case of many virosis in human beings.

EXPERIMENTAL

Use was made of the blood plasma of naturally infected anemia cases, of experimental artificially infected, and, as control, of normal healthy horses. The plasma (using 3.8% sodium citrate solution as an anti-coagulant) was employed as wholeplasma or as the fractions prepared by ammonium sulfate salting-out method, or COHN's fractionations methods of plasmaproteins. Strict precautions against contamination by bacterial pyrogens were observed throughout. Penicillin and streptomycin were used in case of necessity. Healthy male or female white rabbits weighing 2 to 3 kg were used for the fever response test.

On the day before experiments, rectal temperatures of animals were recorded at inter-

vals of 30 minutes for 5 hours and the animals for fever test were limited to those whose extremes of temperature showed less than 0.3°C.

1. Test to Demonstrate a Fever-Producing Factor in Plasma of Equine Infectious Anemia

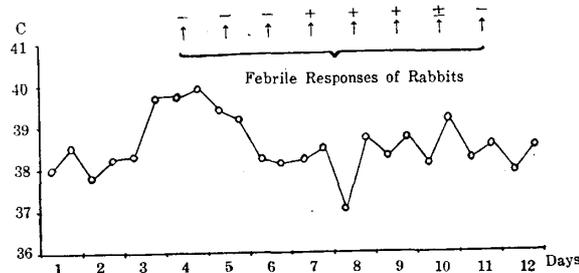
The wholeplasma for the test was prepared from the naturally infected horses which had had a fever fit one day before experiments. Ten ml doses of the material were injected in rabbits intravenously. All 3 rabbits showed 0.7~1.4°C rise in rectal temperature respectively within 2 hours of injection. On the contrary, normal horse plasma showed no sign of a tendency to produce fever.

However, in a second series of experiments with 40 diseased horses, a considerable number of examples failed to show ability to produce a fever. This phenomenon was understood, when the results were classified according to the fever course of diseased horses; the plasma had fever-producing power only when the fever of animals was declining.

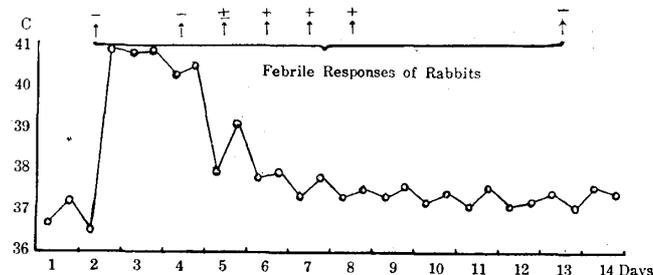
To learn in detail more about the relationship between the appearance of the fever-producing factor and the fever fit of horses, two more horses were tested over a month period. In this period both animals suffered fever fits. As indicated in fig. 1 the results derived from this experiment indicated that the

FIG. 1. *Temperature Curves of Two Diseased Horses and Febrile Responses of Rabbits Injected with Horse Plasma Withdrawn Every Day*

Horse No. 1.



Horse No. 2.



fever-producing factor began to appear only from the beginning of subsidence of the fever, and disappeared in about a week.

2. Extraction or Isolation of the "Pyrogenic Substances"

(1) Extraction of Plasma

In accordance with the methods that are used for bacterial pyrogens, the plasma was treated with several times volumes of organic solvents such as ethanol, ether-ethanol, acetone and chloroform. Table 1 summarizes the results. There was no indication of a fever-producing substance in any of these extracts.

TABLE 1. *Febrile Responses of Rabbits Given Diseased Horse Plasma after Treatment with Organic Solvents*

MATERIALS APPLIED	ANIMALS EMPLOYED	SOLVENTS					
		Ether	Ethanol	Ether- Ethanol Mixture	Acetone	Chloro- form	Benzene
Supernatant Fluid	Numbers of rabbits employed	3	3	3	3	3	3
	Numbs. of rab. reacted with over 0.6°C	0	0	0	0	0	0
	Results	—	—	—	—	—	—
Deposition	Numbers of rabbits employed	3	3	3	3	3	3
	Numbs. of rab. reacted with over 0.6°C	2	3	3	2	2	2
	Results	+	+	+	+	+	+

(2) Isolation of Plasma

a) In conformity with MENKIN'S isolation method of pyrexin, plasma was treated with 1/3 saturated ammonium sulfate solution and separated into 2 parts: the euglobulin fraction precipitated and the residual supernatant solution.

In fever test, the precipitated fraction produced temperature rise in rabbits, as it was a case of pyrexin. MENKIN separated this part by further washing repeatedly with distilled water. Pyrexin remained in the insoluble part of it. There remained, however, nothing insoluble in this part of plasma of infectious anemia, when washed with distilled water. In other experiment, the euglobulin fraction of the normal horse plasma, being different from exudate in dogs, was always soluble in water when sulfate ion was present. From these facts, $(\text{NH}_4)_2\text{SO}_4$ salting-out method was thought not to be suitable for the isolation or purification of pyrogenic substances from horse plasma.

b) Use was made therefore of COHN's method for the above-stated aim.

As can be seen in table 2 fraction II+III by method 6 of COHN's method showed the fever-producing property. In a few materials, fraction IV showed also the same potency. Separating this further into fractions IV-1 and IV-4, the former showing positive results but the latter hardly any. This may be due to shifting of the pyrogenic substance to fraction IV during the procedure. The fever-producing property positive II+III fraction was separated further by method of ONCLEY et al. into fractions III-0 and II+III-0. The former which contains a great part of lipoprotein in plasma, showed positive results.

TABLE 2. *Febrile Responses When Each Fraction of Horse Plasma Had Been Separated by Sulfate Salting-Out and COHN's Method*

METHOD	FRACTION	RESULT
Sulfate Salting Out	{ Euglobulin fraction	+
	{ Euglobulin filtrate	-
COHN's Method	{ Frac. I	-
	{ Frac. II + III	+
	{ Frac. IV	-
	{ Frac. V	-

c) To demonstrate the difference in isolation by COHN's method between the pyrogenic substance in horse plasma and in exudates of the acute inflammation, exudate of aseptic terpentin pleurisy in goat was treated by COHN's method. The result obtained showed the pyrogenic substance in this material existed in fraction III-0 the same as that in horse plasma.

(3) To Extract the Pyrogenic Substance from Fraction III-0

It was treated with organic solvents such as ethanol, ether-ethanol, acetone, and chloroform as the case of plasma, but extracts showed negative results just as plasma did.

3. Attempts to Demonstrate the Change of Normal Plasma into a Pyrogen-producing Substance by Heating

As stated above, the pyrogenic substance was observed in the diseased horse plasma only after the fever fit had declined. So tests were made on the possibility of the change of plasma protein into the pyrogenic substance as a result of heating.

FIG. 2-A. *Temperature Curve of the Normal Horse Given the Sulfur Emulsion and Febrile Responses of Rabbits Injected with the Plasma of That Horse*

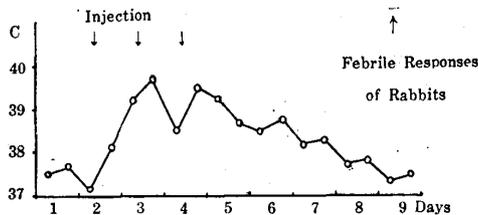
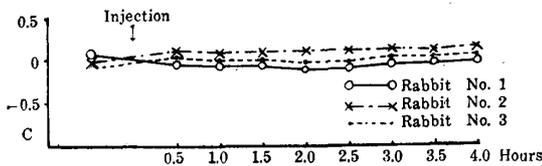


FIG. 2-B. *Fluctuation in Temperature of Rabbits Given Horse Plasma Withdrawn after the Sulfur Emulsion was Injected and Fever Fit was Over*



of pyrogenic fraction II+III or of euglobulin fraction.

5. Effect of pH

Preparation retained potency over a pH range from 4 to 10.5 but beyond that range the potency disappeared.

6. Stability at 20°C (room temperature) and 0°C

Leaving plasma at room temperature for 72 hours or storage for as long as 3 months at 0°C had no detectable effect upon the ability of preparations to cause fever in normal rabbits.

7. Dialysis

After dialysis through cellophane against distilled water or physiological saline for as long as 3 days, the fever-producing material was found to remain within the cellophane bag.

Test in vivo: A normal horse was caused to produce the fever by giving it sulfuric preparation for 3 days and on the 4th day, after its temperature had fallen, its plasma was tested.

Test in vitro: The normal horse plasma which had not the fever-promoting property, was treated at 42°C for 48 hours in incubator.

Fig. 2 summarizes the results. There was no evidence of any acquisition of a fever-producing property by the plasma protein in any of these preparations.

4. Heat Stability

Boiling for 30 minutes did not destroy the fever-producing capacity

FIG. 2-C. *Fluctuation in Temperature of Rabbits Given Non-febrile Diseased Horse Plasma Heated at 42°C for 48 Hours*

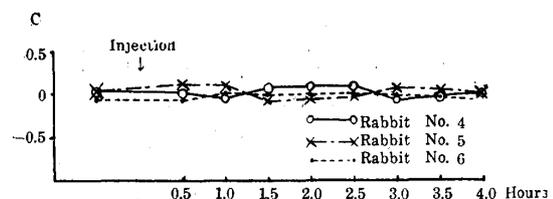
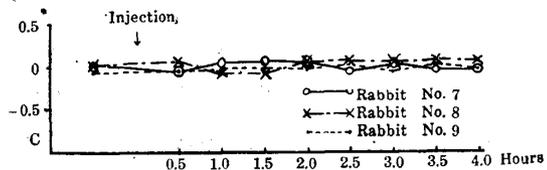


FIG. 2-D. *Fluctuation in Temperature of Rabbits Given Normal Horse Plasma Heated at room temperature for 72 Hours*



8. Electrophoretic Pattern

There are no differences between the pattern of the fever-producing plasma and the normal one. The pattern of the III-0 fraction which has fever-producing potency, revealed that nearly all of it was in β -globulin.

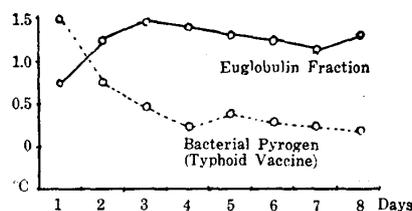
9. Test for Inhibition by X-Ray

To apply the X-ray of 1200 Röntgen units to the material resulted in no change of its potency.

10. Test for Development of Tolerance

Two rabbits were given daily intravenous injections of III-0 fraction for a week and their rectal temperature was recorded for 5 hours after injection daily. Test results were, as can be seen in fig. 3, that the animals showed no sign of tolerance.

FIG. 3. *Febrile Responses of Rabbits Injected with Euglobulin Fraction Repeatedly*



DISCUSSION

The plasma protein denatured by heating *in vivo* or *in vitro* produces no fever-producing factor, and being undialysible and insoluble in certain organic solvents such as ethanol, ether-ethanol, acetone and chloroform, the fever-producing factor which authors found in plasma in diseased horses was not denatured protein and not anemia virus itself nor bacterial pyrogens. This is true because it has been proved that the anemia virus was dialysible through cellophane, and that bacterial pyrogens were soluble in the same organic solvents noted above. The facts that the present substance was heat stable, it was characterised by the short duration of the febrile response, it caused no tolerance to rabbits, and it was found in euglobulin fraction, lead to the conclusion that it is like the pyrexins, which MENKIN, HATTA and YOSHIDA found in exudates in dogs or in human beings.

Although these substances are precipitated in different fractions by $(\text{NH}_4)_2\text{SO}_4$ salting-out, it does not necessarily mean that these substances possess different natures. It must be taken into consideration that the one is in exudate of dog, another is in exudate of man, while the present substance is in horse plasma. As found by using COHN's method, pyrexin in goat and the present substance in horse plasma both are in the same fraction III-0.

According to COHN and ONCLEY, β -globulin of plasma is concentrated by COHN's method in fraction II+III, most of it is deposited as fraction III-0 and a great part of lipoprotein of plasma is in fraction III-0. The present substance is also

shown electrophoretically as a β -globulin. From the results, and taking into consideration the fact reported by KEIDERLING et al. that highly-purified lipopolysaccharide had a fever-promoting property, the fever-promoting factor in horse plasma, perhaps pyrexins too, are thought to be closely concerned with lipoprotein of plasma.

This substance was observed in a diseased horse plasma only after fever fit is over, and in the case of pyrexins the same phenomenon was observed. MENKIN attributing to it the fever which accompanied acute inflammation state: explaining that there is in inflammation at first no lymphatic blockade and material diffuses readily into the circulation, thus affecting the fever centers. Later lymphatic blockade supervenes and material fails to diffuse readily from the site of acute inflammation. The present substance was always isolated from circulating blood which was normal pH, this means that the fever producing factor is not necessarily the fever caused response of diseased animals, but there might be another one or if not, there should lie concealed further secret mechanism in such cases.

SUMMARY

In plasma in equine infectious anemia there was found a pyrogenic substance which caused a febrile response when injected intravenously in rabbits. This substance appeared in plasma of the diseased animals which suffered from fever fit, only when the fever fit was over, and it disappeared in about a week.

The substance was insoluble in certain organic solvents such as ether-ethanol, acetone and chloroform. By salting-out method it was found in euglobulin fraction, and by method 9 of ONCLEY in III-0 fraction. It has been proven undialysible with cellophane, and was not destroyed by boiling for 30 minutes at pH 6.6

It was active in producing fever over a pH range of 4 to 10.5 and maintained potency at room temperature for 72 hours, or at 0°C for as long as 3 months. By COHN's method for fractionation it was treated at -6°C for about 3 days; its potency remained.

To apply the X-ray of 1200 Röntgen units resulted in no change of its activity. The electrophoretic pattern of pyrogenic III-0 fraction revealed that the greater part of this substance was in β -globulin. Rabbits given daily intravenous injection of this substance for one week showed no sign of tolerance.

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