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STUDIES ON MYXOVIRUS PYROGEN (II)
SOME FACTORS AFFECTING FEVER TOLERANCE IN RABBITS

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SUMMARY The fever response and tolerance phenomenon induced by influenza virus in rabbits were compared with those by endotoxin from *E. coli*.

Various pharmacological properties of the virus were also studied. The following results were obtained.

1. The tolerance phenomenon depended on the dosis of virus in the first injection.
2. Cross tolerance was observed between RDE and virus.
3. Virus pyrogen like endotoxin was partially inactivated by DOC (deoxycholate).
4. Recovery of tolerance was in parallel with adsorption of virus onto blood cells and production of leucocytic pyrogen from polymorphonuclear leucocytes in vitro.
5. Some pharmocological properties of the virus were compared with those of endotoxin and it was concluded that in some aspects, virus pyrogen was similar to endotoxin.

INTRODUCTION

The course of the fever reaction of rabbits to myxovirus in the absence of reproduction of the virus is an interesting problem in relation to the pathogenesis of virus infection. Various authors have discussed on this problem (Atkins, 1960, Grossgebauer, 1967, Siegert, 1966).

Previously, we reported (Kanoh, et al., 1966) that the production of leucocytic pyrogen in vitro, described by Atkins (1954), was accelerated by incubation of polymorphonuclear leucocytes (PMN) with the virus and suggested that PMN might be essential mediators of the pathogenesis of virus induced fever.

It is well known that after one injection of influenza virus rabbits do not show any febrile response to a second injection (Atkins, 1956, Bennett, et al., 1949, Siegert, 1964). This fever tolerance phenomenon has also been observed with endotoxin induced fever, but it has not been explained.

This report describes quantitative studies on fever reactions. The explanation of the tolerance phenomenon in the case of influenza virus is discussed in comparison with that of the endotoxin from *E. coli*.

MATERIALS AND METHODS

1. *Animals and determination of body temperature.*

Male rabbits weighing 1.5 to 2.0 kg were used throughtout the experiments. Body temperature

was estimated by rectal measurement as described in the previous report (Kanoh, et al., 1966).

2. *Pyrogenic substances; virus, endotoxin and keucocytic pyrogen(LP).*

Influenza virus strain A_2/Okuda/57 was used. Infected cholioallantoic fluid (CAF) showed 540 HAU/0.5 ml and $10^{8\cdot2}EID_{50}$/0.2 ml. Endotoxin was prepared from *E. coli* UKTB by the method of Westphal (1954). The minimum pyrogenic dose was 0.01 μg per kg in rabbits. LP was prepared from the supernatant of peritoneal PMN incubated in saline in roller tubes at 40 C for one hour (Kanoh, et al., 1966).

3. *Receptor destroying enzyme (RDE).*

Commercial RDE (Takeda Co., Ltd.,) was diluted diluted with non-pyrogenic saline. Its HI activity was 1 unit/0.25 ml.

4. *Haemorrhagic reaction induced by Epinephrine.*

Epinephrine hydrochloride in saline (0.1 mg/ml) was injected subcutanously at a dose of 0.1 ml into the abdomens of tolerant and normal rabbits and then endotoxin or virus was injected intravenously. After injection of endotoxin or virus, the hemorrhagic reaction at the site of epinephrine injection was observed for four hours and the results were recorded as $-$, $\pm$, $++$ etc.

5. *Determination of increased vascular permeability.*

The permeability increasing activity of the test samples was evaluated by subcutanous injection of 0.1 ml or 0.2 ml of sample into the flank of rabbit which had previously received an intravenous injection of Evans blue dye solution (1 ml/kg in 2.5% saline). When necessary the samples were adjusted to the physiological pH value and salt concentration. Reactions were usually read after 20 min and 1, 2 and 3 hours. The activity was expressed as the average diameter of the resulting blue area.

RESULTS

1. *Cross tolerance between virus, endotoxin and leucocytic pyrogen*

A typical cross tolerance experiment between virus, endotoxin and leucocytic pyrogen (LP) in rabbits is shown in Fig. 1 and Table 1.

As shown in the figure, after a first injection of virus, the rabbits did not respond to a second injection of virus, but responded to a second injection of endotoxin or LP. Rabbits received a first injection of virus or LP one day previously responded to a second injection of endotoxin and the second fever reaction was as great as the first. Rabbits which received LP first respond to a second injection of either endotoxin or virus.

These data suggest that cross tolerance only exists in the case of virus induced fever since it was not clearly demonstrated in the case of endotoxin induced fever. The tolerance phenomenon to the virus was analysed in group of two to three rabbits. Rabbits were injected with various doses of virus and one day later each group was re-injected with a constant dose of virus. The FI-5 of each group was calculated.

As clearly demonstrated in Fig. 2, tolerance increased with the dose of virus given in the first injection and with doses of the virus above a certain threshold complete tolerance was observed. The data suggest that some kind of receptor is involved in the virus induced fever reaction. This result is consistent with results in a previous paper (Wagner, et al., 1950).

2. *Relationship between virus and RDE in the fever reaction*

The existence of a receptor for the fever reaction was suggested by the preceeded results, so the relationship between the virus and RDE in the fever reaction of rabbits was tested.

As shown in Fig. 3 and Table 1, when rabbits were injected with RDE a fever response was observed after the same doese of RDE or injection of endotoxin on the second day but not after injection of virus. Commercial RDE may be contaminated with *Vibrio cholerae* endotoxin (Panse, et al., 1963), so the fever reaction caused by RDE may be due to contaminating endotoxin. Fig. 4 shows the quantitative relationship between the fever reactions caused by RDE and by virus. A series of two fold dilutions of RDE were injected intravenously into groups of two to three rabbits and one day

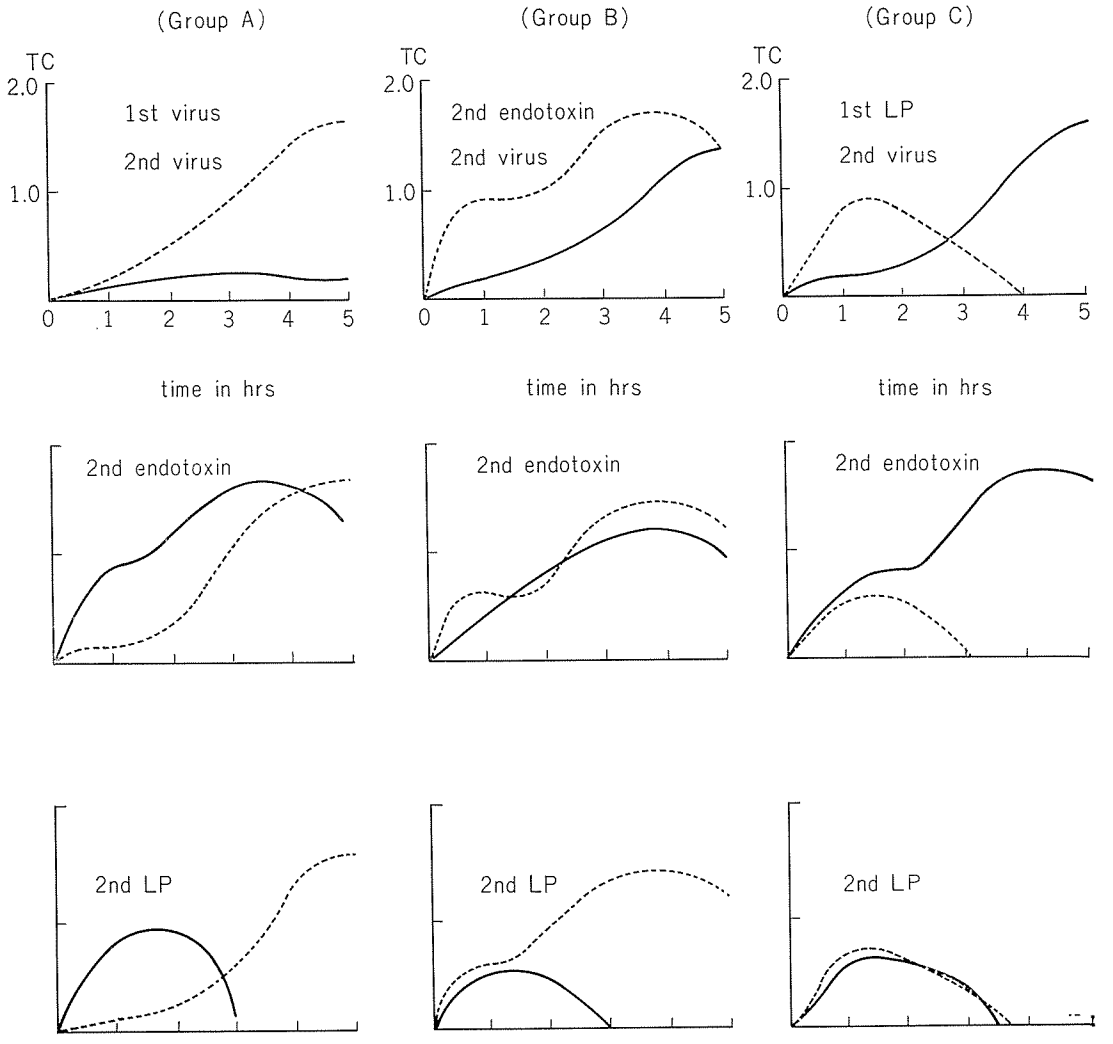

FIGURE 1. *Typical Cross Tolerance Phenomena between Virus, Endotoxin and Leucocytic Pyrogen (LP) in Rabbits. In the 1st i.v. injection rabbits in group A received 640 HAU of virus, in group B they received 0.05 μg/kg of endotoxin and in group C 1 ml/kg of LP.*
--------- : *1st injection,* ———— : *2nd injection*
The 2nd injections were as shown in the figure. The dose of the pyrogens were equal in the 1st and 2nd injections.

later all the animals received a second intravenous injection of 640 HAU/kg of virus. Their mean fever responses were calculated as FI-5 values. The fever response was much the highest after a first injection of 10 ml/kg of RDE.

3. *Effect of Deoxycholate (DOC) on the pyrogenic action of the virus*

The pyrogenicity of endotoxin is destroyed by addition of DOC (Ribi, et al., 1966) because the large diaphoretic molecule of endo-

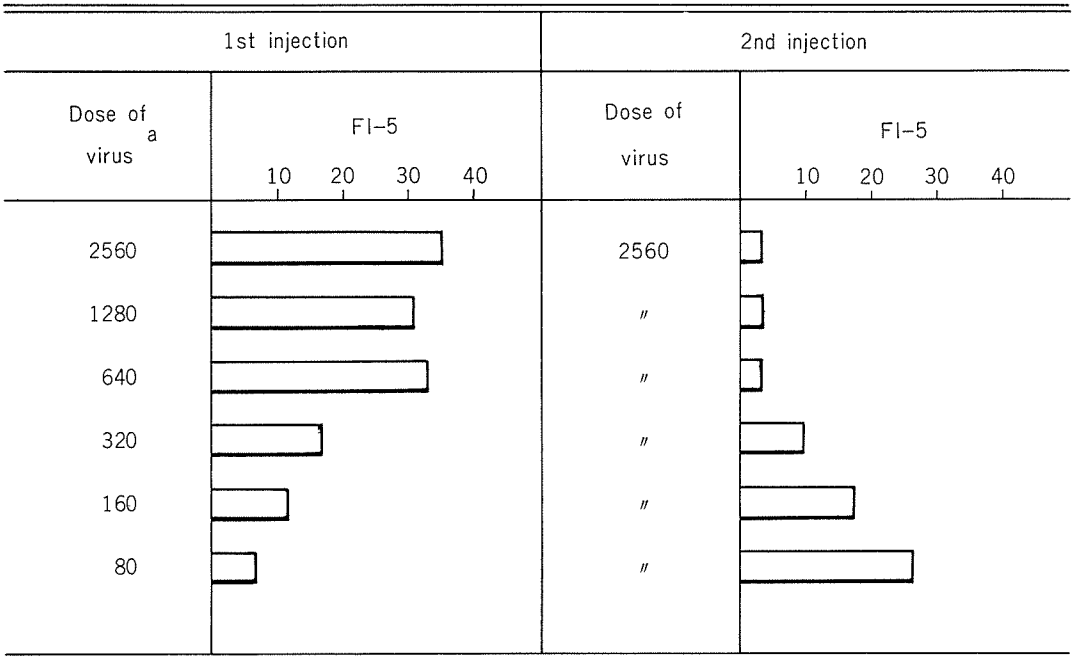

FIGURE 2. *Relationship between Dose of Virus and Fever Response.*
a HAU/kg

TABLE 1. *Cross Tolerance Phenomenon of Fever Response between Virus, Endotoxin and Leucocytic Pyrogen in Rabbits*

1st inj. \ 2nd inj.	Virus	Endotoxin	Leucocytic Pyrogen
Virus	##	—	—
Endotoxixn	—	—[a]	—
Leucocytic Pyrogen	—	—	—

a After a 2nd injection of endotoxin, no biphasic curve was seen.

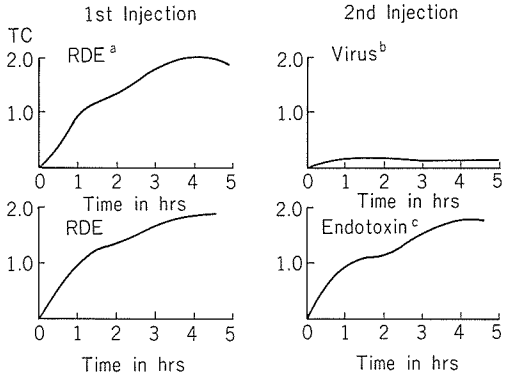

FIGURE 3. *Effect of a 1st injection of RDE on the Fever Response induced by a 2nd injection of Virus or Endotoxin.*
10 *ml/kg of commericial preparation (diluted in saline) injected intravenously*
b 1*ml/kg of virus* (640 *HA*)
c Lipopolysaccharide of E. coli injected intravenously at a dose of 1.0 *μg/kg*

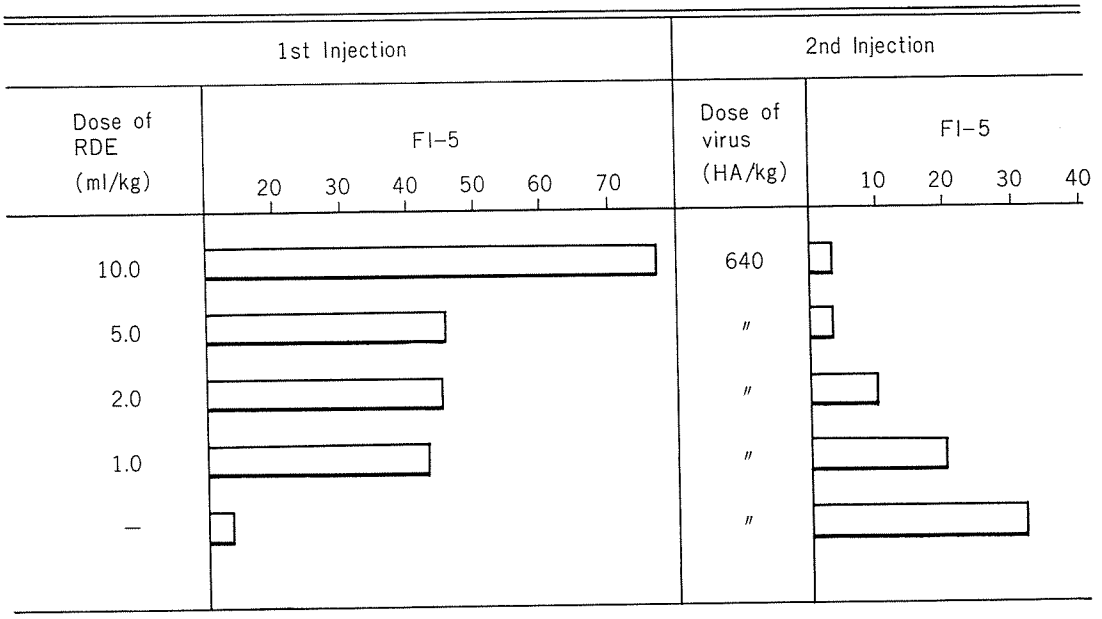

FIGURE 4. *Relationship between Dose of RDE and Fever Response.*

toxin containing both lipophilic and lyophilic radicals, is dissociated by DOC becoming non-pyrogenic.

Using DOC an improved vaccine (HA vaccine) was developed which showed none of the undesirable side reactions sometimes shown by currently used vaccines (Duxbury, et al., 1968).

The effect of DOC on the pyrogenic reaction of the virus was tested. In the first injection, mixtures of various concentrations of DOC with a fixed amount of virus were injected into groups of three rabbits. The next day, a constant dosis of virus was injected. The fever response of each group was estimated. As a control, a mixture of endotoxin and DOC was given in the first injection. The results in Fig. 5, show that the fever reaction to the second injection decreased inversely with the intensity of the first fever reaction. The same relationship was demonstrated in the control group.

4. *Adsorption of virus onto PMN and LP-production after a single intravenous injection of virus*

The above data suggested that there might be a single receptor for fever response by the virus and by RDE. To investigate this, 640 HAU/ml of the virus were injected intravenously into three rabbits and on the first, fourth, sixth, and tenth days, there after peritoneal PMN was collected from the rabbits. Circulating blood cells were collected separately from the rabbits. Circulating blood cells were collected separately from the animals and PMN and blood cells were tested for their ability to adsorb virus on incubation at 37 C for 2 hours with 10% normal rabbit serum. PMN with adsorbed virus were also incubated in roller tubes at 41 C for one hour, and the pyrogenicity of the supernatants of these mixtures was estimated in normal rabbits.

The results in Fig. 6 show that after a first injection of virus, PMN and blood cells did not immediately adsorb the virus, *in* vitro, but they

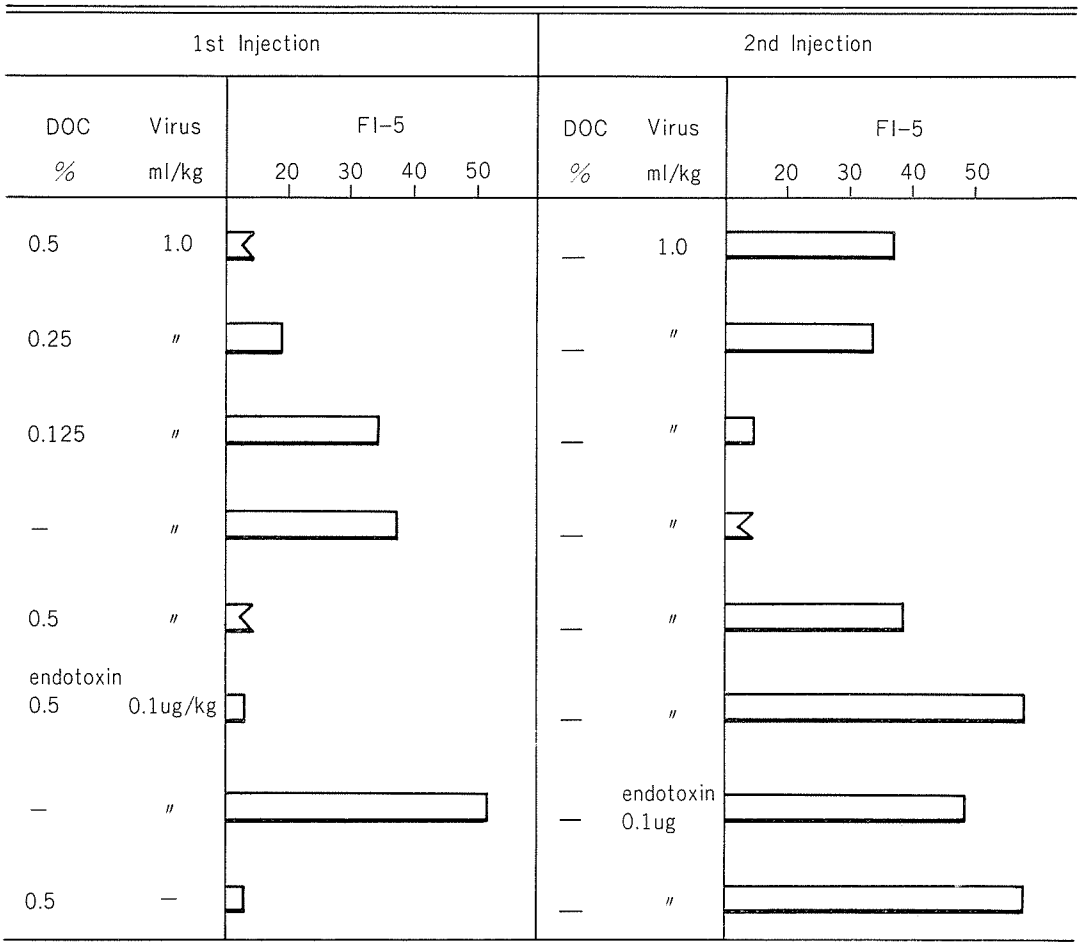

FIGURE 5. *Effect of Deoxycholate (DOC) on the Pyrogenicity of Virus and its Tolerance.*

could gradually adsorb it over a 10 day period. Moreover, production of LP by PMN increased in vitro with increase in adsorption of virus.

5. *Effect of RES blockage on tolerance of rabbits*

Previously, Beeson (1947) reported that rabbits which have developed toleranc following endotoxin injection regain sensitivity to endotoxin when the reticuloendotherial system (RES) is blocked with colloidal carbon, He stated that the tolerance to endotoxin mainly depends on increased activity of the RES. To see whether tolerance induced by virus is influenced by blockage of the RES rabbits were injected first with virus, then with 2 ml of 1% Indian ink and the next day with the same dose of virus as in the first injection. The fever response was observed.

As shown in Fig. 7, tolerant rabbits responsed fairly strongly to the second virus injection after injection of Indian ink. This suggests that virus tolerance was partially regained by blockage of the RES, as in the case of endotoxin tolerance. However, the relationship between

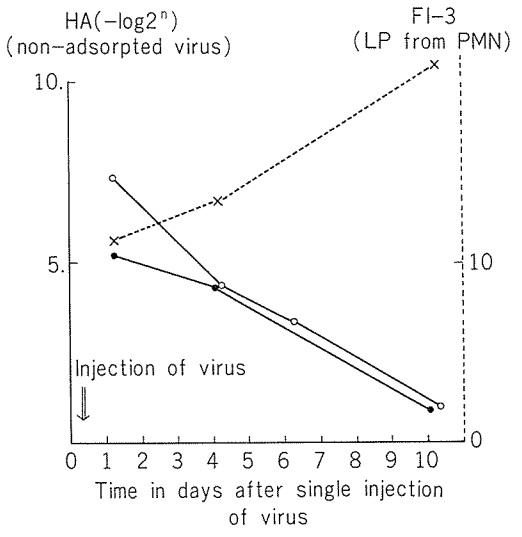

FIGURE 6. *Relationship between Adsorption of Virus to PMN and LP-production after single intravenous injection of Virus.*
×------×: *FI curve of LP released from PMN in vitro*
●——●: *Virus no adsorpted to MPN in vitro*
○——○: *Virus not adsorpted to blood cells*

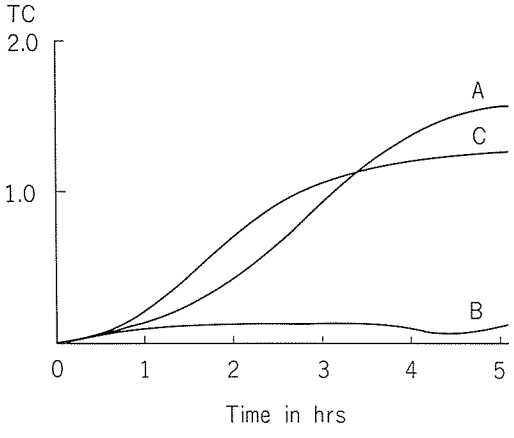

FIGURE 7. *Effect of PES Blockaae on tolerace of Rabbits.*
A: 1*st infection of virus* (1 *ml/kg*)
B: 2*nd injection of virus* (1 *ml/kg*)
C: 2*nd injection of virus* (1 *ml/kg*) *after RES blockage by Indian ink*

fever tolerance and RES activity is probably not a simple one and other pharmacological properties of the virus require further clarification.

6. *Some pharmacological properties of virus pyrogen*

To study the mechanism of the fever response and the tolerance phenomenon for the various pharmacological properties of the virus pyrogen and endotoxin were compared.

1) Epinephrine induced hemorrhagic reaction

Endotoxin causes production of various biological active amines in vitro. Thomas (1956) showed that intravenous injection of endotoxin followed by subcutaneous injection of epinephrine resulted in extensive hemorrhagic necrosis of the skin at the site of injection of epinephrine and the mechanism of this reaction has been discussed by Zweifach, et al. (1956). A similar experiment was carried out with virus.

As shown in Table 2, in normal rabbits on injection of virus epinephrine induced hemorrhagic lesion as in the case of endotoxins, but rabbits which were in tolerant to virus, no lesions were observed. In a rabbit which was partially tolerant to endotoxin a small lesion was seen.

2) Effect of cyproheptidine (CHD)

After subcutaneous injection of various doses of the virus, encotoxin, and a biologically active amine (histamine or serotonin), 5 ml/kg of 0.1% trypan blue and 1 mg/kg of CHD were separately injected intravenously. The average diameter of the resulting blue area at the site of injection was recorded during the following three hours.

The results are shown in Table 3. After histamine or serotonin, the average diameter of the lesions was about 0.5 cm to 1.0 cm, but the blue area disappeared within one day. On the contrary, after injection of CHD, the blue areas caused by injection of endotoxin or virus were 0.4 cm to 1.0 cm in diameter and these areas did not decrease in size on administration of CHD. These data suggest that

TABLE 2. *Epinephrine Haemorrhagic Reaction induced by Virus and Endotoxin in normal and tolerant Rabbits*

Rabbits	Epinephrine (subcutanously) (μg/0.1 ml)	Dose of Virus or Endotoxin (intravenously) (ml/kg)		Haemorrhagic[a] lesions after pyrogen injection 6 hrs	24 hrs
Normal	10.	virus	1.0	±	+
,,	100.	,,	1.0	+	+
Tolerant[b]	10.	,,	1.0	−	−
,,	100.	,,	1.0	−	−
Normal	10.	endotoxin	10.0	±	++
,,	100.	,,	10.0	+	++
Tolerant[c]	10.	,,	100.	±	+
,,	100.	,,	100.	±	++
Normal	10.	—	—	−	−
,,	100.	—	—	−	−

a The extent of the haemorrhagic reaction was expressed as followe: −, no lesion, ±, slightly haemorrhagic. +, ca 1.5×3.5 cm in average diameter. ++, ca 1.5 cm×5.0 cm or larger in average diameter.
b 1.0 ml/kg of virus was injected the day before.
c 50 μg of endotoxin was injected three times at two day intervals.

TABLE 3. *Effect of Cyproheptidioe (CHD)[a] on the vascular Permeability[b] induced by Virus, Endotoxin and some biogenic Amines in normal Rabbits*

Material	Dose subcutanous (μg/0.1 ml)	Av. Diameter withou CHD (cm)	Av. Diameter with CHD (cm)	Dose of CHD (mg/kg)
Histamine	10.0	1.0	0	1.0
Serotonin	1000	0.5	0	1.0
Endotoxin	10.0	1.0	0.9	1.0
,,	1.0	0.4	0.5	1.0
Virus	0.1 ml of CAF	1.0	1.0	0.5
,,	,,	1.0	0.8	1.0
,,	0.1 ml of CAF diluted to 10 folds	0.8	0.8	0.5
,,	,,	0.8	0.6	1.0

a Cyproheptidine: 1-Methyl-4-(5-dibenzo-α, e-cycloheptatrienylindene)-piperidine.
b Vascular permeability was estimated after subcutanous injection.

serotonin and histamine are not mediators of vascular permeability induced by virus or endotoxin.

3) Effect of heating and UV-irradiation on the vascular permeability increasing activity of the virus

It is unknown whether influenza virus has a similar activity to endotoxin as a vascular permeability increasing factor. To study this, virus was heated or irradiated with UV-light and then injected subcutaneously into rabbits and its effect on permeability was measured.

TABLE 4. *Effect of Heat and UV-irradiation on the Vascular Permeability of the Virus*

Treatment of Virus	Dllution[a] (×)	Vascular Permeability[b] time in hrs 1	2	3	4	24
none	1.	+	++	++	++	+
,,	10.	−	+	+	+	±
56 C for 30 min	1.	+	+	+	+	+
,,	10.	±	+	+	+	+
80 C for 30 min	1.	+	+	+	+	+
,,	10.	−	+	+	+	±
100 C for 30 min	1.	−	+	+	+	±
,,	10.	−	±	+	+	±
UV-irradiation for 20 min	1.	−	+	++	++	+
,,	10.	−	+	+	+	+
UV-irradiation for 30 min	1.	−	+	+	+	+
,,	10.	−	+	+	+	±
normal CAF	1.	−	−	−	−	−

a An injection of 0.1 ml was given.

b Vacular permeability was expressed as follows: diameter of blue area: −, none area. +, 0.2×0.4 cm +, 0.5×0.9 cm ++, 1.0×1.5 cm.

Table 4 shows this activity of the virus was not destroyed by heating or UV-irradiation and showed a dose-response relationship. This suggests that the permeability increasing activity of influenza virus is like that of endotoxin.

4) Effect of anti-pyretics on the fever induced by the virus and endotoxin.

Aminopyrine or cortisone were administered intravenously to three rabbits before and after the injection of the pyrogens. The results in Fig. 8 showed that after injection of endotoxin, cortisone decreased the fever reaction but on injection of virus, cortisone did not decrease the fever reaction, even when injected three days before the virus. On the contrary, aminopyrine decreased the fevers resulting from both pyrogen immediately after its injection.

DISCUSSION

The mechanism of the fever reaction caused by endotoxin has been studied on a cellular level with PMN from which LP was liberated (Kaiser, 1962). In this system, endotoxin might activate lysosomal enzyme (Weissmann, 1963). It is important to know whether the mechanism of production of fever by endotoxin is similar to that by virus pyrogen and so we compared some of the properties of virus pyrogen and endotoxin as described in this paper.

With regard to fever tolerance, there may be a cross tolerance between the every response to virus and RDE. This suggested that the tolerance of virus induced fever might be due to destruction of virus receptors on PMN. This was confirmed with respect to the interaction of circulating blood cells with virus. Thus after injection of virus, PMN or circulation blood cells did not immediately adsorb virus and produced little or no LP in vitro, but over a two week period they gradually adsorbed virus and gradually produced LP. At this time, the fever response to the virus was almost completely regained.

It has been stated that the tolerance to endotoxin generally depends on increased activity of the reticuloendothelial system (RES), and its activity is blocked by injection of colloidal carbon (Beeson, 1947).

An effect of RES blockage was also observed on virus tolerance.

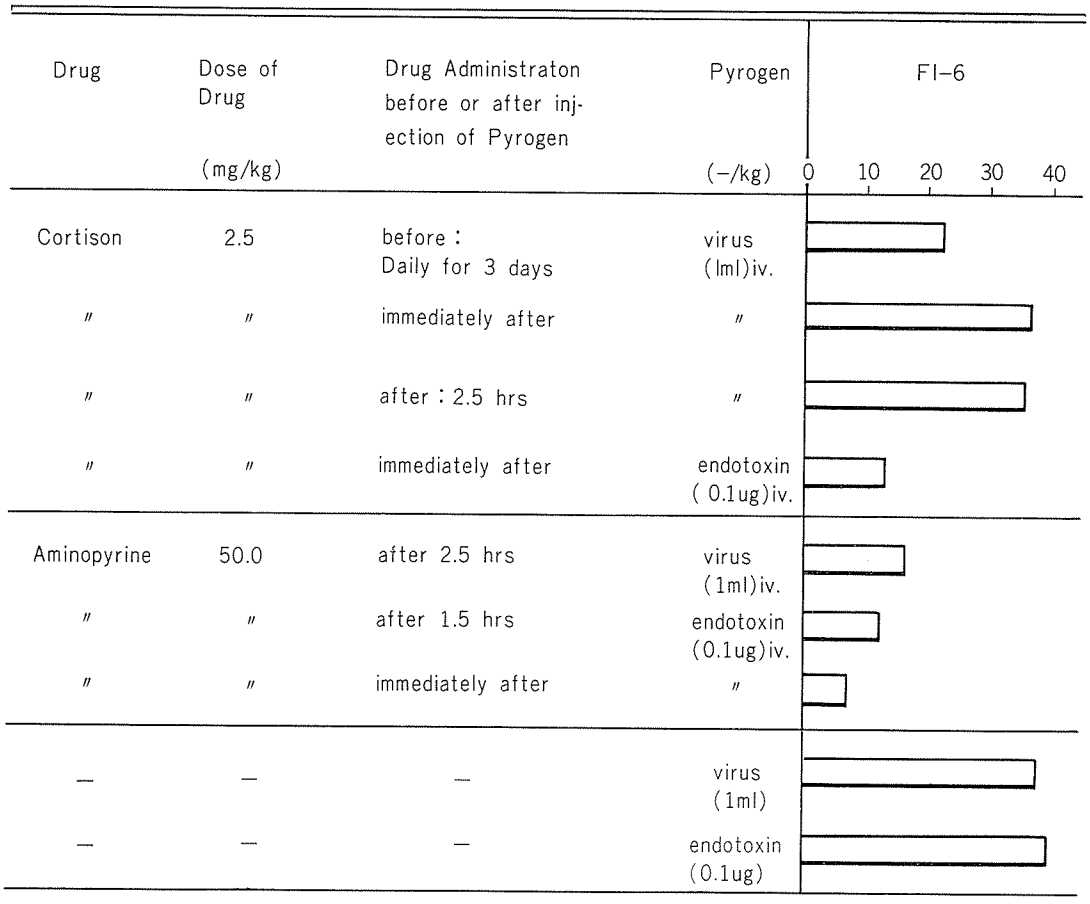

FIGURE 8. *Effect of Anti-Pyretic drugs on Febrile Response induced by Virus or Endotoxin in Rabbits.*

The results suggested that the action of virus pyrogen in vivo in some ways resembles that of endotoxin. There is much information available on the physical and biochemical properties of influenza virus and its mechanism of reproduction, but its pharmacological properties, including its toxicity are still unknown. To study the mechanisms of fever reactions, the difference between the reactions to virus and endotoxin should be compared pharmacologically.

In the present experiments, the effects of endotoxin and virus was compared on the epinephrine hemorrhagic reaction, changes in vascular permeability and in the presence of various anti-pyretics. Results showed that these pharmacological properties of virus and endotoxin were very similar. The hemorrhagic effect of endotoxin with epinephrine was observed by Zweifach, et al. (1956) and found to be less in tolerant rabbits. The same was also true with virus, and this property might be associated to the virus itself. The effect of virus on vascular permeability was also associated with the virus. This phenomenon is generally considered to be due to a factor increasing permeability dependent on the release of histamine or serotonin from the cells, but in the case

of virus it did not depended on these mediators.

Moreover, the activity of the two pyrogens was not inhibited by treatment with CHD, which blocks histamine or serotonin release from the cells and it was not inactivated by heating or UV-irradiation of the virus.

Aminopyrine, a most potent anti-pyretic, reduced the fever induced both by endotoxin and virus, but cortisone only reduced the fever due to endotoxin. Therefore, the pharmacological actions of virus and endotoxin are in some aspects, slightly different in others, so that the differences between endotoxin and virus should be studied further.

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