STUDIES ON THE PATHOGENESIS OF FEVER WITH INFLUENZAL VIRUSES

I. The Appearance of an Endogenous Pyrogen in the Blood Following Intravenous Injection of Virus *

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Although there has been extensive study of various aspects of thermoregulation, the sequence of events which lead to the development of fever following the inoculation of pyrogenic agents has remained obscure. Most investigations of the pathogenesis of fever have been carried out with bacterial endotoxins. These agents are potent toxins and in minute dosages produce cellular damage and a variety of pharmacological effects in addition to fever (1–3). A widely held hypothesis is that endotoxins act indirectly in the pathogenesis of fever by the liberation in the host of some pyrogenic product of tissue injury (4). This substance, in turn, presumably stimulates the thermoregulatory centers directly to cause fever. Although there is an obvious clinical correlation between the presence of inflammation and fever, there has been little experimental evidence for the role of such postulated endogenous substances in the development of the febrile response.

Several recent experiments indicate, however, that endogenous substances with pyrogenic properties are present in normal animals, and under appropriate experimental conditions, can be demonstrated in the circulation during fever.

First, the recovery of a pyrogenic material from both sterile exudates and circulating polymorphonuclear leukocytes of normal animals suggests a possible source for an endogenous pyrogen. In a study of the normal tissues of the rabbit, Bennett and Beeson (5) were able to demonstrate pyrogenic activity only in the polymorphonuclear leukocyte. However, the presence of an endogenous pyrogen in cell-free exudates of leukopenic animals indicated that other sources of endogenous pyrogen might also exist (6).

Second, a pyrogen of apparently endogenous origin has been demonstrated in the circulation of rabbits injected with typhoid vaccine (7, 8). Certain properties of this substance resemble those of leukocytic pyrogen and appear clearly to differentiate it from the injected bacterial pyrogen. More important is the close correlation between the duration of fever and the presence of this material in the circulation. The appear-

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ance of an endogenous pyrogen under these conditions has prompted the search for similar circulating substances in other types of experimental fever.

The intravenous injection of a number of viruses of the influenza group produces a characteristic febrile response in the rabbit although there is little or no virus multiplication in this animal. Virus-induced fever differs in certain important respects from the fever which follows the injection of bacterial endotoxins. There is a latent period of 1 hour or more after inoculation of virus as compared with the relatively short interval of 15 to 30 minutes before onset of fever with endotoxins. Tolerance to the pyrogenic effect of the virus is much more rapidly acquired, appearing with some viruses after a single inoculation. These features of the febrile response as well as certain distinctive properties of virus as a pyrogenic agent have led previous investigators to conclude that there is a fundamental difference in the pathogenesis of fever following the injection of viruses and bacterial endotoxins (9, 10).

Despite the differences which exist between these two forms of experimentally induced fever, evidence to be presented indicates that the inoculation of influenzal viruses is similarly followed by the appearance of a circulating pyrogen originating in the host animal. Furthermore, the properties of the virus-induced pyrogen suggest that it is similar to the endogenous pyrogen obtained in other forms of experimental fever.

Methods

General.—Male and female rabbits weighing 3 to 4 kg. were used in all experiments. The majority were of albino Flemish and New Zealand stock although several other breeds were occasionally employed. The rabbits were housed in an air-conditioned room, and experiments were performed in an adjacent room similarly maintained at 65–70°F.

Procedures used in the selection and handling of rabbits and in the recording of temperatures are similar to those reported elsewhere (11) with the exception that rectal temperatures were obtained by means of the Foxboro rabbit scanning switch and fever recorder.[1] Rabbits with initial temperatures of over 40°C. or showing a variation of more than 0.3°C. in the period prior to inoculation were not injected.

Assay of Circulating Pyrogen.—The essentials of the passive transfer technique for the measurement of circulating pyrogen have been detailed in previous reports (7, 8). In the experiments to be described, this procedure consisted of the inoculation of virus into normal rabbits designated as *donors*. The injection was given over a period of approximately 5 seconds into the marginal ear vein. The rabbits were then exsanguinated by cardiac puncture after appropriate time intervals. The blood was placed in a flask and allowed to clot for approximately 1 hour at 37°C. After remaining overnight at 4°C., the serum was cleared by centrifugation and pooled with other sera of the same time interval. Sera stored at 4°C. were incubated at 37°C. for about 45 minutes prior to use, generally within 2 or 3 days of collection.

The volume of sera injected into recipient rabbits for assay of circulating pyrogen varied between 10 and 50 ml. All injections were given into the marginal ear vein by means of a No. 20 guage needle at the rate of about 1 to 2 ml. per second. In most experiments, recipient rabbits received an injection of the usual dosage of either the PR8 strain of influenza virus or Newcastle disease virus (NDV) on the day prior to challenge with the donor serum. By

[1] Manufactured by Foxboro Co., Foxboro, Massachusetts.

this means they were rendered tolerant to the effects of virus which might be present in the tested samples of serum (10). Temperature readings were made at 15 minute intervals for the first 2 hours following inoculation and every half-hour thereafter for a total of 5 hours. The resulting fever curves were plotted on ⅙ inch graph paper with 5 lines on the vertical axis for each degree C. and 6 lines on the horizontal axis for each hour. In several experiments the area beneath the individual 5 hour fever curves was measured in square centimeters as previously described (7) to give a "fever index" for each recipient, an expression of both the height and duration of fever. Mean fever indices were averaged from a group of 3 or more recipients.[2]

The usual precautions were taken for the exclusion of contaminating bacterial pyrogens from all glassware, syringes, and needles (1). Physiological salt solution (0.85 per cent NaCl) was prepared from doubly distilled water. After being autoclaved, the solution was determined to be non-pyrogenic before use.

Viruses.—The PR8 strain of influenza A virus and Hickman strain of Newcastle disease virus (NDV) were employed.[3] Virus suspensions were obtained by inoculation of 10- to 11-day-old chick embryos with 10^{-3} dilutions of virus in physiological saline. After inoculation, embryos were incubated 40 to 44 hours at 35°C., chilled, and the allantoic fluids were harvested. Pooled fluids were stored in glass ampoules at −20°C. Passages of both viruses were made at monthly intervals to insure supplies of virus with constant titer. The hemagglutinin titer of the pooled allantoic fluids containing PR8 was 1:1024, the titer of NDV was 1:256. In the case of each virus, the amount injected, 1 and 4 ml., was recorded as the volume of suspension with these titers, respectively.

Hemagglutination Titration.—Hemagglutinin (HA) titers were determined by the modified Salk procedure (13). Titrations were carried out with serial twofold dilutions of virus in physiological saline. An equal volume of 1 per cent chicken erythrocytes was added to each dilution. All tests were read at 60 minutes at 4°C.

Infectivity Titration.—Serial tenfold dilutions of the serum sample to be tested were prepared in broth. 0.1 ml. of each dilution was inoculated *via* the allantoic sac to 10- or 11-day-old embryos which were incubated at 35°C. Four or 5 embryos were used per dilution. Allantoic fluids of embryos dying within 2 to 6 days were harvested and tested for the presence of hemagglutinin (14). The LD_{50} was calculated according to the method of Reed and Muench (15).

RESULTS

The Presence of a Circulating Pyrogen after Injection of PR8 Virus

The mean febrile responses of normal rabbits to intravenous injection of 1 ml. PR8 virus suspension in allantoic fluid are shown in the upper half of Fig. 1. The following points are characteristic of fever induced by this virus (9): (*a*) the long latent period of 1 to 1½ hours before onset of fever, (*b*) the development of maximum elevation of 1.5 to 2.5°C. at the 4th hour, (*c*) complete tolerance to the pyrogenic effect of the same dose of virus on the following day.

In the experiments which follow, a technique of passive transfer was em-

[2] For reasons that will be discussed in the following paper (12) normal recipients are more sensitive to the pyrogenic effects of endogenous pyrogen than are virus-tolerant recipients. Accordingly, in all cases in which the *titer* of circulating endogenous pyrogen was determined and recorded as a fever index, normal recipients were used exclusively.

[3] These viruses were obtained through the courtesy of Dr. R. R. Wagner and Dr. A. M. Prince, respectively.

ployed for the detection of circulating pyrogen (see Methods). Virus was injected intravenously into rabbits designated as donors. Samples of sera were

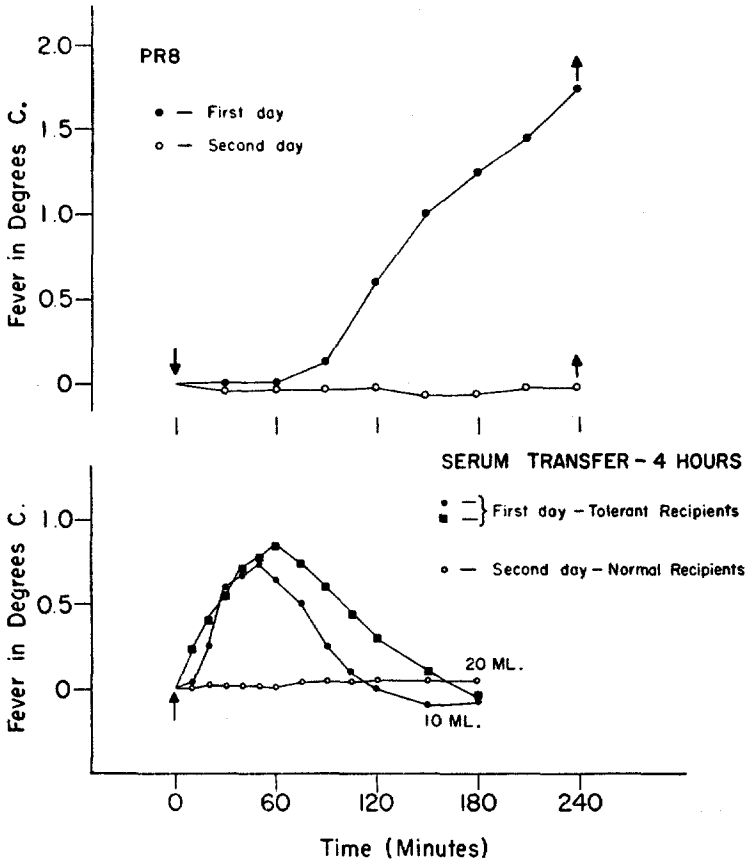

FIG. 1. Upper chart: Mean febrile responses of 6 normal rabbits to initial injection of PR8 and of 3 animals reinjected with virus on the following day. Lower chart: Mean fevers induced in virus-tolerant recipients by injection of 10 and 20 ml. pooled sera drawn from donors 4 hours after single inoculation of PR8. The mean response of normal recipients to 15 ml. unpooled sera from 2 donors reinjected with virus on day 2 and bled at 4 hours is shown for comparison. Average of 4 recipient responses is shown in each case. Upright arrows indicate time of serum transfer.

then obtained at various intervals after inoculation and tested in recipient rabbits for the presence of either endogenous pyrogen or virus. Two aspects of virus fever should be emphasized which make possible by this technique differentiation between endogenous pyrogen and virus in the serum of the donor animal. First, when animals which are tolerant to virus are used as recipients, the fever-inducing effect of endogenous pyrogen can be readily

separated from that of virus *per se* to which these recipients are unresponsive. Second, the difference in onset of fever with endogenous pyrogen and virus (short and long latent periods respectively) affords another means of distinguishing these two pyrogenic agents if present in the same serum sample.

In the lower half of Fig. 1 are shown the mean febrile responses of tolerant recipients to sera from donor rabbits injected on either the 1st or 2nd day with PR8 virus. Sera were drawn 3½ to 4½ hours after inoculation of the donors with the virus. All recipients had been given virus on the preceding day and were thus rendered tolerant to the pyrogenic effect of virus (see 2nd day curve in upper half of Fig. 1).

It is apparent that sera from donors made febrile by an initial injection of PR8 virus produced a prompt monophasic fever in tolerant recipients which were unresponsive to virus.

On the other hand, serum from tolerant donors, which remained afebrile when reinjected with virus on the 2nd day, produced no fever in either tolerant or normal recipients. This serum, therefore, contained neither demonstrable quantities of the serum pyrogen present in the febrile donor on the 1st day nor detectable amounts of circulating virus which should produce fever in normal recipients.

The following features of the febrile response to serum pyrogen resemble those previously described for an endogenous pyrogen appearing in the sera of rabbits given typhoid vaccine (7, 8): (*a*) The short latent period (less than 10 minutes), (*b*) The abrupt monophasic response with a peak in 50–60 minutes, (*c*) The rapid defervescence to normal within 2 to 2½ hours.

The Presence of Transferable Serum Pyrogen after Injection of Newcastle Disease Virus (NDV).—The intravenous injection of 4 ml. of this virus resulted in a febrile response which resembled that occurring with influenza virus but was somewhat higher and more prolonged with elevated temperatures persisting in some instances for more than 24 hours (9). Tolerance to this amount of virus was not complete on the 2nd day as in the case of PR8.

An experiment of similar design to that used with PR8 virus was performed with NDV. Sera withdrawn 3½ to 4½ hours after inoculation of the donor rabbits with NDV produced brisk monophasic fevers in virus-tolerant recipients. The mean responses to three different dosages of sera are shown in Fig. 2.

Sera from NDV-injected rabbits were somewhat more pyrogenic than were sera from donors given PR8. In the case of NDV, the mean fever peak reached 1.2°C. as compared with 0.8°C. for PR8. However, the shape of the fever curve was entirely similar in the two groups (compare Figs. 1 and 2). With the 50 ml. dosage, serum from the donors inoculated with NDV produced a somewhat more prolonged fever with a maximal temperature between 1 and 2 hours and defervescence was proportionately delayed. However, the

responses of the tolerant recipients remained essentially monophasic to all the dosages of serum employed.

Effect of Drug-Induced Modification of Body Temperature on Appearance of Circulating Pyrogen.—Since the presence of circulating pyrogen corresponds with the development of fever following the inoculation of either PR8 virus or NDV, the possibility was considered that fever *per se* might play a role in the production of this substance.

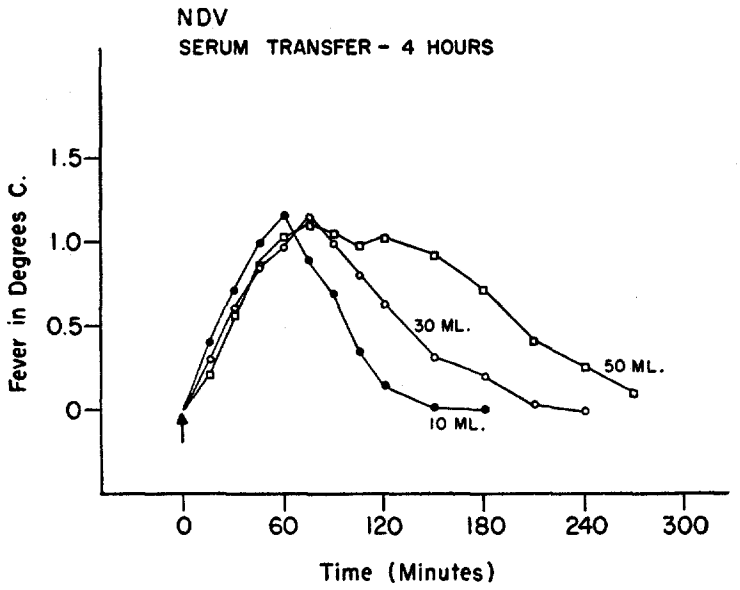

FIG. 2. Mean febrile responses of groups of 3 virus-tolerant recipients to varying dosages of pooled sera obtained from donor rabbits 4 hours after inoculation of NDV.

Accordingly, two different experiments were devised to investigate this possibility.

In the first of these a group of 6 rabbits was given a series of 3 intramuscular injections of epinephrine.[4] The injections, containing a total of 1.5 mg. of the base, were administered at 15 minute intervals (16). A rapid monophasic fever ensued reaching a mean maximum in 4 animals of 2.0°C. in 2½ hours (see Fig. 3). The other 2 rabbits with the highest temperatures (average 2.75°C.) were selected as donors, bled at 1¾ hours, and their sera pooled and transferred in 15 ml. aliquots to each of a group of 4 normal recipients.

The results are charted in the left upper section of Fig. 3. It is apparent that despite the high fever in the donors, their sera contained no transferable pyrogen.

[4] Epinephrine solution, 1:1000 (Winthrop-Stearns) containing 1.2 mg. per ml. of epinephrine hydrochloride equivalent to 1.0 mg. per ml. of the base.

In the second experiment, 2 rabbits were injected with NDV. In order to block the ordinarily occurring fever, a subcutaneous injection of 0.6 gm. antipyrine (in a solution of 200

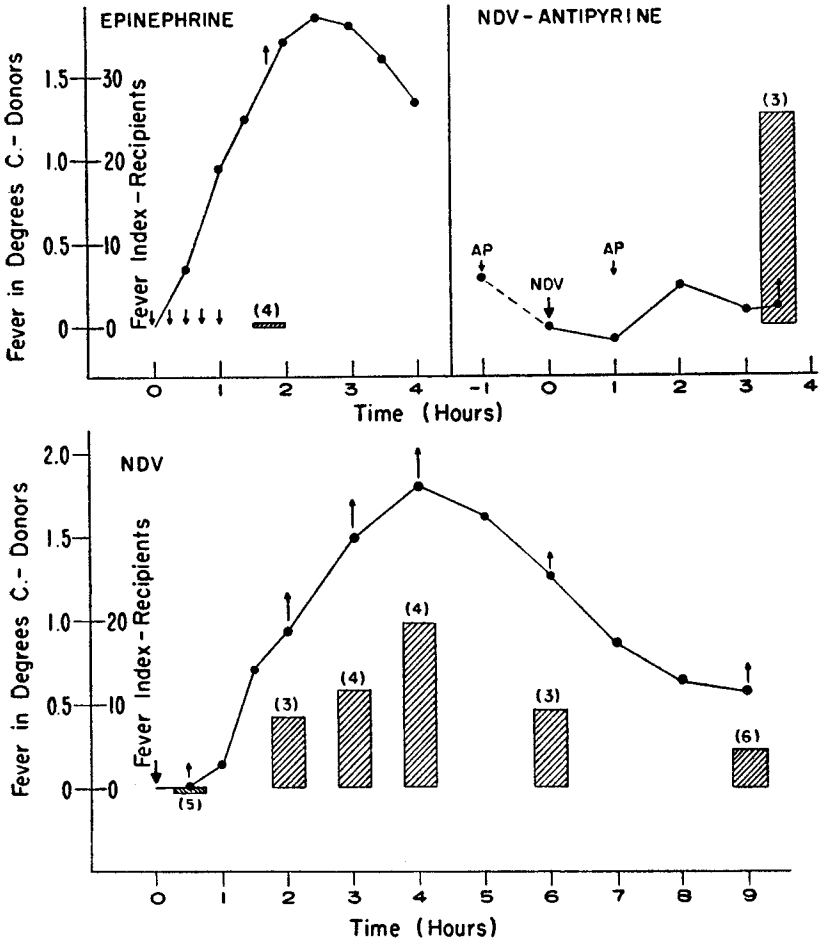

FIG. 3. Correlation between fever and circulating endogenous pyrogen. Relation of mean fevers induced in donor animals by epinephrine and NDV (with or without antipyrine) to circulating amounts of endogenous pyrogen as measured by passive transfer to normal recipients. The average fever indices for each interval are calculated from groups of 3 to 6 animals (shown by numbers above each bar). Upright arrows indicate times at which donors were bled.

mg. per ml.) was given 1 hour before and a second injection of 1.2 gm. was administered 1 hour after the inoculation of virus (10). By this means fever was almost entirely abolished (mean maximal rise of < 0.3°C.). However, despite the absence of fever in the donor animals there was no diminution in the amount of circulating pyrogen present in their sera at 4 hours when tested in normal recipients (see Fig. 3, upper right graph).

The results of these two experiments indicate conclusively that the production of circulating pyrogen is not caused by fever.

Relation of Virus-Induced Fever to Circulating Levels of Endogenous Pyrogen.—The titer of endogenous pyrogen in the sera of animals injected with typhoid vaccine coincides closely with the height and duration of fever produced by the injected vaccine in the donor animal (8). This correlation is strongly suggestive of a causal relationship.

A similar experiment was performed with NDV. Donor animals were given an initial injection of virus. Groups of animals were then bled at one of the following intervals after inoculation: 30 minutes, 2, 3, 4, 6, and 9 hours. The sera from each group of donor animals were then pooled and tested for the presence of pyrogenic activity in normal recipients.[5] The mean fever index of the recipients injected with serum drawn at each time interval was then calculated (see Methods).

The results are shown in the lower half of Fig. 3. The mean fever of 8 donor rabbits to the virus is compared with the level of circulating pyrogen present in their sera at the various intervals tested. It is apparent that the amount of transferable pyrogen is closely correlated with the actual shape of the fever curve. No pyrogen was present during the latent period and maximal amounts appeared with the height of the fever at 4 hours. Finally, by 9 hours, when the temperature had nearly returned to normal, there was a marked diminution in the amount of circulating pyrogen.

Relation of Circulating Virus to the Appearance of Serum Pyrogen.—In order to determine the rate of disappearance of virus from the blood stream a group of normal rabbits was injected with NDV and bled by intracardiac puncture at 30 minutes and 4 hours after the inoculation. These two intervals correspond to the latent period and the height of the fever respectively following inoculation of virus (see Fig. 1).

Infectivity and hemagglutinin titrations were then performed on the pooled sera from each of these two intervals (see Methods).

The results of two such experiments are plotted in Fig. 4. The rapid disappearance of infectious virus from the circulation is evident. At 30 minutes, the titer had fallen to less than 10^1 and at 4 hours no titratable virus was present. HA titers performed on the pooled samples of sera from both intervals were negative.

These results are to be compared with the effects of the 30 minute and 4 hour sera. (See shadowgraphs in Fig. 4.) The 30 minute serum was non-pyrogenic to both normal and tolerant recipients, indicating the absence of both serum pyrogen and pyrogenic amounts of virus.[6] The 4 hour serum, on the other

[5] The use of normal rather than tolerant recipients to determine the titer of endogenous pyrogen in the blood is discussed in Methods.

[6] In another experiment, 2 donor animals were inoculated with NDV and bled at 10 minutes. The pooled heparinized whole blood was immediately transferred in 20 ml. individual doses to 3 normal recipients. No febrile response was obtained, indicating that with this technique the pyrogenic effects of injected virus are undetectable within 10 minutes.

hand, though without demonstrable amounts of virus by infectivity titration, contained circulating pyrogen and caused characteristic brief fevers in virus-tolerant recipients.

The presence of circulating pyrogen, therefore, is not associated with titratable virus. Conversely, serum obtained during the latent period at 30 minutes, though containing small amounts of virus, was non-pyrogenic.

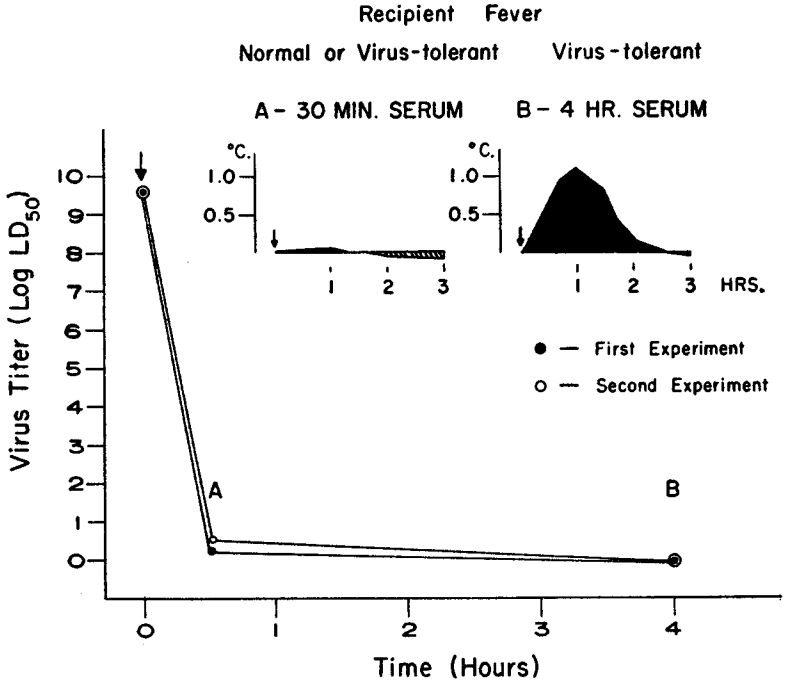

FIG. 4. "Clearance" of NDV from the blood stream of normal rabbits. Data in the first experiment are from pooled sera of the same 3 animals. In the second, sera were pooled from 2 animals at 30 minutes and from 6 at 4 hours. Mean pyrogenic responses of both normal and tolerant recipients to 10 ml. sera drawn at 30 minutes and of tolerant recipients to 10 ml. sera at 4 hours are shown in the shadowgraphs.

Attempt to Produce Endogeneous Pyrogen by in Vitro Techniques.—The intravenous injection of the influenza viruses regularly results in a marked fall in circulating lymphocytes. The development of lymphopenia coincides closely with the rise in temperature, both being maximal at about 4 hours after inoculation of the virus. There is no consistent change in the number of polymorphonuclear leukocytes (9, 17).

The close correlation between the development of fever and lymphopenia suggested the possibility of an interaction between circulating virus and lymphocytes. It was decided to determine whether such a combination *in vitro* might result in the production of endogenous pyrogen.

A normal rabbit was sacrificed by intravenous injection of air. Immediately after death peritoneal and axillary lymph nodes were dissected out and placed in pyrogen-free saline., The nodes were stripped of surrounding fatty tissue and gently macerated with teasing needles to allow release of lymphocytes. The material was then filtered through a No. 80 wire mesh and centrifuged at 2000 R.P.M. for 15 minutes (18). The supernatant was discarded, pyrogen-free saline was added to the sedimented cells to a total volume of 10 ml., and cell count and viability studies performed. Supravital staining with a freshly prepared 1 per cent solution of trypan blue revealed 70 to 80 per cent were alive (19). Except during centrifugation all procedures were carried out in an ice bath. Total lymphocyte count revealed 6×10^9 cells which were then diluted with either pyrogen-free saline or serum to six aliquots of 10 ml. containing 1×10^9 cells each. This number is approximately equal to the total number of circulating lymphocytes in the rabbit (20). 0.8 ml. undiluted NDV virus was added to each of 5 aliquots and incubated for 3½ hours at 37°C. After incubation, the virus-cell mixtures were injected in individual dosages of 10 ml. to 5 tolerant recipients. The sixth aliquot (cell-saline suspension) served as a control and after the same period of incubation was given to a normal recipient.

Additional experiments of similar design were carried out to determine whether other constituents of the blood were responsible for the elaboration of endogenous pyrogen. In these, PR8 virus was added in a final concentration of 1:100 to one of the following substances and incubated at 37°C. for 4 hours: (*a*) fresh heparinized whole blood, (*b*) normal serum or plasma, (*c*) 50 per cent suspension of washed rabbit erythrocytes in physiological saline. 20 ml. of each mixture (with 0.2 ml. virus) were given to each of 3 to 6 tolerant recipients.

In no instance did any of the mixtures containing either NDV or PR8 virus produce a rapid onset of fever when injected into tolerant recipients. Under these conditions, therefore, the incubation of virus with either whole blood, plasma, serum, or lymphocytes *in vitro* did not appear to cause the release of endogenous pyrogen.

Effect of Nitrogen Mustard (HN_2)-Induced Leukopenia.—The pyrogenic properties of endogenous pyrogen present in the circulation of donor animals given either typhoid vaccine or virus resemble closely those hitherto described for an endogenous pyrogen extracted from polymorphonuclear leukocytes. On the other hand, previous studies have indicated that animals made severely granulocytopenic with HN_2 have identical fevers as compared with controls when challenged either with endotoxins or with PR8 virus (21, 22). Recently, a marked decrease in circulating endogenous pyrogen has been demonstrated when such leukopenic animals are injected with typhoid vaccine (23).

It was of interest, therefore, to determine whether animals rendered leukopenic with HN_2 and injected with virus would differ from normal animals in respect to production of endogenous pyrogen.

A group of 12 rabbits was given an intravenous injection 5 or 7 mg. of HN_2. Daily white blood cell counts were performed on all animals. On the 3rd to the 5th day, when there appeared to be a maximal depression of circulating leukocytes, 6 animals with the lowest counts (average $1.15 \pm 0.16 \times 10^3$ per $mm.^3$) were selected as donors and individually injected with 4 ml. NDV. Three and one-half to 4 hours after inoculation, these animals were bled. Unpooled sera from the 6 donors were then given in 15 ml. dosages to 12 normal recipients. The results are shown in Fig. 5.

In confirmation of previous studies (22), there was no evident difference in the mean pyrogenic response of the leukopenic donors to this dosage of virus although there was a somewhat greater variability in response, as compared with controls. In addition, there appeared to be no significant reduction in the amount of transferable endogenous pyrogen in the 4 hour sera of leukopenic donors challenged with virus. Finally, the mean response of a group of similarly leukopenic recipients to 15 ml. endogenous pyrogen from normal donors was

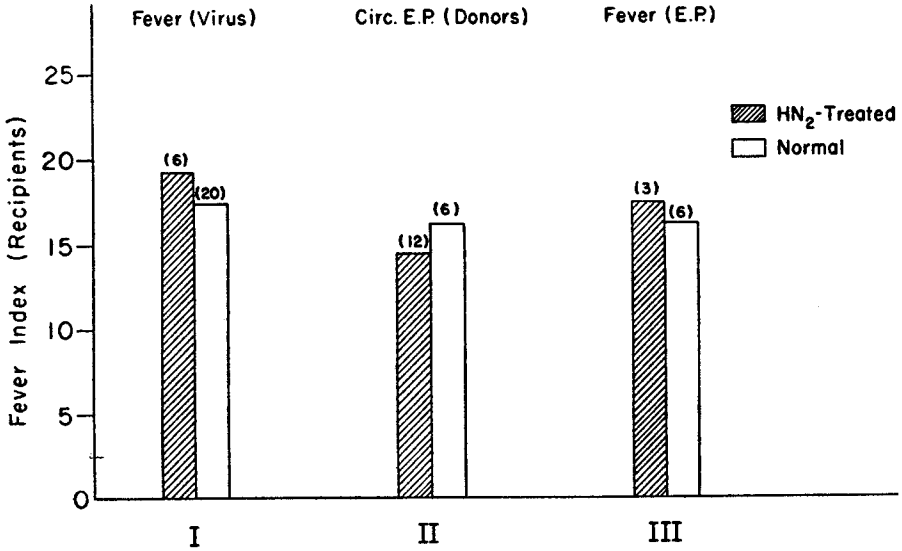

FIG. 5. Comparison of the action of HN_2-induced leukopenia on febrile response to NDV (I), production of endogenous pyrogen after injection of virus (II), and response to injected endogenous pyrogen (III). Mean fever indices of leukopenic animals are compared with normal controls in each case. The titers of circulating endogenous pyrogen in both leukopenic and normal donors (II) were measured in normal recipients. Figures above each bar represent number of animals tested. Virus fever is calculated on the basis of 4 hours. Other fever indices represent 5 hour responses. E.P., endogenous pyrogen.

similar to the average response of normal recipients to the same dosage of endogenous pyrogen.

These results indicate that the febrile response to either virus or endogenous pyrogen does not depend upon a normal number of circulating leukocytes. Moreover, the presence of leukopenia does not impair the production of endogenous pyrogen in donors injected with virus.

Injection of Virus into Prepared Peritoneal Exudates.— Despite the foregoing negative evidence that the polymorphonuclear leukocyte is implicated in virus fever, it was thought advisable to carry out further experiments with this cell since it is the only known source of endogenous pyrogen (5). It was reasoned that if the interaction of virus with polymorphonuclear leukocytes resulted in

the release of endogenous pyrogen, there might be an acceleration in the febrile response when virus was injected into a prepared sterile exudate.

Four rabbits were tied down on their backs and their abdomens shaved with electric clippers. A single injection of 300 ml. of normal saline was then given intraperitoneally in 3 to 5 minutes through a No. 17 gauge needle and the abdomen massaged gently to distribute the injected fluid throughout the peritoneal cavity. Recent studies have demonstrated that this

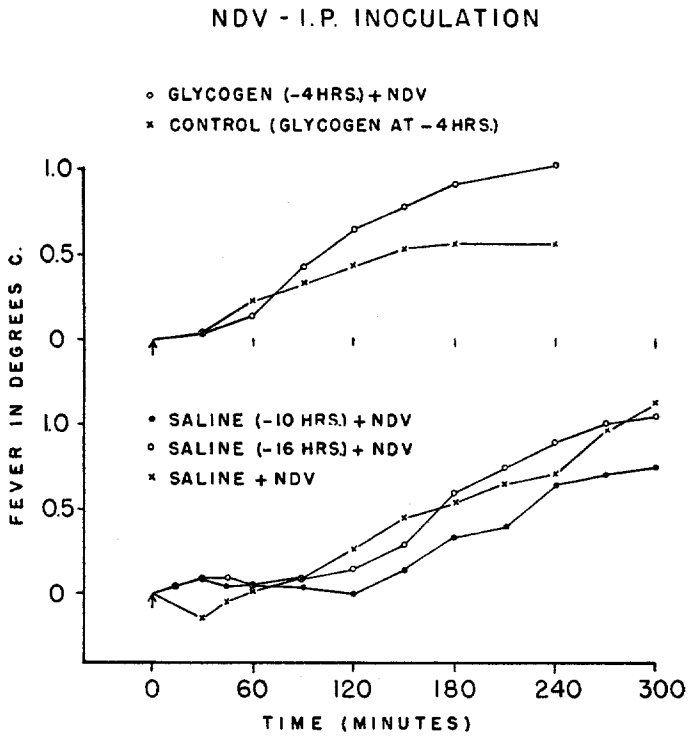

FIG. 6. Mean febrile responses induced by intraperitoneal inoculation of NDV into prepared exudates. Curves shown are averaged from 4 animals in each group. (Groups preinoculated with saline contain 2 animals each.)

procedure regularly evokes in 10 to 15 hours a peritoneal exudate containing 1.1 to 4.8 $\times$ 10^8 cells consisting almost exclusively of polymorphonuclear leukocytes (24).

Accordingly, at 10 and at 16 hours after saline injection, the experimental group (2 animals at each time period) was injected intraperitoneally with 4 ml. NDV. A control group of 4 animals was given the same amount of saline (warmed to 38°C.) and NDV simultaneously by means of a single intraperitoneal inoculation. Both groups of animals were then placed in boxes and their temperatures recorded.

Because of the report of Hirsch (25) that more cellular exudates (5 $\times$ 10^8 to 2 $\times$ 10^9)[7] can be obtained at 4 hours by the addition of small amounts of glycogen (C. P. Amend Drug

[7] This is approximately equal to the total number of circulating polymorphonuclear leukocytes in the normal animal (20).

and Chemical Co., Inc., New York), a second experiment of similar design was performed with the use of a saline solution containing glycogen. The glycogen was added in a concentration of 1 mg. per ml. just prior to the inoculation of the animal. In this modification of the initial experiment, virus was given to the experimental group of 4 animals 4 hours after intraperitoneal inoculation.

The results of both experiments are shown in Fig. 6. The glycogen mixture itself, unlike saline alone, proved pyrogenic as was evident by the gradual rise in temperature in the control group throughout the period of observation. However, in neither experiment did the inoculation of virus into the prepared exudates result in any reduction in the latent period which might indicate the liberation of an increased amount of endogenous pyrogen.

DISCUSSION

The present studies have demonstrated that a circulating pyrogen is present in the sera of rabbits made febrile by the injection of either PR8 strain of influenza A virus or NDV. Several features suggest that this substance is associated with the production of fever. There was a close correlation between the fever curve induced by virus and the amount of transferable pyrogen in the circulation. No pyrogen was detectable in the latent period and there was markedly less during defervescence 9 hours after injection of virus. Furthermore, this substance was absent in the sera of tolerant animals which remained afebrile after a second injection of PR8 virus.

There are certain biological characteristics which indicate that the pyrogen is separable from the injected virus and hence apparently endogenous in origin. Noteworthy is the fact that endogenous pyrogen produced fever in the recipient rendered tolerant to reinjection of virus. The rapid onset of the febrile response is also in contrast to the long latent period following injection of virus in the normal animal. The brief monophasic fevers caused by this pyrogen in virus-tolerant recipients resemble closely those seen with endogenous pyrogen obtained from polymorphonuclear leukocytes and present in the sera of animals given bacterial endotoxins such as typhoid vaccine. Other studies have shown that a circulating endogenous pyrogen with similar properties is present in rabbits made febrile by intrapharyngeal or intradermal inoculation of living streptococci (26, 27) and in hypersensitive animals injected intravenously with ordinarily non-pyrogenic antigens such as old tuberculin (28) and egg albumen (29). Finally, an endogenous pyrogen has recently been detected both in the thoracic duct lymph and in the blood streams of rabbits infected intraperitoneally with pneumococci (30, 27). In all of these latter instances, with the possible exception of streptococci (31), it should be emphasized that the development of fever cannot be attributed to any known endotoxin-like fraction of the injected material which might undergo modification within the host to an apparently "endogenous" pyrogen (32).

Further study will be necessary to determine whether the pyrogens obtained

under these different experimental situations are, in fact, identical. No attempt has as yet been made in this laboratory to characterize biochemically endogenous pyrogen appearing in the sera of animals given either endotoxins or viruses. The lack of tests other than pyrogenicity for the detection of this substance and the necessity of carrying out biochemical techniques in a pyrogen-free environment have made such study difficult. A few studies have been reported by Bennett and Beeson with the endogenous pyrogen extracted from polymorphonuclear leukocytes (6).

The following preliminary observations have been obtained with NDV-induced endogenous pyrogen.

(*a*) The substance appears to be relatively stable. Unlike either of the viruses employed, it is not inactivated at temperatures up to 70°C. for 30 minutes although it does not differ in this regard from leukocytic (6) or other serum endogenous pyrogen (33). Likewise, its fever-inducing capacity is not diminished by remaining at 4°C. for periods up to at least 3 months.

(*b*) The electrophoretic pattern of serum containing endogenous pyrogen is indistinguishable from that of normal serum. This fact has also been reported with endogenous pyrogen derived from sterile peritoneal exudates (6).

(*c*) There is no consistent change in serum properdin titers[8] obtained either before injection of virus, during the latent period, or at 4 hours when maximal amounts of endogenous pyrogen are present.

Since the source of endogenous pyrogen which appears in the circulation after injection of virus still remains unknown, the possibility that endogenous pyrogen represents a pyrogenic fraction of the originally injected virus cannot be excluded. However, the re-emergence of these viruses into the blood stream after initial clearance has not been demonstrated to our knowledge. Furthermore, tests for virus by the standard techniques of infectivity and hemagglutination titrations do not reveal its presence.

It should be pointed out that if there is a single source of endogenous pyrogen, the mechanism by which this substance is released or activated must be different in these various types of experimental fever. There are important biochemical differences in the pyrogenic agents themselves and also in the fevers resulting from an injection of virus and bacterial endotoxin (9, 10). Similarly, the rapid, transient fall in circulating polymorphonuclear leukocytes after injection of endotoxin (1, 2) is unlike the slower but more sustained lymphopenia which is produced by virus (9, 17). The significance of changes in circulating cells in the pathogenesis of fever with either of these agents has not, however, been established with certainty.

The lymphopenia which follows the injection of virus coincides with the height of the fever and suggests the possibility of cellular injury. However,

[8] Kindly performed by the late Dr. L. Pillemer.

endogenous pyrogen was not produced by *in vitro* techniques, involving incubation of virus with either lymphocytes or other elements of the circulation.

Ginsberg *et al.* (34–36) have recently shown that influenza viruses are capable of causing certain biochemical alterations in guinea pig polymorphonuclear leukocytes to which they are adsorbed *in vitro*. The inhibition of phagocytosis caused by these agents suggests cellular injury in the absence of viral multiplication. It is tempting to hypothesize that a similar series of events may take place after injection of virus *in vivo*, resulting in the release of endogenous pyrogen by the damaged leukocytes. However, the evidence presented here does not support the possibility that the polymorphonuclear leukocyte produces the endogenous pyrogen found circulating in this type of fever.

Techniques which markedly alter the number of polymorphonuclear leukocytes at the site of inoculation did not modify virus fever. The injection of virus, either intravenously into leukopenic recipients or intraperitoneally into prepared sterile exudates, did not result in any apparent difference in either the onset or the duration of fever as compared with controls. In addition there appeared to be an approximately equal amount of endogenous pyrogen in the sera of both normal and leukopenic animals given virus. There are certain limitations, however, in all these techniques for modifying the total number of polymorphonuclear leukocytes. The number of these cells present either in the circulation or at a local site of inflammation is an insignificant fraction of the total available in the body (37, 38). Also, since it is virtually impossible to eliminate polymorphonuclear leukocytes entirely from such extravascular sites, the results of the experiments reported here do not justify the exclusion of this cell as a source of endogenous pyrogen.

The possibility was considered that endogenous pyrogen is similar to the tissue polysaccharides recently isolated by Landy *et al.* (39). These materials have been found to reproduce all the biological phenomena which had previously been thought to be uniquely associated with endotoxins. There is suggestive evidence that endogenous pyrogen is different from tissue polysaccharides. The release of endogenous pyrogen in the donor animal given virus is not associated with the characteristic changes in circulating polymorphonuclear leukocytes (initial leukopenia followed by leukocytosis) which occur when either bacterial or tissue polysaccharides are injected (1, 39). Further evidence for the dissimilarity between these substances and endogenous pyrogen obtained in other types of experimental fever is presented elsewhere (8, 27, 30, 40–44).

In conclusion, the experimental data presented here strongly support the hypothesis that the intravenous inoculation of viruses of the influenza group is followed by the release of an endogenous pyrogen from some as yet undefined tissue source. It is the circulation of this latter substance which, in turn, is believed to be responsible for the development of fever. The rapid onset of fever

following injection of viral-induced endogenous pyrogen further suggests that this material like other endogeneous pyrogens acts directly on the thermoregulatory centers of the brain (42).

SUMMARY

A substance with pyrogenic properties appears in the blood streams of rabbits made febrile by the intravenous inoculation of the PR8 strain of influenza A and Newcastle disease viruses (NDV). By means of a technique involving passive transfer of sera from animals given virus to recipient rabbits, the titer of circulating pyrogen was found to be closely correlated with the course of fever produced by virus.

Certain properties of the pyrogen are described which differentiate it from the originally injected virus and suggest that the induced pyrogen is of endogenous origin. These properties resemble those of endogenous pyrogens occurring in other forms of experimental fever.

The source of virus-induced pyrogen is unknown. *In vitro* incubation of virus with various constituents of the circulation did not result in the appearance of endogenous pyrogen.

Granulocytopenia induced by HN_2 failed to influence either fever or the production of endogenous pyrogen in rabbits injected with NDV. Similarly, the intraperitoneal inoculation of NDV into prepared exudates did not modify the febrile response. These findings do not lend support to the possibility that the polymorphonuclear leukocyte is a significant source of endogenous pyrogen in virus-induced fever.

It is concluded that the liberation of an endogenous pyrogen from some as yet undefined source is an essential step in the pathogenesis of fever caused by the influenza group of viruses.

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