THE PRODUCTION OF FEVER BY INFLUENZAL VIRUSES

I. Factors Influencing the Febrile Response to Single Injections of Virus

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(Received for publication, June 14, 1949)

Certain microorganisms are capable of producing toxic effects in animals without causing active infection. The parenteral injection of the "endotoxins" or "pyrogens" of Gram-negative bacilli produces rapid rises in body temperature (1), changes in circulating leucocytes (2), hemodynamic alterations (3), and diffuse vascular changes in sensitized (4) or susceptible (5) tissues. Chemical analysis indicates that the active fractions of these organisms are polysaccharides possessing similar characteristics, prominent among which is marked heat stability (6, 7). Some purified fractions have been found to be non-antigenic and there is no neutralization of their action by immune serum (8).

Due to difficulties in purification, very little is known about the inherent toxic effects of viral products. Henle and Henle (9) have shown that the intracerebral inoculation of influenza virus in mice causes convulsions even when there is no evidence of virus multiplication. Intraperitoneal or intravenous injection of this virus in animals causes death, associated with diffuse hemorrhagic lesions (10). Harris and Henle (11) have described profound lymphopenia in rabbits following intravenous injection of irradiated influenza virus. Although some of these toxic reactions to influenza virus appear to resemble those of bacterial pyrogens, they are distinguished by the neutralizing effect of homologous immune serum for virus. The toxic property is not separable from the virus particle and is more resistant to heat than is the infective capacity (9).

During an investigation of the toxicity of viruses of the influenza group, it was found that the intravenous injection of allantoic fluid containing virus in rabbits was followed by febrile responses (12). This effect on body temperature differs in some respects from that which follows the injection of bacterial pyrogens. The present report describes the febrile response to influenza and Newcastle disease viruses and points out the relation of this reaction to certain *in vitro* properties of the viruses.

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Materials and Methods

Animals.—Male rabbits of mixed breed weighing 2500 to 3000 gm. were used. During experiments they were placed in individual stalls and secured by loose-fitting neckboards. Three rectal temperatures were taken at 30 minute intervals to establish a base line and the virus preparations were injected into the marginal ear veins. Rectal temperatures were then recorded at 30 minute intervals for 6 hours. Animals were given no food or water and were not moved from the stalls during experiments. Room temperature was maintained at 70–73°F. A new group of animals was used in each experiment.

Virus Preparations.—The following egg-adapted viruses were used: PR8 strain of influenza A, Lee strain of influenza B, and the "B" strain of Newcastle disease virus (NDV)[1]. Uniform virus suspensions were prepared by inoculation of 10 day old chick embryos by the allantoic route with 10^{-3} or 10^{-4} dilutions of virus in physiologic salt solution. After incubation at 36.5°C. for 44 hours, viable embryos were chilled for 4 hours at 4°C. and blood-free allantoic fluids were harvested and pooled. After testing for bacterial sterility, pooled fluids were stored in 2.0 ml. lots at −76°C. in sealed glass ampoules. The original hemagglutinin titers of the pooled allantoic fluids varied from 1:1024 to 1:4096. The titers of all fluids were adjusted to 1:1024 by dilution with physiologic salt solution immediately before injection and the amount of virus injected was recorded as the volume of suspension with a titer of 1:1024.

Serology.—Hemagglutinin titers were determined by the method of Salk (13). These titrations were carried out at room temperature and read at 90 minutes for the influenza viruses and 45 minutes for NDV.

Avoidance of Bacterial Pyrogens.—All glassware was sterilized by baking at 170°C. for 2 hours. This is sufficient to destroy any contaminating bacterial pyrogen (7). Physiologic salt solutions were tested at intervals in animals and were always pyrogen-free.

Leucocyte Counts.—During certain of the experiments, total and differential counts of leucocytes in the peripheral blood were performed at 60 minute intervals. Freely flowing drops of blood were obtained by making small incisions over the ear veins, a new site being used for each determination. Mouse WBC pipettes[2] were employed. A coverslip smear was made with each count and one hundred cells were classified for differential counts.

EXPERIMENTAL RESULTS

In the experiments to be described, the temperature curves for individual animals in any given group were remarkably consistent. Therefore, composite curves have been used to compare differences between groups. Using the mean temperature at the time of injection as a base line, average temperature changes were charted at 30 minute intervals.

Effect of Virus in Allantoic Fluid.—All three viruses tested caused fever in rabbits although there were certain variations between strains and for different dosages of the same strain.

Rabbits injected intravenously with 1.0 ml. of PR8 virus suspension showed elevations of temperature which began 1½ to 2 hours after injection, reached a peak of 2.5 to 3.5°F. at the 5th hour, and gradually fell to normal within 24 hours. As little as 0.025 ml. of the virus caused fever, although the temperature

[1]Kindly supplied by Dr. F. B. Bang.

[2]One-fifth the size of human WBC pipettes, manufactured by Thomas M. Walton Co., Philadelphia.

rises were of a lower order. With decreasing amounts of virus, the lag period between injection and the beginning of the temperature rise lengthened to as much as 3 hours. Fig. 1 illustrates the febrile response to varying doses of this virus.

One ml. of the Lee strain produced an average elevation of temperature slightly higher than that seen after PR8 virus. The curves were similar in time of initial rise and duration, however. With larger doses of Lee virus, the temperature rose earlier and reached its peak sooner. Two animals given a lethal dose, 40.0 ml., developed fever within 30 minutes which was sustained until death 18 hours later. Fig. 2 summarizes these results.

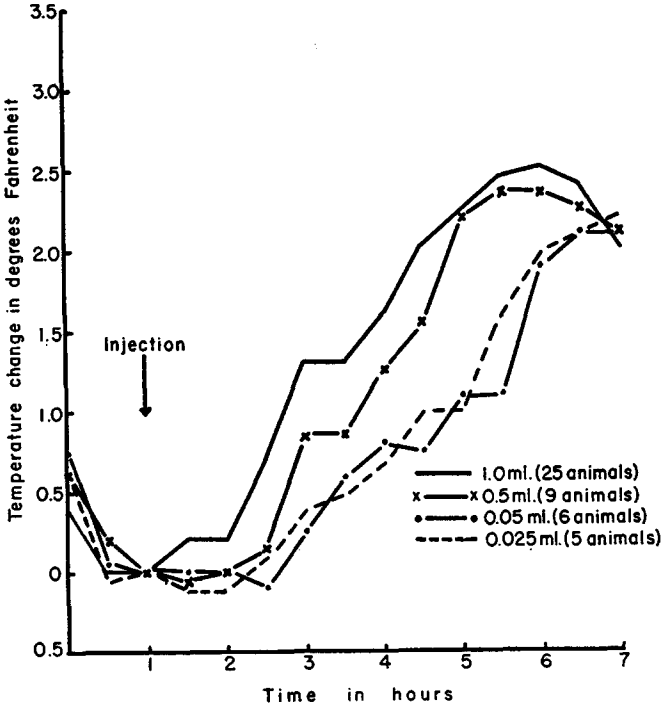

FIG. 1. Temperature responses of groups of rabbits given varying amounts of allantoic fluid containing PR8 virus (hemagglutinin titer 1:1024). With decreasing amounts of virus the lag period between injection and beginning of temperature rise lengthened.

Fig. 3 demonstrates the effect of 4.0 ml. and 8.0 ml. of allantoic fluid containing the "B" strain of NDV. In respect to average time of elevation and to height, the temperature curves of animals receiving this virus resembled those following influenza virus injection. The febrile response, however, was of longer duration, extending to 48 hours. About one-third of these animals died; there was no mortality from comparable amounts of influenza virus.

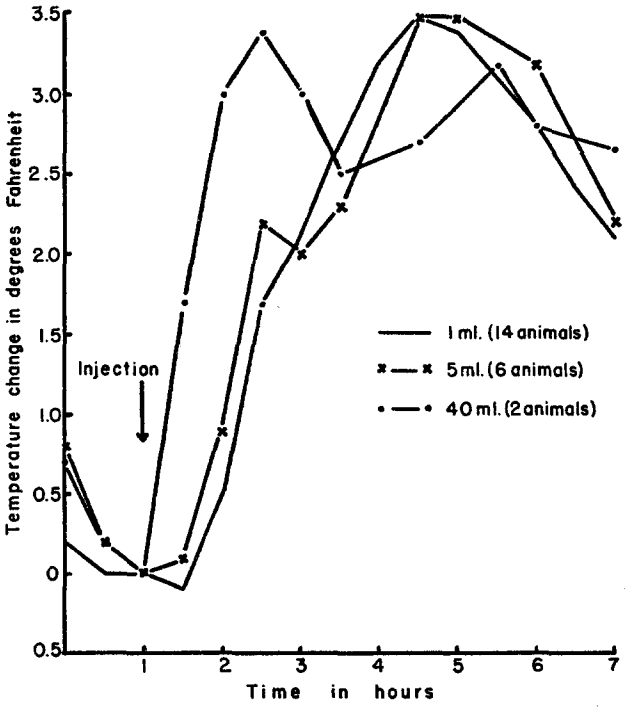

FIG. 2. Temperature records of groups of animals after intravenous injection of allantoic fluid containing Lee virus (hemagglutinin titer 1:1024). Note the rapid rise in temperature after 40.0 ml. of virus suspension; both animals given this dose died in about 18 hours.

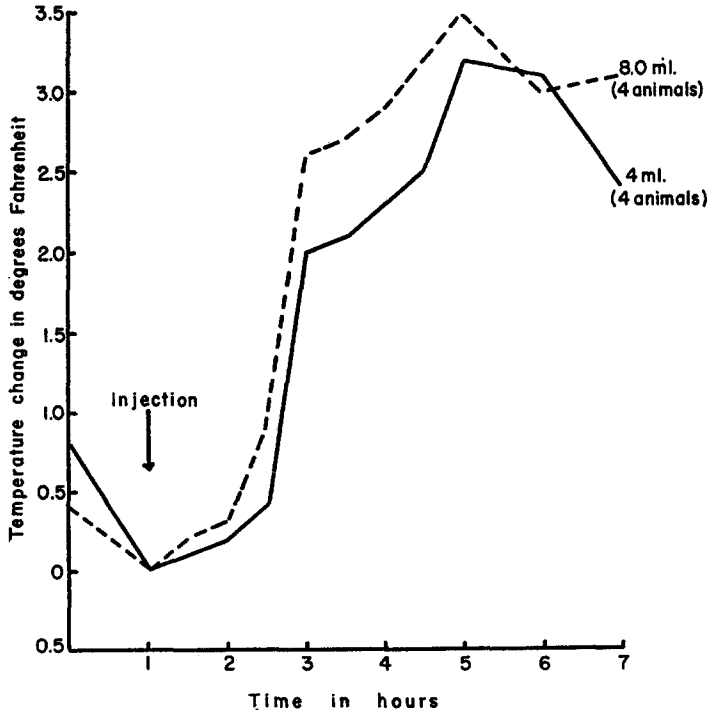

FIG. 3. Fever curves of two groups of rabbits given allantoic fluid suspensions of the "B" strain of Newcastle disease virus (hemagglutinin titer 1:1024). Temperature elevations of these animals were of longer duration than those after influenza virus.

Effect of Saline Suspensions of Virus.—Ultracentrifugal analysis of influenza virus has shown that the infective and hemagglutinating properties of the virus are removed with the 600-S component (14). The toxic factor described by Henle and Henle (9) has not been dissociated from the infective particle. The following experiment was designed to ascertain whether the pyrogenic activity

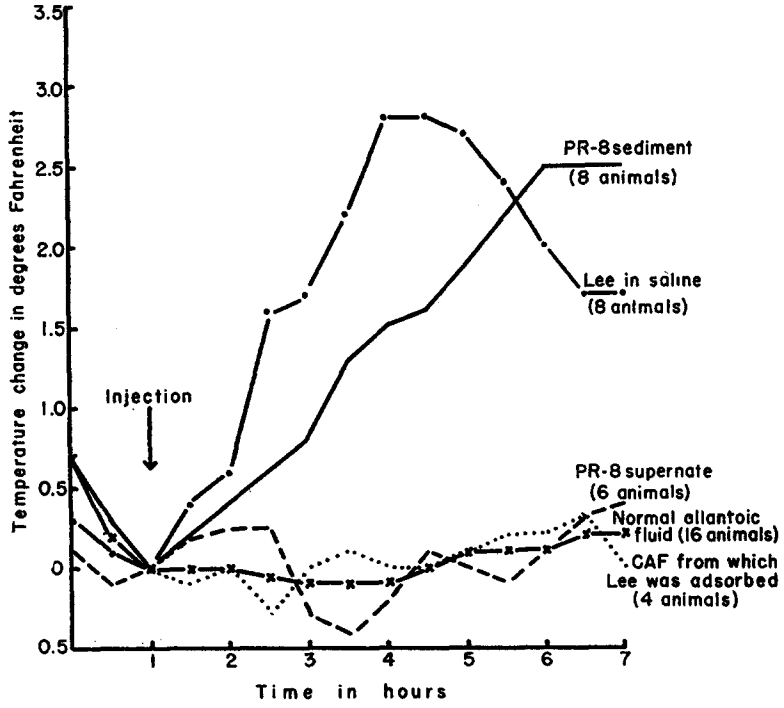

Fig. 4. Temperature records showing the production of fever by PR8 virus separated from allantoic fluid by high-speed centrifugation and Lee virus removed from allantoic fluid by adsorption onto chicken erythrocytes and elution into physiologic saline solution. Neither of the fluids from which virus was removed produced significant temperature elevations. Normal allantoic fluid was non-pyrogenic.

is associated with the virus particle or some soluble product in the allantoic fluid.

Ten ml. of an allantoic fluid suspension of PR8 virus (hemagglutinin titer 1:1024) was centrifuged at 30,000 R. P. M. for 100 minutes. The hemagglutinin titer of the supernatant allantoic fluid was reduced to 1:4 and four rabbits given 1.0 ml. of this material intravenously remained afebrile. The sediment reconstituted in 9.0 ml. of physiologic salt solution showed a hemagglutinin titer of 1:1024; when injected into eight animals in 1.0 ml. amounts, this material produced typical fevers (Fig. 4).

Purification of influenza virus by adsorption on chicken erythrocytes and

elution leaves the properties of the virus unchanged (15). In the following experiment, influenza virus was tested in rabbits after separation from allantoic fluid by this method.

Influenza virus (Lee) was adsorbed from 20 ml. of allantoic fluid (hemagglutinin titer 1:1024) with 5.0 ml. of packed, washed chicken erythrocytes at 4°C for 1 hour. After centrifugation in refrigerated cups at 3,000 R. P. M., the hemagglutinin of the supernatant allantoic fluid had fallen to 1:4 and this material, when injected into six rabbits in 1.0 ml. amounts, produced no fever. The adsorbed virus was allowed to elute into 12.0 ml. of physiologic salt solution at 37°C. for 3 hours and the red cells were removed by centrifugation at 3,000 R. P. M. The hemagglutinin titer of the saline eluate was 1:1024; 11 animals given 1.0 ml. of this preparation showed typical febrile responses (Fig. 4).

Normal allantoic fluids were tested in 16 animals and were non-pyrogenic (Fig. 4).

Effect of Heated Virus.—Certain properties of the influenza viruses are differentially susceptible to heat. In general, the ability to agglutinate red cells is more resistant to heat than the infective capacity (16). The hemagglutinin of Lee withstands higher temperatures than does that of PR8 (17).

Exposure of allantoic fluid containing PR8 virus to a temperature of 56°C. for 30 minutes destroyed its infectivity for chick embryos and reduced its hemagglutinin titer from 1:1024 to 1:64. Six animals injected intravenously with this material in 1.0 ml. amounts showed febrile responses (Fig. 5).

Allantoic fluid preparations of both PR8 and Lee virus were heated simultaneously at 62°C. for 30 minutes. PR8 virus lost both infectivity for chick embryos and hemagglutinating ability and produced no fever in 6 rabbits given 1.0 ml. intravenously. The Lee virus was rendered non-infectious but retained hemagglutinating capacity, its titer falling from 1:1024 to 1:128. A group of 6 animals received 1.0 ml. each of this material and responded with fevers as is shown in Fig. 5.

Heating Lee virus at 70°C. for 30 minutes destroyed infectivity for chick embryos, hemagglutinin, and fever-producing capacity (Fig. 5).

It would appear that the production of fever by these viruses is associated with their capacity to agglutinate erythrocytes. The relative heat lability of the viral fever-producing factor is in marked contrast to the heat resistance of bacterial pyrogens which withstand prolonged autoclaving (7).

Effect of Virus Adsorbed on Chicken Erythrocytes.—Various substances neutralize the hemagglutinin of influenza virus. Saline extracts of erythrocytes and many tissues will inhibit virus hemagglutination (18). The ability of influenza virus to elute from red cells is destroyed by heat before the hemagglutinin is affected (19). The following experiments were designed to determine the effect on fever production of injecting virus adsorbed on erythrocytes.

Five ml. of allantoic fluid containing PR8 virus (hemagglutinin titer 1:2048) was exposed to 2.5 ml. of packed, washed chicken erythrocytes at 4°C. for 2 hours. After centrifugation at 3,000 R. P. M. in refrigerated cups, the hemagglutinin titer of the supernatant allantoic fluid had fallen to less than 1:2. The erythrocytes were resuspended in cold physiologic salt

solution and each of 4 rabbits was given 1.0 ml. of this red cell suspension. All 4 animals responded with fever but the temperature rises were less than would be expected with comparable amounts of free virus. Four animals given 1.0 ml. of a 50 per cent suspension of normal chicken erythrocytes as controls had no fever (Fig. 6).

An allantoic fluid suspension of PR8 virus was heated at 56°C. for 20 minutes, reducing its hemagglutinin titer from 1:4096 to 1:512. Ten ml. of this material was then exposed to 2.5 ml. of packed, washed chicken erythrocytes at 4°C. for 2 hours and centrifuged in refriger-

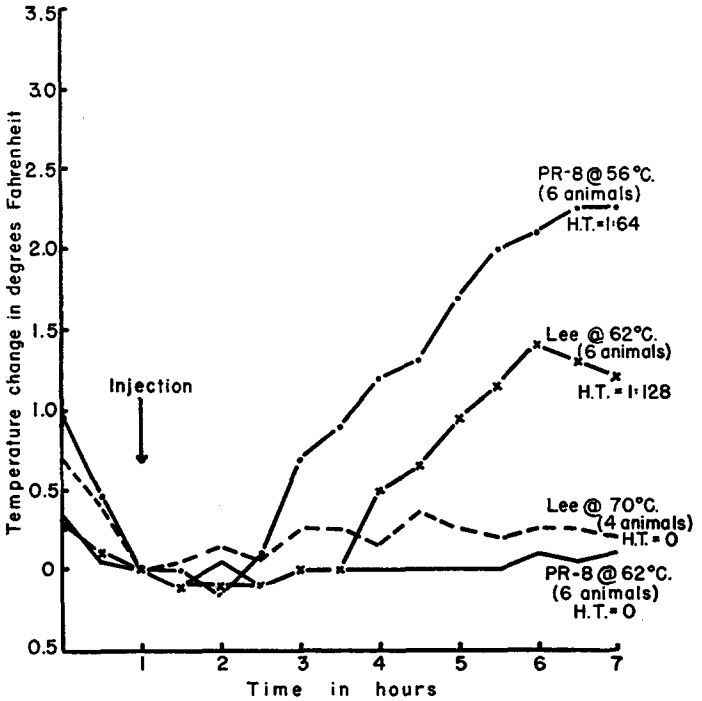

FIG. 5. Temperature responses of rabbits to intravenous injection of 1.0 ml. amounts of heated allantoic fluid suspensions of influenza virus. Note that Lee virus heated at 62°C. for 30 minutes retained hemagglutinating capacity and caused fever while the hemagglutinin titer of PR8 virus subjected to the same temperature fell to less than 1:2 and the virus lost its capacity to cause fever.

ated cups at 3,000 R. P. M. The hemagglutinin titer of the supernatant fluid fell to less than 1:2. The red cells were resuspended in 10 ml. of physiologic salt solution; there was no detectable elution of the adsorbed virus after 3 hours at room temperature, indication that heating had destroyed this property. Four animals received 2.0 ml. of the erythrocyte suspension intravenously. They had no fever. Control animals injected with 1.0 ml. of the original heated preparation showed typical febrile responses (Fig. 6).

Effect of Immune Serum.—Animals given PR8 or Lee virus mixed with homologous immune serum showed no febrile response. Fig. 7 compares the temperature records of these animals with those of animals given the virus without

immune serum. Normal rabbit serum failed to prevent the pyrogenic effect of influenza virus.

Effect of Antipyrine upon Fever Produced by Influenza Virus.—The febrile response after injection of virus in allantoic fluid was abolished by premedication with antipyrine.

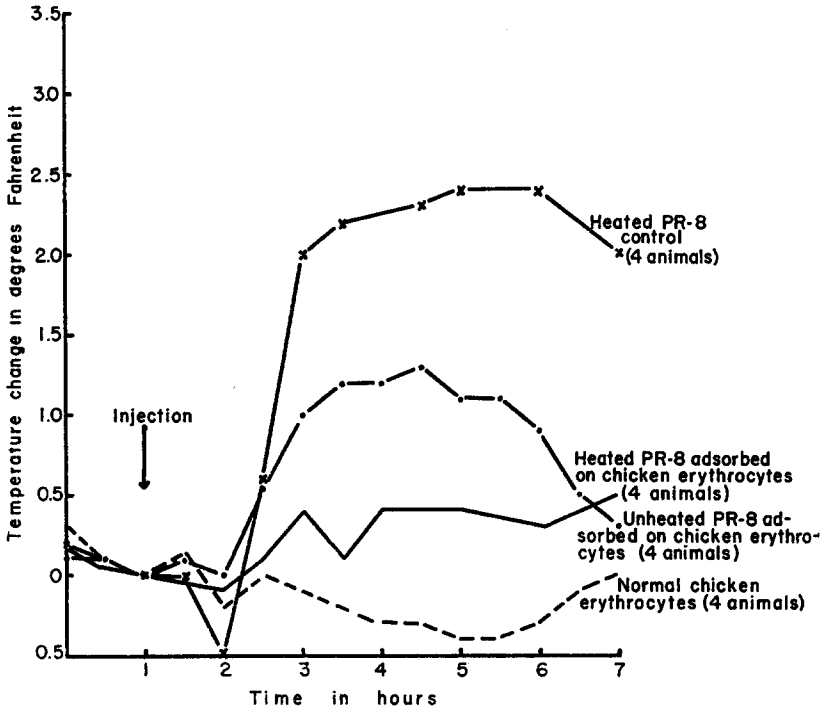

FIG. 6. Temperature records showing the effect of PR8 virus adsorbed onto erythrocytes. The injection of erythrocytes onto which unheated virus was adsorbed produced slight fever. The eluting capacity of heated virus was destroyed, and although it produced fever when injected alone, after adsorption onto erythrocytes, no significant febrile response followed.

Six animals were given 0.2 gm. of antipyrine per kilo by subcutaneous injection 1 hour before the intravenous injection of 1.0 ml. of a suspension of PR8 virus in allantoic fluid (hemagglutinin titer 1:1024). The dosage of antipyrine was repeated 3 hours after administration of the virus. None of the animals showed any elevation of temperature.

Effect of Virus upon Leucocyte Count.—During certain of the foregoing experiments, hourly total and differential leucocyte counts were performed. In most respects, the findings of Harris and Henle (11) were confirmed. Some of these observations are summarized briefly.

Although the injection of these viruses was followed by fluctuations in both total leucocyte and heterophile counts, the only consistent effect noted was

on circulating lymphocytes. Taking the mean of three lymphocyte determinations during the hour before injection as a base line, the hourly changes were charted as per cent of this mean.

The injection of 1.0 ml. of an allantoic fluid preparation of PR8 or Lee virus (hemagglutinin titer 1:1024) was followed by a progressive reduction in the absolute number of circulating lymphocytes amounting to about 80 per cent

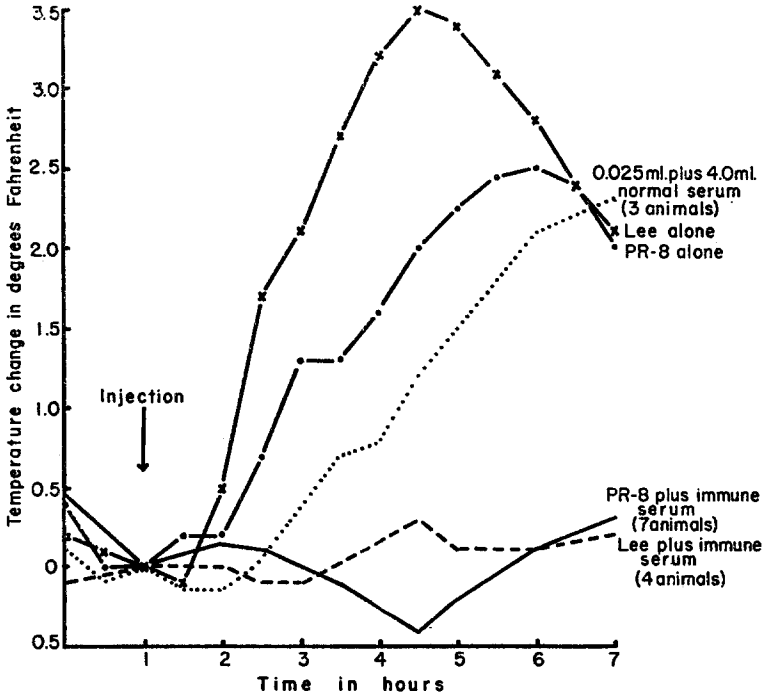

FIG. 7. Temperature records showing the neutralizing effect of mixing PR8 or Lee virus in allantoic fluid with specific immune serum before injection as compared with the febrile responses after virus alone. Normal serum had no effect on the production of fever by 0.025 ml. of PR8 virus.

of the preinoculation mean at the 5th hour. However, as with the pyrogenic effect, the degree of lymphopenia was found to depend upon the amount of virus injected. For example, with 0.05 ml. of the allantoic fluid suspension of PR8 virus, the decrease amounted to less than 60 per cent of the absolute count at the 5th hour (Fig. 8).

Control animals given normal allantoic fluid responded with an average lymphocyte reduction of only 23 per cent at the 5th hour. In studies of spontaneous variations in leucocyte counts of rabbits, Farr (20) has shown that under the conditions of these experiments, there is a spontaneous decrease in

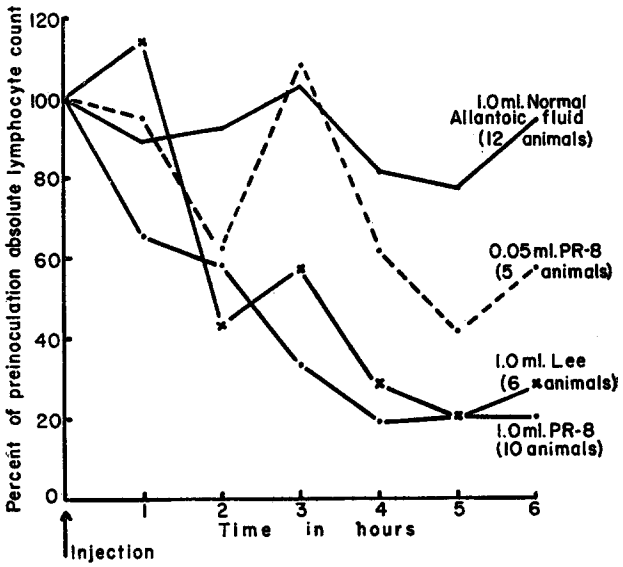

FIG. 8. Changes in circulating lymphocytes after injection of varying amounts of Lee or PR8 virus in allantoic fluid as compared with the effect of normal allantoic fluid. The hemagglutinin titer of the virus suspensions used in these studies was 1:1024.

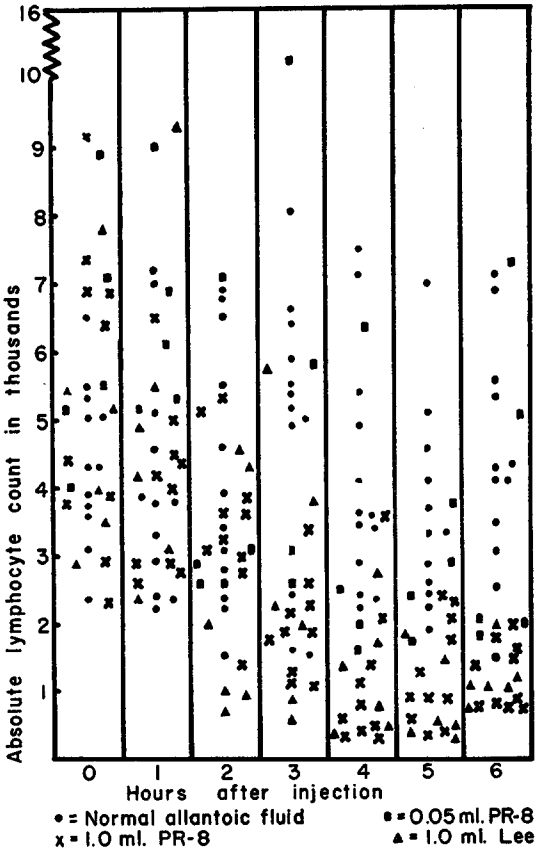

FIG. 9. Distribution of lymphocyte counts obtained on animals given normal allantoic fluid or varying amounts of influenza virus.

circulating lymphocytes which amounts to about 25 per cent of the initial mean at the 5th hour. His animals were placed in stalls of the type employed in the present study. The distribution of counts obtained in these experiments with varying doses of virus and with normal allantoic fluids is shown in Fig. 9.

Virus neutralized with homologous immune serum caused no more lymphopenia than was seen in the normal allantoic fluid controls. PR8 virus heated at 62°C. for 30 minutes lost infectivity, hemagglutinin, and capacity to produce fever, and caused no lymphopenia. Lee virus exposed to the same temperature lost infectivity but retained its hemagglutinating and pyrogenic properties; this treatment did not destroy its ability to produce lymphopenia.

DISCUSSION

The remarkable uniformity of the febrile response in rabbits to injections of these viruses would appear to provide a useful tool for the *in vivo* study of certain virus properties. Although differences were noted in the responses to the three viruses, such as the higher temperatures seen with Lee virus and the longer duration of the fevers of animals given NDV, the evidence seems to indicate that the mechanism of fever production is the same for each strain.

The fever produced by influenzal viruses differs from that following injection of bacterial pyrogens. Although the period between injection and temperature rise is roughly proportional to the amount of injected virus, in general this interval is longer with viruses than with bacterial pyrogens. Two characteristics of the viral fever-producing agent which are in striking contrast to bacterial pyrogens are the relative heat lability of the virus pyrogenic factor and its neutralization by specific immune serum.

The removal of bacteria will not eliminate the pyrogenic activity of a solution. However, it was not possible in these experiments to separate the viral pyrogenic property from the infective particle. Normal allantoic fluids and fluids from which most of the virus had been removed by high speed centrifugation or adsorption on chicken erythrocytes produced no fever. There is a possibility that some single product of tissue injury (21) which is removed by centrifugation or adsorption is responsible for the febrile reaction. This is unlikely since fever-producing capacities of different viruses are differentially susceptible to heat and are neutralized by specific immune serum.

A property common to viruses of the influenza-Newcastle-mumps group is the ability to be absorbed onto certain cell surfaces by union with a specific receptor substance (15). The agglutination of erythrocytes by virus typifies this reaction. The receptor material is ubiquitous, being found in the tissues of many species of animals (18). Production of fever by the viruses used in this study was closely associated with hemagglutinating capacity. The strongest evidence of this relationship is the parallelism of hemagglutinin and pyrogenic property observed with each virus. Virus rendered non-infectious by heat causes

fever provided hemagglutinating activity is retained. Progressive reduction of hemagglutinin by heat or dilution causes a proportional decrease in febrile response. Heated preparations which are no longer capable of agglutinating erythrocytes are non-pyrogenic. This association of the hemagglutinating and fever-producing activities of influenza viruses was further indicated by the experiments with virus adsorbed onto chicken erythrocytes before injection. When virus adsorbed onto red cells is injected into rabbits, their fevers are of a lower order than those following equal amounts of free virus. When virus heated at 56°C., destroying its capacity to elute, is adsorbed onto erythrocytes and injected, animals remain afebrile. Similar amounts of heated virus alone produce fever.

The observations of lymphocyte changes following the injection of influenza virus in rabbits confirm the over-all findings of Harris and Henle (11). In our experiments the lymphopenia produced by the virus seemed to parallel its pyrogenic effect.

It appears then, that these physiologic changes produced by influenza viruses and the closely related Newcastle disease virus may be associated with a common property of this group. However, this is not a single pyrogenic factor for all strains; its physical and serologic characteristics are variable, resembling strain-specific hemagglutinins.

It is of interest to note that fever is one of the commonest untoward reactions which follow prophylactic immunization with influenza virus vaccines in humans. This reaction in adults is relatively mild (22) but may be extremely severe in children. In one reported series (23), the incidence of hyperpyrexia after initial injection of a vaccine containing influenza A and B virus was more than 50 per cent. At least one instance of fatal hyperpyrexia in a child has been attributed to influenza vaccine (24, 25). Although the febrile response observed in rabbits suggests a relation to these vaccine reactions, it is by no means certain that the mechanisms are the same.

SUMMARY

The intravenous injection of the PR8 strain of influenza A virus, the Lee strain of influenza B, and the "B" strain of Newcastle disease virus produces fever in rabbits. This phenomenon has been studied in relation to certain *in vitro* properties of these viruses.

Saline suspensions of virus prepared by centrifugation or elution from chicken erythrocytes produced fever. Fluids from which most of the virus particles had been removed were non-pyrogenic.

Exposure to temperatures which destroyed the infectivity of the virus for chick embryos did not prevent fever. However, heating sufficient to destroy the hemagglutinin also rendered virus non-pyrogenic.

The injection of erythrocytes onto which virus had been adsorbed produced

fever. Heated virus adsorbed onto erythrocytes, which failed to elute, produced no elevation of temperature, although heated virus alone was pyrogenic.

Neutralization of virus with specific immune serum prevented fever.

Antipyrine was capable of abolishing the febrile response to virus.

Certain differences between the febrile response in rabbits to the injection of viruses and that following bacterial pyrogens were noted. The period between injection and beginning of temperature rise is longer with virus than with bacterial pyrogens. Relatively low temperatures inactivate the fever-producing capacity of viruses, whereas bacterial pyrogens withstand prolonged autoclaving, and the neutralization of viral fever by specific immune serum contrasts sharply with the failure of antibody to affect the response to bacterial pyrogens.

Certain previous observations on the lymphopenia produced in rabbits by the injection of influenzal viruses were confirmed. The capacity of virus preparations to induce fever in rabbits closely parallels their capacity to induce lymphopenia.

It was concluded that the fever-producing property of influenzal viruses is closely associated with the capacity to agglutinate erythrocytes.

The authors are grateful for the technical assistance of I. B. Stacy and P. K. Schork, Chief Hospitalmen, United States Navy, and H. P. Cordova, Hospitalman First Class, United States Navy.

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