THE PRODUCTION OF FEVER BY INFLUENZAL VIRUSES

II. Tolerance in Rabbits to the Pyrogenic Effect of Influenzal Viruses

By IVAN L. BENNETT, Jr., M.D.,* ROBERT R. WAGNER, M.D.,‡ and VIRGIL S. LeQUIRE, M.D.§

(*From the Naval Medical Research Institute, Bethesda*)

(Received for publication, June 14, 1949)

Patients given daily injections of typhoid vaccine rapidly become tolerant to its pyrogenic effect unless increasing amounts are used (1, 2). Beeson (3) has shown that rabbits given daily injections of a bacterial pyrogen acquire tolerance to its fever-producing effect and that animals tolerant to the pyrogen of one bacterial species are also insensitive to those of other organisms. These findings have been confirmed (4, 5) and this cross-tolerance has been demonstrated in human subjects (6). Tolerance is of short duration, being lost in 3 weeks when daily injections are stopped. It is unrelated to specific antibody formation (3, 5), but can be abolished by blockade of the reticulo-endothelial system with dyes or thorotrast (7). This acquired resistance to bacterial products is not complete; animals continue to respond with low fevers of short duration and this minimal response remains though daily injections be continued for as long as 6 weeks.

In the preceding report (8) of fever production in rabbits with single injections of the viruses of influenza and Newcastle disease (NDV), differences were pointed out between viral fever and that caused by bacterial pyrogens. There is a longer lag period between injection and temperature rise with viruses and the ability of viruses to cause fever is abolished by specific immune serum. The fever-producing capacity of these viruses is destroyed at temperatures which leave bacterial pyrogens unaffected. Studies with heated virus indicated that the ability to produce fever is closely related to the capacity to cause hemagglutination.

Administration of influenzal vaccines to mice not only protects them against infection with homologous strains, but also renders them insusceptible to the so called toxic effect of intravenously injected virus (9). Presumably, this resistance to the toxic effect is due to specific antibody. In the present study, it is shown that rabbits become tolerant to the pyrogenic and lymphopenic effects

*Present address: Department of Pathology, John Hopkins Medical School, Baltimore.

‡ Present address: Department of Internal Medicine, Yale University School of Medicine, New Haven.

§ Present address: Department of Anatomy, Vanderbilt School of Medicine, Nashville.

of influenza viruses, and evidence is presented that this phenomenon is unrelated to antibody formation.

Materials and Methods

Preparation of viruses, hemagglutinin determinations, and leucocyte counts were performed as outlined in the preceding paper. The method of recording temperatures has been described.

Antibody Titrations.—The level of serum antibody was determined by inhibition of virus hemagglutination. Serum was heated at 56°C. for 30 minutes and added to a series of tubes as follows: 0.5 ml. of a 1:10 dilution to the first tube, and 0.5 ml. of a 1:20 dilution to the remaining tubes. After the addition of 0.5 ml. of virus suspension to the first tube, serial twofold dilutions were made, the last 0.5 ml. being discarded. Five-tenths ml. of a 0.25 per cent suspension of washed chicken erythrocytes was then added and the tests were read after 90 minutes at room temperature.

Typhoid Vaccine.—The typhoid vaccine employed in certain experiments contained 2.0 billion heat-killed *Salmonella typhosa* organisms per ml.; no perservative was added.

EXPERIMENTAL RESULTS

Effect of Homologous Virus on the 2nd Day.—Animals which had received 1.0 ml. of an allantoic fluid suspension of PR8 virus on the 1st day with attendant febrile response were found to show no elevation of temperature after a repeated injection of this virus on the 2nd day.

Ten animals were given allantoic fluid containing PR8 virus (hemagglutinin titer 1:1024) in 1.0 ml. amounts intravenously. They responded with typical fevers. On the following day, they received 1.0 ml. of the same virus suspension and all 10 animals remained afebrile throughout the 6 hour observation period. This experiment was repeated twice with the same results.

Similar 2nd day tolerance to the pyrogenic effect of Lee virus was found. After an injection of 1.0 ml. of an allantoic fluid suspension of this virus (hemagglutinin titer 1:1024) on the 1st day, animals showed slight fever when challenged on day 2. However, when the initial injection was increased to 5.0 ml. of the virus suspension, all animals were completely tolerant to the pyrogenic effect of 1.0 ml. or 5.0 ml. of homologous virus on the 2nd day. Fig. 1 compares the temperature records of a group of 6 animals given 1.0 ml. of Lee virus on day 1 followed by 1.0 ml. of the same virus on day 2 and a group given an initial injection of 5.0 ml. of virus and challenged with 1.0 ml. or 5.0 ml. on the following day.

Because of the long duration of the fever following a single injection of NDV (which often lasted 48 hours) and because of the severity of the injury produced by this strain, it was never possible to demonstrate complete tolerance to its effect. Several experiments indicated, however, that animals rapidly became less responsive to repeated injections. Fig. 2 illustrates two of these experiments in which groups of animals given 4.0 ml. or 8.0 ml. of an allan-

toic fluid suspension of this virus (hemagglutinin titer 1:1024) daily showed decreasing febrile responses with each successive injection.

Duration of the Tolerance and Its Relation to Specific Antibody Formation.— The following experiment was designed to determine the duration of this period of tolerance to the fever-producing effect of influenza virus.

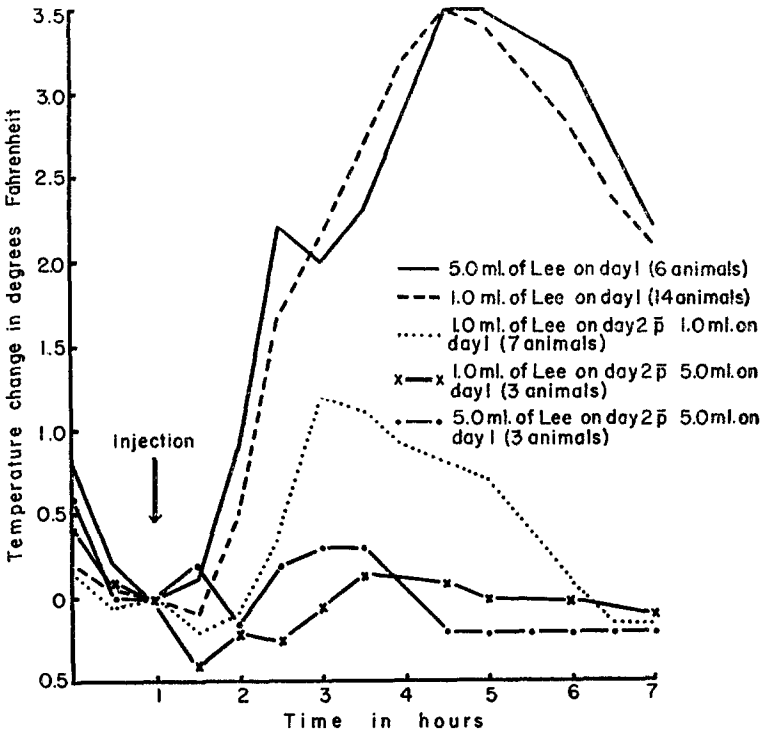

Fig. 1. Fever curves of animals given Lee virus in allantoic fluid (hemagglutinin titer 1:1024) on 2 successive days. The initial injection of 1.0 ml. of virus conferred partial protection on animals against a similar dose on the following day. With an initial injection of 5.0 ml., however, animals were completely tolerant when challenged on the 2nd day with 1.0 or 5.0 ml. of virus.

Nine animals were given 1.0 ml. of PR8 virus in allantoic fluid (hemagglutinin titer 1:1024) and were reinjected in groups of three with the same material on the 4th, 7th, and 11th experimental days. There was slight fever on the 4th day, a greater one on the 7th day, and by the 11th day, the temperature responses were of nearly the same magnitude as at the initial injection. Comparison of the temperature responses of these animals with those of other groups given virus on days 1 and 2 was presented graphically in a previous report (10). Antibody titrations were performed on each of these 9 animals. Blood was obtained by cardiac puncture just prior to the initial injection of virus and again before the injection of virus on the 4th, 7th, and 11th days. These paired specimens were titrated simultaneously and

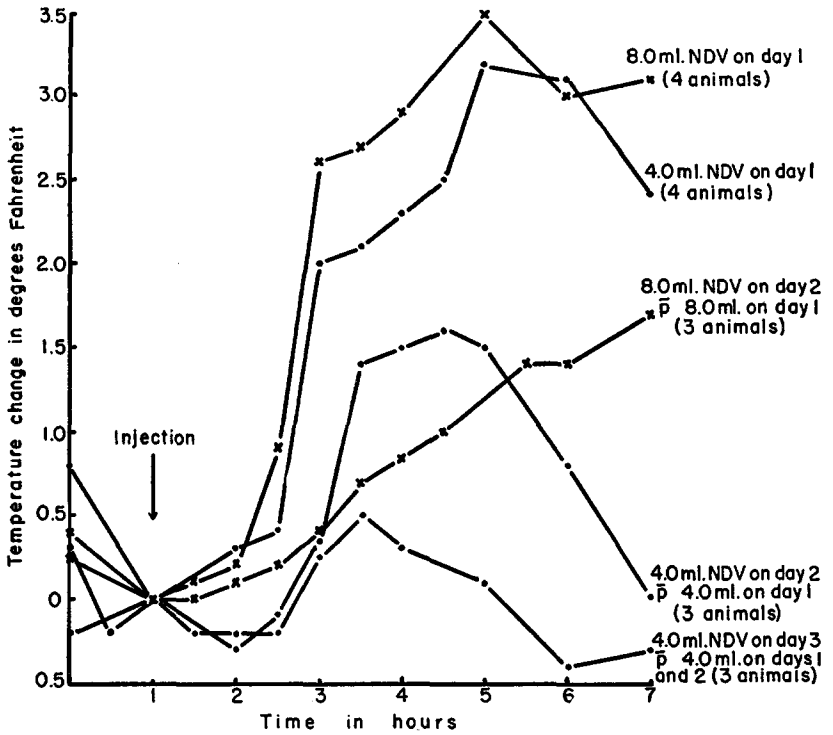

Fig. 2. Temperature records of two groups of animals given repeated injections of an allantoic fluid suspension of Newcastle disease virus (hemagglutinin titer 1:1024). Note the decrease in febrile response with successive injections.

TABLE I

Antibody Titers of Animals Given a Single Injection of PR8 in Allantoic fluid

	Animal	Preinjection*	Postinjection*	Tube difference	Inhibition titer of undiluted serum
	1	1:256	1:32	3	1:160
Day 4	2	1:32	1:4	3	1:160
	3	1:128	1:16	3	1:160
	4	1:128	1:2	6	1:1280
Day 7	5	1:128	<1:2	7	1:2560
	6	1:128	<1:2	7	1:2560
	7	1:256	1:8	5	1:640
Day 11	8	1:512	1:8	6	1:1280
	9	1:256	1:4	6	1:1280

* 1:20 dilution of serum titered against falling twofold dilutions of virus.

differences in hemagglutination inhibition recorded. The titers of the individual animals at each interval varied no more than one tube; results are presented in Table I. The levels of circulating antibody gradually rose over the 11 day period during which tolerance to the febrile reaction was progressively lost.

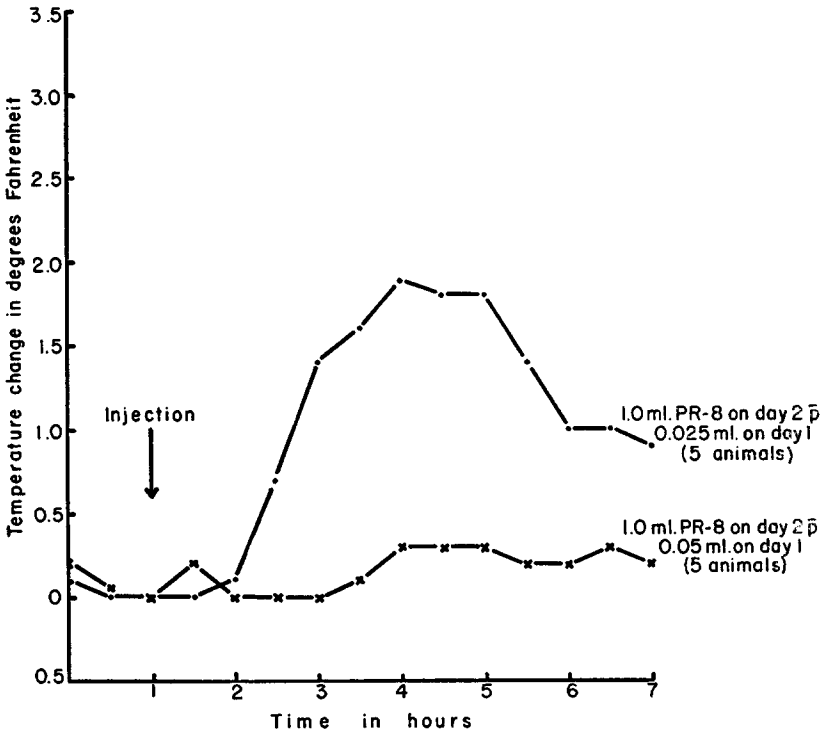

FIG. 3. Temperature curves of two groups of animals after 1.0 ml. of PR8 virus on the second experimental day. Animals given 0.05 ml. of PR8 virus in allantoic fluid (hemagglutinin titer 1:1024) on the 1st day were tolerant to the fever-producing effect of the second injection. The group given only 0.025 ml. on the 1st day, however, were not protected against the 2nd day challenge.

This indicates that the 2nd day tolerance is not a result of the neutralizing effect of circulating antibody. In spite of the relatively high antihemagglutinin titers shown by the 11 day animals, they exhibited little tolerance.

Minimal Protective Dose of Virus on Initial Injection.—In an attempt to determine the minimum amount of virus which on initial injection would protect against the pyrogenic effect of 1.0 ml. of an allantoic fluid suspension of PR8 virus on the 2nd day, a series of experiments was performed in which decreasing doses of virus were given initially.

A suspension of PR8 virus in allantoic fluid with a hemagglutinin titer of 1:1024 was used. Five animals were given 0.05 ml. of this suspension intravenously and 5 others received 0.025

ml. Both groups responded with fevers to this initial injection, the temperature curves differing little in extent. When 1.0 ml. of the virus suspension was given on the 2nd experimental day, their responses contrasted sharply. The animals injected initially with 0.05 ml. showed complete tolerance to the pyrogenic effect of the challenge dose while all the animals in the group which had received only 0.025 ml. initially had fever after the challenge injection. Fig. 3 shows the temperature curves of these two groups after the 2nd day injection of virus.

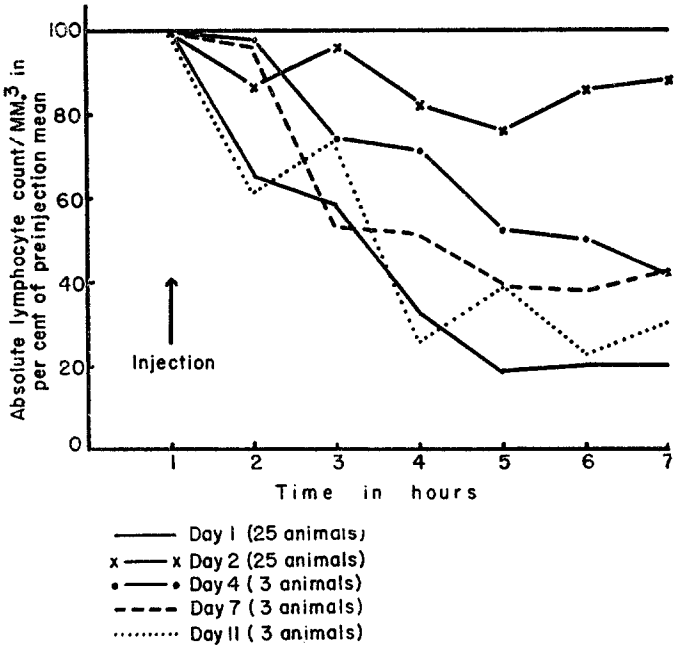

FIG. 4. Changes in circulating lymphocytes of animals given 1.0 ml. of PR8 virus in allantoic fluid (hemagglutinin titer 1:1024) on the first experimental day and then reinjected on the 2nd, 4th, 7th, or 11th day. Note the relative unresponsiveness to the virus on the 2nd day followed by progressive return of lymphopenic responses toward 1st day levels during the next 10 days.

Effect of Repeated Injection of Homologous Virus on Circulating Lymphocytes.—Hourly total and differential leucocyte counts were performed on several groups of animals following initial injection of PR8 virus and after a challenge dose on the 2nd, 4th, 7th, and 11th days. Typical lymphopenia (11) occurred on the 1st day, but on the 2nd day, no significant decrease in lymphocytes was found.

Animals reinjected on day 4 showed mild lymphopenia and by the 11th day, as with the fever, this response approached that seen on the 1st day. Fig. 4 shows the effect on circulating lymphocytes of repeated injections of PR8 virus.

Effect of Heterologous Virus on the 2nd Day.—Immunity to infection with

influenza viruses can be correlated with serum antibody levels. This immunity is specific. Although some cross-protection exists within the A and B groups (12), antibodies for influenza A confer no protection against B strains and *vice versa*.

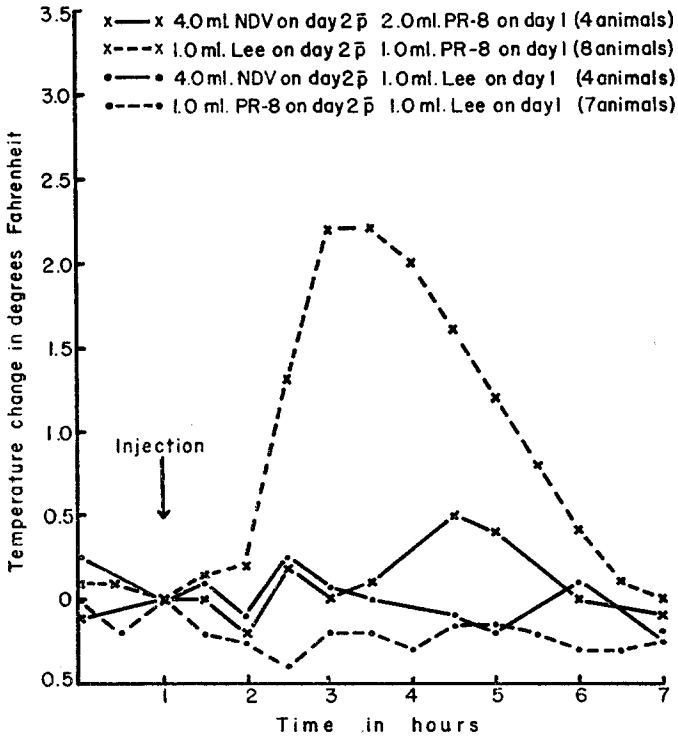

FIG. 5. Temperature records demonstrating the effect of initial injections of PR8 or Lee virus in allantoic fluid upon fever production by heterologous virus on the second experimental day. Animals given PR8 virus on day 1 were tolerant to Newcastle disease virus, but had fever when given Lee virus. Injection of Lee virus on the 1st day protected animals against the pyrogenic effect of both PR8 and Newcastle disease virus on the 2nd day. All virus suspensions used in these experiments had hemagglutinin titers of 1:1024.

Animals given initial injections of 1.0 ml. of allantoic fluid containing PR8 virus responded with fever to an injection of Lee virus on the 2nd day. However, the injection of NDV on day 2 after PR8 virus on day 1 produced no febrile response. Rabbits given 1.0 ml. of Lee virus in allantoic fluid on the 1st day had no fever when challenged with either PR8 or NDV on the 2nd day. Fig. 5 shows the temperature curves of groups of animals challenged with heterologous viruses after initial injections of PR8 or Lee viruses.

It was never possible to demonstrate any protective effect of initial injection

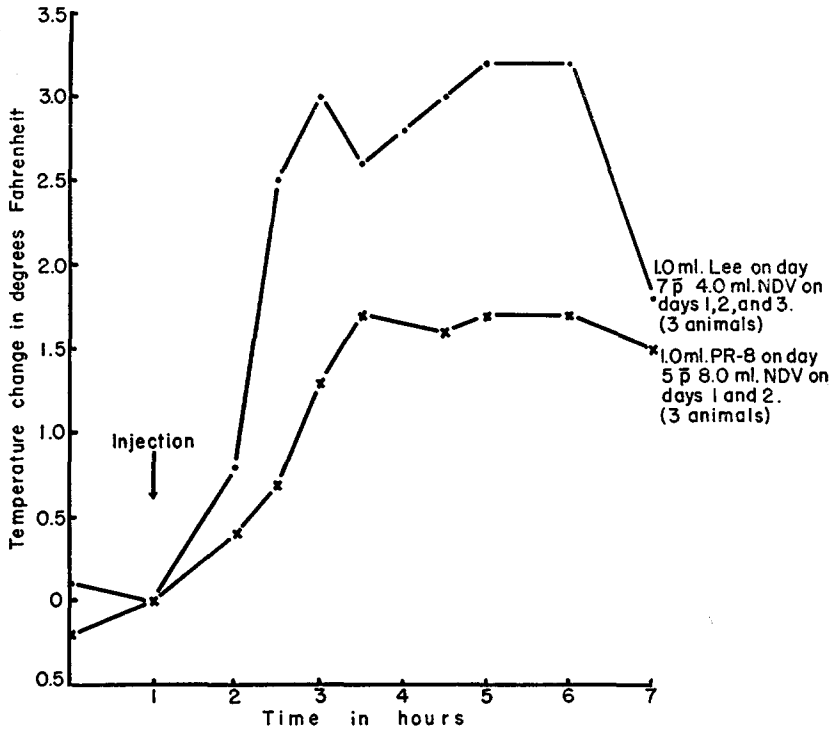

FIG. 6. Temperature responses of rabbits to injections of PR8 or Lee virus after prior administration of varying amounts of Newcastle disease virus. It was not possible to demonstrate a protective effect of Newcastle disease virus against fever production by influenza viruses.

TABLE II

Summary of Results of Experiments with Heterologous Virus on the 2nd Day

Inital injection	Challenge injection		
	NDV	PR8	Lee
NDV	+	−	−
PR8	+	+	−
Lee	+	+	+

+ indicates protection against pyrogenic effect of challenge injection.
− no protection.

of allantoic fluid suspensions of NDV against the pyrogenic effect of either Lee or PR8 viruses (Fig. 6).

Table II summarizes the results of experiments with heterologous viruses.

It can be seen that the viruses tested fall into a definite order of protective effect: NDV, PR8, and Lee.

Effect of Modified Virus.—Several experiments were performed in which animals were given initial injections of virus treated in various ways and challenged with allantoic fluid suspensions on the 2nd day.

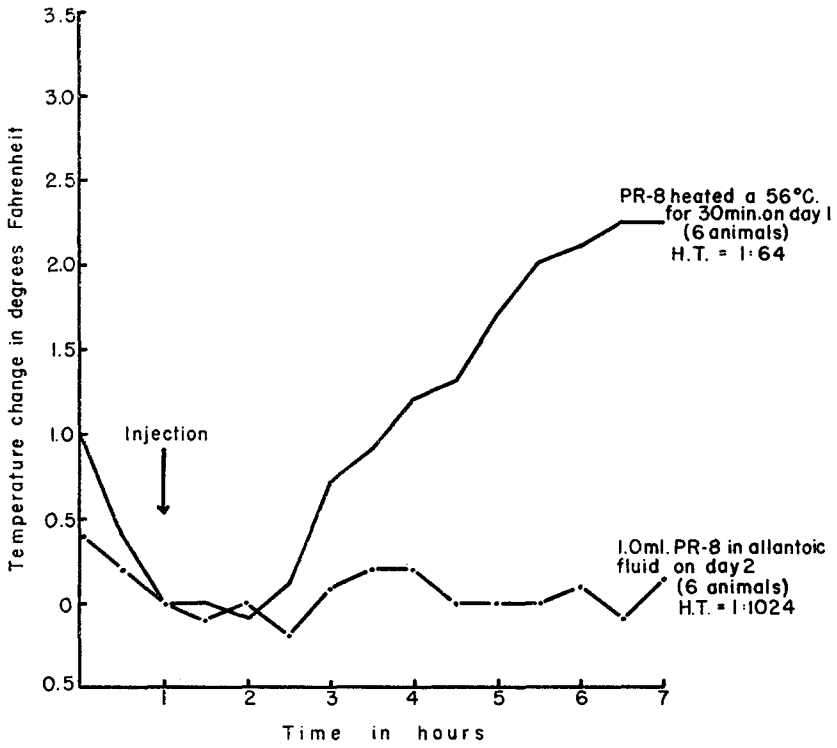

FIG. 7. Temperature records of a group of 6 animals demonstrating that an initial injection of heated virus will protect against the fever-producing effect of unheated virus given on the following day.

The initial injection of saline suspensions of PR8 virus prepared either by high-speed centrifugation or by erythrocyte adsorption and elution protected animals against the pyrogenic action of allantoic fluid preparations on the 2nd day.

Heated virus appeared to confer tolerance as readily as did unheated preparations with comparable hemagglutinin titers. The protective effect of heated virus was lost when its hemagglutinin was destroyed. The following examples illustrate this.

Allantoic fluid containing PR8 virus was heated at 56°C. for 30 minutes, destroying infectivity and eluting capacity and reducing its hemagglutinin titer from 1:1024 to 1:64. Six animals received 2.0 ml. of this material and responded with typical fevers. They were completely tolerant when challenged on the following day with 1.0 ml. of the unheated virus (Fig. 7). Four animals were given an initial injection of PR8 virus in allantoic fluid which had been heated at 70°C. for 30 minutes, reducing its hemagglutinin titer to less than 1:2. These animals had no fever and when given 1.0 ml. of unheated virus on day 2 showed febrile

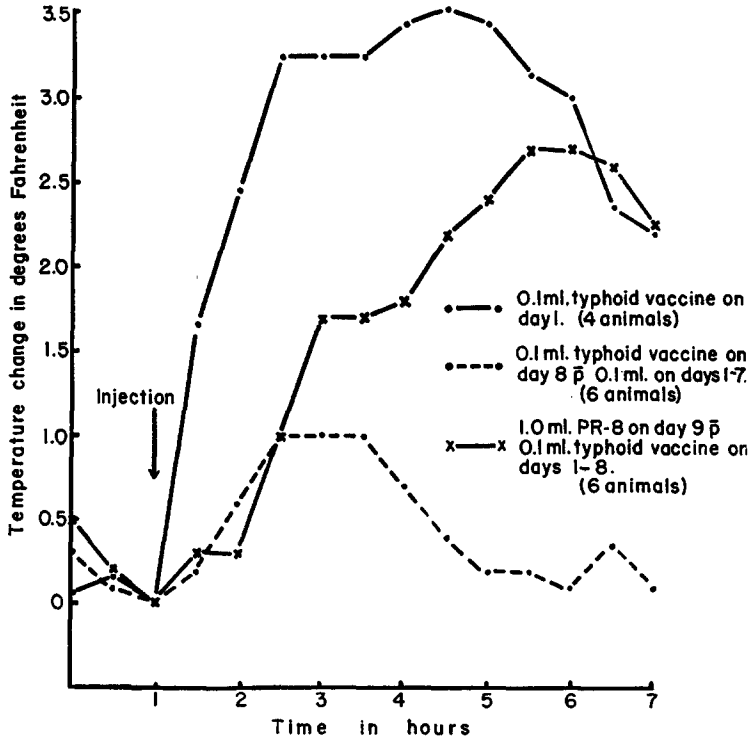

FIG. 8. Fever curves demonstrating the failure of tolerance to bacterial pyrogens (typhoid vaccine) to modify fever production by PR8 virus.

responses. The injection of unheated virus was repeated on day 3 and all animals remained afebrile.

Animals given an initial injection of PR8 virus, the hemagglutinin of which had been neutralized by incubation with homologous immune serum, were not protected against the pyrogenic effect of an allantoic fluid suspension of the virus on the following day.

Effect of Bacterial Pyrogens.—No cross-tolerance with bacterial pyrogens was found in animals given influenza virus.

Four rabbits were given 1.0 ml. of PR8 virus in allantoic fluid on day 1 and challenged on day 2 with 0.1 ml. of typhoid vaccine intravenously. They responded with brisk fevers. An-

other group of 6 animals received daily intravenous injections of 0.1 ml. of typhoid vaccine for 8 days, with progressive diminution in fevers until a "minimal response" was obtained. When challenged with 1.0 ml. of PR8 virus on the 9th day, these animals showed febrile responses which were of the order expected from an initial injection of the virus (Fig. 8).

Effect of Reticulo-Endothelial Blockade.—It has been shown (7) that the tolerance for bacterial pyrogens can be abolished by reticulo-endothelial blockade. This procedure was found to have no effect upon the tolerance for the pyrogenic effect of influenza virus.

Six animals received 1.0 ml. of PR8 virus in allantoic fluid (hemagglutinin titer 1:1024) initially and responded with typical fever. At the end of the observation period on the 1st day, each animal was given 8.0 ml. of thorotrast intravenously. Sixteen hours later, the injection of virus was repeated. All animals remained afebrile, indication that blockade of the reticulo-endothelial cells had no effect upon the development of tolerance to the pyrogenic effect of influenza virus.

Effect of Antipyrine upon Development of Tolerance.—The prevention of the fever following an initial injection of influenza virus by antipyrine did not interfere with the development of tolerance.

Five animals received an initial injection of 1.0 ml. of PR8 virus in allantoic fluid. One hour before the administration of the virus, each animal was given 0.2 gm. of antipyrine per kilo of body weight. This drug was injected subcutaneously as a solution containing 200 mg. per ml. This dose of antipyrine was repeated 3 hours after injection of the virus. None of the animals showed any elevation of temperature after the initial injection of virus and when challenged on the following day with the same amount of homologous virus, they also remained afebrile. This indicated that the development of tolerance to the pyrogenic effect of influenza virus is independent of the temperature rise.

DISCUSSION

It was shown in the previous report (8) that the ability of influenzal viruses to produce fever when injected into rabbits is apparantly closely asociated with their hemagglutinating capacity. The evidence for this may be summarized briefly. Differentially heated virus preparations are pyrogenic until hemagglutinin is destroyed and the febrile response is roughly proportional to the amount of injected hemagglutinin. The heat-lability of the virus fever-producing factor varies between strains as does that of the hemagglutinin. Heated virus adsorbed onto erythrocytes from which it cannot elute in detectable amounts causes no fever when injected and homologous antiserum neutralizes the febrile response.

That the mechanism of tolerance to viral fever differs from that for bacterial pyrogens is illustrated by the complete lack of cross-tolerance between the two and the failure of reticulo-endothelial blockade to modify the tolerance for viruses. As has been shown for bacterial pyrogens (3), the protective effect of an initial injection of virus is not dependent upon the temperature elevation, since the prevention of fever by antipyrine on the 1st day did not interfere with the development of tolerance.

The progressive increase in responsiveness of animals during a time of increasing antibody production argues against any rôle of specific immunity in tolerance despite the fact that incubation of virus with homologous immune serum prevents the production of fever. The protective effect of heterologous virus also makes it unlikely that specific immune mechanisms are involved.

The elution of adsorbed influenza virus from erythrocytes is apparently the result of an enzymatic reaction which destroys the receptor substance (13). Thus, cells from which virus has eluted are no longer capable of adsorbing the homologous strain, although the virus is unchanged and will readily be adsorbed onto fresh cells. The complete unresponsiveness of animals to the fever-producing effect of 2nd day injections of homologous virus suggests another correlation between the fever-promoting capacity of the virus and its hemagglutinin. It may be postulated that union of the virus with receptor plays some part in the production of fever. Possibly, the initial injection destroys or inactivates supplies of a receptor substance that is necessary for the febrile reaction. Return of an animal's capacity to react with fever could be due to restoration of this receptor material. The fact that most of the cells of the animal contain receptor material (14) and that relatively small amunts of virus exert a protective effect makes it difficult to believe that the tolerance is dependent upon destruction of all receptor substance present in the body. The protective effect of heated virus suspensions also argues against complete destruction of receptor as the mechanism of tolerance.

Burnet and his coworkers (15) have described a "receptor gradient" for the viruses of the mumps-Newcastle-influenza group in this order: mumps, NDV, most strains of influenza A, most strains of influenza B, and swine influenza. Erythrocytes from which any given strain has eluted will not adsorb the homologous strain but will adsorb strains below it. For example, red cells from which NDV has eluted will adsorb neither NDV nor mumps virus, but will adsorb influenza virus. The order in which the viruses employed in this study confer tolerance to the pyrogenic effect of heterologous strains conforms to their positions in this *in vitro* gradient. Lee protects against the homologous strain, PR8, and NDV; PR8 protects against the homologous strain and NDV; and NDV confers tolerance for the homologous strain only. This would seem to afford additional evidence that the production of fever by these viruses may be the result of union between the virus particle and a receptor substance.

SUMMARY

The mechanism of the fever caused by the intravenous injection of viruses of the influenza group in rabbits has been studied by observing the effect of repeated injections of the same or heterologous viruses.

An initial injection of the PR8 strain of influenza A, the Lee strain of influenza B, or the "B" strain of NDV conferred tolerance to the pyrogenic effect

of homologous virus administered on the following day. The period of tolerance lasted approximately 11 days.

Prior injection of virus appeared also to protect against the lymphopenic action of homologous strains.

These viruses were found to confer tolerance to the fever-producing effect of heterologous strains in an order corresponding to their positions in the receptor gradient of Burnet.

Heated virus preparations appeared to confer tolerance in proportion to survival of hemagglutinin.

Tolerance is probably unrelated to specific antibody formation since it is lost during a period of rapid immune response and heterologous strains exert a protective effect.

No cross-tolerance was demonstrable between viruses and bacterial pyrogens and reticulo-endothelial blockade with thorotrast failed to modify the unresponsiveness of animals to 2nd day injections of homologous virus.

Prevention of fever with antipyrine did not interfere with the protective effect of initial injections of virus.

Arguments for and against a hypothesis that union of the virus particle with a receptor substance may play a part in the production of fever by these viruses are discussed.

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.

BIBLIOGRAPHY

1. Heyman, A., *Ven. Dis. Inform.*, 1945, **26,** 51.
2. Favorite, G. O., and Morgan, H. R., *J. Lab. and Clin. Med.*, 1946, **31,** 672.
3. Beeson, P. B., *J. Exp. Med.*, 1947, **86,** 29.
4. Bennett, I. L., Jr., *J. Exp. Med.*, 1948, **88,** 267.
5. Morgan, H. R., *J. Immunol.*, 1948, **59,** 129.
6. Morgan, H. R., *J. Clin. Inv.*, 1948, **27,** 706.
7. Beeson, P. B., *J. Exp. Med.*, 1947, **86,** 39.
8. Wagner, R. R., Bennett, I. L., Jr., and LeQuire, V. S., *J. Exp. Med.*, 1949, **90,** 321.
9. Henle, W., and Henle, G., *J. Immunol.*, 1948, **59,** 45.
10. Bennett, I. L., Jr., Wagner, R. R., and LeQuire, V. S., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 132.
11. Harris, S., and Henle, W., *J. Immunol.*, 1948, **59,** 9.
12. Zellat, J., and Henle, W., *J. Immunol.*, 1941, **42,** 239.
13. Briody, B. A., *J. Immunol.*, 1948, **59,** 115.
14. Friedewald, W. F., Miller, E. S., and Whatley, L. R., *J. Exp. Med.*, 1947, **86,** 65.
15. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exp. Path.*, 1946, **27,** 228.