6. Browning, H. C., Larke, G. A., White, W. D., Proc. Soc. Exp. Biol. and Med., 1962, v111, 686.

7. Hagen, E. O., Rawlinson, H. E., Cancer Res., 1964, v24, 59.

8. Boot, L. M., Mühlbock, O., Ropcke, G., Tengberen, W. van Ebbenhorst, ibid., 1962, v22, 713.

9. Harris, G. W., Endocrinology, 1964, v75, 627.

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Tolerance to the Induction of Interferons by Endotoxin and Virus: Role of a Humoral Factor.* (30421)

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A single intravenous administration of bacterial endotoxin or virus induces circulating interferon or interferon-like viral inhibitors in experimental animals(1,2,3). We soon found that the response to a second inoculation of either endotoxin or virus in terms of interferon formation was markedly diminished. A preliminary report of this phenomenon of "tolerance" to the interferon-inducing effect of endotoxin and virus has been made (4). It has been found independently by Youngner and Stinebring(5).

It is well known that under certain circumstances, normal serum may inactivate or detoxify endotoxins(6,7). Whether tolerant serum has an enhanced capacity to do this is less well studied. In this report, in addition to describing tolerance to endotoxin as measured by its interferon-inducing activity, we wish to report on a factor in the serum of tolerant rabbits which inactivates this newly found biological activity of endotoxin.

Materials and methods. Albino rabbits weighing about 1 kg were inoculated intravenously in the marginal ear vein. Serum was obtained from blood collected by cardiac puncture or from the ear. Endotoxin extracted from *E. coli* (strain 0113) according to the Boivin method was provided by Dr. A. I. Braude. The use of endotoxin as well as Sindbis (AR339) virus pellet for induction of interferons has been previously described (2,8). To titer for interferon activity, serum collected 2 hours after injection of endotoxin, and 4 hours after injection of Sindbis virus was used. Tube cell cultures of rabbit kidney or rabbit embryo were incubated at 37°C for 18 hours with 2-fold serial dilutions of serum. These cultures were then challenged with about 1000 TCD_{50} vesicular stomatitis virus (Indiana, VSV) after the serum dilutions were decanted. Interferon effect was measured by the highest dilution in which 50% of the cultures showed inhibition of the cytopathology of VSV, as previously described(2, 8,9). The range of titers obtained was 1:16 to 1:512 for endotoxin induced interferon, and 1:40 to 1:10,000 for virus induced interferon.

The endotoxin inactivating factor (EIF) of rabbit serum was measured by the capacity of serum to eliminate the interferon-inducing capacity of endotoxin. A dose of endotoxin (5 or 10 μg) ordinarily sufficient to produce more than 1:16 interferon was diluted 1:5 in the serum to be tested, making a final volume of 0.5 to 1.0 ml. The mixture was incubated at 37°C for one to two hours, and given intravenously to a test rabbit. Serum was collected 2 hours later and titrated for interferon activity. If no interferon activity was detectable or if the titer was less than 1:8, it was assumed that the serum had eliminated or reduced the interferon inducing capacity of endotoxin. The serum obtained 24 hours after inoculation of endotoxin (*i.e.*, from tolerant animals) was called "tolerant serum."

The hemagglutination test against Type "O" human red blood cells coated with NaOH-treated endotoxin was performed in

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TABLE I. Tolerance to Endotoxin and to Virus.

Groups	Day 1 Inoculum	Day 1 Interferon titer	Day 2 Inoculum	Day 2 Interferon titer
1	Virus	160, 320	Virus	<5, <5
2	"	90, 45	Endotoxin	32, 128
3	Endotoxin	45	"	<1
4	"	128	Virus	<1

Each number represents a titer in reciprocals of dilutions obtained from a separate rabbit. Each rabbit was inoculated twice, on Day 1 and Day 2.

the manner described by Neter *et al*(10).

Results. The phenomenon of tolerance is demonstrated by an experiment described in Table I. On Day 1, six rabbits were divided in 4 groups and inoculated with 10 μg endotoxin or 10^9 PFU Sindbis virus as shown in the Table. On Day 2, these rabbits received a second injection of either endotoxin or virus. As is evident from the Table, the animal which received 2 doses of virus or 2 doses of endotoxin (Groups 1 and 3) failed to respond to the second dose. Preinoculation with virus did not prevent the usual response to endotoxin on the second day (Group 2), but one dose of endotoxin on Day 1 prevented the usual response to virus on the Day 2 (Group 4).

The profiles of interferon titers at various intervals following one and two inoculations of virus are described in Table II. It is apparent that there was a marked decrease in responsiveness in terms of interferon formation when a second dose was inoculated 24, 48 or 72 hours after the first.

To determine the dose of endotoxin required to produce the state of tolerance, 13 rabbits were given varying doses of endotoxin (0-10 μg) on Day 1. On Day 2, they each received 10 μg endotoxin, a dose which consistently induces interferon of a titer greater than 1:16 in untreated animals. Interferon titers were obtained on bloods collected 2 hours after endotoxin injections on each day. As shown in Table III, those animals which received 0.1 μg or more on Day 1 responded to a second dose by forming little or no interferon on Day 2. Tolerance was thus brought about by ≧0.1 μg of endotoxin. The results were equivocal for 0.01 μg but no tolerance could be detected if the animals received as little as 0.001 μg on Day 1.

To determine how long tolerance to endotoxin would persist, and the effect of repeated doses, an experiment using 9 rabbits is summarized in Fig. 1. The following points may be made: As shown in Table I, 24 hours after an inoculation of 5 μg of endotoxin, the animals became tolerant. Tolerance was still evident if the second dose was given in 3 days. However, if the interval between 2 doses was prolonged to 6 days, tolerance had largely disappeared. This was also true when an animal which had received 2 or more doses

TABLE III. Dose of Endotoxin and Development of Tolerance.

Day 1 Dose, μg	Day 1 Interferon, 1/dilution	Day 2 Dose, μg	Day 2 Interferon, 1/dilution
10	90	10	<1
5	>128, 45	10	<1, <1
1	16, 8	10	4, <1
.1	<1, <1	10	1, <1
.01	<1, NT	10	16, <1
.001	<1, NT	10	>128, 16
0	NT, NT	10	45, 32

NT = not tested. Each entry represents an animal which was injected on both Day 1 and Day 2 except for 2 rabbits which received no endotoxin on Day 1.

TABLE II. Interferon Titers After One and Two Doses of Sindbis Virus.

Hr after 1st inoculation 1.5	4	7	24	Interval between 2 inoculations	Hr after 2nd inoculation Pre	1.5	4	7
10*	5120	5120	20	24	20	20	5	20
<20	160	20	<5	24	<5	<5	<5	<5
< 5	320	5120	<5	48	<5	<5	<5	<5
10	80	20	<5	48	<5	<5	<5	<5
5	5120	>5120	80	72	<5	<5	<5	<5

* Each figure represents reciprocal of interferon titer of serum samples from separate rabbits, a different one being represented by each row.

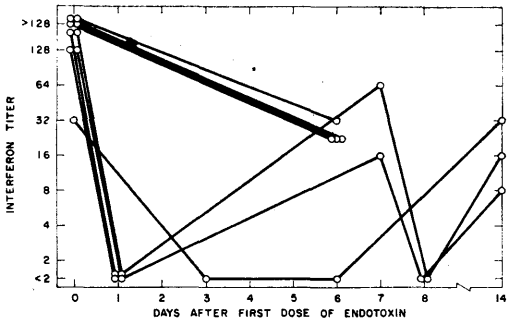

FIG. 1. Tolerance to endotoxin and intervals between inoculations of endotoxin. Each circle (O) represents interferon titer 2 hr following an injection of separate rabbits; the courses of the same rabbits are connected by lines.

was allowed to rest for 6 days; but tolerance was again produced in 24 hours if endotoxin was again given.

To test whether or not the serum of tolerant rabbits contains an endotoxin inactivating factor (EIF), 11 serums from untreated normal rabbits, and 11 serums from rabbits which had each received 10 μg endotoxin 24 hours previously ("tolerant serum") were tested for EIF. These serums represented different rabbits except 4 which contributed both normal and "tolerant" serum. After incubating each serum sample with 5 or 10 μg endotoxin for one to two hours at 37°C as described in *Materials and methods,* each serum-endotoxin mixture was inoculated into 22 separate rabbits. Two hours thereafter, sera were collected from these rabbits and titrated for interferon (Fig. 2). It is evident that normal serum did not decrease the interferon inducing capacity of endotoxin, in contrast to the "tolerant serum," which eliminated the interferon inducing capacity of endotoxin in 9 out of 11 cases, and markedly reduced the amount of interferon formed in the remaining two. There is therefore a serum endotoxin inactivating factor in tolerant rabbits, which is not found using the described method in normal animals.

To determine how soon tolerance develops and to what extent EIF is associated with tolerance, the following 2 experiments were done.

1. The response of animals to 2 closely spaced doses of endotoxin and the rapidity with which EIF develops was investigated. Four rabbits each received the first 10 μg dose of endotoxin at 0 hour. Four hours later serum was obtained from one of them, and a second 10 μg dose of endotoxin was inoculated. Six, 10 and 12 hours after the first dose, the 3 other rabbits were also bled and similarly inoculated. All animals were bled for a second time 2 hours after their second dose. The serum interferon titers obtained 4, 6, 10 and 12 hours after the first dose were 1:64, 1:16, 1:4 and $<$1:2. This indicates that, as previously described(*2*), circulating interferon had largely disappeared 6 to 10 hours after one dose of endotoxin. The interferon titers 2 hours following the second dose and given 4, 6, 10 and 12 hours after the first were respectively, 1:16, 1:4, $<$1:2 and $<$1:2. These values indicate that the animals were already poorly reactive or tolerant to the effect of a second dose of endotoxin at most 6 hours after an injection of the first. It is not possible to say whether the animal is tolerant 4 hours after the first dose, since residual circulating interferon may mask a response to a second dose. Sera collected 4, 6 and 10 hours after the first dose all contained EIF as defined in *Materials and methods.*

2. To test whether or not EIF disappeared with the loss of the tolerant state, 4 rabbits were rested for 6 days after each received 5 μg of endotoxin. As shown previously, tolerance was largely lost by this time (Fig. 1).

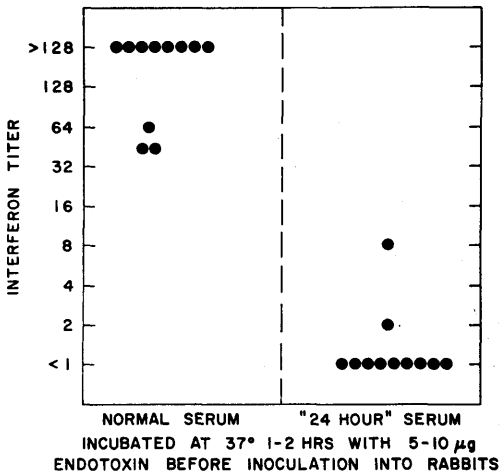

FIG. 2. Development of endotoxin inactivating factor after one dose of endotoxin.

Four sera were obtained at this time and tested for EIF by incubating each sample with 10 μg of endotoxin. The endotoxin-serum mixtures induced interferon titers of >1:128, >1:128, >1:128 and 1:40, indicating that no EIF was evident. Each animal then received another 10 μg dose of endotoxin to make them tolerant in 24 hours. Sera were obtained and tested for EIF again. They all were able to inactivate the interferon inducing activity of 10 μg endotoxin (interferon titers <1:1). This experiment shows that EIF disappears and appears corresponding to the state of tolerance.

Since EIF may appear in 6 hours and disappear 6 days after one dose of endotoxin, it appears unlikely that it is an immune globulin. To test this point further, the rise of antibodies against endotoxin was compared to that of EIF. An animal received 7 daily injections consisting of 1, 2, 3, 5, 10 and 20 μg endotoxin according to the schedule of Freedman to obtain "tolerant" serum(11). One, 3, 4, 5, 6 and 11 days after the first dose, the animals were bled and the sera were titrated for hemagglutinin antibody against endotoxin and for EIF. Antibody titers on these days were <1:4, 1:64, 1:256, 1:512, 1:512 and 1:1024. To measure EIF, the 6 sera were each incubated with 10 μg endotoxin and injected in rabbits. Circulating interferon titers were obtained in 2 hours. These were 1:6, 1:2, 1:8, 1:6, 1:20 and 1:40. It is evident that hemagglutinin titers were elevated by the 4th day and remain elevated. On the other hand, EIF was already evident in 24 hours, but did not increase with repeated inoculations. It disappeared after a lapse of endotoxin inoculations between the 6th and 11th days. These data show that antibodies and EIF do not develop concurrently and are probably unrelated.

In view of the fact that "endotoxin detoxifying component" of normal blood is reportedly potentiated by addition of citrate (7), the following experiment to determine whether the addition of citrate to tolerant serum would potentiate EIF was undertaken. Two sets of 2-fold serial dilutions each consisting of 0.8 ml were made. To Set 1 consisting of 1:2, 1:4, 1:8, 1:16 and 1:64 serum dilutions, 0.1 ml of 0.2 M sodium citrate was added. To Set 2 consisting of undiluted, 1:2, 1:4 and 1:8 serum dilutions, 0.1 ml buffered physiological saline was added. Tubes from both sets then received 0.1 ml endotoxin (10 μg), and were incubated at 37°C for one hour. Each dilution was inoculated into a separate rabbit, from which a 2-hour blood sample was taken for titration of interferon. The interferon titers of those rabbits that received fluids of Set 1 were: <1:1, <1:1, 1:3, 1:16 and 1:45. This shows that endotoxin inactivating effect was evident up to the 1:8 serum dilution. On the other hand, rabbits which received fluids of Set 2 had the following interferon titers: <1:1, 1:16, 1:11, 1:16 and 1:64. This indicates that there was no definite endotoxin inactivating effect beyond undiluted serum. It was evident from this and similar experiments that the addition of citrate accentuated the serum endotoxin inactivating effect as brought out by Skarnes *et al*(7).

Discussion. The development of tolerance to the febrile response of a single inoculation of virus has been well demonstrated by Bennett *et al*(12). The present work measures a different biological parameter of viral activity, *i.e.*, induction of interferon. While the demonstration of such a tolerance phenomenon is not surprising, its mechanism is at present just as obscure. Bennett *et al* did not show any crossed tolerance with endotoxin (12).

The development of tolerance to endotoxin after a single dose has also been described by measuring various biological effects of endotoxin. Thus Creech *et al* found that mice receiving 100 μg endotoxin were protected in 24 hours against its lethal effect(13). Similar protection has been shown by Chedid *et al* 3 hours after 0.3 μg endotoxin in adrenalectomized mice(14). Braude showed that 2 hours after a rabbit had received endotoxin, it was completely refractory to the pyrogenic effect of a second dose; and 12 hours afterward, the animal would develop a typical "tolerant" fever curve if rechallenged(15). The development of tolerance to the interferon inducing effects of endotoxin as soon as 6 hours after a dose is consistent with these facts.

The present study has also brought out the interesting fact that there is a humoral endotoxin inactivating factor associated with the state of tolerance. Since it is already well known that normal rabbit serum will decrease the lethal activity of endotoxin(6, 7), it is quite likely that the serum factor herein described represents an increase of what is normally already present. This hypothesis is strengthened by two further considerations. First, while normal rabbit serum does not inactivate the interferon inducing capacity of endotoxin after one to two hours of incubation at 37°C, occasional normal serum has been found to do so if incubation is prolonged to 18 hours. Second, addition of citrate to "tolerant" serum markedly potentiates the serum inactivating effect, although we have not been able to show this with normal serum under our conditions.

Our inability to demonstrate consistent endotoxin inactivating effect in normal serum may be a reflection of the sensitivity of our method. Alternatively, it could also be due to the simultaneous presence of an endotoxin augmenting factor in normal serum, which may be decreased in the state of tolerance (16).

The precise role of the endotoxin inactivating factor (EIF) in tolerance remains to be ascertained. While its presence correlates well with that of tolerance, its activity is low, being easily diluted out in the absence of citrate. We have not been able to transfer tolerance by passive transfusion of 10 to 20 ml of "tolerant serum" into rabbits. Large amounts (100 μg) of endotoxin cannot be inactivated by "tolerant" serum. The titer of the tolerant serum does not increase with multiple inoculations, which is the usual regimen for development of tolerance to pyrogens(17). Finally we have found no effect of such tolerant serum on viruses. From these considerations, it is unlikely that EIF explains the tolerance phenomenon completely. However it strengthens the concept that a humoral detoxifying system plays a role in tolerance to endotoxin(14,18).

The chemical nature of EIF and its mechanism of action are still under investigation. Previous work has shown that it is a protein residing in the Fraction II and III of serum fractionated according to Cohn's method(6), and that it acts like an enzyme(19). On the basis that it is labile at 56°C for one hour, that it is not affected by mercaptoethanol, and that it does not parallel the development of anti-endotoxin antibodies, we assume that it is not an antibody.

Summary. The intravenous inoculation of either endotoxin or Sindbis virus rendered a rabbit tolerant to the interferon-inducing action of endotoxin or virus. There was crossed tolerance in the sense that animals first inoculated with endotoxin became tolerant to virus. By 6 hours after receiving an inoculation of endotoxin, the animals became tolerant to endotoxin and remained so for at least 3 days, but by the 6th day, sensitivity to endotoxin had been regained. Concurrent with the development of tolerance to endotoxin, a serum humoral factor which could inactivate or decrease the interferon-inducing capacity of endotoxin was found. It is suggested that this factor may play a role in producing tolerance to endotoxin.

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1. Stinebring, W. R., Youngner, J. S., Nature, 1964, v204, 712.

2. Ho, M., Science, 1964, v146, 1472.

3. ———, Bact. Rev., 1964, v28, 367.

4. ———, Kono, Y., J. Clin. Invest., 1965, v44, 1059.

5. Youngner, J. S., Stinebring, W. S., private communication (Interferon Information Exchange).

6. Ho, M., Kass, E. H., J. Lab. & Clin. Med., 1958, v51, 297.

7. Skarnes, R. C., Rosen, F. S., Shear, M. J., Landy, M., J. Exp. Med., 1958, v108, 685.

8. Kono, Y., Ho, M., Virology, 1965, v25, 162.

9. Ho, M., Kono, Y., Proc. Nat. Acad. Sci., 1965, v53, 220.

10. Neter, E., Westphal, O., Luderitz, O., Gorzynski, E. A., Eichenberger, E., J. Immunol., 1956, v76, 377.

11. Freedman, H. H., J. Exp. Med., 1960, v111, 453.

12. Bennett, I. L., Jr., Wagner, R. R., LeQuire, V. S., ibid., 1949, v90, 335.

13. Creech, H. J., Hankwitz, R. F., Jr., Wharton, D. R. A., Cancer Research, 1949, v9, 150.

14. Chedid, L. Parant, M., Boyer, F., Skarnes,

R. C., in Bacterial Endotoxins, M. Landy and W. Braun, eds., 1964, p500.
15. Braude, A. I., Zalesky, M., Douglas, H., J. Clin. Invest., 1958, v37, 880.
16. Cluff, L. E., Mulholland, J. H., Scheder, E. P., Bull. Johns Hopkins Hosp., 1959, v104, 51.
17. Beeson, P. B., Proc. Soc. Exp. Biol. and Med., 1947, v64, 146.
18. Rutenburg, S. H., Rutenburg, A. M., Smith, E. E., Fine, J., ibid., 1965, v118, 620.
19. Keene, W. R., Landy, M., Shear, M. J., J. Clin. Invest., 1961, v40, 302.

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Catecholamines in Organs of Immunosympathectomized Mice.* (30422)

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Levi-Montalcini and Angeletti(1) have reported upon the norepinephrine content of several organs in immunosympathectomized mice. Berk *et al*(2) have studied both the norepinephrine and epinephrine contents of a few organs in comparably treated rats. In preparation for studies on the physiology of the immunosympathectomized animals we have analyzed a number of organs in immunosympathectomized and control mice for both of the above catecholamines. The data to be reported here confirm and extend the observations noted above.

Methods. The sympathetic nerve growth promoting factor was isolated from mouse submaxillary glands, purified, and its activity assayed by chick embryo dorsal root ganglia. Antiserum was prepared from rabbit immunized with this factor and assayed for potency by inhibition of the growth factor on chick embryo ganglia. All these procedures were adapted from Cohen(3) and Levi-Montalcini and Angeletti(4). Newborn C_3H strain male mice were injected with the antiserum daily for one week, about 0.05 ml serum (titer about 1:1000 by the inhibition test *in vitro*) per gram body weight per day. A group of similar newborn control mice were injected with normal saline in the same manner. The mice were housed in an air-conditioned room and cared for according to our standard methods. When the animals were 4 weeks old, various organs were assayed for epinephrine and norepinephrine content. The effectiveness of the immunosympathectomy of the experimental mice was checked by examination of the sympathetic chain of ganglia, and the cervical ganglia were studied histologically. The extensive reduction of the neurones in the experimental group was verified.

The animals, 13 (11-16) g in weight, were sacrificed by cervical dislocation. The hearts, brain, adrenal glands, spleen and kidney were removed immediately, fat and connective tissue trimmed away, blood washed off with a small amount of redistilled water, and blotted with filter paper. The tissues were then minced with scissors and transferred to a pre-weighed flask with a measured amount of 10% trichloroacetic acid. Organs from 8 to 20 mice were pooled as a single sample. The average weights of the organs from individual mice were: heart 58 (48-70) mg, brain 320 (290-380) mg, spleen 35 (28-45) mg, and kidney 45 (38-52) mg. The adrenal glands were not weighed. The preparation of tissue homogenates and the methods of determination of norepinephrine and epinephrine were essentially the same as those described in a previous paper from this laboratory(5).

Results. As shown in Table I, the catecholamine content of the heart, spleen, kidney, and especially the brain of the normal mouse is mainly norepinephrine, while the adrenal glands contain both epinephrine and norepinephrine, the level of the former being higher than that of the latter. In the im-

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