sponse to cold exposure or reserpine administration. Iproniazid significantly reduced the ability of reserpine to stimulate ACTH release while it did not block ACTH release in response to cold exposure, as judged by adrenal and thymus weights. Postpartum lactation was significantly reduced by iproniazid, without producing any significant loss of body weight in the mother rats. These results suggest that iproniazid may inhibit the release of prolactin, ACTH or oxytocin in response to electrical stimulation of the uterine cervix, reserpine injections or suckling.

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1. Shore, P. A., *Pharmacol. Rev.,* 1962, v14, 541.
2. *Advances in Neuroendocrinology,* A. V. Nalbandov, Ed., 1963, Univ. of Ill. Press, Urbana.
3. Botros, M., Robson, J. M., *J. Endocrinol.,* 1960, v20, X.
4. Setniker, I., Murmann, W., Magistretti, M. J., *Endocrinology,* 1960, v67, 511.
5. Lindsay, D., Poulson, E., Robson, J. M., *J. Endocrinol.,* 1961, v23, 209; *ibid.,* 1962, v25, 53.
6. Poulson, E., Botros, M., Robson, J. M., *Science,* 1960, v131, 1101; *J. Endocrinol.,* 1960, v20, XI.
7. Lindsay, D., Poulson, E., Robson, J. M., *J. Endocrinol.,* 1963, v26, 85.
8. Spector, W. G., *Nature,* 1960, v187, 514.
9. Fluckiger, E., Salzmann, R., *Experientia,* 1961, v17, 130.
10. Meites, J., in *Proc. of First Intern. Pharmacol. Meeting,* R. Guillemin, Ed., Pergamon Press, Oxford, 1962, vI, p151.
11. Meites, J., Nicoll, C. S., Talwalker, P. K., Chap. 8 in *Advances in Neuroendocrinology,* A. V. Nalbandov, Ed., Univ. of Ill. Press, Urbana, 1963.
12. Siegel, S., in *Non-parametric Statistics,* 1956, McGraw-Hill Book Co., Inc., New York, p116.
13. Gaunt, R., Renzi, A. A., Chart, J. J., *Endocrinology,* 1962, v71, 527.
14. Ratner, A., Meites, J., *Am. J. Physiol.,* 1963, v204, 268.
15. Gertner, S. B., *Nature,* 1959, v183, 750.

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Role of Humoral Mediator in Tolerance to the Pyrogenicity of Bacterial Endotoxin. (28983)

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Previous studies from this laboratory(1-3) demonstrated unequivocally that humoral factors play a role in endotoxin tolerance. The mechanisms by which tolerance develops, however, remain unknown.

Our findings suggested that the humoral mediator of tolerance, demonstrated by passive transfer, effected an altered state in the tissues rather than acting within the blood as an inhibitor of, or antibody to, the endotoxin. Recent studies by Greisman *et al*(4) have suggested that the humoral mediator of tolerance acts as an opsonin with high endotoxin specificity. The experiments reported here, based upon our earlier observation of the retention of passively acquired tolerance (2), demonstrate that rabbits given amounts of tolerant donor plasma too small to be effective against a first test dose of endotoxin display a remarkable degree of tolerance, compared to identically endotoxin-treated controls, when rechallenged with a larger dose of endotoxin the following day. The results are inconsistent with hypotheses invoking direct inhibition, detoxification, or opsonization of the endotoxin by the humoral mediator, and, most important, suggest that the events leading to the expression of tolerance by the animal are initiated by neither the endotoxin alone nor the humoral mediator alone, but by a combination of the two.

Materials and methods. The procedures used for preparing tolerant donor rabbits, collecting plasma, and performing pyrogen tests in normal recipient rabbits, and the precautions observed, have been described(2). The

Boivin endotoxin used was derived from *Salmonella typhosa* O-901. All experiments were done with one pooled lot of tolerant donor plasma which at a 10 ml dose allowed the demonstration of passive tolerance previously reported(2,5). Since, as described below, the basic experimental model required the use of amounts of tolerant plasma demonstrably ineffective against a first test dose of endotoxin, a number of experiments were done using from 1 to 5 ml volumes of plasma followed directly or at various times up to 2 hours with a 2.5 μg dose of endotoxin. Rechallenge the following day was with a dose of endotoxin 4 or 5 times larger. Control and experimental groups, comprising 3 or 4 rabbits each, were examined at the same time in all instances. All injections were made via a marginal ear vein. Male albino rabbits weighing 1 to 1.2 kg were obtained from a local breeder.

Results. Results of a representative experiment are given in Fig. 1. On the first day the experimental animals each received 2 ml of tolerant donor plasma and 5 minutes later were injected with 2.5 μg of endotoxin; the controls received only the endotoxin. From the mean febrile responses shown it is clear that there was no significant effect of this small amount of tolerant plasma on the pyrogenicity of the endotoxin test dose. The following day, both groups were tested with 10 μg doses of endotoxin. This increase in dose was sufficient to produce in the controls another biphasic fever, of greater magnitude than that seen the first day. The experimental group, however, despite the ineffectiveness of the tolerant plasma pre-treatment against the lesser challenge the previous day, responded this second day with a fever significantly smaller than both that suffered by the controls and the previous day's response.

For the various modifications of method by which this experiment was done (*vide supra*), there were instances where the second day biphasic response of the controls closely approximated that seen the first day, in which case the experimental group showed a monophasic fever the second day. Similarly, where there was an apparent small effect of the tolerant donor plasma on day 1 (which, in any one experiment, could barely be considered significant), the experimental group's response on day 2 was monophasic. Thus, this striking difference in febrile response upon rechallenge with an increased dose of

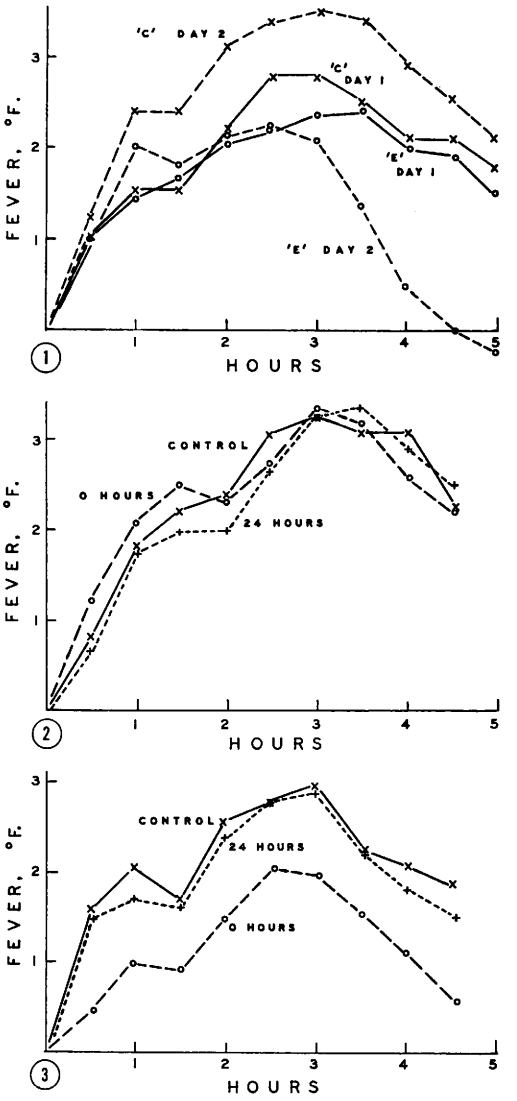

FIG. 1. First and second day mean febrile responses of control (C) and experimental (E) groups of 4 rabbits each, given 2.5 μg endotoxin on day 1 and 10 μg endotoxin on day 2; experimental group received 2 ml tolerant donor plasma 5 min before endotoxin on day 1.

FIG. 2. Mean febrile responses to 2.5 μg endotoxin given at 0 and 24 hr after 2 ml pooled tolerant donor plasma.

FIG. 3. Mean febrile responses to 2.5 μg endotoxin given at 0 and 24 hr after 4 ml pooled tolerant donor plasma.

endotoxin, 24 hours after an initial test with an amount of tolerant plasma ineffective against the lesser challenge used at that time was seen consistently.

Since passive tolerance may be easily demonstrated on direct test by using larger volumes of tolerant donor plasma, *e.g.*, 10 ml (2,4), it appeared possible that the initially ineffective smaller volumes used here might require a longer interval between administration and testing. Groups of 4 to 6 rabbits each were given 2 ml of tolerant donor plasma and were tested with the 2.5 μg dose of endotoxin after intervals of 0, ½, 1, 2, and 24 hours, with controls given only the endotoxin. In Fig. 2 are shown the mean febrile responses of the groups tested at 0 and at 24 hours after treatment. This dose of plasma was ineffective at all test intervals, so the tolerance seen on day 2 in the experiment described in Fig. 1 cannot be attributed to a direct late effect of the plasma, even disregarding the 4-fold increase in dose of endotoxin used in the first experiment.

The results of the experiments represented by Fig. 1 might be a consequence of an additive effect, *i.e.*, a combination of the passively supplied humoral mediator plus an amount contributed by the animal as a result of the first endotoxin dose. This latter portion would also occur in the controls, constituting the base-line against which differences are measured. The sum would exceed some critical value, making possible the second day tolerant response to the larger dose of endotoxin, when neither portion, separately, is responsible. For hypotheses involving any direct inactivation or opsonization of endotoxin by mediator, it would be imperative that the passive contribution of humoral factor to the postulated critical sum be present at the time of rechallenge the second day.

To study this point under conditions applicable to the present investigation the following experiment was done. A group of 3 rabbits was given 4 ml volumes of the pooled tolerant donor plasma used for the present studies. Twenty-four hours later a second group of 3 was given the same treatment, and directly thereafter both groups, plus a third untreated group, were tested with 2.5 μg doses of endotoxin. The mean febrile responses are given in Fig. 3. The limited effectiveness of this pre-treatment with tolerant donor plasma apparent against immediate challenge had disappeared by the following day. Any contribution of humoral mediator 24 hours after an even lesser dose and tested with an even greater challenge would appear most unlikely.

Discussion. The results of the present investigation raise the following question: if the humoral mediator acts directly upon the endotoxin by any particular mechanism—inhibition, detoxification, opsonization—to effectively lower the dose of endotoxin for the animal, how can the mediator in a small amount of plasma have no effect upon 2.5 μg of endotoxin exposed to it by injection minutes later, but be remarkably effective against a larger dose given the same animal 24 hours later? The tolerance seen on the second day in the experiments represented by Fig. 1 cannot be attributed to the endotoxin itself, for this is the treatment given the controls to which comparison is made. The humoral factor cannot be acting directly in a delayed or additive manner for the control experiments disprove this. It appears most reasonable that what we recognize as tolerance is the result of an ill-understood change in the animal, taking some time to develop, and initiated by neither endotoxin alone nor humoral mediator alone, but requiring both the endotoxin and the factor that appears (at least in increased amount) in the blood of an endotoxin-treated animal (made available here by passive transfer).

The experiments reported here in no way rule out an immunological mechanism for endotoxin tolerance. With the invalidation(4,6, 7) of most of the assumptions that led to the belief that tolerance does not have an immunological basis the possibility that the humoral mediator discussed here is an antibody must be considered, although no direct evidence for this is available. Recently, Watson and Kim(8) have offered impressive experimental evidence consistent with an im-

munological mechanism. If antibody is assumed, our results would suggest that tolerance is a host response to an antigen-antibody interaction, a circumstance which has not been demonstrated for any endotoxic activity.

Summary. Rabbits given volumes of endotoxin-tolerant donor plasma too small to modify significantly the course of fever produced by a first test dose of endotoxin injected shortly thereafter, display a remarkable degree of tolerance to a larger dose of endotoxin given 24 hours later, compared to identically endotoxin-treated controls. The results are inconsistent with hypotheses invoking any direct effect of the humoral mediator upon the endotoxin.

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1. Freedman, H. H., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 504.
2. ———, *J. Exp. Med.*, 1960, v111, 453.
3. ———, *Ann. N. Y. Acad. Sci.*, 1960, v88, 99.
4. Greisman, S. E., Carozza, F. A., Jr., Hills, J. D., *J. Exp. Med.*, 1963, v117, 663.
5. Freedman, H. H., *ibid.*, 1960, v112, 619.
6. Watson, D. W., Kim, Y. B., in *Symposium on Bacterial Endotoxins, Inst. Microbiol.*, Rutgers, Sept. 4-6, 1963, in press.
7. Freedman, H. H., Sultzer, B. M., *ibid.*
8. Watson, D. W., Kim, Y. B., *J. Exp. Med.*, 1963, v118, 425.

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Protein-Polysaccharide Complexes of Normal and Herniated Human Intervertebral Discs.* (28984)

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Changes in the chemical composition of the intervertebral disc with aging or prolapse have been studied by a number of investigators(1,2,3,4). They reported the following changes with increasing age: a decrease in total polysaccharide content, an increase in collagen content and an increase in ratio of keratosulfate to chondroitin sulfate-C. It has also been shown that, in rabbits, growth hormones, androgens and estrogens are able to alter the composition of mature tissue toward that representative of a younger age.

Very little work has been published on the chemical and physical properties of the mucopolysaccharide-protein complexes from either the nucleus pulposus or annulus fibrosus of the human intervertebral disc, despite the fact that this is very probably the form in which these compounds exist *in vivo*. This paper presents results of a study of some of the chemical and physical properties of these complexes and their changes with increasing age and in disc prolapse.

Methods. Material for the "normal" specimens was obtained from subjects of ages 15 to 88 years, who were autopsied within 6 hours after death. In each case a large section of the vertebral column was removed and immediately frozen at the temperature of dry ice. When frozen solid, the bone was removed from the disc with an electric band saw, the material from the disc placed in a polyethylene bag and stored over dry ice until ready for extraction. Samples of herniated disc were obtained at surgery and frozen immediately in dry ice until ready for extraction.

The annulus fibrosus and nucleus pulposus were separated and extracted separately. Thin sections were freeze-dried and weighed. The freeze-dried tissue was minced and extracted at 0 to 5°C with water for 1.5 hours in a Virtis "23" homogenizer running at half speed. Soluble collagen was eliminated by precipitation with 2 volumes of ethanol in the

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