between the results.

Previous studies in this laboratory using Fe-59 indicated plasma volumes comparable to those reported by the investigators previously mentioned; however, in these studies the whole curve over a period of 2½ hours was used. In the present study the early part of the curve (the first 12 minutes) was used and as can be seen in Table II these volumes agreed with those found using ICG. It can, therefore, be concluded that even though the absolute values of the plasma volumes vary between studies, depending on the technique used, the ICG method of estimating the volume of distribution is a reliable one. The sex and T ½ values are included in this Table also to show that the additional data support the sex difference found in this study.

This report also indicates that the single injection method using ICG is a relatively simple and reliable one for evaluating liver function. The somewhat longer plasma clearance time and no evidence of extra-hepatic clearance are decided advantages of the ICG method over the BSP method.

Summary. 1. The mean plasma clearance half time of indocyanine green (ICG) was found to be 5.6 ± 0.8‡ minutes in male beagles, and 7.0 ± 1.48 in female beagles. 2. Female dogs in this study cleared ICG significantly more slowly than the males. 3. Plasma volume estimated using ICG is 37.7 ± 6.0 ml/kg. This figure agrees with 37.5 ± 6.1 ml/kg found using radioiron (Fe-59).

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Relationship of Endogenous Pyrogen and Serum Augmenting Factor To Endotoxin Tolerance.* (30141)

JONAS A. SHULMAN AND ROBERT G. PETERSDORF
Department of Medicine, University of Washington and King County Hospital, Seattle

Following intravenous injection of Gram-negative bacterial endotoxin, a characteristic febrile response occurs in rabbits and in man(1). Repeated daily injection of endotoxin produces a state of tolerance manifested by a decreasing febrile response(2). The mechanism of pyrogen tolerance is poorly understood. Initially tolerance was attributed to more rapid reticuloendothelial (RES) clearance of endotoxin because rabbits made tolerant to typhoid vaccine again became fully susceptible to the pyrogenic action of endotoxin following administration of thorium dioxide, thorotrast, a RES blocking agent(3). The relationship of tolerance to RES clearance has recently been challenged with evidence that endotoxin-tolerant rabbits developed pyrogen tolerance independently of their capacity to clear heterologous colloids from the blood(4). It was also shown that RES blockade did not reverse tolerance to the pyrogenic effects of endotoxin when normal-blockaded were compared with tolerant-blockaded rabbits, and that pyrogen tolerance can be passively transferred(4,5). These studies suggested that some factor present in serum was responsible for endotoxin tolerance. It is conceivable that this substance is related in some manner to the "endotoxin-augmenting" factor (AF) in serum described

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by Farr *et al*(6). These investigators showed that incubation of endotoxin in normal serum *in vitro* produces an augmentation of the febrile response. Serum from tolerant animals failed to augment the pyrogenic effect of endotoxin. Not only did tolerant serum not enhance the febrile response, but it also inhibited the augmenting effect of normal serum when added to a pyrogen-serum mixture.

The experiments to be described were performed to reexamine the possible role of these serum factors in production of endotoxin tolerance and to determine if passive transfer was associated with inability of the recipient animals to augment the pyrogenic action of endotoxin or to release endogenous pyrogen (EP).

Materials and methods. Male and female albino rabbits weighing 2-3 kg were used in all experiments. Animals were housed unrestrained in an air-conditioned room and food and water were withheld on the day of experiment. The endotoxin employed in all studies consisted of heat-killed *E. coli* vaccine containing approximately 10^8 cells per ml. EP was prepared in donor rabbits which were injected intravenously with 1.0 ml endotoxin and were bled 120 minutes later by cardiac puncture. Blood was allowed to clot overnight and serum cleared by centrifugation at 4°C. Serum was cultured and used only if bacteriologically sterile. Assay for EP was performed in 3 normal recipient rabbits. Temperatures were measured by means of an indwelling rectal thermister (Telethermometer, Yellow Springs Instrument Co.). Temperatures were recorded at 15-minute intervals. Fever curves were plotted on standard graph paper and a fever index was determined by planimetry.

Tolerance was produced by daily injection of 1.0 ml *E. coli* endotoxin for 14 days. Febrile responses were measured in 9 animals on the 1st, 8th, and 14th days to confirm presence of tolerance.

Augmenting factor (AF) in normal and tolerant serum was assayed by incubating 4.0 ml aliquots with 1.0 ml *E. coli* endotoxin for 30 minutes at 37°C. The serum-endotoxin mixture was then tested in 4 tolerant recipients. The degree of augmentation for each sample was quantified by subtracting the fever index produced by endotoxin alone from the fever index produced by the serum-endotoxin mixture.

To avoid contamination by bacterial pyrogens, all needles, glassware, and instruments were sterilized in hot air ovens at 170° C for 3 hours. All serum and other injectable agents were cultured in thioglycollate broth for 72 hours and discarded unless sterile.

Results. Decrease in EP and AF with development of tolerance. Fig. 1 shows typical development of endotoxin tolerance with a progressive decrease in both height and duration of fever between the 1st and 14th days. As fever decreased, animals experienced a parallel reduction in both AF and EP (Fig. 2).

Effect of passive transfer of tolerant serum elaboration of EP and AF. Fig. 3 demonstrates that passive transfer of tolerant serum from normal recipients produced a state of tolerance similar to that of animals made tolerant by repeated daily injections of pyrogen. Animals made tolerant by transfer produced no more EP than animals made tolerant by repeated injection of endotoxin (Fig. 4). Serum from these "passively transferred" tolerant donors failed to augment endotoxin (Fig. 4).

Effect of thorotrast on pyrogen tolerance and release of EP. RES blockade was produced by administration of thorotrast 6 hours before challenge with endotoxin. When thorotrast-treated normal animals were compared to thorotrast-treated tolerant animals, a marked degree of pyrogen tolerance was still demonstrable (Fig. 5), confirming previous observations that RES blockade does not completely reverse endotoxin clearance(4). Fig. 6 shows that thorotrast-treated tolerant animals produced some EP but considerably less than was elaborated by thorotrast-treated normal animals, emphasizing that when tolerance is expressed in terms of an animal's ability to release EP, RES blockade is associated with only a partial reversal of tolerance. Presence of AF paralleled release of EP.

Discussion. Recently evidence suggesting that humoral factors are important in the

mechanism of pyrogen tolerance has been gathered and two groups of investigators have been able to transfer pyrogen tolerance passively in rabbits(4,5). The present studies confirm these observations and also demonstrate that 2 other parameters of endotoxin

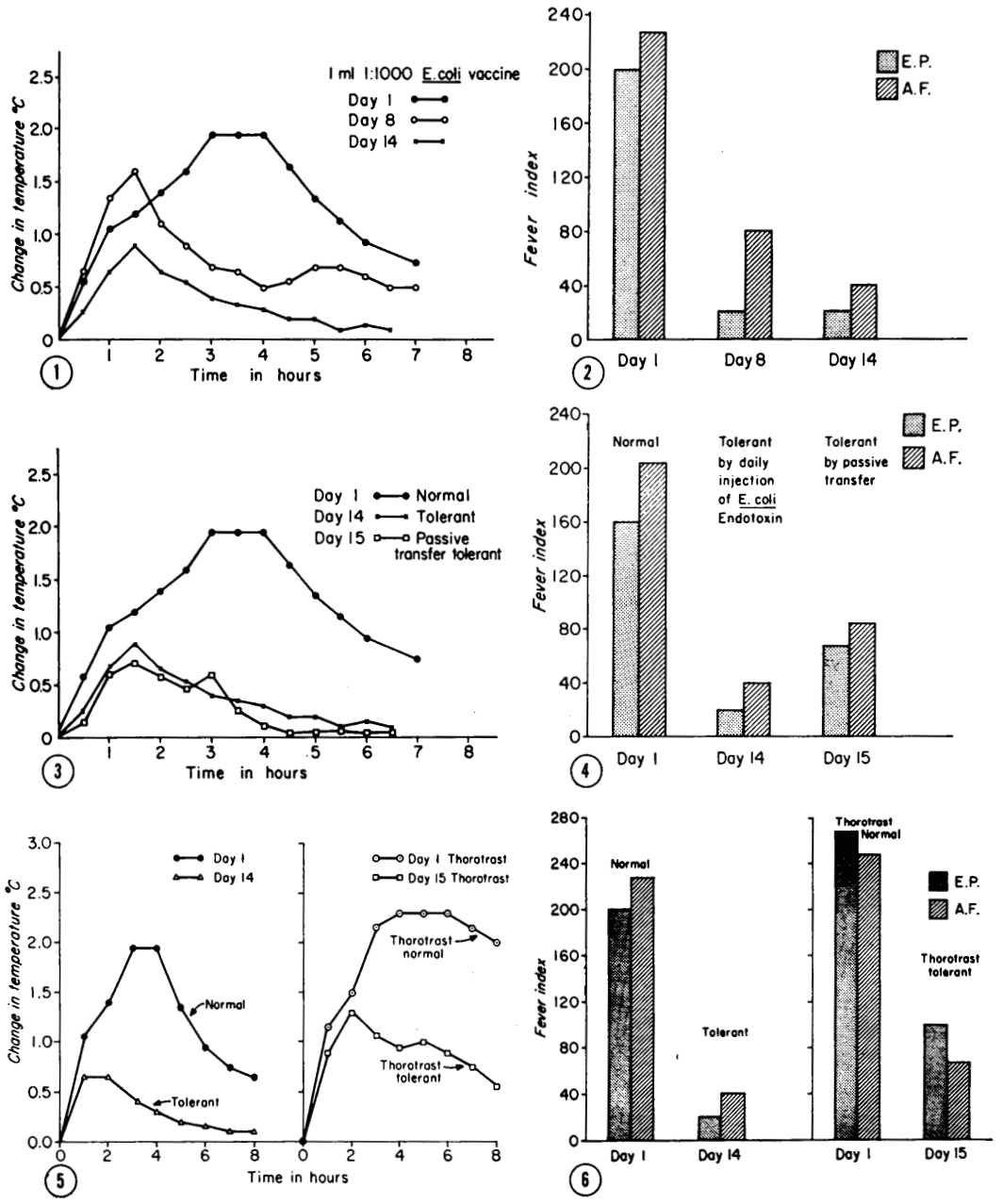

FIG. 1. Development of tolerance by daily injection of *E. coli* vaccine in 9 rabbits.

FIG. 2. EP and AF during development of tolerance.

FIG. 3. Fever curves of normal rabbits and rabbits made tolerant by daily injection of endotoxin and by passive transfer.

FIG. 4. EP and AF in normal animals, animals made tolerant by daily injection of endotoxin and animals made tolerant by passive transfer.

FIG. 5. Fever curves of normal and tolerant rabbits before and after RES blockade.

FIG. 6. EP and AF in normal and tolerant animals before and after RES blockade.

tolerance, diminished release of EP and disappearance of AF in serum, occur in animals made tolerant by passive transfer. There was a close temporal relationship between development of pyrogen tolerance and loss of AF and reduction in EP. Furthermore, following RES blockade, tolerance was not abolished and neither AF nor EP reappeared in serum.

The parallelism between pyrogen tolerance, AF and EP raises two questions. First, what is the role of augmenting factors, or inhibitors in tolerant animals, in the pathogenesis of tolerance? Second, what is the relationship of these serum factors of release of EP? Since tolerant serum *in vitro* blocks the augmentation of endotoxin by normal serum, and does not inhibit endotoxin directly, it is conceivable that *in vivo* the inhibitor in tolerant serum may act not against endotoxin *per se* but against the complex of endotoxin plus serum augmenting factor. This argument gains some support by the close association between the pyrogenicity and antigenicity of some endotoxins(7). It is possible that endotoxin fever is mediated by an antigen-antibody reaction, *i.e.*, a reaction in which the endotoxin-normal serum mixture acts as the antigen-antibody complex. If this were true, tolerance would be due to the appearance of an inhibitor or blocking antibody.

The relationship of AF to EP is not clear. It is unlikely that the two are identical simply because normal serum which contains AF fails to produce fever. On the other hand, the biological similarities between AF and EP resurrect the argument that EP produced following injection of endotoxin may be only the toxin modified by contact with serum. Previous studies have shown that there are a number of similarities between endotoxin-in-serum and EP. Both substances produce similar fever curves in normal and tolerant recipients when similar doses are employed(8). With both, there is a short latent period between time of injection and onset of fever(9), neither is associated with demonstrable tolerance in the usual doses employed(10,11), and both are heat-labile(11). The present studies extend these observations indirectly because they show that in each of 3 situations in which augmenting factor is absent from serum and in which interaction between this factor and endotoxin presumably cannot take place, EP is also lacking. While the data should not be interpreted to mean these endotoxin-in-serum and EP are identical, they emphasize that the relationship of these substances to one another and to leukocytic pyrogen cannot be settled with certainty until they are characterized more precisely by biochemical techniques.

Summary. Pyrogen tolerance was produced by repeated daily injections of endotoxin. As tolerance developed, the ability of the serum to augment the pyrogenic effect of endotoxin and production of endogenous pyrogen were diminished. When tolerance was produced by passive transfer, these 2 parameters were similarly affected. RES blockade did not reverse tolerance nor did it restore augmenting factor or markedly increase the animal's ability to elaborate endogenous pyrogen following injection with endotoxin. These observations suggest that serum inhibitors may mediate tolerance by inactivating the ability of serum to augment endotoxin.

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