be responsible for the protection. The studies of Iversen *et al*(9) showing decreased cardiac uptake of H^3-norepinephrine in reserpinized rats at 24 hours were performed with a comparable dose of norepinephrine. Likewise, Pokrovskaya(10) found decreased myocardial sensitivity to cardiac glycosides in both intact and decapitated frogs which were treated with reserpine. However, Tanz and Marcus(11) have questionel whether the myocardial catecholamine content necessarily reflects the physiological responses of this organ. In addition, Spann *et al*(12) have more recently noted that depletion of cardiac norepinephrine stores by reserpine did not alter the inotropic response of cat papillary muscles to strophanthidin.

The data of Table I indicate that the depletion of catecholamines from the adrenal medulla cannot suffice as the sole explanation for these observations. The depletion of brain catecholamines by reserpine may explain this protection although other mechanisms cannot yet be disregarded.

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Antibody Formation at Various Times After Previous Treatment of Mice With Endotoxin. (32152)

HENRY H. FREEDMAN, ALFRED E. FOX, AND BENJAMIN S. SCHWARTZ
Department of Microbiology, Warner-Lambert Research Institute, Morris Plains, N. J.

The number of hemolysin-forming spleen cells increases after injection of bacterial endotoxin into normal mice(1). This increase fails to occur in mice previously made endotoxin-tolerant by a series of daily doses, although the response to specific antigen, sheep red blood cells, is unimpaired(2). In addition, endotoxin has an adjuvant effect, elevating the specific immune response when injected together with sheep red blood cells(2). There are reasons for believing that these stimulatory effects of endotoxins on pre-existing hemolysin-forming cells may not be attributable to cross-reacting antigens, but rather to the cytotoxicity of endotoxins with release of stimulatory DNA breakdown products(2-5). Whether cytotoxicity of endotoxins reflects a direct primary toxicity or has an immune basis is presently unresolved(6). The present report extends previous studies on the influence of endotoxin on antibody formation and supports the hypothesis that reactivity to these ubiquitous antigens is conditioned by previous exposure.

Materials and methods. Female CD-1 or C57Bl/6 mice, weighing 18-20 g, were used. Protein-containing Boivin type endotoxins (ET) of *Salmonella abortus equi, Salmonella typhosa, Serratia marcescens* and *Escherichia coli* (Difco), and protein-containing and pro-

TABLE I. Influence of Previous Treatment with *S. abortus equi* Endotoxin on Numbers of Hemolysin-Forming Spleen Cells 48 Hours After Injection of Sheep Red Blood Cells (sRBC) or Homologous Endotoxin (ET) in CD-1 Mice.

Treatment	No. hemolysin-forming spleen cells (mean ± S.E.) Interval, pretreatment to challenge 1 day	10 days
None	3 ± 1.0	1 ± 1.0
Pretreated, only*	4 ± .6	3 ± 1.7
sRBC, only	72 ± 5.3	49 ± 15.7
Pretreated, sRBC	157 ± 4.6	32 ± 3.3
ET, only	42 ± 2.6	30 ± 2.2
Pretreated, ET	11 ± 3.9	125 ± 25.0
ET + sRBC, only		285 ± 80.4
Pretreated, ET + sRBC		769 ± 65.7

* Mice given 0.01 γ ET and spleens harvested and assayed 3 or 12 days later.

tein-free endotoxins of *Salmonella enteritidis* (kindly provided by Dr. Edgar Ribi) were injected i.p. Sheep red blood cells (sRBC) were injected i.v. at a dose of 10^8/mouse. Numbers of antibody-forming spleen cells were determined at 48 hours after challenge with sheep red blood cells or injection of 10 γ of endotoxin. Pretreated mice received 0.01 γ of endotoxin i.p., at 1 to 12 days before challenge with sheep red cells, endotoxin, or both. Responses to challenge of pretreated mice were compared in each assay to responses of non-pretreated control mice given the identical challenge. Mean ± standard error was computed for each group and significance of difference between means accepted for "p" values less than 0.01. Hemolysin formation was assayed by the technique of localized hemolysis in agar(7), using sheep red blood cells and 1/5 of the cell suspension of the whole spleen as previously described(2). Individual assays employed control and experimental groups of 5 mice each, and all experiments were repeated several times. Dilutions for injection were made in non-pyrogenic saline and rigorous precautions to avoid contamination with extraneous endotoxins were observed.

Results. The data in Table I demonstrate that when mice treated with 0.01 γ endotoxin the previous day were injected with sheep red blood cells the numbers of hemolysin-forming spleen cells found 48 hours later were significantly increased compared with the responses in mice not previously treated with endotoxin. In contrast, when endotoxin was substituted for the specific antigen the numbers of hemolysin-producing cells 48 hours later were depressed in pretreated mice compared with responses to the test dose of endotoxin in control mice which had not been pretreated. The number of hemolysin-forming cells in the spleens of mice treated with 0.01 γ endotoxin 10 days prior to injection with sheep red cells was about the same as that of mice not pretreated with endotoxin. In contrast, when endotoxin was substituted for the specific antigen, the number of hemolysin-forming cells was increased by pretreatment with endotoxin. This effect was also found for the adjuvant effect of endotoxin given together with the specific antigen. Mice assayed after only the pretreatment dose of endotoxin had numbers of hemolysin-forming cells not significantly different from the numbers found in untreated mice. The influence of pretreatment with each of 5 endotoxins on hemolysin formation following subsequent injection of the same endotoxin is shown in Table II. Responses to the test dose given 1 day after pretreatment were significantly depressed for all but the *E. coli* and protein-free *S. enteritidis* endotoxins. At 10-12 days after pretreatment, injection of any but the protein-free endotoxin resulted in greater than normal increases in numbers of hemolysin-forming spleen cells. The effect of endotoxins on numbers of hemolysin-forming spleen cells after pretreatment with heterologous endotoxin is given by the data in Table III. Pretreatment with *S. abortus equi* endotoxin 10 days earlier resulted in elevated responses to the homologous and to the *E. coli* endotoxins, but not to the *S. marcescens* endotoxin which was equally active in control mice not pretreated. In Table IV are given data from experiments in which the increase in numbers of antibody-producing cells after injection of endotoxin was investigated in mice given serum or spleen cells of donors pretreated with endotoxin. Normal mice or mice given 0.01 γ endotoxin 10 days earlier served as donors. Serum (0.25 ml) or spleen cells (1.7×10^8 cells) was injected i.p. into normal

recipient mice which were then given endotoxin. Only transfer of viable spleen cells of pretreated donors resulted in greater than normal increases in numbers of hemolysin-forming cells in response to endotoxin. In additional experiments serum of pretreated donors failed to influence the response to endotoxin given within 4 hours of transfer, and spleen cells from donors pretreated with the *S. abortus equi* endotoxin did not provide elevated responses in recipient mice tested with the *S. marcescens* endotoxin (see also Table III). When only spleen cells of pretreated donors were injected into normal mice, the numbers of antibody-producing cells found either 4 or 6 days later (the times of

TABLE II. Influence of Previous Treatment with Endotoxin on Numbers of Hemolysin-Forming Spleen Cells 48 Hours After Injection of the Same Endotoxin in CD-1 Mice.

Treatment	Pretreatment to challenge, interval, days	No. hemolysin-forming spleen cells (mean ± S.E.) Endotoxins* S.a.e.	S.t.	E.c.	S.e.	S.e.P-F
None	—	2 ± 1.3	1 ± .9	3 ± 1.6	1 ± .7	1 ± .7
Challenged, endotoxin, only	—	34 ± 5.2	22 ± .6	28 ± 2.2	29 ± 1.3	16 ± .8
Pretreated, challenged	1	7 ± 2.6	4 ± 1.5	35 ± 2.9	3 ± 1.2	17 ± 1.6
" , "	10–12	112 ±13.0	78 ± 8.9	104 ±13.0	76 ± 3.6	18 ± 1.9
" , only†	—	1 ± 1.5	4 ± 2.2	1 ± .8	2 ± 1.2	1 ± 1.3

* Endotoxins: S.a.e., *S. abortus equi*; S.t., *S. typhosa*; E.c., *E. coli*; S.e., *S. enteritidis*; S.e.P-F, *S. enteritidis* protein-free.
† Assayed 12-14 days after pretreatment.

TABLE III. Influence of Treatment 10 Days Earlier with *S. abortus equi* Endotoxin on Numbers of Hemolysin-Forming Spleen Cells 48 Hours After Injection of Homologous or Heterologous Endotoxins in CD-1 Mice.

Treatment	No. hemolysin-forming spleen cells (mean ± S.E.) Endotoxins* S.a.e.	S.m.	E.c.
None	1 ± .4	6 ± 5.9	1 ± .4
Challenged, only	36 ± 2.8	36 ± 6.2	44 ± 8.9
Pretreated, challenged	120 ± 16.5	19 ± 5.6	121 ± 13.3
" , only†	1 ± 1.1	2 ± .9	1 ± 1.1

* Endotoxins: S.a.e., *S. abortus equi*; S.m., *S. marcescens*; E.c., *E. coli*.
† Assayed 12 days after pretreatment dose.

TABLE IV. Influence of Transfer of Serum or Spleen Cells Harvested from Normal or Pretreated Donor Mice on Numbers of Hemolysin-Forming Spleen Cells 48 Hours After Injection of *S. abortus equi* Endotoxin, in C57Bl/6 Mice.*

Treatment of assayed recipients	No. hemolysin-forming spleen cells (mean ± S.E.) Exp A	Exp B
None	1 ± .8	1 ± 1.0
Challenged, only	21 ± .8	23 ± 1.4
Normal donor serum, challenged	19 ± 3.1	
" " cells, "	17 ± 1.2	
Pretreated donor serum, challenged	24 ± 1.8	
" " cells, "	81 ± 8.3	113 ± 10.8
" " " , heated,† challenged		39 ± 14.6
" " " , frozen,‡ "		23 ± 1.4
" " " , only, 4 days		2 ± 1.2
" " " , " , 6 "		2 ± 1.1

* Pretreated donors received homologous endotoxin 10 days before harvest, and recipient mice were injected with endotoxin 4 days after transfer.
† Spleen cells heated at 56°C for 30 min before transfer.
‡ Spleen cells subjected to 5 cycles of freezing-thawing before transfer.

endotoxin injection or assay in tested recipients) did not differ from the numbers found in untreated mice.

Discussion. It has previously been shown (2) that the increase in number of hemolysin-forming spleen cells in mice injected with endotoxin fails to occur in mice given a series of daily doses of endotoxin, whereas the increase in numbers of antibody-forming spleen cells after injection of specific antigen, sheep red blood cells, becomes far greater in mice given a series of daily injections of specific antigen. The present report extends these findings to show that mice pretreated with a single small dose of endotoxin, which does not by itself significantly modify the numbers of hemolysin-forming spleen cells, are initially refractory to the influence of a test dose of endotoxin on numbers of hemolysin-forming spleen cells. In contrast, such pretreated mice respond to sheep red cells with greater than normal increases in numbers of antibody-producing spleen cells. At a later time after pretreatment with endotoxin, mice respond to sheep red blood cells with only normal increases in numbers of hemolysin-forming spleen cells whereas injection of endotoxin results in significantly greater responses than are produced by the same dose of endotoxin in not pretreated mice. This increased reactivity to endotoxin is found for both endotoxin given alone and for the adjuvant effect of endotoxin given together with sheep red cells. Clearly, host reactivity to endotoxin is conditioned by previous exposure of normally reactive mice. The *S. marcescens* endotoxin is fully active in increasing the numbers of hemolysin-forming spleen cells in normal mice, yet, unlike the *E. coli* endotoxin, it does not produce a greater than normal increase in mice pretreated with the heterologous *S. abortus equi* endotoxin.

Results with the protein-free endotoxin preparation of *S. enteritidis,* which contains 0.2% nitrogen accounted for by hexose-amine (8), are of particular interest. This endotoxin differs from the protein-containing Boivin or *S. enteritidis* endotoxins in that only mice pretreated with it do not respond with greater than normal increases in numbers of hemolysin-forming spleen cells when subsequently injected with the same endotoxin 10-12 days later. Whether widespread reactivity of normal animals to the many effects of endotoxin, including cytotoxicity and stimulation of antibody formation, has a basis in acquired hypersensitivity resulting from exposure to Gram-negative bacteria is unresolved(6). The data reported here for antibody formation are compatible with results of studies demonstrating induced hyperreactivity to other endotoxic effects(9-11) by previous treatment with endotoxin, including transfer by cells (11). The hypothesis that a naturally acquired hypersensitivity of the delayed type contributes to normal host reactivity to endotoxin, and the problems in accepting this hypothesis have been discussed critically(12). While protein-free lipopolysaccharide endotoxins are reported to be no less active than conventional preparations in normal animals (8) the protein associated closely with endotoxic activity in the native state is present during the residence of relevant bacteria in the host, and thus the present findings with the protein-free endotoxin take on added significance. From the present and continuing studies the current estimate of the protein moiety in immunological responses to the macromolecular endotoxins will require re-evaluation.

Summary. The effects of sheep red blood cells or endotoxins on numbers of hemolysin-forming spleen cells have been studied in mice previously injected with a small dose of endotoxin. One day after pretreatment the response to sheep red cells was greater than normal and the response to endotoxin was smaller than normal. At 10-12 days after pretreatment, the response to sheep red cells was normal but the response to endotoxin, given alone or together with sheep red cells, was greater than normal. Of 5 endotoxin preparations studied, only a protein-free endotoxin failed to alter reactivity to a subsequent injection of the same endotoxin.

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Effect of Evisceration on the Development of Tourniquet Shock.* (32153)

JOHN KABAL AND ESTELLE R. RAMEY (Introduced by L. S. Lilienfield)
Department of Physiology and Biophysics, Georgetown University, Schools of Medicine & Dentistry, Washington, D. C.

The whole concept of the role of 'toxic' factors in the development of the shock picture has been widely argued. For example, the lethality of bilateral limb ischemia is said to be caused by toxic factors arising from the injured limbs. This view is based on the observation that survival time of tourniquet stressed animals is extended following the release of the tourniquet if the tourniquet is reapplied within a critical period(1). This could be interpreted to mean either that the toxic factor theory is untenable or that a critical quantitative relationship exists between the amount of toxic factor released and the precipitation of the shock state. None of these so-called toxic factors have been identified from traumatized limbs and the question remains unresolved. The most widely held theory of the mechanism leading to the development of shock relates to the pooling of fluid in the traumatized area. There is an extensive literature to support this concept(2). We have data (to be reported later) which tends to support the hypothesis that massive extravasation of fluid from limb blood vessels damaged by prolonged anoxia, leads to a diminution in circulating blood volume with subsequent hypotension and vascular collapse.

Another line of evidence suggests that the mesenteric hyperemia and local intestinal hemorrhages observed following traumatic shock result in anoxic damage to gut cells leading to increased permeability of toxic substances(3). If this were the case, it might be postulated that removal of the source of such factors, *i.e.*, evisceration, could alter the survival time of tourniquet stressed animals and extend this to the survival time of the eviscerated animal without tourniquet. This study is an attempt to verify this hypothesis. We eviscerated tourniquet stressed animals and compared their survival time to non-eviscerated animals with hind limb ischemia. We included adrenalectomized animals in this study because of their well known stress sensitivity. It was felt that if the gut toxins were contributing significantly to the development of tourniquet shock, this might be markedly demonstrated in an animal with a very small range of homeostatic responses.

Methods. Male, albino rats of Sprague-Dawley strain, 225 ± 25 g, were used throughout the experiment. The animals were about 10 weeks old.

The animals were maintained on Purina Rat Chow and drinking water *ad libitum*. The room temperature was maintained at 72 ± 2°F. The rats were kept in individual cages.

Limb ischemia was produced by ligation of the hind limbs at the inguinal level with a double rubber band under light ether anesthesia. The ligature was kept in place for 5 hours.

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