Antibody Formation in Endotoxin-Tolerant Mice.* (31012)

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Bacterial endotoxins, when injected into an animal together with a specific antigen, can elevate the specific immune response(1). In addition, endotoxins can elevate preexisting low levels of a large variety of specific antibodies and this effect can be elicited when endotoxin is administered without antigen (2,3,4). There is good reason to believe that such multiple effects of endotoxin on preexisting antibody-forming cells cannot be attributed to cross-reacting antigens(5) but may involve the release, by endotoxin, of a stimulator from intracellular sites(5); this stimulator, in turn, can trigger the multiplication of antibody-forming cells. It is now known that oligodeoxyribonucleotides are capable of producing such stimulatory effects in the presence of either specific antigen or permeability-altering agents(4). This has led to the suggestion (6) that the cytotoxic effects of endotoxins may release such stimulatory DNA breakdown products and that the effects of endotoxins on antibody formation are thus indirect rather than direct. Recently, it has become feasible to assay the number of antibody-forming cells with the aid of Jerne's plaqueing technique(7). This technique, especially when applied to tests with endotoxin-tolerant animals, has now provided us with an opportunity to determine more accurately to what extent the stimulatory effects of endotoxin on antibody production are dependent on events involving the antibody-forming cells themselves or involve events at a prior stage of suspected release of stimulators. It is well-known that the "tolerance" resulting from repeated injections of endotoxin is accompanied by a decrease in toxic, pyrogenic, and adjuvant effects(8,9). It, therefore, became of interest to determine, a) whether in the tolerant animal the number of hemolysin-forming spleen cells (which increases following injection of endotoxin into the non-tolerant animal) will fail to increase, and b) whether an absence of such effects is accompanied by any alteration in responses to a specific antigen (sheep red blood cells). Also, if the inability of endotoxin-tolerant animals to respond to endotoxin should involve a destruction of the effector that triggers the release of a stimulator for antibody-forming cells, then the suspected stimulator itself, *i.e.*, oligodeoxyribonucleotides, should still be able to elevate the response to an antigen. Accordingly, normal and tolerant mice were exposed to endotoxin, sheep red blood cells, oligodeoxyribonucleotides, or a combination thereof, and their spleens were assayed for numbers of hemolysin-forming cells.

Materials and methods. Female AKR mice, weighing 20-25 g, were used. Sheep red blood cells (sRBC) were injected i.v. at a concentration of 1×10^8/mouse; endotoxin (Difco, *Serratia marcescens*, Control No. 469775) was given i.p.—DNA digest was prepared and administered i.v. and i.p. as described previously (1). Carbon ("Pelikan" C11/1431, Günther Wagner), when used, was injected i.v. (0.25 ml) at a concentration of 30 mg/ml in sterile 0.6% gelatine (Charles B. Knox Co.). For production of endotoxin tolerance, 10 γ of the endotoxic lipopolysaccharide were injected i.p. daily for 10 days; in a second series of experiments (Table IV), 10 γ were given on days 1 and 2, 50 γ on days 3 and 4, 100 γ on days 5, 6, 7, 8 and 9, and 10 γ on day 10. Hemolysin production was assayed, on 1/5 of a cell suspension prepared from an entire spleen, using the technique of localized hemolysis in agar (4,7). Groups of 5 animals were used in all spleen cell assays.

Results. The data in Table I and IV confirm that in the normal mouse endotoxin can elevate the response to a specific antigen and also can cause a response in the absence of antigen (which, as in the present case, can occasionally surpass the response to specific antigen, at least at certain doses). In the endotoxin-tolerant mouse endotoxin fails to

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TABLE I. Influence of Endotoxin-Tolerance on Number of Hemolysin-Producing Spleen Cells 48 Hr After i.p. Injection of Endotoxin or i.v. Injection of Sheep Red Blood Cells.

Treatment of spleen donors	Avg No. (±S.E.) of hemolysin-producing cells per 1/5 spleen
None	3.0 ± .9
Endotoxin (1x)	110.0 ± 26.1
" (10x)	16.4 ± 6.9
" (1x) + sRBC (1x)	441.2 ± 71.1
" (10x) + " "	116.8 ± 40.9
sRBC (1x)	47.0 ± 4.6
" (10x)	1500 ±215.0

produce its usual effect, but specific antigen (sRBC) still produces an undiminished effect. Table I also indicates that, in contrast to what occurs following daily injections of endotoxin, the repeated i.v. injection of sRBC resulted in a striking elevation of the number of hemolysin-forming spleen cells.

To determine whether a reestablishment of endotoxin susceptibility could result in an immediate reestablishment of the stimulatory effects of endotoxin on hemolysin formation, tolerant mice were subjected to a type of RES "blockade" known to result in renewed sensitivity to endotoxin. As shown in Table II, the administration of carbon 3 hours prior to the last injection of endotoxin restored the ability of endotoxin-tolerant animals to react to endotoxin like a normal unblocked animal. It should be noted that the carbon preparation, when used alone, had some effect on hemolysin production, possibly due to a contamination of the carbon with endotoxin. It is unlikely, however, that such possibly additive endotoxin effect could account for the effect of carbon on the spleen response in the tolerant animal.

TABLE II. Effect of Administration of Carbon on Number of Hemolysin-Producing Spleen Cells in Endotoxin-Tolerant and Normal Animals. Carbon (i.v.) was injected 48 hr prior to spleen harvest; when given to endotoxin-exposed mice, it was injected 3 hr prior to the last or only dose of endotoxin (i.p.).

Treatment of spleen donors	Avg No. (±S.E.) of hemolysin-producing cells per 1/5 spleen
None	8.6 ± 3.2
Endotoxin (1x)	49.2 ± 8.0
" (10x)	11.0 ± 5.0
" " + carbon	40.6 ± 3.0
Carbon	28.0 ± 3.5

Prior data on hemolysin-forming cells(4) had indicated that oligodeoxyribonucleotides, when given to normal mice in conjunction with endotoxin, can enhance the stimulatory effects of endotoxin. Results shown in Table III con-

TABLE III. Influence of DNA Digest (DD) on Number of Hemolysin-Producing Spleen Cells in Endotoxin - Tolerant and Non - Tolerant Mice. Spleens were harvested 48 hr after one endotoxin injection or 48 hr after the last of 10 daily i.p. injections with 10 γ of endotoxin.

Treatment of spleen donors	Avg No. (±S.E.) of hemolysin-producing cells per 1/5 spleen
None	6.0 ± 1.6
Endotoxin (1x)	51.6 ± 14.3
" (10x)	14.4 ± 3.8
" (1x) + DD	115.6 ± 14.6
" (10x) + "	47.4 ± 18.1
DD	9.4 ± 1.4

firm such observations and indicate that a sufficient trace of an endotoxin effect can remain in endotoxin-tolerant animals to support the stimulatory effects of oligodeoxyribonucleotides. [It should be recalled that oligodeoxyribonucleotides are without effect when administered without endotoxin(4)]. However, as illustrated in Table IV, if endotoxin tolerance is achieved by daily endotoxin dosages that are higher than those used in the animals of Table III, oligodeoxyribonucleotides given *without antigen* are as inactive as they are in a normal antigen-free animal. Table IV further shows that in the *presence of antigen,* oligodeoxyribonucleotides can retain their stimulatory effects in the endotoxin-tolerant mouse. For as yet unknown reasons, the magnitude of such stimulation is below that obtained in the non-tolerant mouse.

Discussion. The finding that hemolysin-forming spleen cells can still increase when sRBC are administered to an endotoxin-tolerant animal, or when carbon is injected prior to the last of a series of daily endotoxin injections, strongly indicates that antibody-forming cells are not lost or altered in an endotoxin-tolerant animal. The failure of the tolerant animal to show the usual response to endo-

TABLE IV. Influence of DNA Digest, Given With or Without Antigen, on Number of Hemolysin-Producing Spleen Cells in Endotoxin-Tolerant and Non-Tolerant Mice. Spleens were harvested 48 hr after one, or the last of 10 daily i.p. injections with increasing dosages (see *Materials and methods*) of endotoxin.

Treatment of spleen donors	Avg No. (±S.E.) of hemolysin-producing cells per 1/5 spleen
None	4.6 ± 1.0
sRBC (1x)	78.4 ± 7.5
" + DNA digest	317.2 ± 45.6
Endotoxin (1x)	149.2 ± 28.1
" + sRBC (1x) + DNA digest	652.4 ± 53.2
Endotoxin (10x)	64.2 ± 6.1
" + sRBC (1x)	97.4 ± 7.8
" + DNA digest	50.0 ± 25.9*
" + DNA digest + sRBC (1x)	142.0 ± 18.7

* The reason for unusually wide variations among individual animals of this group is unknown and is currently being investigated.

toxin, in terms of antibody-forming spleen cells, may therefore be attributed to a failure of endotoxin to elicit the necessary stimulator in the tolerant animal. The simplest conclusion would be that this failure is due to an absence of active endotoxin at sites from which the stimulator can be released. In other words, in accordance with the suggestions of others (9), endotoxin is probably inactivated in the tolerant animal, and therefore becomes unavailable for the reactions that cause the release of stimulators for plasma cell populations. This conclusion is further supported by the observation that the suspected stimulators, in the form of oligodeoxyribonucleotides, can still function in the endotoxin-tolerant animal.

It is of interest to note that in the tolerant animal resistance to certain bacterial infections increases with each injection of endotoxin (10), whereas data such as those presented here demonstrate that in tolerant animals endotoxin fails to produce increases in pre-existing antibody-forming cell populations. This includes the production of bactericidins since recent observations (11) have revealed that bactericidins behave in the tolerant animal exactly the same way as hemolysins do. Such findings support the prior conclusion of others that there is no necessary correlation between enhanced resistance and elevated antibody formation.

Summary. The red cell plaqueing technique reveals that hemolysin-forming cells do not increase after endotoxin injection into an endotoxin-tolerant animal. In contrast such increases occur following endotoxin injection into non-tolerant mice or following sheep red blood cell injections into endotoxin-tolerant animals. It was also shown that the injection of carbon into an endotoxin-tolerant animal immediately restores the animal's capacity to react to endotoxin with increases in hemolysin-forming spleen cells. These data suggest that endotoxin tolerance interferes with the ability of endotoxin to release a stimulator for plasma cells, but such tolerance does not interfere with the ability of antibody-forming cells to respond to the injection of specific antigen. Also, stimulatory oligodeoxyribonucleotides still can function in the endotoxin-tolerant animal. The bearing of these observations on the problem of relationships between elevated host-resistance and elevated antibody formation have been discussed.

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