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Lack of Relationship Between Toxicity and Bone Marrow Cell Colony Stimulating Activity of Endotoxin Preparations (40714)

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An elevation of serum colony stimulating factor (CSF) after administration of endotoxin (or LPS) has been demonstrated in many studies (1–4). Several laboratories reported that the lipid-rich moiety of LPS contains active sites for toxicity as well as for a number of other endotoxic reactions, while the polysaccharide region is responsible only for the serological specificity. Studies done in our laboratory indicated that lipid-free and nonendotoxic fraction (called PS) obtained by partial hydrolysis of the LPS was active in CSF release (5, 6). Apte and co-workers reported (7) that the PS is much less active than LPS, but still considerable CSF release could be induced by its injection. Urbaschek reported strong induction of colony forming units using a similar polysaccharide rich preparation obtained by acetic acid hydrolysis (Freeman polysaccharide), thus confirming our findings (VIII International Congress of the Reticuloendothelial Society, Israel, 1978).

In this report, we present further data which indicate that hitherto unidentified nontoxic and periodate sensitive components are responsible for the generation of CSF in mice.

Materials and methods. Endotoxin preparations. Endotoxin was extracted from *Serratia marcescens* by the trichloroacetic acid method of Boivin as described elsewhere (8). Five batches, namely, 649, 627, 598, J-4, and 697, were selected. Preparation 725 has been obtained from heat-killed and centrifuged *S. marcescens* cells. The cell sediment has been resuspended in distilled water, vigorously stirred for 2 hr at 37°C, and centrifuged again at 5000*g* for 60 min. The clear supernate was dialyzed and lyophyllized.

Polysaccharide-rich fraction (PS) was prepared as previously described (5, 6). Briefly, the acid-soluble supernate obtained by partial hydrolysis of lipopolysaccharide was neutralized in a beaker by adding to it Dowex 1 ion-exchange resin in OH^- form. The resin was filtered, washed, and the filtrate was lyophilized.

Periodate oxydized PS was prepared by dissolving 10 mg PS in 10 ml distilled water to which 2 ml 0.1 *M* $NaIO_4$ has been added. The mixture was kept in the cold room for 24 hr wrapped in aluminum foil. The reaction mixture was subsequently dialyzed for 2 days at +4°C, changing the outer fluid twice daily. The dry weight content of the dialyzed solution has been determined and the preparation was kept frozen until used. A control PS solution has been treated in identical fashion, including dialysis, but without periodate.

Chemically detoxified endotoxins have been prepared by two procedures. One of these was the $KOCH_3$ treatment, which deacylates the lipid moiety of the LPS and reduces its toxicity to 1 to 3% of the original activities (9). Several of the beneficial effects of endotoxins were maintained in such detoxified preparations, which we also called endotoxoids (9–11).

The other chemical detoxification involved alkaline hydrolysis. LPS solution (1 mg/ml) was prepared and made alkaline to 1.0 *N* final concentration using 10 *N* NaOH. The solution was heated in boiling water bath for 60 min, then neutralized to pH 7.0

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using HCL. The final concentration of alkali-treated LPS in the test solution was adjusted to 0.1 mg/ml.

The above preparations were obtained from five different cultures of *S. marcescens*. All these were kindly prepared for us by the Merck Sharp and Dohme Company in Rahway, New Jersey. The authors gratefully acknowledge here the generous donations of the bacteria.

Determination of LD_{50} in mice. The LD_{50} of the preparations in ICR female mice was determined by a procedure described elsewhere (8).

Preparation of CSF-rich serum. A 100 μg/ml suspension of each preparation in pyrogen-free saline was prepared. Two-tenths of a milliliter (20 μg) of each preparation was injected iv in each of the groups of ICR mice. Two hours later, the mice were bled. The blood collected from each group (five mice per group) was pooled and the serum separated.

Assay for CSF. Each of the pooled sera were tested in three separate assays for CSF content. The assay system described by Metcalf and Moore (13) was used. Bone marrow plugs from a single femur of two ICR mice were collected in Eagle's 1010 medium. The cell concentration was adjusted to 2.5×10^7/ml. Equal volumes of double strength Eagle's medium and 0.6% agar solution were mixed together. One-tenth of a milliliter of bone marrow cell suspension was added to every 10 ml of this agar-containing medium. One milliliter of the resulting mixture was pipetted into each 35-mm Falcon plastic dish. Fifty microliters of the serum to be tested was added to the mixture before it hardened. The dishes were cultured in a CO_2 incubator at 37°C in a moisture-saturated atmosphere. Seven days later the number of colonies was counted as a measure of the colony stimulating activity of the test serum. Stimulation index was calculated by dividing the number of colonies in the test cultures by the number of colonies found in the control cultures, to which 50 μl normal mouse serum was added.

We counted as colonies the aggregation which contained more than 20 cells.

Limulus lysate assay. The presence of endotoxin in CSF-rich serum was tested for by the Limulus lysate assay. Limulus amoebocyte lysate (LAL) was obtained from Associates of Cape Cod, Inc., Woods Hole, Massachusetts. A lyophilized vial of LAL was suspended in 5 ml of pyrogen-free water. As a standard, fourfold dilutions of endotoxin, ranging from 0.01 to 0.0004 μg/ml, were prepared. One-tenth of a milliliter of each dilution was added to each 8 × 75-mm disposable pyrogen free test tube. One-tenth of a milliliter of undiluted CSF serum, or 0.1 ml of a 1:5 or 1:10 dilution of CSF serum, was added to other tubes. One-tenth of a milliliter of pyrogen-free water was added to a test tube and served as a negative control. One-tenth of a milliliter of LAL was added to each of the tubes. The tubes were shaken and incubated at 37°C in a water bath for 1 hr. The endpoint was the lowest concentration of endotoxin which triggered clot formation.

The concentration of endotoxin in CSF serum was approximated by comparing the endpoint obtained with CSF serum with that obtained with the endotoxin standards.

Results. Toxicity of different endotoxin preparations. The results obtained are given in Table 1. The first five preparations differed from each other to a considerable degree. Preparation Nos. 598 and 627 were more toxic than 649 or J-4. The PS preparation and the two chemically detoxified preparations were not lethal at 4 mg/mouse. Preparation No. 725, which has been obtained from the bacterial cells by washing them under extensive agitation at 37°C with distilled water, was also nontoxic at the 2-mg level. The periodate treated PS was without any detectable toxicity.

Induction of CSF release. CSF inducing activity of the samples showed no correlation whatsoever with the mouse lethality measurements. The nontoxic preparation, such as J-4, 649, 725, $KOCH_3$-469, or NaOH-649 were active in CSF release *in vivo*.

Size of colonies. As far as the morphology of the colonies are concerned there are three types of growth one can observe. One is a large cluster, consisting of several hundred cells, most of which are closely packed, forming a rather dense center of

TABLE I. LD_{50} AND CSF ACTIVITY OF DIFFERENT ENDOTOXIN PREPARATIONS AND THEIR DERIVATIVES

Preparation[a]	LD_{50}[b]	No. of colonies	Colony index[c]
Saline	Nontoxic	62 (±12)[d]	1.0
649	0.80	140 (±21)	1.8
725	>2.00	223 (±19)	3.6
J-4	2.20	197 (±26)	2.5
598	0.54	93 (±21)	1.2
627	0.54	103 (±13)	1.3
PS	Nontoxic	190 (±19)	2.4
IO_4-PS	Nontoxic	62 (±7)	0.8
$KOCH_3$-649	>4.00	122 (±10)	1.6
NaOH-649	>4.00	161 (±14)	2.1

[a] 20 μg (or 0.2 ml) injected iv.
[b] Determined in ICR mice, expressed as mg/mouse.
[c] Number of colonies obtained divided by the number of colonies in the saline control.
[d] Numbers in parentheses are standard deviations.

the colony. Sometimes such colonies are so large that one can see them in the agar with the naked eye. Another morphological type consists of a loose aggregation of cells where the distance between the proliferating cells is large as if they could migrate away from their place of origin. Such colonies can not be seen without a 40–80× magnification. Both of above types of colonies can be seen frequently if one uses sera of mice injected with toxic LPS. The third type of colony consists of a much smaller number of cells, usually between 20 to 50. The production of these small, rather tight colonies is elicited by using PS preparation, or by chemically altered "endotoxoids" given ip. This latter observation has been confirmed by Apte *et al.* (7) in 1977. PS or other nontoxic derivatives given iv will act similarly in CSF induction as toxic LPS preparations.

These findings may indicate that either there are more than one cell type which respond to the colony stimulating factor, or there are several colony stimulating factors in the CSF sera with different stimuli on the various bone marrow cells. Whether this assumption is correct remains to be investigated.

Effect of periodate oxydation of the CSF inducing activity of PS. A complete destruction of the CSF activity was seen indicating that the component in the PS which is responsible for CSF release is sensitive to IO_4 oxydation. The control PS solution which was treated in an identical fashion but without IO_4, maintained its original activity.

Level of endotoxin in CSF serum. The endotoxin level in all the CSF sera prepared in this study was approximated by the Limulus lysate assay. All the specimens tested contained less than 4×10^{-6} μg/ml.

Discussion. It has been observed in our laboratory that different batches of endotoxin differ quantitatively in their biological properties, although similar extraction procedures for their preparation are used. This may be due partly to a greater amount of nontoxic components obtained in the less active preparations (14).

In this study, we investigated the CSF activity of several endotoxin preparations and their derivatives which differed in their mouse lethality. As our results indicate, the less toxic preparations, namely 694 and J4, and the nontoxic PS preparation were more active in releasing CSF than the more toxic 598 and 627 preparations. The endotoxoid preparation was nontoxic at 4 mg/mouse dose level. Using the much more sensitive chick embryo lethality assay, the LD_{50} was 1.25 μg, while the parent endotoxin preparation had an LD_{50} of 0.03 μg/ml. In the CSF assay, the endotoxoid was as active as the parent LPS preparation.

The NaOH detoxified endotoxin had no detectable activity in mouse LD_{50} assay, and in chick embryo lethality the LD_{50} was 10 μg/embryo. The same preparation had a considerable CSF activity. The differences in CSF activity of these preparations could

not be due to differences in the clearance of endotoxin from the circulation of the mice since endotoxin was absent from all the CSF sera as shown by the *Limulus* lysate assay.

As far as the alterations caused in the chemical structure of LPS by the various treatments are concerned only general comments can be made at this moment. Treatment with dilute alkali cleaves all alkali sensitive groups, among others ester-bound fatty acids and phosphate groups. Detoxification with $KOCH_3$ is more selective to O-acyl cleaves as we showed in 1964 (10). Acidic hydrolysis acts in a random fashion on glycosidic as well as on other acid labile linkages. Periodate oxydation is a classical procedure to degrade polysaccharide moieties, but other compounds having vicinal diol or similar functional groups may be also oxydized.

What seems to be clear from the results of this as well as of earlier publications is that while alkaline or $KOCH_3$ treatments quickly diminish toxic properties of LPS, they do not alter the CSF inducing activity of the LPS to the same extent. This is in full accord with earlier publications (9–11, 15) where comparisons were made between toxic and other biological activities of endotoxin and their chemically altered derivatives.

The component of the LPS which releases CSF *in vivo* is apparently nontoxic. It is present not only in some less toxic batches of endotoxins, but also in nontoxic derivatives of chemically altered preparations. These chemical treatments involved relatively mild partial acidic hydrolysis, deacylation by anhydrous $KOCh_3$ and alkaline degradations.

Summary. The release of colony stimulating factor (CSF) by endotoxin was studied using several endotoxin preparations differing greatly in their toxicities measured as mouse LD_{50}. No correlation was found between lethality and CSF release. It is concluded that the toxic effect of endotoxin is not required for the generation of CSF.

Furthermore, the component in the polysaccharide rich and lipid free, nonendotoxic, partially hydrolyzed endotoxin breakdown product (called PS), which can stimulate the CSF release in mice, is sensitive to periodate oxydation.

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