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STUDIES ON THE PATHOGENESIS OF THE EDEMA DISEASE OF SWINE

I. THE EFFECT OF THE PURIFIED LIPOPOLYSACCHARIDE ENDOTOXIN OF ESCHERICHIA COLI ON MICE

By

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Recently the role of hemolytic *Escherichia coli* strains in the pathogenesis of the edema disease of swines was studied by several authors (BOUCHAERT, 1960; DAVIS et al. 1961; GELENCZEI, 1959; GREGORY, 1960; HARTWIGK, 1962; ITO, 1960; KERSTEN and BECK, 1960; KELEN et al., 1959; PHILIP and SHONE, 1960; REDEL, 1959; SZABÓ, 1958). However, no exact data are available as yet on the pathophysiological mechanism of this disease as elicited by endotoxins. The present studies were performed to elucidate the nature and development of pathological changes occurring after injection of lipopolysaccharide (hereafter referred to as LPS) endotoxins prepared from both hemolytic and non-hemolytic *E. coli* strains. Further to this attempt was made to reveal the pathophysiological changes directly responsible for endotoxin shock. In this connection the possibility of protection against endotoxin shock was also investigated.

Materials and methods

In the present experiments hemolytic *E. coli* strains isolated from swines expired of edema disease and dyspepsia-type *E. coli* strains isolated from calves were used. These strains belonged to serological types 0 141, 0 101 and 0 78. Endotoxin was prepared from mass bacterium cultures in Roux flasks on a solid medium containing 2 per cent agar dissolved in horse broth, 5 per cent Witte peptone, 0.25 per cent Proteose-peptone (Difco) and 0.5 per cent NaCl. Strains were cultivated in 100 Roux flasks each. The pH of the medium was adjusted to 7.6. As an inoculum 5 ml each of 6 hours old cultures was withdrawn from every Roux flask. The cultures were incubated for 60 hours at 37° C and subsequently over 24 hours at room temperature. Bacteria were washed off with 20 ml sterile distilled water per flask. The bacterium suspension was repeatedly washed by centrifugation. From the final suspension germ count was determined; the dry material content of the same was found to fluctuate between 3 and 4 per cent when examined after drying at 100° C. From the total amount of bacterium suspension — corresponding to about 60 to 70 g dry material — endotoxins were prepared by the phenol method of WESTPHAL et al. (1952). From the non-purified endotoxin nucleic acids were removed by precipitation with alcohol; on repeated alcoholic precipitation of the residuum, fairly pure LPS fraction could be harvested. At this grade of purity the quantity of LPS endotoxin amounted to 3 per cent of the dry material content of bacteria. Endotoxin preparations were stored lyophylised.

Our experiments were performed on 400 albino mice of 15 to 25 g body weight, belonging to the same strain.* The animals received commercial mouse diet and some green food. When calculated for mice according to the method of BEHRENS (1932), the DL_{50} of our LPS-endotoxin preparations was 50 μg/g. However, for testing toxicity higher doses were given: 75 μg/g, i.e. 1.5 mg dissolved in saline was injected intraperitoneally per animal. The effect of endotoxin was checked 24 hours after administration.

For histological examinations organs were fixed in formaline. In order to prevent autolysis, the fixation of the intestinal tract was performed immediately after exit, by the following procedure: the intestinal tract was cut open in the longitudinal direction, then laid on a wet filter paper strip, rolled in and put into hot formaline for a few minutes. Thus on the paraffine-embedded cuts prepared subsequent to the above pretreatment, the entire cross-section of the intestinal tract was visible.

Results

The influence of LPS endotoxins on mice

The lethal dose of endotoxin prepared from the strains used in the present experiment caused a rapidly proceeding lethal disease of mice on both intraperitoneal and intravenous administration. A few hours after injection the animals became adynamic and the majority of them expired within 8 to 14 hours succeeding endotoxin injection. At autopsy characteristic changes were revealed (see Fig. 1). The abdomen remarkably enlarged as a result of a manifold dilatation of the stomach and duodenum. The dilatated duodenum had yellow colour. In the majority of cases remarkable dilatation of the jejunum, ileum and large intestines was also observed. Enlarged spleen was found in a part of the animals only; however, spleen was bacteriologically sterile in every instance. In several cases small amounts of exudate were found in the abdominal cavity. Sometimes also small necrotic foci could be observed in the liver. The heart of the animals was generally pale, displaying the colour of cooked meat.

It was revealed by histological examinations that the epithelium of the mucous membrane of the small intestines emerged from the pars villosa of the propria, most probably because of the edema (see Fig. 2). A similar phenomenon was observed on the mucous membrane of the large intestines, i.e. the epithelium was separated from the propria and was consequently elevated. At the tip of intestinal villi cell detritus were visible. When exit was protracted for 20 to 24 hours after injection, necrobiotic areas were visible in the liver, and sporadic lesions and small necrotic foci were observed in the heart muscle.

The above pathological changes are elicited in mice only when LPS endotoxine is injected intraperitoneally or intravenously. If the same dose is given subcutaneously, mostly no toxicosis is developed and generally 80—90 per cent of the animals remain free of toxic symptoms. It was supposed that the reason for this might be the too slow absorption of endotoxin from the subcutaneous connective tissue. In order to clarify this matter, the speeding up of the absorption process was attempted by adding hyaluronidase enzyme to the LPS-toxin injected (Table 1). As visible in the Table, as a result of hyaluronidase activity toxic symptoms developed in about the same ratio as on i.p. administration of the endotoxin and also autopsy findings were essentially similar.

Fig. 1

Table 1

The effect of hyaluronidase enzyme on the absorption of LPS endotoxin

Group	Treatment	No. of animals	Ratio of survival Survirvors/total number of animals after 24 hours
I.	1.5 mg LPS i.p.	20	0/20
II.	1.5 mg LPS s.c.	20	18/20
III.	1.5 mg LPS + 30 U Hyase* s.c.	20	1/20

* "Hyase", Pharmaceutical Factory "Kőbányai Gyógyszergyár", Budapest

* Supplied by the Institute of Nutrition (OÉTTI), Budapest.

Fig. 2

Studies on the mechanism of action of LPS endotoxins

In this part of our experiments the most important factors of the pathomechanism of the disease elicited by LPS-endotoxins were investigated. Since according to literary data the liberation of histamine has an important role in the mechanism of action of LPS endotoxins (GREISMAN, 1960; HINSHAW et al., 1960; HINSHAW et al., 1961), protection against endotoxin effect was attempted with anti-histamines. Results are presented in Table 2. As visible in the Table, the effect of LPS endotoxin can be counteracted by several pharmacons. On the basis of the observation that anti-histamine preparations or anti-inflammation materials prolong the time of survival, it is supposed that the liberation of histamine has an important role in calling forth the clinical picture of endotoxin shock. However, anti-histamine compounds are rapidly excreted and therefore their effect is not durable. This is the reason why protection with "Suprastin" is inadequate. The favourable effect of the sedative

Table 2

The protective effect of different pharmacons against endotoxin shock of mice elicited by LPS endotoxin of *E. coli* O 141

Group	Treatment	No. of animals	Ratio of survival Survivors/total number of animals after 24 hours
I.	1.5 mg LPS i.p.	20	0/20
II.	1.5 mg LPS i.p. + 1 mg "Suprastin"[1] i.m.	20	10/20
III.	1.5 mg LPS i.p. + 0.5 mg "Hibernal"[2] i.m.	20	8/20
IV.	1.5 mg LPS i.p. 1 mg "Deltacortril"[3] i.m.	20	8/20
V.	1.5 mg LPS i.p. 1 mg "Pipolphen"[4] i.m.	20	0/20
VI.	1.5 mg LPS i.p. 1 mg "Regitine"[5] i.m.	20	0/20
VII.	1.5 mg LPS i.p. "Pipolphen" + "Novurit"[6] (l. to mixture 1 mg + 1 mg) i.m.	20	15/20
VIII.	1.5 mg LPS i.p. 2 mg histidine i.m.	20	8/20

[1] "Suprastin" (N-dimethyl-aminoethyl-N-p-chlorobenzyl-aminopyridium-hydrochloride) Kőbányai Gyógyszerárugyár
[2] "Hibernal" (Promethazin hydrochloride) Kőbányai Gyógyszerárugyár
[3] "Deltacortil" (Prednisolon) Pfizer
[4] "Pipolphen" (N-2-dimethylamino-l-prophyl-phenothiazin-hydrochlorid) Egyesült Gyógyszer- és Tápszergyár
[5] "Regitine" (2-N-p-tolyl-N-p-oxy-phenyl-aminomethyl-imidazoline-metasulphonic acid) Ciba
[6] "Novurit" (Camphoric acid-allylamid-metoxy-mercuriteophylline-sodium) Chinoin

"Hibernal" is due to its anti-histamine properties. The effectivity of "Deltacortril" is also suggestive of the involvement of histamine-release in the toxic process. Attempts to use "Pipolphen" and "Regitine" for protection purposes failed altogether; this might be explained by the toxicity of the large doses given. The physiological anti-histamine histidine had favourable effect, which also indicates that histamine has an important role in the pathomechanism of diseases elicited by LPS endotoxins. With regard to the fact that anti-histamines are rapidly excreted, attempt was made to create anti-histamine reserves in the organism, in order to ensure the presence of a sufficient anti-histamine level for a longer period. For this purpose a 4 : 1 mixture of "Pipolphen" and "Novurit" was used, according to the practical observations of KÉKESI (1961). The mixture of the above preparations is a micro-crystalline substance and most probably the diuretic effect of "Novurit" has a favourable influence on the anti-histamine properties of Pipolphen.

Immunization experiments with LPS endotoxin

In the course of our experiments attempts were made to immunize mice with LPS endotoxin against the fatal effect of lethal doses of the same. Results are summarized in Table 3. As visible in the Table, immune response was highest when LPS endotoxin was administered on one occasion together with complete Freund adjuvant (Difco). When applied after injections of different quantities of endotoxin it was found that a mixture of endotoxin and Freund adjuvant elicited the increase of immune response in inverse relation to the amount of the dose originally injected for immunization purposes.

Table 3

Immunization experiments with the LPS endotoxin of *E. coli* O 141

Group	No. of animals*	Immunization	Ratio of survival survivors/total number of animals after 24 hours	Percentage of protection
I.	20	—	0/20	0
II.	20	LPS vaccine** 1 × s.c.	12/20	60 per cent
III.	20	50 μg LPS s.c. + LPS vaccine s.c. 14 days later	10/20	50 per cent
IV.	20	100 μg LPS s.c. + LPS vaccine s.c. 14 days later	6/20	30 per cent
V.	20	200 μg LPS s.c. + LPS vaccine s.c. 14 days later	6/20	30 per cent
VI.	20	400 μg LPS s.c. + LPS vaccine s.c. 14 days later	5/20	25 per cent
VII.	20	800 μg LPS s.c. + LPS vaccine s.c. 14 days later	5/20	25 per cent
VIII.	20	900 μg LPS s.c. + LPS vaccine s.c. 14 days later	4/20	20 per cent
IX.	20	1000 μg LPS s.c. + LPS vaccine s.c. 14 days later	2/20	10 per cent

*Mice of both sexes with 22 g average body weight
**LPS vaccine: 25 μg LPS endotoxin in complete Freund adjuvant

Discussion

On the basis our of results it is obvious that by the method of WESTPHAL et al. (1952) LPS-endotoxin with remarkable toxic effect on mice can be prepared from both hemolytic and dyspepsia-type *E. coli* strains. The toxicosis elicited in mice by this substance results in extraordinarily characteristic section picture and pathohistological changes. Regardless of the type of the *E. coli* strains used — i.e. hemolytic or dyspepsiae — these changes are identical. Presumably, the development of changes is remarkably influenced by the histamine liberated by the action of LPS endotoxins, as it was also found by GREISMAN (1960) and HINSHAW et al. (1960, 1961). Our observation that the survival of mice given lethal doses of LPS endotoxin can be prolonged by anti-histamine treatment, supports the above supposition on the role of histamine in endotoxin shock. The nature of histamine release could not be elucidated as yet. According to the results of SCHAYER (1960) it is highly possible that LPS endotoxins have a stimulating effect on the enzymatic activity of histidine decarboxylase and consequently they speed up the splitting of histamine from histidine. Besides histamine other factors may also have influence on the development of clinical appearance, as suggested by the results of DAVIS et al. (1961), DESPREZ et al. (1961), GELLER et al. (1954) and SPINK et al. (1961). According to these authors the shock elicited by LPS endotoxins can be prevented or mildered by pharmacons of entirely different nature. However, our observation that out of all substances tested the anti-histamine given for reserve-creating purposes had the most favourable effect, supports the supposition that in the clinical process of endotoxin shock histamine has the most important role. The pathophysiological analysis of the shock elicited by bacterial LPS endotoxins also indicates that the liberation of histamine or histamine-like substances, resulting in a series of biochemical reactions, has a decisive role in the clinical appearance of endotoxin shock. This is also the most plausible explanation for the hemodynamic changes observed in attendance of the shock (GILBERT, 1960; HINSHAW and NELSON, 1962; SPINK and VICK, 1961; STETSON, 1961; VICK and SPINK, 1961). Our results with the adrenal cortex preparation "Deltacortril" are in accordance with the observation of GELLER et al. (1954) i.e. in endotoxin shock cortison has marked protective effect.

The results of our immunization experiments with mice show that there is possibility to immunize against LPS endotoxins; however, the quantity administered for that purpose has a decisive role. The possible explanation for this is that large quantities of endotoxin cause a block of the reticulo-endothelial system, while small ones have a stimulating effect on its activity by way of biological counter-regulation as it was also demonstrated by PILLEMER et al. (1955) in their studies on relations between the properdin system and polysaccharides.

SUMMARY

The authors produced lipopolysaccharide endotoxin from a hemolytic. *E. coli* 0 141 strain isolated from swine, as well as from the dyspepsia-type *E. coli* strains 0 101 and 0 78, isolated from calves. Endotoxin was prepared according to the method of WESTPHAL et al. In the present studies the effect of lipopolysaccharide endotoxin on mice was examined. It was found that the the lipopolysaccharide endotoxin prepared from any of the above strains elicited in mice a fatal shock attended by characteristic intestinal edema. The lethal effect of

lipopolysaccharide endotoxins can be reduced or prevented with anti-histamine compounds or prednisolon. Protection was highest when an anti-histamine reserve was created in the organism of animals. Immunization experiments with lipopolysaccharide endotoxins were also performed on mice. It was observed that when immunized 1 month prior to inoculation with a lethal dose of lipopolysaccharide endotoxin, 60 per cent of mice can be protected against a fatal outcome of the disease.

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REFERENCES

BEHRENS, H.: Arch. exp. Path. **167** (1932), 356. BOUCHAERT, J. H., OYAERT, W. and SIERENS, R.: DTW. **67** (1960), 349. DAVIS, J. W., ALLEN, R. C. and SMIBERT, R. M.: Am. J. Vet. Res. **22** (1961) 736. DAVIS, R. B., MEEKER, W. R. and BAILEY, W. L.: Proc. Soc. Exp. Biol. Med. **108** (1961) 774. DES PREZ, R. M., FOLLON, N. and HOOK, E. W.: Proc. Soc. Exp. Biol. Med. **107** (1961), 529. GELENCZEI, E. F.: Allied Vet. **30** (1959), 36. GELLER, P., MERRILL, E. R. and JAWETZ, E.: Proc. Exp. Biol. Med. **86** (1954), 716. GILBERT, R. P.: Physiol Rev. **40** (1960), 245. GREGORY, D. W.: Am. J. Vet. Res. **21** (1960), 88. GREISMAN, S. E.: Proc. Soc. Exp. Biol. Med. **103** (1960), 628. HARTWIGK, H.: Tierärztliche Umschau, 1962, 293. HINSHAW, L. B., JORDAN, M. M. and VICK, J. A.: J. Clin. Invest. **40** (1961), 1631. IDEM: Am J. Physiol. **200** (1961), 987. HINSHAW, L. B. and NELSON, D. L.: Am. J. Physiol. **203** (1962) 870. HINSHAW, L. B., VICK, J. A., CARLSON, C. H. and YON-LING FAN: Proc. Soc. Exp. Biol. Med. **104** (1960), 379. ITO, A.: Jap. J. Vet. Res. **8** (1960), 215. KELEN, A. E., CAMPBELL, S. G. BARNUM, D. A.: Canad. J. Publ. Health **50** (1959), 27. KÉKESI, B.: Magyar Állatorvosok Lapja, 1961, 47. KERSTEN, W. and BECK, G.: Mhf. Tierheilk. **12** (1960), 116. PHILIP, J. R. and SHONE, D. K.: J. S. Afr. Vet. Med. Ass. **31** (1960), 427. PILLEMER, L., SCHOENBERG, M. D., BLUM, L. and WURZ, L.: Science **122** (1955) 545. REDEL, E.: Proc. XVIth Int. Vet. Congr. Madrid, 1959, 253. SCHAYER, R. W.: Science **131** (1960), 226. IDEM: Am. J. Physiol. **198** (1960), 1187. SPINK, W. W. and VICK, J. A.: Proc. Soc. Exp. Biol. Med. **106** (1961), 242. STETSON, C. A., JR.: Bull. N. Y. Acad. Med. **37** (1961), 486. SZABÓ, I.: Magyar Állatorvosok Lapja, 1958, 295. VICK, J. A. and SPINK, W. W.: Proc. Soc. Exp. Biol. Med. **106** (1961), 280. WESTPHAL, O, LÜDERITZ, O. and BISTER, F.: Z. Naturforsch. **7b** (1952), 148.

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