Newer Data on the Role of Complement System in Toxicity of Endotoxin

Recently, experimental evidence has been increasing in support of SPINK and VICK's[1] earlier hypothesis that the complement system plays a substantial role in the development of endotoxin shock. BLADEN et al.[2] observed full in vitro activation by endotoxin of the entire serum complement system.

Previous studies in this laboratory have shown that activation of complement by endotoxin takes place also in vivo[3]. It seemed worthwhile to investigate how the titres of the 4 'classical' complement components changed in animals rendered tolerant to endotoxin by serial pretreatment and consequently exhibiting increased resistance to lethal doses of endotoxin.

Material and method. The experiments were performed on randomized female Wistar rats, weighing 140–160 g. The endotoxin used for pretreatment was prepared from *Serratia marcescens* according to BOIVIN and MESROBEANU[4]. 1 ml of this preparation contained active substance from 4.10^9 germs, and 0.5 ml/100 mg of it corresponded to 1 rat MLD.

Tolerance to endotoxin was conferred by treating the animals with rising doses of the preparation on 6 occasions at 48-h intervals. The total amount was 2.2 ml/100 g. The last dose, which was twofold the 1 rat MLD, caused no symptoms, indicating the development of endotoxin tolerance in the meantime. Blood sampling and later on challenge with endotoxin were made 48 h after the last treatment.

The titers of the complement factors were determined with R reagents prepared from guinea-pig serum, according to the standard method. The titration procedure was described in detail previously[5]. According to the new nomenclature of complement[6], the components C1, C4 and C2 detectable by R reagents correspond to components designed by the same symbols, while the 'classical' C3 detectable by R3 corresponds to the C3, C5 and C6 components[7].

Results and discussion. The Table shows the titers of the 4 'classical' components of complement in sera from rats pretreated and not pretreated with endotoxin. The total complement level as well as the titers of components C1, C4 and C2 were significantly lower in the treated than in the untreated control group, whereas the component C3 showed no significant change.

Subsequently, change of the apparently stable component C3 was examined 1 h after challenge with medium (0.1 ml/100 g) and sure lethal doses (1.0 ml/100 g) of endotoxin in rats treated and not treated previously with endotoxin.

The endotoxin concentrations used in the applied in vitro system corresponded to the in vivo levels produced by administration of the above doses. The incubation period was 60 min (Figure).

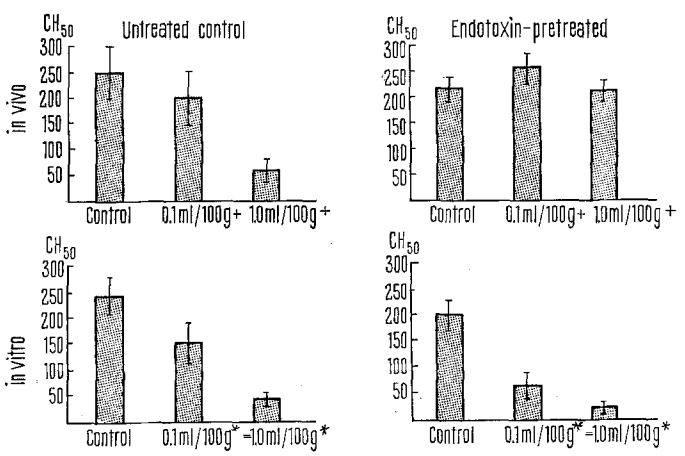
As can be seen from the Figure, in the control animals treatment with 0.1 ml/100 g endotoxin caused a moderate, whereas treatment with 1.0 ml/100 g a considerable, decrease of the serum C3 level. In the in-vitro system, the corresponding concentrations of endotoxin produced an essentially similar decrease of C3.

[1] W. W. SPINK and J. VICK, J. exp. Med. *114*, 501 (1961).
[2] H. A. BLADEN, H. GEWURZ and S. E. MERGENHAGEN, J. exp. Med. *125*, 767 (1967).
[3] G. FÜST and M. KERESZTES, Acta microbiol. hung. *16*, 135 (1969).
[4] A. BOIVIN and L. MESROBEANU, Rev. Immun. *1*, 554 (1935).
[5] G. FÜST and P. KERTAI, Acta microbiol. hung. *14*, 345 (1967).
[6] *Nomenclature of Complement*, Bull. Wld Hlth Org. *39*, 935 (1968).
[7] P. KLEIN and U. HADDING, Arzneimittelforsch. *20*, 167 (1970).

Titrations of total complement and complement components in untreated and endotoxin pretreated (-tolerant) rats

	No. of sera examined	T[a]		C1		C4		C2		C3	
		CH_{50}	± S.D.	CH_{50}	± S.D.	CH_{50}	± S.D.	CH_{50}	± S.D.	CH_{50}	± S.D.
Untreated control	10	60.9	9.7	342	133	809	205	116	29.0	249	55
Endotoxin pretreated	10	37.0[b]	9.6	106[b]	26	258[b]	140	41[b]	29.7	215	24

[a] T, total complement. [b] The difference is significant ($p < 0.001$).



Effect of *Serratia marcescens* endotoxin on C3-titer of sera of untreated and endotoxin-pretreated rats. +, C3-titer in 60 min sera. *, 0.4 ml endotoxin-dilution, 60 min/37 °C (the in vitro concentration of endotoxin is equivalent to the in vivo endotoxin concentration in blood).

The C3 titre of sera from rats pretreated with endotoxin showed in vitro a marked decrease on addition of the lower endotoxin dose and practically disappeared on addition of the higher dose. In contrast, in vivo even 1.0 ml/100 g of endotoxin failed to reduce the C3 level.

SNYDERMAN et al.[8] and LICHTENSTEIN et al.[9] showed that the titre decrease of excusively of C3 complement component in vitro is associated with formation of those biologically active substances – anaphylatoxin, chemotactic substances – which may play a role in the development of endotoxin shock. Our experiments indicate that in rats prepared with endotoxin, provocation fails to activate the full complement system and accordingly neither anaphylatoxins, nor chemotactic substances are formed.

Conclusions as to the nature of the mechanism responsible for the inactivity of the provoking endotoxin would be premature.

Based on the above findings, the C3 reducing action of endotoxin may be utilized for the study of the mechanism of endotoxin tolerance induced by serial treatment.

Résumé. L'effet d'une dose létale d'endotoxine, qui abaisse in vivo le taux du C3 du rat ne se manifeste pas chez les rats rendus tolérants à cette substance.

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National Institute of Public Health, and State Institute of Pharmacy, Budapest (Hungary), 8 April 1970.

[8] R. SNYDERMAN, H. GEWURZ, S. E. MERGENHAGEN, J. exp. Med. *128*, 259 (1968).

[9] L. M. LICHTENSTEIN, H. GEWURZ, N. F. ADKINSON, H. S. SHIN and S. E. MERGENHAGEN, Immunology *16*, 327 (1969).

Antigenic Components of Normal and Burned Mouse Skin

The presence of specific newly formed toxic antigens in burned tissues has been suggested by several authors[1–4]. Other investigators have failed to demonstrate either antigens or antibodies which uniquely arise following thermal injury[5,6]. Additional studies showed that both burned and normal skin extracted under sterile conditions yielded substances toxic in either normal or burned mice[7–9]. The present study was undertaken to determine if there were differences in the antigenic components in normal and burned mouse skin.

Material and method. Female CF-1 mice (Carworth Farms) weighing 22–24 g were shaven and anesthetized with penthrane. An asbestos board with 1×1.5 inch window was pressed firmly against the shaven back. Ethanol (0.5 ml) was evenly spread over the area of the back outlined by the window, ignited and allowed to burn for 15 sec. This caused a burn of approximately 35% of the body surface. 10–15 min post burn, the mice were sacrificed and the burned skin excized. The skin tissue was ground with saline and sand (0.75 g) using a mortar and pestle; the ratio of tissue to saline being 4.5 g/15 ml. After 10 min maceration, the ground tissue was passed through several layers of gauze to remove large particles and was then centrifuged at 15,000 *g*/60 min in a refrigerated International Model B 20 centrifuge. The pellet and floating lipid layer were discarded and the burned tissue extract was filtered through a 0.45 μ Millipore filter and refrigerated. The same procedures were used with normal mouse skin to prepare normal tissue extract. These materials were used as antigens for double diffusion gel precipitation tests.

To obtain a greater quantity of antigen, the extraction procedure was modified and 30 g of burned skin were homogenized in a Waring blender (Model 1042) for 10 min at highest speed. This procedure was carried out at 4 °C. At this point, the extract was treated in an identical manner to that described above. Similar procedures were used to prepare normal tissue extracts. While the total protein values per ml of extract prepared in this manner was 3 times higher than those prepared using hand grinding, the ratio of protein was similar in both methods.

Rabbits were immunized as follows: Lyophilized normal or burned tissue extract which contained 18 g of protein was emulsified in 2.5 ml of saline plus an equal volume of complete Freund's adjuvant. Groups of 6 Dutch Belted rabbits, 300–500 g, were inoculated s.c. and i.m. in several places. After a 45 day resting period the respective groups of rabbits were given 2–3 ml of burned or normal tissue extract s.c. and i.m. in 0.5 ml amounts and the rabbits were bled 10 days later. The antisera obtained from the 6 rabbits were pooled and used in gel diffusion studies.

To adsorb out antibodies to mouse serum from the rabbit serum, 0.1 ml of normal mouse serum was added to 2 ml of antisera, incubated at 37 °C for 1 h, refrigerated overnight and any precipitate formed removed by centrifugation. This procedure was repeated until no further precipitation occurred. Similarly 2 ml of tissue extracts were treated with 0.1 ml of goat anti-mouse serum and treated as outlined above. This procedure removed the normal mouse serum components from the tissue extracts.

The antiserum treated as outlined above was then subjected to ammonium sulfate fractionation to obtain γ-globulin[10] and the γ-globulin was concentrated 4-fold.

Double diffusion gel precipitation tests were performed in petri dishes using 1% agarose in borate-buffered saline at pH 7.8. Wells were filled once every 24 h for 3 days, incubated 48 h and then filled once every 24 h for 3 more days. Incubation was done at room temperature. Refilling the wells in this manner gave the best results of the various filling procedures tested.

[1] N. A. FEODOROV and S. U. SKURKOVICH, *Research in Burns* (Am. Inst. biol. Sci. and F. A. Davis Co. 1962), p. 226.

[2] S. R. ROSENTHAL, J. B. HARTNEY and W. A. SPURRIER, J. Am. med. Ass. *174*, 957 (1960).

[3] O. J. MALM and G. J. M. SLAWIKOWSKI, *Research in Burns* (Am. Inst. biol. Sci. and F. A. Davis Co. 1962), p. 282.

[4] M. DOBRKOVSKY, J. DOLEZALOVA and L. PAKOVA, *Research in Burns* (Am. Inst. biol. Sci. and F. A. Davis Co. 1962), p. 260.

[5] W. T. NEWTON, K. FUJII and C. A. MOYER, Arch. Surg. *85*, 912 (1962).

[6] P. MATTER, K. CHAMBLER, B. BAILY, S. R. LEWIS, T. G. BLOCKER and V. BLOCKER, Ann. Surg. *157*, 725 (1963).

[7] C. L. FOX, I. A. HOLDER and L. L. MALIN, J. Am. med. Ass. *187*, 655 (1964).

[8] C. L. FOX, *Research in Burns* (Am. Inst. biol. Sci. and F. A. Davis Co. 1962), p. 58.

[9] C. SHUN-SCHINELLA, J. W. STANFORD, J. GREENBERG, F. ANNECCHIARICO and C. L. FOX, Ann. N.Y. Acad. Sci. *150*, 816 (1968).

[10] D. H. CAMPBELL, J. S. GARVEY, N. E. CREMER and D. H. SUSSDORF, *Methods in Immunology* (W. A. Benjamin, Inc., New York 1963).