Modification of an Endotoxin Effect by Esters

The endotoxins of Gram-negative bacteria affect both the pre-existing immune and unresponsive states, the former by decreasing the lag period of the immune response and by elevating antibody titers[1], and the latter by causing a conversion to the immune state characterized by antibody formation[2]. The mechanism by which the conversion is brought about is not known, but the recent evidence that the reticuloendothelial system participates in the early phase of antibody formation[3] suggests that this system of cells may be involved in termination of unresponsiveness as well. In the present report experiments are described in which the peritoneal phagocytes of unresponsive animals are inhibited in activity by esters of aliphatic acids. Animals treated in this manner show a decreased capacity to respond to the endotoxin. In this report we also compare endotoxin with a zirconium salt, which possesses similar properties, i.e. of causing cell proliferation and stimulation of the RES[4].

The immunoassay used for studying the effects of esters on the phagocytic cells was that of quantitative radial immunodiffusion[5]. Samples of peritoneal fluid were drawn at intervals from a pool of animals injected with ethyl stearate 24 h prior to injection of endotoxin. The esters were given in emulsion with Tween 80 (0.8% v/v) and glucose (5% w/v)[6]. The fluids were placed in the wells of a plate having a layer of agar in which anti-endotoxin serum was contained. By comparing the pattern of precipitin bands with plates employing known concentrations of endotoxin, it was observed (Table I) that the ester-treated animals cleared the colloid more slowly (12 h or greater, compared with 8 h for controls). Qualitative immunodiffusion tests in which peritoneal washings from ester-treated and control animals were tested simultaneously showed a similar relationship when washings from both groups of animals were spot-tested against rabbit antiserum (Table II). In this assay also, fluids of animals injected with either ethyl stearate or ethyl ricinoleate contained endotoxin 12 h after injection, while controls had cleared the colloid by 8 h.

The cell counts taken at the time the fluids were drawn show that the decrease in endotoxin content could not be due to dilution by exudation, since the concentration of endotoxin remaining after a preliminary equivalent drop was higher in the fluids showing the higher number of cells. The data suggest that a decrease in phagocyte activity had been produced by the esters.

Ethyl stearate emulsion was then introduced into the peritoneal cavities of mice which had been paralyzed immunologically by injection of 500 μg type 8 pneumococcal polysaccharide (S8). Fourteen days after such an injection of S8, only 1 of 11 mice had serum antibody when assayed by indirect red cell hemolysis, with complement as lytic factor (Table III). Another group of paralyzed mice received 50 μg of endotoxin 20 h before challenge with an immunizing dose of antigen (0.5 μg); 67% of these animals showed the appearance of antibody. A third group of paralyzed animals was given ethyl stearate (60 mg) before the endotoxin (24 h prior); only 30% of these animals had antibody after challenge. Further, when the total dose of ester was increased to 200 mg none of the animals produced antibody. These

Table I. Suppressed clearance of endotoxin[a] following treatment with 60 mg ethyl stearate (i.p.); quantitative test

Treatment	Test interval (h post-endotoxin)	Average No. cells × 10^7/ml	Endotoxin remaining (μg/ml)
Control	0	189	200
	2.5	26.5	100
	5	25.0	50
	8	35.7	0
	12	84.1	0
Stearate	0	460	200
	2.5	275	100
	5	420	100
	8	404	100
	12	357	50

[a] Administered i.p. 24 h following i.p. injection of the ethyl stearate.

Table II. Differences in clearing of endotoxin from the peritoneal cavities of ester-treated and untreated mice; qualitative test

Experiment	Treatment	Immunodiffusion test for endotoxin at times indicated (h)			
A		0	24	48	
	Ethyl stearate (60 mg)	+	+	—	
	None	+	—	—	
B		0	4	8	12
	Ethyl stearate (60 mg)	+	+	+	+
	None	+	+	—	—
C		0	4	8	12
	Ethyl ricinoleate (60 mg)	+	+	+	+
	None	+	+	—	—

Table III. Inhibition of endotoxin-induced conversion by ethyl stearate in immunologically unresponsive mice

Treatment[a]		No. Reactors total	Reciprocal mean hemolysin titers	Average weight spleens (g/100 g wt.)
A	None	1/11	58	–
B	Endotoxin only	6/9	250	–
C	Stearate (60 mg) + endotoxin	3/10	40	–
D	Stearate (200 mg) + endotoxin	0/10	< 20	–
E	Zirconium	0/13	< 20	0.88
F	Stearate (200 mg) + zirconium	0/10	< 20	0.56[b]

[a] Injection schedule: A, S8, 500 μg day 1. B, S8, 500 μg day 1; endotoxin, 50 μg day 14; S8, 0.5 μg 20 h post-endotoxin. C, S8, 500 μg day 1; stearate, 20 mg days 15, 16, 17; endotoxin, 50 μg day 16; S8, 0.5 μg day 17, 20 h post-endotoxin. D, S8, 500 μg day 1; stearate, 40 mg days 15, 16, 17, 18, 19; endotoxin, 50 μg day 18; S8, 0.5 μg day 19, 20 h post-endotoxin. E, S8, 500 μg day 1; zirconium, 7.8 mg day 14. F, S8, 500 μg day 1; stearate 40 mg days 15, 16, 17, 18, 19; zirconium 7.8 mg day 18. All injections i.p. [b] $P = < 0.01$. (Comparison groups E and F.)

[1] S. G. Bradley and D. W. Watson, Proc. Soc. exp. Biol. Med. *117*, 570 (1964).
[2] M. S. Brooke, Nature *206*, 635 (1965).
[3] J. Fishman, J. exp. Med. *114*, 837 (1961).
[4] I. M. Braverman, J. invest. Derm. *43*, 509 (1964).
[5] J. G. Feinberg, Int. Archs Allergy appl. Immun. *11*, 129 (1957).
[6] G. N. Cooper and A. E. Stuart, J. Path. Bact. *83*, 227 (1962).

findings suggest that the reticuloendothelial system is involved in the endotoxin-induced termination of immunologic unresponsiveness. Unlike endotoxin, however, zirconium does not cause such a termination even though it produced splenic hyperplasia indicative of phagocyte proliferation. The ethyl stearate decreased the average weight of the spleens (10–12 spleens) to a near-normal level and there was no evidence that ethyl stearate was cytotoxic, judging from trypan blue tests for viability of the peritoneal phagocytes.

In earlier work by one of us (S.M.) it was shown that endotoxin could cause increased resistance to challenge with viable *D. pneumoniae* even when the mice were immunologically paralyzed to the capsular antigen[7]. Two interpretations were possible: (a) the resistance was independent of the immunologic state, and did not, therefore, require antibody for expression, and (b) the observed effect was due to a conversion of the paralytic state to the immune state by the endotoxin. The present experiments cannot decide between the 2 alternatives, since resistance to *D. pneumoniae* may also involve antigens other than the capsular polysaccharide antigen[8]. But the data do extend the findings of BROOKE, who observed termination by endotoxin in similarly paralyzed animals. Unlike the findings by that author, the titers in the present experiments are high, and suggest an active synthesis of antibody rather than a release from antibody-forming or carrying cells by a cyto-allergic mechanism. Further, they show that a blockade of the phagocytic cells of the peritoneal cavity decreases the capacity of endotoxin to produce the conversion. They also suggest that the mechanism by which the effect is brought about is through the antigenicity of endotoxin, since the similarly acting zirconium did not cause the conversion[9].

Zusammenfassung. Immuntolerante Mäuse können durch Endotoxin in immunologisch reaktive Tiere verwandelt werden. Eine zu wiederholten Malen verabreichte Substanz (Äthylstearat) hemmt die Umwandlung.

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S. MARGHERITA and H. FRIEDMAN, J. Bact. *89*, 277 (1965).
[8] C. A. NEEPER and C. V. SEASTONE, J. Immun. *91*, 378 (1963).
[9] This work was supported by PHS Training Grant No. 5T1AI162-05.

Two Different Iodinating Systems in Isolated Thyroid Cells

The cellular site of iodide-binding to thyroglobulin in the thyroid has been a much discussed topic between biochemists and morphologists[1]. Arguments have been presented for its location in the follicular lumen[2–4], in the thyroid cell[5–7] and at the cell colloid interface[4–8]. NUNEZ et al.[9] have suggested the existence of 2 iodination sites: 1 in the follicular lumen for the iodination of preformed molecules, the other in the thyroid cell for the iodination of newly synthesized molecules. In this communication we report evidence of the existence of 2 iodinating systems in isolated thyroid cells.

Suspensions of isolated sheep and horse thyroid cells were prepared by means of an adaptation of the continuous flow trypsinization technique of TONG[6–10]. The incubation procedure, and the collection of isolated cells, after the incubation, have been described previously[10]. The duration of the incubation was 2 h for the measurements of iodide uptake and iodide organification, and 6 h for the measurements of ^{125}I iodide incorporation in the iodoamino acids of proteins. For the latter experiments, the incubation medium contained ^{125}I iodide 4 μM (specific activity 750 C/M). Iodide trapping was evaluated by the C/M ratio, where C and M are the radioactivities of ^{131}I iodide in identical volumes of cells and medium[6–10]. At $10^{-7} M$, the C/M ratios were about 10 for horse cells and about 40 for sheep cells. Iodide organification was measured by the incorporation of ^{131}I iodide in the twice washed 10% trichloracetic acid precipitate of the cells. Incorporation of ^{125}I iodide into iodothyronines was calculated from the radioactivity of the cell homogenate and from the relative radioactivities of the iodothyronine spots on chromatograms of the cells hydrolysates (chromatographic systems: tertiary isopentanol-NH_4OH 2.5 N, and n-butanol-ethanol-NH_4OH 0.5 N (5:1:2))[10]. When results of separate experiments were pooled, the means, the standard deviation of the mean, and the Student's t-values were calculated from the common logarithms of these results. In this case, results are expressed as antilogarithms of the means and of the means $\pm$ the standard deviation of the mean[11]. Thyrotropin (thytropar) and twice crystallized bovine liver catalase were obtained respectively from Armour (Kankakee, USA) and Sigma (Saint Louis, USA).

$NaClO_4$ 2 mM completely inhibited the trapping of iodide by isolated thyroid cells at all the concentrations of iodide which were used (C/M = 0.8). The iodide organification which proceeds in the presence of perchlorate may therefore be called 'organification independent' of iodide trapping while the organification which is suppressed by $NaClO_4$ may be called 'organification dependent' on iodide trapping. Above concentrations of iodide of $10^{-6} M$ for horse thyroid cells and of $10^{-7} M$ for sheep thyroid cells, the addition of $NaClO_4$ did not depress iodide organification; i.e. the organification of iodide was independent of iodide trapping; below these concentrations the organification of iodide was partially inhibited by $NaClO_4$, i.e. partially dependent on iodide uptake.

'Dependent' and 'independent' ^{131}I iodide organification have been studied in horse isolated thyroid cells for

[1] L. J. DEGROOT, New Engl. J. Med. *272*, 243 (1965).
[2] N. J. NADLER and C. P. LEBLOND, Brookhaven Symp. Biol. *7*, 40 (1955).
[3] S. H. WOLLMAN and I. WODINSKY, Endocrinology *56*, 9 (1955).
[4] O. STEIN and J. GROSS, Endocrinology *75*, 787 (1964).
[5] L. J. DEGROOT and E. CARVALHO, J. biol. Chem. *235*, 1390 (1960).
[6] W. TONG, P. KERKOF and I. L. CHAIKOFF, Biochim. biophys. Acta *60*, 1 (1962).
[7] R. PITT-RIVERS, J. S. F. NIVEN and M. R. YOUNG, Biochem. J. *90*, 205 (1964).
[8] I. PASTAN, Endocrinology *68*, 924 (1961).
[9] J. NUNEZ, J. MAUCHAMP, V. MACCHIA and J. ROCHE, Biochim. biophys. Acta *107*, 247 (1965).
[10] F. RODESCH and J. E. DUMONT, Expl Cell Res. *47*, 386 (1967).
[11] J. E. DUMONT, Bull. Soc. Chim. biol. *46*, 1131 (1964).