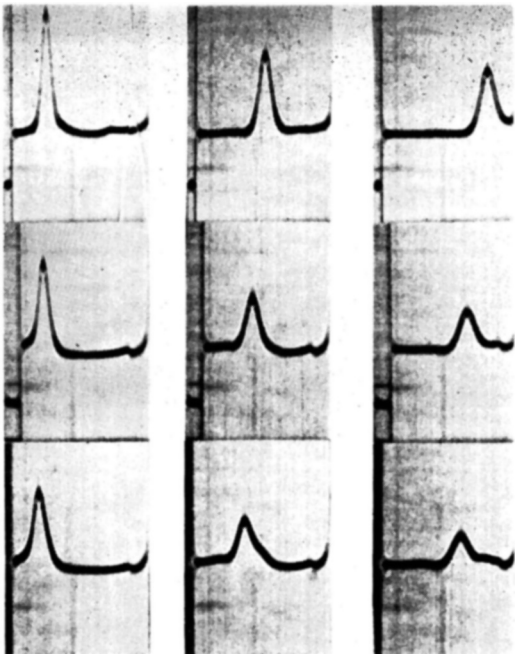

30 min. 60 min. 90 min.

FIG. 2. Ultracentrifugation patterns of 33% globulin fraction, bleeding 2 (top), and digests prepared from it: 60% fraction of 1:100 pepsin:protein (middle), and 60% fraction of 1:200 pepsin:protein (bottom). 59,780 rpm, 26°C, 1% protein in 0.9% NaCl.

fate fractionation of pooled sera of rabbits immunized with formalin-killed *Salmonella typhosa*, were digested with pepsin. Extent of digestion was estimated by tyrosine present in dialyzable digest fragments. Digest fractions, which precipitated between 40%-60% ammonium sulfate saturation, had agglutinin titers equal to, or perhaps greater than, titers of undigested globulin. The major component of the 40%-60% fraction also migrated more slowly in the ultracentrifuge. This suggests that it has been possible to digest rabbit antibody globulin so that smaller molecules are obtained in a digest fraction which still maintains its agglutinin antibody activity.

1. Kirk, J. S., Sumner, J. B., *J. Immunol.*, 1934, v26, 495.
2. Rosenheim, A. H., *J. Path. and Bact.*, 1935, v40, 75.
3. ———, *Biochem. J.*, 1937, v31, 54.
4. Kleczkowski, A., *Brit. J. Exp. Path.*, 1950, v31, 145.
5. Porter, R. R., *Biochem. J.*, 1950, v46, 479.
6. ———, *Nature*, 1958, v182, 670.
7. Whitehouse, F., Jr., *Bact. Proc.*, 1958, 61.
8. Hartley, P., *Proc. Roy. Soc., London, s.B.*, 1951, v138, 499.
9. Deutsch, H. F., Petermann, M. L., Williams, J. W., *J. Biol. Chem.*, 1946, v164, 93.
10. Haugaard, G., Roberts, R. M., *J. Am. Chem. Soc.*, 1942, v64, 2664.

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Effect of Zymosan on Endotoxin Toxicity in Mice.* (24781)

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Animals made tolerant to the lethal effects of endotoxin by pretreatment with these bacterial extracts were found to show an increased phagocytic capacity of the reticulo endothelial system (R.E.S.)(1,2). These findings, together with reports that endotoxin tolerance to fever(3) and lethal effects(4) were abolished in rabbits by reticulo endothelial blockade, suggested(1,2) that the reticulo endothelial system, which phagocytizes these macromolecular toxins(5), plays an important role in protecting against their toxicity. However, Suter *et al.*(6) and Halpern *et al.*(7) reported recently that mice infected with B.C.G. become 50 to 100 times more susceptible to the lethal effects of endotoxin, despite the fact that R.E.S. responds to the infection with increased phagocytic activity(8). These observations emphasize that factors other than phagocytic activity of the R.E.S. are concerned with the resistance to endotoxin. The increase in susceptibility to toxic effects of endotoxin in tuberculous animals, when first observed, was believed by Bordet(9) to be related to presence of inflam-

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matory lesions. Another experimental model which would not involve infection or tuberculin hypersensitivity could be helpful in determining the mechanism of this increased susceptibility to endotoxins. During our experience with the stimulating effect of the yeast extract, zymosan, on phagocytic function of the reticulo endothelial system(2,10), a similar state of increased susceptibility to endotoxins was observed in mice receiving repeated injections of zymosan. Such a treatment causes also considerable hypertrophy of the R.E.S. due to proliferation of macrophage elements(2) with granulomata formation in the liver and spleen(10). The results of our experiments on increased toxicity to endotoxin in zymosan treated animals and on the effect of cortisone on this phenomenon are reported here; the relationship between hypertrophy of R.E.S. and adrenal function is discussed.

Methods. Experiments were performed on white male Swiss Webster mice weighing 25 to 30 g. *Endotoxin toxicity*: *E. coli* endotoxin (0.111 B4) from Difco Laboratories was dissolved in 0.9% sterile saline and the proper dose injected intravenously in the tail vein in 0.2 ml volume. Mortality was recorded at 24 hours. *Zymosan treatment*: Zymosan, lot No. 7B-340 from Standard Brands was used. 5 mg/ml suspension in 0.9% saline was prepared, boiled in a water bath 10 minutes, then shaken with glass beads to ensure an homogeneous suspension. One mg in 0.2 ml was injected intravenously every other day for a maximum of 5 doses. Endotoxin toxicity was investigated in groups of these mice 24 hours after the last injection of zymosan. In other zymosan treated mice, phagocytic activity of R.E.S. was investigated by measuring rate of clearance of carbon from blood. *B.C.G. infection*: B.C.G., Phipps strain, was grown on Dubos' liquid medium and harvested after 12 days. Mice were injected intravenously in the tail vein with 1 mg B.C.G. wet weight. Two weeks later, sensitivity to endotoxin was investigated. *Carbon clearance*: Phagocytic activity of the R.E.S. was investigated by measuring rate of clearance of carbon from the blood by the liver and spleen(11,12). Clearance of colloids by R.E.S. follows an exponential function of time $K = \frac{\text{Log } C_1 - \text{Log } C_2}{T}$; the constant K of this equation, called phagocytic index, measures phagocytic activity of the macrophages of the liver and spleen provided a suitable dose is used. K varies with relative size of the liver and spleen according to a third power relationship $\alpha = \frac{W}{WLS}\sqrt[3]{K}$, $\frac{W}{WLS} = \frac{\text{Weight of body}}{\text{Weight of liver and spleen}}$; α, called corrected phagocytic index, measures phagocytic activity per unit weight of liver and spleen(12). Carbon suspension C_{11} 1431a manufactured by Gunther Wagner, Hanover and suspended in gelatin as described(11) was used. This preparation contains particles homogeneous in size, measuring about 250 Å. Sixteen mg carbon per 100 g body weight were injected intravenously, then 0.025 ml blood samples were withdrawn at suitable times from the retroorbital venous plexus, with a calibrated glass pipette previously washed with heparin, and lysed in 2 ml 0.1% sodium carbonate; carbon concentration was read with a spectrophotometer at 675 mμ. After disappearance of carbon from the blood, the mice were sacrificed by decapitation and liver and spleen weighed in the wet state. The clearance curve was drawn in each case and phagocytic indexes K and α were calculated. *Adrenalectomy*: Bilateral adrenalectomy was performed under ether anesthesia by the dorsal route in control animals and in animals having received 4 injections of zymosan. Endotoxin toxicity was investigated 48 to 72 hours after adrenalectomy. *Cortisone treatment*: Control animals, B.C.G. infected, and zymosan treated animals (4 injections) were pretreated with cortisone acetate or hydrocortisone before investigating endotoxin toxicity. They received either 2.5 mg cortisone or 1 mg hydrocortisone 24 hours and again 3 hours before testing.

Results. Phagocytic activity of the R.E.S. is greatly stimulated by repeated intravenous injections of zymosan (Table I). This effect is at its maximum level after 4 injections and is associated with considerable hypertrophy of the liver and spleen due to hyperplasia of

TABLE I. Effect of 1 mg of Zymosan Every Other Day on Phagocytic Activity of Reticulo Endothelial System in Mice, Measured by Rate of Clearance (K) of Carbon Particles from Blood. Carbon, 16 mg/100 g K = Phagocytic Index α = Corrected Phagocytic Index. $\frac{W}{WLS} = \frac{\text{Body wt}}{\text{Wt of liver and spleen}}$.

Zymosan, mg		K	$\frac{W}{WLS}$	α
1		.050	13.3	4.9
		.039	13.9	4.7
		.074	12.5	5.2
		.144	12.9	6.7
	*	.077	13.1	5.3
2		.049	12.6	4.6
		.086	11.9	5.2
		.114	11.3	5.5
		.140	9.4	4.9
		.158	9.8	5.3
	*	.109	11.0	5.1
3		.035	13.6	4.4
		.084	10.8	4.8
		.100	11.3	5.2
		.160	9.8	5.3
		.160	12.6	6.8
	*	.108	11.6	5.3
4		.033	15.4	4.9
		.047	13.4	4.9
		.102	14.7	6.9
		.106	8.8	4.1
		.112	13.3	6.4
		.112	12.4	6.0
		.114	10.5	5.1
		.360	7.9	5.6
	*	.123	12.0	5.4
5		.230	9.0	5.4
		.130	13.8	7.0
10 controls	*	.021	14.6	4.0

* Mean values.

phagocytic elements(10,13). Susceptibility of mice to the lethal effect of endotoxin is considerably enhanced by zymosan injections and attains a level comparable to that observed in B.C.G. infected animals(6,7) (Table II). The effect of zymosan treatment is progressive and seems to attain its maximum after 4 to 5 injections over a period of 7 to 9 days; it is negligible a few hours after the first injection when the greatest decrease in properdin titer is generally observed. To understand the nature of this phenomenon, the increased susceptibility (50 to 100 times) to the lethal effect of endotoxin brought about by zymosan or B.C.G. infection is compared with that which follows adrenalectomy, which is of the order of 5,000 times. Cortisone protects mice nearly completely against the increased susceptibility of animals treated with zymosan or B.C.G., while in normal mice it shows only a minor protection against the lethal effects of endotoxin, a finding in accord with previous reports(14).

An attempt was also made to compare the level of endotoxin toxicity in adrenalectomized controls and in animals adrenalectomized after 4 injections of zymosan. While control mice withstand bilateral adrenalectomy without mortality, 16 out of 20 zymosan treated mice died within 24 hours after adrenalectomy. The 4 remaining animals were injected with 0.1 μg endotoxin without further mortality showing that when these animals survived adrenalectomy, they were not more susceptible to endotoxin than the adrenalectomized controls.

Discussion. Increased susceptibility to endotoxin could be explained either: 1) on the

TABLE II. Effect of 1 mg of Zymosan Every Other Day and of B.C.G. Infection on Endotoxin Toxicity in Mice. Endotoxin *E. coli* 0111 B4.

		Mortality						
		Zymosan, total dose and duration of treatment						B.C.G.
Endotoxin, mg	Controls	1 mg 5 hr	1 mg 24 hr	2 mg 3 days	3 mg 5 days	4 mg 7 days	5 mg 9 days	1 mg 2 wk
2	10/10							
1	11/13							
.5	11/19							
.25	6/26	3/5	2/4	4/5	4/5	10/10		
.1	1/21	0/5	3/5	8/11	"	"	6/6	
.05	0/8	"	"	2/5	"	13/14	"	
.025		"	0/5	0/5	"	11/14	"	5/5
.010						2/4	3/6	2/5
.005						1/4		"
.001								1/5

TABLE III. Effect of 2 × 2.5 mg of Cortisone or of 2 × 1 mg of Hydrocortisone on Hypersensitivity to Endotoxin in Mice Brought About by B.C.G. Infection or Zymosan Injections.

	Mortality								
	Controls				B.C.G.		Zymosan (4 mg, 7 days)		
E. coli endotoxin, mg	Untreated	Cortisone	Hydrocortisone	Adrenalectomized	Untreated	Cortisone	Untreated	Cortisone	Hydrocortisone
2	10/10	10/10	5/5						
1	11/13	7/10	4/5					6/6	
.5	11/19	4/10	3/5					5/6	
.25	6/26	0/10	1/5			5/5	10/10	4/11	3/5
.1	1/21					3/10	"	0/13	2/5
.05	0/8					0/5	13/14	0/5	0/5
.025					5/5	0/5	11/14	"	"
.010					2/5		2/4		
.005					"		1/4		
.001				7/7	1/5				
.0005				4/5					
.0001				3/8					
.00004				1/5					
.00001				0/3					

basis of hypersensitivity to endotoxins of the very cells of the R.E.S. which have been made to proliferate after B.C.G. infection or zymosan treatment, or 2) in view of the predominant role played by the adrenal in resistance against endotoxin, on the basis of decreased resistance due to interference with the level of adrenal hormones.

The data presented favor this last interpretation, since pretreatment with cortisone for 24 hours before endotoxin to a large extent abolishes increased susceptibility to endotoxin, while affecting only slightly endotoxin toxicity in normal mice. These experiments suggest, as far as utilization is concerned, a relative deficiency of adrenal hormone in these animals. If this hypothesis is correct, chronic infections or granulomatous inflammation, such as follows zymosan injections, are associated with decreased secretion or increased utilization of corticosteroids. Thus, as a corollary to the known effects of hormones on R.E. function and inflammation, it is suggested that such alterations can in turn also affect adrenal function. The data presented do not exclude the possibility that cortisone exerts an inhibiting effect on a hypersensitivity of the R.E.S. elements to endotoxin, as postulated by the first hypothesis. Further experiments are required to explore these possibilities.

Summary. Zymosan renders mice highly susceptible to the lethal effect of endotoxin at a time when activity of the R.E.S. is greatly stimulated. This effect is similar to that following B.C.G. infection. Pretreatment with cortisone abolishes nearly completely the increased toxicity to endotoxin caused by zymosan or B.C.G. infection.

1. Biozzi, G., Benacerraf, B., Halpern, B. N., *Brit. J. Exp. Path.*, 1955, v36, 226.

2. Benacerraf, B., Sebestyen, M. M., *Fed. Proc.*, 1957, v16, 861.

3. Bennett, I. L., Jr., Beeson, P. B., *J. Exp. Med.*, 1948, v88, 267.

4. Good, R. A., Thomas, L., *ibid.*, 1952, v96, 625.

5. Howard, J. G., Rowley, D., Wardlaw, A. C., *Immunol.*, 1958, v1, 181.

6. Suter, E., Ullman, G. E., Hoffman, R. G., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 169.

7. Halpern, B. N., Biozzi, G., Howard, J., Stiffel, C., Mouton, D., *C. R. Soc. Biol.*, 1958, v152, 899.

8. Biozzi, G., Benacerraf, B., Grumbach, F., Halpern, B. N., Levaditi, J., Rist, N., *Ann. Inst. Pasteur*, 1954, v87, 291.

9. Bordet, P., *ibid.*, 1936, v56, 525.

10. Gorstein, F., Benacerraf, B., *Fed. Proc.*, 1959,

11. Biozzi, G., Benacerraf, B., Halpern, B. N., *Brit. J. Exp. Path.*, 1953, v34, 441.

12. Benacerraf, B., Biozzi, Halpern, B. N., Stiffel, C., *Physiopathology of the R. E. S.*, Blackwell, Oxford, 1957.

13. Kelly, L. S., *Fed. Proc.*, 1958, v17, 84.

14. Spink, W. W., Anderson, D., *J. Clin. Invest.*, 1954, v33, 540.

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