Size of Antibody and Role of Autonomic Nervous System in the Adjuvant Action of Endotoxin. (28305)

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This paper concerns results of study of (a) the effect of mercaptoethanol on the antibody formed following a single injection of a protein antigen under the adjuvant stimulus of endotoxin, and (b) the relationship of the autonomic nervous system to this antibody enhancing effect of endotoxin(1). The antibody formed to soluble protein in the primary response appears initially to be a macroglobulin of the 19S class, followed later by the appearance of 7S antibody(2). The pattern of this response to bovine gamma globulin (BGG) in endotoxin-stimulated rabbits was studied utilizing the observation that mercaptoethanol will dissociate high molecular weight antibodies of the 19S class into smaller fragments, with loss of antibody activity(2,3,4). In addition, investigation of the hemodynamic aspects of endotoxin suggests that some of its activities may be mediated through the autonomic nervous system(5,6,7,8,). To determine if the antibody-enhancing effect of endotoxin is also mediated through, or associated with, the autonomic nervous system, the relationship of the adrenergic blocking agent, dibenzyline, to this enhancing action was studied.

Materials and Methods. Rabbits. White, New Zealand rabbits 12 to 14 weeks old, 2.5 to 3.0 kg were used. *Antigen.* Bovine gamma globulin (BGG), Armour, was given in a dose of 10 mg/ml 0.85% NaCl via the marginal ear vein of the rabbit. *Endotoxin. Serratia marcescens* or *Salmonella typhosa* endotoxin (Boivin) was given intravenously in a dose of 5-10 μg in 0.85% NaCl mixed with BGG. *Dibenzyline hydrochloride* Lot no. 60273 (Smith, Kline, and French), was given intravenously 1 hour prior to injection of antigen in dosage of 3 mg/kg body weight. The original concentration of 50 mg/ml was diluted to 9 mg/ml with 20% ethylene glycol and 10% ethyl alcohol, plus water to reach final volume. *Epinephrine hydrochloride.* This drug was mixed with 10 mg BGG prior to injection and given intravenously in a dosage of 5 μg/kg in 0.15 M NaCl, to give a total injected volume of 1 ml. *Hemagglutinating antibody determinations* were carried out essentially by the method of Boyden(9). *Antibody nitrogen* was determined by the micro-Kjeldahl method of Heidelberger and Kendall, as outlined by Kabat and Mayer(10). Two ml of rabbit antiserum were used except in rabbits 4, 8, and 9 (3 ml) and rabbits 5, 21, and 26 (4 ml) in Experiment B. *Mercaptoethanol* was added to antiserum diluted 1:10 in 0.01 M phosphate buffered saline, pH 7.2, to make a final concentration of 0.1 M mercaptoethanol. Mixtures were incubated at room temperature for 4 hours, then vigorously dialyzed against 0.15 M NaCl for 16 to 24 hours at 4°C to remove the mercaptoethanol. Earlier work in this laboratory showed that the hemagglutinating activity was not regained once it was lost after treatment with mercaptoethanol, irrespective of whether dialysis to remove mercaptoethanol was with 0.15 M NaCl or iodoacetamide. Control antisera were treated similarly except for omission of mercaptoethanol.

Results. (A) *Effect of mercaptoethanol on antibody formed with and without endotoxin stimulation.* Sixteen rabbits were divided into 2 equal groups. Group 1 rabbits each received 10 mg BGG intravenously, while Group 2 rabbits each received 10 mg BGG plus 5 μg endotoxin intravenously. Blood was drawn from the central ear artery or by cardiac puncture on days 4 to 9, 13, 16, following injection of antigen. The blood was allowed to clot 2 to 4 hours at room temperature, the serum removed, heated at 56°C for 30 minutes, and hemagglutinating antibody titers then determined. All antisera containing antibody activity were treated with mercaptoethanol, and

TABLE I. Effect of Mercaptoethanol on Antibody Formed to BGG in Association With Endotoxin Enhancement.

Group	Product	Rabbit*		Hemagglutination titer (reciprocal) Days after injection of antigen 4	5	6	7	8	9	13	16
1	BGG, 10 mg i.v.	1	C	0	0	0	0	80	40	80	†
			ME					0	0	0	
		2	C	0	0	0	0	0	0	0	0
			ME								
		3	C	0	0	0	0	0	0	0	0
			ME								
		4	C	0	0	0	0	0	20	10	0
			ME						0	0	
		5	C	0	0	0	40	160	160	160	80
			ME				0	20	20	0	0
		6	C	0	0	0	0	40	80	80	80
			ME					0	20	10	20
		7	C	0	0	0	0	20	0	0	0
			ME					0			
		8	C	0	0	0	0	0	80	20	20
			ME						0	0	0
	Geo. mean of control antisera			0	0	0	0	10	15	10	5
2	BGG, 10 mg i.v. and endotoxin, 5 μg	9	C	0	0	80	640	640	640	320	160
			ME			0	80	160	80	20	20
		10	C	0	0	0	0	40	40	80	0
			ME					0	0	0	
		11	C	0	0	0	40	160	160	80	40
			ME				0	40	40	10	10
		12	C	0	0	0	0	20	40	80	0
			ME					0	0	0	
		13	C	0	0	0	160	160	1280	320	320
			ME				0	0	40	10	0
		14	C	0	0	80	160	160	320	160	160
			ME			0	40	10	40	40	20
		15	C	0	0	0	160	80	80	40	20
			ME				0	0	0	0	0
		16	C	0	0	80	160	160	160	20	0
			ME			0	10	20	20	0	
	Geo. mean of control antisera			0	0	5	45	115	175	95	15

* C = Control Antiserum.
ME = Mercaptoethanol-treated antiserum.

† = Not done (Rabbit expired).
0 = Titre less than 1:10.

hemagglutinating antibody titers on these and control sera were again determined. All studies were carried out on fresh, unfrozen antisera within 36 hours after being drawn. The results are given in Table I. In Group 2 (BGG plus endotoxin), where a definite adjuvant effect occurred as seen in the geometric mean titers, the initial hemagglutinating antibody response was completely lost after addition of mercaptoethanol. Subsequently, hemagglutinating activity was retained in part in 5 of 8 rabbits despite mercaptoethanol treatment. This sequence was also seen in 2 of 6 rabbits in Group 1 which received only BGG. In 4 of 6 rabbits in Group 1 and 3 of 8 rabbits in Group 2, the entire hemagglutinating antibody response was made up of only mercaptoethanol-sensitive antibody. In all rabbits where mercaptoethanol-resistant antibody was present, mercaptoethanol-sensitive antibody was formed earlier. Thus, the initial hemagglutinating antibody response to the soluble protein BGG given together with endotoxin, as well as to BGG alone, appears to be a macroglobulin of the 19S class, with subsequent hemagglutinating activity being due at least in part to antibody of the 7S class.

(B) *Relationship of autonomic nervous system to antibody-enhancing effect of endotoxin.* Twenty-eight rabbits were divided into 4

TABLE II. Effect of Dibenzyline on Antibody-Enhancing Action of Endotoxin.

Group	Rabbit No.	Product received	Hemagglutination titer (reciprocal) — Day 10 — Titer	Day 10 — Geo. mean	Day 14 — Titer	Day 14 — Geo. mean
	1	BGG 10 mg i.v.	5,120		640	
	2		5,120		640	
	3		10,240	3,800	640	230
1	4		5,120		160	
	5		640		10	
	6		10,240		640	
	7		1,280		160	
	8		10,240		2,560	
	9	BGG 10 mg i.v.	2,560		640	
2	10	endotoxin 10 μg	10,240	6,200	N.D.*	1,100
	11		20,480		2,560	
	12		2,560		N.D.*	
	13		20,480		2,560	
	14		1,280		160	
	15	Dibenzyline 3 mg/kg	10,240		1,280	
	16	i.v. 1 hr before	2,560		320	
3	17	BGG 10 mg i.v. and	20,480	12,700	20,480	1,300
	18	endotoxin 10 μg	20,480		N.D.*	
	19		20,480		1,280	
	21		20,480		320	
	22	Dibenzyline 3 mg/kg	2,560		320	
	24	i.v. 1 hr before	1,280		320	
	25	BGG 10 mg i.v.	1,280	2,000	160	250
4	26		640		20	
	27		2,560		640	
	28		10,240		1,280	

Rabbits No. 20 and 23 died 4 hr after injection of dibenzyline.

* N.D. – Not done – rabbit expired.

equal groups: Group 1 received 10 mg BGG intravenously, Group 2 received 10 mg BGG and 10 μg endotoxin intravenously, Group 3 received dibenzyline, 3 mg/kg intravenously 1 hour before receiving 10 mg BGG and 10 μg endotoxin intravenously, and Group 4 received dibenzyline, 3 mg/kg intravenously 1 hour before receiving 10 mg BGG intravenously. Blood was drawn by cardiac puncture on days 10 and 14 after injection of the antigen, the serum removed and frozen at –20°C until ready for testing. Antiserum obtained on day 10 was tested both for hemagglutinating antibody and antibody-nitrogen content, while that taken on day 14 tested only for hemagglutinating antibody.

There was no inhibition of the antibody-enhancing effect of endotoxin by prior treatment of the animal with dibenzyline (Table II). Antibody-enhancing activity was present in both groups of rabbits receiving endotoxin, and in fact was greater in Group 3 where dibenzyline was given prior to BGG and endotoxin. It is of interest that no correlation between the hemagglutinating antibody titer and the precipitating antibody titer was found when antisera obtained on day 10 was analyzed (Table III).

In an additional experiment, designed to determine whether or not the endotoxin enhancing effect was mediated through the release of epinephrine, 10 rabbits were divided into 2 groups, Group 1 receiving 10 mg BGG intravenously and Group 2 receiving epinephrine, 5 μg/kg and 10 mg BGG together intravenously. Blood was drawn by cardiac puncture or from the marginal ear vein on days 7, 10, and 14 after injection of antigen, and the sera removed and frozen at –20°C until tested for hemagglutinating antibody. There was no enhancement of antibody to BGG by epinephrine given intravenously (Table IV).

Discussion. A significant amount of evidence is available indicating that loss of antibody activity after treatment with sulfhydryl-con-

TABLE III. Comparison of Hemagglutinating and Precipitating Antibodies in Day 10 Antisera.

Group	Rabbit No.	Product received	Hemagglutination titer (reciprocal)	Maximum μg Ab N/ml
	1		5,120	12
	2	BGG 10 mg i.v.	5,120	36
1	3		10,240	55
	4		5,120	0
	5		640	0
	6		10,240	34
	7		1,280	5
	8		10,240	0
	9	BGG 10 mg i.v.	2,560	7
2	10	endotoxin 10 μg	10,240	0
	11		20,480	18
	12		2,560	0
	13		20,480	30
	14		1,280	0
	15	Dibenzyline 3 mg/kg	10,240	0
	16	i.v. 1 hr before	2,560	0
3	17	BGG 10 mg i.v. and	20,480	26
	18	endotoxin 10 μg	20,480	9
	19		20,480	47
	21		20,480	<5
	22	Dibenzyline 3 mg/kg	2,560	0
	24	i.v. 1 hr before	1,280	0
4	25	BGG 10 mg i.v.	1,280	5
	26		640	0
	27		2,560	0
	28		10,240	0

taining compounds, such as mercaptoethanol, is a property of antibodies of the 19S class (2, 3,4). One recent report by Rockey and Kunkel(11) describing loss of activity of 2 intermediate-sized antibodies (8-11S and 9-15S) after mercaptoethanol treatment suggests that this group of large-sized mercaptoethanol-sensitive antibodies may be further divided as more refined ultracentrifugation methods are developed. However, at this point it appears that in most instances where loss of antibody activity is noted upon treatment with sulfhydryl-containing compounds, such an antibody can be considered as other than 7S antibody and most likely to be of the 19S class. In this study, evidence is presented that the antibody elevated during the primary response by endotoxin follows a pattern similar to that seen with soluble protein alone(2), to particulate antigens(12), and to bacteriophage(13), in that 19S antibody appears first and is followed later by the appearance of 7S antibody. A similar sequence has been noted in newborns(14), suggesting that, with certain ex-

TABLE IV. Effect of Epinephrine HCl on Antibody Response to BGG.

			Hemagglutination titer (reciprocal)					
Group	Rabbit No.	Product received	Day 7	Geo. mean	Day 10	Geo. mean	Day 14	Geo. mean
	1		20,480		1,280		640	
	2	BGG 10 mg i.v.	10,240	1930	5.120	1940	1,280	845
1	3		2,560		5,120		1,280	
	4		20		160		80	
	5		2,560		5,120		5,120	
	6		5,120		1,280		1,280	
	7	BGG 10 mg i.v.	80	175	320	640	1,280	540
2	8	epinephrine HCl	80		640		640	
	9	5 μg/kg i.v.	0		80		80	
	10		5,120		5,120		N.D.*	

* N.D. = Not done.

ceptions (for example 0 agglutinins, cold agglutinins and heterophile antibodies which remain as 19S antibodies), this is probably the normal sequence of antibody formation.

Although the autonomic nervous system is thought to be involved in some of the toxic manifestations of endotoxin, our experiments present evidence that this system is not involved in the antibody-enhancing effect of endotoxin. The adrenergic blocking agent, dibenzyline, did not inhibit the endotoxin from elevating antibody levels, nor did epinephrine *per se* given intravenously potentiate antibody formation. According to Harvey and Nickerson, adrenergic blockade in the rabbit, as determined by the presence of epinephrine reversal to 5 μg of epinephrine hydrochloride/kg, was considered present one hour after intravenous administration of dibenzyline in the dose of 3 mg/kg(15). Gourzis *et al.*(5) also found this dosage and time interval adequate to protect rabbits against very large doses (300-500 μg) of *E. coli* endotoxin. Goodman and Gilman stated that the duration of adrenergic blockade by the dibenamine-group of drugs (which includes dibenzyline) may last 3 to 4 days or longer(16), but specific data on the duration of blockade using dibenzyline, 3 mg/kg in the rabbit, is not available. However, in this regard, intravenously injected endotoxin is rapidly removed from the blood stream. Braude *et al.* found in rabbits that 50% of the dose of endotoxin is removed in 2 hours(17). In addition, Seligman *et al.*(18) found only 25% of *Serratia marcescens* endotoxin present in the plasma of a rabbit 2.3 hours after injection. Kind and Johnson, using human serum albumin and endotoxin in rabbits, found the periods over which endotoxin exerts its antibody-enhancing activity to be rapid and transient. Antibody enhancement was not found when endotoxin was given 5 or more hours before injection of antigen(19). This rapid and transitory effect of endotoxin is also in agreement with its known hemodynamic, leukopenic, and pyrogenic effects which are initiated in the first hour after injection of endotoxin. On the basis of this evidence it is felt that the dibenzyline blockade in our experiment was sufficient to extend over the time period when endotoxin was capable of enhancing antibody formation.

In contrast to Ungar's findings, in which he demonstrated significant antibody enhancement in guinea pigs given epinephrine together with toxoid antigen as a subcutaneous injection(20), in the present study, no antibody enhancement was found with intravenously administered epinephrine and antigen. One possible explanation for this discrepancy is that the adjuvant effect noted by Ungar may be related to the *local* vascular effects produced by subcutaneous injection of epinephrine, since sympathomimetic drugs, which did not possess vasoconstrictive activity, had no adjuvant action(20).

Conclusions. (1) Stimulation by endotoxin of the primary antibody response to bovine gamma globulin in rabbits results initially in antibody of the 19S class, and later, in part, of antibody of the 7S class. In some instances, 19S antibody constituted the entire primary response. (2) In the rabbit the sympathetic nervous system did not appear to be involved in the antibody-enhancing effect of endotoxin, in that the adrenergic blocking agent, dibenzyline, did not inhibit antibody enhancement, nor did epinephrine given intravenously potentiate antibody formation. (3) The hemagglutinating antibody titer did not correlate with the precipitating antibody titer.

1. Johnson, A. G., Gaines, S., Landy, M., *J. Exp. Med.*, 1956, v103, 225.

2. Bauer, D. C., Stavitskey, A. B., *Proc. Nat. Acad. Sci.*, 1961, v47, 1667.

3. Fudenberg, H. H., Kunkel, H. G., *J. Exp. Med.*, 1957, v106, 689.

4. Deutsch, H. F., Chan, P. C. Y., *Fed. Proc.*, 1958, v17, 210.

5. Gourzis, J., Hollenberg, M., Nickerson, M., *J. Exp. Med.*, 1961, v114, 593.

6. Zweifach, B. J., Nagler, A. L., Thomas, L., *ibid.*, 1956, v104, 881.

7. Thomas, L., *ibid.*, 1956, v104, 865.

8. Boquet, P., Izard, Y., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 254.

9. Boyden, S. V., *J. Exp. Med.*, 1951, v93, 107.

10. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry*, Charles C Thomas, Springfield, Ill., 1961, ch. 2.

11. Rockey, J. H., Kunkel, H. G., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 101.

12. Stelos, P., Talmage, D. W., *J. Infect. Dis.*, 1957, v100, 126.

13. Mathies, M., Stavitskey, A. B., *Fed. Proc.*, 1962, v21, 26.

14. Smith, R. T., in *Ciba Foundation Symposium on Cellular Aspects of Immunity*, G. E. W. Wolstenholme and M. O'Connor, Eds., Little Brown and Co., Boston, 1960, p348.

15. Harvey, S., Nickerson, M., *J. Pharm. Exp. Ther.*, 1953, v108, 281.

16. Goodman, L. S., Gilman, A., *The Pharmacologic Basis of Therapeutics*, Macmillan Co., N. Y., 1956, p572.

17. Braude, A. I., Carey, F. J., Zalesky, M., *J. Clin. Invest.*, 1955, v34, 858.

18. Seligman, A. M., Shear, M. J., Leiter, J., Sweet, B., *J. Nat. Cancer Inst.*, 1948, v9, 13.

19. Kind, P., Johnson, A. G., *J. Immunol.*, 1959, v82, 415.

20. Ungar, J. M. D., *Brit. Med. J.*, 1955, v2, 20.

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Serotonin and Histamine: Effects of a Single Injection on the Mouse Testis and Prostate Gland.* (28306)

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Serotonin and histamine occur together in high concentrations in localized regions, such as in mast cells(1,2) and in the hypothalamus (3). These 2 compounds, classified as hormones, frequently have similar pharmacological effects as evidenced by studies on vascular permeability(4,5) and vasoconstriction (6,7). It has been suggested that serotonin may act as a synergist with histamine or that part of its action may result from a release of endogenous histamine(8). Recently, while examining organ systems in mice that had been given an injection of histamine and serotonin combined in a single dose, gross changes in the size and color of the prostate glands were observed. Following these preliminary observations, additional mice were treated identically to augment the data for this presentation.

Methods. Male Swiss Yale albino mice, 6 to 8 weeks of age at the beginning of the experiment, were injected subcutaneously with serotonin (*5HT*) and/or histamine (*H*) in 0.25 ml physiological saline. Dosages were fractions of the established effective dosage of serotonin creatinine sulfate and histamine diphosphate (CalBiochem.)(9,10). Group I, killed 7 days after a single injection, consisted of: (a) saline controls, (18 mice); (b) *5HT*, 6.25 mg/kg (8 mice); (c) *H*, 200 mg/kg (8 mice); (d) *5HT*, 3.13 mg/kg, plus *H*, 100 mg/kg (10 mice). Group II, killed 30 days after a single injection, consisted of: (a) saline controls (10 mice); (b) *5HT*, 3.13 mg/kg plus *H*, 100 mg/kg (10 mice); (c) *5HT*, 6.25 mg/kg, plus *H*, 200 mg/kg (10 mice).

At autopsy the testes and ventral prostate of each mouse were weighed and then fixed in Bouin's fluid. Sections were stained with hematoxylin and eosin, Mallory's trichrome stain, or PAS (periodic acid-Schiff). Organ weights were expressed in milligrams per 100 g of body weight (Table I).

Results. Prostate glands from mice that received either histamine or serotonin, or a combination of the 2 drugs, had mean weights which significantly exceeded those of the saline controls; the greatest increase was displayed following injection of the 2 drugs in combination (Table I). At 30 days mean weights of experimental prostate glands still were greater than controls; however, the only statistically significant change was in the group of 9 mice injected with 6.25 mg serotonin and 200 mg histamine per kg body weight. The most frequent histological changes in the prostates were an increased

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