Formation of Antibody to a Protein Antigen in Adult Rabbits Given Salmonella Lipopolysaccharide. (32289)

ARNOLD J. SPURGASH* AND LOUIS R. SIBAL†
(Introduced by Mary Alexander Fink)
Department of Microbiology, University of Illinois College of Medicine, Chicago

Intravenous administration of Salmonella somatic polysaccharide to adult rabbits has been shown to induce the production of large numbers of plaque-forming cells specific for this antigen in the spleen. Significant numbers of such cells were also detected in the peripheral blood and thymus(1-3). Whether the presence of plaque-forming cells in the thymus was related to the unique antigenic properties of this polysaccharide or the physiological changes elicited by this group of toxic substances was not determined. Therefore, the present work was undertaken to study the effect of the administration of Salmonella lipopolysaccharide (LPS) on the pattern of response of rabbits to an unrelated protein antigen, bovine serum albumin (BSA). The results showed that significant levels of antibody to LPS were found in the spleen and thymus, antibody formation to BSA in the spleen was accelerated and increased by LPS and antibody was found in the thymus when BSA was given simultaneously with LPS.

Materials and methods. Seventy-nine adult New Zealand rabbits of both sexes and weighing 1.8 to 2.3 kg were divided into 3 experimental groups: (I) BSA alone, 10 mg/kg body weight (KBW); (II) LPS of *Salmonella abortus equi* alone, 5 μg/KBW; (III) BSA, 10 mg/KBW plus LPS, 5 μg/KBW, simultaneously. The LPS (Pyrexal) was prepared in the laboratory of Dr. Otto Westphal(4). All materials for injection were prepared in pyrogen free saline and given intravenously in 1.0 ml amounts. Representative rabbits were bled or sacrificed and tissue removed at daily intervals for 10 days after antigenic stimulation.

Preparation of organ extracts. Immediately after removal from the animal, the spleen or thymus was rinsed in cold 0.85% NaCl, blotted, and weighed. Each organ was gently minced and pressed through a monel-metal wire cloth (150 mesh). The resulting cells were collected in Hanks' balanced salt solution and washed 3 times. Then the cells were suspended in 2.0 ml of distilled water which had been adjusted to pH 7.6 with NaOH. They were then frozen and thawed 5 times(5). To the final cell lysate 20% NaCl solution was added until the concentration was 0.85%. Following the removal of cellular debris by centrifugation, the supernatant fluid was kept at −70°C until tested.

Antibody titrations. The anti-BSA content of serum and organ extracts was determined by the tanned cell hemagglutination technic (6); anti-LPS was measured by a modified hemagglutination technic originally developed by Neter *et al*(7). LPS antigen for sensitization of sheep erythrocytes (SRBC) was prepared as follows: *S. abortus equi* was cultured in Difco brain heart infusion agar for 24 hours at 37°C. The growth was harvested in 25 ml of sterile phosphate buffered saline (PBS), pH 7.2, and heated for 90 minutes at 100°C. Following centrifugation at 1400 × *g* for 30 minutes, the supernatant fluid was diluted with PBS to a final volume of 30 ml and kept at −20°C. For sensitization, an equal volume of washed 2.5% SRBC was added to a 1:100 dilution of the LPS antigen and incubated for 15 minutes at 37°C. The sensitized SRBC were washed 3 times in PBS and resuspended to a final concentration of 2.5%.

For both tests, serial 2-fold dilutions of inactivated serum and extracts were carried out in duplicate. To each tube containing 0.5 ml of material to be tested, 0.05 ml of SRBC sensitized with either BSA or LPS were added. The tubes were shaken and allowed to settle at room temperature; agglutination patterns were read after 3 hours. Evidence for speci-

* Present address: National Dairy Products Corp., Glenview, Ill.

† Present address: Nat. Inst. Health, Nat. Cancer Inst., Bethesda, Md.

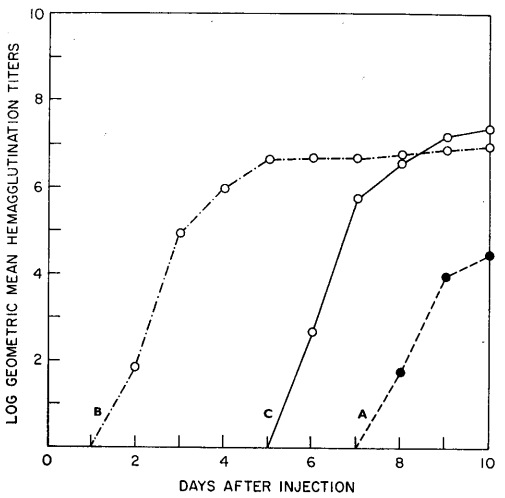

FIG. 1. Serum antibody response of rabbits given either (A) 10 mg BSA/KBW, (B) 5μg LPS/KBW, or (C) 10 mg BSA/KBW plus 5μg LPS/KBW, intravenously.

ficity was obtained by inhibition of the specific reactions. Appropriate controls consisting of standard positive and negative serums and cell controls were included in each assay. Serum titers are expressed as the $\log_2$ geometric mean of the highest dilution of serum showing complete hemagglutination or as the $\log_2$ geometric mean of the number of hemagglutinating units/g of tissue extracted.

Results. The results of preliminary experiments showing the adjuvant effect of LPS on the primary response of rabbits to BSA, as measured by this technic, are given in Fig. 1. Antibody to BSA appeared in the serum of LPS-stimulated rabbits on the 6th day after injection, 2 days earlier than in controls given BSA alone. The anti-BSA titers were markedly higher in the LPS-stimulated animals. Antibody to LPS appeared in the sera of animals given LPS alone and LPS plus BSA on the 2nd day after injection. The titers of both groups were identical, reaching a plateau on the 4th day after LPS stimulation.

The content of antibodies in the spleen and thymus was then determined and the results of these experiments are presented in Tables I, II, and III. Rabbits given BSA alone showed antibody in spleen extracts on the 7th day, 1 day earlier than the serum. The experiment was terminated on the 10th day before peak levels of antibody formation were attained. No antibody could be detected in thymic extracts at any of the times studied (Table I). As seen in Table II, antibody to LPS was detected in spleen extracts on the 1st day and in thymic extracts on the 3rd day after stimulation. The content of antibodies increased in both organs on succeeding days, but the amount of anti-LPS was considerably greater per unit weight of spleen than of thymus. Maximal levels of anti-LPS in the spleen and thymus were approached by the 4th and 5th days, respectively. This response was similar in the groups given LPS alone and LPS plus BSA (Table II and III). The appearance and content of anti-BSA in LPS-stimulated animals is also shown in Table III. Antibody to BSA was detected in the spleen on the 4th day and in the thymus and serum on the 6th day. The concentration of antibody in both organs and serum increased on succeeding days and the amounts of anti-BSA in the spleen were considerably greater per unit weight of spleen than of thymus. Also given in Tables I, II, and III are the wet weights of the organs. Spleen weights were not markedly altered following the injection of BSA or LPS. However, an increase following

TABLE I. $\log_2$ Geometric Mean Hemagglutination Titers of Serum and Organ Extracts of 23 Rabbits Given 10 mg BSA/KBW, Intravenously.

Days after injection	Serum anti-BSA	Spleen		Thymus	
		Wt, g*	Anti-BSA	Wt, g*	Anti-BSA
6	0	1.0	0	4.2	0
7	0	1.1	1.6	4.6	0
8	1.9	1.0	3.3	3.9	0
9	4.0	1.2	4.0	4.3	0
10	4.5	1.4	5.1	3.7	0

* Average wet wt in g.

TABLE II. $\log_2$ Geometric Mean Hemagglutination Titers of Serum and Organ Extracts of 23 Rabbits Given 5 μg LPS/KBW, Intravenously.

Days after injection	Serum anti-LPS	Spleen		Thymus	
		Wt, g*	Anti-LPS	Wt, g*	Anti-LPS
1	0	1.0	1.8	2.3	0
2	1.6	1.3	3.2	2.8	0
3	4.9	1.1	5.9	3.1	1.1
4	6.0	1.2	6.5	3.5	2.6
5	6.6	1.3	6.7	4.0	4.3

* Average wet wt in g.

TABLE III. Log_2 Geometric Mean Hemagglutination Titers of Serum and Organ Extracts of 23 Rabbits Given 10 mg BSA/KBW and 5 μg LPS/KBW, Intravenously.

Days after injection	Serum		Spleen			Thymus		
	Anti-BSA	Anti-LPS	Wt, g*	Anti-BSA	Anti-LPS	Wt, g*	Anti-BSA	Anti-LPS
4	0	5.9	1.2	1.9	6.4	3.2	0	2.6
5	0	6.6	1.4	4.3	7.5	3.6	0	4.2
6	2.6	6.7	1.5	5.3	7.5	4.1	.7	4.3
7	5.7	6.7	1.9	6.5	7.6	4.4	2.2	4.3
8	6.7	6.8	2.3	6.8	7.7	4.0	3.0	4.3

* Average wet wt in g.

the response to BSA in LPS-treated rabbits was noted on the 7th and 8th days after antigenic stimulation. A diminution in thymic weights occurred during the first 4 days after LPS injection, after which there was a return to normal values.

Discussion. The finding of antibodies to LPS and BSA in thymic extracts of adult rabbits following simultaneous injection of these antigens provides evidence that involvement of this organ is not uniquely related to antigenic properties of LPS. In these experiments, conditions were chosen which were optimal for the demonstration of enhancement of the primary antibody response to BSA. This was evident by the decrease in the induction period and marked increase in the antibody content of the spleen and serum. As expected, no response could be detected in the thymus following administration of BSA alone. The presence of antibody to LPS in both spleen and thymus extract supports the previous finding of plaque-forming cells in these organs by Landy and his co-workers(2,3).

The presence of antibodies to both antigens in the thymus may be accounted for in several ways. One possibility is that immunocompetent cells present in the thymus participated in the immune response. The administration of LPS could either have permitted access to or substantially increased the amounts of these antigens reaching the thymus. Hemodynamic changes relating to the toxic nature of the LPS or interference in the removal of injected material from the circulation by cells of organs not associated with antibody formation have been suggested as explanations for this response(8). Another possibility is that the presence of antibodies to BSA in the thymus was the result of infiltration of this organ by immunocompetent cells or their precursors from other more active antibody-forming organs (*e.g.*, spleen). The consistent appearance of antibodies to both antigens in the spleen at least 2 days prior to their detection in the thymus would support this hypothesis. The spleen weight increase and thymus weight decrease associated with the early antibody response may be related to this. The present data do not permit a clear choice between these two alternatives.

Our findings demonstrate that LPS administration influences the distribution of antibody or antibody-forming cells to a protein antigen in the rabbit. Landy and Baker(3) have reported that when Salmonella somatic polysaccharide and SRBC were simultaneously given to rabbits by the intravenous route, the numbers and organ distribution of plaque-forming cells for each antigen remained unchanged. Although direct comparison of the results of these two studies is difficult, it does not necessarily follow that a soluble protein antigen (BSA) and a cellular antigen (SRBC) would incite the same pattern of response. Our experiments do not suggest that the thymus plays an essential role in the adjuvant action of LPS since the contribution of this organ to the total antibody response of the animal was minimal. Campbell *et al*(9) have recently shown that thymectomy of adult mice failed to diminish enhancement by endotoxin.

Summary. Experiments were designed to study the immune response of the rabbit spleen and thymus following systemic administration of bovine serum albumin (BSA), Salmonella lipopolysaccharide (LPS) and BSA plus LPS. Significant amounts of antibody to LPS were found in the spleen and thymus. The formation of antibody to BSA in the spleen was accelerated and enhanced

by LPS; antibody was found in thymus when BSA was given with LPS.

1. Sorkin, E., Landy, M., Experientia, 1965, v21, 677.
2. Landy, M., Sanderson, R. P., Bernstein, M. T., Lerner, E. M. II., Science, 1965, v147, 1591.
3. Landy, M., Baker, P. J., J. Immunol., 1966, v97, 670.
4. Westphal, O., Luderitz, O., Bister, F., Z. Naturforsche, 1952, v7b, 148
5. Stavitsky, A. B., J. Immunol., 1955, v75, 214.
6. ———, ibid., 1954, v72, 360.
7. Neter, E., Gorzynski, E. A., Gino, R. M., Westphal, O., Luderitz, O., Canad. J. Microbiol., 1956, v2, 232.
8. Munoz, J., in Adv. in Immunol., Dixon, F. J., Humphrey, J. H., ed., Academic Press, New York, 1964, v4, 411.
9. Campbell, P. A., Rowlands, D. T., Jr., Harrington, M. J., Kind, P. D., J. Immunol., 1966, v96, 849.

Received April 20, 1967. P.S.E.B.M., 1967, v125.

A Fluorometric Method for Measurement of Sperm Capacitation. (32290)

R. J. ERICSSON
Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

The fact that rabbit sperm require several hours' residence in the female reproductive tract before acquiring the capacity to fertilize ova was reported independently by two investigators in 1951(1,2). Termed capacitation, this phenomenon has since been investigated in various species, in different anatomical structures of the female, and under the influence of several sex hormones (3,4,5). All work done to date has one thing in common, capacitation is measured by the ability of sperm to fertilize ova. Cleavage in recovered ova provides an accurate measure of capacitation, but this technique consumes time and, therefore, limits the number of animals and species which can be studied. Work in *in vitro* capacitation has been hampered for lack of a simpler method to measure capacitation.

In this paper, we describe a method to identify capacitated sperm fluorometrically.

Materials and methods. This technique is based on the observation that sperm bind with a fluorescent compound, tetracycline HCl (T-HCl). Steps involved in this procedure and the reaction of mammalian sperm to it have been reported(6). Sixteen experiments were performed with rabbits over a 15-month period. General procedures for these experiments were: 1) T-HCl in Tyrode's solution (3 mg/ml) mixed with semen at 6 $\mu g/10^6$ sperm for 10 minutes; 2) seminal fluid and excess T-HCl removed by centrifugation at 2900 rpm for 10 minutes; 3) sperm resuspended in Tyrode's solution for deposition in reproductive tract of estrous and pseudopregnant does. Twenty to fifty $\times$ 10^6 T-HCl-labeled sperm were injected through a 25-gauge needle into each uterine horn (.25 ml) and in some experiments 10 $\times$ 10^6 sperm in each oviduct (.10 ml). Laparotomy was performed under sodium pentobarbital and ether anesthesia. In one experiment 10% of the T-HCl bound to sperm was labeled with tritium. Sperm radioactivity was determined according to a procedure already described (7).

Sperm recovery took place 6-7 hours after incubation *in vivo*. Each uterine horn was clamped at both ends and 1.0 ml of Tyrode's solution injected into the lumen and after manual manipulation the fluid was recovered in a syringe; oviducts were flushed with 0.25 ml of Tyrode's solution. Recovered sperm cells were checked for fluorescence, motility and number. Sperm fluorescence was determined at 320 magnification with a Leitz Ortholux microscope equipped with a high pressure mercury vapor lamp. The microscope was also fitted with a BG 12 fluorescence filter for UV-blue light and a darkfield condensor.

Experiments consisted of: 1) comparison of