Addition of NEAA to the EAA diet resulted in significantly lower PAN levels but elevated liver fats.

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γ G Antibodies Appearing Early in the Primary Response of the Mouse.* (31179)

R. T. CUSHING† AND A. G. JOHNSON
Departments of Internal Medicine and Microbiology, University of Michigan, Ann Arbor

The sequence of appearance of molecular species of antibody following immunization with particulate and soluble antigens has been studied by ultracentrifugation of antisera and localization of antibody activity within the globulin fractions. Gel filtration has also been used to fractionate serum proteins according to molecular weight and sedimenting characteristics, and antibody activity can be localized in the globulins of the 7S or 19S species. Using the passive hemagglutination test of Boyden(1) to measure antibody activity, various investigators(2,3) have concluded that the primary immune response in various animal species to bovine serum albumin (BSA) consists of 19S macroglobulin formation followed by the 7S variety. In the course of studying the primary immune response to BSA in the inbred mouse with and without the adjuvant effect of endotoxin, the presence of an early appearing 7S antibody response was detected. This antibody response was easily demonstrable by the Farr antigen binding capacity (ABC) technique(4), but poorly evident using passive hemagglutination. Rosenquist and Guilden(5) previously reported finding early 7S antibody activity to BSA in chickens, and since our studies were undertaken, reports of similar findings in the chicken and rabbit by Benedict and coworkers(6,7) and by Wei and Stavitsky(8) in the rabbit have been published. We wish to report the evidence in yet a third species and discuss the significance of these findings in relation to the general immune response to BSA.

Materials and methods. Antigen. BSA (Calbiochem, lot 43648) dissolved in saline and endotoxin (lot SMTCA AA-1) prepared from *Serratia marcescens* by Dr. A. Nowotny was injected intraperitoneally into mice 7-10 weeks old. Dosage was 3 mg BSA plus 10 μg endotoxin in a volume of 0.5 ml. The mice were maintained on Purina Mouse Checkers

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† Present address: St. Louis Park Medical Center, St. Louis Park, Minnesota.

and water *ad libitum*. Mice were caged in groups of 4 or 5, and groups were exsanguinated under ether anesthesia at varying intervals. The serum was separated by centrifugation, quick-frozen, and kept at —20°C until tested.

Antibody determinations. Tests for antibody utilized the tanned cell hemagglutination test of Boyden(1) and the antigen binding capacity technique described by Farr(4) as modified by Vredevoe(9). Slight modification of the method of Banerjee and Ekins (10) was used to label antigen with I^{131}, with separation of non-protein radioactivity by column chromatography using Sephadex G25. Counts were made in a well-type scintillation counter.

Farr test. Tagged BSA (I-BSA) was assayed for protein concentration by the method of Waddell(11) and diluted in 1:100 normal mouse serum-borate buffer (pH 8.3-8.4) to a concentration of 1 or 2 μg I-BSA per ml. Test antisera were diluted to 1:10 in duplicate using 1:10 NMS-borate buffer as diluent, the final volume in each tube being 0.5 ml; 0.5 or 1.0 μg of I-BSA (0.5 ml) then was added to each tube and thoroughly mixed. After incubation for 30 minutes at 37°C, 1.0 ml saturated ammonium sulfate was added to each tube and the tubes reincubated at 37°C for 30 minutes and at 4°C for 20 minutes. The resulting precipitate containing the serum globulins was separated by centrifugation in the cold at 2000 rpm for 45 minutes and the supernatant decanted carefully into a second tube for counting of non-precipitated radioactivity. The precipitate was dissolved in 2 ml water before counting. Calculations of antigen binding by the test antisera were made following Farr's method (4) and are reported as per cent of added radioactivity precipitated by antibody.

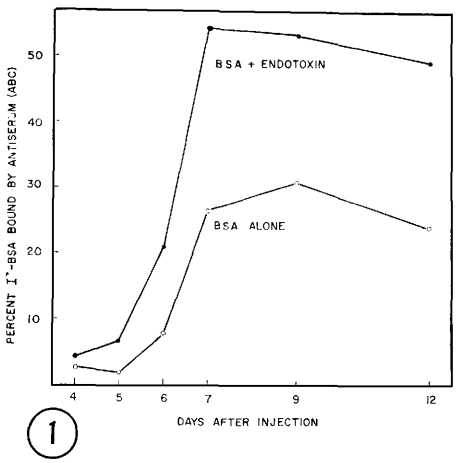
Fractionation of mouse antiserum. One ml antiserum was fractionated by gel filtration in the cold, utilizing a Sephadex G200 column measuring 100 × 4.3 cm. The eluant was tris HCl buffer, pH 8.0. Protein fractions were determined spectrophotometrically at 280 μ, and the peaks were pooled. These pools were concentrated to approximately 1 ml by dialysis against 10% polyethylene glycol and the concentrates then assayed for antibody activity by the Farr technique.

Results and discussion. Antibody titers on whole mouse sera as determined by the hemagglutination test ranged from 1:10 to 1:40. Hemagglutination inhibition tests with BSA confirmed the validity of these titers, but the titers were so low as to be considered uninterpretable within the experimental limitations of the test.

On the other hand, antibody activity as measured by antigen binding capacity (ABC) was consistently measurable. Fig. 1 depicts typical ABC curves produced by antisera collected at varying time intervals after injection of BSA with and without endotoxin. Significant ABC values were detectable by 6 days and reached a peak between 7 and 9 days before gradually diminishing. Antibody in significant amounts, however, persisted for the duration of the study, *i.e.*, 28 days.

Seven-day serum from mice injected with BSA plus endotoxin repeatedly and consistently showed the highest ABC values of any tested; consequently, the molecular species of globulin-containing antibody participating in the ABC was determined for this serum using Sephadex G200. With such filtration, the first peak included 19S macroglobulins, the second peak 7S globulins, and the third peak primarily 4S albumin as reported by Fireman *et al*(12) (Fig. 2). Concentrated pooled fractions comprising the 19S peak, the intermediate area, and the 7S peak were then tested for ABC. ABC was found only in the 7S peak.

This finding of localization of all the ABC in the 7S peak of a serum sample obtained at a time when antibody is just beginning to appear can be interpreted in several ways. An earlier 19S response may have been missed. This is unlikely because on repeated experiments significant ABC values (greater than 10%) were not found until 6 days after injection, and 19S antibody activity would be expected to persist at least to day 7. On the other hand, 19S antibody may have been present, but not measurable by this technique, or lost in the fractionation or concentration procedures. This possibility cannot be ruled out, but even if it were true, the presence of



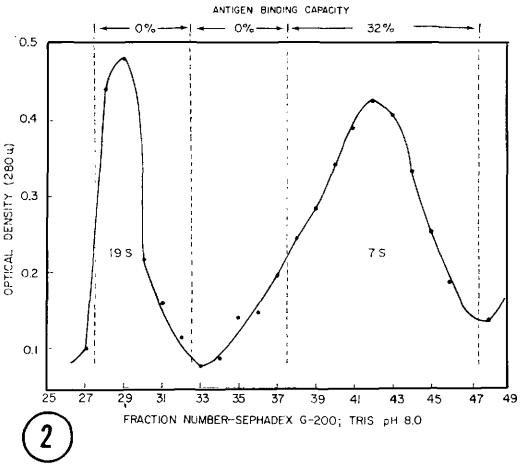


FIG. 1. Enhancement by endotoxin of early antibody synthesis to bovine serum albumin (BSA) as measured by antigen binding capacity.

FIG. 2. Antigen binding capacity of the 7S fraction of anti-BSA serum appearing 7 days after antigen injection.

a significant amount of early 7S antigen binding capacity indicates that the concept of 19S to 7S sequence of antibody formation may not hold for all antigens or species. Our findings are in accord with those of Rosenquist and Gilden who found no evidence of 19S antibody activity in chickens immunized with BSA as measured by the Farr technique on gel filtered and ultracentrifuged fractions (5). On the other hand, Dreesman *et al*(6) did find 19S antibody to BSA in early primary chicken antisera, but the greatest amount of ABC resided in the 7S fraction of 6-day serum. The low hemagglutination titers and lack of ABC in the 19S peak are also consistent with Benedict's finding in the rabbit that the hemagglutination test may preferentially measure 19S antibody, when, in fact, significant levels of 7S antibody are also present in the early primary sera(7).

The finding of an early 7S antibody in the mouse which differs from later 7S antibody by its lack of reactivity in the passive hemagglutination test represents the third species in which this has been demonstrated. This pattern of response would appear to be part of the general antibody response to BSA. If tests comparable to the Farr procedure were available to measure the immune response to other purified protein antigens, it is likely that this early 7S antibody response would be demonstrated. If so, this finding represents an added dimension to the primary immune response.

Summary. Evidence is presented using the Farr technique that a 7S gamma G antibody represents a significant portion of the early primary antibody response to BSA in the inbred mouse. The adjuvant effect of endotoxin with respect to this early 7S antibody was demonstrated. Correlation of this finding with the method selected for determining antibody activity is discussed.

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