

Immune responses of human adults after oral and parenteral exposure to bovine serum albumin

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Investigations were carried out to examine the possible causes of the low incidence and low levels of anti-BSA in human adults as compared to those of normal children. To determine if the reduced amount of BSA in the adult diet was an important factor, childhood levels of dietary BSA in the form of oral supplemental doses were administered to 17 normal human volunteers for 21 days. To learn if the manner in which dietary protein was catabolized by the gastrointestinal tract of the adult was a determining factor, selected individuals were also stimulated by intradermal injections of BSA. When anti-BSA was present before the experiment, there was a measureable antibody response following both methods of stimulation. The responsiveness of this group indicates that, among adults who retained the capacity to produce anti-BSA, the dose of antigen was an important variable in determining the amount of antibody actually produced. When no anti-BSA or very low levels existed before the experiment, the adults were unresponsive or hyporesponsive to both methods of stimulation. At least 80 per cent of these unresponsive adults had been synthesizing anti-BSA during childhood and should have resumed antibody production had their adult unresponsiveness been due to either a low dietary intake of BSA or the gastrointestinal conditions unique to adults. Circulating antigen was demonstrated in the sera of individuals without anti-BSA for as long as 21 days after intradermal injections of BSA. In subjects with trace amounts of anti-BSA prior to the intradermal injection of BSA, both BSA and anti-BSA were demonstrated to exist simultaneously in the serum for a minimum of 21 days. These data suggest that antigen-antibody complexes can sometimes persist in the circulation for prolonged periods following the injection of relatively small doses of antigen into human subjects. None of the subjects demonstrated an immediate wheal-and-flare response, an Arthus reaction, or a tuberculin type of response when tested either before or after the experiment. Thus it appears that the low incidence of detectable anti-BSA among adults is due in part to an acquired immunological hyporesponsive state to BSA.

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Circulating antibodies to dietary antigens have been demonstrated in many human sera, suggesting that immunization to proteins ingested as food is a frequent and natural occurrence.¹⁻³ In one study, antibodies against a protein in cow's milk, bovine serum albumin (BSA), were found in the sera of more than 80 per cent of normal children between 4 months and 5 years of age.³ In contrast, anti-BSA activity was found in only 29 per cent of sera from adults between 16 and 40 years of age and in only 7 per cent of persons over age 40. Similar observations were also made with a second milk antigen, alpha-lactalbumin.³ Possible explanations for the decline of anti-milk antibodies in the mature age groups are (a) a decrease in consumption of milk and milk products among the older age groups; (b) changes in the gastrointestinal tract that would reduce the availability of antigen from the lumen of the gastrointestinal tract; and (c) establishment of an immunologic hyporeactive state following long-term ingestion of antigen. The present study was designed to examine which, if any, of these mechanisms may be responsible for the reduction of anti-milk protein antibodies in persons over 20 years of age.

METHODS

Subjects

The persons involved in this study were 17 adults, 9 men and 8 women, ranging in age from 22 to 50 years. Three persons (P. H., P. F. K., and L. G.) had allergic rhinitis or bronchial asthma associated with reagin and 14 were in good health and free of allergic disorders. P. F. K. and L. G. were receiving hyposensitization injections with antigens not of mammalian origin. A history of recent daily ingestion of foods containing BSA was obtained from each subject, and the daily BSA intake was calculated on the basis of previous immunologic measurements of the BSA content of certain beef products.⁴ Subject B. S. had carefully avoided milk and milk proteins for 10 years before this experiment, and the remaining 16 individuals ingested between 0.1 and 1.5 mg. per pound per day, as noted in Tables I, II, and III.

Oral immunization

Fifteen subjects were given supplemental dietary BSA* (fraction V) for 21 days, and in two subjects (B. S. and K. H.) it was continued for 35 days. The supplemental dose was 12 mg. per pound per day, which represents one and one-half times the BSA per pound per day that would be ingested by a two-year-old child drinking the minimal daily recommended amount of milk.⁵ These calculations were based upon the average weight of a two-year-old child as 27 pounds⁵ and the concentration of BSA in skimmed milk as 0.29 mg. per milliliter.⁴ This supplemental dose represents considerably more milk than the average adult ingests in 24 hours. For example, a person would have to drink 8.3 L. of milk every 24 hours to ingest this calculated dose of BSA. Other sources of BSA in the adult diet are negligible, since cooking destroys up to 95 per cent of the native antigenicity of BSA extractable from beef.⁴ It is

*Armour Pharmaceutical Co., Kankakee, Ill.

also unlikely that these individuals were exposed to other mammalian albumins that cross react with BSA, such as sheep or horse albumin. Sera were obtained from each individual prior to ingestion of supplemental BSA and on the seventh, fourteenth, and twenty-first days of the experiment. Selected individuals were also bled on the twenty-eighth, thirty-fifth, and sixty-fifth days.

Parenteral immunization

One month following the cessation of supplementary BSA ingestion, 7 subjects were bled and skin tested with 0.02 ml. intradermal injections of a solution containing 1 mg. of crystallized BSA per milliliter of normal saline. No immediate wheal-and-flare, Arthus, or delayed types of reactions were observed. These subjects were then given intradermal injections of 0.1 mg. of crystallized BSA solution in ten separate sites (total dose = 1.0 mg. of BSA), and sera were obtained on days 7, 14, and 21. Two weeks after the day 21 bleeding, 4 subjects who had failed to respond to either the oral or intradermal challenges of BSA were again bled and skin tested. These individuals were then given further intradermal injections of 1 mg. of crystallized BSA (ten sites of 0.1 ml. each) every other day until they had received a total dose of 3 mg. of BSA. Sera were obtained at 2 and 72 hours following the last injection and weekly for 3 weeks.

Antibody detection

Each serum was studied in duplicate for anti-BSA by a modification⁶ of the ammonium sulfate technique.⁷ Crystallized BSA* was labeled with I¹³¹ by a modified chloramine-T method⁸ and diluted in 1:100 normal human serum in

*Armour Pharmaceutical Co., Kankakee, Ill.

Table 1. *BSA binding in sera from eight individuals having no anti-BSA prior to experiment*

<i>Subject</i>	<i>Age</i>	<i>BSA content of regular diet (mg./ pound/ day)</i>	<i>Day 0</i>	<i>Day 7</i>	<i>Day 14</i>	<i>Day 21</i>	<i>Day 28</i>	<i>Day 35</i>	<i>Day 65</i>
J.M.	47	1.1	0.6* (-) †	0.2	0.3	0.0 (-)			0.1 (-)
V.L.	28	0.1	0.4 (-)	0.2	0.3	0.1 (-)		0.1 (-)	
P.F.K.	31	1.5	0.4 (-)	0.1	0.1	0.2 (-)	0.3 (-)	0.5 (-)	0.8 (-)
T.N.	23	0.7	0.2 (-)	0.2	0.6	0.9 (-)		0.6 (-)	0.8 (-)
S.C.	23	0.5	0.9 (-)	0.6	0.9	0.7 (-)	0.7 (-)		
P.K.	30	0.1	0.8 (-)	0.3	0.6	1.4 (-)	2.7 (-)		
K.H.	24	0.6	4.0 (-)	2.5	3.8	4.1 (-)	4.3 (-)	5.6 (-)	5.6 (-)
B.S.‡	35	0.0	2.6 (-)	2.5	5.7	4.8 (-)	6.6 (+)	8.4 (+)	8.4 (+)

*Per cent of 0.02 µg of I-BSA N specifically bound by 0.5 ml. of a 1:5 dilution of antisera.

†The presence (+) or absence (-) of the capacity to bind BSA as demonstrated by radioimmuno-electrophoresis.

‡This subject had not ingested foods containing BSA during the 10 years preceding this experiment.

borate buffer so that the final concentration of I^{131} -labeled BSA (I-BSA) was 0.04 μg of I-BSA nitrogen (N) per milliliter. The per cent of labeled BSA bound by a 1:5 dilution of each test serum was calculated and compared with the per cent of labeled antigen bound by antiserum to which unlabeled BSA antigen had been previously added. The antigen specifically bound by the antisera was determined by subtracting the per cent I-BSA N bound by the aliquots to which unlabeled antigen had been added from the per cent of I-BSA bound by the test antiserum. Anti-BSA activity was considered present when this value was greater than 5 per cent, and capacity to bind I-BSA was confirmed by radioimmuno-electrophoresis. All samples from a given individual were studied on the same day with the same lot of I-BSA to minimize experimental variations. Under these circumstances, 5 separate determinations performed on 4 serum samples from different subjects varied from 0.2 to 2.5 per cent. When the quantity of antibody was sufficient to bind greater than 33 per cent of the antigen employed, the antigen-binding capacity of the serum (ABC-33) was also determined with the use of 0.04 μg of I-BSA N per milliliter as the test antigen.⁷

Immuno-electrophoresis and radioimmuno-electrophoresis were carried out as previously described.⁹

Detection of circulating antigen

Studies to detect the presence of BSA or antigenic BSA fragments were performed on selected sera following oral and parenteral immunization. The specific inhibition of a standard human anti-BSA:I-BSA reaction by each serum sample was compared with similar inhibition by normal sera to which known amounts of unlabeled BSA had been added. Under these experimental conditions, when 0.5 ml. of a 1:2 dilution of the test serum produced more than 20 per cent inhibition, it was considered to indicate the presence of at least 0.08 μg of BSA per milliliter of undiluted serum. Details of this procedure have previously been described.⁶

RESULTS

Oral immunization

Data from eight subjects whose sera contained no detectable anti-BSA activity prior to ingesting supplemental BSA are presented in Table I. The ingestion of BSA did not stimulate the production of detectable anti-BSA in any of these subjects by day 21. However, two (K. H. and B. S.) sera bound 4.1 and 4.8 per cent of labeled antigen. Since this was only slightly less than the 5 per cent end point indicative of antibody activity, BSA ingestion was continued in these two subjects for another 14 days. The serum of K. H. eventually bound 5.6 per cent of the I-BSA, but this did not represent antibody because anti-BSA activity was not confirmed by radioimmuno-electrophoresis. In contrast, antigen bound by B.S. increased from 2.6 to 6.6 per cent on day 28 and to 8.4 per cent by day 35. Thirty days after the cessation of supplemental BSA, serum obtained from B. S. continued to bind 8.4 per cent, and antibody activity was confirmed by radioimmuno-electrophoresis. Thus B. S., the only individual on a BSA-free diet previous to the experiment, was the only individual

in Table I to initiate or reinstitute production of detectable anti-BSA following the oral challenge.

Data from four subjects whose sera contained trace amounts of anti-BSA prior to the ingestion of supplemental BSA are presented in Table II. Two of these (M. Y. and P. H.) demonstrated small but significant increases of anti-BSA activity.

Sera from five subjects bound enough I-BSA prior to the experiment to allow ABC-33 determinations. Data from these sera are presented in Table III. Four of these individuals responded with greater than a 25 per cent increase in antibody activity. A brisk rise characteristic of the temporal pattern of an anamnestic response was observed in one serum (D. R.). In contrast, the increase in three of the individuals was not detected until 14 to 21 days after the initiation of the oral challenge. The fifth serum (R. M. R.) did not demonstrate a significant increase in anti-BSA activity during this phase of the experiment, but a significant increase of antibody activity was observed 44 days after the cessation of high doses of supplemental BSA.

Table II. *BSA binding in sera from four individuals having trace amounts of anti-BSA prior to experiment*

Subject	Age	BSA content of regular diet (mg./pound/ day)	Day 0	Day 7	Day 14	Day 21	Day 28
R.F.	44	0.5	5.2* (+) †	5.8	5.9	7.3	7.5 (+)
M.Y.	50	0.6	9.0 (+)	9.0	9.5	12.4	18.4 (+)
N.B.	50	1.1	15.9 (+)	14.3	17.6	18.6	16.9 (+)
P.H.	35	0.4	18.9 (+)	17.6	18.1	20.5	24.4 (+)

*Per cent of 0.02 μ g of I-BSA N specifically bound by 0.5 ml. of a 1:5 dilution of antisera.

†The presence (+) or absence (–) of the capacity to bind BSA as determined by radio-immuno-electrophoresis.

Table III. *BSA binding in the sera from five individuals having larger amounts of anti-BSA prior to experiment*

Subject	Age	BSA content of regular diet (mg./ pound /day)	Day 0	Day 7	Day 14	Day 21	Day 28
S.R.	22	1.5	0.05* (+) †	0.05	0.12	0.13	0.15 (+)
D.R.	53	1.2	0.38 (+)	0.47	0.46	0.42	0.40 (+)
L.G.	26	1.4	0.59 (+)	0.54	0.59	0.60	0.99 (+)
R.T.R.	35	0.4	0.53 (+)	0.54	0.57	0.90 (+)	
R.M.R.‡	33	0.2	0.10 (+)	0.11	0.12	0.12	0.12 (+)

*Micrograms of I-BSA N bound per milliliter of undiluted serum (ABC-33 at 0.02 μ g of I-BSA N).

†The presence (+) or absence (–) of the capacity to bind BSA as demonstrated by radio-immuno-electrophoresis.

‡On day 44 this serum had ABC-33 of 0.15 μ g of I-BSA N, a significant increase in antibody activity.

As noted in Tables I, II and III, radioimmuno-electrophoresis was used to confirm the presence or absence of antibody in the sera of all subjects. With the one exception previously discussed (K. H., Table I), γ G with capacity to bind I-BSA was detected in all persons whose sera bound more than 5 per cent of the antigen added in the ammonium sulfate test. No antibody activity was observed by radioimmuno-electrophoresis in any of the subjects whose sera bound less than 5 per cent. In one subject (L. G., Table III), I-BSA binding was also noted in the γ A immunoglobulins, but in no instance was anti-BSA activity observed in the γ M immunoglobulins.

Since it has been demonstrated that trace amounts of antigenically recognizable BSA or BSA fragments can be detected in the sera of rabbits fed BSA,⁶ similar studies were performed on the day 7 and day 14 sera presented in Tables I, II, and III. Free BSA was not detected in any of these sera.

Parenteral immunization

Seven of the subjects presented in Tables I, II, and III were injected intradermally with 1 mg. of BSA, and subsequent studies of their sera are shown in Table IV. Three subjects (J. M., T. N., and P. F. K.) who had no anti-BSA before the experiment and who had not responded following the ingestion of BSA again failed to manifest an increase in anti-BSA after the intradermal injections. B. S., who had developed an antibody response after oral BSA, did not show further increase in antibody production after intradermal challenge. The other subject (R. F.), who had only trace amounts of anti-BSA before and after the oral challenge, also failed to respond to the intradermal challenge. The two remaining subjects, D. R. and R. M. R., who had considerable anti-BSA before the experiment and responded to the oral challenge, again demonstrated significant increases in anti-BSA activity after intradermal stimulation.

Four of the five subjects in Table IV who did not respond to the intradermal injections of 1 mg. of BSA were given additional doses of 1 mg. of BSA intradermally every second day for a total of 3 mg. Their sera were studied for the

Table IV. *Effect of intradermal injection of 1 mg. of BSA on the I-BSA-binding capacity*

Subject	Time after intradermal BSA			
	Day 0	Day 7	Day 14	Day 21
J.M.	1.1* (-) †	0.2	0.5	0.1 (-)
T.N.	0.8* (-)	0.5	0.4	0.5 (-)
P.F.K.	0.5* (-)	0.4	0.5	0.6 (-)
R.F.	7.5* (+)	3.5	7.6	8.2 (+)
B.S.	8.4* (+)	2.8	7.7	7.6 (+)
R.M.R.	0.15 ‡ (+)	0.12	0.17	0.19 (+)
D.R.	0.32 ‡ (+)	0.25	0.48	0.53 (+)

*Per cent of 0.02 μ g of I-BSA N specifically bound by 0.5 ml. of a 1:5 dilution of antisera.

†The capacity to bind I-BSA as demonstrated by radioimmuno-electrophoresis.

‡Micrograms of I-BSA N bound per milliliter of undiluted serum (ABC-33 at 0.02 μ g of I-BSA N).

Table V. *Effect of intradermal injection of 3 mg. of BSA on the I-BSA-binding*

<i>Time after last injection of BSA</i>	<i>P.F.K.</i>		<i>T.N.</i>
	<i>BSA in serum*</i>	<i>Anti-BSA determinations</i>	<i>BSA in serum*</i>
Pre-bleed	Negative	0.5† (-)‡	Negative
2 hours	ND	0.7	Positive
3 days	Positive	0.3	Positive
7 days	Positive	0.6	Positive
14 days	Positive	0.5	Positive
21 days	Positive	1.1	Positive
25 days	Negative	0.8 (-)	Negative

ND = not done.

*A positive result represents a minimum of 0.08 μ g of unlabeled BSA per milliliter of undiluted serum.

†Per cent of labeled antigen specifically bound by 0.5 ml. of a 1:5 dilution of antisera.

‡The capacity to bind I-BSA as demonstrated by radioimmuno-electrophoresis.

presence of free circulating antigen as well as for the presence of antibody, and results are presented in Table V. Free antigen was expressed as either positive or negative, and a positive result signified the presence of at least 0.08 μ g of BSA per milliliter of undiluted serum. Despite the presence of free antigen in the serum of all four individuals for a minimum of 21 days, only one (J. M.) responded with antibody production. The presence of anti-BSA in this 35 day serum was demonstrated by both the ammonium sulfate test and radioimmuno-electrophoresis. One subject (R. F.), who had not had urticaria in more than 20 years, developed a transitory facial urticarial reaction after the last dose of BSA. Serum samples from this individual contained both BSA and anti-BSA on the two occasions studied, suggesting the possibility that circulating antigen-antibody complexes may have been responsible for the urticaria.

None of the subjects responded with an immediate wheal-and-flare reaction, an Arthus reaction, or a tuberculin-type response when tested by the intradermal injection of BSA (1 mg. per milliliter), either before or after oral and parenteral stimulation.

DISCUSSION

Seven of the eight individuals in Table I who had no detectable circulating anti-BSA prior to these experiments were unresponsive to BSA despite the fact they had been ingesting 0.1 to 1.5 mg. of BSA per pound per day. The eighth subject (B. S.) was different in that she had not been exposed to oral BSA for 10 years. It has been previously reported that detectable amounts of anti-BSA are not present unless there is BSA in the diet.¹⁰ On the basis of previous studies concerning the incidence of antibodies to BSA as it varies with age,³ it may be assumed that an average of five of any seven unresponsive adults in the age group presented in Table I had been producing anti-BSA during childhood. Accordingly, if the amount of BSA in the diet were the controlling factor that determined the *incidence* of circulating anti-BSA in adults, approximately five of the seven unresponsive individuals in Table I should have resumed production of detectable antibody after the oral dose of BSA was restored to childhood

capacity

<i>Anti-BSA determinations</i>	<i>J.M.</i>		<i>R.F.</i>	
	<i>BSA in serum*</i>	<i>Anti-BSA determinations</i>	<i>BSA in serum*</i>	<i>Anti-BSA determinations</i>
0.5† (-)‡	ND	0.3† (-)‡	Negative	7.5† (+)‡
0.2	Positive	0.2	Positive	6.5 (ND)
0.3	Positive	0.3	ND	7.2 (ND)
0.0	Positive	0.2	Positive	7.6 (+)
0.0	Positive	1.2	Positive	6.8 (ND)
0.3	Positive	2.3	Positive	7.4 (+)
0.8 (-)	Negative	5.8 (+)	Negative	5.7 (+)

luted serum.

levels. With the use of chi-square with the Yates correction for continuity because of the small sample size, the difference between the prediction that five individuals should have resumed detectable antibody production and the observation that none actually did so is significant ($P < 0.05$). These data indicate the low incidence of anti-BSA in adults is not the result of merely a low dietary content of BSA.

Only two of the four adults in Table II who were producing trace amounts of anti-BSA prior to the experiment could be induced to have even feeble anamnestic responses when the ingested dose of BSA was restored to childhood levels. Neither of these individuals produced enough additional anti-BSA to reach an ABC-33 end point, and thus the persons in Table II must be considered hyporesponsive to BSA. It is known that sera from one of these subjects (R.F.) had enough anti-BSA to reach an ABC-33 end point eight years before the present experiments, at a time when he was ingesting comparable amounts of BSA. Thus, it would appear that this individual may be in the process of converting from a moderately responsive type of person as presented in Table III to an unresponsive state as represented by the individuals in Table I.

Four of the five persons in Table III responded relatively well to the oral challenge of childhood doses of BSA, indicating that oral administration of antigen can be effective in most adults who are currently producing moderate amounts of antibody. Antibody production was also stimulated by orally administered antigen in Subject B. S. (Table I), who had not ingested BSA for the past ten years. Although the data in Table I would implicate factors other than total daily dose of BSA as the reason for the low incidence of detectable anti-BSA among adults, the data in Table III strongly indicate that the daily dose of BSA is an important variable with regard to the amount of anti-BSA produced once antibody production was underway.

The experiments reported in Tables IV and V were designed to learn if representative subjects from Tables I, II, and III would respond to parenterally injected BSA in a different way from their response to orally ingested BSA. If the manner in which the gastrointestinal tract of the adult catabolized

dietary protein antigens was the major factor that determined the absence of detectable anti-BSA among adults in Table I and the hyporesponsive state observed in Table II, then parenteral injection of BSA to bypass the gastrointestinal tract should have resulted in a marked immune response. However, none of the three individuals tested from Table I responded to 1 mg. (Table IV) and only one (J. M.) had a weak response to the 3 mg. intradermal course of injections (Table V). Neither of the two hyporesponsive individuals (R. F. and B. S.) had significant responses to 1 mg. of BSA intradermally (Table IV). On the other hand, two of the moderately responsive individuals tested from Table III (R. M. R. and D. R.) had modest responses following 1 mg. of intradermal injections (Table IV). Thus it would appear that neither gastrointestinal factors or the relatively low dietary intake of BSA play a major role in determining the low incidence of anti-BSA among adults.

It is of interest that detectable circulating antigen was present in the sera of all four subjects for 21 days following the intradermal injection of 3 mg. of BSA (Table V). This long half-life for BSA injected into humans without detectable anti-BSA is compatible with a previously reported case in which the half-life was 24 days.¹¹ It is also significant that the sera of Subject R. F. had detectable amounts of both antigen and antibody for at least 18 days. This phenomenon had occasionally been observed in rabbits following single injections of milligram amounts of BSA¹² and more recently has been observed in many rabbits following large doses of parenteral BSA during immunological unresponsiveness.¹³

The capacity of a given individual to maintain or reinstate anti-BSA production is a multifaceted phenomenon subject to many variables, such as: (A) immunological capacity to respond to the antigen in question,^{12, 14} (B) exposure of gastrointestinal lymphoid tissue to antigen,^{3, 10, 15} (C) denaturation of antigen due to cooking,⁴ (D) the presence or absence of concomitant symptomatic allergic states associated with reagin to antigens other than BSA,¹⁶ (E) gastric acidity,¹⁰ and (F) the age of the individual.³ The present experiments indicate that in adults where antibody production is underway, another significant variable is the dosage of antigen.

The data presented above suggest that the hyporesponsiveness of most adults to BSA cannot be accounted for by a reduced dietary intake or by changes in the gastrointestinal tract. As a possible explanation for the hyporesponsiveness observed in these experiments, consideration is warranted of a recent proposal by Talmage and Claman¹⁷ that acquired immunologic tolerance may occur following continuous minimal antigenic stimulation. According to this postulate, active antibody synthesis by mature antibody-producing cells is associated with an inhibition of replication of this cell population with reduction in capacity to synthesize the antibodies in question. The circumstances under which the present data were obtained suggest the attractive but unproven possibility that prolonged minimal antigenic stimulation via the oral route may be an important factor that in some way is responsible for the hyporesponsiveness of adults to BSA.

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