

Antibodies in rabbits fed milk and their similarities to antibodies in some human sera

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Four of six adult rabbits drinking skimmed cow's milk produced antibody against BSA and ALA. Anti-BSA was first detected in some sera 14 days after the initiation of milk feedings and anti-ALA was present when first studied on the thirty-fifth day. Three of the four antisera bound more I¹²⁵BSA than I¹²⁵ALA during the entire study. The specificities of the antibodies which bind ALA and BSA in human serum were very similar to the specificities of the respective antibodies in the sera from rabbits drinking milk and in the sera from rabbits immunized with purified BSA or ALA. No shared antigenic groups could be demonstrated between ALA and BSA, HSA or ovalbumin. As measured by immunological methods, commercial skimmed milk contained an average of 0.29 mg. BSA per milliliter and an average of 1.3 mg. ALA per milliliter. Raw beef contained 1.36 mg. extractable BSA per gram and cooked beef contained only 0.08 mg. extractable BSA per gram. Cow's milk is probably the major source of antigenic stimulation for both the anti-BSA and anti-ALA in human serum. Even when compared on a weight-for-weight basis rather than on a mole-for-mole basis, BSA appears to be a generally more effective oral antigen than ALA in both humans and rabbits. As judged by the few parameters studied, the immune response of rabbits drinking milk was similar to the human immune response to milk.

The finding of antibodies which react with the milk proteins in the sera of many persons with chronic respiratory¹⁻⁴ or gastrointestinal illness⁵⁻⁹ has raised the possibility that these antibodies may sometimes cause disease. How frequently these antibodies do cause disease is not known, since similar antibodies have also been found in apparently healthy people.¹⁰⁻¹⁴ In a quantitative study of the incidence and amount of antibody to two of the milk proteins,

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bovine serum albumin (BSA) and alpha laetalbumin (ALA), it was found that the sera of 74 per cent of normal children, 4 and 5 years old, had anti-BSA activity and 41 per cent of the same sera had anti-ALA activity. If an individual made antibodies to both antigens, the serum usually bound more BSA than ALA.^{10, 11} In view of the observation that there is more ALA than BSA in milk,¹⁵ the relatively high levels of anti-BSA suggested that BSA may be a more efficient oral antigen and/or that humans receive additional BSA stimulation from eating beef or beef products. Further, since these antisera reacted with both BSA and ALA, the assumption was made that both of these milk proteins were also *immunizing* antigens. This assumption need not be true. For example, the serum from rabbits immunized with BSA will react with similar antigens from a variety of other mammalian species.¹⁶ Therefore, it is possible that some of the anti-BSA or anti-ALA activity of human sera was the result of immunization by only one of these antigens.

These considerations suggested the present studies to: (1) learn if rabbits fed milk would produce antibodies against both BSA and ALA, and if they did make these antibodies, would they produce more anti-BSA despite the trace amounts of BSA in skim milk; (2) compare the specificities of antibody from rabbits immunized with purified BSA or ALA with the same antibodies present in the sera of humans and rabbits drinking milk; (3) accurately quantitate and compare the amount of BSA and ALA in pasteurized skimmed milk; and (4) estimate the amount of extractable BSA in raw and cooked beef.

MATERIALS AND METHODS

Antigens and protein solutions

Crystallized bovine plasma albumin* was labeled with I^{131} as previously described.¹⁷ The labeled protein was diluted in either 1:100 normal rabbit or human serum in borate buffer, depending upon the source of the antiserum to be studied, and the final concentration of I^{131} -labeled BSA (I^* BSA) was 0.04 γ N per milliliter.

Five times recrystallized alpha laetalbumin, prepared by the method of Aschaffenburg and Drewry¹⁸ and supplied by Dr. S. Saperstein, was labeled with I^{131} and diluted in 1:100 normal serum in borate buffer in the same manner as BSA.

Antibody specificity was studied by measuring the capacity of the following unlabeled protein solutions to inhibit the interaction between I^* BSA or I^{131} -labeled ALA (I^* ALA) and their respective antibodies:

- (a) Crystallized BSA.*
- (b) Crystallized ALA.†
- (c) Crystallized human serum albumin (HSA).‡
- (d) Ovalbumin recrystallized twice following its preparation.§

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§Worthington Biochemical Corporation.

- (e) Fat-free milk (No. 1) containing 3.1 mg. N per milliliter diluted with an equal volume of borate buffer.
- (f) Fat-free milk (No. 2) containing 2.37 mg. N per milliliter diluted with an equal volume of borate buffer.
- (g) Extract of raw ground round steak containing 2.1 mg. N per milliliter prepared by macerating 5.0 gm. of meat in 10 ml. of normal saline, freezing, and thawing the suspension, and removing the solid phase by filtration.
- (h) Extract of cooked ground round steak containing 1.2 mg. N per milliliter prepared in the same manner as the raw beef extract. The meat had been fried until brown throughout prior to extraction.
- (i) Extract of pureed beef* containing 2.5 mg. N per milliliter prepared in the same manner as the raw beef extract.

Oral immunization, rabbits

New Zealand white rabbits weighing between 2.5 and 4.0 kilograms were fed a grain chow diet† and skim milk (No. 1) was substituted for water in the drinking cups for the three-month duration of the experiment. The milk was offered *ad libitum* and the rabbits drank between 100 and 250 ml. per day. These animals were bled before the milk feeding was instituted and then at weekly intervals during the experiment. Serum was separated from the cellular elements and stored at -20° C.

A second group of New Zealand white rabbits was orally immunized to BSA in the same manner as the milk immunized group except that a 0.1 per cent solution of BSA was substituted for drinking water. This group of animals is discussed in greater detail elsewhere.¹⁹

Adjuvant immunizations, rabbits

BSA- and ALA-adjuvant emulsions were prepared in a Sorvall Omnimixer by adding 10 mg. of either antigen in 10 ml. of saline to a previously emulsified mixture of 9 ml. of Bayol F and 1 ml. of Arlael C. Each animal was immunized with a total of 2 ml. of the final emulsion injected in equal parts into each of the four foot pads. The sera from each animal were then collected on the twenty-fifth day.

Human sera

The human sera used in these studies were chosen from a previously reported group of 1,000 sera tested for I*BSA and/or I*ALA binding capacity.^{10, 11} Each of the five sera selected bound more than 0.4γ I¹³¹-labeled antigen. Four of the five individuals were children younger than 10 years of age.

Laboratory procedures

The presence or absence of detectable amounts of anti-BSA in a given serum was determined by mixing 2 ml. of a 1:10 dilution of antiserum with 0.5 ml. of

*Commercial Baby Food.

†Purina W. O.

borate buffer and then adding 0.5 ml. of a solution containing 0.04 γ I*BSA N per milliliter. The 0.5 ml. of borate buffer was included to provide a compartment in which to add unlabeled proteins for the specificity studies described below. After overnight incubation at 4° C., 1 ml. aliquots were mixed with equal volumes of saturated ammonium sulfate to precipitate the I*BSA-antibody complexes. Details of this procedure and the calculations have been described elsewhere.^{11, 20} A serum was considered to have specific antibody activity if it precipitated more than 10 per cent of the I* antigen added. The maximum antigen binding capacities of these antisera were not investigated.

The specificities of the antibodies in the various sera were studied by making two modifications of the above procedure. First, to be certain that the test was not performed in antibody excess, the antisera were diluted in 1:10 normal serum so that a 2 ml. aliquot bound approximately 50 per cent of the I*BSA added. Second, to measure the effect of the various unlabeled proteins on the I*BSA-anti-BSA reaction, 0.5 ml. of a 1 mg. per milliliter solution of each unlabeled protein was substituted for the 0.5 ml. of borate buffer in the above procedure. The unlabeled antigens were incubated with the antisera for 30 minutes prior to the addition of the I*BSA. After further incubation overnight at 4° C., the per cent I*BSA precipitated by ammonium sulfate was determined in the usual manner and compared to the per cent I*BSA precipitated by a control mixture containing the same dilution of antiserum but without unlabeled protein in the borate buffer compartment. A decrease in the per cent I*BSA precipitated indicated that either the unlabeled protein in the borate buffer compartment shared antigenic groups with BSA, or that the unlabeled protein contained some BSA. To discriminate between shared antigenicity and the presence of BSA, the decreases in I*BSA binding caused by the addition of known amounts of unlabeled BSA to the borate buffer compartment were compared with the decreases in I*BSA binding caused by the addition of serial dilutions of unlabeled protein. Under these conditions, an inhibition curve with a slope similar to the slope of the curve obtained with unlabeled BSA indicates the presence of BSA, and an inhibition curve with a decreased slope indicates shared antigenicity. This method has been previously described and discussed in detail elsewhere.^{21, 22}

The concentration of BSA in skim milk was obtained from inhibition curves by multiplying the reciprocal of the dilution of skim milk necessary to prevent 50 per cent of the I*BSA binding by the concentration of unlabeled BSA required to cause the same degree of inhibition. The BSA concentrations of the beef extracts were determined in a similar manner.

To determine the amount of anti-BSA in a given serum, an antigen-binding capacity (ABC-33) with the use of 0.02 γ I*BSA N was determined by procedures described previously,²⁰ except that the precipitates were washed with 3 ml. of 50 per cent saturated ammonium sulfate in borate buffer prior to counting.

The maximum differences between normal serum and serum containing anti-ALA were demonstrated by fractionating the I*ALA-antibody complexes with 40 per cent rather than 50 per cent saturated ammonium sulfate. Therefore, all experimental procedures employed in the study of I*ALA-anti-ALA com-

plexes were the same as for I*BSA-anti-BSA complexes except that they were fractionated in 40 per cent saturated ammonium sulfate.

The precipitin reaction was studied with the use of a microimmunodiffusion technique. A 1 mm. layer of Ionagar No. 2* was applied to microscope slides, and wells were cut with a template.† After the wells were filled with serum or antigen, the slides were incubated in a humidified chamber at 4° C. for 72 hours, washed with normal saline for 48 hours, dried, and the precipitin bands stained with amido black.

RESULTS

BSA and ALA binding in the sera of rabbits drinking milk

Six rabbits were fed skim milk for three months and the sera studied for anti-BSA and anti-ALA. The results of this experiment, summarized in Table I, demonstrate: (1) circulating antibody to both BSA and ALA was detected in four of the six rabbits, the remaining two animals were not immunized by either antigen; (2) anti-BSA activity was first detected between the fourteenth and twenty-second days; (3) the concentration of both antibodies increased during the first two months and then varied slightly from week to week during the third month; (4) three rabbits bound significantly more BSA than ALA; and (5) one rabbit bound more ALA than BSA.

A comparison of the specificities of BSA and ALA binding found in human and rabbit antisera

Anti-ALA specificity. The specificity of the I*ALA binding by sera from humans and from rabbits drinking milk was compared with the specificity of the I*ALA binding by sera from rabbits immunized with purified ALA or BSA. Table II demonstrates: (1) the I*ALA binding of the rabbit and human sera was completely inhibited by a 1:5 dilution of skim milk or a solution containing 0.5 mg. of unlabeled ALA; (2) the I*ALA binding was not inhibited by the prior addition of solutions containing 0.5 mg. of BSA or HSA or ovalbumin, suggesting that these proteins do not share antigenic groups with ALA; (3) sera from rabbits immunized with BSA did not bind I*ALA; and (4) the specificity of the anti-ALA activity in the human sera and in the sera from rabbits drinking milk was identical to the specificity of the antibody from rabbits immunized with purified ALA.

Anti-BSA specificity. The specificity of the I*BSA binding by sera from humans and from rabbits drinking milk was compared with the specificity of the I*BSA binding by sera from rabbits immunized with purified BSA or ALA. Table III demonstrates: (1) the prior addition of 0.5 mg. of unlabeled BSA or a 1:5 dilution of skim milk completely prevented the subsequent binding of I*BSA by all sera studied; (2) the prior addition of 0.5 mg. of ovalbumin, a protein that does not share antigenic groups with BSA,²³ did not affect I*BSA binding by any serum studied; (3) HSA, a protein known to share approxi-

*Consolidated Laboratories, Inc.

†LKB Products, Sweden.

Table I. BSA and ALA binding in the sera of 6 rabbits drinking milk

Animal	Antibody	Day antibody first detected	ABC-33 at 0.02 γ I*antigen N per milliliter						Average ABC-33 (between days 56 and 84)
			Day 35	Day 49	Day 56	Day 61	Day 71	Day 84	
R-1	Anti-BSA	14	1.1	3.3	8.7	10.8	9.2	9.2	9.5
	Anti-ALA	NS*	†	0.3	0.5	0.7	0.4	0.5	0.5
R-2	Anti-BSA	22	†	0.4	0.4	0.4	Died		0.4
	Anti-ALA	NS*	†	0.6	1.2	0.9	Died		1.1
R-3	Anti-BSA	†	—	—	—	—	—	—	—
	Anti-ALA	†	—	—	—	—	—	—	—
R-4	Anti-BSA	14	2.5	4.4	8.7	8.3	7.9	6.4	7.8
	Anti-ALA	NS*	†	0.3	0.5	0.3	0.3	0.2	0.3
R-5	Anti-BSA	22	1.7	2.6	5.8	7.8	9.5	11.6	8.7
	Anti-ALA	NS*	†	†	†	†	†	†	†
R-6	Anti-BSA	†	—	—	—	—	—	—	—
	Anti-ALA	†	—	—	—	—	—	—	—

*Not studied until Day 35.

†The sera contained antibody but in insufficient amounts to bind 33 per cent of the 0.02 γ I*antigen N added.

‡Antibody not detected during entire study period.

Table II. The effect of various unlabeled proteins on the I*ALA-binding capacity of antisera from humans and rabbits

Sera	Immunizing antigen	Per cent of 0.0066 γ I*ALA N precipitated					
		Control*	0.5 mg. BSA	1:5 dilu- tion of skim milk	0.5 mg. ALA	0.5 mg. oval- bumin	0.5 mg. HSA
Human 4	Milk (oral)	46	47	3	0	48	—
Human 5	Milk (oral)	25	24	0	0	26	—
Rabbit 4	Milk (oral)	41	40	5	1	41	41
Rabbit 1	Milk (oral)	40	40	3	2	40	40
Rabbit 8	ALA (Subcutaneous)†	45	47	7	3	46	44
Rabbit 7	ALA (Subcutaneous)†	42	41	6	2	42	40
Rabbit 12	BSA (oral)	0	0	0	0	0	0
Rabbit 9‡	BSA (Subcutaneous)†	0	0	0	0	0	0

Two milliliters test serum dilution + 0.5 ml. of borate buffer with unlabeled proteins added + 0.5 ml. of I*ALA.

*Borate buffer without unlabeled proteins in solution.

†Subcutaneously in incomplete Freund's adjuvant.

‡ABC-33 @ 0.02 γ I*BSA N = 1.32 γ /ml.

mately 15 per cent of the antigenic groups of BSA,¹⁶ decreased the capacity of rabbit sera to bind I*BSA by approximately 23 per cent; (4) HSA did not affect the I*BSA binding by human sera; (5) approximately 75 per cent of the I*BSA binding was inhibited by 0.5 mg. ALA; (6) the specificity of the I*BSA binding of sera from rabbits immunized with purified BSA was identical to the specificity of the I*BSA binding in sera from rabbits drinking milk; and (7) the specificity of the I*BSA binding of the serum from a rabbit immunized with 1 mg. of ALA was similar to the I*BSA binding of sera from animals immunized

Table III. *The effect of various unlabeled proteins on the I*BSA binding capacity of antisera from humans and rabbits*

Sera	Immunizing antigen	Per cent of 0.0066γI*BSA N precipitated					
		Control*	0.5 mg. BSA	1:5 dilu- tion of skim milk	0.5 mg. ALA	0.5 mg. oval- bumin	0.5 mg. HSA
Human 1	?Milk (oral)	23	0	0	7	22	21
Human 2	?Milk (oral)	51	0	2	16	53	—
Human 3	?Milk (oral)	58	0	0	9	56	—
Rabbit 4	Milk (oral)	67	0	0	10	67	46
Rabbit 10	Milk (oral)	57	0	0	11	56	47
Rabbit 11	BSA (oral)	64	0	3	9	64	50
Rabbit 12	BSA (oral)	55	0	3	13	56	48
Rabbit 8	ALA (Subcutaneous)†	45	0	5	11	45	30

Two milliliters test serum dilution + 0.5 ml. of borate buffer with unlabeled proteins added + 0.5 ml. of I*BSA → 1 ml. aliquot + 1 ml. SAS.

*Borate buffer without unlabeled protein in solution.

†Subcutaneously with 1 mg. of ALA in incomplete Freund's adjuvant.

with crystalline BSA. The serum from a second rabbit immunized with ALA also bound I*BSA, but not in sufficient amounts to permit investigation of specificity by the techniques employed in these experiments.

As noted above, ALA inhibited I*BSA binding (Table III) but BSA did not inhibit I*ALA binding (Table II). Further, antisera from rabbits immunized with BSA did not bind I*ALA (Table II), but antisera from rabbits immunized with ALA bound I*BSA with the specificity of antibody produced against purified BSA (Table III). These observations suggest the possibility that the highly purified ALA used in these experiments contained trace amounts of BSA. Therefore, the capacity of ALA to inhibit I*BSA binding was studied in greater detail to learn if this inhibition was similar to the inhibition caused by unlabeled BSA, or similar to the inhibition caused by a protein such as HSA, that only shares some antigenic groups with BSA. Increasing amounts of unlabeled BSA, HSA, or ALA were added to three different rabbit anti-BSA sera, three different rabbit antimilk sera, and one rabbit anti-ALA serum which had been previously diluted so that each bound approximately 50 per cent of the I*BSA to be added. Each mixture was then studied in the usual manner and the decrease in the amount of I*BSA in the precipitate determined.

The inhibition curves obtained with rabbit anti-BSA sera were identical to those obtained with rabbit antimilk and anti-ALA sera, and a representative set of inhibition curves is shown in Fig. 1. These demonstrate: (1) the prior addition of unlabeled BSA completely inhibited the binding of I*BSA when the concentration of unlabeled BSA was greater than 16 times the concentration of the I*BSA added; (2) when progressively less unlabeled BSA was added, the inhibition decreased with a slope which was characteristic for all sera studied; (3) the addition of increasing amounts of HSA resulted in an inhibition curve with a flat slope, which was different from the slope of the unlabeled BSA inhibition curve; and (4) the slope of the curve obtained with increasing amounts of ALA was closely related to the slope of the BSA inhibition curve. The near

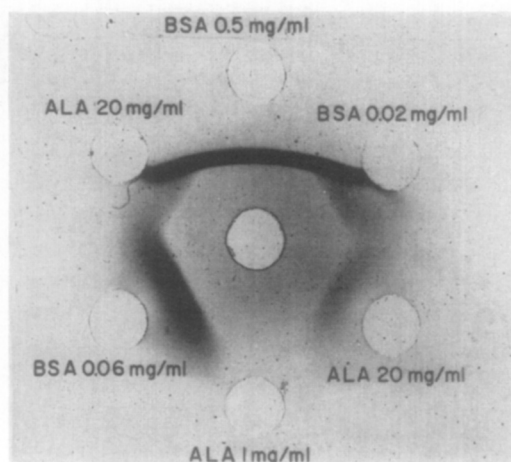
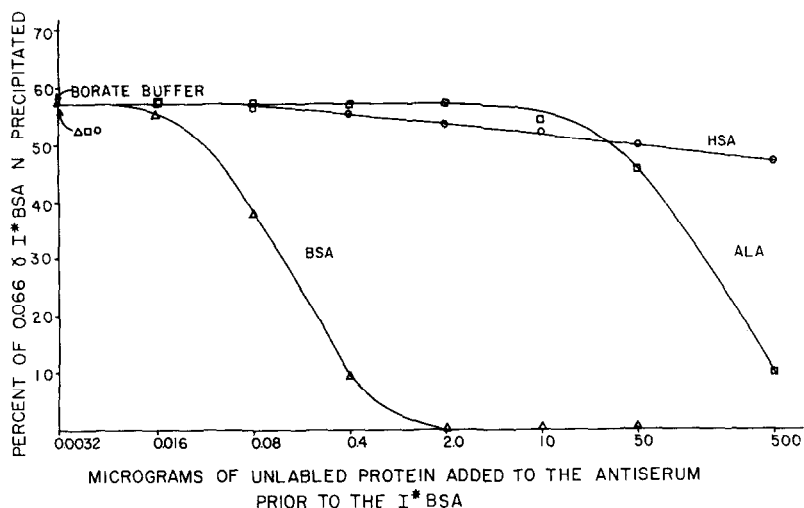


Fig. 1

The inhibition of ^{125}I -BSA binding by rabbit anti-BSA following the addition of increasing concentrations of unlabeled BSA, ALA, and HSA to the antiserum prior to the addition of the ^{125}I -BSA.



which developed 48 hours after anti-BSA had been placed in the central well and dilutions of both BSA and ALA were placed in the peripheral wells. The heavy precipitin line between the 0.5 mg. BSA per milliliter well and the central well coalesced as a line of identity with the rather diffuse precipitin bands that developed between the central well and the 20 mg. ALA per milliliter well on the left side, and the 0.02 mg. BSA per milliliter well on the right. The latter diffuse line also coalesced as a line of identity with a similar precipitin band which formed between a second 20 mg. ALA per milliliter well and the central well. A similar coalescence of bands was associated with the 20 mg. ALA per milliliter and 0.06 mg. BSA per milliliter wells. No reaction was detected between the 1 mg. ALA per milliliter well and the central well. Although it was not possible to obtain sharp precipitin bands between the anti-BSA in the central well and the 20 mg. ALA per milliliter well, the 0.02 mg. BSA per milliliter well or the 0.06 mg. BSA per milliliter well, the similarity and complete coalescence of the reactions obtained with the dilute BSA solutions and the concentrated ALA solution further demonstrate that the ALA contains trace amounts of BSA.

The antigenic concentration of BSA and ALA in cow's milk and beef

The concentration of BSA and ALA in skim milk was determined in a manner similar to that used to determine the amount of BSA in ALA. The top of Table IV shows that the results obtained by this method approximate the results obtained previously by other methods,^{15, 24} and that the concentration of ALA is four times as great as the concentration of BSA in skim milk.

The extractable BSA content of raw and cooked beef was also determined and the results are presented in the bottom of Table IV. The raw ground round steak contained significant amounts of BSA but 95 per cent of the antigenicity of this BSA was destroyed by routine cooking. The Commercial Baby Food had no detectable BSA activity.

DISCUSSION

The present experiments demonstrate that the addition of skim milk to the diet of adult rabbits can immunize rabbits against BSA and ALA. Three of

Table IV. *BSA and ALA contents of foods of bovine origin as determined by the immune inhibition technique*

<i>Food</i>	<i>Number of determinations</i>	<i>Average concentration</i>	<i>Range</i>	<i>Previously reported concentrations</i>
Skim milk BSA	5	0.29 mg. BSA/ml.	0.24-0.34 mg./ml.	0.36 mg./ml. ¹⁵
Skim milk ALA	4	1.3 mg. ALA/ml.	1.0-1.5 mg./ml.	1.4-3.2 mg./ml. ²⁴
Raw ground round steak	2	1.36 mg. extractable BSA/Gm. of beef	1.28-1.44 mg./Gm.	
Cooked ground round steak	2	0.08 mg. extractable BSA/Gm. of beef	0.08-0.08 mg./Gm.	
Commercial pureed beef (baby food)	2	0.00 mg. extractable BSA/Gm. of beef	0.00-0.00 mg./Gm.	

the four rabbits immunized against BSA and ALA remained well while drinking milk for 90 days. The fourth rabbit died after drinking skim milk for 63 days, and this serum was different from the sera of the other three immunized rabbits in that it bound more I*ALA than I*BSA. No evidence was obtained that milk ingestion was related to this one death, and it will require a carefully controlled study involving many animals to learn if drinking milk can lead to death in some rabbits.

Despite species differences, the immune response to ALA and BSA of rabbits drinking milk was similar to what is known about the human immune response to milk in the following ways: (1) these antibodies appeared in the circulation of rabbits and humans 14 to 22 days after the initiation of cow's milk feedings (Table I)²⁵; (2) these antibodies occurred in apparently healthy rabbits and humans drinking milk¹¹⁻¹⁴; (3) the specificities of both the anti-ALA and anti-BSA in the sera from rabbits drinking milk were identical to the specificities of the respective antibody activities found in human sera (Tables II and III). These specificities were also identical to the specificities of the anti-ALA or anti-BSA from rabbits immunized with the purified proteins (Tables II and III). The only difference noted in the specificities was the lack of effect of HSA on the I*BSA binding by human sera, and this exception was to be expected; and (4) if a given serum bound both antigens, it usually, but not always, bound more BSA than ALA. This similarity is demonstrated in Fig. 3 in which the BSA and ALA binding by sera from the six rabbits drinking milk is compared with similar human data previously reported.¹¹

The inhibition studies also demonstrated that ALA itself does not share antigenic groups with BSA, HSA, or ovalbumin and that the highly purified

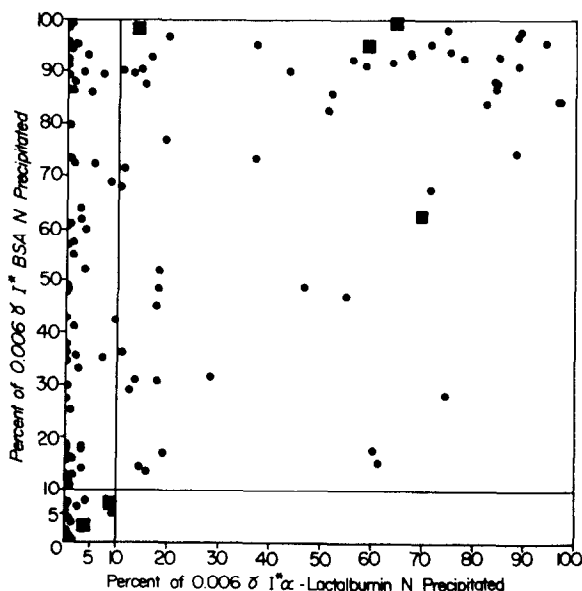


Fig. 3

A comparison of the I*BSA and I*ALA binding by sera from 153 humans and from the 6 rabbits drinking milk. Dots, human sera; squares, rabbit sera.

ALA used for these studies contained trace amounts of BSA. The presence of BSA in the ALA was confirmed by a microimmunodiffusion technique (Fig. 2). The biological activity and experimental difficulties that can be caused by 0.19 per cent BSA in the ALA were demonstrated by the capacity of 0.5 mg. of this ALA to inhibit I*BSA binding significantly and the capacity of 1.0 mg. in incomplete Freund's adjuvant to immunize a rabbit against BSA. The 1.9 γ BSA per milliliter in the aqueous phase of the incomplete Freund's adjuvant was slightly above the previously reported minimal antigenic dose of BSA in rabbits.²⁶

Skim milk, the only source of ALA and BSA for the rabbits, contained four times more ALA than BSA by weight. Since the molecular weight of ALA is 16,000²⁷ as compared to 60,000 for BSA, the rabbits were exposed to approximately 16 molecules of ALA for every molecule of BSA. When comparing the amount of antibody produced by the rabbit against BSA and ALA as measured by a primary binding test, the smaller molecular weight of ALA must also be considered. Assuming that the anti-BSA and anti-ALA were both bivalent antibodies with approximately equal affinity for their respective antigens, the concentration of anti-ALA molecules would have to be approximately four times greater than the concentration of anti-BSA molecules, were a given serum to bind equal amounts of I*ALA and I*BSA. Taking these considerations into account, three of the four rabbits immunized against BSA and ALA made more anti-BSA than anti-ALA. Thus, BSA appears to be a more effective antigen than ALA when fed to rabbits, perhaps, partly because of the lower molecular weight of the ALA.

The similarity in the relative capacities of human and rabbit serum to bind I*BSA and I*ALA (Fig. 3) suggests that either BSA is also a better oral antigen for humans, or that the generally higher anti-BSA levels found in humans were the result of antigenic stimulation by BSA in beef products other than milk. In an attempt to answer this question, the amount of beef that would have to be consumed to equalize the daily dose of BSA and ALA was calculated. Because milk and meat intake varies in different age groups, these calculations were made for a 5-year-old child, an age known to have a high incidence of both antibodies.¹¹ Assuming that the child drinks the minimal milk recommendation of $\frac{3}{4}$ quart per day,²⁸ 21 grams of milk protein²⁴ containing 923 mg. of ALA and 206 mg. of BSA (Table IV) would be consumed. Raw beef contained approximately 1.36 mg. extractable BSA per gram, 95 per cent of which was destroyed by routine cooking (Table IV). To supplement the BSA content of milk so that the daily intake of BSA would equal, by weight, the 923 mg. daily intake of ALA, the child would have to eat approximately 1 pound of raw or 24 pounds of cooked ground round steak. Although the amount of BSA in the meat extract could be quantitated with less than 5 per cent error, it is not known how much of the actual BSA content in the beef was extracted by the procedures employed. Since the average daily intake of animal protein by a 5-year-old child is only 0.09 pound,²⁹ the extraction procedures could have been less than 10 per cent efficient and these data would still indicate that BSA is a more efficient oral antigen than ALA in the human as well as in the rabbit.

Although beef may indeed provide an additional source of BSA in the human diet, especially in adults who eat rare meat, it appears that cow's milk is probably the major source of antigenic stimulation for both the anti-BSA and anti-ALA found in human sera.

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REFERENCES

1. Heiner, D. C., Sears, J. W., and Kniker, W. T.: Multiple Precipitins to Cow's Milk in Chronic Respiratory Disease, *Am. J. Dis. Child.* **103**: 634, 1962.
2. Heiner, D. C., Wilson, J. F., and Lahey, M. E.: Sensitivity to Cow's Milk, *J. A. M. A.* **189**: 563, 1964.
3. Holland, N. H., Hong, R., Davis, N. C., and West, C. D.: Significance of Precipitating Antibodies to Milk Proteins in the Serum of Infants and Children, *J. Pediat.* **61**: 181, 1962.
4. Nelson, T. L.: Spontaneously Occurring Milk Antibodies in Mongoloids, *Am. J. Dis. Child.* **108**: 494, 1964.
5. Taylor, K. B., and Truelove, S. C.: Circulating Antibodies to Milk Proteins and Ulcerative Colitis, *Brit. M. J.* **2**: 924, 1961.
6. Taylor, K. B., Truelove, S. C., Thomson, D. L., and Wright, R.: An Immunologic Study of Coeliac Disease and Idiopathic Steatorrhea, *Brit. M. J.* **2**: 1727, 1961.
7. Sewell, P., Cooke, W. T., Cox, E. V., and Meynell, M. J.: Milk Intolerance in Gastrointestinal Disorders, *Lancet* **2**: 1132, 1963.
8. Kivel, R. M., Kearns, D. H., and Liebowitz, D.: Significance of Antibodies to Dietary Proteins in the Serums of Patients With Nontropical Sprue, *New England J. Med.* **271**: 769, 1964.
9. Alarcon-Segovia, D., Herskovic, T., Wakim, K. G., Green, P. A., and Scudamore, H. H.: Presence of Circulating Antibodies to Gluten and Milk Fractions in Patients With Nontropical Sprue, *Am. J. Med.* **36**: 485, 1964.
10. Rothberg, R. M., and Farr, R. S.: Incidence and Amount of Antibovine Serum Albumin and Anti-alpha-lactalbumin in Serum of Children and Adults, *Clin. Res.* **10**: 219, 1962. Abst.
11. Rothberg, R. M., and Farr, R. S.: Antibovine Serum Albumin and Anti-alpha-lactalbumin in the Serum of Children and Adults, *Pediatrics* **35**: 571, 1965.
12. Gunther, M., Aschaffenburg, R., Mathews, R. H., Parrish, W. E., and Coombs, R. R. A.: The Levels of Antibody to the Proteins of Cow's Milk in the Serum of Normal Human Infants, *Immunology* **3**: 296, 1960.
13. Peterson, R. D. A., and Good, R. A.: Antibodies to Cow's Milk Proteins: Their Presence and Significance, *Pediatrics* **31**: 209, 1963.
14. Saperstein, S., Anderson, Jr., D. W., Goldman, A. S., and Kniker, W. T.: Milk Allergy. III. Immunological Studies With Sera From Allergic and Normal Children, *Pediatrics* **32**: 580, 1963.
15. McMeekin, T. L.: The milk proteins, in *The Proteins*, 1954, Academic Press, Inc., vol. 2, chap. 16, p. 391.
16. Weigle, W. O.: Immunochemical Properties of the Cross-reactions Between Anti-BSA and Heterologous Albumins, *J. Immunol.* **87**: 599, 1961.
17. Talmage, D. W., Baker, H. R., and Akeson, W.: The Separation and Analysis of Labeled Antibodies, *J. Infect. Dis.* **94**: 199, 1954.
18. Aschaffenburg, R., and Drewry, J.: Improved Method for the Preparation of Crystalline Beta-Lactoglobulin and Alpha-Lactalbumin From Cow's Milk, *Biochem. J.* **65**: 273, 1957.
19. Farr, R. S., Dickinson, W., and Smith, K.: Quantitative Aspects of Oral Immunization Against Bovine Serum Albumin, *Fed. Proc.* **19**: 199, 1960. (Abst.)
20. Farr, R. S.: A Quantitative Immunochemical Measure of the Primary Interaction Between I*BSA and Antibody, *J. Infect. Dis.* **103**: 239, 1958.
21. Linscott, W. D.: Contamination of Commercial Rabbit Albumin Preparation by Bovine Albumin, *Science* **142**: 1170, 1963.
22. Lidd, D., and Farr, R. S.: Similarities Between Antigens Derived From Ragweed Pollen and From Other Botanical Sources, *J. ALLERGY* **34**: 48, 1963.
23. Weigle, W. O., and McConahey, P. J.: Serologic Cross-reactions Between Bovine Serum Albumin and Anti-ovalbumin, *J. Immunol.* **88**: 121, 1962.
24. Altman, P. L., and Dittman, D. S.: Blood and Other Body Fluids, *Fed. Am. Soc. Exper. Biol.*, p. 460, 1961.
25. Lippard, V. W., Schloss, O. M., and Johnson, P. A.: Immune Reaction Induced in

- Infants by Intestinal Absorption of Incompletely Digested Cow's Milk Protein, *Am. J. Dis. Child.* **51**: 562, 1936.
26. Farr, R. S., and Dixon, F. J.: The Effect of Antigen Concentration on the Initiation of Detectable Antibody Synthesis in Rabbits, *J. Immunol.* **85**: 250, 1960.
 27. Gordon, W. G., and Semmett, W. F.: Isolation of Crystalline Alpha-lactalbumin From Milk, *J. Am. Chem. Soc.* **75**: 328, 1953.
 28. Nelson, W. E.: *Textbook of Pediatrics*, ed. 8, Philadelphia, 1964, W. B. Saunders Company, p. 123.
 29. Jackson, R. L., Hanna, F. M., and Flynn, M. A.: Nutritional Requirements of Infants and Children, *Pediat. Clinics N. Am.* **9**: 879, 1962.