Some Immunological Studies with Heat-Denatured Bovine Serum Albumin and Its Peptic, Tryptic, and Chymotryptic Peptides[1] (34712)

B. R. Das,[2] P. H. Maurer, and C. T. Liu[3]

Department of Biochemistry, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

It was shown in a previous study (1) that the controlled hydrolysis of heat-denatured bovine serum albumin (HDBSA) with crystalline pepsin, trypsin, and chymotrypsin resulted in the formation of peptide fractions which no longer precipitated with specific rabbit anti-HDBSA antiserum. However, these peptides inhibited the precipitation reaction of HDBSA and anti-HDBSA and elicited passive cutaneous anaphylaxis (PCA) in guinea pigs injected with anti-HDBSA antiserum. The degree of inhibition and the PCA activities of these peptides depended on the enzyme used and on the molecular size of peptides formed.

The present studies were extended to include the effects of HDBSA and its enzyme digests on other immunological phenomena, *i.e.*, immediate and delayed hypersensitivity reactions in guinea pigs and immune tolerance in rabbits. A study of this nature involving chemical and immunological properties of a protein and its peptide fractions obtained by action of proteolytic enzymes of different specificities has not been reported before, to our knowledge. The investigations of Gell and Benacerraf (2), and Nelson and Boyden (3) have dealt with delayed hypersensitivity reactions in guinea pigs with native and denatured proteins but not with enzyme digests of these proteins. Similarly, the reports of Weigle (4, 5) and Smith (6) have dealt mainly with the induction and abrogation of tolerance to BSA by heterologous albumins, as well as chemically and physically modified BSA.

Materials and Methods. Antigens. HDBSA was prepared by heating a 1% aqueous solution of BSA at pH 7.5 for 1 hr in a boiling water bath and then rapidly cooling the solution. HDBSA was stored as a lyophilized preparation. The dialyzable and non dialyzable peptide fractions were prepared by the action of crystalline pepsin, trypsin, and chymotrypsin on HDBSA according to the methods described in the previous study (1). Briefly, pepsin hydrolysis was done using an enzyme: substrate ratio of 1:10 (w/w) for 1 hr. Tryptic and chymotryptic hydrolyses employed enzyme: substrate ratio of 1:100 for 18–24 hr.

Studies in guinea pigs. Passive anaphylaxis. Hartley strain guinea pigs (250–300 g) were given intraperitoneal injections of 1 ml of rabbit anti-HDBSA (70 μg of N antibody/ml). Forty-eight hr later, they were challenged by an intravenous injection (1 or 10 mg in 1 ml of 0.15 *M* NaCl) to determine whether the previously injected peptides inhibited subsequent anaphylaxis.

Sensitization of guinea pigs for delayed hypersensitivity. Albino guinea pigs (300–400 g) were injected into hind foot pads (0.1 ml/foot pad) with HDBSA or BSA (100 μg/ml or 1000 μg/ml emulsified with equal volumes of Freund's complete adjuvant). A total of 10 and 100 μg antigen was injected. The guinea pigs were skin tested 9 and 21 days after sensitization. The skin of the flanks was shaved 3–4 hr before intradermal injection of the test antigens (10 μg/0.1 ml of 0.15 *M* NaCl). Ten injections (BSA,

[1] Supported by Grants AI-07825 and TIAI-334 from the National Institutes of Allergy and Infectious Diseases.

[2] Present Address: Department of Biochemistry, University of Bristol Medical School, Bristol, England.

[3] Present Address: Public Health Research Institute, Inc., New York, New York.

HDBSA, nondialyzable and dialyzable peptides), five on either side of the flanks, were given to each animal. Skin reactions were measured at 4, 6, and 24 hr. The area (mm diameter) of skin erythema and induration was recorded.

Studies in rabbits. Induction of tolerance. Newborn New Zealand albino rabbits were grouped on a littermate basis and injected as follows with various antigens (100 mg of antigen/ml): HDBSA, nondialyzable tryptic peptides, dialyzable tryptic peptides, and BSA. A control group received injections of 0.15 *M* NaCl. Two intraperitoneal injections were given on day 1 (0.5 ml) and day 4 (0.25 ml) followed by an intravenous injection on day 45 (0.25 ml). The animals were then allowed to rest for 30 days.

Immunization. Hind foot pads of rabbits were injected (0.1 ml/foot pad) on day 75 with an emulsion of HDBSA or BSA in Freund's complete adjuvant. Each animal received a total of 8 mg of protein or digest. They were bled on days 7, 14, and 21 after the injection. Sera from each rabbit was pooled and absorbed with an unrelated antigen–antibody precipitate to remove complement components. This was termed course I serum. Immunization was repeated two more times as above to obtain antisera from courses II and III. The interval between the injection of each course was about 1 month. Antibody levels in the sera were estimated by the method of Heidelberger and Kendall (7) as described before (1).

Results. Passive anaphylaxis studies. Results on the effect of HDBSA and its enzyme digests, on the production or inhibition of immediate anaphylactic reactions in guinea pigs sensitized passively with injections of rabbit anti-HDBSA antiserum, are presented in Table I. The reaction noted with the 3 guinea pigs in each group were the same. Challenge with 1 mg of HDBSA caused fatal anaphylactic shock. Challenge with the nondialyzable tryptic peptides, and to a less extent the peptic peptides, caused mild shock in animals given 1–5-mg quantities. When 1 mg of HDBSA was subsequently injected intravenously 20 min later, the guinea pigs did not exhibit symptoms of shock. Challenge with 1–5 mg of chymotrypic nondialyzable peptides of HDBSA resulted in fatal anaphylatic shock.

TABLE I. Effect of HDBSA and Enzyme Digests of HDBSA on Passive Anaphylaxis in Guinea Pigs Sensitized with Rabbit Anti-HDBSA Antisera.[a]

Test substance[b]	Amount injected (mg)	Symptoms of anaphylaxis[c] Immediate	After 20 min following HDBSA (1 mg in ml) injection
HDBSA	1	4+	
NDC	1	4+	
	5	4+	
NDT	1	3+	0
	5	3+	0
NDP	1	2+	0
	5	2+	0
ND C/T	1	3+	0
	5	3+	0
DC	1	2+	4+
	10	4+	
DT	1	+	4+
	10	+	4+
DP	1	+	4+
	10	+	4+
D C/T	10	0	4+

[a] 70 μg of N rabbit anti-HDBSA antisera was used for passively sensitizing guinea pigs.

[b] HDBSA = Heat denatured bovine serum albumin; D = dialyzable peptides; ND = nondialyzable peptides; C = chymotrypsin; T = trypsin; P = pepsin; C/T = chymotrypsin digestion of tryptic nondialyzable peptides.

[c] Symbols for anaphylaxis: 0 = negative reaction; + = questionable reaction; 2+ = mild anaphylaxis, no death; 3+ = severe anaphylaxis, without death; 4+ = anaphylactic shock and death.

Challenge with the tryptic and peptide dialyzable peptides neither caused significant shock nor inhibited the subsequent anaphylaxis caused by challenge with HDBSA. However, injection of the chymotryptic dialyzable peptides elicited significant symptoms of shock and caused death of animals when injected at the 10-mg level.

Immediate and delayed hypersensitivity studies. In Tables II and III, results are

TABLE II. Effect of BSA, HDBSA, and Enzyme Digests of HDBSA on Arthus (6 hr) and Delayed Hypersensitivity (24 hr) Reactions in Guinea Pigs Sensitized with HDBSA (10 μg).

Guinea pig no.	Day of testing	Diameter (mm) of skin erythema and induration at 6 and 24 hr after intradermal injection of test substances (10 μg)[a]																			
		HDBSA		BSA		NDC		NDT		NDP		ND C/T		DC		DT		DP		D C/T	
		6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24
1	9	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5
	21	8	9	8	10	8	0	12	0	10	0	15	10	10	6	10	0	5	0	10	0
2	9	0	10	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	21	14	18	13	15	12	10	15	12	14	0	13	14	8	9	9	6	9	8	6	6
3	9	11	16	10	15	8	10	10	10	0	10	5	10	8	8	0	9	0	5	5	0
	21	15	15	15	13	10	8	15	15	10	8	15	14	10	0	10	6	10	0	10	6
4	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	21	9	7	10	12	10	10	9	0	9	10	9	10	0	8	0	0	0	0	0	0
5	9	10	14	6	15	8	10	8	0	6	8	0	0	7	0	4	0	0	5	0	0
	21	15	10	15	12	10	5	10	0	10	5	15	10	10	6	5	0	0	0	6	0

[a] BSA = bovine serum albumin; HDBSA = heat-denatured bovine serum albumin; D = dialyzable peptides; ND = nondialyzable peptides; C = chymotrypsin; T = trypsin; P = pepsin; C/T = chymotrypsin digestion of tryptic nondialyzable peptides.

TABLE III. Effect of BSA, HDBSA, and Enzyme Digests of HDBSA on Arthus (6 hr) and Delayed Hypersensitivity (24 hr) Reactions in Guinea Pigs Sensitized with BSA (10 μg).

Guinea pig no.	Day of testing	Diameter (mm) of skin erythema and induration at 6 and 24 hr after intradermal injection of test substances (10 μg)[a]																			
		HDBSA		BSA		NDC		NDT		NDP		ND C/T		DC		DT		DP		D C/T	
		6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24
1	9	10	10	8	11	7	7	10	6	8	8	10	8	7	9	8	4	7	6	0	6
	21	14	12	16	12	10	0	9	0	10	0	11	10	9	0	8	0	9	0	6	0
2	9	9	11	10	10	10	10	5	10	5	6	0	0	0	0	4	5	0	0	0	5
	21	0	0	10	10	5	5	0	0	5	5	12	0	5	5	0	0	5	5	0	0
3	9	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	21	10	10	13	10	10	0	7	5	5	0	14	8	5	0	0	0	5	0	10	3
4	9	12	20	12	20	10	11	10	12	11	0	10	13	0	6	10	12	5	4	7	0
	21	15	15	13	14	10	0	13	10	10	0	12	10	10	0	13	10	10	0	0	0
5	9	8	8	5	13	5	10	8	0	5	8	5	0	5	7	8	0	5	0	5	0
	21	15	15	15	14	15	8	15	10	10	8	12	10	10	—	15	10	10	5	12	5

[a] BSA = bovine serum albumin; HDBSA = heat-denatured bovine serum albumin; D = dialyzable peptides; ND = nondialyzable peptides; C = chymotrypsin; T = trypsin; P = pepsin; C/T = chymotrypsin digestion of tryptic nondialyzable peptides; — = not determined.

given on the effect of BSA, HDBSA, and its various peptide fractions on elicitation of Arthus and delayed hypersensitivity reactions in guinea pigs. Guinea pigs sensitized with HDBSA (10 μg) (Table II) showed the following reactions when tested with various peptide fractions of HDBSA and unaltered BSA: At day 9, $\frac{3}{5}$ animals in each group of guinea pigs injected with HDBSA and BSA showed erythema and induration at 4–6 hr which continued to increase in intensity over the next 24 hr. At 24 hr, one additional animal exhibited a good delayed reaction with both proteins. Neither the dialyzable nor the nondialyzable peptides elicited any reactions at 4 hr. However, reactions began to appear at 6 hr. Two of five animals injected with *non dialyzable* peptides developed moderate delayed reactions (24-hr readings). The chymotryptic peptides (NDC) were most effective followed by peptides of pepsin (NDP), trypsin (NDT) and "simultaneous chymotryptic-tryptic reaction" (NDC/T), only one reaction. None of the dialyzable peptides elicited delayed reactions of any significance.

At day 21, 4–6-hr Arthus reactions were noted in all animals injected with HDBSA and BSA. The nondialyzable peptides caused Arthus reactions in 5/5 animals in each group and delayed reactions were observed in 2–5 animals of each group. The "chymotryptic-tryptic" peptides were most active and the peptic peptides the least active. The dialyzable peptides elicited Arthus reactions in only 2–4/6 animals. In general, peptic peptides were least active, whereas, chymotryptic and tryptic peptides behaved in a similar way. Similar results were obtained when the guinea pigs were sensitized with 100 μg of HDBSA.

Guinea pigs sensitized with BSA (10 μg) (Table III) reacted as follows to intradermal injections of BSA, HDBSA, and peptides of HDBSA: At day 9, no reactions were seen at 4 hr. However, by 6 hr, definite reactions began to appear and by 24 hr 4/5 animals challenged with BSA or HDBSA exhibited delayed reactions of more intense erythema. Of the nondialyzable peptides of HDBSA, the chymotryptic peptides were most active (4 reactions) and the peptic peptides were least active (2 reactions) save for 1 reaction with the tryptic peptide, none of the groups injected with the dialyzable peptides elicited significant delayed reactions.

At day 21, 5/5 animals in the group injected with BSA and 4/5 animals in the HDBSA group showed both good Arthus and delayed reactions. The nondialyzable peptides elicited some good Arthus reactions followed by moderate delayed reactions. The most significant reactions (3/5) occurred in the group challenged with the "chymotryptic-tryptic" peptides and the least reactions occurred with the peptic peptides (1/5). The Arthus reactions elicited by the dialyzable peptides were weak and no delayed reactions were noted. Essentially similar results were obtained with the group of guinea pigs sensitized with 100 μg of BSA.

Immune tolerance studies in rabbits. The antibody levels in sera of rabbits injected during neonatal and adult life with BSA, HDBSA, tryptic nondialyzable and dialyzable peptides, and subsequently immunized with HDBSA, are presented in Table IV. Values for a control group of rabbits which were treated with 0.15 *M* NaCl in early life and immunized later on with HDBSA, are also included. Rabbits injected neonatally with BSA were also immunized with BSA.

All the animals in control groups elicited a fair amount of antibody. An increase in antibody level was noted following each booster injection. Significantly reduced antibody levels were noted in each course in the group of animals injected neonatally with HDBSA relative to the control group. Rabbits injected neonatally with the tryptic nondialyzable peptides had levels of antibody greater than those injected with HDBSA neonatally but less than the control group. Rabbits which received tryptic *dialyzable peptides* in early life showed an interesting pattern in that there was little precipitating antibody in Course I sera but the antibody levels in Courses II and III were similar to those in the control group.

4/5 rabbits injected neonatally with BSA and subsequently immunized with BSA in later life did not make precipitating antibody

TABLE IV. Response of Normal and Tolerant Rabbits to Injections of BSA, HDBSA, and Tryptic Nondialyzable and Dialyzable Peptides of HDBSA.[a]

Group	Rabbit no.	Anti-HDBSA antibody (μg of N/ml of serum) Course I	Course II	Course III
I. Control	4-75	20	118	133
	4-77	20	86	132
	4-78	75	143	312
	4-79	23	44	140
	4-98	17	50	148
	4-99	35	113	136
	5-00	10	82	152
II. HDBSA	4-70	4	52	53
"tolerant"	4-72	14	26	130
	5-54	0	14	30
	5-55	11	16	109
	5-56	38	58	86
	5-57	19	17	61
III. NDT	4-86	16	60	62
"tolerant"	4-87	7	37	58
	4-88	23	46	96
	4-89	25	31	66
	5-59	16	7	71
	5-60	55	106	180
IV. DT	4-91	0	82	172
"tolerant"	4-92	3	78	228
	4-93	0	31	106
	4-96	4	33	174
	4-97	0	108	264
V. BSA	5-38	0	0	0
"tolerant"	5-39	0	0	0
	5-40	0	0	0
	5-41	8	14	0
	5-43	29	159	—

[a] BSA = bovine serum albumin; HDBSA = heat-denatured bovine serum albumin; NDT = nondialyzable tryptic peptides; DT = dialyzable tryptic peptides; — = not determined.

in Courses I, II, or III, indicating complete tolerance unlike the rabbits in other groups.

Two rabbits from the BSA tolerant group, (Nos. 5-40 and 5-41) were subsequently immunized with HDBSA. The amounts of detectable precipitating antibody (either anti-BSA or anti-HDBSA) was exceedingly low. However, when these same rabbits were later immunized (a month after Course IV) with BSA they formed 35 and 21 μg of N anti-HDBSA and 105 and 84 μg of N anti-BSA antibody, respectively.

Similarly, when rabbits Nos. 5-54 and 5-57 from the HDBSA tolerant group were immunized with BSA (Course IV), their sera contained 43 and 132 μg of N anti-BSA and 36 and 123 μg of N anti-HDBSA antibody, respectively. When these rabbits were now immunized a month later (Course V) with HDBSA, their serum contained 41 and 104 μg of N anti-BSA and 31 and 83 μg of N anti-HDBSA, respectively.

Discussion. The availability of definitive data on the immunochemical properties of HDBSA and its enzyme digests prompted the present extension to the study of hypersensitivity and immune tolerance phenomena. Experiments were therefore done to obtain information on the following questions: (i) what is the relationship between the whole protein molecule and its immunologically active peptide fragments in terms of production or inhibition of immediate anaphylaxis, delayed hypersensitivity, and immune tolerance; (ii) what is the contribution, if any, of the specificity of the proteolytic enzymes used in this study; and (iii) what conclusions can be drawn on the physical nature, size and composition of HDBSA and its peptides and their immunological activities.

Experiments on passive anaphylaxis (Table I) appeared to demonstrate the importance of both the molecular size of the active peptides and the specificity of enzymes with which they were obtained. Nondialyzable peptides of HDBSA were as a rule more potent than dialyzable peptides indicating a correlation between the molecular size and activity. Among the nondialyzable peptides, the chymotryptic peptides were most active in that they caused immediate anaphylaxis in sensitized guinea pigs whereas other nondialyzable peptides elicited much reduced symptoms of anaphylaxis but inhibited the subsequent anaphylactic reaction of HDBSA. Among the dialyzable peptides, only the chymotryptic peptides at a 10-mg level caused significant anaphylaxis. The possibility of trace contamination of the highly active nondialyzable chymotryptic peptides in the chy-

motryptic dialyzable peptides is, however, not ruled out. Although chymotrypsin and pepsin have similar substrate specificities, *i.e.*, both cleave the peptide bonds attached to aromatic amino acids, their action on HDBSA lead to the formation of peptides of widely differing anaphylactic activities.

The delayed hypersensitivity (Tables II and III) reactions elicited 9 days after sensitization of guinea pigs were generally weak and did not follow any immediate reactions. However, at day 21 there was a definite Arthus component (6-hr reaction) elicited by all the test substances followed in some instances by delayed reactions. Both HDBSA and BSA evoked similar immediate Arthus reactions. They also cross reacted equally well. Previously Gell and Benacerraf (2) observed no difference between heat-denatured and native proteins in the production of delayed hypersensitivity reactions. This similarity in cross reactions between HDBSA and BSA in delayed hypersensitivity was not seen in our earlier studies (1) using the precipitation reaction. The nondialyzable peptides elicited a definite Arthus response but only a slight delayed response, whereas the dialyzable peptides produced some Arthus response and no delayed reaction. Of the fractions, the nondialyzable peptides formed by the simultaneous reaction of chymotrypsin and trypsin (NDC/T) were most active. A possible explanation for this activity may be that the simultaneous attack of two enzymes exposed more of the "buried" antigenic sites of HDBSA than when only one of the enzymes was used.

Since the dialyzable peptides produced an Arthus response but no delayed reaction, it would appear that the peptide fractions of smaller molecular size can form complexes with circulating antibody but are poorly immunogenic in themselves. However, the peptide fragments of larger molecular size which are immunogenic are involved in both Arthus and delayed reactions.

Under the experimental conditions in which almost complete tolerance against BSA could be induced in rabbits, it was not possible to induce complete tolerance to HDBSA by neonatal injections of HDBSA or its tryptic nondialyzable or dialyzable peptides (Table IV). On the basis of the antibody levels produced, HDBSA was slightly more effective than the nondialyzable tryptic peptides in inducing tolerance and the dialyzable tryptic peptides were completely ineffective. However, an interesting finding was that the dialyzable peptides which were poor immunogens appeared to prolong a state of tolerance (Course I) which was broken within 4–5 weeks (Course II).

Cross immunization of BSA-tolerant rabbits with HDBSA did not effect their tolerant state but subsequent immunization of these rabbits with BSA terminated the tolerant state. Injection of BSA to partially HDBSA-tolerant rabbits caused further breakdown in the tolerance. This effect was enhanced with a further injection of HDBSA. Although, only two animals in each group was studied, these results confirm those of Weigle (4) on the termination of BSA tolerance.

Summary. The effect on passive anaphylaxis and delayed hypersensitivity in guinea pigs and immune tolerance in rabbits of injections of heat-denatured bovine serum albumin (HDBSA) and its dialyzable and nondialyzable peptides, formed by the action of crystalline trypsin, chymotrypsin, and pepsin has been presented. In some experiments, a comparison has been presented with the effect of native bovine serum albumin (BSA). A direct relationship was observed between the molecular size of the peptides and their *in vivo* immunological activities. There were also some differences in the immunological activity of peptides formed by the different enzymes. Chymotryptic peptides were most effective in eliciting passive anaphylaxis; whereas, there were no significant differences in the ability of nondialyzable products of various enzymes in causing Arthus and delayed hypersensitivity reactions in guinea pigs. Heat denaturation did not affect the ability of BSA to cause Arthus and delayed reactions. In fact, HDBSA and BSA behaved very similarly and also cross reacted equally well in the above reactions. Dialyzable peptides produced some Arthus reactions but no delayed reactions, whereas nondialyzable peptides elicited good Arthus reaction

and some delayed hypersensitivity. Under the conditions in which tolerance to BSA was induced in rabbits, neither HDBSA nor its tryptic digests were able to establish complete tolerance in rabbits.

1. Liu, C. T., Das, B. R., and Maurer, P. H., Immunochemistry **4**, 1 (1967).

2. Gell, P. G. H., and Benacerraf, B., Immunology **2**, 64 (1959).

3. Nelson, D. S., and Boyden, S. V., Int. Arch. Allergy Appl. Immunol. **25**, 279 (1964).

4. Weigle, W. O., J. Exp. Med. **116**, 913 (1962).

5. Weigle, W. O., J. Exp. Med. **114**, 111 (1961).

6. Smith, R. T., *in* "Advances in Immunology" (W. H. Taliaferro and J. H. Humphrey, eds.), Vol. 1. Academic Press, New York (1961).

7. Heidelberger, M., and Kendall, F. E., J. Exp. Med. **62**, 697 (1935).

Received Nov. 5, 1969. P.S.E.B.M., 1970, Vol. 133.