Internal Antigenic Determinants in Protein Molecules.* (25394)

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For approximately 15 years, the problem of fragmentation of foreign antigens after injection, and the role of such fragments in immune responses has been of interest to us. Much of the original basic research work has been reviewed by Campbell and Garvey(1) and speculations and theories regarding the significance of such fragments have been presented by Campbell(2). If these antigen fragments play a role in immune mechanisms and antibody formation, then regardless of what theory of antibody formation one prefers, certain consequences can be expected. For example, one would predict that since the actual specific antigenic determinant is relatively a small part of the total molecule, antigen fragments should present determinants which do not occur on the surface of the native antigen molecule. Consequently, antibodies would be formed against internal structures of the native antigen molecule which do not occur on the surface of the native antigen molecule and would not be detected when antiserums were tested with the native intact antigen. Antibodies to such internal molecular structures ("hidden determinants") could be detected only by tests with partially degraded antigens. The problem is complicated by the fact that *in vitro* degradation might be different from that which occurs in the body and that simple inhibiting haptens might be formed. Lapresle and his collaborators(3) have shown that antibodies are produced against digested proteins which are not present in antiserums against native proteins, but the question arises as to the significance of the difference, and whether the difference in specificity is an artifact as a result of *in vitro* digestion. Probably the first evidence of "hidden" antigenic determinants on a molecular level was shown by Bartel and Campbell(4) using a dissociable hemocyanin as an antigen. Their data demonstrated that antiserums from rabbits immunized with hemocyanin contained antibodies which reacted with the dissociated molecular units, but not with the intact associated hemocyanin molecule. Our investigation was undertaken to see if an antigen such as bovine serum albumin (BSA) could be broken down *in vitro* into fragments which would react with rabbit anti-BSA serums that had been absorbed with intact native BSA. The results of these experiments are of a preliminary nature, but are very provocative and clearly suggest the reality of "hidden" valencies in immune mechanisms.

Materials. Our studies deal with crystalline BSA (Armour and Co.) and rabbit anti-BSA serums. Degraded BSA was prepared by digestion with crystalline pepsin (Worthington Biochemical Corp.) as will be described later. Anti-BSA serums were prepared by immunization of rabbits with BSA following 4 different schedules. The 6 rabbits of the first group were injected intravenously with 10 mg BSA in saline 3 times a week for 6 weeks. The 3 rabbits of the second group were immunized with the same antigen 3 times a week for 8 weeks. The 4 rabbits of the third group were immunized by intravenous injections 4 times a week for 4 weeks with increasing amounts of a neutral suspension of alum precipitated BSA. The 3 rabbits of the fourth group were immunized with BSA suspended in Freund's adjuvant, and 12.5 mg of protein was injected subcutaneously once a week for 8 weeks. These rabbits were bled 7 days after the last injection. The amount of anti-BSA in these antiserums, determined by quantitative precipitin analysis, is shown in Table I. The antibody against intact BSA molecules in these antiserums was absorbed with BSA. An amount of BSA corresponding to the equivalence point was added

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TABLE I. Reactions between Absorbed Antiserums and Fractions of the Digested BSA.

Group*	Rabbit No.	Conc. of anti-BSA, mg/ml	Reactions with absorbed antiserums					
			Ring test			PCA		
			Fraction 1	Fraction 2	BSA	Fraction 1	Fraction 2	BSA
1	1	.93	+	—	—	—	—	—
	2	.78	+	—	—	—	—	—
	3	.24	+	—	—	—	—	—
	4	.21	+	—	—	—	—	—
	5	.41	+	—	—			
	6	.07	+	—	—			
2	1	.62	+	—	—	+	—	—
	2	.73	+	—	—	+	—	—
	3	.78	+	—	—	+	—	—
3	1	.58	—	—	—			
	2	.75	—	—	—			
	3	.83	—	—	—			
	4	.67	—	—	—			
4	1	1.18	—	—	—			
	2	.77	—	—	—			
	3	.73	—	—	—			

* Group 1 and 2, immunized with BSA in saline (see text); Group 3, immunized with neutral suspension of alum precipitated BSA; Group 4, immunized with BSA suspended in Freund's adjuvant.

to each antiserum. After 48 hours at 4°C, the specific precipitates were centrifuged off. The supernatants were tested for presence of anti-BSA and of BSA by ring test. The antibody was not detected in any supernatant, although a trace of BSA was present in some of them. These supernatants are the absorbed antiserums used in subsequent experiments. Purified anti-BSA was prepared by dissociation of specific BSA-anti-BSA precipitates(5). The gamma globulin fraction of anti-BSA serum was first removed by precipitation with ammonium sulfate, at ⅓ saturation (pH 7.8), and purified by repeated precipitation. The final precipitate was redissolved in saline, and dialyzed against borate buffered saline at pH 8.4. After dialysis the antibody was precipitated at the equivalence point with BSA. After 24 hours at 4°C, the precipitate was removed, washed 3 times with and resuspended in saline. The pH of the suspension was adjusted to 3.2. After shaking for one hour at room temperature to dissolve the precipitate, the insoluble residue was removed by centrifugation in a Spinco preparatory centrifuge at 78,000 g for one hour. The supernatant was fractionated with saturated sodium chloride at the same pH. The precipitate was dissolved in and dialyzed against a borate buffer of pH 8.4. Nitrogen content in the final preparation was 0.11 mg N/ml. About 60% of the total protein was precipitable with BSA at the equivalence point.

Results. Degradation of BSA and separation of antigen fragments reacting with absorbed antiserums. A degraded preparation of BSA was prepared by pepsin digestion. A 1% solution of BSA in saline was adjusted to pH 4.2, and 1 mg/ml of crystalline pepsin was added. The solution was allowed to stand for 24 hours at room temperature. To prevent further peptic digestion, the solution was heated to 58°C for 45 minutes and filtered to remove the coagulated protein. The solution was then adjusted to pH 8.6, and dialyzed against several changes of saline for 3 days at 4°C. The preparation was then layered on absorbed antiserums from Group 1 rabbits, and on normal rabbit serums as a control. After one to 2 hours white rings appeared at the interfaces with absorbed antiserums, whereas such a reaction was never observed with normal serums. On the other hand, it was found that the absorbed antiserums did not give any reaction with 0.01 to 10 mg/ml of BSA layered on the antiserums. These results suggested that anti-BSA rabbit serums contained some antibody which reacted with digested BSA, but not with intact BSA molecules.

In view of this result, and the fact that the digestion product was very heterogeneous, attempts were made to separate the solution of digested BSA by starch block electrophoresis and to study the various fractions. Zonal electrophoresis on a starch block was carried out in the usual manner, using barbital buffer (pH 8.6, $\mu = 0.1$). The solution of digested BSA was concentrated by pervaporation at 4°C and dialyzed against saline. Protein concentration of the preparations was about 3%. A volume of about 7 ml of each sample was allowed to migrate on starch for about 19 hours at 300 volts. The starch block was then cut into one cm segments, and each segment was eluted with 7 ml of saline. Each eluate was tested for protein concentration by the biuret reaction(6), and for the presence of antigen by ring test with the absorbed

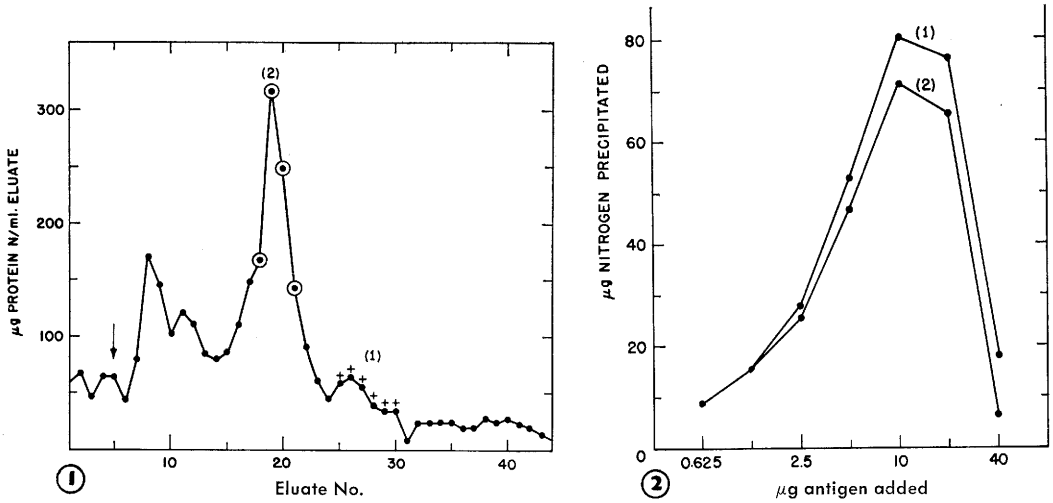

FIG. 1. Starch electrophoretic pattern of digested BSA. (1) represents Fraction 1 (No. 24 to No. 30). (2) represents Fraction 2 (No. 18 to No. 21). Plus represents presence of reactive substance(s) against absorbed antiserums. Arrow indicates the origin.

FIG. 2. Inhibition of the BSA-anti-BSA precipitin reaction with Fraction 2. Curve 1 represents precipitin curve between BSA and anti-BSA. Curve 2 represents precipitin curve in presence of Fraction 2.

antiserums of Group 1. A representative starch block electrophoresis pattern is shown in Fig. 1. Several eluates that had a mobility greater than the highest peak, definitely reacted with the absorbed antiserums. These eluates (from 23-30 cm) were pooled and designated as Fraction 1. The eluates from the segments corresponding to the highest peak were pooled separately and designated Fraction 2. By ring test, Fraction 1 gave a definite reaction with the absorbed antiserums, whereas it gave no reaction with either specifically purified anti-BSA or normal rabbit serum. To study the possibility that the reactive substance in Fraction 1 might be pepsin, heated pepsin was tested for reactivity with the absorbed antiserums. A saline solution containing 1 mg/ml of pepsin was heated at 56°C for 30 minutes, and 2-fold dilutions of the solution were layered on the absorbed antiserums. However, no positive ring tests were observed in any of the tubes, indicating that the reactive substance in Fraction 1 was not pepsin. The digestion of BSA and fractionation of the degraded material by starch block electrophoresis was repeated 9 times. The antigen fragments reactive with the BSA-absorbed antiserum were always detected only in the eluates from similar segments of the starch block, as described above.

In some preparations Fraction 2 contained materials that gave positive ring tests with specifically purified anti-BSA. In other preparations, however, Fraction 2 did not give reaction with the purified anti-BSA. It became apparent that Fraction 2 of these preparations contained substances which inhibited the BSA-anti-BSA precipitation. 0.25 ml volumes of Fraction 2 were added to equal volumes of anti-BSA serum. After 15 minutes at room temperature, 0.5 ml volumes of 2-fold dilutions of BSA were added and incubated for 48 hours at 4°C. The precipitates were centrifuged, washed 3 times with cold saline, and precipitated nitrogn was determined. The BSA-anti-BSA precipitates at the equivalence point, and especially in the region of antigen excess, were decreased by the addition of Fraction 2 (Fig. 2).

To get some idea of the properties of the reactive antigen in Fraction 1, the digested material, without further purification by starch block electrophoresis, was analyzed by free boundary electrophoresis, using barbital buffer (pH 8.6, $\mu = 0.1$). The mobility of the highest peak was -6.9×10^{-5} cm^2 volt^{-1}sec^{-1}, which is the same as the mobility of intact BSA. As the Fraction 1 migrates faster than the highest peak (Fraction 2) in starch block electrophoresis, the mobility of the reactive

antigen in Fraction 1 is probably faster than the intact BSA. This finding suggested that the antigen is the fragment of a BSA molecule.

Dependence of the presence of antibody reactive to Fraction 1 on immunization schedule. The absorbed antiserums from Group 1, 2, 3, and 4 rabbits were tested for the presence of antibody against the degraded BSA (Fraction 1 and 2) by ring test. The results are shown in Table I. All absorbed serums from Groups 1 and 2 gave definite reactions with Fraction 1, whereas none of the absorbed serums from Groups 3 and 4 reacted.

The gamma globulin fractions of the absorbed antiserums from Group 1 were also tested for presence of antibody. The gamma-globulin fractions were obtained by precipitation 3 times with 1/3 saturation ammonium sulfate (pH 7.8). A definite reaction was observed with Fraction 1, but not with Fraction 2.

As the first appearance of the reaction by ring test takes a long time (1 to 2 hours) as compared with the BSA-anti-BSA reaction, the passive cutaneous anaphylaxis (PCA) reaction and a complement fixation test were applied to detect antibody against the degraded substances. In the PCA reaction(7), guinea pig skin was sensitized locally with intradermal injections of 0.1 ml volumes of the absorbed antiserums from Groups 1 and 2. Three hours later these guinea pigs were injected intravenously with Evans blue followed by intradermal injections of 0.1 ml volumes of Fractions 1 and 2, 0.1% of BSA solution, and 0.9% saline as control into the sensitized areas. The guinea pigs sensitized with the absorbed serums from Group 1 did not give reaction with either the fractions of digested BSA or intact BSA, while the guinea pigs sensitized with the absorbed serums of Group 2 gave a definite reaction with Fraction 1.

Complement fixation tests were performed by the method of the U.S. Army Medical School, using veronal buffer containing an adequate concentration of calcium and magnesium ion(8) in place of saline. The absorbed antiserums of Groups 1 and 2 were tested using Fraction 1 and intact BSA (0.01 mg/ml) as antigens. Three series of 2-fold dilutions of the antiserums were made with the buffer, and an adequate amount of complement was added to each tube. Fraction 1 was added to the first series; to the second series intact BSA was added. To the third series an equal volume of buffer in place of the antigen was added as the antiserum control.

The complement fixation reactions between the absorbed antiserums and Fraction 1 were not definitely positive; however they were slightly different from the completely negative results obtained both between the absorbed antiserums and intact BSA or antiserum control.

Discussion. The results presented here indicate the presence of an antibody which reacts with degraded BSA but not with the intact BSA molecules in some of the rabbit anti-BSA serums. The reactive antigen in the degraded materials did not give reaction with the specifically purified anti-BSA, while the absorbed antiserums, which gave a reaction with the degraded substances, did not contain anti-BSA. These findings indicate that the specificity of the reaction between the degraded substances and the absorbed antiserums is quite different from that of the BSA-anti-BSA reaction.

The appearance of the precipitin reaction (ring test) between the degraded substances and the antiserums absorbed with BSA took a long time (1 to 2 hours), and the white ring on the interface was not as sharp as that of the BSA-anti-BSA reaction. The possibility arises that the degraded materials might react with serum protein nonspecifically, and thus give a reaction. This possibility is unlikely because the degraded substances gave no reaction with normal rabbit serums. Another possibility, which may explain the delay in appearance of the reaction, is the presence of inhibitors in the digested material. Although the reactive antigen was partially purified by starch block electrophoresis, there might be some inhibiting substances present, such as simple haptens in the fraction, and the precipitation might be inhibited by them. Moreover, the reactive antigen in the degraded material might not be the same fragment of BSA as that which would be present

in vivo, participating in formation of the antibody as a template. In other words, the reaction between the degraded protein and the absorbed antiserums might be a kind of cross reaction. These might be the reasons why the reaction was weak and took a long time to appear. The positive PCA reactions with Fraction 1, but not with either Fraction 2 or intact BSA in the guinea pigs sensitized with the absorbed antiserums, also suggest the presence of antibody against fragments of antigen.

The presence of antibody, directed toward the "hidden sites," in the antiserums does not parallel the concentration of anti-BSA (Table I). The antiserums prepared by immunization with soluble antigen reacted with Fraction 1, whereas the antiserums prepared with either the alum-precipitated antigen, or the antigen mixed with Freund's adjuvant did not, indicating that the presence of the antibody relates to the properties of the antigen injected, and/or the schedule of immunization. These findings suggested to us that the rate of destruction of antigen *in vivo* might be delayed by addition of the adjuvants, and that production of antibody against the internal structure of the antigen might have some relation to the process of antigen destruction.

Summary. 1. Positive ring tests and PCA reactions were observed between pepsin-degraded BSA and some of the BSA absorbed anti-BSA rabbit serums, suggesting the presence of the antibody against antigenic determinants which are not on the surface of the native protein molecule. 2. The presence of the antibody to the "hidden sites" did not parallel the concentration of anti-BSA in the serum but rather seemed to be related to the state of the antigen used in the immunization, and to the immunization schedule.

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Early Response of Plasma Volume, Red Cell Mass and Plasma Proteins To Massive Hemorrhage.* (25395)

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The response of blood volume and plasma proteins to massive hemorrhage in the rat has been reported(1). Measurements were made at 12, 24, 48 and 72 hours after bleeding. After hemorrhage of about 50% of measured blood volume, plasma volume was replenished within the first 12 hours. Arterial hematocrit was lowest at 12 hours, and although rising at 72 hours after hemorrhage, had returned to only 67% of control level by that time. Plasma protein concentration was fully restored by 48 hours, and was already 88.5% of control value at 12 hours, which was the earliest post-hemorrhagic measurement made. Since it was apparent that major plasma volume and protein concentration changes occurred prior to 12 hours, the experiments have been repeated with measurements of circulating volumes and plasma proteins at 2, 4, and 8 hours after hemorrhage. Such measurements were not made in the earlier study for 2 reasons. First, the purpose of that study was

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