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Further Studies on Cold Insoluble Circulating Immune Complexes in Rabbits Immunized with Bovine Albumin (39060)

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Circulating bovine serum albumin (BSA) antigen becomes cold insoluble when it is undergoing immune catabolism (1). As much as 43% of the antigen present in the fresh serum may become insoluble when it is refrigerated (2). The BSA antigen precipitates in association with very high titer anti-BSA antibody (3). After the antigen precipitates, there is a corresponding decrease in the amount of soluble antibody bound antigen in the serum (2). These data suggest that cold insoluble antigen represents circulating immune complexes of antigen and antibody.

This study was done to define further the nature of cold insoluble circulating BSA antigen. The range of temperature, rate of precipitation and the redissolvability of cold insoluble antigen were studied.

Materials and Methods. General. Rabbits were immunized with 250 mg/kg 125I BSA intravenously and 10 mg/kg BSA in Freund's complete adjuvant subcutaneously.

Blood samples were obtained from the central ear artery or by cardiac puncture Blood was allowed to clot at 37° for 2 hr. The blood was centrifuged at 5000 rpm and the serum decanted. The serum was recentrifuged to remove cells completely.

Serum 125I BSA concentration was determined by counting the radioactivity precipitated by 10% trichloracetic acid.

Serum 125I BSA complexed to antibody was determined by the ammonium sulfate precipitation technique of Farr (4).

Effect of temperature on antigen precipitation. Serum obtained from animals 12 days after immunization was used. Two ml serum samples were placed in centrifuge tubes and incubated at one of several temperatures: (a) 37° incubator, (b) 30° incubator, (c) 24° in a thermostatically controlled room, (d) 16° in a refrigerated ultracentrifuge, (e) 8° in a cold room, (f) 4° in a refrigerator, and (g) 0° in an ice bath. Temperatures were checked daily and were consistently within ±1° of the stated value. Merthiolate was added as a preservative.

After incubation for 5 days the tubes were centrifuged and the precipitates washed by centrifugation three times in PBS. The radioactivity in the precipitates was then determined. 95% of the radioactivity was TCA precipitable.

Rate of antigen precipitation. Serum from animals obtained 12 days after immunization was used. Samples were placed at 0° and 24° for times varying from 2 hr to 7 days. The amount of antigen in the precipitate was assayed as described under effect of temperature.

Redissolvability of antigen in the cryoprecipitates. Precipitates obtained from samples incubated at 4° for 5 days were suspended in one ml of the following solutions: (1) 0.5 *M* citrate pH 3.1, (2) 0.1 *M* glycine: HCl pH 3.2, (3) 3 *M* NaCl, (4) 0.05 *M* barbital pH 8.6, (5) 0.01 *M* phosphate in 0.14 *M* NaCl pH 7.4, and (6) 0.05 *M* Tris: HCl pH 7.4.

The total radioactivity was determined by counting the entire precipitate. Samples were then incubated at 37° for 2 hr. Each sample was mixed in a vortex mixer twice during incubation. The samples were centrifuged at 5000 rpm for one hour. An 0.1 ml aliquot of the supernate was carefully removed and assayed for 125I BSA. The per cent 125I

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BSA which could be redissolved was calculated according to this formula: Percent 125I BSA redissolved = 10 × cpm in aliquot of supernate/Total cpm in precipitate × 100.

Results. Effects of temperature. Figure 1 shows the effect of temperature on antigen solubility in animal 88. In this sample 2.3% of the complexed antigen was cold insoluble.

Similar patterns were observed in two other animals studied. If a large fraction of complexed antigen was cold insoluble, large amounts also precipitated at 24°. For example, in serum from animal 101, 36% of the complexed antigen was insoluble at 0°, but 27% was insoluble at 24°. Precipitation was not seen at 30° and above in any of the three samples studied.

Rate of precipitation. Studies on the rate of precipitation are shown in Fig. 2. Antigen nsoluble at 0° after seven days represented

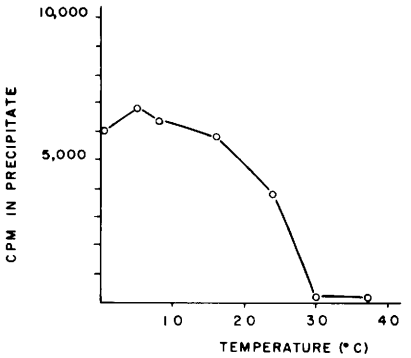

FIG. 1. The effect of varying temperature on the amount of antigen precipitation.

14.3% of the complexed antigen in this sample. Similar patterns were observed in two other animals studied.

Redissolvability of antigen in precipitates. Cryoprecipitates were dissolved for 1 hr at 37° in a variety of buffers. The percent antigen which was dissolved is shown in Table I. Buffers known to dissociate immune complexes, such as low pH citrate or glycine, dissolved more of the precipitate than did neutral buffers.

Discussion. Cold insolubility can cause large errors in the measurement of antigen. These errors can be avoided if blood and serum are handled by special methods. Antigen determinations should be done on fresh serum. Antigen may become cold insoluble in human sera as well. This phenomenon has been observed with hepatitis antigen (5) and with renal tubular epithelial antigen (6).

Buffers most effective in redissolving antigen from the cryoprecipitates are those which dissociate immune complexes. Low pH glycine or citrate will dissolve 60–70%.

Antigen cryoprecipitation is an important laboratory phenomenon. It can cause error in the measurement of antigens in serum. In the rabbit model the occurrence of antigen cryoprecipitation is a reliable sign of immune elimination of antigen. The extent of cold insolubility of antigens present in human sera should be carefully analyzed, especially where the antigen may be in the form of circulating immune complexes.

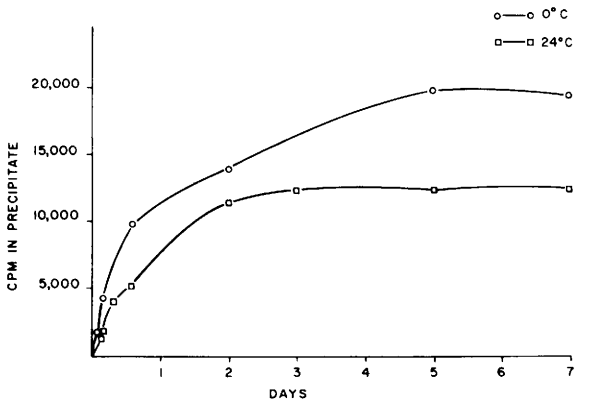

FIG. 2. The rate of antigen precipitation in samples incubated at 0° and 24°.

TABLE I. REDISSOLVABILITY OF CRYOPRECIPITATES.

Buffer	Percentage of antigen redissolved
0.5 *M* Citrate pH 3.1	74%
0.1 *M* Glycine HCl pH 3.2	66%
3 *M* NaCl	51%
0.05 *M* Barbital pH 8.6	42%
0.01 *M* Phosphate in 0.14 *M* NaCl pH 7.4	39%
0.05 *M* Tris: HCl pH 7.4	13%

Summary. Cold insoluble circulating immune complexes of BSA and anti-BSA antibody are formed *in vivo* while immune catabolism of antigen is occurring. The effects of temperature, rate of precipitation and redissolvability of the cold insoluble antigen were studied in this model.

Circulating BSA is soluble at 37°, but may precipitate when the temperature is reduced. The solubility of antigen decreases at 24° and below. Complete precipitation occurs in 5–7 days. The antigen in the cryoprecipitate is difficult to redissolve unless low pH citrate or glycine buffers are used.

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