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The specific *in vitro* antibody-mediated retention of bovine serum albumin by porcine hyaline articular cartilage

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SUMMARY

Porcine hyaline articular cartilage (HAC) has been used to investigate the interaction of bovine serum albumin (BSA) and anti-BSA with articular collagenous tissues *in vitro*. There was a marked retention of ^{125}I-labelled BSA by plugs of HAC (a) exposed to rabbit anti-BSA for 1–2 h at 37°C prior to a similar incubation with the antigen, or (b) exposed to the antigen and then to antibody. The specifically retained radiolabelled BSA was localized in or at the articular surface of the plugs. In the absence of specific antibody a relatively small amount of the antigen was retained. Exposure of HAC to multiple cycles of antibody and antigen treatment resulted in an increased retention of the ^{125}I-BSA. There was a concomitant increase in the retention of the anti-BSA and the capacity of the treated plugs to fix complement. The forces that maintained the labelled antigen in the tissue were not readily reversed by excess unlabelled BSA. Pre-formed, soluble BSA/anti-BSA complexes did not appear to penetrate the tissue unless the HAC was first exposed to anti-BSA. The results suggest that the antibody-mediated, surface oriented retention of ^{125}I-BSA results from the formation of immune complexes in the tissue.

INTRODUCTION

Articular collagenous tissues are sites of antigen, antibody and complement deposition, and a chronic inflammatory response, when previously immunized rabbits are challenged intra-articularly with the immunizing antigen (Cooke & Jasin, 1972; Cooke *et al.*, 1972; Hollister & Mannik, 1974; Cooke *et al.*, 1975). This experimental antigen-induced arthritis serves as a useful model for rheumatoid arthritis (Cooke & Jasin, 1972; Cooke *et al.*, 1972). Antigen localizes and persists in the collagenous tissues of the joint, probably as complement fixing, insoluble immune complexes (Cooke *et al.*, 1975). It has been suggested that in antigen-induced arthritis and in rheumatoid arthritis the immune complexes localized in articular collagenous tissues might play a significant role in sustaining a chronic synovitis (Cooke, 1981). The mechanisms by which immune complexes become deposited or form within articular collagenous tissues are not well defined. This present communication reports an *in vitro* investigation of the antibody-mediated retention of bovine serum albumin (BSA) by hyaline articular cartilage (HAC) from the knee joints of young pigs.

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MATERIALS AND METHODS

Antigen and antibody preparations. The BSA was labelled with ^{125}I as previously described (Cooke & Jasin, 1972). More than 98% of the ^{125}I-labelled protein was specifically precipitated by anti-BSA. Antisera to BSA and ovalbumin were prepared in rabbits as previously described (Cooke & Jasin, 1972). All antibody preparations were heated at 56°C for 30 min and used routinely as the total immunoglobulin fraction. Further fractionation on DEAE-cellulose (Fahey & Terry, 1973) and on BSA covalently linked to Sepharose 4B (Johnson & Garvey, 1977) yielded an IgG preparation of the anti-BSA. A sample of this IgG was labelled with ^{125}I (Cooke & Jasin, 1972) and had a specific activity of $4{\cdot}4 \times 10^7$ ct/min/mg protein.

Tissue source. Shavings of HAC were obtained from the knee joints of recently sacrificed young pigs. The tissues were washed extensively with phosphate-buffered saline (PBS), pH 7·2 at 4°C and used within 2 days of sacrifice.

Interaction of HAC with antibody and antigen. HAC shavings, 1–2 mm thickness were cut into circular plugs of 5 mm diameter, each plug having an articular and a non-articular surface. Unless otherwise stated in the text, the antigen was used at a concentration of 0·1 mg/protein/ml PBS and the antibody at an immunochemically equivalent concentration. The plugs were sequentially incubated at 37°C in 0·15 ml of the various reactants. The optimum incubation time was between 1 and 2 h. The plugs were rinsed briefly in four 500 ml volumes of PBS between each treatment. After the final treatment the rinsed plugs were leached in 15 ml of PBS for 24 h at 4°C to remove any unbound reactants and the amount of firmly retained radiolabelled material determined. Control experiments employed heat inactivated normal rabbit serum, PBS or anti-ovalbumin. The same basic procedure was used to investigate the interaction of soluble pre-formed immune complexes with the HAC.

Complement fixation. Treated and untreated plugs of HAC were incubated with 3 CH_{50} units of fresh human complement for 1 h at 37°C. The residual haemolytic activity was determined by a standard assay (Rapp & Borsos, 1970).

Elution of specifically retained ^{125}I-BSA. Plugs of HAC exposed first to anti-BSA and then to labelled antigen were counted after the usual rinsing and leaching, then submerged for a further 24 h at either 37°C or 4°C in 15 ml of various concentrations of unlabelled BSA. The plugs were rinsed and the amount of persistent radiolabelled material determined. For comparative purposes, plugs of the same dimensions were cut from agarose gels (2% in PBS) and treated as above.

Preparation of preformed soluble immune complexes. Soluble antigen–antibody complexes were prepared at 5 times antigen excess by incubating 1 ml of anti-BSA with 1 ml of ^{125}I-BSA, containing 0·5 mg protein/ml for 1 h at room temperature. The mixture was clarified and then dialysed for 18 h at 4°C in 0·02 M Tris-HCl, pH 8·0 buffer containing 0·028 M NaCl. Excess antigen was removed by passing the mixture through a column ($0{\cdot}8 \times 10$ cm) of DEAE-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, California, USA) equilibrated and eluted with the Tris-HCl buffer. The specific activity of the recovered soluble complexes was determined.

RESULTS

Antibody-mediated retention of antigen

HAC plugs exposed to 15 μg of ^{125}I-BSA ($3{\cdot}3 \times 10^7$ ct/min/mg protein) for 2 h at 37°C and then to an equivalent amount of anti-BSA (2 h, 37°C) retained 3–4% of the labelled antigen prior to the leaching. The ^{125}I-BSA that was firmly associated with the tissue after the leaching period represented about 1% of the labelled antigen present in the initial incubation step. A comparable result was observed when the HAC was treated with the antibody prior to the labelled antigen. In contrast, less than 0·1% of the initial antigen dose persisted in control plugs treated with normal rabbit serum of PBS in place of antibody. The results in Table 1 summarize these *in vitro* experiments and show that the anti-BSA treatment was effective either prior to, or after, exposure to the antigen. A non-cross-reacting antibody preparation, anti-ovalbumin, was not effective.

Table 1. Specific antibody mediated retention of ^{125}I-BSA in HAC

HAC treatment*		^{125}I-BSA retention†
Reactant 1	Reactant 2	(ct/min ± s.d.)
^{125}I-BSA	anti-BSA	4,833 ± 2,669
anti-BSA	^{125}I-BSA	4,468 ± 1,334
^{125}I-BSA	NRS‡	311 ± 48
NRS	^{125}I-BSA	463 ± 70
anti-OVA‡	^{125}I-BSA	296 ± 31

* Plugs exposed to reactant 1 for 2 h, 37°C, rinsed then submerged in reactant 2 for 2 h, 37°C, rinsed and finally leached for 24 h, 4°C before counting.

† Mean ct/min ± s.d. of five separate plugs.

‡ NRS = normal rabbit serum; OVA = ovalbumin.

Distribution of the retained labelled antigen

The results in Table 2 show that the specific antibody-mediated retention of ^{125}I-BSA has an orientation to the articular surface of the HAC plugs. Plugs of HAC exposed first to anti-BSA or normal rabbit serum and then to labelled BSA were sectioned laterally through the centre to produce articular surface and non-articular surface halves. Antigen that persisted in HAC plugs, previously exposed to anti-BSA, was localized predominantly in the articular surface half. Whereas the relatively smaller amount of ^{125}I-BSA that persisted in control plugs was evenly distributed between the two halves.

Repeated exposure of HAC to antibody and antigen

Table 3 shows that there was a marked and progressive increase in the amounts of both antigen and antibody retained by cartilage exposed to repeated cycles of the antibody–antigen treatment. In comparison with a single antibody–antigen treatment there was an 8·7-fold increase in the persistence of ^{125}I-BSA in tissues exposed to two successive treatment cycles, and a 21·2-fold increase following three sequential treatments. Repeated exposure of HAC to ^{125}I-BSA without prior and intervening treatments with anti-BSA caused only a small increament in the amount of labelled antigen retained.

A single exposure of the cartilage to BSA, after a single treatment with the labelled antibody promoted the persistence of the ^{125}I-IgG. Exposure of HAC to three successive cycles of the antibody–antigen resulted in a 13-fold increase in the amount of persistent ^{125}I-IgG. In the absence

Table 2. Distribution of ^{125}I-BSA retained by the HAC plugs

	Percentage ^{125}I-BSA retained*	
	Articular surface half	Non-articular surface half
Antibody-mediated retention†	79·4 ± 5·0	20·6 ± 5·0
Non-specific retention‡	47·1 ± 6·0	52·9 ± 6·0

* Mean ± s.d. of five separate determinations.

† HAC plugs exposed to anti-BSA and ^{125}I-BSA.

‡ HAC plugs exposed to normal rabbit serum and ^{125}I-BSA.

Table 3. The effect on the retention of reactants by HAC repeatedly and sequentially exposed to specific antibody and antigen

HAC treatment*		Number of sequential exposures	^{125}I retained (ct/min ± s.d.)†
Reactant 1	Reactant 2		
anti-BSA	^{125}I-BSA	1	3,101 ± 1,973
		2	26,832 ± 10,981
		3	65,753 ± 17,164
PBS	^{125}I-BSA	1	1,341 ± 375
		2	1,977 ± 758
		3	2,670 ± 864
^{125}I-anti-BSA	BSA	1	2,615 ± 266
		3	33,914 ± 1,797
^{125}I-anti-BSA	PBS	1	1,487 ± 261
		3	5,027 ± 456

* HAC plugs exposed to reactant 1 for 2 h, 37°C, rinsed then exposed to reactant 2 for 1 h, 37°C, rinsed again and then either leached for 24 h, 4°C and counted or recycled through further treatments with reactants 1 and 2 before leaching and counting.

† Mean ct/min ± s.d. of five separate plugs

of intervening treatments of the cartilage with BSA there was a much smaller increase (3·4-fold) in the retention of labelled protein following three successive exposures to the ^{125}I-IgG.

Complement fixation

The results in Table 4 show that (a) as the HAC was exposed to four successive treatments of anti-BSA and BSA there was a progressive increase in the complement fixing capacity, (b) untreated

Table 4. The complement fixing capacity of HAC plugs repeatedly and sequentially exposed to antibody and antigen

HAC treatment		Number of sequential exposures	CH_{50} units fixed
Reactant 1*	Reactant 2†		
anti-BSA	BSA	1	0·66
		2	0·72
		3	0·96
		4	1·32
PBS	BSA	4	0·33
anti-BSA	PBS	4	0·37
PBS	PBS	4	0·15

* HAC plugs exposed to the first reactant for 2 h, 37°C and rinsed in saline before treatment 2.

† Plugs exposed to second reactant for 1 h, 37°C and then leached for 24 h, 4°C or recycled through treatments 1 and 2 before the leaching and final exposure to three CH_{50} units of human complement for 1 h, 37°C.

Table 5. Elution of specifically retained ^{125}I-BSA from plugs of HAC and agarose by unlabelled BSA*

Unlabelled BSA concentration (mg/ml)	Percentage ^{125}I-BSA remaining			
	HAC		Agarose	
	4°C	37°C	4°C	37°C
0	98·7	76·0	90·8	80·5
0·5	88·3	53·4	68·4	21·0
1·0	83·3	42·7	46·8	9·9
2·0	81·0	40·4	28·3	3·6
10·0	59·0	18·8	10·1	0·4

* Plugs first exposed to anti-BSA (2 h, 37°C) and to ^{125}I-BSA (1 h, 37°C), leached (24 h, 4°C) and counted. Then submerged in solutions of unlabelled BSA (300 vol.) for 24 h at 4°C or 37°C and recounted.

plugs had a comparatively low complement fixing capacity and (c) plugs treated with either anti-BSA and PBS or PBS and BSA, for between 1 and 4 times also had relatively little complement fixing activity.

Elution of the retained ^{125}I-BSA

The interaction between BSA and its specific antibody is potentially reversible. The results shown in Table 5 compare the effect of excess unlabelled BSA on the persistence of the specifically retained ^{125}I-BSA in plugs of HAC and 2% agarose. The specifically retained ^{125}I-BSA was more readily liberated from 2% agarose plugs than from HAC. The amount of radiolabelled antigen eluted from both HAC and agarose plugs increased with increasing concentrations of unlabelled BSA in the leaching solution. The effect was more pronounced at 37°C than at 4°C. Whether the eluted antigen was free or complexed with antibody as small soluble immune complexes was not determined. These results suggest a limited reversibility of the forces that maintain the ^{125}I-BSA, probably as complement fixing immune complexes within or at the articular surface of the cartilage plug.

Interaction of soluble immune complexes with HAC

Each HAC plug was exposed to 2·4 μg of ^{125}I-BSA, either in the form of soluble immune complexes

Table 6. Retention of preformed ^{125}I-BSA/anti-BSA soluble immune complexes by HAC plugs

HAC treatment		^{125}I-BSA retained (ng/plug)
Reactant 1*	Reactant 2†	
PBS	IC‡	1·4± 0·2
anti-BSA	IC	34·6±15·8
PBS	^{125}I-BSA	1·8± 0·9
anti-BSA	^{125}I-BSA	42·7±13·6

* 2 h, 37°C.

† 2 h, 37°C. Reactant 2 (0·15 ml) contained 2·4 μg of antigen either associated with anti-BSA (IC) or as free antigen.

‡ IC = immune complex.

or as free antigen. The results are shown in Table 6. Tissue plugs not exposed to the specific antibody retained a relatively small amount of immune complexes and the free antigen. However, prior treatment of the tissue with specific antibody resulted in more than a 20-fold increase in the retention of both the soluble immune complexes and the free antigen. These results indicate that the soluble immune complexes formed from ^{125}I-BSA and anti-BSA in 5 times antigen excess, have difficulty penetrating or being retained by HAC in the absence of specific antibody.

DISCUSSION

Hyaline articular cartilage provides a suitable matrix for investigating the *in vitro* interaction of a protein antigen (BSA) and its specific antibody with articular collagenous tissues. Treatment of the cartilage first with one reactant and then with the other, leads to a specific retention of the BSA and the anti-BSA which is greatly increased by the repeated and sequential exposure to the both reactants. It is most probable that the BSA and the anti-BSA interact to form immune complexes which get larger or become more numerous as the tissue is exposed to repeated cycles of the antibody–antigen treatment. The specificity of the interaction, the easy elution of antigen in the absence of specific antibody, and the increase in complement fixing capacity that parallels the increasing BSA and anti-BSA retention, suggest that immune complexes are formed.

It has been suggested that there is a direct interaction between immune complexes and articular collagenous tissues *in vivo* (Ohno, Tateishi & Cooke, 1978) which might relate to both antigen persistence and matrix degradation observed in antigen-induced arthritis (Cooke & Maeda, 1979). The stability of the immune complexes which become localized in porcine HAC *in vitro* is also influenced, to some extent, by the collagenous tissue. In the presence of excess unlabelled antigen the ^{125}I-BSA/anti-BSA complexes associated with the cartilage do not dissociate or become liberated from the matrix as readily as comparable complexes formed in agarose. The nature of the interaction between the immune complexes and the tissue is at present unknown. Possible factors involved include fixed charge density (Maroudas, 1979) and Fc receptors. Somewhat similar observations to those presented in this manuscript have been demonstrated by Hollister & Mannik (1974) with human serum albumin and rabbit menisci, and by Teucher & Donaldson (1979) with rabbit non-articular collagenous tissues. In both of these investigations it was shown that the Fc portion of rabbit IgG antibody did not influence the specific retention of the antigen.

Whether the *in vitro* interaction of the anti-BSA and its antigen takes place directly on the articular surface of HAC to form surface associated immune complexes or whether it occurs within the matrix to yield subsurface immune complexes is not yet firmly established. Both BSA and IgG molecules are capable of penetrating the superficial layers of hyaline cartilage (Maroudas, 1979). Immunofluorescence studies have shown that persistent BSA in the HAC from knee joints of rabbits with antigen-induced arthritis, localizes within the superficial layers of the articular surface (Cooke *et al.*, 1972). Ohno *et al.* (1978) also demonstrated the *in vivo* localization of ferritin and IgG in the subsurface layers of rabbit HAC.

The data of the present *in vitro* investigation support the idea that *in vivo* immune complexes become deposited within the collagenous tissues by the *in situ* interaction of antigen and antibody (Hollister & Mannik, 1974; Jasin, 1975). Pre-formed soluble BSA/anti-BSA immune complexes do not readily interact with porcine HAC *in vitro*, unless the cartilage has been exposed previously to anti-BSA. Antigen-induced arthritis is developed in a previously immunized animal so it is conceivable that, as well as the *in situ* formation of immune complexes, the binding of preformed complexes by collagenous tissues, previously exposed to the antibody, may occur.

The presence and persistence of immune complexes in articular collagenous tissues may play a central role in sustaining a chronic synovitis by promoting inflammatory responses, tissue degradation and by providing a continous immunogenic stimulus (Cooke, 1981). There are many features in common between the observations presented in this manuscript and the characteristic retention of antigen by articular collagenous tissues during antigen-induced arthritis (Cooke & Jasin, 1972; Cooke *et al.*, 1972; Cooke *et al.*, 1975; Jasin, 1975; Ohno *et al.*, 1978; Chu, Cooke & Aston, 1982). *In vitro* models such as the one presented in this investigation can provide a basis for

the systematic study of mechanisms whereby immune complexes form, deposit and persist in articular collagenous tissues.

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