Enzyme-linked Immunosorbent Assay Analyses of the Hyaluronate-binding Region and the Link Protein of Proteoglycan Aggregate

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An enzyme-linked immunosorbent assay, in combination with an independent inhibition step, was established to quantitate two components of the proteoglycan aggregate, namely link protein and hyaluronate-binding region, at concentrations below 100 ng/ml. The presence of other components of the aggregate in the samples to be tested influenced quantitation in a specific manner. The apparent antigenicity of link protein increased 2-5 times when either purified proteoglycan monomer, purified hyaluronate-binding region, or purified hyaluronate (macromolecular or oligomers) were present in the link protein samples. These findings are interpreted as showing different states of conformation or degree of association of the link protein with other components of aggregate in solution. In separate experiments, a 2-4-fold increase in the apparent antigenicity of purified hyaluronate-binding region was observed when hyaluronate molecules with at least 20 disaccharides were present in the samples. Co-incubation of the hyaluronate-binding region or proteoglycan monomer with either purified link protein or with smaller hyaluronate oligomers did not change its antigenicity in the assay. However, when hyaluronate oligomers with 8 disaccharides were included in a mixture of macromolecular hyaluronate with hyaluronate-binding region, the increase in apparent antigenicity was blocked. The results illustrate the inherent difficulties in using the enzyme-linked immunosorbent assay for the quantitation of link protein or proteoglycan monomers in samples where these macromolecules can associate with themselves or other components of proteoglycan aggregates.

The majority of proteoglycans in hyaline cartilages exist as large macromolecular aggregates containing proteoglycan monomers, link proteins, and hyaluronate (1-3). Proteoglycan monomers consist of a core protein to which a large number of glycosaminoglycan chains and oligosaccharides are covalently attached (4-5). One end of the core protein, referred to as the hyaluronate-binding region, lacks glycosaminoglycan chains and contains an active site which binds noncovalently and very specifically with hyaluronate (7-9). The link protein (10-13) stabilizes the interaction of the proteoglycan with hyaluronate by binding to an adjacent length of hyaluronate (14) and to a portion of the core protein of the proteoglycan near the hyaluronate-binding region (15, 16). When aggregates are treated with proteases such as trypsin or clostripain, the hyaluronate-binding region of the core protein and the link protein resist digestion and remain bound to the hyaluronate while the rest of the core protein with the glycosaminoglycan chains is removed (8, 16-18). The complex consisting of a strand of hyaluronate with a large number of associated hyaluronate-binding region-link protein pairs can be purified from such digests; subsequently, the hyaluronate-binding region and link protein can be separately purified by molecular sieve chromatography in dissociative solvents (16, 18).

For the experiments described in this paper, the complex of hyaluronate-binding region-link protein-hyaluronate was purified from clostripain digests of aggregates from the Swarm rat chondrosarcoma (19) and was used directly to raise antibodies in rabbits. The antisera in combination with highly purified preparations of hyaluronate-binding region and of link protein was used to develop sensitive and specific enzyme-linked immunosorbent assays able to detect hyaluronate-binding region or link protein in the 30-200 ng/ml range. The assays were shown to be highly sensitive to such parameters as conformation of antigen, self-association of antigen, and interaction with other components of aggregate structure.

**EXPERIMENTAL PROCEDURES**

*Materials.*—Flat-bottom micro-ELISA plates (Immulon-treated, M 129A) were obtained from Dynatech, dithiothreitol from Bethesda Research Laboratories, Tween 20 (polyoxyethylene sorbitan monolaurate), o-phenylenediamine, and umbilical cord hyaluronate from Sigma, peroxidase-labeled anti-rabbit IgG (goat), lot 61-202, S566, from Miles-Yeda, and clostripain (*Clostridium perfringens*) from Boehringer Mannheim.

*Antigen Preparation.*—Proteoglycan aggregate was prepared from an associative isopycnic density gradient of an associative extract of the Swarm rat chondrosarcoma and proteoglycan monomer was prepared from the aAl preparation with a dissociative isopycnic density gradient as previously described (19). The aggregate preparation was digested with clostripain to produce ternary complexes of hyaluronate-binding region-link protein-hyaluronate which were isolated and purified using an associative isopycnic density gradient and

*The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; aAl and aAlD1, aggregate and monomer fractions as defined in Ref. 19; R-98, antiserum raised in a rabbit against the complex of hyaluronate-binding region-link protein-hyaluronate (a paper describing this antiserum is in preparation, A. R. Poole, personal communication); R-101 and R-1610, antisera raised in rabbits against purified hyaluronate-binding region; HAs, HAs, HAs, HAs, and HAs, oligomers of hyaluronate containing 6, 16, 40, 45, and 50 monosaccharides per molecule, respectively.*
molecular sieve chromatography on Sepharose 6B in an associative solvent as previously described (18). The hyaluronate-binding region and link protein were then separated from each other and from hyaluronate by molecular sieve chromatography on Sepharose CL-6B in 4 mM guanidine HCl as previously described (18, 20) to yield preparations which were greater than 98% pure when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified antigens were stored at 200–500 µg/ml at 4 °C in 4 mM guanidine HCl, 0.05 M sodium acetate, pH 6.8, for subsequent use. Concentrations were determined by ultraviolet absorption at 278 nm and by amino acid analysis as previously described (18).

Antiserum—The purified complex of hyaluronate-binding region–link protein was used to immunize a rabbit as has been previously described (20). This antiserum, R-98, has been shown to contain antibodies directed against both the hyaluronate-binding region and the link protein. Antiserum against purified hyaluronate-binding region, R-101, and R-1610 were raised in rabbits using essentially the same procedure. Antiserum were stored at 4 °C in the presence of 0.02% w/v sodium azide.

Hyaluronate Oligomers—Hyaluronate oligomers of defined sizes were prepared by partial digestion of umbilical cord hyaluronate with testicular hyaluronidase followed by molecular sieve chromatography as described elsewhere (6, 14, 21).

ELISA Technique—Both ELISA technique in itself (22, 23) and inhibition assays done in conjunction with it (22, 23) have been well described and were used without any major modification. Polystyrene plates were used throughout the study. Because of well-to-well variation in final absorbance (A₄₉₂) reading (data not shown), testing was restricted to the inner 32 wells, leaving out the two outer rows along each axis. The antigen solutions for coating were prepared immediately prior to use by diluting an aliquot of the stock solution in 4 mM guanidine HCl with 20 mM carbonate/bicarbonate buffer, pH 9.2, containing 0.02% w/v sodium azide. Plates were coated using 200 µl of antigen at 80–2,500 ng/ml. For standard assays, final concentrations of 1,000 ng/ml were used for both hyaluronate-binding region and link protein. This represents dilutions of stock of 1:470 and 1:220, respectively, introducing negligible amounts of guanidine HCl into the coating solutions. Plates containing the coating solution were successfully stored at 4 °C in a humid chamber for up to 4 months. All subsequent incubations at the indicated concentrations, and washes performed on the plates, were done in the presence of 0.05% Tween 20, using phosphate-buffered saline, pH 7.0, whenever dilution was required. The presence of the detergent prevents nonspecific absorption of antibody and of additional antigen (in the inhibition assays) to the polystyrene.²

The standard test was performed as follows: coated plates were washed three times with phosphate-buffered saline, pH 7.0, ionic strength = 0.17, containing 0.05% Tween 20, to remove excess antigen and then incubated with 200 µl of a specific rabbit antiserum at an appropriate dilution in phosphate-buffered saline/Tween 20. At the end of the incubation period (30–60 min), excess unbound antibody was removed by washing 3 times as described above, following which 200 µl of a second antibody diluted 1:1,000 with phosphate-buffered saline and containing peroxidase-labeled goat antibody directed against rabbit IgG was placed in the wells. Following incubation for 30–60 min, unbound antibody was removed by washing the plates three times (5 min each) with phosphate-buffered saline, 0.05% Tween 20 and 200 µl of substrate, H₂O₂ + o-phenylenediamine (22), was placed into the wells for 10–60 min. The production of chromophore was stopped by the addition of 50 µl of 2 M H₂SO₄. The plates were read in a Titertek Multiscan instrument (Flow Laboratories) at a wavelength of 492 nm.

The incorporation of an inhibition stage prior to the addition of the specific antibody to the coated plates allows the quantitation of antigen present in unknown samples (22). The conditions yielding optimal quantitation will be discussed under "Results."

RESULTS

Enzyme-linked immunosorbent assays are based on the sandwich technique and have been widely used to determine titers and specificity of antisera as well as to measure antigens both qualitatively and quantitatively. Briefly, the test consists of first incubating a specific antibody with antigen that has been adsorbed on the surfaces of a polystyrene microtiter plate thereby binding some antibody to the plate; second, incubating with a second antibody which contains covalently coupled peroxidase and which is directed against the first antibody; third, quantitating the amount of second antibody bound to the plate by monitoring the amount of colored end product formed during a final incubation with substrate for the peroxidase. Washes are used between each step to remove any antibodies which are not specifically bound. The concentrations of the various interactants, times of incubation, and other conditions of the test must be determined to optimize for sensitivity, accuracy, reproducibility, and convenience. The effect of some of these parameters on the ELISA assays for hyaluronate-binding region and link protein are presented below.

Coating of the Polystyrene Plates—Binding of hyaluronate-binding region or link protein was more efficient at pH 9.2 using 20 mm carbonate/bicarbonate than at lower pH and was inhibited by the presence of guanidine HCl at concentrations greater than 0.1 mM and by the detergents Tween 20, Triton X-100, and Zwittergent 3–12 at concentrations as low as 0.01%. Overnight incubation with the coating antigen at 4 °C was sufficient to ensure maximal binding for any given set of conditions. Fig. 1 shows the results obtained when a fixed amount of antiserum R-98 raised against the complex hyaluronate-binding region-link protein-hyaluronate was incubated with plates coated with purified hyaluronate-binding region or purified link protein at concentrations varying from 12.5–2,500 ng/ml. Sufficient antibody was present so that we could determine the coating concentration which should be used to ensure that small changes in the amount of antigen that bound in individual wells did not reflect large changes in color value (A₄₉₂). The binding curves for the two antigens were different. Half-maximal binding to the polystyrene was obtained at lower concentrations of hyaluronate-binding region (50–100 ng/ml) than of link protein (300–600 ng/ml). A coating concentration of 1,000 ng/ml was used for both antigens for all subsequent experiments. This concentration of purified antigen did not result in any significant well-to-well variation (standard deviation of duplicates <2%) for the amount of first

![FIG. 1. Binding of R-98 antiserum to plates coated with varying concentrations of antigen.](image)

Plate was coated by incubating wells with 200 µl of the indicated concentration of antigen and stored at 4 °C overnight. Antiserum diluted 1:10,000 was then incubated with the coated antigens (link protein (LK) C3, hyaluronate-binding region (HA-BR) C5)) for 60 min. Subsequent incubations with peroxidase-labeled anti-rabbit IgG (1:1,000) and substrate were performed as described in the text. The amount of colored end product was determined by absorbance at 492 nm.
antibody interacting with each well. Since the wells were coated with 200 μl of solutions, 200 ng of antigen was used for each test point.

**Incubation with Specific First Antibody**—The data presented in Fig. 2a show the effect of incubation time on antibody binding when fixed amounts of antiserum R-98 (1:4,000 or 1:10,000) were reacted at room temperature with plates coated with hyaluronate-binding region or link protein, respectively. The lower dilution (1:4,000) of antiserum yielded, in hyaluronate-binding region-coated plates, A492 values similar to those generated using a higher dilution (1:10,000) in link protein-coated plates. Using identical conditions, but different concentrations of the R-98 antiserum, antibody became limiting at dilutions of 1:1,000 (hyaluronate-binding region-coated plates) and 1:5,000 (link protein-coated plates), respectively.

This may indicate that antiserum R-98 contains higher titers of antibodies against the link protein or that more molecules of link protein than of hyaluronate binding region are absorbed onto the polystyrene surface during coating. The amount of colored end product obtained for both antigens by 60 min under these conditions was >80% of that obtained when the interaction reached equilibrium by 120 min (Fig. 2a). For convenience, all subsequent assays were done using a 60-min incubation time with the first antiserum. Reproducibility was good (standard deviation of duplicates <2%).

**Incubation with Peroxidase-coupled Anti-rabbit IgG**—The time required for effective binding of the peroxidase-coupled second antibody to the first antibody (R-98 used at 1:4,000) was estimated under standard conditions on plates coated with hyaluronate-binding region (Fig. 2b). The concentration of second antibody (1:1,000) was not limiting (data not shown). The binding of the second antibody was rapid (% maximum value ≈ 15 min). An incubation period of 60 min at room temperature was used in all subsequent experiments; reproducibility was good (standard deviation of duplicates <2%). When serum from a nonimmune rabbit (1:2,000) was used instead of antiserum R-98 as the first antibody and standard conditions applied for all subsequent steps, the colored end product values were below 0.10.

**Incubation with Substrate**—o-Phenylenediamine was dissolved in methanol at 10 mg/ml and diluted 1:100 with 0.003% (v/v) H2O2 in water just before use (22). Following the addition of 200 μl of substrate to each well, production of chromophore was linear up to 40 min of incubation at room temperature with the rate decreasing slightly thereafter (data not shown). The reaction was stopped by adding 50 μl of 2 M H2SO4. The duration of the incubation with the substrate was left constant for all wells within each polystyrene plate, but varied from experiment to experiment to yield final A492 values between 0.2 and 1.8. The plates were read in a Titertek Multiscan instrument (Flow Laboratories).³

**Inhibition Assay**—The incorporation of an inhibition stage in the ELISA allows the quantitation of antigen present in unknown samples. The test samples (prepared by dilutions of stock into phosphate-buffered saline/Tween 20 immediately prior to use) were preincubated in glass tubes for 1 h with a known amount of first antibody. This inhibition mixture (first antibody and test sample) was then added to the insolubilized antigen on the plates followed by incubation for 60 min. The A492 values obtained at the end of the assay were compared with the values obtained for known amounts of purified soluble antigens treated in identical inhibition tests.

Typical inhibition curves for purified hyaluronate-binding region and link protein standards are shown in Fig. 3. The inhibition mixtures (500 μl final volumes) each contained 250 μl of R-98 antiserum prepared at 1:4,000 for plates coated with hyaluronate-binding region or 1:10,000 for plates coated with link protein and 250 μl of test antigens at the indicated concentrations. The mixtures were incubated for 1 h at room temperature before subsequent incubations on the plates (200 μl/well, duplicates). In both cases, as little as 30 ng of hyaluronate-binding region/ml or 60 ng of link protein/ml were sufficient to give, with good reproducibility, a reduction in the amount of chromophore produced when compared with antiserum incubated with phosphate-buffered saline/Tween 20 alone. At the concentrations of R-98 antiserum used in this experiment, ½ inhibition was obtained at concentrations of 125 ng of hyaluronate-binding region/ml and 170 ng of link protein/ml, respectively. The data in Fig. 3 show that the inhibition is specific in that link protein produced no significant inhibition when tested on plates coated with hyaluronate-binding region while hyaluronate-binding region produced no significant inhibition when tested on plates coated with link protein. The data support the observations by others (24, 25) that the hyaluronate-binding region and link proteins have no common antigenic determinants. Inhibition can be detected in this assay with lower antigen concentrations by lengthening the time of incubation of first antibody with soluble antigen prior to the introduction of the inhibition mixture into the wells. However, this significantly reduces the slopes of the curves, a disadvantage if accurate quantitation is sought.

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³ Dilutions of the colored end product with distilled water can be read in a spectrophotometer (A405).
Incubation of the first antibody (R-98 antiserum) with purified hyaluronate at concentrations up to 50 \( \mu \text{g/ml} \) had no inhibitory effect in either the hyaluronate-binding region or link protein assay. Inhibition curves generated using higher starting concentrations of R-98 antiserum (see below) yielded similar results with \( \frac{1}{2} \) inhibition occurring, however, at higher concentrations of hyaluronate-binding region or link protein, respectively (data not shown).

The results thus far indicate that the technique can be used to quantify two of the three components of the purified complex. The following sections present data which indicate that the precise quantitation of either antigen is difficult if a sample contains other macromolecular components of the aggregate.

**Quantitation of Link Protein** — Fig. 4 shows the effect of preincubation of purified link protein antigen at 500 \( \text{ng/ml} \) with varying amounts of \( \text{aAID1 proteoglycan} \) upon the quantitation of link protein in a subsequent inhibition-ELISA assay. The proteoglycan concentration for the \( \text{aAID1 sample} \) is reported in terms of hyaluronate-binding region equivalents as calculated in a separate inhibition assay. The extent of inhibition and hence antigenicity of link protein (present in all cases at 500 ng/ml) increased nearly 4-fold as the amount of monomer in the mixtures increased to 100 ng/ml of hyaluronate-binding region equivalent (4,000 ng of proteoglycan/ml); about 1 hyaluronate-binding region eq for 8 link protein molecules assuming molecular weights of 67,000 and 42,000 for hyaluronate-binding region and link protein, respectively. The half-maximum increase occurred at about 25 ng of hyaluronate-binding region equivalent/ml. The proteoglycan by itself at the highest concentration used gave no inhibition in the link protein inhibition assay and hence showed no link protein antigenicity (data not shown). The increased antigenicity of link protein in mixtures with proteoglycan monomer was consistently observed. The magnitude of the increase was constant for samples treated concurrently, using identical conditions. The magnitude (2-5-fold) of the increase, however, varied when different batches of antigen, different batches of coated plates, or different R-98 antiserum or antigen dilutions were used.

**Similar increases in measured antigenicity of link protein were induced by preincubating constant amounts of link protein with increasing amounts of either purified hyaluronate-binding region, umbilical cord hyaluronate, or \( \text{HA}_{16} \) oligomers** (Table I). The half-maximum increase for mixtures with purified hyaluronate-binding region was 65 ng/ml; the magni-
tude of the increase (nearly 4-fold) was similar to that obtained with proteoglycan aAlD1 monomer. Under identical conditions, umbilical cord hyaluronate and HA_{35} oligomers produced half-maximum increases at 45 ng/ml and 40 ng/ml, respectively (about 10 disaccharides of hyaluronate per link protein assuming a molecular weight of 400 for the disaccharide). Both hyaluronate preparations produced an increase in antigenicity of link protein of the same magnitude (4-fold) as that produced by purified hyaluronate-binding region.

Preincubation of link protein with 1% bovine serum albumin for 24 h at 4 °C had no effect on the assay and, further, the incorporation of 1% bovine serum albumin during the preincubation of link protein with either hyaluronate-binding region or hyaluronate did not reduce the magnitude of the increase in antigenicity of link protein in the mixtures (data not shown). This suggests that the effect of hyaluronate-binding region and hyaluronate on link antigenicity is related in some way to interactions which are probably involved in proteoglycan aggregation, as discussed below.

Quantitation of Hyaluronate-binding Region and of Proteoglycan Monomer—Fig. 5 shows the results obtained when constant amounts of hyaluronate-binding region (C) or of equivalent amounts of aAlD1 monomer (■) were preincubated overnight, in phosphate-buffered saline, 0.05% Tween 20 at 4 °C with different amounts of macromolecular hyaluronate prior to interaction with the antibody and testing in the inhibition assay for hyaluronate-binding region. As was observed for the antigenicity of the link protein in the experiments described above, the antigenicity of the hyaluronate-binding region increased approximately 3.5-fold (half-maximum increases occurred at hyaluronate concentrations of 13 ng/ml when hyaluronate-binding region was used and 20 ng/ml when aAlD1 proteoglycan monomer was used).

Assuming molecular weights of 67,000 and 400 for hyaluronate-binding region and hyaluronate disaccharide, respectively, half-maximum increase is attained with about 13 hyaluronate disaccharides per hyaluronate-binding region equivalent for monomer (maximum increase at ≈35 hyaluronate disaccharides/hyaluronate-binding region equivalent). As was observed for the link protein assay, the magnitude of the increase in the hyaluronate-binding region assay was constant for samples treated concurrently using identical conditions, but varied (2-4-fold magnitude of increase) when different batches of plates, antigens, or different R-98 antisera or antigen dilutions were used. Unlike the results with the link protein assay, however, preincubation of hyaluronate-binding region with link protein at concentrations up to 1500 ng/ml or with HA_{35} oligomer up to 1280 ng/ml did not change the antigenicity of the hyaluronate-binding region (data not shown). Neither the link protein nor hyaluronate, alone or in combination, gave any significant inhibition of antibody (R-98) binding to the coated well in the hyaluronate-binding region assay.

A number of experiments were devised to study in some detail the specificity of the modulation by macromolecular hyaluronate of the antigenicity of the hyaluronate-binding region. Because smaller oligomers of hyaluronate, HA_{35}, had no effect even in large excess, oligomers of larger size were tested. Oligomers with an average of 50 monosaccharide residues (HA_{50}) were the smallest to produce a response identical with that obtained using equivalent amounts of umbilical cord hyaluronate (Fig. 6). Oligomers with an average of 40 monosaccharides gave a much smaller response.

The results shown in Fig. 7 indicate that the increase in antigenicity of the hyaluronate-binding region in the presence of macromolecular hyaluronate is related to the ability of the hyaluronate-binding region to interact specifically with hya-

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**Figure 5.** Effect of preincubation of hyaluronate-binding region or of aAlD1 proteoglycan with macromolecular hyaluronate in the hyaluronate-binding region inhibition assay. Aliquots of hyaluronate-binding region (HA-BR) (C) (250 ng/ml) or aAlD1 proteoglycan (■) (250 ng of hyaluronate-binding region equivalent/ml) were preincubated with varying amounts of umbilical cord hyaluronate at 4 °C, overnight in phosphate-buffered saline/Tween 20 prior to interaction with the antiserum R-98 at 1:1000 in the hyaluronate-binding region inhibition assay. All concentrations reported are those present in the final assay mixture prior to interaction with R-98 antiserum. The amounts of “HA-BR antigen detected” represent amounts of purified hyaluronate-binding region yielding equivalent A_{492} values when treated in an identical fashion. The arrow represents the value obtained for hyaluronate-binding region or proteoglycan preincubated without hyaluronate.

**Figure 6.** Effect of preincubation of hyaluronate-binding region with hyaluronate oligomers in the hyaluronate-binding region inhibition assay. Aliquots of hyaluronate-binding region (HA-BR) (250 ng/ml) were preincubated with different amounts of umbilical cord hyaluronate or hyaluronate oligomers of various sizes as outlined in the legend of Fig. 5 prior to interaction with R-98 antiserum at 1:1000 in the inhibition assay as described in the legend to Fig. 5. HA_{uc}, hyaluronate from umbilical cord.
The hyaluronate-induced increase in antigenicity of hyaluronate-binding region was not restricted to assays in which the R-98 antisera raised against the complex was used as the source of the first antibody. It was still present when either R-101 or R-1610, antisera raised against purified hyaluronate-binding region, was used as the source of first antibody (data not shown).

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**DISCUSSION**

This paper describes the development of inhibition tests using enzyme-linked immunosorbent assays and immune serum to detect link protein and hyaluronate-binding region at concentrations below 100 ng/ml. The assay is rapid (4 h), highly specific, requires small amounts of purified antigen (200 ng/well), and allows many tests to be run at the same time. The high degree of specificity results from the purity of the antigens used to coat the plates and does not rely on the purity of the antisera, a consideration also discussed by Wieslander and Heinegård (25) for radioimmune inhibition assays. This was demonstrated by using an antisera (R-98) which was raised against the complex of hyaluronate-binding region and hyaluronate and which contained high titers of antibodies directed against both hyaluronate-binding region and link protein. The presence of the antibodies against link protein did not influence the inhibition assay for hyaluronate-binding region and vice versa. As reported by others (24, 25, 27), the link protein and the hyaluronate-binding region do not share any detectable antigenic determinants. The ELISA method also provides a convenient method for comparing antibody titer and for determining the extent to which an antisera raised against an antigen such as monomer proteoglycan may have been contaminated with link protein.

Other immunoassays have been developed for estimating contents of link protein, proteoglycan, or subcomponents of the core protein of proteoglycans. These include radioimmunoassays against link protein (28), monomer (29), and hyaluronate-binding region (25); Laurel rocket assays for link protein (24) and monomer (24, 27); and ELISA procedures for intact or chondroitinase-digested proteoglycans (22) and for link protein (30). The ELISA procedures described in this paper are nearly as sensitive as the most sensitive of the radioimmune procedures described (25, 28, 29) and they avoid the necessity for iodinating the antigen and determining radioactivity in the large number of tests required. Further, larger numbers of samples can be more rapidly handled with a higher degree of reproducibility than can be obtained with most of the other methods previously described.

Many aspects of the application of ELISA methods to quantitation of proteoglycans which were not discussed in this report are presented in a previous study using antisera raised against monomer proteoglycans from the rat chondrosarcoma (22). These include the effects of serum dilutions and coating antigen concentrations on the sensitivity and accuracy of the test. An appreciation of these parameters could be used to increase the level of sensitivity of the assays described above to that required for specific applications. The use of hyaluronate-binding region as the test antigen in the present study confers higher specificity to the assay and avoids some of the problems described in the earlier study (22) for coating plates with intact proteoglycans.

The antigenicity of the link protein increased significantly when other components of the aggregate, namely hyaluronate or proteoglycan (or hyaluronate-binding region), were added prior to testing in the inhibition assay. Bovine serum albumin, on the other hand, had no effect. This suggests that some of the antigenic determinants in purified link protein samples are masked, possibly because of self association of link protein molecules. Ultracentrifugal experiments have suggested that purified link protein samples in 1 M Tris do, in fact, form aggregates (31). Other results to be presented elsewhere indicate that purified link protein molecules form tetramers in a variety of solvents including 6 M urea. Such aggregate structures would be likely to exist in the isotonic solutions used in the interaction of link protein and R-98 antisera during the inhibition step of the ELISA assay presented here. Some workers have also shown that the link protein will bind to hyaluronate in the absence of monomer proteoglycan (25) and to monomer proteoglycan in the absence of hyaluronate (25, 32). The results presented here suggest that the addition of either hyaluronate or proteoglycan to a link protein solution results either in the disruption of link aggregates or in a

*Personal communication from A. R. Poole, manuscript in preparation.*
conformation change leading to unmasking of antigenic determinants.

It was also shown that the antigenicity of the hyaluronate-binding region or of intact monomer proteoglycan increased significantly when macromolecular hyaluronate was added prior to testing in the inhibition assay. Divalency of the antibody was not essential since the effect occurred to an equal extent when monovalent R-98 antiserum prepared by reduction with dithiothreitol (33) was used instead (data not shown). In this case, however, addition of link protein or of smaller oligomers of hyaluronate (less than about 40 monosaccharide units) had no effect on the antigenicity. Furthermore, hyaluronate oligomers sufficiently large to bind tightly to the binding site in the hyaluronate-binding region but too short to accommodate more than one molecule (HA_{10-30}) were able to reverse the effect of molecular hyaluronate.5 A mechanism of self-association by the purified hyaluronate-binding region could also provide an explanation for these results. Previous investigations have in fact suggested that purified proteoglycans can form at least dimers in low salt solutions and that the interactions involved are protein-protein associations (34, 35). Our results could indicate that such interactions mask some antigenic determinants on the hyaluronate-binding region and that rearrangement of proteoglycan monomers or hyaluronate-binding region molecules along macromolecular hyaluronate would unmask these determinants. A small oligosaccharide would be able to occupy a binding site without disturbing the self-association of the hyaluronate-binding region molecules and reversal of the interaction with macromolecular hyaluronate by adding such small oligosaccharides would allow the molecules to once again associate with each other. The interaction of hyaluronate-binding region with link protein, which was clearly demonstrated in the case of the link protein assay, would not reverse the self-association since it had no effect on the antigenicity of the hyaluronate-binding region. The magnitude of the response with hyaluronate (2-4-fold) and the amount of hyaluronate required for half-maximum response (about 13 disaccharides per hyaluronate-binding region equivalent for monomer) would be consistent with this interpretation. The observation (see Fig. 5) that more hyaluronate was required to accommodate the same number of monomers as purified hyaluronate-binding region molecules may be explained by steric effects (8).

Whether or not the above hypotheses are in fact correct, the results illustrate an inherent difficulty that will be encountered for using any inhibition immunological assay for precise determination of proteoglycan or link protein concentrations. The antigenic determinants in the test samples may be expressed differently if the molecules are partially or entirely aggregated; in this case, antigenicity cannot be directly related to purified standards. More likely, the effects reported here represent, at least in the case of the hyaluronate-binding region, unmasking of one of the major antigenic sites capable of interacting with most antisera raised against preparations containing proteoglycan. Standardization of the conditions of the assay, such as denaturation of link protein in sodium dodecyl sulfate (28, 30) prior to testing may circumvent this.

\footnote{Experiments performed after the original submission of this paper have shown that HA_{10-30} is, like HA_{10-20}, able to compete with and reverse the effect of macromolecular hyaluronate. In contrast, HA_{10-10} and \textsuperscript{1}HA do not do so. These results are consistent with and supportive of the interpretation of the data in the discussion.}

**REFERENCES**

Immunology of Proteoglycan Aggregate

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