CHARACTERIZATION OF MONOCLONAL ANTISERUM TO HUMAN GAMMA
CRYSTALLIN IN AGING HUMAN LENSES

By

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A MASTER'S THESIS
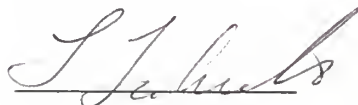
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ACKNOWLEDGMENTS

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SECTION II

LITERATURE REVIEW

LITERATURE REVIEW

INTRODUCTION

The lens is a very unique organ that serves two
functions. One is to change its shape according to the
requirements of the accomodative process. The other,
which is of our major concern, is to transmit light, or
be transparent. To accomplish transparency lens tissue
contains no blood, connective tissue or nerve cells. This
simple tissue consists almost entirely of enucleated fiber
cells with high concentrations of protein, making it an
excellent subject for protein and membrane researchers.

These fiber cells differentiate from a monolayer of
epithelial cells which cover the anterior surface of the
fibers. Epithelial cells are gradually displaced toward
the lens equator where they enucleate and elongate to give
rise to the fiber cells.

Protein synthesis occurring in the epithelial layer
has been demonstrated by tritiated thymidine
autoradiography (26). Longer periods of incubation show
rapidly decreasing amounts of synthesis toward the equator.
The nucleus, or center, of the lens contains no protein
synthetic activity (70). In other words, the cellular
history of protein synthesis is preserved in the adult
tissue. The proteins synthesized in early lifetime are
still present in the lens nucleus of the adult. These

proteins are suspended in the lens for decades without turnover. They are subjected to a variety of post-synthetic modifications, many of which can result is a drastic change in chemical properties, or cataract.

CATARACTS

Cataracts are classified into three major groups according to the region of the lens which opacifies. Posterior subcapsular cataracts are an opacification that occurs to only a few cell layers. Cortical cataracts are generally considered to be caused by a change in the environment, such as an alteration in the salt and water balance. They are somewhat rare. Nuclear cataracts, the most common type occurring in the aged or senile lens, is characterized by the accumulation of large amounts of insoluble colored proteins. A likely source of these insoluble colored proteins appears to be the water-soluble proteins (25,53).

Oxidation is considered a prime cause of protein modification in many oxidative mechanisms which include photo-oxidation (10), ionizing radiation (18), high concentrations of hydrogen peroxide (2), the presence of naphthalene (47), and the presence of quinones (42). The proteins altered by oxidation or other processes can change in solubility, sulphydryl content (16), fluorescence (37), isoelectric point (67), crosslink content (61), and amino acid content (60). The severity of protein modification is expecially apparent when comparing solubilities. Van Haard et al. has shown that 70% of the protein in brunescent (severely discolored) lenses was water

insoluble while normal lenses of comparable ages contained only 11% insoluble protein (66).

Another area of cataract research is the study of protective mechanisms. Protective mechanisms act by detoxifying reactive intermediates capable of oxidizing or by reversing any damage done by them. A list of these mechanisms found in lens tissue includes glutathione (20), superoxide dismutase (3), catalase (2), glutathione peroxidase (44), and ascorbic acid (14).

Much of this research has led to the development of a host of anticataract drugs. These medications usually try to prevent oxidation from occurring with the use of cysteine, glutathione, or ascorbic acid (48). Their ineffectiveness and the conclusions of researchers point out some of the problems involved in cataract research. The lens is actually a very complex tissue. Cararactogenesis is probably caused by a combination of factors. A great deal more needs to be known about the structures of the lens proteins, their organization, and their relationship to lens metabolism.

LENS COMPOSITION

As in most tissues the lens is mostly water. Klethi et al. (35) have determined the water content of a variety of species. Fish lenses contain about 50% water, mammals range from 60% to 70%, and birds' lenses have about 75% water. This range is probably due to an animal's need for the lens to accomodate (35).

For all practical purposes, the remainder is all protein. The total organic nonprotein substances, which include glutathione, lipids, ascorbic acid, and inositol, amount to only one to two percent (51).

As mentioned previously, the protein fraction is separated on the basis of its solubility. Typical procedures involve homogenization in dilute buffer and then centrifugation to separate the water soluble fraction (WSF) from the water insoluble fraction. The water insoluble fraction can undergo several more steps of homogenization and centrifugation to purify it from the WSF. Treatment with 8 M urea is used in some procedures to yield an urea soluble fraction. Water insoluble proteins represent about 11% of the total protein in old normal human lenses (11).

WSF is comprised of 90% crystallins (5). The crystallins constitute the structural proteins in the vertebrate eye lens. When the WSF is run through a

gel filtration column or a size exclusion column of HPLC three basic fractions are eluted. These three fractions elute from the WSF of all species of vertebrates tested (8). The first two consist of a mixture of multimeric proteins while the last fractions, the lowest molecular weight, is made up of monomeric proteins. On the basis of highest molecular weight first and the order in which they elute the fractions are named alpha, beta, and gamma. In birds and reptiles the final eluting fraction is designated delta (28). Birds in particular have lenses which are capable of a high degree of accomodation. This special requirement might account for the difference in protein composition.

Beta crystallin is the major protein in all except very young lenses and generally increases gradually throughout life (63). It is the major protein in mature lenses of many mammals. Gamma crystallin, on the other hand, is a minor component in all but very young lenses. The lens nucleus is rich in gamma while the cortex, expecially the young cortex, has a low percentage of gamma (38).

WATER INSOLUBLE PROTEINS

Being insoluble and very heterogeneous makes this component of the lens proteins the most difficult to analyze. It is mainly made up of membrane and high molecular weight (HMW) aggregates (initially termed matrix proteins or albuminoid). Aging of the lens results in increasing amounts of insoluble protein (52,57). The extreme of this process is probably the senile nuclear cataract lens where up to 90% of the total protein is insoluble (1,45).

The membranes can be separated from the HMW aggregates by washing with 8 M urea (41), or by gradient centrifugation (9). Analyzing these membranes by SDS-PAGE reveals the major lens membrane proteins. In humans there are two major intrinsic protein populations: 26,000 daltons and 22,000 daltons (50). The 26K population is highly conserved throughout evolution (62), while the 22K seems present only in the human lens, perhaps due to decades of aging. Garner et al. have shown that the 22K protein is a degradation product of 26K (21).

HMW aggregates are usually a heterogeneous mixture of many different molecular weights. The polypeptides can be held together by noncovalent or covalent bonds (24). It is nearly impossible to separate into a homogeneous sample. HMW aggregates

can be found in the WSF, in the urea-soluble fraction, or, of course; the water insoluble fraction (22). Almost any protein in the lens can be found in the HMW aggregates.

Particular attention is paid to the aggregates resulting from cataractogenesis. Harding found aggregates formed by protein-glutathione and protein-protein disulfide bonds (27). Membranes disintegrate and release polypeptides into both the WSF and insoluble fractions. The membrane and membrane fragments are involved in covalent high molecular weight aggregates with an extrinsic membrane protein (43,000 daltons) and a cytoplasmic protein (gamma crystallin) (22).

ALPHA CRYSTALLIN

Alpha crystallin is the easiest crystallin to purify.
It elutes in a single peak and contains very little if any
contaminants from the beta crystallin peaks. Understandably,
it has been well characterized. This multimeric lens
protein consists of large aggregates composed of similar
subunits with marked variations in electrophoretic
mobility (40). In bovine lens these aggregates can be
separated into two peaks (54). Molecular weights of the
aggregates can range from 800,000 to more than 5×10^7 (55).

The subunits comprising alpha crystallin are believed
to originate from two related gene products termed alpha A_2
and alpha B_2 (65). These two are the predominant
constituents of alpha crystallin from the calf lens,
whereas the deamidated analogs are abundant in the old
bovine lens (69). Calf cortex contains four subunits of
alpha crystallin: A_1, A_2, B_1, and B_2. Older bovine lens
cortex contains an additional subunit, N_1. The nucleus of
both ages contains four more subunits, designated as
AA_1, AA_2, AA_3, and N_2. All of these subunits have
molecular weight of about 20,000 (5). Much work has been
done on these subunits. A_1 and B_1 are products of a
deamination _in vivo_ of A_2 and B_2 respectively (6,58).
AA_1 and AA_3 result from an intracellular degradation
of A_1 and A_2 respectively (68). Other subunits appear
to undergo similar posttranslational modifications (69).
The observed specific limited degradation of subunits

increases with aging of the tissue. Deamination, however;
does not appear to be related to aging (68). These studies
have given us a clearer picture concerning the
heterogeneity and modification related to crystallin subunit
structure.

Immunological interspecie studies of alpha A and
alpha B polypeptides have shown these subunits to be
remarkably conserved. This conservation over a wide
evolutionary range reflects stringent constraints on the
three-dimentional structure of alpha crystallin (46).

BETA CRYSTALLIN

In terms of molecular weight and isoelectric point beta crystallin is the most heterogeneous crystallin. Much of the research of beta crystallin has been done on bovine lenses. In this multimeric protein, beta crystallin can be separated into two classes, termed $beta_H$ (H=high) and $beta_L$ (L=low) (63). These two classes have molecular weights of 200,000 and 50,000, respectively (72). Their composition consists of identical polypeptide chains with molecular weights ranging from 24,000 to 35,000 daltons (29).

This composition of beta crystallin is highly conserved throughout evolution (74). There appears to be more complexity in the human lens. Beta crystallin from human lens contains three peaks called $beta_1$, $beta_2$, and $beta_3$ from the nomemclature of Kramps et al. (32,36,49). These peaks have molecular weights of 140,000, 82,000, and 40,000 respectively (32), consisting of identical subunits of 18,000, 23,000, and 28,000 daltons (36). Zigler et al. have found all three peaks to be similar with small differences when examined on SDS and isoelectric focusing gels. Immunologically all three peaks cross-react with each other and with gamma crystallin (71). This indicates that there is at least one identical antigenic site found in both human beta crystallin and gamma crystallin.

Circular dichroic spectra for B_1, B_2, and B_3 are all quite similar. They appear to consist primarily of beta pleated sheet and random coil conformation without significant alpha-helical structure (31,39).

Also of interest in beta crystallin is the presence of a 43,000 dalton polypeptide. It is found only in human beta crystallin and appears to increase with age (23). Coincidentally, a 43,000 dalton component is also found in the water insoluble human lens that is immunologically similar. Spector has observed that this polypeptide contains a high concentration of non-tryptophan fluorescence and has suggested that it becomes associated with the lens plasma membranes as an extrinsic membrane protein (56).

Beta crystallin contains at least five different primary gene products (30,64). In bovine lens $beta_H$ there are at least nine chains and in $beta_L$ there are at least eight as shown by basic urea gel electrophoresis. SDS gel electrophoresis shows five bands for $beta_H$ and four for $beta_L$ (15). One polypeptide chain in particular occurs often and throughout all beta crystallin from all species tested (73). This major beta crystallin chain is termed beta B_p (B-basic; p=principal). The partial primary structure of the beta B_p chain has been resolved (17). It contains not only an internal duplication but reveals also homology with gamma crystallin.

GAMMA CRYSTALLIN

The water soluble low molecular weight protein of lenses
is called gamma crystallin. These are the monomeric proteins
which are the last proteins to elute from the gel filtration
column. Gamma crystallin has been of increasing interest
to lens researchers ever since Papaconstantinou's first
observation that a new gamma crystallin emerges with age (43).

Human gamma crystallin can be separated into four low
molecular weight protein fractions. In order of decreasing
molecular weight they are designated as $beta_s$ ($beta_s$ = slow
moving), gamma H (H=heavy molecular weight), gamma L_1 (L_1=
first light molecular weight), and gamma L_2 (L_2=second light
molecular weight). They have molecular weights of 28,000,
24,000, 20,000, and 11,000 respectively (34). Bjork was
able to separate gamma crystallin into six peaks. Four of
the peaks ($beta_s$ was probably not included) behaved as
single components by gel electrophoresis and
immunoelectrophoresis. These were probably homologous
proteins, but did differ distinctly according to sulphydryl
group content, chromatographic properties, and amino acid
compositions (4). N-terminal sequences were similar for
all of the fractions crystallized (4). The structure of
gamma crystallin has been determined by x-ray diffraction
of 5.5 $\overset{o}{A}$ resolution. The molecule is arranged in two
globular domains with a radius of approximately 25 $\overset{o}{A}$. The
whole molecule appeared to be an ellipsoid with main axes

of about 55 x 30 x 25 $\overset{o}{A}$ (7).

The beta$_s$ protein has characteristics of the beta crystallins and definitely is a distinct protein from gamma H or gamma L$_1$. In older human lenses gamma H and beta$_s$ increase while gamma L$_1$ decreases. This observation has also been made for the bovine lens (33). The 11,000 dalton peptide (gamma L$_1$) increases with age in the human lens (43).

Several researchers have found evidence that the low molecular weight protein content decreases during the formation of cataract (12,13,19,40,59). This observation can easily be made by examining elution profiles. Other experiments also implicate gamma crystallin's involvement with cataractogenesis. Clark et al. have observed that alpha crystallin increases while gamma crystallin decreases in both aging and cataract formation. This change is more pronounced in the cataract. Gamma crystallin are surprisingly much more liable to precipitation while alpha and beta crystallins, being composed of polymers, change very little (19). There is evidence that gamma crystallin can be lost from the senile cataract by leakage (12). Garner has found the presence in cataracts of a number of unique complexes containing cytosol and membrane components. These disulfide linked aggregates contained gamma crystallin as the major cytosol species (24).

SECTION III

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SECTION IV

MANUSCRIPT: CHARACTERIZATION OF MONOCLONAL
ANTISERUM TO HUMAN GAMMA
CRYSTALLIN IN AGING
HUMAN LENSES

ABSTRACT

A monoclonal antiserum was produced which, unlike polyclonal antiserum, reacts preferentially to one of the major gamma crystallin polypeptides found in the lens. Further characterization demonstrates that this monoclonal antiserum is species-specific, binding only to gamma crystallin of human origin. The antigenic site was found to increase dramatically in number between the ages of two and eight. Western blot analysis with the probe was able to identify the proteins containing the antigenic site, suggesting relationships between these proteins which were previously indistinguishable.

INTRODUCTION

Most of the research done on the lens involves separation of components and their analysis. Identification of a protein has usually been made according to its molecular weight in SDS gel electrophoresis, its elution profile in gel filtration chromatography, or how well it reacts to a polyclonal antiserum. Accurate identification in cataract research is critical since the very process of cataractogenesis involves changes in proteins. Lens transparency is dependent on protein structure and resultant protein-protein interactions (3,15).

Aging and/or cataractogenesis alters these parameters, as shown by quantitation of accessible sulfhydryl (6) and amino groups (5). A need exists for specific probes that can identify a protein when its physical properties are altered.

Several researchers have found evidence that the gamma crystallin fraction decreases in protein content during the formation of cataract (1,2,4,10). Another has demonstrated changes in gamma crystallin's antigenic sites during cataractogenesis with the use of monospecific antisera purified from polyclonal antisera (13).

In this mnauscript the development of a monoclonal antibody specific to a major polypeptide of gamma crystallin is documented. This specific probe is used in a spot blot assay to measure the presence of the antigenic site in lenses of different ages. To identify the proteins containing

this site Western blot analysis was performed. These methods demonstrate the usefullness of monoclonal antibodies in furthering our understanding of changes in proteins of the lens.

METHODS AND MATERIALS

PURIFICATION OF GAMMA CRYSTALLIN: Normal human lenses were
obtained from the National Diabetes Research Interchange
and kept frozen at $-85^{\circ}C$ until use. Bovine lenses were
donated by a local slaughterhouse and were immediately
frozen at $-85^{\circ}C$ following dissection. Mice were sacrificed
for their lenses just prior to use.

To purify gamma crystallin decapulated lenses were
homogenized in approximately 10X volume of .1 M Na_2SO_4 and
0.06 M NaH_2PO_4 at pH 7.0. The homogenate was centrifuged
at 27,000xg for 15 minutes. Fractionation of the
supernatant was performed isocratically with the same
buffer on HPLC. A TSK 3000SW column (Altex was used with
a flow rate of one ml/min. The crystallin peaks (fig. 3)
of several runs were pooled, dialyzed in 1 mM NH_4HCO_3,
and lyophilized.

PRODUCTION OF POLYCLONAL ANTISERUM: Polyclonal antiserum
to human gamma crystallin was produced in rabbits as
described (6).

PRODUCTION OF MONOCLONAL ANTISERUM: Immunization of Balb/C
mice for fusion was accomplished with injections of 100
micrograms of human gamma crystallin. The antigen dissolved
in 100 microliters of 50% complete Freund's adjuvant was
injected interperitoneally every 30 days. Four days after
the third injection the spleen was removed for fusion. The

fusion was performed according to the protocol of Kohler
and Milstein (8). Fused cells were plated into 4, 24 well
plates. After two weeks, cell growth was observed in 17
of 96 wells, indicating statistically that these were
single colonies. After another two weeks of growth the
supernatants were tested for reactivity to human gamma
crystallin spotted on nitrocellulose. Of the 17 original
wells, only one reacted positively and could be visualized
with radioiodinated protein A. This well was cultured until
approximately 3×10^5 cells could be injected interperitoneally
into each of 3 Balb/C mice. After 40 days one of the mice
showed growth of a solid subcutaneous tumor near the site
of injection. Antisera from tail bleedings of this mouse
contained high concentrations of the monoclonal antibody.

ISOELECTRIC FOCUSING: Isoelectric focusing was performed
in slab gels using 5% polyacrylamide and 2% ampholines
(Servalyt 4-9T, Serva Fine Biochemicals). SDS, NP-40,
urea, an d 2-mercaptoethanol were omitted from all solutions
to prevent denaturation of antisera. Otherwise, the basic
protocaol of O'Farrell was used (11).

GEL ELECTROPHORESIS: 7.5% polyacrylamide gels were run
essentially according to the protocol of Laemmli (9). SDS
was omitted from all solutions. 6 M urea was included in
the stacking and resolving gels. The reservoir buffer
was adjusted to pH of 8.0 and electrophoresis was performed
at 20MA.

IODINATION OF PROTEIN A AND GAMMA CRYSTALLIN: Proteins to be iodinated were dissolved in 200 microliters of .05 M Tris pH 7.4. 200 microcuries of ^{125}I (Amersham) and 20 microliters of 1 mg/ml. chloramine T (Sigma) were added. The reaction was allowed to proceed for one minute before quenching with 20 microliters of 1 mg/ml. sodium metabisulfide. The resulting mixture was dialyzed against .05 M Tris pH 7.4.

WESTERN BLOTTING: Transfer of proteins from isoelectric focusing and polyacrylamide gels to nitrocellulose (Schleicher and Schuell) was performed in an electroblot chamber (E-C Apparatus Corporation) according to Towbin et al. (14). After transfer, the sheet of nitrocellulose was blocked for at least one hour, incubated with antisera for at least two hours, and incubated with radiolabeled protein A (or radiolabeled crystallin for testing focused antisera) for at least one hour.

SPOT BLOTTING: This immunoassay method was performed as described by Jahn et al. with slight modifications (7). A sheet of nitrocellulose 8.8 x 2cm was squared off into .8 x 1cm sections with a pen and ruler. Lyophilized samples were diluted in distilled water at predetermined concentrations. Five microliters of a sample were spotted to each .8 x 1cm square. The nitrocellulose sheet was

allowed to dry at room temperature before incubation in blocking buffer (2% bovine serum albumin, 0.1% NaN_3, 50mM EDTA, and 10mM Tris-HCl pH 7.5). After blocking for one hour the sheet was incubated for another hour in blocking buffer, this time containing either a 1:333 dilution with monoclonal antiserum, or a 1:3333 dilution with polyclonal antiserum. The sheet was then washed four to five times in blocking buffer without bovine serum albumin or sodium azide. It was left in this solution with 3×10^6 cpm of radioiodinated protein A (5 micrograms) for one hour. Finally, after extensive washing, the blot was either exposed to x-ray film overnight, or the squares cut out and counted on a gamma counter and averaged.

RESULTS

Protocol for the development of a monoclonal antibody generally calls for two clonings of the cells in positively tested wells. In this particular fusion the low number of wells with hybridoma growth (17 of 96) is statistically well within the range to be deemed a clone. Unfortunately, cloning, as well as growing, of this cell line in tissue culture proved difficult, if not impossible. Isoelectric focusing was performed to further verify that the antibodies, available in ample supply from murine serum, are actually of monoclonal nature. To prevent denaturing of the antibodies, SDS, NP-40, urea, and 2-mercaptoethanol were left out of all solutions. Several antisera were focused, transferred to nitrocellulose, blocked and probed with radioiodinated gamma crystallin or radiolabeled protein A. The results (figure 1) show that the labeled antigen reacts to only one band of focused antibodies (lane E). This indicates the presence of monoclonal antibodies to gamma crystallin. Lane B, monoclonal antiserum probed with radiolabeled protein A shows the presence of a large contingent of antibodies. Other controls show normal mouse sera with a similar contingent of antibodies (lane C) reacting negatively to radiolabeled gamma crystallin (lane G). Rabbit polyclonal antiserum to gamma crystallin reacts well to both radiolabeled probes as expected (lanes a and d).

Immunological interspecies studies of lens proteins
with polyclonal antiserum generally have shown that they
are highly conserved. When the monoclonal antibody is
tested against crystallins from three different mammalian
species it reacts with only the gamma crystallin from
human lens (Figure 2).

Figure 3 shows elution profiles of an old and a
young lens WSF from HPLC. The peaks from the old lens
WSF show less resolution as expected due to aging, but
do correspond well with the peaks from the young lens WSF.
Some peaks show decreases or increases with aging. The
gamma crystallin peak from the old lens elutes slightly
ahead of the gamma crystallin peak of the young lens as
previously reported (6).

Gel electrophoresis as described in Materials and
Methods is designed to separate the gamma crystallins
in their native state according to each protein's electrical
charge. Gamma crystallin from young lens is clearly
separated in two components, designated gamma A and gamma
B (Figure 4, left lane A). A narrow band is observed
between these two which is probably protein caught at the
interface of the resolving and stacking gels. In lane B
(left) the old lens gamma crystallin is seen as a series
of bands with increasingly negative charges as they proceed
down the lane. Gamma A has nearly disappeared. The more
negatively charged species could be related to gamma A or
gamma B. Peptide mapping of the two show related but

distinct patterns (Zigler, S., Takemoto, L., Hansen, J., and Horwitz, J., submitted for publication).

When the same two lanes are blotted onto nitrocellulose and probed with polyclonal antiserum (Figure 4, center) and monoclonal antiserum (Figure 4, right) more information is gained. The polyclonal antiserum binds to all species of gamma crystallin while the monoclonal antiserum binds to only gamma B and the negatively charged species. This suggests a common antigenic site between gamma B and the negatively charged species. Gamma A does not contain this site.

The binding of antigen by radioactive probes can easily be measured quantitatively (7). The results of figure 5 verify that the methods in this report are similarly quantifiable. This graph shows a linear relationship for both antisera over a range of different concentrations of antigen spotted on nitrocellulose. The remaining experiments will use .4 micrograms per square, as these data show that this concentration is well within the linear range.

Quantitative spot blotting was performed with gamma crystallin from several different aged human lenses. The results (table 1) demonstrate that the monoclonal antiserum can recognize subtle changes in the gamma crystallin that the polyclonal antiserum cannot. The polyclonal antiserum binds to all ages at about the same intensity. In contrast, the monoclonal antibody binds poorly to the youngest lenses and binds much better to older lenses.

Experimental error, background, and binding inconsistencies of individual lenses can be reduced by comparing the values of polyclonal bound to monoclonal bound in a ratio. The graphed results show dramatically that the monocloanl antiserum recognizes a change in gamma crystallin. This change occurs between the ages of two and eight (figure 6).

DISCUSSION

Proteins have been the primary focus of lens researchers, since it is believed that proteins are responsible for the property of transparency in the lens. Consequently, cataractogenesis could easily be a change in the same protein responsible for transparency. Research to date indicates that these intricate relationships or changes, whether involving long range lattice structure (15) or short range protein-protein interactions (3), need to be understood on a structural basis.

Structural probes, like antibodies, can identify small regions of a protein's structure and any changes that may occur. Monoclonal antibodies would be the most accurate of structural probes since they recognize only one site with a single type of antibody. In this report a monoclonal antiserum is used to demonstrate a methodology that recognizes subtle changes in a polypeptide. This method, adapted from Jahn et al. (13) with minor variations, is very sensitive and can be used with experiments where variables occur in individual lenses or individual sections of lenses (12). As low as .1 microgram of protein can be quantitatively assayed as shown in figure 5.

Gel electrophoresis and the consequent blots show that the monoclonal antiserum binds to a site specific to only gamma B. Further aging of the lens creates a series of more negatively charged bands. Many if not all of these

species react to monoclonal antiserum, indicating that gamma B and these species have an antigenic site in common. This demonstrates that these proteins are similar, and distinct from gamma A.

Increased binding to gamma B by the monoclonal antiserum is probably related to aging. Table 1 demonstrates a three-fold increase when comparing young lenses (less than 2 years) to old lenses (eight years and more). Two possibilities exist that can explain the molecular nature of this increased binding. The first is that there simply is a higher percentage of gamma B in the gamma fraction originally analyzed. This could mean increased synthesis of the gamma B polypeptide. The other alternative is that age-related structural changes occur to gamma B. These changes, or perhaps only one of them, allow the monoclonal antiserum to bind to the antigenic site on gamma B. Gamma B, as well as the more negatively charged species, could be the result of proteolysis, deamination, or other covalent mechanism. When considering the low rate of synthesis and the small gain in lens volume during this period of increased binding, the latter possibility appears to be more likely.

According to the results of figure 6, the change creating the more negatively charged series occurs between the age of two and eight. It remains constant for the lifetime of the patient. A follow-up experiment would be to determine the amino acid sequence that contains this change. The production of additional hybridomas could greatly further the understanding of how these relate to each other.

SECTION III

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Fig. 1. Western blot of isoelectric focusing gel
containing focused antisera. Each lane containing 50
micrograms of protein. a and d) polyclonal antiserum
to human gamma crystallins; b and e) monoclonal antiserum
to human gamma crystallins; c and f) normal sera taken
from a Balb/C mouse; a, b and c) lanes reacted to
radioiodinated protein A; d, e and f) lanes reacted to
radioiodinated human gamma crystallin.

$$\alpha \quad \beta \quad \gamma$$

HUMAN •

BOVINE

MURINE

Fig. 2. Spot blot of all three crystallin (alpha, beta, and gamma) from three mammalian species probed with monoclonal antiserum. Approximately 0.4 micrograms of each sample was applied to a 1 cm. x 1 cm. square sectioned from a 3 cm. x 3 cm. sheet of nitrocellulose. Visualization was by incubation with 3×10^6 cpm of radioiodinated protein A followed by overnight antoradiography.
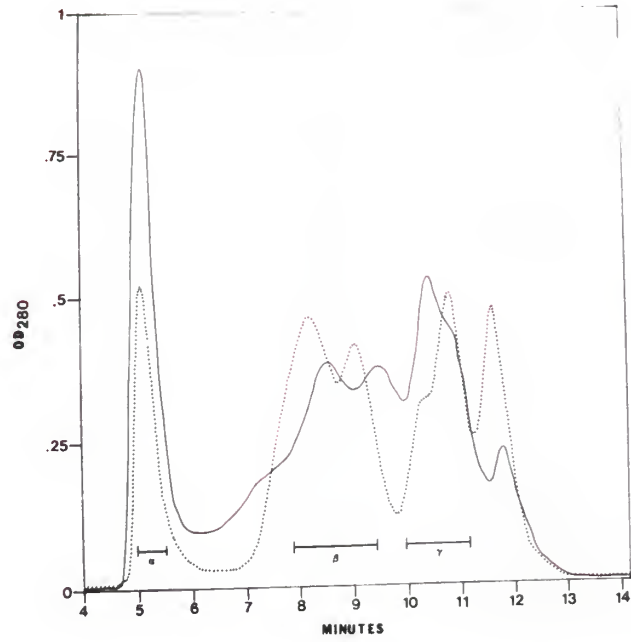
Fig. 3. HPLC elution profile of young versus old human
lens water soluble fractions. The dotted line represents
crystallin profiles from two month old human lens. The
solid line represents crystallin profiles from 51 year old
normal human lens. Bars represent the alpha, beta, and
gamma fractions taken for further analysis.

$\gamma_A \rightarrow$

$\gamma_B \rightarrow$

$\leftarrow \gamma_A$

$\leftarrow \gamma_B$

a   b
STAINED
GEL

a   b
POLYCLONAL
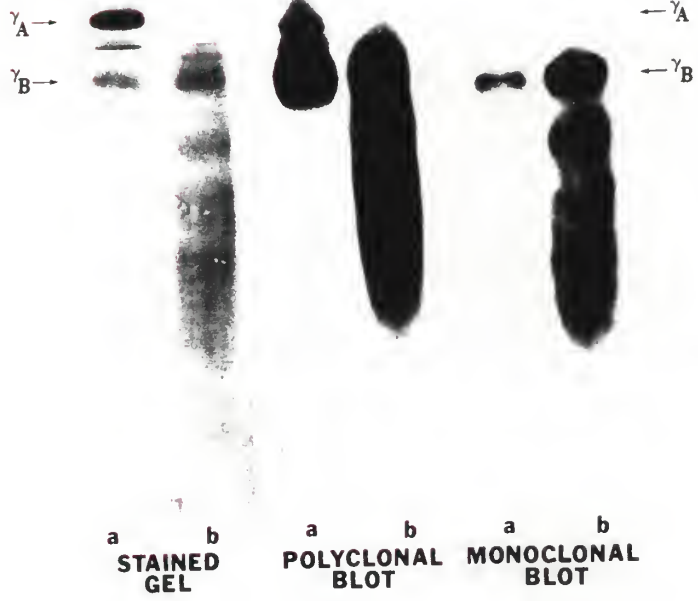BLOT

a   b
MONOCLONAL
BLOT

Fig. 4. Gel electrophoresis of young versus old gamma
crystallin. Approximately 15 micrograms of protein applied
to each lane of a 7.5% acrylamide gel without SDS,
containing 6 M urea. a) gamma crystallin from 3 month
old lens; b) gamma crystallin from 66 year old normal lens.
The left two lanes show a commassie blue stained gel of
the two samples. The center two lanes are a western blot
of the two samples probed with polyclonal antiserum to
gamma crystallin. The right two lanes are a western
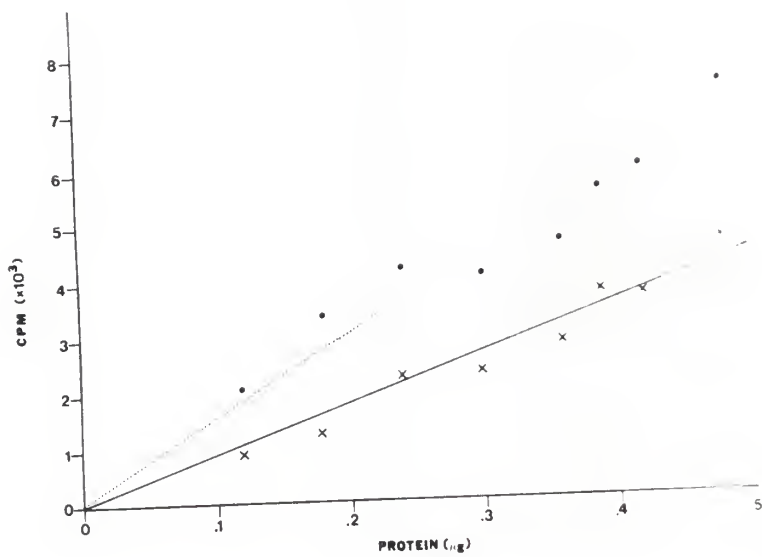blot probed with the monoclonal antiserum.

Fig. 5. Linearity of spot blot assay. Two sets of
increasing amounts of protein from the gamma crystallin
of a 51 year old normal human lens were used. Each
point is the average of two values. The dotted lines
represents the values obtained from one set probed with
polyclonal antiserum. The solid line represents the
values obtained from the other set probed with monoclonal
antiserum.

Table 1. Binding of monoclonal and polyclonal antisera to
         gamma crystallin of different age.

Age Human Lens (yrs)	Binding Polyclonal (cpm)	Binding Monoclonal (cpm)
0.12	8079	2090
0.5	8598	2076
0.75	9516	2428
1.9	6856	1882
8	7304	5530
19	7256	4624
27	8314	7988
37	9002	6414
51	10374	8526
66	8566	5790
85	9558	7362

A series of lenses from young to old ages were prepared
as described in Materials and Methods for gamma crystallin.
The dialyzed and lyophilized samples were dissolved in
water to a concentration of .08 micrograms per microliter
and 5 microliters were spotted onto nitrocellulose in
duplicate. These spotted squares were then treated
with antiserum and radioiodinated protein A before being
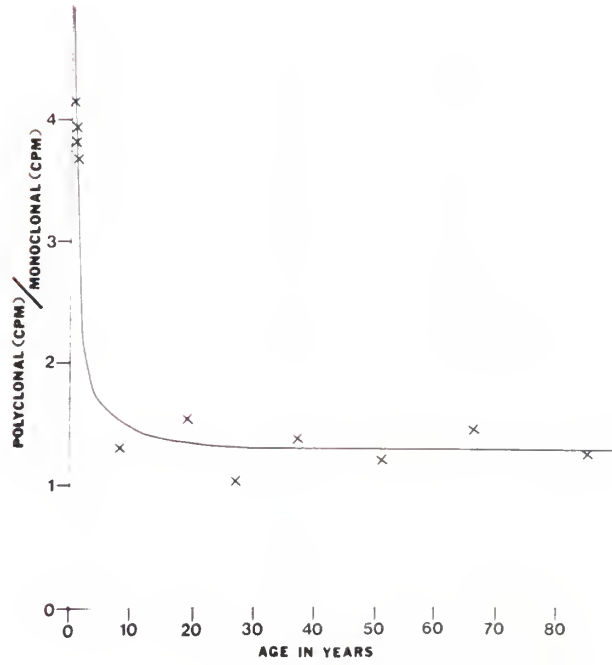counted on a gamma counter.

Fig. 6. The values from table 1 are compared by graphing the ratios of polyclonal/monoclonal binding to gamma crystallin for each lens of different age.

CHARACTERIZATION OF MONOCLONAL ANTISERUM TO HUMAN GAMMA
CRYSTALLIN IN AGING HUMAN LENSES

by

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B.S., Iowa State University, 1976

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ABSTRACT

A monoclonal antiserum was produced which, unlike polyclonal antiserum, reacts preferentially to one of the major gamma crystallin polypeptides found in the lens. Further characterization demonstrates that this monoclonal antiserum is species-specific, binding only to gamma crystallin of human origin. The antigenic site was found to increase dramatically in number between the ages of two and eight. Western blot analysis with the probe was able to identify the proteins containing the antigenic site, suggesting relationships between these proteins which were previously indistinguishable.