THE EFFECTS OF ANTI-LINK PROTEIN ANTIBODY ON RAT MIDFACIAL GROWTH AND DEVELOPMENT

by

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ABSTRACT

THE EFFECS OF ANTI-LINK PROTEIN ANTIBODY ON RAT MIDFACIAL GROWTH AND DEVELOPMENT

The effects of anti-link protein monoclonal antibody on rat midfacial growth and development were examined. Anti-link protein monoclonal antibody (mAb8A4) has been found to bind to all forms of link protein and has been used to identify and quantify link protein on electrophoretic gels. The antibody was injected into growing rats from birth to 30 days.

Cephalometric analysis of rats injected with anti-link protein monoclonal antibody did not demonstrate a statistically significant ($p \leq .05$) reduction in midfacial dimensions.

These studies indicate that some other antibody present in the anti-rat cartilage antibody used by Hans (1981) caused significant reduction in rat midfacial development. Further studies should be conducted to determine what monoclonal antibodies directed at molecules in the extracellular matrix of cartilage will cause significant growth effects. These experimental approaches provide the means for better

understanding the cellular and molecular basis for craniofacial growth and abnormalities.

DEDICATED TO

My parents, William and Catherine.

My wife to be, Ana.

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INTRODUCTION

The mechanisms controlling facial growth have been a subject of intense interest in the field of orthodontics and craniofacial biology for many decades. Although various factors have been demonstrated to have an influence on facial growth, the precise signals which initiate cell division and differentiation have remained a mystery.

Early investigators hypothesized that the craniofacial cartilages (nasal septal, condylar and synchodrosal) acted as "growth centers" for controlling growth of the midface, mandible and cranial base, respectively. Current theories of facial growth generally recognize multiple influences on craniofacial growth such as genetic, intrinsic and extrinsic growth factors and environmental influences.

Studies have been done with the goal of controling facial growth by targeting individual growth cartilages within the craniofacial complex with specific antibodies. The first of these was done by Brigham in 1976. He demonstrated antigenic differences among the cephalic cartilages using

indirect immunoflurescence. Palmer (1978) injected growing rats with anti-rat nasal septal cartilage antisera raised in rabbits. He reported histologic evidence of cytopathic effects on the nasal septum chondrocytes and the localization of the antisera within the nasal septal cartilage. Hans (1981) injected a similar anti-nasal septum antisera into growing rats. He observed a statistically significant reduction in midfacial growth of the rats. In 1991, Nguyen characterized the proteins in the extracellular matrix of rat nasal septal and condylar cartilages using electrophretic gels. He then tested the antisera used by Hans (1981) on these proteins to determine which ones were reactive with the antisera. The anti-nasal septum antisera was found to be reactive with small proteoglycans containing dermatan sulphate, large proteoglycans and link protein. In addition, Nguyen tested monoclonal antibodies directed against known structural proteins of cartilage.

The present study evaluates the effects of monoclonal antibody 8A4, specific for link protein, on the midfacial growth

of rats from birth to 30 days.

LITERATURE REVIEW

Cartilage is a specialized form of connective tissue consisting of chondrocytes embedded in an extracellular matrix. The chondrocytes reside in lacuna and synthesize and secrete all of the macromolecular components of cartilage. They are a classic example of protein secretory cells with an elaborate rough endoplasmic reticulum and a well developed golgi complex. Chondrocytes have surface indentations and protrusions that serve to increase cell membrane surface area which is important since cartilage is avascular and is nourished by diffusion from capillaries of the perichondrium or by means of synovial fluid (articular cartilage)(Godman and Porter, 1960; Goel, 1970; Searls et. al., 1972; Thorogood and Hinchliff, 1975; Junqueira et. al.,1986). Likewise, no lymphatics or nerves are found within cartilage (Junqueira et. al., 1986). The extracellular matrix is composed of collagen fibrils, which gives cartilage its tensile strength and maintains its shape, and proteoglycan molecules that gives the tissue its osmotic properties.

The extracellular matrix gives cartilage the firm consistency and resilience which allows the tissue to withstand mechanical stresses. Its gel-like consistency depends on

electrostatic bonds that occur between collagen fibers and the glycosaminoglycan side chains of matrix proteoglycans. There is also an affinity for water to the negatively charged glycosaminoglycan chains extending from the proteoglycan core proteins.

The extracellular matrix of cartilage consists predominantly of water, collagen and proteoglycan. Water makes up 60-80% of cartilage and the remaining 20-40% is composed of collagen, proteoglycans, glycoproteins, lipids and cells. Type II collagen, consisting of 3 alpha I type II chains, makes up 40% of the dry weight of hyaline cartilage. Proteoglycan (constituting approximately 25% of the dry weight) consists of two polysaccharide moieties, chondroitin sulphate and keratan sulphate, attached to a core protein and N- and O-linked oligosaccharides (Myers and Mow, 1983). Many of the core proteins are attached non-covalently to hyaluronic acid chains, stabilized by link proteins, which form a proteoglycan aggregate.

Proteoglycan aggregates are made up of approximately 95% polysaccharide and 5% protein. The glycosaminoglycans, chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate, are disaccharide repeating units with at least one of the sugars having a negatively charged sulphate. This allows binding of large amounts of water. The glycosaminoglycans

are covalently linked to the core protein resembling bottle brushes (core protein=backbone, radiating glycosaminoglycan chains=bristles). It is believed that about 30-60 keratan sulphate chains are bound to a core protein in an average proteoglycan (Hascall and Heinegard, 1975; Hascall, 1977). Approximately 140 core proteins are non-covalently bound at intervals of 300 A to a filament of hyaluronic acid (Stryer, 1988). This interaction between the core protein and hyaluronic acid is promoted and stabilized by link protein . The resulting proteoglycan aggregate , with a mass of approximately 2 x 10 daltons and a length up to 4 um, is thought to be stiffly extended and entrapped in the collagen II fibrillar network.

Chondrocytes differentiate from mesenchymal cells derived from mesoderm if they give rise to limb and vertebral chondrocytes whereas cranial cartilage chondrocytes arise from the ectodermally-derived neural crest. The superficial mesenchyme becomes fibrochondrocytes of the perichondrium while the remaining cells differentiate into chondroblasts and subsequently into chondrocytes. The chondrocytes in turn synthesize and deposit all components of the extracellular matrix as well as other molecules. Differentiation of cartilage occurs from the

center outward with the peripheral cells being chondroblasts and the centrally located cells being chondrocytes.

This organization allows for the two types of growth observed in cartilage: interstitial growth due to mitotic division of chondrocytes and appositional growth due to differentiation of perichondral cells. Interstitial growth occurs during early phases of cartilage formation, at epiphyseal plates and in articular cartilage as cells and the matrix are worn away (no perichondrium present). As the extracellular matrix becomes increasingly rigid because of cross-linking of matrix components, interstitial growth becomes less pronounced.

Moss and Moss-Salentijn (1983) described three functional demands of cartilage:1) protection and support of related non-skeletal tissues and organs, 2) articulations between skeletal elements and 3) certain dynamic processes related to skeletal growth. These specific functional demands are directly related to the specific organization of the cells and extracellular substance.

Three forms of cartilage exist and each type is specialized for a particular function. Hyaline cartilage, the most common cartilage found in mammals, consists of type II collagen (Miller and Matukas, 1969; Miller, 1976; Trelstad et. al., 1970). In adult mammals, hyaline cartilage is found at articular surfaces, walls of larger respiratory passages (nose, larynx, trachea,

bronchi) and the ventral ends of ribs. Hyaline cartilage serves as a temporary skeleton in the embryo until it is replaced by bone. Hyaline cartilage is also found in the epiphyseal plate of growing long bones and is responsible for longitudinal growth of these bones. Elastic cartilage is another type of cartilage and is composed of type II collagen and elastic fibers. It is found in the auricle of the ear, the walls of the external auditory canals, the eustachian tubes, epiglottis and the cuneiform cartilage of the larynx. Fibrocartilage is the third type of cartilage and consists of collagen type I. It is a tissue with characteristics intermediate between those of hyaline cartilage and dense connective tissue and is found in intervertebral discs (annulus fibrosus), the symphisis pubica and in attachments of particular ligaments to bones.

Link Protein

Link protein functions in stabilizing the proteoglycan aggregate and plays a role in protecting a part of the proteoglycan monomer that binds to hyaluronic acid from proteolytic attack. It is believed that there is a 1:1 ratio of link protein with the proteoglycan monomer (Neame and Barry, 1993).

Link protein consists of three disulphide-bonded loops, an immunoglobulin fold near the N-terminus and two proteoglycan tandem repeat loops. These same three configurations are seen in the proteoglycan monomer and is called the G1 domain. Two additional tandem repeats are located in the G2 domain of proteoglycan monomer. The two tandem repeat loops of the link protein and the G1 domain of proteoglycan monomer bind to hyaluronic acid while the immunoglobulin fold of link protein interacts with the immunoglobulin fold of proteoglycan monomer (Perin et. al., 1987). The proteoglycan repeat is a repeat of 99 residues and is positively charged in link protein and negatively charged in proteoglycan monomer while the immunoglobulin folds of link protein and the G1 domain are neutrally and negatively charged respectively. The immunoglobulin fold resembles the variable domain in immunoglobulins (Bonnet et. al., 1986) and may be involved with recognition events (Williams, 1987; Williams and Barclay, 1988). It was also found that both the proteoglycan G1 domain and link protein consist of two distinct globular components that may supply the two functional areas of these proteins in proteoglycan aggregation (Perkins et. al., 1989; Hardingham and Fosang, 1992).

Human articular cartilage contains three link proteins based on their electrophoretic mobility on SDS polyacrimide gels, LP1

(48kDa), LP2 (44 kDa) and LP3 (41kDa) with LP1 predominating (Roughley et. al., 1982). LP1 has an additional N-linked oligosaccharide at residue 6 that is not present in LP2. It is the high mannose oligosaccharide structures that account for the link proteins microheterogeneity observed in SDS polyacrimide gels. LP3 is believed to be proteolytically derived from either LP1 or LP2 by the removal of 16 amino acid residues from the N-terminus (Nguyen et. al., 1989). Proteolytic cleavage within the N-terminal disulphide-bonded loop is also thought to give rise to link protein fragments (26-30 kDa)(Mort et. al., 1983) which along with LP3 accumulate throughout life. It is interesting to note that the metalloproteinase stromolysin cleaves link protein at the site identical to that accounting for the production of LP3 in the human neonate (Nguyen et. al., 1989). There is no evidence that these proteolytically derived forms are less capable in forming proteoglycan aggregates.

Maturation of chondrocytes is accompanied by an increase in the proportion of free monomer. This is not due to insufficient synthesis of link protein but may be related to a low hyaluronic acid supply or a lowered binding affinity of newly secreted monomer for hyaluronic acid (Plaas et. al., 1988). Flannery et. al. (1990) also found no evidence of marked degenerative changes in the structure of link proteins

with maturation in the rabbit. There was some evidence of fragmented link proteins in mature rabbits similar to those seen in humans but the proportion was much less (5%). This may be due to the extended life span of humans.

Link protein from equine, bovine, porcine, chicken, rabbit, canine and rat chondrosarchoma have been studied. All except rat chondrosarchoma exhibit two types of link protein while rat chondrosarchoma proteoglycan possessed only one link protein (Liu et. al., 1992; Fife et. al., 1985; McKeown-Longo et. al., 1982; Plaas et. al., 1988). The two link proteins in the above species are LP1 and LP2 forms while the single link protein found in rat chondrosarchoma is an LP2 form (Liu et. al., 1992).

Link protein amino acid sequence is highly conserved between species with the differences being at the extreme N-terminus of the molecule. The complete amino acid sequences of chicken (Deake et. al., 1986), rat (Neame et. al., 1986), rat chondrosarchoma (Rhodes et. al., 1988) and pig (Perkins et. al., 1989) have been identified and a partial link protein sequence for human (Perkins et. al., 1989) and bovine (Bonnet et. al., 1986) have been identified.

Osborne-Lawrence et. al. (1990) have concluded that the human gene for cartilage link protein is localized at position 5q13-q14.1. To date, no disorders associated with

abnormalities of the human cartilage link protein gene have been mapped to the long arm of chromosome 5.

Link Proteins Found in Other Tissues

Link protein and associated molecules have been found in several different cells and tissues. Link proteins have been reported in the aorta (Gardell et. al., 1980; Vijayagopal et. al., 1985; Barday et. al., 1985), synovium (Fife et. al., 1985) and eye (Poole et. al., 1982) although their function has not been entirely identified. Different proteoglycans capable of aggregating with hyaluronic acid have been found to be synthesized by smooth muscle cells, colon cells, tendon, sclera, skin fibroblasts, lung tissue, embryonic bone and glial cells (Kimura et. al., 1987). Herring and Sandell (1991) have found link protein mRNA in canine synovial fibroblasts, human dermal fibroblasts and abundant levels in human notocord. The finding of abundant link protein mRNA in human notocord suggests a possible role of link protein in development.

Embryonic Origin of the Nasal Septum and Associated Structures

Between the fourth and sixth week of human embryonic development the nasal septum begins to develop from the frontal prominence forming a vertical zone of tissue between the merging medial nasal swellings. At the same time, the paired palatal shelves form from each side of the maxillary arch with the tongue interposed. Due to this tongue position, the shelves enlarge downward in an oblique direction. The downward growth of the lower face brings the tongue with it. This allows for the palatal shelves to expand medially. By differential growth, the shelves rotate upward in order to contact one another (Enlow, 1982). The nasal septum then merges with the superior surface of the palate separating the two nasal chambers.

Throughout fetal life, the cartilage of the cranial base and nasal septum are continuous (Young, 1960). On the ventral surface of the nasal septum, the vomer develops within its perichondrium. Once the presphenoid ossification begins it spreads forward into the nasal septum forming a portion of the perpendicular plate of the ethmoid. This structure continues to grow in a downward and forward direction until it unites with the vomer. This growth occurs at the expense

of the cartilage of the nasal septum. By age 7 only the anterior portion of the nasal septum remains as cartilage (Scott, 1953).

Differential growth of the various parts of the two nasal chambers and the oral cavity occurs to different extents and at different rates as the whole midface increases in size. The result is a balanced, harmonious face that continues to grow even through adulthood (Behrents, 1986).

The Role of Cartilage in Craniofacial Growth

Craniofacial cartilage functions in conjunction with particular growing bones in the form of the nasal septum, the synchondrosis of the cranial base and the condylar cartilage of the mandible. Several theories have been set forth to explain the intimate association between bone and cartilage. Early studies centered around the belief that craniofacial cartilages possessed an intrinsic growth potential that is relatively unaffected by local environmental factors.

Wolff's law of bone transformation (1899), stated that bone accomodates its structure to its functions. Most attempts to apply Wolff's law have not distinguished between the physical forces operating on a bone and forces operating on the periosteum or cartilage. In 1968, Enlow found that it is

actually the forces acting on the periosteum or growth cartilage that form and model the bone.

Other investigators believed that the growth of bone followed an intrinsic genetic program. This genetic program was thought to reside in the nucleus of bone producing cells of the sutures, periosteum and related cartilages. The mandibular condyles, sutures and synchondroses were considered master control centers that regulated the tissue separating force that resulted in the displacement and enlargement of bones. The downward and forward growth of the maxillary complex was once thought to be directed by innate growth at the bony sutures (Keith and Campion, 1922; Weinman and Sicher, 1955; Sicher and Debrul, 1970). Enlow's (1982) elaborate characterization of depository and resorptive fields in the skull has shown that many of the facial bones actually undergo resorption on their anterior aspect. In fact, the majority of periosteal deposition that occurs suggests an upward and backward growth pattern.

In 1953, Scott proposed his nasal septum theory which stated that sutures were traction-adapted rather than pressure adapted. According to this theory, the cartilagenous nasal septum provided the tissue separating force to drive the maxillary complex downward and forward while the bones of the midface responded by deposition at the sutures. Scott

later expanded this theory to include the condylar cartilage and the synchondrosis as additional control centers generating tissue separating force. Scott continued to look for other mechanisms of the downward and forward growth of the maxillae. After examining the relationship of the retro-maxillary pad and orbital mass of fat to the maxillae in human specimens, Scott postulated that a basic biologic principle of multiple assurance applies to facial growth. That is, facial growth involves more than one mechanism so that if one does not operate there are other mechanisms available (Latham and Scott, 1970).

Regional differences in mitotic activity of chondrocytes of the nasal septum of rabbits and rats have been found using autoradiographic techniques. There are significantly higher levels of mitotic activity in the postero-inferior, postero-superior and antero-inferior areas with the first two zones being associated with areas of endochondral ossification (Long et. al., 1968). Searls (1975) found that there were age specific differences in rats, nasal septal growth being most rapid at 5 days. In another autoradiographic study Roberts and Blackwood (1984) found that the period of most active antero-posterior growth of the nasal septum occured between 4 and 14 days postnatal.

Moss et. al. (1960, 1962, 1964, 1968,) gave rise to the functional matrix theory in which the head and neck consist of independent yet integrated functions. Each function was carried out by a cranial component which consists of tissues, organs, spaces and skeletal parts necessary to accomplish that particular function. The functional cranial component is then divided into functional matrices and skeletal units. The functional matrices are either 1) Capsular, such as the brain or eyeball; 2) Periosteal, such as muscles or blood vessels, or 3) Functioning spaces, such as the nasopharyngeal airway. The capsular matrix and functioning spaces were responsible for translative changes while the periosteal matrix was reponsible for transformative changes (Moss and Young, 1960; Moss and Salentign, 1969; Moss, 1969). The skeletal unit was strictly compensatory to the functional matrix which it supported and the tissue which it protected. The functional matrix theory describes the combination of actions, reactions and feedback interplay that occurred during facial growth.

The composite concept (Van Limborgh, 1972) stated that growth control was brought about by a variety of factors. Van Limborgh believed that local epigenetic factors, intrinsic genetic factors and environmental facors combined to control the facial growth process. He further states that cartilage received the most input from local epigenetic factors.

Transplantation studies were done to determine the intrinsic capability of chondrocytes to divide and cartilage to grow in the absence of local extrinsic factors. Autotransplantation yielded the most consistent results and it was reported that the transplanted cartilages continue to grow at a rate comparable to controls (Kvinsland, 1973; Engelsma et. al., 1980).

Many investigators have attempted to verify the various theories of facial growth by surgical removal of the nasal septal cartilage and the other craniofacial cartilages from experimental animals (Moss, 1968: Sarnat and Wexler, 1970; Kremanak and Searls, 1971; Kvinsland, 1974). Reduction in growth was exhibited in all. According to Sarnat (1988) the important factors were loss of anatomical and physiological integrity. However, surgical trauma results in a destruction of the local environment confounding the results of these studies and cast doubt on their validity. A better model would be to render the proposed cephalic cartilage non-functional without surgical intervention.

Use of Anti-Cartilage Antibodies in Facial Growth Studies

Brigham (1976) was able to investigate antigenic and chemical differences between nasal septal cartilage, epiphyseal

cartilage and condylar cartilage. Using antigenic extracts from bovine nasal septum, epiphysis and condyle, he produced crude antisera in rabbits directed at each of these cartilages. By cross adsorption and indirect immunofluorescence, Brigham showed that antigenic differences existed among the nasal septal, epiphyseal and condylar cartilages as well as regional differences in the location of antigenic determinants found in the nasal septal cartilage. He also reported that all three cartilages possessed some common antigenic determinants but condylar cartilage posessed at least one antigenic determinant distinct from nasal septal and epiphyseal cartilage. Chemical differences among these three craniofacial cartilages were investigated using polyacrylamide gel disc electrophoresis. The results revealed compositional differences among the cartilages with the differences of the condylar cartilge being the most extensive. Brigham concluded that generalized statements for cartilage could not be supported because it appears that all cartilages may not behave identically.

Palmer (1978), working in the same laboratory, extracted the protein components from rat nasal septum and bovine condylar cartilage. Each of these antigenic extracts were injected into a different rabbit to produce an antisera against rat nasal septum and bovine condylar cartilage. Isolation of the IgG portion from the rabbits antisera by cellulose

chromatography was done so unwanted toxic side effects were minimized. Each antibody was injected every four days into a different litter of growing rats. At thirty days the rats were killed, radiographed and the nasal septum and condylar cartilage were removed and fixed for light microscopy and SEM examination.

Palmers results are as follows:

1. The IgG fraction of the anti-cartilage antibody became bound to cartilage in vivo and in vitro as judged by indirect immunofluorescence. The greatest immunolocalization was seen with rabbit anti-rat nasal septum antibody and rat nasal septum.

2. Histologic examination of the nasal septal cartilage from rats treated with rabbit anti-rat nasal septum antibody showed some indication of cytopathic effects (stellate, pleomorphic cells).

3. Two craniofacial measurements, anterior nasal position (I-N) and sphenoid length (BA-SO), demonstrated the least litter variance and had significant treatment effects as compared to the other measurements used.

Palmers work produced an antibody that specifically bound to craniofacial cartilage in vivo and in vitro and may alter translational growth of the craniofacial complex.

Hans (1981) examined the effects of anti-cartilage antibodies on midfacial growth in vivo and in vitro. Rat nasal septum chondrocytes were grown in cell culture (system developed by Hans) and the effects of anti-nasal septum antisera was assessed. The antisera was found to cause a complement-dependent cytotoxic effect in cultured rat chondrocytes. By indirect immunofluorescence he demonstrated that the antisera was localized to the cell surface and the extracellular matrix of the chondrocytes. The in vivo portion of the study consisted of injecting a polyclonal antisera raised in rabbits to rat nasal septum into growing rats from birth to thirty days. The results demonstrated a statistically significant reduction in midfacial growth. The combined results of this study suggested that it was possible to develop specific cytotoxic agents specific for each cephalic cartilage which could be useful in the study of facial growth.

Slodov (1991) examined whole cartilage and cultured chondrocytes obtained from the nasal septum, mandibular condyle and epiphyseal plates of rats. Using one dimensional SDS gel electrophoresis and silver stain, Slodov found that the chondrocytes from these three locations had similar membrane

proteins but the extracellular matrices exhibited several unique protein bands. Slodov concluded that it should be possible to produce monoclonal antibodies to each of these cartilages for studying the role of craniofacial cartilages in facial growth.

Due to the rapid pace in technology in molecular biology, Nguyen (1992) conducted a study to analyze rat nasal septal and condylar cartilage matrix and assess the reactivity of the rabbit anti-rat nasal cartilage antisera used by Hans (1981). Western Blot analysis, Dot blot analysis, 3%-16% gradient SDS-PAGE electrophoresis and 2D electrophoresis were used to identify reactive matrix antigens of rat nasal septal cartilage and condylar cartilage. The protein bands of nasal septum and condyle matrix proteins were found to be grossly similar but some differences existed in quantity and quality of the proteins between the two. Once the protein component of the rat nasal septum and condyle were separated, the antisera used by Hans was tested on these proteins to determine which ones reacted with the antisera. Nguyen also used monospecific monoclonal antibodies (mAb8A4, specific for link protein; mAb3B3, specific for chondroitin-6-sulfate; mAb5D4, specific for keratan sulfate; mAb2B6, specific for chondroitin-4-sulfate) as probes to identify antigens common to the proteins and to the anti-nasal septum antisera. The anti-nasal septum antisera was found to

be reactive with small proteoglycans containing dermatan sulphate, large proteoglycans and link protein. Owing to the critical role link protein plays in stabilizing cartilage extracellular matrtix, a specific monoclonal antibody (8A4) was chosen as the experimental reagent in the present study.

Monoclonal Antibody 8A4

Monoclonal antibody 8A4 belongs to the immunoglobulin class G2b and recognizes two identical epitopes residing in the C-terminal half of link protein. Goetinck et. al. (1987) showed that the epitopes for mAb8A4 bind to 10 amino acid regions situated between the 2 cysteine residues in the paired tandem repeat loops. They also demonstrated that these two regions are involved in binding to hyaluronic acid.

Monoclonal antibody 8A4 has been found to bind to all link proteins and link protein fragments of 20 kDa or greater from different animal species (Caterson et. al., 1985). Hoedt-Schmidt et. al. (1993) demostrated positive binding of mAb8A4 to the interterritorial matrix of normal bovine articular cartilage. The fact that mAb8A4 binds to all link proteins and link protein fragments from different species indicates that the determinant recognized by mAb8A4 may be a part of a conserved sequence of the link protein polypeptide unique to

all link proteins and may be an important functional or structural domain of the primary sequence of link protein.

With the advent of monoclonal antibody methods, antibodies can be raised that recognize specific structural determinants on the polypeptide backbone of proteins. These monoclonal antibodies are being used to characterize and isolate peptide fragments for primary structure analyses.

Since many antigens of the extracellular matrix have been identified (small proteoglycans containing dermatan sulfate, large proteoglycans and link protein) the next logical step would be to test monoclonal antibodies targeted at these antigens in vivo. An immunologic tool as powerful as this could be used in the study of mechanisms of facial growth as well as clinically to restrict or enhance growth potentials of the craniofacial cartialges.

STATEMENT OF THESIS

The purpose of this investigation is to determine if anti-link protein monoclonal antibody will cause significant effects on the growth and development of the rat midfacial complex. Randomized block design will be used to include treatment and control groups in the same litter. Newborn rats will be injected every four days with anti-link protein monoclonal antibody over a 30 day period. At the end of this time period, the animals will be sacrificed and the effects of anti-link protein monoclonal antibody on craniofacial dimension will be assessed by cephalomentric radiography.

MATERIALS AND METHODS

It has been shown that immunoglobulins may be passively transferred from animal to animal and that these specific antibodies may be localized in tissues (Beutner et. al., 1975; Toyka et. al., 1977). Palmer (1978) found that injecting rabbit anti-rat nasal septum antibody into growing rats resulted in binding of the antibody and apparent cytopathic changes. Hans (1981) found that in addition to the binding and the cytotoxic effects on nasal septum chondrocytes, there was also statistical evidence of a small reduction in midfacial growth.

With this particular study it was anticipated that the size of the expected growth alteration may be small owing to the inherent number of extraneous variables encountered in such an in vivo study and all treatment and control animals would be included within each litter because Palmer (1978) demonstrated that Sprague-Dawley rats exhibit a significant amount of inter-litter variability regarding craniofacial dimensions.

Prior to any experimentation, the protocol for this experiment was submitted and approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

Twenty-nine neonates were obtained from three timed pregnancy Sprague-Dawley rats. There were fourteen rats in the first litter, five rats in the second and ten in the third. Each litter was randomly separated into control and experimental groups in accordance with the block design. Rat pups were kept with their mothers until day 21 when they were separated to three animals per cage. The experimental animals were injected with rabbit anti-rat link protein immunoglobulin G (mAb 8A4). The control animals were injected with normal rabbit immunoglobulin G. Since the half life of rabbit IgG injected into a rat is four days (Brigham, 1976), the injections began on the first day postpartum and continued every fourth day. At birth the rats immunological system is immature and starting the injections on the first day postpartum allows the rat to become immunotolerant to rabbit IgG. The first three injections (days 1, 4 and 8) were given subcutaneously between the scapulae due to practical limitations of size, the remaining injections were given intraperitoneally.

The rabbit IgG and the rabbit anti-rat link protein was diluted to 16 ug/ml of saline prior to the beginning of the experiment. This dilution was determined by the amount of antibody that we could obtain divided by the number of injections multiplied by the anticipated weight of the animals

at each injection (total Ab/(# of injections X total weight of rats at each injection). Five one-hundredths ml/gram body weight was determined as the dose by the actual physical ability of an adolescent rat to survive an intraperitoneal injection greater than 7 ml (maximum amount injected). The rats were weighed prior to each injection to determine the amount of solution to be injected. After each injection session the remaining antibody was filtered through a micropore filter.

The experimental time period was set at thirty days following Palmers methodology (1978). Beherents (1975) and Baer (1951) determined that most growth of the rat facial complex occurs between 15-20 days of age corresponding to the human growth spurt between the ages of nine and eleven years. Massler and Schour (1951) found that the major facial change from round to elongated occured before thirty days in the rat.

On day 8, one control and one experimental animal were randomly selected from the largest litter (litter 1). They were killed by an overdose of sodium pentobarbital. Their nasal septa were dissected free by bilateral incisions through the nares extending to the frontal bone. This allowed for the removal of the overlying nasal bones to expose the septum. Using tissue forceps, the septum was freed from its sphenoid and ethmoid articulations. Following their removal, the septa

were scraped free of all adherent soft tissue using a dulled #15 scapel blade. The septa were rinsed with 0.15 M phosphate buffered saline (PBS) at pH 7.0 and then immediately placed in saline and placed on dry ice. The septa have been kept frozen until a later date when direct immunofluorescence will be performed.

The remaining rats were killed on day 30 with an overdose of sodium pentobarbital, sexed, individually weighed and radiographed in both the dorsoventral and lateral orientation.

Radiographic Technique

Radiographs were made using the Broadbent Bolton cephalometer (series 1K550, Bolton Fund, Cleveland, Ohio) and standard dental occlusal film (Kodak, Rochester, N.Y.) at 70 kV, 20 mA with a three second exposure time. A cephalostat for rats designed by Palmer (1978) was used to insure constant source-film and object-film distances. Each rat was radiographed in both norma dorsalis and norma lateralis for cephalometric analyses.

Cephalometric Landmarks

The following reproducible landmarks were adapted from Hans' study (1981). These points are illustrated in figure 1.

Dorsoventral

1)UM: The interface between the maxilla and the mesial surface of the maxillary first molar root.

2)MX: The most anterior point on the maxillary bone at the infra-orbital foramen.

3)I: A point in the median plane that lies on a line tangent to the inner curvature of the maxillary incisor.

4)N: The most anterior point on the nasal bone.

5)O: The most posterior point on the external occipital protuberance.

6)AB: A tangent drawn through the most posterior point on the external occipital protuberance.

7)CO: The most posterior point on the mandibular condyle.

8)C: The tip of the coronoid process.

9)ZG: The most lateral point on the zygomatico-maxillary suture.

10)BA: The most anterior point on the outline of the foramen magnum.

11)P: The most posterior point on the palatal bone at the midline.

12)LI: A point in the median plane denoting the border between the mandible and lingual surface of the mandibular incisors.

Lateral

1)so: The most superior (dorsal) point on the outline of the sphenoccipital synchondrosis.

2)is: The most superior (dorsal) point on the outline of the intersphenoidal synchondrosis.

3)i: A point that lies on the inner curvature of the maxillary incisor where it intersects the superior surface of the hard palate.

4)um: The intersection of the hard palate and the anterior surface of the averaged maxillary right and left first molar roots.

5)nf: The most superior point on the frontonasal suture.

6)ba: The most anterior point on the margin of the foramen magnum.

7)jl: The most inferior (ventral) point on the image of the jugum limitans fossa olfactoria.

8)n: The tip of the nasal bone.

9)mn: The average of the right and left intersections of the mandibular alveolar process and the anterior surface of the mandibular first molars.

10)pns: The most posterior point on the palatal process of the palatine bone.

11)co: The most posterior point on the curvature of the averaged outline of the left and right condyles.

12)ap: The most inferior and posterior point on the outer surface of the angular process.

Cephalometric Analysis

Sixty-six measurements were used to characterize the craniofacial dimensions of each animal (Hans, 1981). A discription of each measurement along with its construction are found in Tables I through V and figures 2 through 6. This analysis emphasizes measurements that reflect changes in midfacial dimension (Palmer, 1978; Hans, 1981).

All lateral and dorsoventral radiographs were enlarged three times using a photographic enlarger (Besler, East Orange, N.J.) and landmarks located for comparison of radiographic structures. All landmarks were indicated by pinholes made in a single sheet of bond paper.

Data reduction was accomplished by digitizing all of the landmarks. Each sheet of bond paper was securely fastened with tape to a digitizing board. Points were then entered in the same order for each lateral and dorsoventral radiograph. After all points were entered the measurements were done using the Dentofacial Planner (Toronto, Ontario).

Data Reduction

The Statistical Package for the Social Sciences (SPSS.Chicago , Illinois) was used to examine differences between experimental and control groups. Student t tests were used to detect significant differences between the control and experimental group. The Bonferoni correction was used to correct for the multiple testing that was done. Using this correction, an alpha value (.05/66) of $\leq$.0008 was used to assign statistical significance.

Error Study

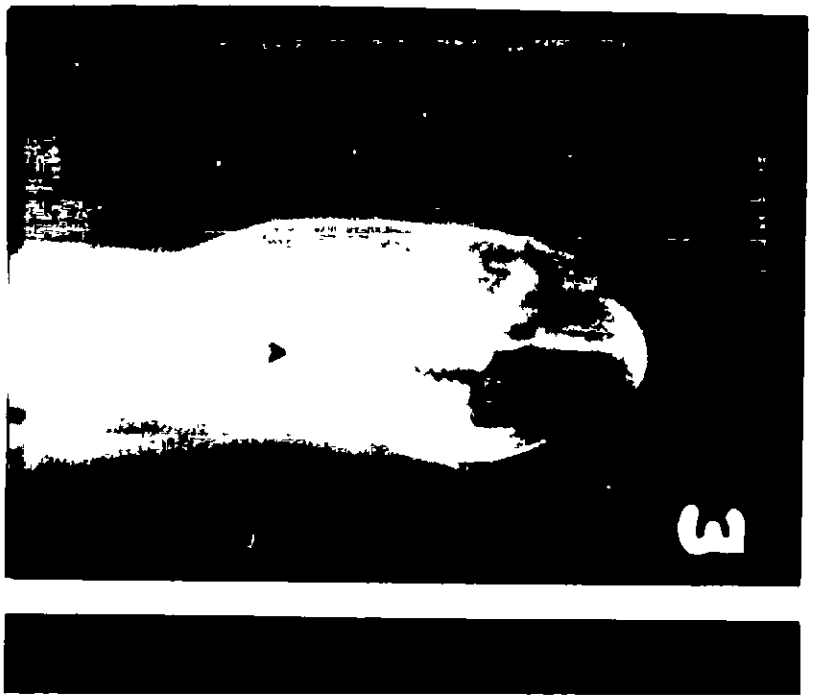
The reliability of each measurement was determined by double determination. All 25 landmarks were located on ten animals' dorsoventral and lateral radiographs. Measurements were digitized a second time and a coefficient of linear correlation and a paired t-test was calculated between the first and second determination of each dimension.

PLATE I

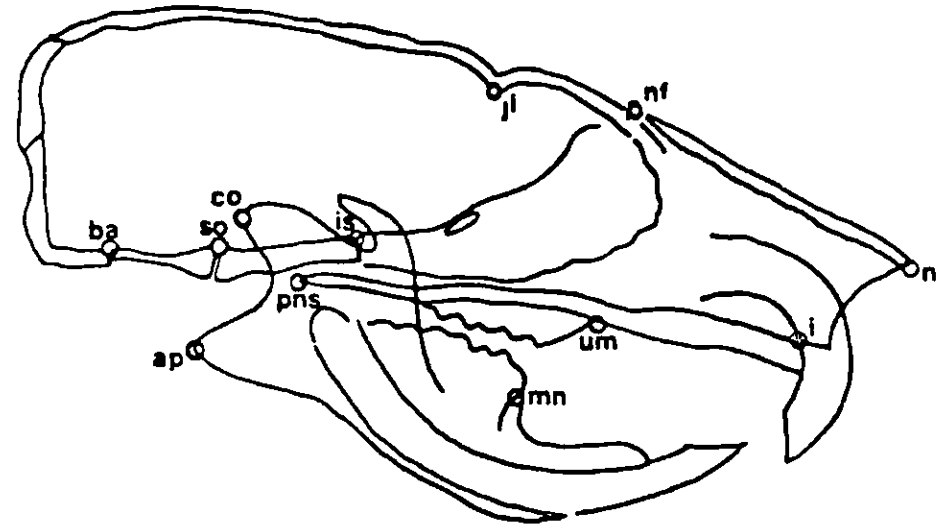
Lateral and dorsoventral cephalograms of a 30 day old Sprague-Dawley rat (not to scale).

Lateral cephalometric orientation

Dorsoventral cephalometric orientation






jl
nf
co
ba
so
is
n
pns
i
um
ap
mn

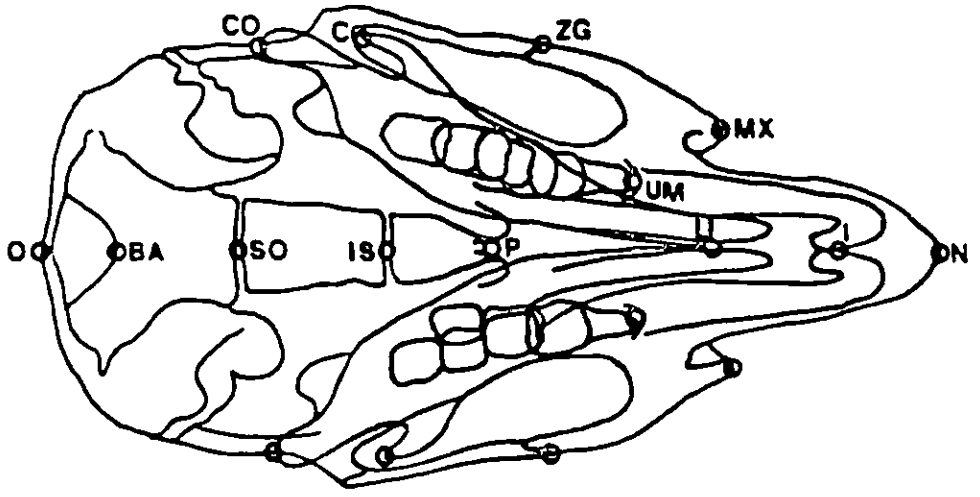

CO
C
ZG
MX
UM
O
BA
SO
IS
P
I
N

TABLE I

VARIABLES TO MEASURE ANTEROPOSTERIOR CHANGES IN MIDFACIAL BODY SIZE OR DISPLACEMENT OF THE MIDFACIAL COMPLEX

Lateral View

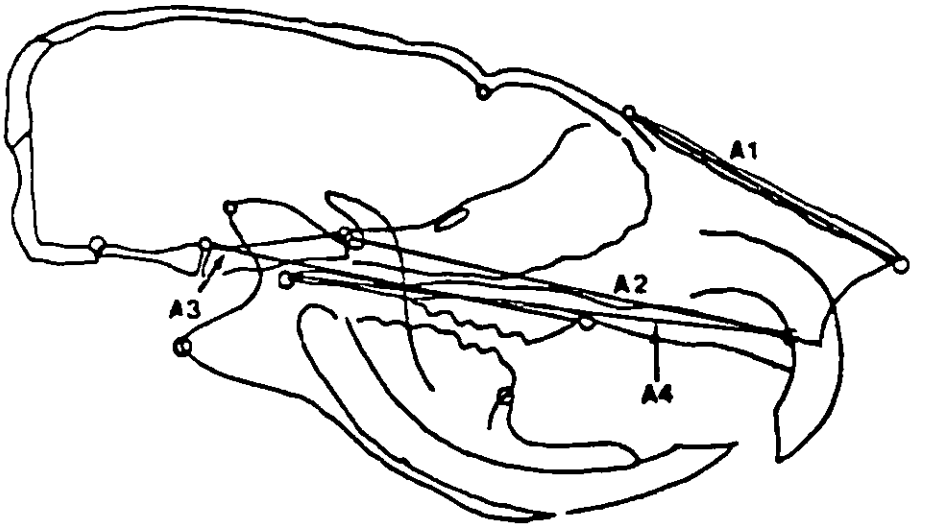
Variable	Landmarks Employed	Measures
A1	nf-n	Nasal bone length
A2	is-i	Premaxillary maxillary advancement
A3	um-so	Maxillary advancement
A4	pns-i	overall palatal length
A5	so-i	Premaxillary, maxillary, sphenoid advancement
A6	ba-i	Overall cranial base and premaxillary
A7	um-i	Length of premaxilla

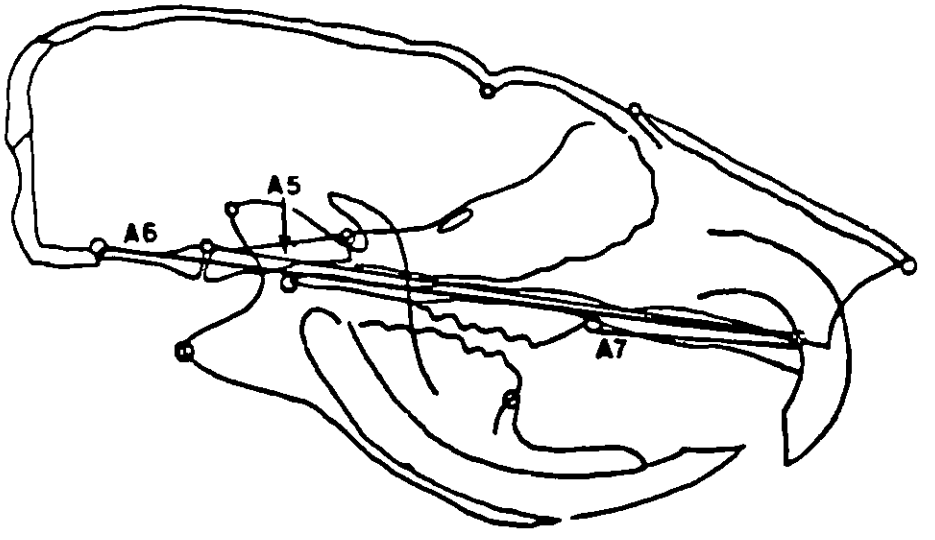
TABLE I (continued)

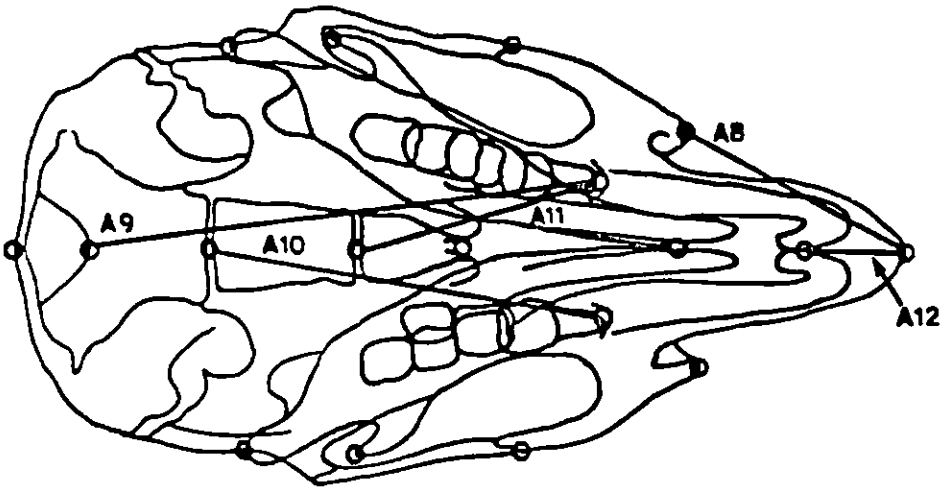
Dorsoventral View

A8	MX-N*	Rostral development
A9	BA-UM*	Maxillary dental-cranial base development
A10	SO-UM*	Sphenoid-maxillary dental development
A11	IS-UM*	Maxillary dental advancement
A12	P-I	Palatal length
A13	AB-ZG*	Anterior zygomatic development
A14	AB-MX*	Anterior maxillary development
A15	AB-UM*	Anterior maxillary dental development
A16	BA-MX*	Maxillary-cranial base development
A17	SO-MX*	Sphenoid-maxillary development
A18	IS-MX*	Maxillary advancement
A19	I-N	Anterior nasal position
A20	O-MX*	Maxillary advancement

*Bilateral measurements


A1
A2
A3
A4


A5
A6
A7


A8
A9
A10
A11
A12

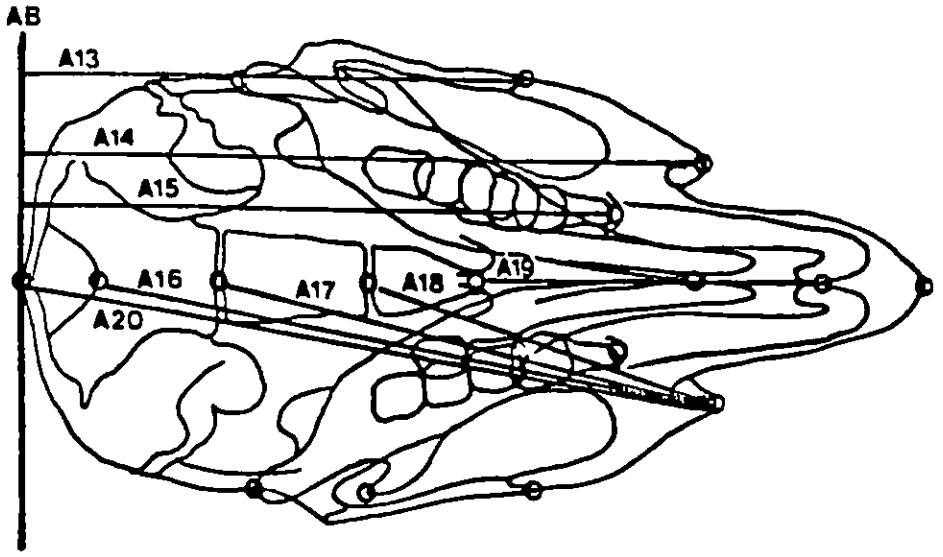

AB
A13
A14
A15
A16
A17
A18
A19
A20

TABLE II
VARIABLES TO MEASURE VERTICAL CHANGES IN MIDFACIAL DIMENSIONS

LATERAL VIEW

Variable	Landmarks employed	Measures
V1	j l - i	Premaxillary advancement and vertical change
V2	um-nf	Posterior nasal height
V3	nf-i	Nasal-premaxillary vertical change
V4	(pns-i)-nf	Posterior nasal height from (pns-i)
V5	i-n	Anterior nasal height
V6	(pns-i)-n	Anterior nasal height from (pns-i)

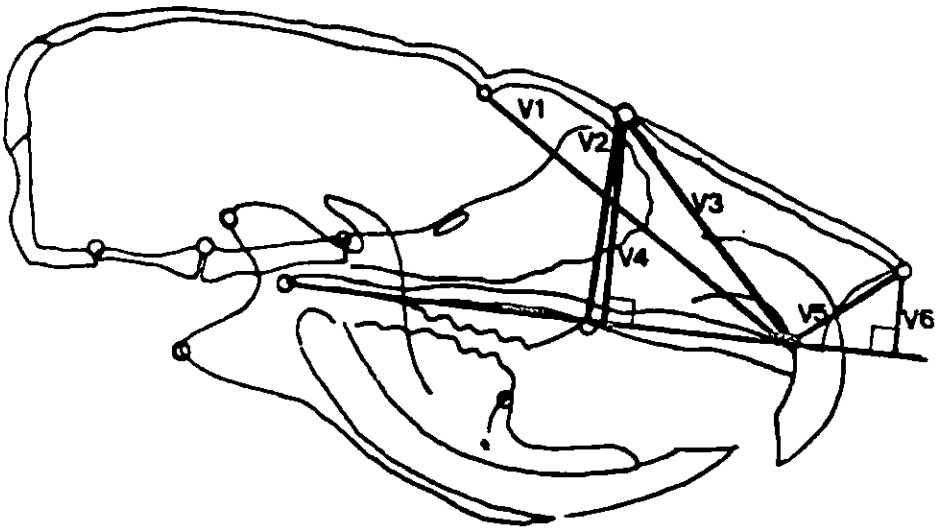
TABLE III

VARIABLES TO MEASURE TRANSVERSE CHANGES IN MIDFACIAL DIMENSION

Dorsoventral View

Variable	Landmarks Employed	Measures
T1	ZG-ZG	Bizygomatic width
T2	UM-UM	Maxillary dental width
T3	MX-MX	Maxillary width
T4	ZG-MX*	Maxillary zygomatic bone length

*Bilateral measurement


V1
V2
V3
V4
V5
V6

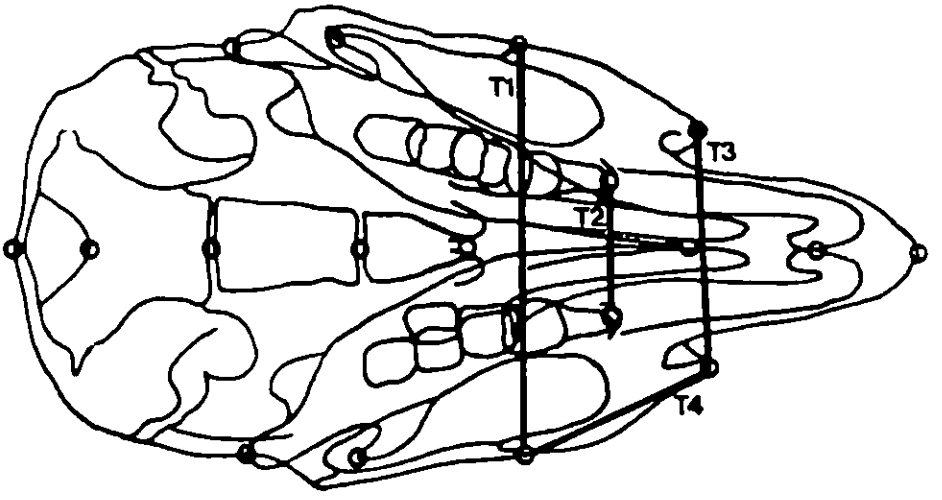

T1
T2
T3
T4

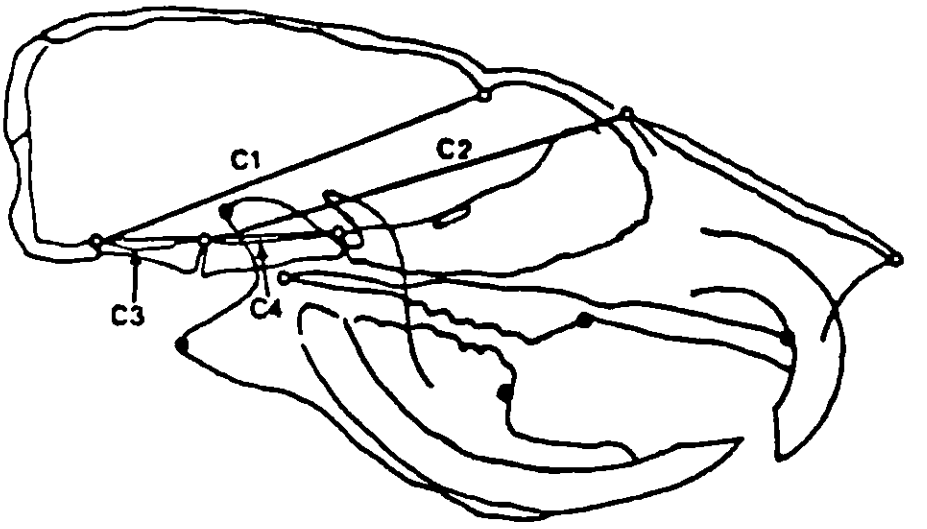
TABLE IV
VARIABLES TO MEASURE CHANGES IN THE CRANIAL BASE OR CRANIUM

Lateral View

Variable	Landmarks employed	Measures
C1	ba-jl	Transcranial length
C2	nf-so	Cranial base anterior cerebrum length
C3	ba-so	Basioccipital length
C4	so-is	Basispenoid length

Dorsoventral View

C5	O-BA	Sphenoid advancement
C6	BA-SO	Sphenoid length
C7	IS-P	Palatal advancement
C8	BA-IS	Pre-sphenoid advancement


C1
C2
C3
C4

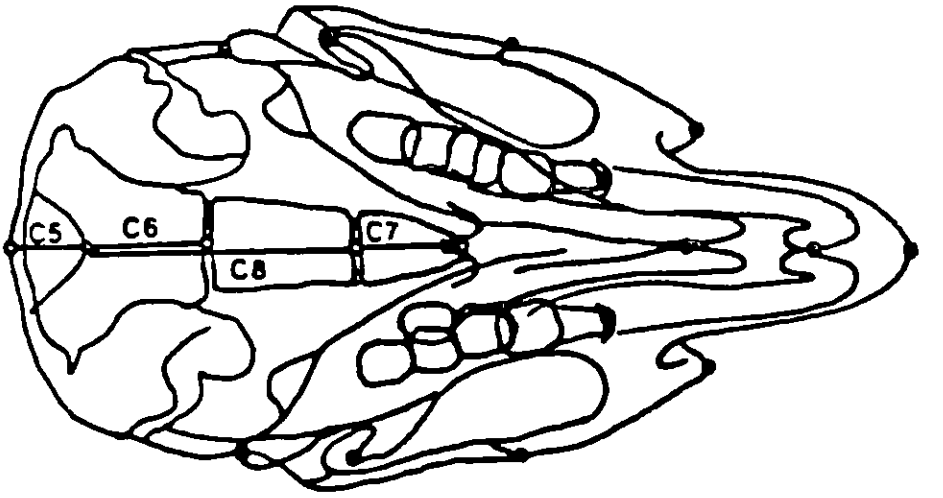

C5
C6
C7
C8

TABLE V

VARIABLES TO MEASURE CHANGES IN THE MANDIBLE

Lateral View

Variable	Landmarks Employed	Measures
M1	co-mn	Mandibular ramus height
M2	ap-mn	Mandibular body length
M3	ap-co	Ramal height

Dorsoventral View

M4	AB-CO*	Anterior condylar position
M5	CO-CO	Bicondylar width
M6	C-C	Bicoronoid width
M7	O-CO*	Condylar advancement
M8	CO-LI*	Mandibular length
M9	C-LI*	Mandibular body length
M10	CO-C*	Coronoid advancement

*Bilateral measurements

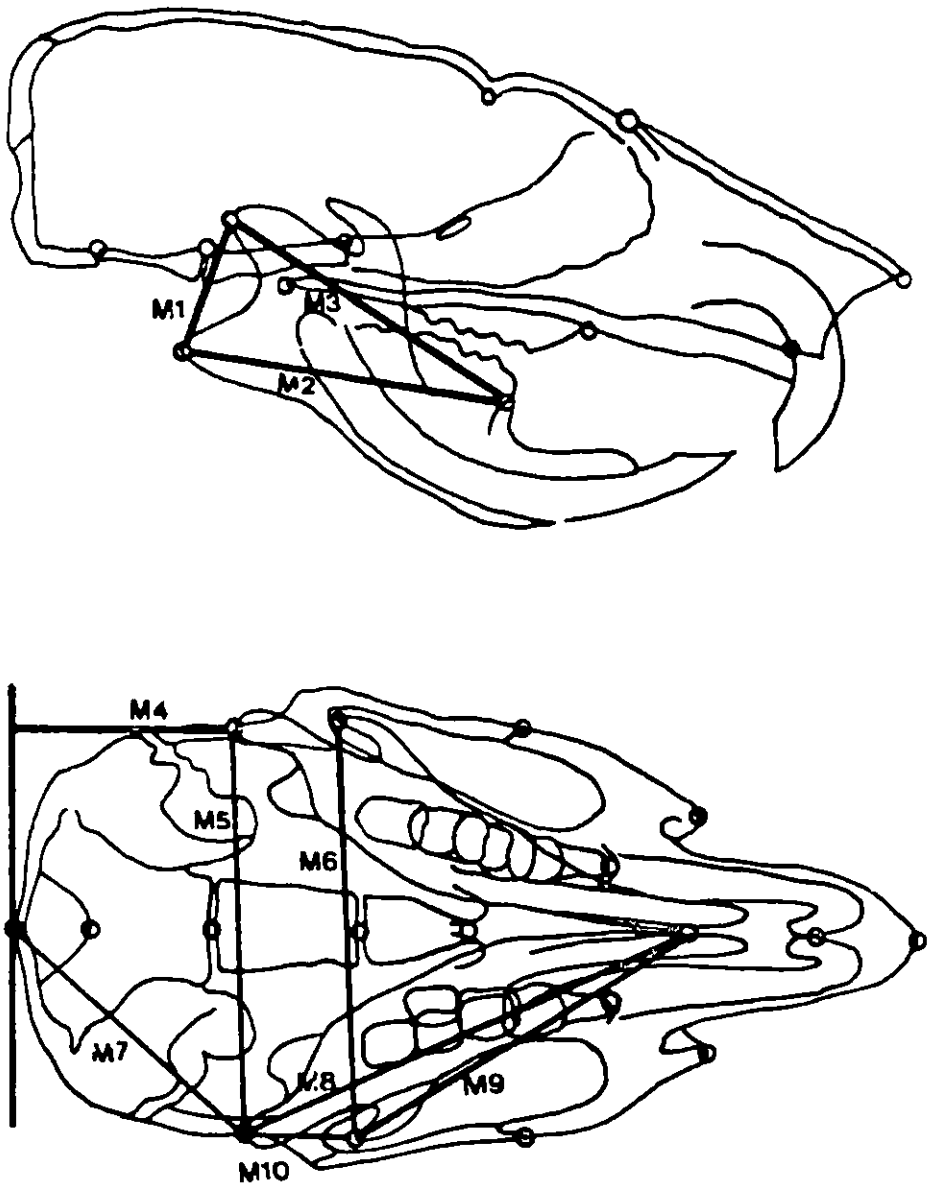

M1
M3
M2
M4
M5
M6
M7
M8
M9
M10

TABLE VI

INITIAL TEN MEASUREMENTS DONE REPRESENTING ANTEROPOSTERIOR CHANGES IN THE MIDFACE

Lateral View

Landmark Employed	Measures
is-i	Premaxillary maxillary advancement
um-i	Length of premaxilla

Dorsoventral View

I-N	Anterior nasal position
AB-MX*	Anterior maxillary development
O-N	Total head length

*Bilateral measurement

VERTICAL CHANGES IN THE MIDFACE

Lateral View

um-nf	Posterior nasal height

TRANSVERSE CHANGES IN THE MIDFACE

Dorsoventral View

UM-UM	Maxillary dental width
MX-MX	Maxillary width

CHANGES IN CRANIAL BASE

Lateral View

so-is	Basisphenoid length

RESULTS

Description of the resulting sample

The original experimental design included three litters in which each animal was randomly assigned to the experimental or control group. The demographic information pertaining to the original sample is presented in Table VII. The demographic information pertaining to the final sample used for analysis is presented in Table VIII.

Reliability of cephalometric determination

The results of the ten double determinations for each of the sixty-six measured variables are presented in Table IX. In this table the higher the value of r, the more accurate the point was able to be located (r = 1.0 is a perfect correlation). Using the paired t-test, there was no significant difference between the first and second measurements.

General appearance, physical condition and body weight

Upon gross physical examination of the experimental animals there was no detectable anatomic differences between treatment and control groups. The coat was normal in color and appearance and the eyes and incisor relationship also appeared normal. The mean body weights of the two groups

at the end of the experiment are reported in Table X. A student t test revealed no significant ($p \leq .05$) weight difference among the groups.

Cephalometric Analysis

The 66 measurements were divided into six functional groups on an anatomic basis in order to categorize significant treatment effects. These groups along with the rationale for including measures within each group are listed below:

1. Anteroposterior midfacial dimension-Thirty-two variables were included in this group. Some of these variables reflect changes in the anteroposterior length of bones within the midface. Others measure the anteroposterior displacement of the midfacial complex relative to the neurocranium (See Table XI).
2. Vertical midfacial dimension-Six variables were included in this group. All of these variables were used to estimate changes in midfacial height from the lateral cephalometric projection (See Table XII).
3. Transverse midfacial dimension-Five variables were placed in this group on the basis that they measure

changes in width of the midfacial complex as determined from the dorsoventral cephalograms (See Table XIII).

4. Cranial base and cranium dimensions-Seven cranial base and one transcranial measurements were included in this group. These seven cranial base measurements reflect changes in the synchondroses (See Table XIV).

5. Mandibular dimension-The variables in this group reflect changes in mandibular dimension and cranial mandibular relative positioning (See Table XV).

6. The initial ten measurements done representing: Anteroposterior changes in the midface, vertical changes in the midface, transverse changes in the midface and changes in cranial base (See Table XVI).

The means and standard deviations for all variables as well as the t-values and p-values are also given.

The variables used show no general trend between treatment and control groups. Three variables exhibited statistically significant ($p \leq .05$) results, IS-UMr, IS-UMl and COr-Cr describing the anteroposterior midfacial changes and mandibular changes, respectively. When subjected to the Bonferoni correction (alpha-value/number of tests=.0008) these variables were no longer significant. Forty-one of the sixty-six variables were found to be actually larger in the experimental group when compared to the control group.

TABLE VII

ORIGINAL SAMPLE DEMOGRAPHICS

(M = Male, F = Female)

Treatment	Cage 1 M	Cage 1 F	Cage 2 M	Cage 2 F	Cage 3 M	Cage 3 F	Total by treatment
IgG	1	4	2	1	2	3	13
mAb8A4	3	4	1	1	3	2	14
Total by Sex	4	8	3	2	5	5	- - -
Total by Cage	12		5		10		27

TABLE VIII

RESULTING SAMPLE DEMOGRAPHICS (M = Male, F = Female)

	Cage 1	Cage 1	Cage 2	Cage 2	Cage 3	Cage 3	Total	Total
Treat-ment	M F	Weight in Grams	M F	Weight in Grams	M F	Weight in Grams	M F	Weight in Grams
IgG	1 4	96.80	2 1	151.90	2 3	118.80	5 8	117.98
mAb8A4	3 4	103.40	1 1	149.20	3 2	120.20	7 7	115.94
Total	4 8	- - -	3 2	- - -	5 5	- - -	12 15	- - -
Total	12	100.65	5	150.82	10	119.50	27	116.92

TABLE IX

DOUBLE DETERMINATION FOR TEN ANIMALS

VARIABLE	r	Mean	t-value	p-value
AB-MXl	.996	-.020	.23	.823
AB-MXr	.988	-.010	-.09	.929
l-N	.981	-.04	-.39	.705
is-i	.997	-.050	-1.05	.322
MXr-MXl	.897	.110	2.70	.024
O-N	.997	-.220	-.275	.022
so-is	.984	.030	.67	.520
um-i	.985	-.020	-.43	.678
nf-um	.965	-.160	-.233	.045
UMr-UMl	.986	.000	.00	1.00
(pns-i)-n	.993	-.16	-1.6	.145

TABLE IX continued

VARIABLE	r	Mean	t-value	p-value
(pns-i)-nf	.987	.010	.14	.893
AB-COl	.998	-.060	-1.00	.343
AB-COr	.881	.100	.52	.614
AB-UMl	.990	-.050	-.52	.614
AB-UMr	.989	-.020	-.19	.853
AB-ZGl	.897	-.100	-1.40	.195
AB-ZGr	.979	.15	.91	.387
ap-co	.982	.070	.53	.610
ap-mn	.992	-.060	-.65	.531
ba-i	.998	-.060	-.62	.551
BA-IS	.990	-.210	-3.12	.012

TABLE IX continued

VARIABLE	r	Mean	t-value	p-value
ba-jl	.997	.040	.71	.494
BA-MXl	.992	-.060	-.63	.541
BA-MXr	.893	-.160	-1.60	.145
ba-so	.997	-.050	-1.05	.323
BA-SO	.991	.220	2.11	.064
BA-UMl	.990	-.050	-.68	.513
BA-UMr	.990	-.070	-.92	.382
Cl-Ll	.998	-.160	-1.95	.083
Cr-Ll	.897	-.080	-.84	.423
COl-Ll	.998	-.190	-2.35	.043
COr-Ll	.895	-.290	-1.98	.079

TABLE IX continued

VARIABLE	r	Mean	t-value	p-value
co-mn	.996	-.14	-2.49	.034
COl-Cl	.886	-.070	-.96	.363
COr-Cr	.887	-.040	-.52	.613
COr-COl	.988	.130	1.57	.152
Cr-Cl	.865	-.150	-1.24	.248
i-n	.881	-.17	-1.27	.237
IS-MXl	.987	.010	.14	.893
IS-MXr	.976	-.050	-.44	.673
IS-P	.903	-.020	-.21	.838
IS-UMl	.975	.040	.55	.599
IS-UMr	.889	.010	.18	.859

TABLE IX continued

VARIABLE	r	Mean	t-value	p-value
jl-i	.984	-.120	-1.13	.288
MXl-N	.987	-.070	-1.91	.089
MXr-N	.985	.020	.33	.751
nf-i	.992	-.120	-2.34	.044
nf-n	.992	-.050	-.60	.563
nf-so	.990	-.010	-.12	.904
O-BA	.990	-.12	-1.69	.126
O-COl	.972	-.370	-2.88	.018
O-COr	.946	.140	.66	.524
O-MXl	.996	-.210	-3.19	.011
O-MXr	.994	-.200	-2.41	.039

TABLE IX continued

VARIABLE	r	Mean	t-value	p-value
P-I	.972	-.12	-1.02	.336
pns-i	.998	-.060	-.90	.394
so-i	.998	-.070	-1.35	.209
SO-MXl	.982	.000	.00	1.00
SO-MXr	.988	-.090	-.89	.399
SO-UMl	.958	.000	.00	1.00
SO-UMr	.961	.020	.16	.879
um-so	.993	-.070	-1.05	.322
ZGl-MXl	.998	-.010	-.15	.882
ZGr-MXr	.991	.220	2.11	.064
ZGr-ZGl	.984	.070	.69	.506

TABLE X

MEAN WEIGHT AND STANDARD DEVIATION (S.D.) FOR EACH GROUP; T-VALUE AND P-VALUE INCLUDED

TREATMENT	MEAN WEIGHT (grams)	S.D.	t-value	p-value
IgG	117.98	23.75		
mAb8A4	115.94	19.11	-.25	.806

TABLE XI

MEAN VALUES (in m.m.), STANDARD DEVIATIONS (s.d.), T-VALUES AND P-VALUES FOR VARIABLES REFLECTING ANTEROPOSTERIOR CHANGES IN MIDFACIAL BONY SIZE OR DISPLACEMENT OF THE MIDFACIAL COMPLEX

VARIABLE	IgG N = 13; wt. = 117.98		mAb8A4 N = 14; wt. = 115.94		t-value	p-value
	Mean	S.D.	Mean	S.D.		
nf-n	40.185	1.540	40.514	1.503	.56	.579
pns-i	65.415	3.310	64.779	2.602	-.56	.582
um-i	30.077	.827	29.786	1.041	-.80	.431
um-so	49.031	2.111	48.500	1.582	-.74	.464
so-i	79.062	2.615	78.264	2.490	-.81	.425
is-i	60.769	1.921	60.221	1.785	-.77	.450
ba-i	91.154	2.369	89.986	3.337	-1.04	.308
AB-MXr	89.000	2.542	89.800	2.987	.75	.462
AB-MXl	88.339	2.967	89.157	2.545	.77	.448
AB-UMr	75.185	2.521	76.257	2.518	1.11	.280
AB-UMl	74.323	2.790	76.129	2.600	1.74	.094

TABLE XI continued

VARIABLE	IgG N = 13; Wt. = 117.98 Mean	S.D.	mAb8A4 N = 14; Wt. = 115.94 Mean	S.D.	t-value	p-value
O-MXr	90.208	2.676	90.821	2.890	.57	.573
O-MXl	90.015	2.988	90.529	2.620	.48	.639
AB-ZGr	61.177	2.961	62.357	3.893	.88	.387
AB-ZGl	60.100	3.273	60.771	4.682	.43	.672
P-I	48.992	1.954	49.514	2.917	.54	.593
MXr-N	32.623	1.493	33.079	1.161	.89	.383
MXl-N	32.046	1.416	32.807	1.450	1.38	.180
I-N	11.054	1.052	11.243	1.348	.40	.690
BA-MXr	79.823	2.108	79.736	2.384	-.10	.921
BA-MXl	79.408	2.199	79.229	2.028	-.22	.827
BA-UMr	64.923	1.619	65.407	1.946	.70	.491

TABLE XI continued

VARIABLE	IgG N = 13; Wt. = 117.98 Mean	S.D.	mAb8A4 N = 14; Wt. = 115.94 Mean	S.D.	t-value	p-value
BA-UMI	64.469	1.593	65.407	2.056	1.32	.200
SO-MXr	63.608	1.919	63.729	1.885	.17	.870
SO-MXI	63.231	1.764	63.150	1.432	-.13	.897
SO-UMr	48.500	1.293	49.207	1.722	1.20	.242
SO-UMI	48.077	1.347	49.150	1.636	1.85	.076
IS-MXr	46.131	1.623	46.500	1.624	.59	.560
IS-MXI	45.639	1.247	45.693	1.306	.11	.913
IS-UMr	30.631	1.048	31.629	1.300	2.19	.038
IS-UMI	30.239	1.229	31.457	1.421	2.38	.026
O-N	117.469	3.303	119.029	3.611	1.17	.254

TABLE XII

MEAN VALUES (in m.m.), STANDARD DEVIATIONS (S.D.), T-VALUES AND P-VALUES OF VARIABLES REFLECTING VERTICAL CHANGES IN MIDFACIAL DIMENSION

VARIABLE	IgG N = 13; Wt. = 117.98		mAb8A4 N = 14; Wt. = 115.94		t-value	p-value
	Mean	S.D.	Mean	S.D.		
(pns-i)-n	11.623	.613	11.614	.620	-.04	.971
(pns-i)-nf	26.877	1.100	26.607	.957	-.68	.502
jl-i	53.462	1.468	52.829	1.722	-1.02	.316
nf-um	27.777	1.314	27.336	.847	-1.04	.306
nf-i	36.269	1.293	36.286	1.324	.03	.974
i-n	17.323	.722	17.400	.828	.26	.800

TABLE XIII

MEAN VALUES (in m.m.), STANDARD DEVIATIONS (S.D.), T-VALUES AND P-VALUES FOR VARIABLES REFLECTING TRANSVERSE CHANGES IN MIDFACIAL DIMENSION

VARIABLE	IgG N = 13; Wt. = 117.98		mAb8A4 N = 14; Wt. = 115.94		t-value	p-value
	Mean	S.D.	Mean	S.D.		
MX-MX	29.300	1.239	29.250	1.213	-.11	.916
UM-UM	15.908	.994	16.043	.929	.37	.718
ZG-ZG	53.508	2.400	53.786	1.556	.36	.718
ZG-MXr	30.569	3.531	30.014	3.283	-.42	.676
ZG-MXl	30.639	3.894	30.879	4.410	.15	.882

TABLE XIV

MEAN VALUES (in m.m.), STANDARD DEVIATIONS (S.D.), T-VALUES AND P-VALUES FOR VARIABLES REFLECTING CHANGES IN THE CRANIAL BASE OR CRANIUM

VARIABLE	IgG N = 13; Wt. = 117.98		mAb8A4 N = 14; Wt. = 115.94		t-value	p-value
	Mean	S.D.	Mean	S.D.		
ba-so	12.377	1.114	11.979	.891	-.66	.515
so-is	18.954	.971	18.614	.961	-.96	.347
ba-jl	53.900	1.444	52.993	2.404	-1.18	.250
nf-so	60.069	2.099	59.021	1.754	-1.41	.170
BA-SO	16.592	.848	16.407	.960	-.53	.601
BA-IS	45.492	2.051	45.786	1.956	.38	.704
IS-P	18.246	.746	18.007	.782	-.81	.425
O-BA	10.723	1.727	11.407	1.766	1.02	.319

TABLE XV

MEAN VALUES (in m.m.), STANDARD DEVIATIONS (S.D.), T-VALUES AND P-VALUES FOR VARIABLES REFLECTING CHANGES IN MANDIBULAR DIMENSION

VARIABLE	IgG N = 13; Wt. = 117.98		mAb8A4 N = 14; Wt. = 115.94		t-value	p-value
	Mean	S.D.	Mean	S.D.		
ap-mn	42.446	2.178	43.286	3.215	.79	.438
co-mn	44.339	1.967	43.750	2.033	-.76	.452
ap-co	22.323	2.398	20.686	2.078	-1.90	.069
AB-COr	31.631	2.248	32.486	2.438	.94	.354
AB-COI	30.285	2.005	30.521	1.963	.31	.759
Cr-CI	50.362	1.829	51.350	1.645	1.48	.152
COr-COI	51.369	1.426	51.929	1.859	.87	.391
COr-LI	61.546	4.478	61.271	3.170	-.19	.855
COI-LI	61.615	4.443	62.007	4.077	.24	.813
COr-Cr	14.362	1.639	13.043	1.289	-2.33	.028
COI-CI	13.769	.996	13.914	1.843	.25	.803

TABLE XV continued

VARIABLE	IgG N = 13; Wt. = 117.98		mAb8A4 N = 14; Wt. = 115.94		t-value	p-value
	Mean	S.D.	Mean	S.D.		
O-COr	40.146	1.611	41.307	1.893	1.71	.100
O-COl	40.508	1.563	40.514	1.520	.01	.991
Cr-Ll	48.685	3.716	49.879	2.885	.94	.358
Cl-Ll	49.139	4.495	49.364	3.124	.15	.880

TABLE XVI

MEAN VALUES (in m.m.), STANDARD DEVIATIONS (S.D.),T-VALUES AND P-VALUES OF INITIAL TEN MEASUREMENTS

(M=Male, F=Female)

VARIABLE	IgG N=13 Wt.+117.98 Mean	S.D.	mAb8A4 N=14 Wt.=115.94 Mean	S.D.	t-value	p-value
is-i	60.769	1.921	60.221	1.785	-.77	.450
um-i	30.077	.827	29.786	1.041	-.80	.431
I-N	11.054	1.052	11.243	1.348	.40	.690
AB-MXr	89.000	2.542	89.800	2.987	.75	.462
AB-MXl	88.339	2.967	89.157	2.545	.77	.448
O-N	117.469	3.303	119.029	3.611	1.17	.254
um-nf	27.777	1.314	27.336	.847	-1.04	.306
UM-UM	15.908	.994	16.043	.929	.37	.718
MX-MX	29.300	1.239	29.250	1.213	-.11	.916
so-is	18.954	.971	18.614	.961	-.96	.347

DISCUSSION

The purpose of this investigation was to determine if a monoclonal antibody specific for link protein can effect the process of facial growth in experimental rats. This was the first time mAb8A4 has been injected into experimental animals. Its use thus far has been to identify and quantify link protein on electrophoretic gels and in tissues by immunohistochemistry(Roberts and Pare, 1991; Nguyen et. al., 1991; Caterson et. al., 1985). The control group received injections of an IgG preparation isolated from rabbit serum. The reason for using IgG rather than saline was that IgG is normally found in rats and was believed to have no effect on facial growth and development. Statistical analysis of the effects of the anti-link protein monoclonal antibody on craniofacial growth were compared to the control group. Statistically significant differences ($p \leq .05$) between control and experimental animals were seen for three variables, IS-UMr, IS-UMl amd COr-Cr. However, when these three variables were subjected to the Bonferoni correction (alpha-value/number of tests=.0008) they were found not to be significant.

The difficulty in demonstrating a significant difference between the control and experimental groups was the possibility that IgG may have caused a reduction in craniofacial growth of the control animals. In fact, over half of the variables were larger in the experimental group when compared to the control group. Terasaki et. al. (1967) reported that IgG fractions isolated from mature animal serum contain antibodies that will cross-react with connective and epithelial tissue components. Hans (1981) found a small cytotoxic effect (6%) on chondrocytes grown in culture with normal rabbit serum.

The findings of this study actually confirm Hans' in vivo results (1981). If Hans would have used the Bonferoni correction he also would have shown a lack of effect on midfacial dimensions in his experimental animals.

Although link protein is found in all cartilages of the body, the midface was the focus in this study because the rat nasal septum is extremely thin and highly vascularized. This high vascularity of the rat nasal cavity is due to a rich capillary network responsible for thermoregulation. Therefore, diffusion of the antibody from the bloodstream to the chondrocytes of the nasal septum could readily occur. In addition, the midface has been shown to be highly susceptible to alteration by metabolic disturbances (McClure et. al., 1979).

There are several possibilities as to why there was no cephalometric evidence of midfacial change. Latham and Scott (1970) stated the theory of multiple assurance asserting that more than one process is usually involved in growth mechanisms, so that if one does not operate there are other processes that make up for it. This could explain our lack of effect on midfacial growth. The amount of monoclonal antibody injected may have been insufficient to bind to all available link protein produced by chondrocytes throughout the body. There were no previous studies done to provide guidelines as to the amount of antibody required to cause significant growth effects. In addition, Searls (1976) reported that chondrocyte proliferation in the rat nasal septum in situ is greatest 5 days before birth. Injections began on the first day postpartum in this study. We have inferred from Hans' results (1981) that the antibody reached the target as he showed this by indirect immunofluorescence. The possibility does exist that the antibody did not bind to link protein although the chance that this occured was extremely low.

One final possibility that deserves further study is that link protein may not play an important role in the growth of cartilage but may actually stabilize the structure once growth is complete. Roberts and Pare (1991) have shown that there is a low link protein concentration in one year old human tracheal

cartilage with a rapid increase in link protein concentration occuring between birth an seventeen years. They suggest that this may permit remodeling of the extracellular matrix and as the tissue growth and change in architecture decrease, the stabilization of proteoglycan aggregates by link proteins would favor the accumulation of irreversibly aggregated proteoglycans in the extracellular matrix of cartilage. In another study (Buckwalter et. al., 1994), it was shown that articular cartilage contains two distinct populations of proteoglycan aggregates and aggrecan clusters. The aggrecan clusters have no associated hyaluronic acid filaments and may or may not contain link protein. One of the proteoglycan aggregates has a reduced concentration of link protein as compared to the other proteoglycan aggregate population. The proteoglycan with the reduced concentration of link protein predominated in the superficial layer of articular cartilage while the other population of high link protein concentration proteoglycan was found in the middle and deep layers of the articular cartilage. These results further substantiate the possibility that link protein maintains the integrity of cartilage once growth and the change in architecture decrease. The superficial layer of articular cartilage maintains a low concentration of link protein so it may continue to be remodeled or repair itself.

Additional studies to be performed range from increasing the amount of antibody injected to eliminating the gene for link protein in transgenic mice. The possibility that the amount of antibody injected was insufficient to cause significant effects justifies repeating this experiment with increased doses of antibody. Immunohistochemical studies should then be done on the nasal septa of the experimental animals verifying the antibody reaching its target. Other monoclonal antibodies aimed at different molecules of the extracellular matrix such as the small proteoglycans containing dermatan sulfate and the large proteoglycans that Nguyen (1991) found anti-nasal septum antisera to be reactive with should be investigated. Searls (1976) results suggest that injecting the pregnant rat might produce alteration of the offsprings midface. Prenatally link protein may be important in development while postnatally environmental effects may predominate. The targeted mutation of a specific gene in the mouse has allowed investigators to analyze vertebrate gene function in great depths (Thomas and Capecchi, 1987; Doetschman et. al., 1988; Kohler et. al., 1989; Schwartzberg et. al., 1989; Thompson et. al., 1989b). It is possible to knockout the gene for link protein in transgenic mice thereby ruling out any extraneous variables such as an antibody not reaching its target or not reaching its target at the key developmental stage.

The lack of significant effects on rat midfacial development in this study should not discourage further studies using monoclonal antibodies in in vivo studies. Antibodies offer a unique and exciting mode for studying facial growth and development at the cellular level.

SUMMARY AND CONCLUSIONS

This research investigated the effects of injecting a monoclonal antibody into experimental animals. Anti-link protein monoclonal antibody (mAb8A4) has been found to bind to all forms of link protein of many species and we anticipated that it would bind to link protein in the craniofacial cartilage of a growing rat. This approach to the study of cartilage and its role in craniofacial growth could be done without altering adjacent tissues.

Cephalopmetric analysis was done and no significant changes in midfacial dimensions were observed. The results of this study suggest that anti-link protein monoclonal antibody does not effect midfacial development in a growing rat. Additional studies using monoclonal antibodies specific for cartilage components should be done to increase the present understanding of growth control mechanisms operating at the cellular level.

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