THE EFFECTS OF ANTI-NASAL SEPTAL CARTILAGE ANTIBODIES ON MIDFACIAL GROWTH AND DEVELOPMENT

by

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ABSTRACT

THE EFFECTS OF ANTI-NASAL SEPTAL CARTILAGE ANTIBODIES ON MIDFACIAL GROWTH AND DEVELOPMENT

The effects of anti-rat cartilage antibodies on rat midfacial growth were examined. Antibodies were developed in rabbits against the cartilaginous portion of the rat nasal septum. The immunoglobulin G fraction of the anti-rat nasal septum antisera was tested *in vitro* on monolayer cultures of rat nasal septum chondrocytes and *in vivo* by injection into rats from birth to 30 days.

The anti-nasal septum antibodies bound to the cell surface and extracellular matrix of the cells *in vitro* and caused complement-dependent cytotoxicity and reduced plating efficiency. Cephalometric analysis of rats injected with anti-rat nasal septum antibody demonstrated a statistically significant ($p \leq .05$) reduction in midfacial dimensions. Premaxillary length, total snout length, and relative midfacial advancement were reduced in rats treated with anti-nasal septum antibodies.

These studies indicate that cytotoxic anti-nasal septum antibodies modulated midfacial growth and development *in vivo*. Furthermore, it is suggested that the use of other tissue specific antibodies may provide a useful experimental approach for studying growth and development.

INTRODUCTION

Many mechanisms have been suggested for controlling the initiation, modulation, and termination of the growth process. Further, any control mechanism ultimately must be operative at the cellular level. Thus, the crucial questions regarding the understanding of growth and its manner of control are then reduced to what "signals" cells to grow, reproduce, function, die, etc. Unfortunately, these signals have not been characterized to a point where a universal model for growth regulation at the cellular level can be proposed. However, it is generally accepted that mediators of cell behavior are of two general classes, extracellular factors and intracellular factors, with the interface between these two groups being the cell membrane. Examples of extracellular mediators which influence cell activity are hormones, such as somatotropin, the somatomedins and insulin, and steroids. Intracellular mediators of cellular function would include cyclic adenosine monophosphate (cAMP), enzymatic reactions, and the genome. The importance of the cell membrane as a transducer, converting extracellular signals to intracellular actions, or vice versa, makes this structure an important link in understanding the control process.

One cell surface phenomenon that has recently been examined in regard to the control of facial growth in general and

cartilage in particular is that of anti-cartilage antibodies. The existence of antigenic differences among cephalic cartilages was demonstrated by Brigham (1976). Using indirect immunofluorescent staining, he found bovine nasal septal cartilage to be antigenically similar to epiphyseal cartilage. In addition, Brigham demonstrated that condylar cartilage contained at least one antigen not found in either epiphyseal or nasal septal cartilage. Owing to the importance of these cartilages in understanding craniofacial growth, additional studies were undertaken to determine the extent to which growth might be modulated via antigen-antibody interaction. In 1978, Palmer tested this concept by injecting growing rats with antibodies directed against rat nasal septum cartilage. Due to retrospective incompleteness in experimental design, the cephalometric analysis of his results led to inconclusive findings. However, it was positively demonstrated by direct immunofluorescent examination that the antibodies were localized within the nasal septum cartilage. In addition, there was some evidence of cytopathic effects (disturbances of normal cell morphology) when tissue sections of the treated animals were examined histologically.

The present study evaluates the effects of anti-cartilage antibodies in midfacial growth using both *in vivo* and *in vitro* experimental methodologies. Based upon the two previous studies, it was decided to redesign the earlier *in vivo* study of Palmer using a randomized block design which would allow a more precise

examination of treatment effects when growing rats are injected with anti-nasal septum antibody. In addition, rat nasal septum chondrocytes were isolated and grown in tissue culture. The in vitro portion of the present study allowed analysis of antigen-antibody relationships in a more controlled experimental model.

LITERATURE REVIEW

Descriptive Anatomy

Downward and forward growth of the maxillary complex and mandible has been a central theme in craniofacial biology. Serial radiographic studies of the growing child have shown this growth vector to be consistent within the bounds of biologic variation (Broadbent, 1975; Riolo et al., 1974). The underlying mechanism(s) responsible for the normal maturation and development of the facial complex is an area under intense investigation by modern craniofacial biologists. Enlow (1975) has provided a detailed descriptive analysis of the skeletal morphogenic alterations accompanying facial change. He has defined two terms in order to be more precise when describing osseous change. The first, "drift", involves the "deposition of bone on the side pointed toward the direction of growth; resorption usually occurs on the opposite side of that particular bony cortex." A second term, "displacement", is "a separate movement of the whole bone, in toto, away from its contact with other bones." Enlow has shown that the anatomical alterations in bony form associated with growth can be effected by either cortical drift, or displacement in combination with cortical drift.

Theoretical Models

It is generally accepted that facial growth involves periosteal and endosteal activity in combination with bodily translation of bones. However, attempts to establish a cause and effect relationship between these well documented skeletal changes and the process of facial growth have not met with unanimous approval. Early attempts to understand the underlying mechanism responsible for osseous change focused attention on the bony skeleton. Keith and Campion (1922) theorized that growth at the maxillary sutures provided the force responsible for maxillary displacement. The idea of bony sutures providing growth directing force was expanded by later authors to include additional sutural areas of the skull (Weinman and Sicher, 1955; Sicher and Debrul, 1970). Brash (1934) offered another bone-based hypothesis. He argued that accretion on the anterior facial skeleton, not sutural growth, was primarily responsible for the downward and forward growth vector. These early investigators relied heavily on experimental evidence drawn from vital dye studies of bone deposition patterns using laboratory animals and craniometric analysis of dried human and animal skulls. Advances in methods for assessing growth and in cell biology have placed some limitations on the role of bone in skeletal growth. Studies involving the response of bone to physiologic forces have shown that bone as a tissue responds to pressure by resorption and to tension by depo-

sition. Any sutural expansion theory would necessarily require that bone exert and withstand a compressive force, and respond to such force by deposition. Osteoblastic activity at the sutural interface under these conditions seems highly unlikely. Similarly, Enlow's (1975) extensive characterization of depository and resorptive fields in the facial skeleton has shown that many facial bones actually undergo resorption on their anterior aspect. In fact, the majority of periosteal deposition occurs in a manner suggesting an upward and backward growth pattern. In light of these findings it would seem prudent to consider bone as primarily a reactive tissue, subject to morphologic modification in response to extrinsic factors.

Two additional theories of facial growth will be outlined. Both of these theories require bone to respond to a tensile stress by deposition. This is physiologically a sound assumption. The difference between these theories relates to the source of the tissue-separating force which results in the tensile stress. Scott (1953) is generally credited with what is popularly known as the "nasal septum theory." He theorized that the growth of the cartilaginous nasal septum "thrusts" the midfacial complex downward and forward. The bones of the midface respond by deposition at sutural margins. This theory has been expanded to include other growth directing cartilaginous structures. In the mandible, the condylar cartilage, and in the cranial base, the synchondroses

were ascribed tissue-separating capability. According to Scott's hypothesis expansile growth by these cartilaginous sites results in separation of the bones, which allows their concommitant growth.

The physical properties of cartilage are important to "Scott's theory." Cartilage, unlike bone and its attendant periosteum, is uniquely a pressure-tolerant tissue. The high water content (approximately 70%) held within the extracellular, amorphous ground substance, by hydrogen bonds allows this tissue to function as a physiologic "shock-absorber." In addition, cartilaginous tissue is generally avascular and has the ability to grow both intersitially and appositionally. These physiologic properties would allow it to "push" on bony structures to cause translation growth.

An alternative to "Scott's theory" has been offered by Moss and his coworkers (1960, 1962, 1964, 1968, 1970, 1972). They have constructed an elaborate scheme whereby "functions" (respiration, speech, neural integration, balance, digestion, vision) are carried out by "functional cranial components." Each functional cranial component consists of two parts, a "functional matrix" and a "skeletal matrix." Tissues necessary to carry out a particular function are termed "functional matrices." Those tissues which serve to protect or provide support for the functional matrix are termed "skeletal units." Skeletal units are comprised of macro-

skeletal units, defined as the entire bone viewed as an intact organ. These macroskeletal units are in turn made up of contiguous osseous subunits, called microskeletal units. Importantly, skeletal units are capable only of secondary growth in response to the influences of the functional matrices.

Functional matrices are also subdivided into two types. The first, "periosteal functional matrices" include tissues and structures which cause the skeletal unit to respond with a tissue surface reaction. This response is termed "transformation" by Moss and corresponds to Enlow's term "remodeling" or "cortical drift" if applied strictly to the response of bone to "periosteal matrices." Muscles, teeth, glands, fat pads, blood vessels, and nerves constitute examples of Moss' periosteal matrices. Moss' second classification of functional matrices are termed "capsular matrices." Included in this group are encapsulated organs such as the brain and the orbits. In addition, Moss has included a non-encapsulated, loosely defined category termed "functioning spaces." Included in this category are the nasopharyngeal airway and the oral cavity. In the hierarchy of functional matrices it is the capsular variety which are responsible for translational growth.

Moss believes that growth of the capsular matrix, the brain for example, precedes growth of its attendant skeletal matrix, the calvariae in this instance. This volumetric expansion

of the brain during growth produces a tensile stress at the sutural margins. The flat bones of the skull and the synchondroses of the cranial base respond to this tension by deposition of new bone. In summary, it is Moss' contention that soft tissue is the primary causative factor directing skeletal growth, and that hard tissues (bone and cartilage) are responsible only for the structural integrity of the system.

The experimental evidence supporting or countering these various theoretical models will be reviewed under two general headings. The first will include research conducted primarily by investigators in the field of craniofacial biology, and will attempt to outline the methodology used to analyze growth concepts at the macroscopic level. Where possible, an attempt has been made to refer to work based primarily on midfacial growth and the nasal septum. The second section will provide information related to growth control mechanisms operative at the cellular level. Although much of this research has not dealt directly with midfacial and craniofacial growth, the general nature of the topic allows for a broad application of the control concepts.

Experimental evidence of growth modulation at the macroscopic level

Extirpation Studies

Many investigators have attempted to alter growth by surgical removal of one of the proposed cephalic growth cartilages

from experimental animals. Surgical ablation of the nasal septum in rabbits (Sarnat and Wexler, 1970) resulted in a significant reduction in snout length and a relative mandibular prognathism. The extent and severity of the reduction was proportional to the amount of septum removed and the length of time the animal survived after the surgical procedure. Similar results have been reported by other investigators (Kremenak and Searls, 1971; Kvinsland, 1974). Recently Siegel (1979) reported that resection of the nasal septum in a non-human primate, Papio cynocephalus, resulted in a dimunition of midfacial growth. His results supported earlier works suggesting that growth reduction was proportional to the amount of septum removed. In addition, his study showed that the younger the animal was at the time of surgery the greater the effect on subsequent midfacial growth. Using an experimental method analogous to septal extirpation, Gange and Johnston (1974) severed the fibrous attachment of the nasal septum from the premaxilla in growing rats by electrocautery. Presumably this procedure is less damaging to the structural integrity of the septum. Their results showed a statistically significant reduction in both "snout length and relative maxillary advancement" in animals receiving the electrocautery treatment.

Moss (1968) also presented results of an experiment involving extirpation of the nasal septum in infantile rats. He noted a marked collapse of the nasal roof, and a downward "decli-

nation" of the palatal plane. Similar "midfacial collapse" was reported by Stenstrom and Thilander (1970, 1972) when the varying amounts of nasal septum were removed from growing guinea pigs.

Interpretation of these seemingly similar results has been varied depending on the theoretical point of view of the investigator. Extirpation, while certainly reducing the "inherent cartilaginous growth potential" of the septum, necessarily alters the "skeletal unit's" structural integrity and interferes with its attendant "functional matrix." Hence the experimental design does not allow for a mutually exclusive type of conclusion from the results. Objective analysis of the results indicates that growth of the midface does not proceed in the same manner or at least to the same degree in the absence of the nasal septum. Whether this is due to the loss of "structural support" as Moss has reported or due to the loss of "growth potential" as concluded by those who support Scott's theory cannot be determined solely on the basis of extirpation studies.

Transplantation and Organ Culture

Transplantation of cartilage to remote sites has been undertaken in an effort to determine the intrinsic capability of cartilage to grow in the absence of extrinsic factors. Autotransplantation has yielded the most consistent results as opposed to the varied host response to homologous or heterologous transplan-

tation. Kvinsland (1973) transplanted rat nasal septum to an interperitoneal site in the same animal. He reported that the transplanted septa continued to grow at a rate comparable to in situ controls. Recently, rat condylar cartilage was transplanted to a functional site in the same animal (Engelsma et al., 1980). Using a microsurgical technique, the condyle was transplanted to the middle phalangeal area of the rat's forepaw. These transplants not only grew at a comparable rate to in situ controls, but also maintained their functional orientation. In addition, Petrovic and coworkers (1968) have demonstrated the ability of nasal septum to grow in organ culture.

The results of these studies would indicate that there exists some intrinsic capability of chondrocytes to divide and cartilage to grow in the absence of local extrinsic factors. While this ability is vital to Scott's theory, it can, at best, be considered only circumstantial evidence of the verity of his conclusions.

Autoradiography

Recently, autoradiography has been used to study regional variation in nasal septum chondrocyte proliferation (Searls and Kinser, 1972; Searls, 1975, 1976, 1979). Tritiated thymidine was injected into growing rats, rabbits, guinea pigs and beagles. These animals were sacrificed and their nasal septa removed for autoradiographic analysis. Their results demonstrated a lack of a

generalized metabolic pattern that is consistent from species to species. In addition, within each species there was no consistently discernible locus of increased cellular division. Instead, different areas of the septa were actively synthesizing DNA at different time periods during the growth of the animal. In light of these results, Searls (1979) rather objectively concluded:

> This picture could very well indicate a passive response, by the septum, to active growth sites in other tissues, rather than an active growth force within the septum itself...there also remains the possibility that similar growth patterns still might be seen among the four animals, but at different times within the life cycles of each.

Experimental evidence of growth modulation at the cellular level

Somatotropin and Somatomedin

Growth hormone, a polypeptide hormone secreted by the anterior lobe of the pituitary gland, has been shown to have a regulatory effect on skeletal growth. Daughaday and coworkers (1957, 1966, 1968, 1971, 1972) have suggested that growth hormone does not act directly on cells to regulate growth. Earlier studies of the effect of growth hormone on radioactive sulfate incorporation by cartilage cells grown in vivo had shown that uptake is reduced in hypophysectomized rats. Administration of exogenous somatotropin restores the ability of chondrocytes to incorporate $S^{35}O_4$. In an attempt to develop a more sensitive assay for serum growth hormone titers Salmon and Daughaday (1957)

studied the effect of radioactive sulfate incorporation into chondrocytes grown in vitro. Using costal cartilage obtained from hypophysectomized rats and incubated in nutrient medium containing $S^{35}O_4$, they were able to show that the addition of serum obtained from normal rats stimulated sulfate uptake. In contrast, serum taken from hypophysectomized rats or hypopituitary children demonstrated no effect on sulfate incorporation. Administration of exogenous growth hormone to a hypopituitary dwarf subsequently restored the ability of his serum to stimulate sulfate incorporation when added to the in vitro test system. However, direct addition of growth hormone to the cultured chondrocytes did not produce this expected increase in sulfate incorporation (Daughaday et al., 1959; Daughaday and Kipnis, 1966; Daughaday, 1971). Their results led to the conclusion that growth hormone was not directly responsible for the observed increase in sulfate incorporation.

Subsequent studies have demonstrated that "sulfonation factor" enhances a variety of cellular metabolic processes. This "secondary messenger" stimulates the incorporation of amino acids into proteins, uridine into RNA, thymidine into DNA, and proline into collagen (Daughaday and Reeder, 1967; Salmon and Duvall, 1970). The discovery of these additional actions of sulfonation factor led Daughaday (1972) to rename this mediator somatomedin.

A considerable body of information has been accummulated over the last decade pertaining to the relationship between somatomedin and cellular function. Van Wyk et al. (1971, 1972) and

Uthyne (1973) are credited with much of the work dealing with the purification and chemical characterization of somatomedin. Their combined results have yielded the following information. First, that at least three distinct types of somatomedin exist, designated with a letter suffix, somatomedin A and B (Uthyne, 1973) and C (Van Wyk et al., 1974). Somatomedin A was found to be a neutral polypeptide with a molecular weight of approximately 7000 daltons. Its biologic activity per milligram of protein has been shown to be about three million times greater than that of normal plasma. Somatomedin B is slightly smaller in molecular weight and acidic in nature. This peptide stimulates thymidine incorporation into fibroblasts. Both somatomedin A and B are growth hormone dependent. Somatomedin C, most recently discovered by Van Wyk (1974), has been characterized as a basic peptide rich in arginine, with a molecular weight in the same range as somatomedin A. Variations in plasma concentration of somatomedin C have been shown to be associated with skeletal growth disturbances. Acromegalic patients have increased plasma levels, and hypopituitary children demonstrate a decreased plasma concentration.

The site of synthesis of the somatomedins and their precise interaction with growth hormone have not been clearly defined. Several theories have been offered, but at this time, there have been no conclusive findings (Van Wyk et al., 1973; Daughaday, 1971). However, on the basis of the preceding find-

ings, it would seem evident that the somatomedins need to be considered in any theoretical model pertaining to the control of cellular metabolism.

Insulin

The islets of Langerhans in the pancreas secrete two polypeptide hormones, glucagon and insulin. Although both hormones function to regulate intermediary cellular metabolism of proteins, carbohydrates and fats, only insulin has a demonstrated effect on skeletal growth. Insulin has been shown to stimulate growth in hypophysectomized rats to almost the same degree as growth hormone. The activity of insulin in biologic systems has been demonstrated to act by binding to specific cell membrane receptor sites. This binding generally results in stimulation of anabolic cell processes.

Hajek and Solursh (1974) have shown stimulation by insulin of growth and mucopolysaccaride synthesis in chick embryo chondrocytes grown in monolayer culture. Salmon and Daughaday (1957) and Salmon et al. (1968) have shown an increased sulfate incorporation and other effects similar to that seen with growth hormone, when costal cartilage cells from hypophysectomized rats were treated with insulin *in vitro*. However, insulin does not seem to exert these effects on cartilage growth *in vitro* when administered at physiologic concentrations. There is also experimental evidence

suggesting a correlation between insulin and the somatomedins. Hintz et al. (1972) reported that the somatomedins compete for insulin binding sites on adipocyte, liver and chondrocyte cell membranes. This competitive binding suggests not only a functional, but also a structural homology between insulin and somatomedin.

Cyclic Adenosine Monophosphate (cAMP)

Cyclic adenosine monophosphate has been shown to be an important factor in the regulation of cellular metabolism and proliferation. This molecule is present, in varying concentrations, in all known cellular organisms. It was first discovered by Sutherland (1951) to act as a cofactor in the conversion of the enzyme phosphorylase from an inactive to an active form.

Since its initial discovery, cAMP, has been postulated to act as a "secondary messenger" in a variety of cell types. Variations in cytoplasmic levels of cAMP appear to exert a modulating effect on enzymatic reactions. Although the effect of cAMP is itself non-specific, its intracellular release usually triggers a specific biologic reaction sequence. Studies have shown that the action of many non-steroid hormones are mediated by cAMP. The hormone binds to specific receptors on the target cell membrane and this binding leads to the non-specific release of adenylate cyclase which catalyzes the conversion of adenosine triphosphate

to cAMP. The cellular response to cAMP levels is variable depending on the functional and genetic differentiation of that cell type. Recently, Drezner et al. (1976) have shown that chick cartilage is a tissue in which cAMP stimulates anabolic processes. Their study showed that increased intracellular levels of cAMP stimulated the incorporation of radioactive precursors into cartilage proteoglycans.

The interdependence between circulating mediators and intracellular cAMP levels suggests that cAMP may be involved as a cofactor in the initiation, modulation or termination of the growth process.

Anti-cartilage Antibodies

Antibodies against bovine cephalic cartilages and epiphyseal cartilage were developed in rabbits by Brigham (1976) from this laboratory. Utilizing a dissociative extraction technique, developed for use in studying proteoglycan structure in cartilage, Brigham obtained the antigenic components of bovine cartilage. He injected the lyophylized antigenic extract of either bovine nasal septum, condylar or epiphyseal cartilage into experimental rabbits to elicit an immunologic reaction. Subsequent isolation of the immunoglobulin G (IgG) fraction of the immune serum yielded antibodies directed against either bovine nasal septum, condylar or epiphyseal cartilage. Using cross adsorption and indirect immuno-

fluorescent labeling, a technique which allows visible demonstration of antibody binding to tissue sections when viewed under fluorescent light, Brigham determined the following characteristics pertaining to anti-cartilage antibodies:

1. There exist antigenic differences among the condylar, the nasal septal and epiphyseal cartilages. In addition, there are regional differences in location of antigenic determinants in nasal septal cartilage.

2. All three cartilages possess some common antigenic determinants.

3. Condylar cartilage possesses at least one antigenic determinant not found in nasal septal or epiphyseal cartilage.

Brigham's work supports the contention that cartilage tissues exist in at least two antigenically different configurations. This difference is important because all three cartilages are actively involved in endochondral bone formation. Prior to this study it was assumed that these so called "growth cartilages" were antigenically and possibly biologically identical. Brigham's results suggest that these cartilaginous sites while having some antigenic similarity in structure, are not identical. This would imply that there may be a similar, but not identical, functional relationship between these tissues.

Based upon this previous work on anti-cartilage antibodies Palmer (1978) tested this antibody binding in vivo. He extracted the protein components (antigens) of rat nasal septum and obtained like extracts from bovine nasal septum and condylar cartilage.

Each of these antigenic extracts was injected into a different rabbit to produce an immunologic response. Isolation of the IgG fraction from the rabbit's serum yielded antibodies specific for each cephalic cartilage. Each of these antibody preparations was injected into a different experimental population of growing rats in order to determine the effect of anticartilage antibodies on the process of craniofacial growth and development. In addition, histologic examination of nasal septal and condylar cartilage at both the cellular and ultrastructural level was conducted. The results of Palmer's study may be summarized as follows:

1. Anti-cartilage antibodies became bound to cartilage in vivo. In addition, the greatest localization was seen in the nasal septum with anti-rat nasal septum antibody, suggesting a high correlation between species and tissue homology.

2. Histologic examination showed some indication of cytopathic effects (stellate, pleomorphic cells) in paraffin embedded sections of nasal septum taken from the experimental group treated with anti-rat nasal septum antibody.

Palmer's results suggest that these antigenic determinants evidenced in bovine nasal septum by Brigham are also present in situ. Additionally, Palmer's work provided an animal model in which circulating anti-cartilage antibodies became localized to their target tissue.

It appears that anti-cartilage antibodies offer the basis for an experimental methodology that allows specific localization

of potentially cytotoxic agents. Such an experimental tool could prove beneficial to the study of mechanisms controlling growth of the craniofacial skeleton.

Department of Orthodontics

STATEMENT OF THESIS

The purpose of these investigations is to further characterize the effects of anti-nasal septum cartilage antibody on midfacial growth *in vivo* and on isolated rat nasal septum chondrocytes grown in tissue culture. Of primary interest is the effect of anti-rat nasal septum antibody on chondrocyte viability and/or metabolism *in vitro*. In the second part of this study the effect of anti-rat nasal septum antibody will be evaluated *in vivo*. For this purpose, the present study will use a randomized block design to include treatment and controls in the same cage. Newborn rats will be injected with anti-rat nasal septum antibody over a 30 day period. At the end of this time period, the animals will be sacrificed, and the effects of anti-cartilage antibody on craniofacial dimension will be examined by cephalometric radiography.

MATERIALS AND METHODS

I. Preparation of Rat Nasal Septum

Dissection of Septa

Eighty, six week old Sprague-Dawley rats were killed by intrapleural injection of 20 mg. Diambutal (Sodium Pentabarbital, Diamond Laboratories, Inc.) and the cartilaginous components of their nasal septa were dissected free by the following method. Bilateral incisions through the nares extending to the frontal bone were performed on each animal. This permitted removal of the overlying nasal bones to expose the septum and associated tissues. Tissue forceps were used to stabilize the septum, and the cartilage was separated from its sphenoid and ethmoid articulations using a fine #15 scapel. Following extirpation the septa were scraped free of all adherent soft tissue using a dulled #15 scapel blade. The septa were then pooled and stored in cold, isotonic, phosphate buffered saline (PBS) pH = 7.4 until used for isolation of the antigenic components.

Isolation of Antigenic Components

Following a modified technique described by Sadjera and Hascall (1969) the septa were finely minced, suspended in PBS, and the specimen was then homogonized at 4°C using a Virtis 45 homogenizer (Gardner, New York). The homogenate was then centrifuged

at 27,000 x g for one hour on a Sorvall model RC 2-B refrigerated centrifuge. The supernate (PBS soluble proteoglycans) was removed, dialyzed exhaustively against 2x distilled water, lyophylized and stored at -20°C. The insoluble proteoglycan fraction (PG-I) was suspended in 0.5 M Tris HCl buffer, pH = 8.5, containing 0.3 M β-mercaptolethonol and 8 M Urea (10 ml solution/gm of septa). Reduction of the PG-I fraction was carried out under a positive 100% nitrogen atmosphere for 6 hours after which time the concentration of β-mercaptoethanol was increased to 0.6M and the reaction allowed to continue for 18 additional hours. Following centrifugation (27,000 x g for 15 min.) the supernate was treated with 1.2 M iodoacetic acid to acetylate any free sulfhydryl groups; the pH was maintained at 8.5 by addition of 10 M NaOH. The reaction was quenched by addition of a two-fold molar excess of β-mercaptoethanol after no further change in pH was noted. The extracted proteins were dialyzed against distilled water, lyophylized and stored at -20°C. One hundred and thirty-nine mg of solubilized material was recovered from the reduced and alkylated extract, fraction PG-I.

II. Preparation of Antisera

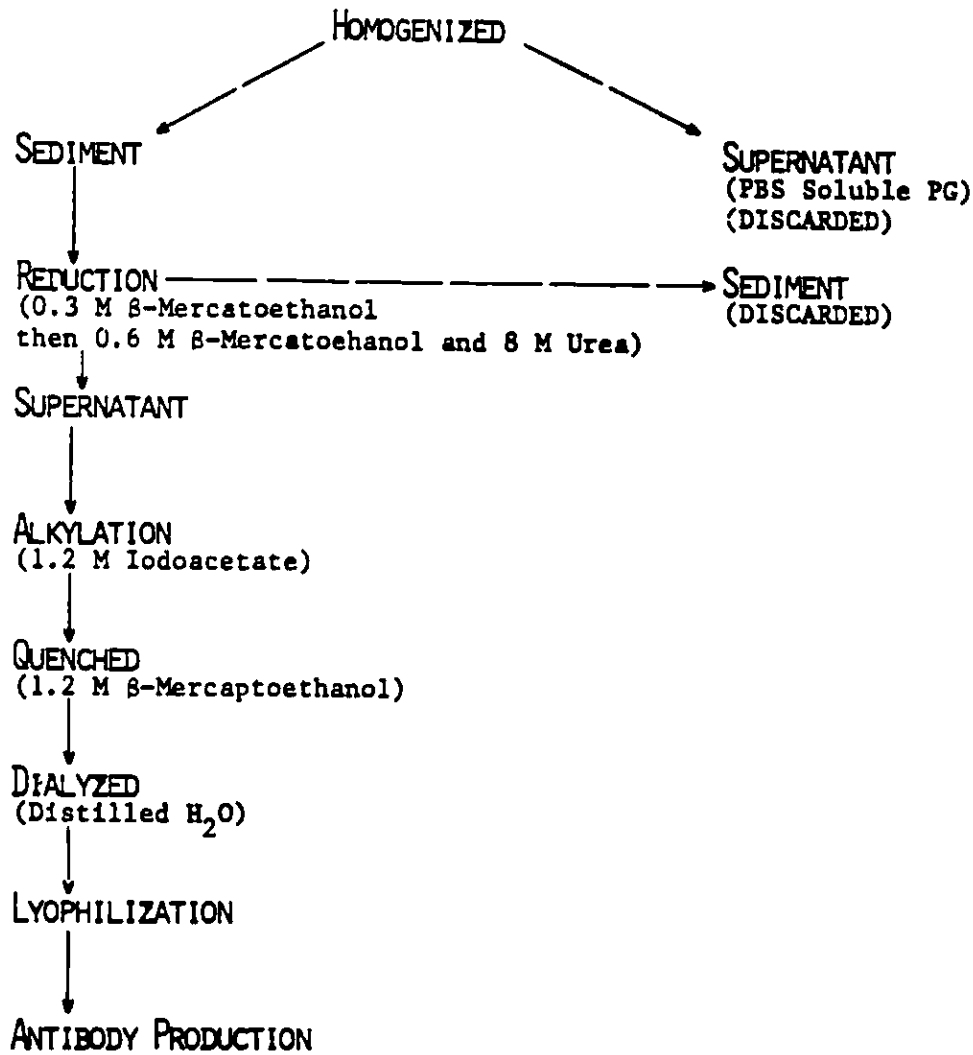
Immunization Procedures

Five male New Zealand white rabbits (Lab Animals, Akron, Ohio) were obtained for rat nasal septum antibody production.

Figure 1. Flow sheet representing the preparation of antigen for immunization.

ANTIGEN PREPARATION

RAT NASAL SEPTUM



Each animal was sedated with 3 mg azapromazine/kg body weight and injected with 1 mg of antigen in 0.5 ml of a 1:1 mixture of PBS and complete Freund's adjuvant in the region of the popliteal lymph nodes. Two additional injections of antigen (1 mg in 0.5 ml PBS) were given at three week intervals (Palmer, 1978). Antibody titers were maintained with monthly intramuscular injections of 1 mg antigen in 0.5 ml PBS. Antibody titer was determined for each animal prior to beginning experimentation, and each rabbit maintained at an antibody titer level of approximately 80 as determined by serial dilution and indirect immunofluourecence (IIF) (Coons et al., 1950). (See section III, Indirect Immunofluorescent Technique). Fresh frozen cryostat sections reacted with each serially diluted antiserum were used to estimate the antibody titer.

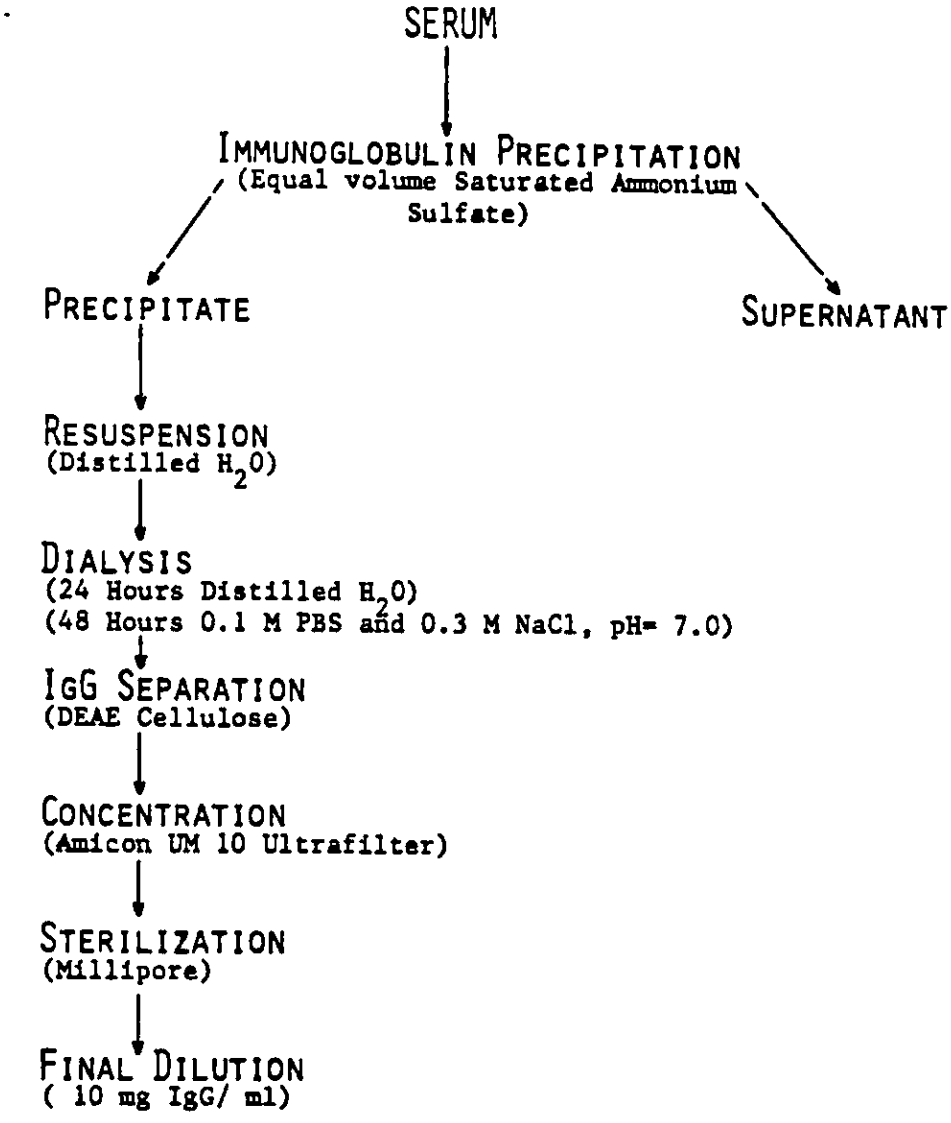
Serum Collection and Storage

The rabbits were sedated with 3 mg azapromazine/kg body weight and bled by intracardial puncture. A total of 45 ml whole blood per animal was obtained weekly. The serum was prepared and pooled for subsequent isolation of the immunoglobulin G (IgG).

Isolation of the crude IgG fraction was done utilizing approximately 500 ml of pooled sera. The globulin portion of the serum was precipitated by addition of an equal volume of saturated ammonium sulfate to yield a final concentration of 50% ammonium

Figure 2. Flow sheet representing the fractionation of antisera.

IgG PREPARATION



sulfate. The resulting precipitate was dissolved in 100 ml of distilled water and dialyzed against 0.1 M PBS and 0.03 M NaCl, at pH 7.0, to remove ammonium sulfate. After exhaustive dialysis against this buffer, the dialisate was centrifuged (30,000 x g for 45 min.) to remove particulate matter and the IgG fraction was chromatographically purified on a DEAE (diethylaminoethyl) cellulose column (Whatman DE 23, W. R. Balston, Co., England) as described by McDonough and Inman (1977). The IgG fraction, spectrophotometrically monitored at 280 nm eluted in the void volume (Peak A) and the remaining globulins were eluted with 1 M NaCl. Both fractions were concentrated to 10 mg/ml using an Amicon UM10 ultra filter (Amicon Co., Lexington, Mass., USA) sterilized by filtration through a 0.2 micron pore size milipore filter and stored at -20°C. Acrylamide gel electrophoresis under standard (pH = 8.9) conditions was used to confirm that the IgG fraction was contained in Peak A. The IgG fraction from normal rabbit serum (NRS Type III Pel Freez, Ark.) was prepared in the same manner and used as the control for subsequent experimentation.

III. In Vitro Cell Culture

Primary Cultures

The cartilaginous portion of the nasal septum was carefully extirpated from 9 four-day-old Sprague Dawley rats by the

previously described method. Removal of all bony fragments and adherent soft tissue was verified by inspection under a dissecting microscope. The cleaned septa were minced into approximately 1 mm^2 sections and placed in a digestion chamber (courtesy Dr. Charles Malemud, CWRU). The minced cartilage was digested at 37°C for 30 minutes in 10 ml PBS (pH = 7.2) containing 0.1% Trypsin (Grand Island Biological Co. (GIBCO), Grand Island, New York). This enzyme solution was decanted and discarded. The undigested sections of cartilage were then reacted for 40 minutes at 37°C in a 0.1% solution of crude collagenase (Sigma Chemicals Co., St. Louis, Missouri) dissolved in PBS (pH = 7.2). The supernatant enzyme solution containing chondrocytes liberated from the cartilage matrix was centrifuged at low speed (1700 x g) for 5 minutes and carefully decanted. The remaining pellet of chondrocytes was suspended in Ham's nutrient mixture F-12 (GIBCO), which was supplemented with 10% fetal calf serum (FCS) (GIBCO), penicillin (10 µg/ml) and streptomycin (10 µg/ml). These chondrocytes were then inoculated in T-30 culture flasks (Falcon Plastics, Los Angeles, California) at a concentration of 2 x 10^6 cells/5 ml of the same culture medium. The cultures were equilibrated to pH = 7.2 with 5% CO_2 in air and incubated at 37°C. After twenty-four hours, approximately ninety percent of the cells were attached and numerous mitotic figures were evident. Cultures were fed twice weekly with increasing volumes of the cultivation medium to main-

tain good cell growth. Confluent cultures were obtained within 72 hours, but some were maintained for a period of one week to increase the yield of cells.

Subculture and Freezing

Confluent primary cultures were trypsinized (0.1% trypsin, for 5 minutes at 37°C) and cells collected by centrifugation (5 minutes at 1700 x g). Confluent cultures contained approximately 5×10^6 cells/T-30 flask. The trypsinized cells were either placed in ampules containing 4×10^6 cells/ml in Hams F-12 supplemented with 15% FCS and 10% dimethylsulfoxide (DMSO) and frozen for storage at -96°C or subcultivated for an additional *in vitro* passage. These secondary cultures were used for production of additional stocks of cells for freezing and for the initiation of coverslip cultures for immunohistochemical evaluation. Secondary cultures were either initiated in T-30 culture flasks at a density of 5×10^5 cells/5 ml or in 60 mm culture dishes (Falcon Plastics) with 1.5×10^5 cells/4 ml of the standard cultivation medium. These cultures were also fed at regular intervals. Subsequent experimentation utilized either frozen primary cultures or cells frozen in like manner from first passage cultures.

Indirect Immunoflourescent Technique

Antibody specificity was assessed by indirect immunofluorescence (Coons et al., 1950) using different substrates

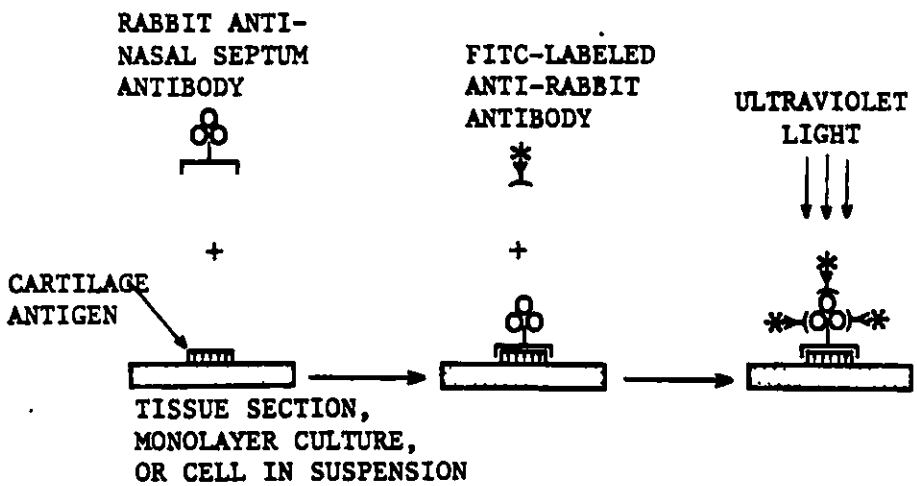
including 6 µm cryostat sections of rat nasal septum, monolayer cultures of rat nasal septum, chondrocytes grown on glass coverslips, or trypsinized rat nasal septum chondrocytes in suspension. Cryostat sections were incubated with 2 drops of control or immune serum for 35 minutes at 37°C. The sections were then rinsed in distilled water and washed in three changes of PBS (Phosphate buffered saline) for 10 minutes each. The slides were then dipped into distilled water and allowed to air dry. Two drops of goat anti-rabbit IgG (heavy chain specific) conjugated with fluorescein isothiocyanate (FITC, Cappel Laboratories, Cochranville, Pa.) were applied to each section and incubated at 37°C for thirty-five minutes in a humid container. Each slide was then washed as previously described. Sections were mounted using a 1:9 mixture of 0.1 M carbonate buffer (pH = 9.0) and glycerol and examined microscopically with a Zeiss fluorescent microscope equipped with the appropriate excitation and barrier filters. Coverslip cultures of primary chondrocytes were fixed in cold 95% ethanol and then treated identically to determine antibody binding. Trypsinized chondrocytes in suspension were not fixed prior to incubation with antisera to determine antibody binding sites.

IV. Antibody Cytotoxicity In Vitro

Viability

Four log-phase cultures from first passage frozen stock

INDIRECT IMMUNOFLUORESCENCE TECHNIQUE



were grown 48 hours in the presence of 1 μci/ml tritiated thymidine (New England Nuclear, Boston, Massachusetts) in Ham's F-12 medium (GIBCO) with 10% fetal calf serum (GIBCO), penicillin and streptomycin. At the end of this 48 hour period essentially all of the cells contained radioactively labelled nuclei. This prelabelling of the cell nuclei with radioactive thymidine was carried out to allow estimation of the number of cells by assay of radioactivity in a liquid scintillation counter (Beckman LS 230). The labeling medium was then decanted and discarded and five milliliters of fresh incubation medium containing one of the following antibody, medium, serum combinations was added to each flask:

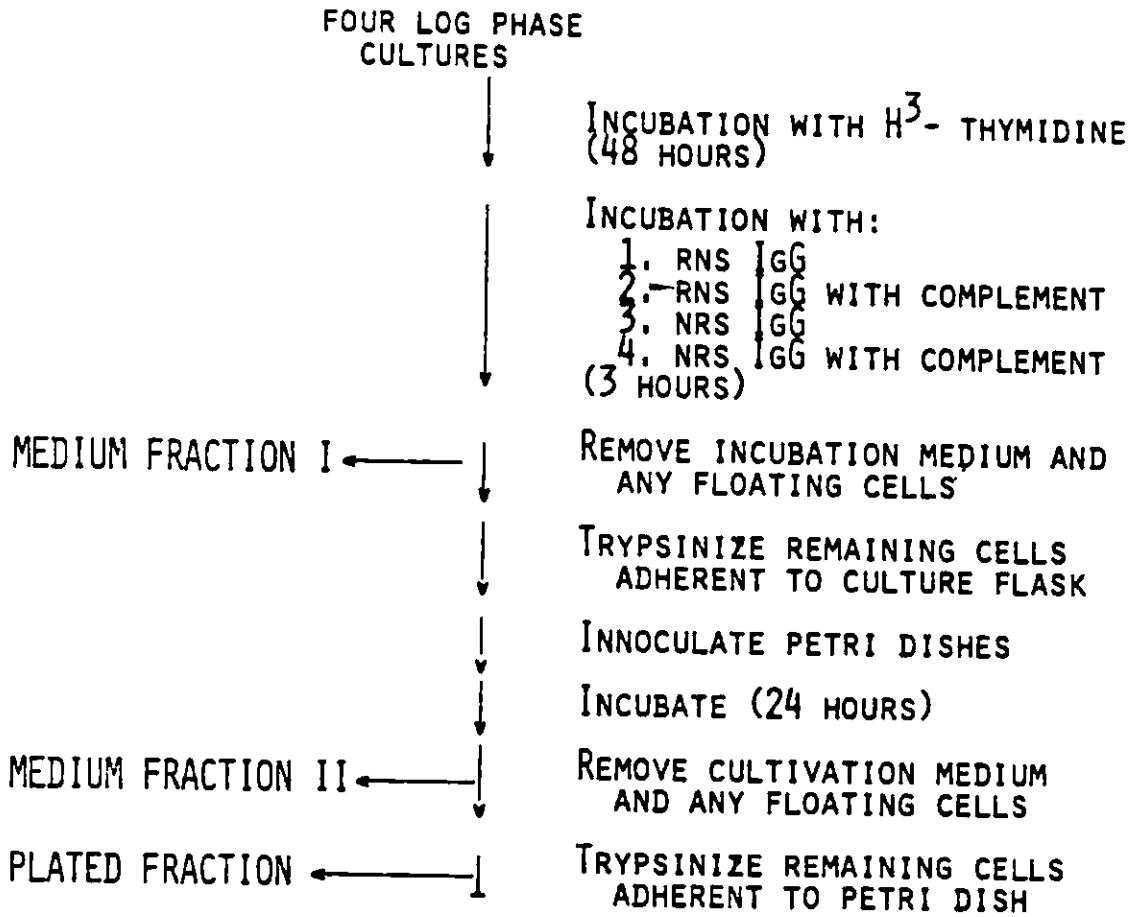
1. Rabbit anti-rat nasal septum IgG (7 mg/ml) and 10% fresh rabbit serum, in Hams F-12 medium;
2. Rabbit anti-rat nasal septum IgG (7 mg/ml) and 10% fresh rabbit serum heated to 56°C for 30 minutes to inactivate complement in Hams F-12 medium;
3. Normal rabbit IgG (7 mg/ml) and 10% fresh rabbit serum in Hams F-12 medium;
4. Normal rabbit IgG (7 mg/ml) and 10% heat inactivated fresh rabbit serum, in Hams F-12 medium.

Cultures were incubated in a humid incubator for three hours at 37°C in 5% CO_2 and air. At the end of the three hour incubation period the medium and any cells which had been dissociated from the monolayer were removed and saved for radioactivity determination (Medium Fraction I). The remaining attached cells were

trypsinized with a solution of .025% trypsin, 1% ethylene diamine tetraacetate (EDTA) in PBS, and the cells collected by centrifugation. Cells of the four treatment groups were suspended in fresh Ham's F-12 medium and innoculated into petri dishes. After 18 hours in a 5% CO_2 and air, humid incubator at 37°C these cultures were examined microscopically and plating efficiency was computed by the following method.

The medium containing any chondrocytes which were not adherent to the culture dish was decanted and saved (Medium fraction II). The cells adherent to the petri dish were collected by trypsinization and comprised the second fraction (Plated fraction). Plating efficiency was determined by computing the ratio of the number of attached cells (plated fraction) to the total number of cells contained in both fractions (Medium Fraction II and Plated Fraction). In addition, cell viability was estimated for the entire experimental population of chondrocytes. Chondrocytes which were not attached to the culture flask after the initial incubation with antibody (Medium fraction I) were included with the chondrocytes which did not adhere after subcultivation (Medium fraction II) for viability estimation. The cytotoxic effect of the antisera was estimated by taking the ratio of the cells in medium fractions I and II which were presumably non-viable to the total number of chondrocytes in the original labeled sample (see flow chart, Figure 3).

Figure 3. Flow sheet for viability and plating efficiency study.



$$\text{NON-VIABLE CELLS (PERCENT)} = \frac{\text{MEDIUM FRACTION I}}{\text{TOTAL CELLS}} \times 100$$

$$\text{PLATING EFFICIENCY (PERCENT)} = \frac{\text{PLATED FRACTION}}{\text{PLATED FRACTION + MEDIUM FRACTION II}} \times 100$$

Trypan Blue Viability

The cytotoxic action of the RNS IgG was also examined on trypsinized cells in an isotonic PBS suspension. A culture of chondrocytes in the log phase of growth was trypsinized and the cells collected by centrifugation as previously described. The cells were then re-suspended and divided equally into 4 test groups. One of the following 4 antibody serum combinations in PBS was added to each group after centrifugation and removal of the diluting medium (Ham's F-12):

1. Anti-nasal septum (rat) IgG (4 mg/ml) and 10% fresh rabbit serum.
2. Anti-nasal septum (rat) IgG (4 mg/ml) and 10% heat inactivated fresh rabbit serum.
3. Normal rabbit IgG (4 mg/ml) and 10% fresh rabbit serum.
4. Normal rabbit IgG (4 mg/ml) and 10% heat inactivated fresh rabbit serum.

Following a two hour incubation period at 37°C the chondrocyte suspension was reacted for five minutes with 0.2% trypan blue (Allied Chemical, Morristown, N.J.). One drop of the trypan blue treated suspension was placed on a hemocytometer and examined microscopically. At least 100 cells in each group were counted and scored viable (unstained) or non-viable (stained). The cytotoxic effect of the antibody was determined by the ratio of stained cells to total cells in each experimental group.

Radioactive Protein Accumulation

Analysis of protein accumulation was conducted to determine the effects of the RNS antibody on cellular metabolism. A mixture of tritiated amino acids (New England Nuclear, NET 250) was used to assess changes in accumulation rate. No attempt was made to identify the specific proteins being accumulated. Twenty-four 25 mm petri dishes (Falcon Plastics) were each inoculated with 1.5×10^5 nasal septum chondrocytes from second passage frozen stock. Cells were grown for two days prior to the beginning of the experiment in our standard cultivation medium to allow for establishment of the culture. Prior to incubation with the isotope this medium was decanted and the cultures washed with Hank's balanced salt solution to remove unlabelled amino acids adhering to the surface of the cells and culture dishes. One of the following antibody-serum combinations in Ham's F-10 nutrient medium (GIBCO) containing 0.01 times the normal concentrations of amino acids and 5 μci/ml tritiated amino acid mixture was added to the cultures:

1. Anti-nasal septum (rat) IgG (8 mg/ml) with 10% fresh rabbit serum.
2. Anti-nasal septum (rat) IgG (8 mg/ml) and fresh rabbit serum heated to 56°C for 30 minutes to inactivate complement.

At half-hour intervals, duplicate cultures were removed from the incubator, the cells scraped into the medium and the proteins

precipitated using a solution of 5% trichloroacetic acid (TCA) -0.25% tannic acid which effectively terminated further protein synthesis. At the end of the three hour period the precipitates were washed repeatedly with 5% TCA -0.25% tannic acid to remove free labeled amino acids. The precipitate was solubilized by addition of 0.5 ml Nuclear Chicago Solubilizer (NCS, Amersham Searle) and agitated in a water bath at 56°C for six hours. The digest was neutralized by addition of 20 µl glacial acetic acid, added to a Triton X 100 based aqueous scintillation fluid and radioactivity was determined in a Beckman LS 230 scintillation counter. Counts per minute (CPM) were converted to disintegrations per minute (DPM) by use of the external standard.

V. In Vivo Experimental Design

Rationale

Beutner (1975) and Toyka (1977) have shown that immunoglobulins can be passively transferred from one animal to another. In addition, these antibodies can be localized in specific target tissues and cytotoxic antigen-antibody reactions can be demonstrated. Palmer (1978) demonstrated in vivo binding and apparent cytopathic changes by injecting rabbit anti-rat nasal septum antibody into growing rats.

The experimental design was chosen for this study based upon the following considerations:

1. The magnitude of expected growth alteration may be expected to be small owing to the inherent amount of extraneous variables involved in such an in vivo experimental model.

2. Palmer (1978) found that Sprague-Dawley rats exhibit a significant amount of interlitter variability in craniofacial dimensions thus necessitating the inclusion of all treatments and controls within each litter (random assignment).

Timed-pregnancy Sprague-Dawley rats were obtained and from them 60 newborn rats were obtained for this experiment. Ideally, the biologic mother should have remained with her offspring; however, due to variability in the number of each offspring in the various litters this experimental design was not used. Instead, the 60 newborns were randomly divided into three experimental groups, each containing 20 animals and then randomly assigned to one of 5 cages. Five of the 6 pregnant females were used as "cage mothers", the sixth female was not used for experimentation. Each cage housed 12 animals; 4 received isotonic saline injections, 4 received injections of the IgG fraction isolated from normal rabbit serum (NRS) and 4 received injections of the isolated IgG fraction from serum of rabbits immunized with rat nasal septum antigen (RNS). The assignment of the three treatments among the 12 animals in each cage was performed randomly in accordance with the block design.

Injection Schedule

In order to retain the balanced design of the experiment,

all antibody preparations and control saline for injection were prepared in advance and frozen in aliquots at -20°C. The injection schedule was based upon that used by Palmer (1978). All injections commenced on the first day postpartum. At this time the immunological system of the neonate is immature, and tolerance to the rabbit immunoglobulin can be effected. Because the half-life of rabbit IgG injected into an adult rat is four days, (Pelley, 1977) all animals were injected every fourth day after the initial injection. Based on the work of Vogel and Stoeckert (1963), 0.35 mg of IgG per gram of body weight was used to provide total plasma volume replacement of the immunoglobulin G component. Prior to each injection the animals were weighed and the amount of IgG computed for that day's injection. Due to practical limitations of size, the first three injections (days 1, 4 and 8) were administered subcutaneously between the scapulae. Beginning on day 12, the injections were administered intraperitoneally until completion of the study.

Following the methodology established by Palmer (1978), the experimental time was set at thirty days. This thirty-day time period encompasses the majority of postnatal facial growth of the rat skeleton which occurs between 15 and 20 days of age (Behrents, 1975; Baer, 1951), corresponding to the growth spurt seen in human subjects between ages nine and eleven. In addition, the change in facial form from round to a more elongated appear-

ance was reported by Massler and Schour (1951) to occur prior to 30 days of age.

On day 30, the animals were killed by overdose with Diambutol, individually weighed, sexed, and radiographed in both the lateral and dorso-ventral orientation.

Radiographic Technique

Each animal was oriented in both norma lateralis and norma dorsalis for cephalometric analyses. A modified cephalostat designed by Palmer (1978) was used to insure reproducible object-film and source-film distances. Radiographs were taken utilizing the Broadbent Bolton cephalometer (Series 1K550, Bolton Fund, Cleveland, Ohio) and standard dental occlusal film (Kodak, Df46, Rochester, N.Y.) at 70kv, 20 mA with an exposure time of 3 seconds. Cephalometric records were taken in duplicate and developed for maximum contrast.

Cephalometric Landmarks

The following relevant and reproducible cephalometric landmarks were used to analyze facial changes. These points are illustrated on the diagram in Figure 4.

Dorso-ventral

1. The most posterior point on the external occipital protuberance (0);

2. The most posterior point on the mandibular condyle (CO);

3. The tip of the coronoid process (C);

4. The most lateral point on the zygomatico-maxillary suture (ZG);

5. The most anterior point on the maxillary bone at the infra-orbital foramen (MX);

6. The most anterior point on the nasal bone (N);

7. The most anterior point on the outline of the foramen magnum (BA);

8. The middle of the sphenoccipital synchondrosis (at the midline) (SO);

9. The middle of the intersphenoidal synchondrosis at the midline (IS);

10. The most posterior point on the palatal bone at the midline (P);

11. The interface between the maxilla and the mesial surface of the maxillary first molar root (UM);

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

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

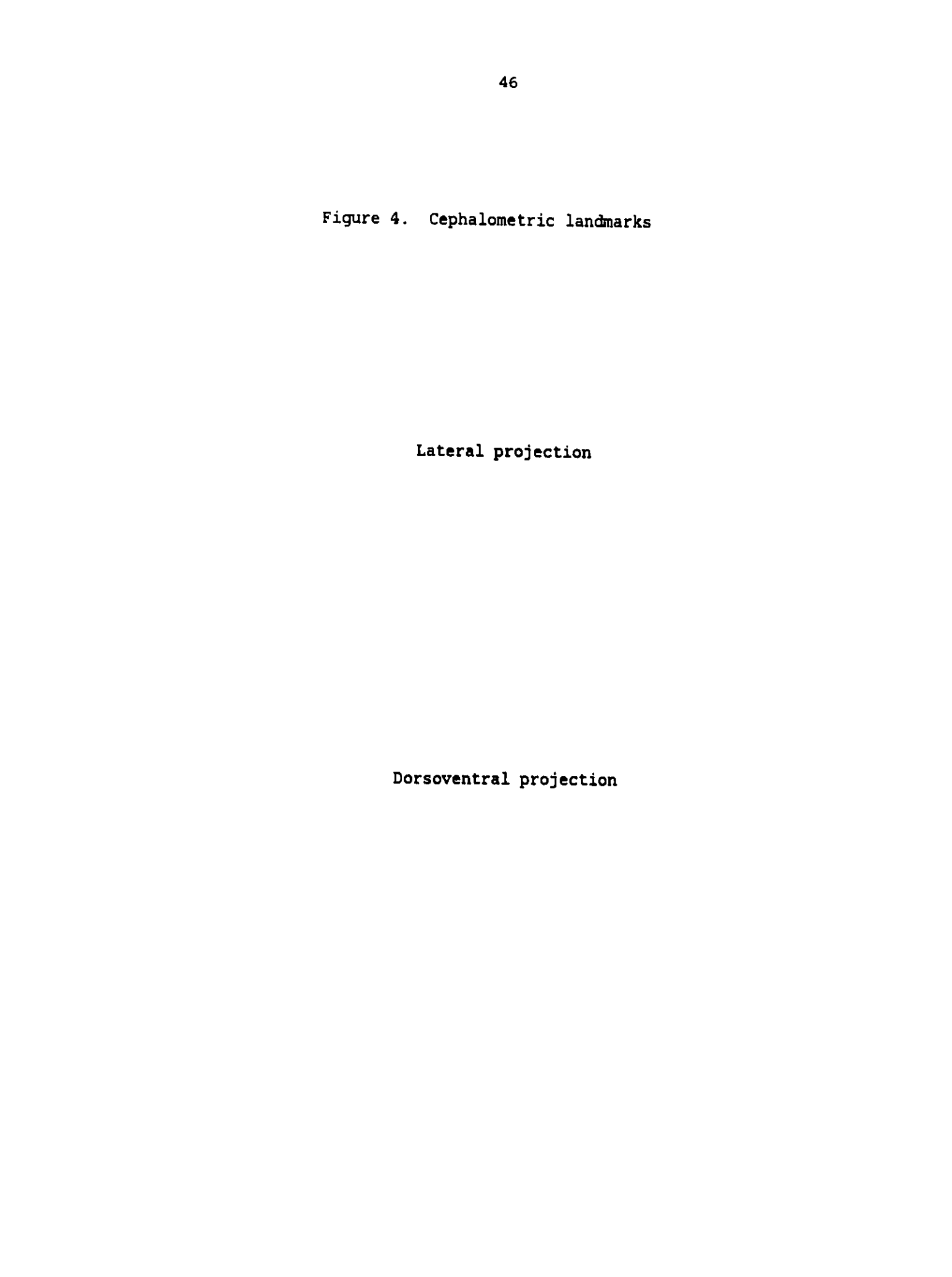
Lateral

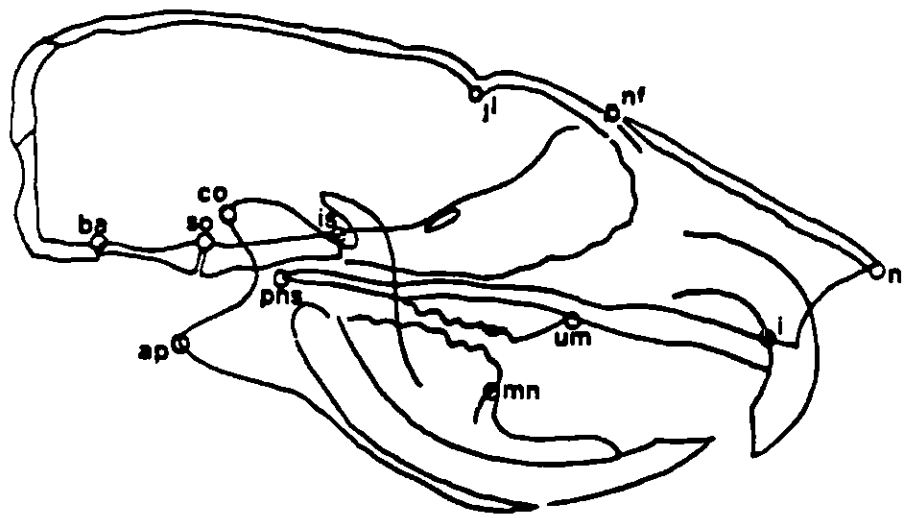
1. The most anterior point on the margin of the foramen magnum (ba);

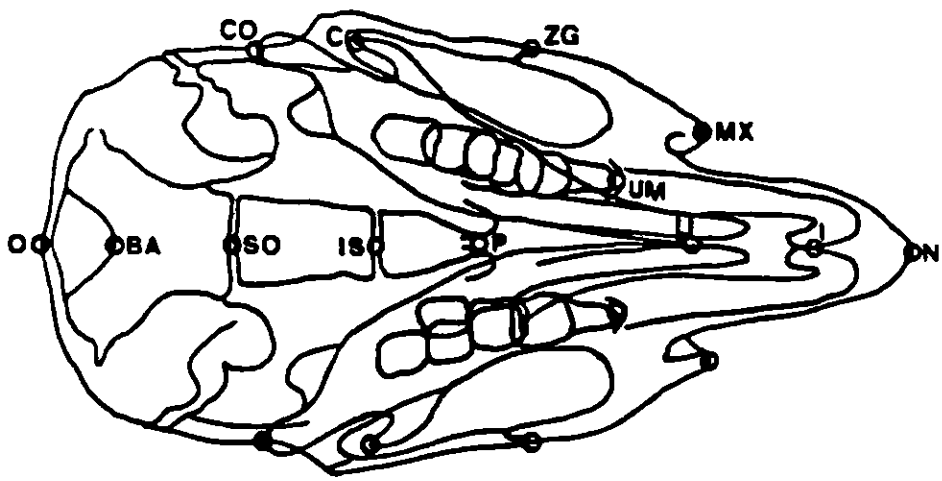
2. The most superior (dorsal) point on the outline of the sphenoccipital synchondrosis (so);

3. The most superior (dorsal) point on the outline of the intersphenoidal synchondrosis (is);

Figure 4. Cephalometric landmarks




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


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

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

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

6. The tip of the nasal bone (n);

7. The intersection of the lingual periodontal membrane of the maxillary incisors and the superior (dorsal) plane of the bony palate (mx);

8. The intersection of the hard palate and the anterior surface of the averaged maxillary right and left first molar roots (um);

9. The most posterior point on the palatal process of the palatine bone (pns);

10. The most posterior point on the curvature of the averaged outline of the left and right condyles (co);

11. The most inferior and posterior point on the outer surface of the angular process (ap);

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

Cephalometric Analysis

Sixty-five linear measurements were used to characterize the craniofacial dimensions of each animal. A description of each measure can be found in Tables I through V along with its construction shown in Figures 5 through 9. This analysis was adapted from earlier works (Gianelly and Moorrees, 1965; Stenstrom and Thilander, 1970; Gange, 1973; Behrents, 1975; Bernabei, 1975; Paulus, 1976; Palmer, 1978) with an emphasis on measures that reflected changes in midfacial dimension.

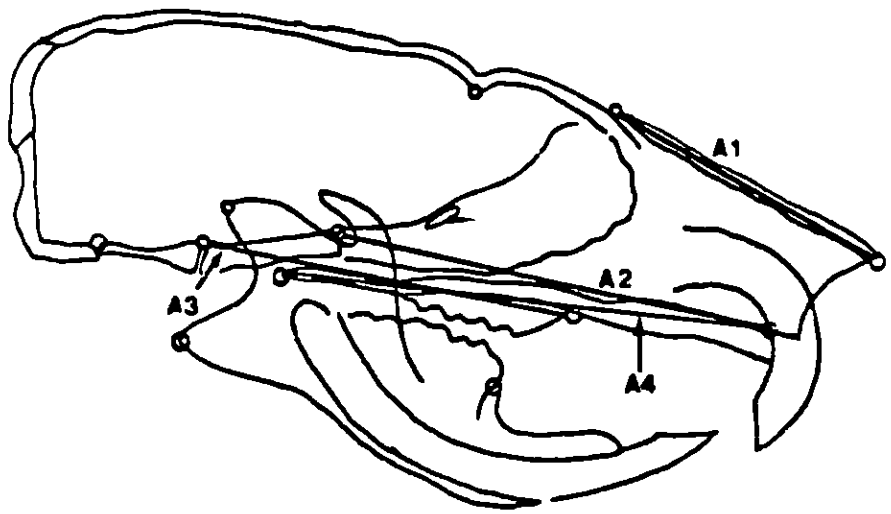
TABLE I

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

Variable	Landmarks Employed	Measures
	Lateral View	
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
	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

Figure 5. Construction of variables to measure anteroposterior changes in midfacial bony size or displacement of the midfacial complex. Lateral cephalometric orientation.


A1
A2
A3
A4

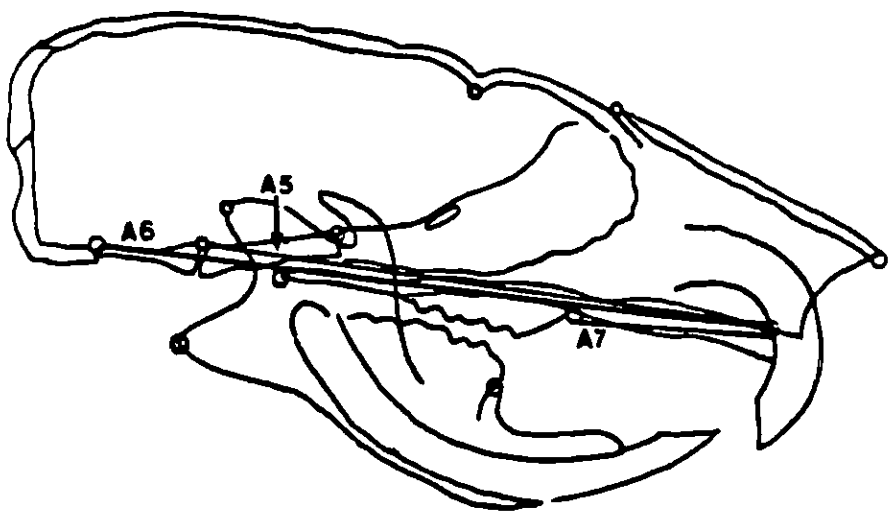
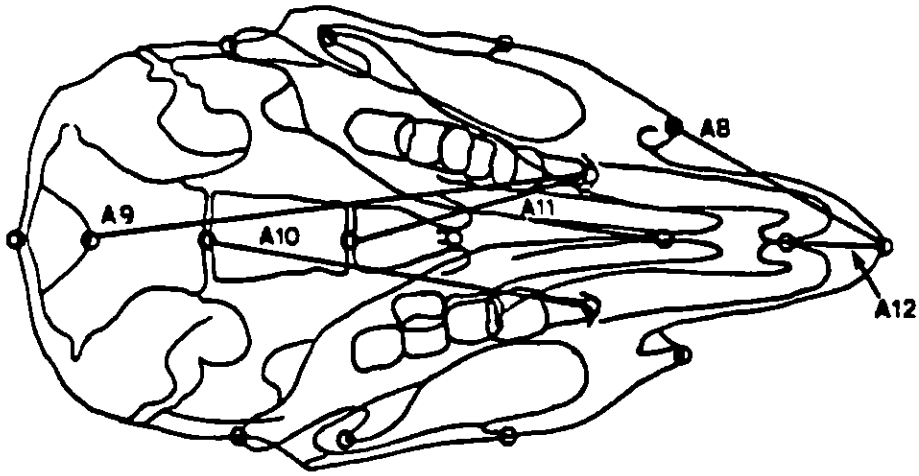

A5
A6
A7

Figure 6. Cephalometric analysis. Variables to measure anteroposterior changes in midfacial bony size or displacement of the midfacial complex. Dorso-ventral cephalometric orientation.


A8
A9
A10
A11
A12

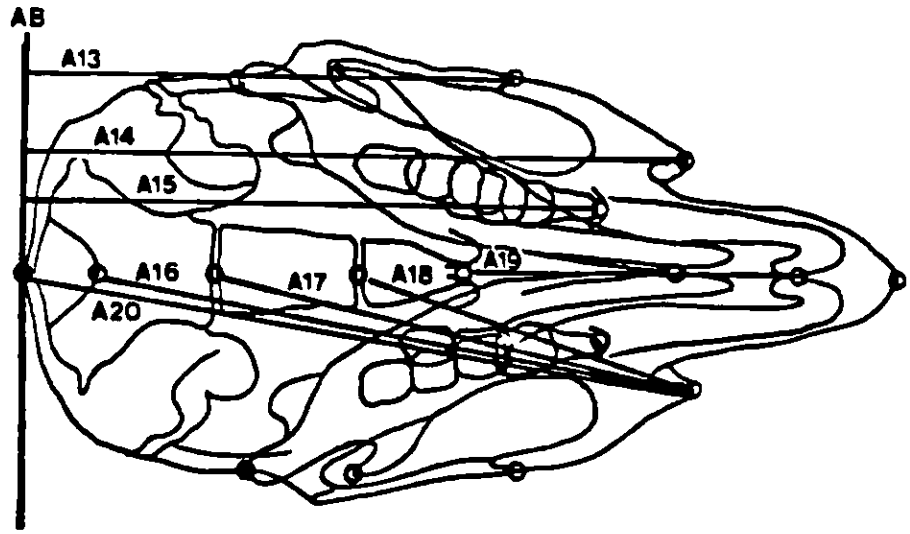

AB
A13
A14
A15
A16
A17
A18
A19
A20

TABLE II

VARIABLES TO MEASURE CHANGES IN THE MANDIBLE

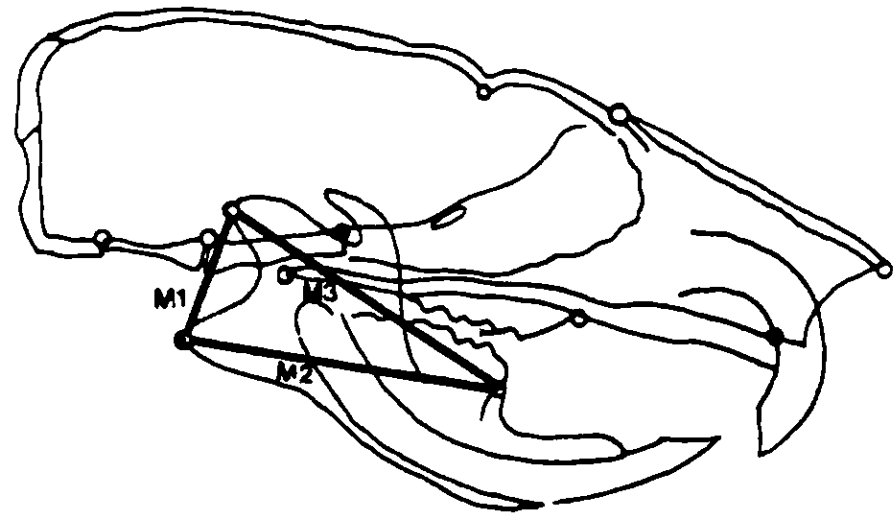
Variable	Landmarks Employed	Measures
	Lateral View	
M1	co-mn	Mandibular ramus height
M2	ap-mn	Mandibular body length
M3	ap-co	Ramal height
	Dorsal Ventral View	
M4	*AB-CO	Anterior condylar position
M5	CO-CO	Bicondylar width
M6	C-C	Bicoronoid width
M7	*O-CO	Condylar advancement
M8	*CO-LI	Mandibular length
M9	*C-LI	Mandibular body length
M10	*CO-C	Coronoid advancement

*Bilateral measurements

Figure 7. Construction of variables to measure changes in the mandible.

Lateral cephalometric orientation.

Dorsoventral cephalometric orientation.


M1
M3
M2

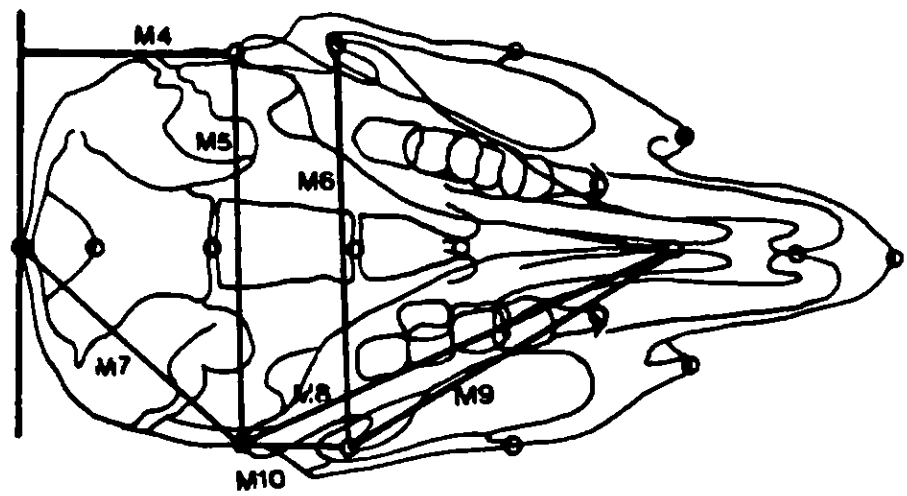

M4
M5
M6
M7
M8
M9
M10

TABLE III

VARIABLES TO MEASURE VERTICAL CHANGES

IN MIDFACIAL DIMENSIONS

Variable	Landmarks Employed	Measures
	Lateral View	
V1	jl-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)-nf
V5	i-n	Anterior nasal height
V6	(pns-i)-n	Anterior nasal height from (pns-i)-n

TABLE IV

VARIABLES TO MEASURE TRANSVERSE CHANGES

IN MIDFACIAL DIMENSION

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

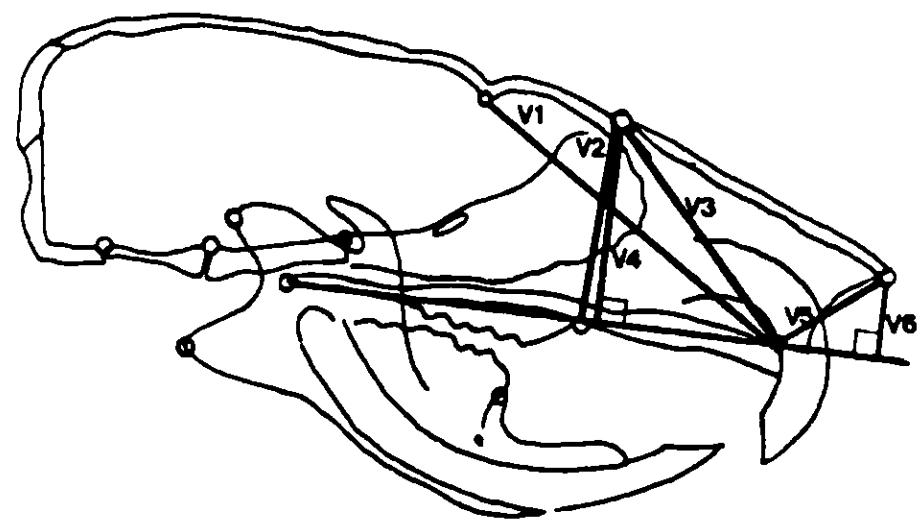
*Bilateral measure

Figure 8. Construction of variables to measure vertical changes in midfacial dimensions.

Lateral cephalometric orientation.

Construction of variables to measure transverse changes in midfacial dimension.

Dorsoventral cephalometric orientation.


V1
V2
V3
V4
V5
V6

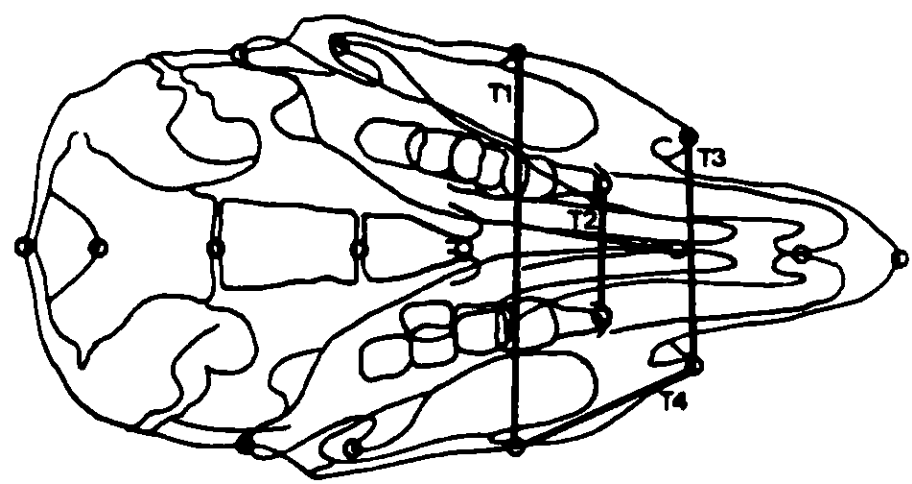

T1
T2
T3
T4

TABLE V

VARIABLES TO MEASURE CHANGES IN THE

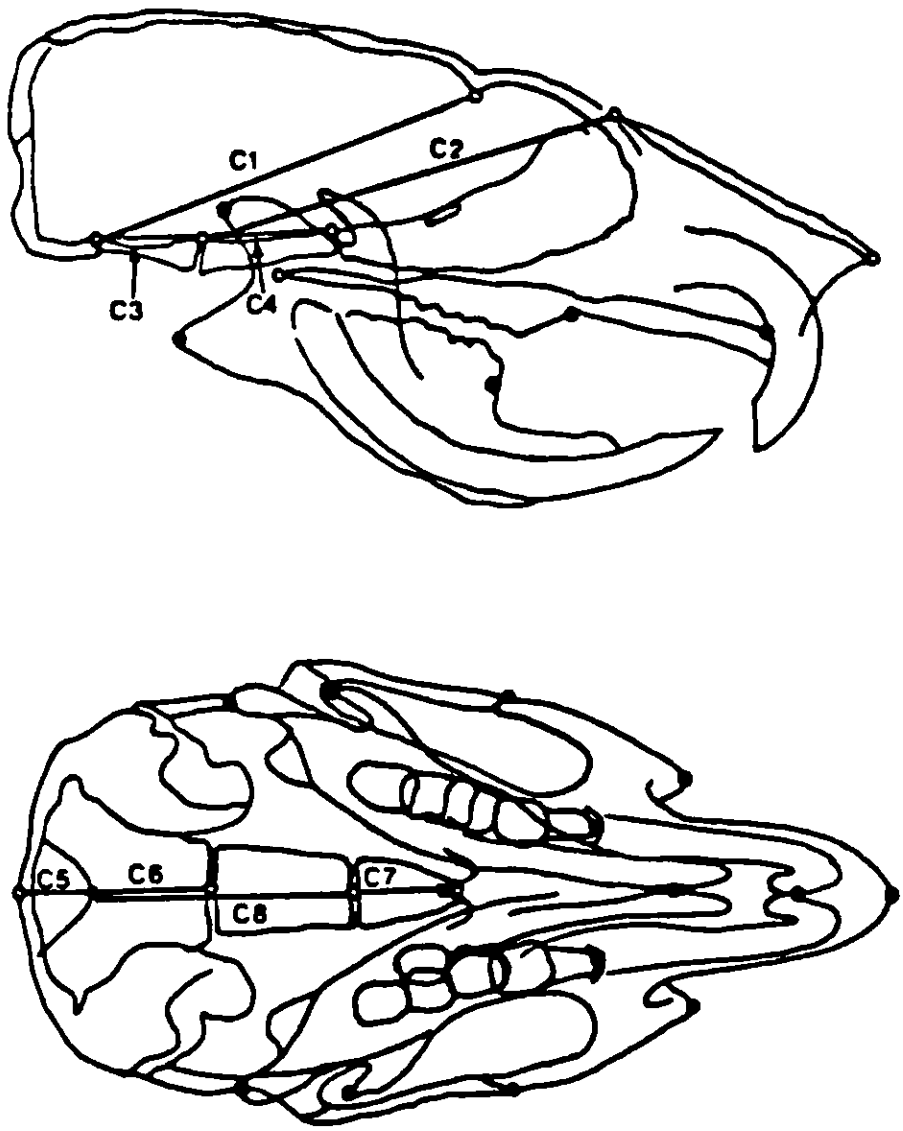
CRANIAL BASE OR CRANIUM

Variable	Landmarks Employed	Measures
	Lateral View	
C1	ba-jl	Transcranial length
C2	nf-so	Cranial base anterior cerebrum length
C3	ba-so	Basioccipital length
C4	so-is	Basisphenoid length
	Dorsal Ventral View	
C5	O-BA	Sphenoid advancement
C6	BA-SO	Sphenoid length
C7	IS-P	Palatal advancement
C8	BA-IS	Pre-sphenoid advancement

Figure 9. Construction of variables to measure changes in the cranial base or cranium.

Lateral cephalometric orientation.

Dorsoventral cephalometric orientation.


C1
C2
C3
C4
C5
C6
C7
C8

Each pair of lateral and dorsoventral radiographs was enlarged three times using a photographic enlarger (Plate I) and landmarks on both films were located simultaneously to allow comparison of radiographic structures. Landmarks for both radiographs were indicated by pinholes placed on a single sheet of bond paper. These pinholes provided a positive seat for the stylus used in subsequent data reduction.

Data reduction was facilitated by using a process known as "digitization" which involved the following procedure. Each sheet of bond paper was securely fastened to an electronic sensor board (digitizing board). A pencil stylus was then placed directly above each point to be entered into the computer. By depressing the stylus the x-y coordinates of each point were entered into the computer and stored on magnetic tape. Before data collection began each lateral and dorsoventral radiograph was registered on the lateral view using points ba and i to serve as the abscissa. A perpendicular constructed to this line formed the ordinate to complete the Cartesian coordinate system. Point ba was arbitrarily assigned the coordinates (1,1) to place all cephalograms in the positive quadrant of the coordinate system. Points were then entered in the same sequence for each animal. The accuracy of landmark determination was within the limits of resolution of the digitizing board (.004 in.). After all points were entered the measurements were executed using the University of Michigan,

PLATE I

Lateral and dorsoventral cephalograms of a 30 day old Sprague-Dawley rat magnified 3 times normal.

Lateral cephalometric orientation (3X)

Dorsoventral cephalometric orientation (3X)

Center for Human Growth and Development computer system (Tridea System, Miller, 1971).

Data Reduction

The Michigan Interactive Data Analysis System (MIDAS) was used to examine differences among treatment groups. A one way analysis of covariance was used to analyze the data with animal weight serving as the covariat. The use of a covariat was suggested by the systematic variability associated with the difference in size of the animals within treatment groups. Weight was chosen as the covariate because it exhibited no treatment effect while maintaining a high correlation with craniofacial dimensions in a controlled population of experimental animals (Paulus, 1976). The correlation coefficients for weight against each variable measured are included in Tables XIV through XVIII. A linear model describing fixed treatments covarying for weight was used in the analysis and may be expressed as follows:

$Y_{ij} = \mu + \alpha_i + \beta(X_{ij} - X_{++}) + \Sigma_{ij}$, where,

Y_{ij} = a measurement for the ith treatment for the jth rat. i = 1 2 3; j = 1, 2...n.

μ = the population mean.

αi = the effect of the ith treatment.

β = the slope of the common regression line for the weight covariate in the population.

$(X_{ij} - X_{++})$ = the deviation of the weight covariate associated with the ith treatment and the jth rat, from the total mean, and

Σ_{ij} = the experimental error associated with the ith treatment, and jth rat.

Error Study

The reliability of each measurement was estimated by selecting 12 animals and repeating the following sequence. Each cephalogram was enlarged three times and the landmarks were located by pin-holes placed in bond paper. All measurements were recomputed and a coefficient of linear correlation was calculated between the first and second determination of each dimension. The square of this coefficient, r_{xx}^2, estimates the reliability associated with each measured variable, and is an estimate of ". . . the proportion of their variance that is true variance" (Guilford, 1965; Gange and Johnston, 1974).

RESULTS

The results of the present study will be presented in three sections: I. Confirmational Results of the Experimental Method, II. *In Vitro* Tissue Culture Results, and III. *In Vivo* Experimental Results.

I. Confirmational Results of the Experimental Method

Description of the resulting sample

The original experimental design included 12 animals in each cage which were randomly assigned to one of three treatment groups. The demographic information pertaining to the original sample is presented in Table VI. During the course of the experiment two animals were lost due to premature death. In addition, 12 other animals did not have both a lateral and dorsal ventral radiograph of sufficient quality to allow for precise cephalometric evaluation. The demographic information pertaining to the final sample used for analysis is presented in Table VII. As seen in Table VII, a majority of the missing data parts were members of the normal rabbit serum control group.

Reliability of cephalometric determination

The results of the twelve double determinations for each of the sixty measured variables are presented in Table VIII. In

TABLE VI

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	Cage 4 M	Cage 4 F	Cage 5 M	Cage 5 F	Total by Treatment
Saline	2	2	1	3	1	3	1	3	1	3	20
NRS	3	1	3	1	1	3	2	2	2	2	20
RNS	3	1	3	1	2	2	1	3	2	2	20
Total by Sex	8	4	7	5	4	8	4	8	5	7	. . .
Total by Cage	12		12		12		12		12		60

TABLE VII

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

Treatment	Cage 1			Cage 2			Cage 3			Cage 4			Cage 5			Total		
	Sex		wt.in grams	Sex		wt.in grams	Sex		wt.in grams	Sex		wt.in grams	Sex		wt.in grams	Sex		wt.in grams
	M	F		M	F		M	F		M	F		M	F		M	F	
Saline	2	2	59.50	0	3	91.17	1	2	79.67	1	3	80.50	0	2	69.50	4	12	75.28
NRS	3	1	60.75	2	1	92.00	1	1	78.00	0	2	79.00	0	0	...	6	5	75.72
RNS	2	1	62.00	3	1	88.25	2	2	81.75	1	3	78.25	2	2	73.75	10	9	77.58
Total	7	4		5	5		4	5		2	8		2	4		20	26	
	11		60.64	10		90.25	9		80.22	10		79.30	6		72.33	46		76.34

TABLE VIII

RELIABILITY COEFFICIENTS FOR TWELVE

DOUBLE DETERMINATIONS

Variable	r	r^2	Variable	r	r^2
pns-nf	.890	.792	ZG-ZG	.734	.539
pns-n	.385	.148	C-C	.878	.771
ba-so	.684	.468	CO-CO	.771	.594
so-is	.814	.663	O-BA	.801	.641
nf-n	.663	.440	BA-~~SO~~	.447	.200
pns-i	.837	.701	BA-IS	.697	.486
um-i	.949	.901	PI	.622	.387
um-so	.921	.848	CO-LI R	.640	.410
so-i	.981	.962	CO-LI L	.828	.686
jl-i	.956	.914	MX-N R	.083	.007
ap-mn	.543	.295	MX-N L	.261	.068
co-mn	.065	.004	CO-C R	.673	.453
ap-co	.080	.006	CO-C L	.670	.449
ba-jl	.795	.632	O-CO R	.674	.454
nf-um	.858	.736	O-CO L	.933	.870
nf-i	.901	.812	IS-P	.431	.186
nf-so	.955	.860	C-LI R	.746	.557
is-i	.996	.992	C-LI L	.783	.613
ba-i	.940	.884	IN	.184	.034
I N	.856	.733	BA-MX R	.833	.694
AB-MX R	.937	.878	BA-MX L	.888	.789
AB-MX L	.931	.866	BA-UM R	.837	.701
AB-UM R	.859	.738	BA-UM L	.873	.762
AB-UM L	.825	.681	SO-MX R	.872	.760
AB-CO R	.653	.426	SO-MX L	.958	.918
AB-CO 1	.911	.830	SO-UM R	.855	.731
AB-ZG R	.571	.326	SO-UM L	.971	.943
AB-ZG L	.582	.339	IS-MX R	.805	.648
MX-O R	.872	.760	IS-MX L	.927	.859
MX-O L	.842	.709	IS-UM R	.830	.689
UM-UM	.703	.494	IS-UM L	.849	.705
MX-MX	.755	.570			

this table the higher the value of r, the more accurately that point was able to be located, i.e., r = 1.0 is a perfect correlation and would indicate that that point was located in exactly the same position on both tracings.

Indirect Immunofluorescent Staining

Cryostat sections of rat nasal septum were examined by indirect immunofluorescence using IgG obtained from rabbits immunized against the reduced and alkylated extract of rat nasal septum (RNS). Immunoglobulin G isolated from normal rabbit serum (NRS) was used as the control for all indirect immunofluorescent staining. The septa treated with anti-RNS antibody showed bright immunofluorescent staining in both the regional chondrocyte territorial matrix and perichondrium. Similar tissue sections treated with control antisera exhibited weak fluorescence confined to the pericellular area and cytoplasm. Plate II shows the typical immunofluorescent staining pattern for both the immune and control sera.

Chondrocytes grown in nutrient medium on glass coverslips were also tested for antibody binding by indirect immunofluorescence. The resulting binding patterns were similar to that seen with the frozen sections. The anti-rat nasal septum antibody demonstrated the same bright fluorescence in the extracellular matrix; whereas, the control anti-sera showed a pericellular staining pattern with no localization in the matrix. Plate III

PLATE II

Indirect immunofluorescence (IIF) labeling of cryostat sections nasal septa from 5 day old rats treated with (A) normal rabbit (NRS) IgG, and (B) anti-rat nasal septum (RNS) IgG (720X)

A. IIF labeled cryostat section treated with NRS-IgG. Note faint localization of label in the perochondrium and in the cytoplasm of chondrocytes. No localization of antibody is found in the cartilage matrix.

B. IIF labeled cryostat section treated with RNS-IgG. Antibody localizes strongly in the perichondrium and in the regional chondrocyte territorial matrix.

PLATE III

Indirect immunofluorescence (IIF) labeling of monolayer cultures of rat nasal septum chondrocytes treated with (A) NRS-IgG and (B) anti-RNS-IgG (720X)

A. Monolayer culture of rat nasal septum chondrocytes treated with NRS-IgG (control). Note the faint perinuclear staining of chondrocyte cytoplasm.

B. Identical cell cultures treated with anti-RNS-IgG. Strong antibody localization is observed in the filamentous extracellular chondrocyte matrix in addition to cell surface and cytoplasmic staining.

shows the typical staining pattern seen in the monolayer cultures. In order to verify that the nasal septum antigens were located on the cell membrane, as well as in the matrix, anti-rat nasal septum antibody was reacted with trypsinized cells in suspension. Microscopic examination under fluorescent light of chondrocytes treated with the anti-rat nasal septum antibody exhibited the typical staining pattern seen in Plate IV. The cell membrane showed discrete sites of localization of the antibody on the cell membrane. However, there was no identifiable area exhibiting greater localization of antibody. Instead the antibody was evenly dispersed on the membrane surface. The control serum did not evidence significant fluorescence, and although not totally negative this fluorescence was not bright enough to allow routine photographic reproduction. It could not be determined if this binding was non-specific or the result of normal rabbit anti-cartilage antibodies.

II. In Vitro Tissue Culture Results

Cell Viability (as determined by trypan blue exclusion)

The cytotoxic effect of anti-RNS antibody on trypsinized cells in suspension was examined microscopically utilizing Trypan Blue. After two hours incubation in the presence of anti-RNS antibody (4 mg/ml) and fresh rabbit complement, 35% of the chondrocytes examined showed intense blue staining. Those cells which

PLATE IV

Indirect immunofluorescent (IIF) labeling of rat nasal septum chondrocytes in suspension treated with anti-rat nasal septum (RNS) IgG

Note the discrete localization of antibody on the cell surface. Nasal septum chondrocytes treated with control antibody did not produce sufficient fluorescence to permit photographic reproduction (1200X).

TABLE IX

VIABILITY AS ASSESSED BY

TRYPAN BLUE EXCLUSION

Treatment	Total Cells Counted	Dead Cells	Percent Killing
RNS with complement	166	60	36.14
RNS without complement	180	4	2.22
NRS with complement	146	1	0.68
NRS without complement	175	0	0.00

could not exclude the dye from their cytoplasm were considered non-viable. In addition, other chondrocytes in the microscopic field displayed a light blue color, but these cells were not included when counting dead cells. The chondrocytes treated with normal rabbit IgG (NRS, control sera) displayed about 1% killing in the presence of complement. The results of this experiment are presented in Table IX.

Neither the IgG control (NRS) or anticartilage IgG (RNS) demonstrated significant cytotoxic effects in the absence of complement. These results indicate that anti-rat nasal septum antibody exerts a cytotoxic effect on chondrocytes in suspension and the toxicity is complement-dependent.

Cytotoxicity Results--Tritiated Thymidine Label (H^3-Thymidine)

H^3-Thymidine labeled cells were treated with either control IgG (NRS) or anti-RNS IgG, with or without complement. Microscopic observation after a three hour period showed that the culture treated with anti-RNS (7 mg/ml) and complement had only 25% of the cells still attached to the culture dish. In contrast, the cells treated with control antibody and complement evidenced approximately 90% of the cells still adherent to the flask. There were no significant microscopic findings in the cultures treated with either antibody in the absence of complement. This confirmed

TABLE X

VIABILITY AS ASSESSED BY RELEASE
OF H^3-THYMIDINE-LABELLED CELLS
FROM THE CULTURE DISHES

Treatment	Total Cells (DPM/ Culture)	Cells Floating in Medium (DPM/ Culture)	Percentage of DPM in Medium Non Viable Cells (%)*
RNS with complement	256,168	185,722	72.50
RNS without complement	700,530	5,994	0.86
NRS with complement	338,831	21,314	6.29
NRS without complement	338,874	5,990	1.77

* Non-viable Cells = $\frac{\text{Floating cells in medium (DPM/culture)}}{\text{Total cells (DPM/culture)}}$

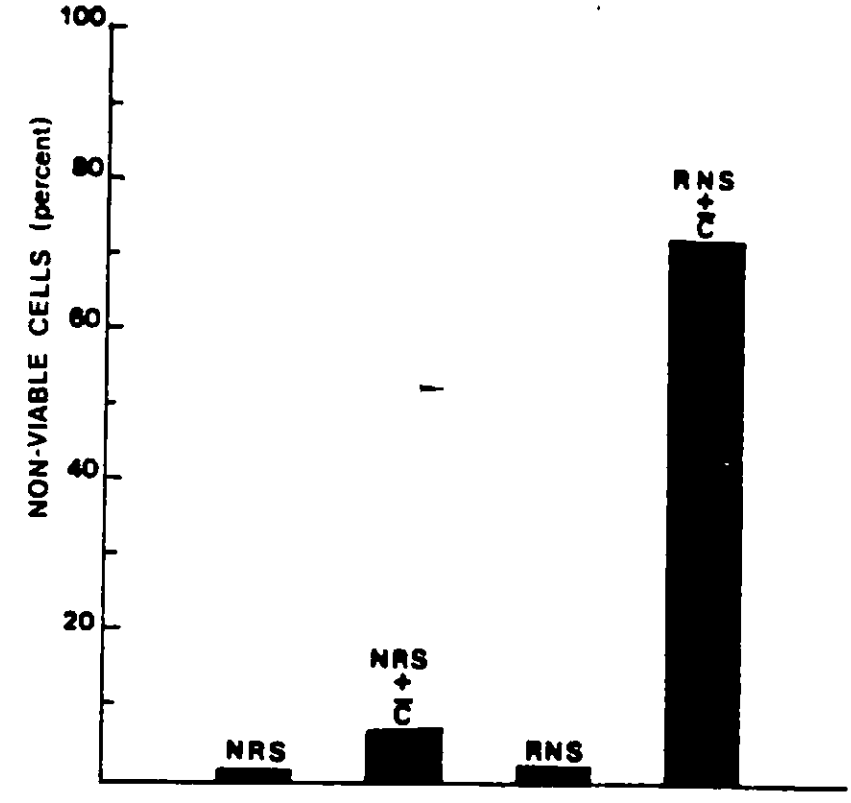
previous results suggesting that neither antibody exerts cytotoxic effects in the absence of complement. To confirm these microscopic findings data analysis of the number of "floating cells" and "attached cells" was quantitated using a liquid scintillation counter. The results of this experiment are reported in Table X and shown as a percentage of non-viable (floating) cells to total cells in Figure 10. This graph demonstrates two important findings. First, normal rabbit IgG (control) had a small (6%) effect on chondrocyte viability. And second, that anti-rat nasal septum IgG, exhibits a much greater (75%) cytotoxic effect under similar experimental conditions. These cytotoxic effects were again demonstrated to be complement-dependent.

Plating Efficiency

To determine the effects of antibody binding on plating efficiency, chondrocytes were grown in the presence of anti-nasal septum antibody and control antibody for a three hour period. As in previous experiments complement was added at the start of the incubation period to half of the cultures, one with anti-RNS IgG and one containing control anti-NRS IgG. The ability of these cells to reattach to the culture flask following trypsinization is an estimate of their viability and to some extent the disturbance to the cell surface caused by the binding of antibody to the cell membrane. In this experiment, only 22% of the cells treated with

Figure 10. Bar graph depicting viability of chondrocytes as assessed by release of H^3-thymidine labeled cells into the medium.

Plating efficiency of nasal septum chondrocytes after treatment with immune and control IgG.


NON-VIABLE CELLS (percent)
100
80
60
40
20
NRS
NRS + C
RNS
RNS + C

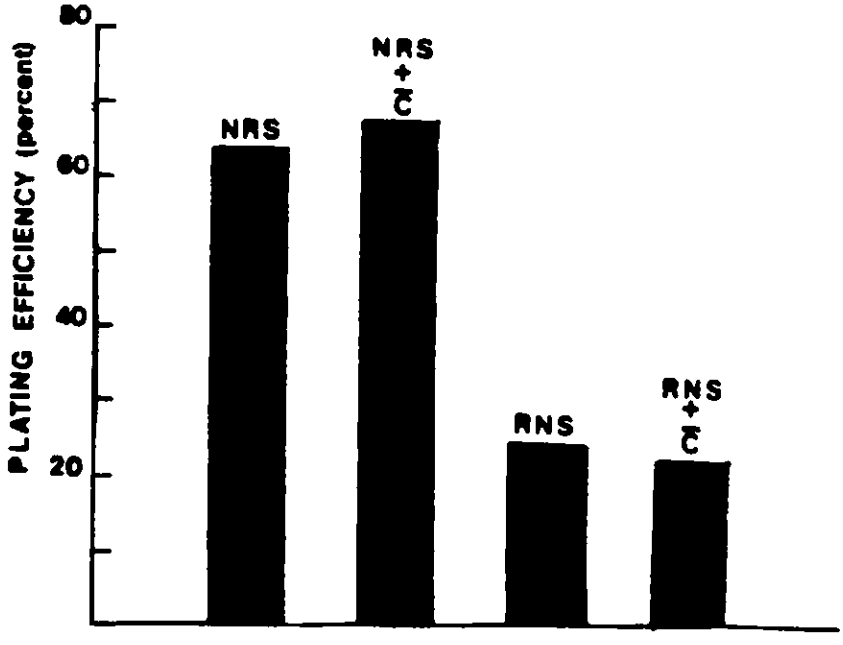

PLATING EFFICIENCY (percent)
80
60
40
20
NRS
NRS + C
RNS
RNS + C

TABLE XI

PLATING EFFICIENCY

Pre-treatment	Attached Cells DPM/Culture	Non-attached Cells DPM/culture	Plating Efficiency* (percent)
RNS with complement	15,846	54,600	22.49
RNS without complement	179,391	515,145	25.83
NRS with complement	212,007	105,510	66.77
NRS without complement	214,428	118,452	64.42

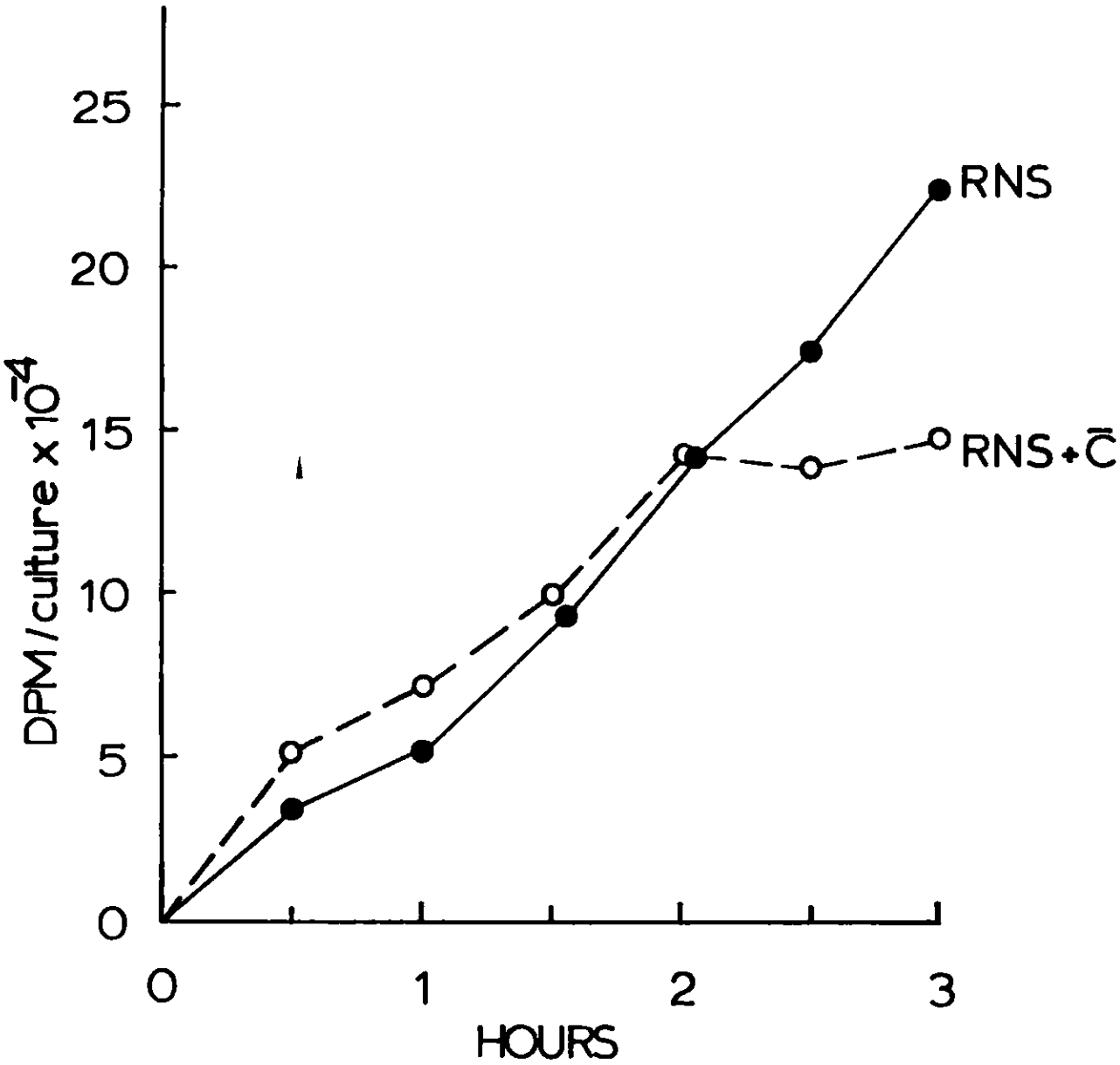
$$*\text{Plating efficiency} = \frac{\text{Non-Attached cells}}{\text{Attached + Non-Attached cells}} \times 100$$

anti-RNS were attached to the culture dish after 18 hours. In contrast, 66% of the cells treated with control antibody were attached within the same time period. Interestingly, there was no difference between the cultures incubated in the presence of complement and those without complement in the medium. Figure 10 depicts plating efficiency as a percentage of attached cells to total trypsinized cells innoculated in the culture.

Protein Accumulation

The effect of anticartilage antibodies on chondrocyte protein accumulation was determined by monitoring the incorporation of a tritiated amino acid mixture into proteins. Monolayers cultures of chondrocytes were incubated in the presence of anti-RNS IgG (8 mg/ml) for three hours. Analysis at half hour intervals of the total amount of accumulated radioactive protein showed that chondrocytes incubated in medium which did not contain active complement demonstrated a linear increase in total labeled protein. Chondrocytes incubated with anti-RNS IgG in the same medium containing active complement exhibited a linear increase in labeled protein accumulation for the first two hours, but then accumulation ceased begining at two hours. This leveling off indicates that some time after two hours the cells were no longer incorporating amino acids into protein. These results support earlier findings that anti-RNS IgG requires active complement to

Figure 11. Graph protein accumulation vs. time.


25
20
15
10
5
0
DPM / culture x 10^{-4}
RNS
RNS + $\bar{C}$
0
1
2
3
HOURS

affect cellular metabolic function. Further, these results suggest that the effects of anti-RNS antibodies are time-dependent. Figure 11 illustrates these results graphically.

III. In Vivo Experimental Results

General appearance, physical condition and body weight

Gross physical examination of the experimental animals showed no detectable anatomic differences between treatment and control groups. The incisor relationship, eyes, coat and color all appeared normal. The mean body weights of the three groups are reported in Table XII. An analysis of variance revealed no significant ($p \leq .05$) weight difference among groups (Table XIII).

Cephalometric Analysis

Descriptive Statistics

In order to categorize significant treatment changes the 65 variables were divided into 6 functional groups on an anatomic basis. These groups along with the rationale for including measures within each group are listed below:

1. Anteroposterior midfacial dimension--Thirty-one 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 XIV).

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 XV).

TABLE XII

MEAN WEIGHT AND STANDARD DEVIATION

(s.d.) FOR EACH TREATMENT GROUP

Treatment	Mean Weight (grams)	S.D. (grams)
Saline	75.28	12.81
NRS	75.72	13.55
RNS	77.58	10.28

TABLE XIII

SUMMARY TABLE FOR ANALYSIS OF VARIANCE FOR

WEIGHT AMONG TREATMENTS

Source	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F Statistic	Signifi-cance
Treatments	2	5,122.9	2561.5	0.17767	0.8378
Within	43	619,930	14417	...	...
Total	45	625,050			

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 XVI).

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

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

The means and standard deviations for all variables adjusted for the weight covariate by the following formula (Snedecor, 1966) are listed by functional group in Tables XIV, XV, XVI, XVII and XVIII:

$Y_A = Y_m - b(X_m - X_T)$, where

Y_A = adjusted mean value

Y_m = the mean measurement before adjustment,

b = the regression coefficient for the weight covariate,

X_m = the mean body weight,

X_T = the total mean of the body weights.

Also included in each table is the correlation coefficient between weight and each variable.

The grouping of the data in this manner allows identification of some resulting trends. Examination of Table XIV for variables reflecting anteroposterior changes in midfacial dimensions shows a generalized decrease in values when saline treated animals are compared with those animals treated with anti-RNS

TABLE XIV

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

Variable	Correlation with weight	SALINE N=16; wt=74.4		NRS N=11; wt=75.72		RNS N=19; wt=77.5	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
nf-n	.74	12.124	.076	11.985	.092	11.957	.070
pns-i	.76	21.141	.110	21.198	.132	20.873	.101
um-i	.84	* 8.977	.053	8.831	.064	* 8.769	.049
um-so	.81	15.068	.072	15.122	.087	14.985	.060
so-i	.89	*24.031	.091	23.943	.109	*23.746	.083
is-i	.91	*18.606	.063	18.486	.076	*18.302	.058
ba-i	.86	28.254	.124	28.269	.150	27.958	.114
AB-MX R	.78	24.003	.117	24.074	.141	23.776	.108
AB-MX L	.74	24.005	.127	24.091	.153	23.817	.117
AB-UM R	.83	*27.560	.125	*27.579	.150	*27.167	.115
AB-UM L	.81	*27.669	.130	27.399	.158	*27.178	.120
O-MX R	.84	*28.001	.121	*27.993	.146	*27.593	.111
O-MX L	.81	*27.998	.128	27.735	.154	*27.499	.117
AB-ZG R	.57	9.563	.238	9.996	.286	10.119	.219
AB-ZG L	.57	10.004	.206	9.886	.249	10.277	.190

TABLE XIV continued

Variable	Correlation with weight	SALINE N=16; wt=74.4		NRS N=11; wt=75.72		RNS N=19; wt=77.5	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
P-I	.57	15.102	.134	15.092	.162	14.768	.123
MX-N R	.36+	10.172	.177	10.017	.214	10.268	.163
MX-N L	.34+	9.937	.168	10.055	.202	10.111	.154
IN	.72	3.718	.179	3.542	.216	3.924	.110
BA-MX R	.84	24.734	.119	24.522	.143	24.435	.110
BA-MX L	.85	24.920	.120	24.876	.145	24.627	.110
BA-UM R	.79	20.962	.107	21.047	.129	20.911	.098
BA-UM L	.82	21.036	.102	21.098	.122	20.916	.093
SO-MX R	.84	19.908	.104	19.644	.126	19.640	.096
SO-MX L	.85	20.117	.104	20.016	.126	19.858	.096
SO-UM R	.79	16.101	.088	16.135	.106	16.084	.084
SO-UM L	.83	16.175	.085	16.188	.102	16.104	.078
IS-MX R	.76	*14.508	.109	14.244	.131	*14.175	.100
IS-MX L	.79	*14.719	.105	14.603	.126	*14.437	.094
IS-UM R	.63	10.650	.098	10.686	.117	10.570	.090
IS-UM L	.74	10.688	.084	10.702	.101	10.588	.077

+ - low correlation with the weight covariat.
* - statistically significant difference ($p \leq .05$) assessed by Analysis of Covariance with weight as the covariat.

TABLE XV

MEAN VALUES (in mm) AND STANDARD DEVIATION (s.d.) OF VARIABLES REFLECTING VERTICAL CHANGES IN MIDFACIAL DIMENSION

Variable	Correlation with weight	SALINE N=16; wt=74.4		NRS N=11; wt=75.72		RNS N=19; wt=77.5	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
pns-n	.52	3.283	.041	3.369	.049	3.288	.037
pns-nf	.82	8.183	.040	8.156	.048	8.159	.036
jl-i	.88	*15.874	.072	15.735	.087	*15.674	.066
nf-um	.79	8.440	.051	8.385	.062	8.367	.047
nf-i	.78	10.704	.059	10.742	.071	10.583	.054
i-n	.72	5.335	.036	5.231	.044	5.321	.033

* - statistically significant difference ($p \leq .05$).

TABLE XVI

MEAN VALUES (in mm) AND STANDARD DEVIATIONS (s.d.)

FOR VARIABLES REFLECTING TRANSVERSE CHANGES

IN MIDFACIAL DIMENSION

Variable	Correlation with weight	SALINE N=16; wt=74.4		NRS N=11; wt=75.72		RNS N=19; wt=77.5	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
um-um	.30+	5.824	.069	* 5.912	.083	* 5.672	.063
mx-mx	.62	* 9.255	.074	9.077	.091	* 9.003	.069
ZG-ZG	.80	16.657	.077	16.641	.093	16.647	.071
ZG-MX R	.11+	9.563	.238	9.996	.286	10.119	.219
ZG-MX L	.19+	10.004	.206	9.866	.249	10.277	.190

+ - low correlation with the weight covariat.
* - statistically significant difference ($p \leq .05$) assessed by Analysis of Covariance with weight as the covariat.

TABLE XVII

MEAN VALUES (in mm) AND STANDARD DEVIATIONS (s.d.)

FOR VARIABLES REFLECTING CHANGES IN THE

CRANIAL BASE OR CRANIUM

Variable	Correlation with weight	SALINE N=16; wt=74.4		NRS N=11; wt=75.072		RNS N=19; wt-77.5	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
ba-so	.29+	4.302	.085	4.422	.102	4.282	.078
so-is	.62	5.564	.055	5.625	.066	5.611	.051
ba-jl	.65	17.011	.092	17.434	.110	17.200	.084
nf-so	.85	18.574	.096	18.469	.115	18.528	.088
BA-SO	.46	4.925	.047	4.980	.057	4.881	.043
BA-IS	.48	10.534	.103	10.590	.124	10.532	.094
IS-P	.48	3.944	.117	4.007	.141	4.027	.108
O-BA	.03+	3.287	.084	3.249	.101	3.084	.077

+ - low correlation with weight.

TABLE XVIII

MEAN VALUES (in mm) AND STANDARD DEVIATIONS (s.d.)

FOR VARIABLES REFLECTING CHANGES

IN MANDIBULAR DIMENSION

Variable	Correlation with weight	SALINE N=16; wt=75.13		NRS N=11; wt=74.79		RNS N=19; wt=77.4	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
ap-mn	.43	12.873	.134	12.640	.162	12.728	.123
co-mn	.10+	13.583	.195	13.648	.235	13.896	.179
ap-co	.20+	6.271	.143	5.977	.172	5.886	.131
AB-CO R	.59	9.174	.108	9.535	.131	9.359	.099
AB-CO L	.51	9.550	.116	9.522	.140	9.453	.107
C-C	.73	*16.063	.072	*16.346	.087	*16.051	.066
CO-CO	.75	15.992	.081	16.143	.097	16.075	.074
CO-LI R	.45	*19.510	.192	19.404	.231	*19.033	.176
CO-LI L	.52	18.766	.181	18.959	.218	18.439	.166
CO-C R	.28+	4.665	.085	4.466	.102	4.424	.078
CO-C L	.18+	4.459	.089	4.431	.107	4.399	.082
O-CO R	.69	*12.114	.090	*12.486	.109	12.322	.083
O-CO L	.61	12.508	.107	12.490	.129	12.425	.098
C-LI R	.35+	15.635	.208	15.725	.250	15.338	.191
C-LI L	.44	14.680	.188	14.953	.227	14.430	.171

+ - low correlation with the weight covariat.

* - statistically significant difference ($p \leq .05$) assessed by Analysis of Covariance with weight as the covariat.

antibody (RNS). The variables in this group which do not follow this trend have either a low correlation with the covariate (MX-N R, MX-N L) or a low reliability estimate (see Table VIII of estimates of reliability) or both (AB-ZG R, AB-Z L). It is also apparent that animals treated with normal rabbit IgG (NRS) have mean values generally between those of saline and RNS.

Tables XV and XVI describing the vertical and transverse midfacial changes, respectively, also demonstrate this decreasing trend among Saline, NRS and RNS treatment groups. Again, low correlations with weight alone or in combination with a low reliability estimate is seen with the variables which do not follow the trend.

The variables measuring changes in the cranial base and cranium and in the mandible, Tables XVII and XVIII, respectively, do not show a general trend between treatment groups. Differences between treatments seem to be randomly dispersed with RNS being largest as often as NRS or Saline. Some of these variables also have low correlations with weight and/or low reliability coefficients.

Inferential Statistics

Results of the analysis of covariance (ANCOVA) for the 15 variables demonstrating a significant ($p \leq .05$) difference between treatments are listed in Table XX and a sample calculation of ANCOVA for one variable (um-i) is shown in Table XIX. Also re-

TABLE XIX

SAMPLE CALCULATION OF ANALYSIS OF COVARIANCE (ANCOVA) WITH WEIGHT AS THE COVARIAT FOR VARIABLE um-i

Source	df	SS*	MS	F	Significance**
Adjusted treatment means	(J-1) 2	(SS'y between) 0.3931	0.1966	4.00	.05
Error	(N-j-1) 42	(SS'y error) 2.0650	0.0492		
Adjusted Total	(N-2) 44				

* $SS'y\ between = SSy\ between + \frac{(SP_{xy}\ within)^2}{SS_x\ within} - \frac{(SP_{xy}\ total)^2}{SS_x\ total}$

$SS'y\ error = SSy\ within - \frac{(SP_{xy}\ within)^2}{SS_x\ within}$

** $F_{.95}\ (2,42) = 3.22$

TABLE XX

RESULTS OF ANALYSIS OF COVARIANCE (ANCOVA) AND ANALYSIS OF VARIANCE (ANOVA) FOR SIGNIFICANT ($p \leq .05$) VARIABLES

Variable	Weight Correlation r	Reliability r^2	F ANOVA[1]	F ANCOVA[2]	Significant difference pairwise comparison S-R [3]	Significant difference pairwise comparison N-R [4]	Significant difference pairwise comparison S-N [5]
um-i	.84	.90	.5393	4.1887	.007	...	...
so-i	.89	.96	.1135	2.7954	.03	...	...
is-i	.91	.99	.3434	6.4249	.001	.06	...
AB-UM R	.83	.74	.4174	3.5982	.03	.03	...
AB-UM L	.81	.68	.5720	3.7790	.009	...	...
O-MX R	.84	.76	.4170	4.002	.01	.03	...
O-MX L	.81	.71	.6002	4.092	.007	...	...
IS-MX R	.76	.65	.5899	2.691	.03	...	...
IS-MX L	.79	.86	.2483	1.987	.05	...	...
jl-i	.88	.92	.1364	2.097	.05	...	...

TABLE XX continued

Variable	Weight Correlation r	Reliability r^2	F ANOVA[1]	F ANCOVA[2]	Significant difference pairwise comparison S-R [3]	Significant difference pairwise comparison N-R [4]	Significant difference pairwise comparison S-N [5]
um-um	.30+	.49	2.2597	2.929	...	.03	...
mx-mx	.62	.57	1.2441	3.062	.02	...	...
ba-jl	.65	.63	2.6200	4.3288	...	...	.005
c-c	.73	.77	1.6340	4.2518	...	.01	.02
o-co R	.69	.45	1.8916	2.3760	.04	...	...

1 F ANOVA--F statistic for Analysis of Variance
2 F ANCOVA--F statistic for Analysis of Covariance
3 S-R--Saline vs. RNS pairwise comparison
4 N-R--NRS vs. RNS pairwise comparison
5 S-N--Saline vs. NRS pairwise comparison

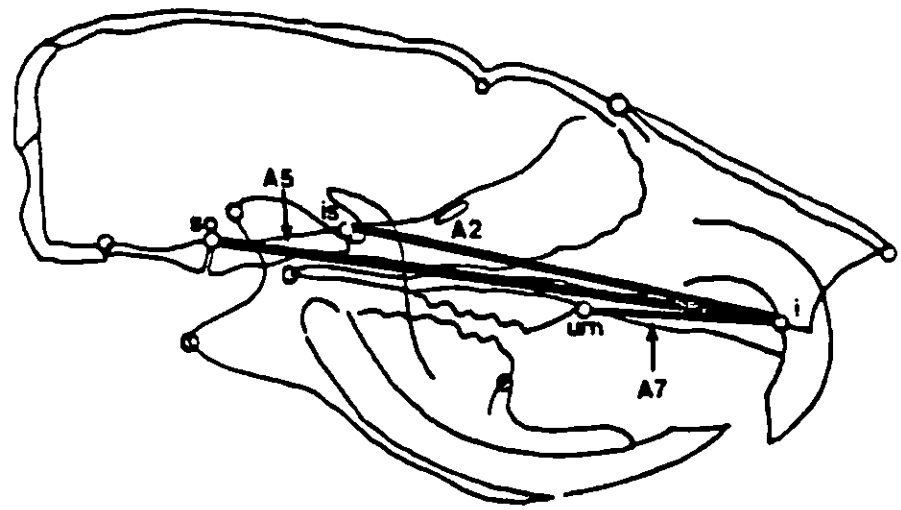
ported in Table XX are the results of the analysis of variance (F ANOVA) and pairwise comparisons by the Scheffe method between the treatments that evidenced significant ($p \leq .05$) effects. Twelve of these 15 variables were midfacial measurements. Nine variables (um-i, so-i, is-i, AB-UM R, AB-UM L, OMX R, OMX L, IS MX L, IS MX R) demonstrated a relative maxillary retrusion of the midfacial complex in animals treated with RNS antibody. One variable (jl-i) showed a relative decrease in nasal height and two variables demonstrated a decrease in width of the midface across the molars (um-um) and maxilla (MX-MX) in the RNS treatment group. One measurement (jl-ba) showed a relative increase in the transcranial dimension in animals treated with NRS. Two mandibular dimensions bi-coronoid width (c-c) and condylar position on the right side (O-CO R) showed a decrease in those animals treated with RNS antibody.

Figures 12 and 13 depict the midfacial variables which showed a significant reduction in length when animals treated with RNS are compared to controls.

Figure 12. Significant Variables.

Anteroposterior measures; Lateral view.

Dorsoventral view.


A5
A2
A7

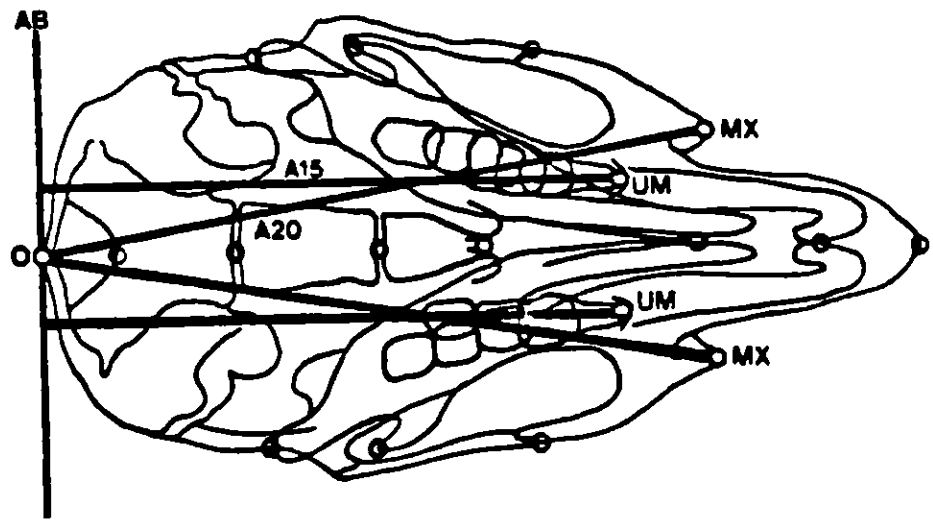
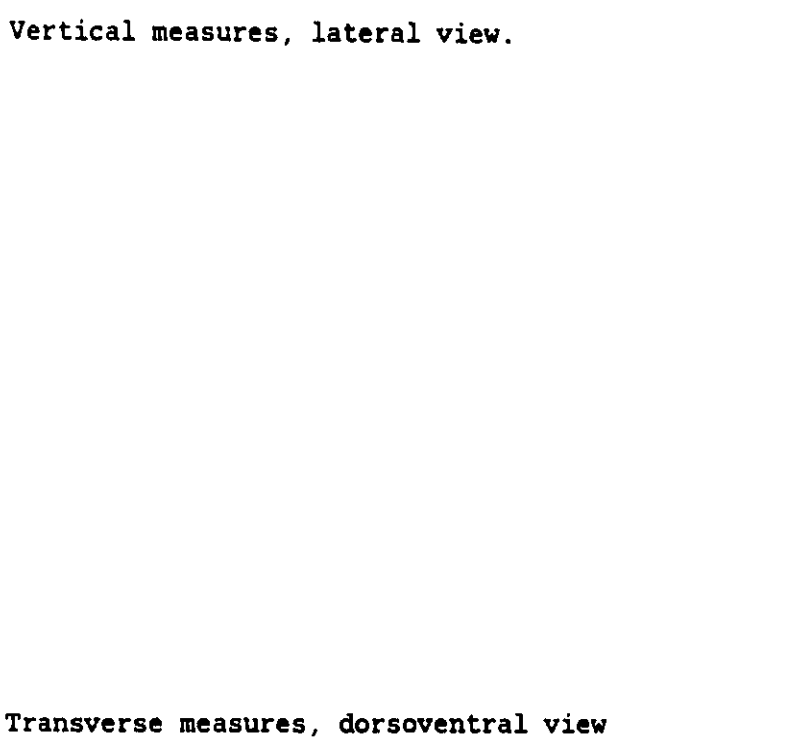
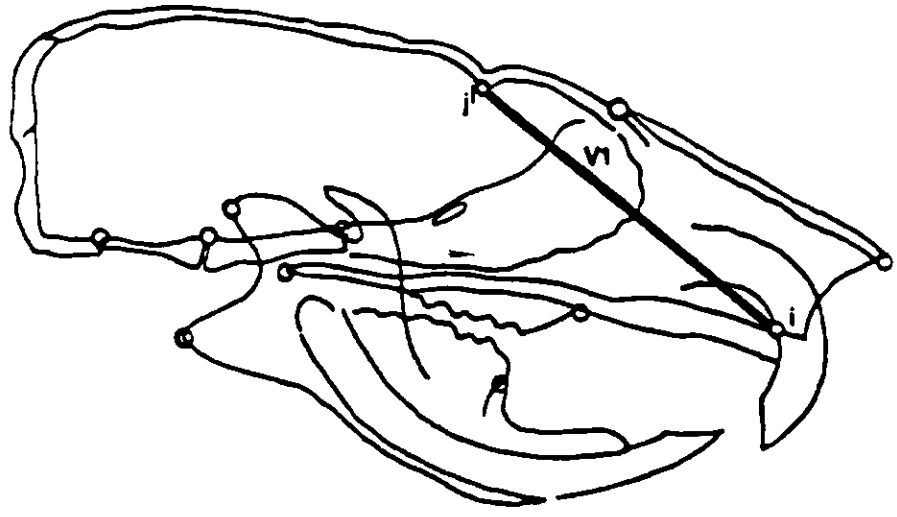
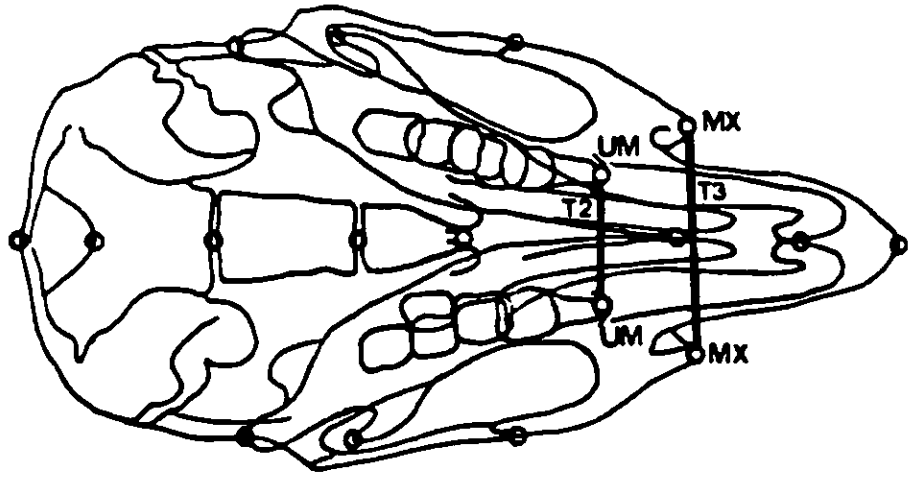

AB
MX
A15
UM
A20
UM
MX

Figure 13. Significant variables




i
VI
i


MX
UM
T3
T2
UM
MX

DISCUSSION

The purpose of this investigation was two-fold: first, to determine if anti-cartilage antibodies can alter cellular functions of chondrocytes grown in tissue culture; and, second, to determine the effect of anti-cartilage antibodies on the process of facial growth and development in experimental rats. Of primary interest was the question, can antibodies control or affect growth at the cellular level?

The *in vitro* portion of this study evaluated the effects of anti-rat nasal septum antibody on isolated rat nasal septum chondrocytes. Tissue culture provided a model system in which many of the extrinsic factors inherent in any *in vivo* model such as rates of diffusion, localization of antibody in target tissues, and vascular supply are eliminated. With these factors controlled, the specific effect of the antibody on cell metabolism can be less ambiguously elucidated. Earlier studies (Brigham, 1976; Palmer, 1978) had reported that anti-cartilage antibodies are bound to the interterritorial matrix and perilucunar area of paraffin embedded tissue sections of nasal septum. Accordingly, it had not been determined if the cartilage antigens were also located on the cell membrane in addition to the extracellular matrix. In the present study it has been shown, by immunohistochemical examination of isolated rat nasal septum chondrocytes in

suspension, that the RNS antibody localizes on the cell membrane surface. Chondrocytes in suspension are necessarily free of their extracellular matrix, whereas monolayer cultures of chondrocytes elaborate extracellular matrix in much the same manner as chondrocytes in situ. Because the cartilage antigens were located on both the cell surface and the extracellular matrix, characterization of the effects of anticartilage antibodies could be carried out on chondrocytes in monolayer culture or suspension. When anti-RNS antibody was reacted with monolayer cultures, there was a significant (75%) cytotoxic effect on the chondrocytes in the presence of complement. Chrondocytes which were dissociated from their extracellular matrix by trypsinization and placed in an isotonic PBS suspension were also killed by anti-RNS antibody with complement. The cytotoxicity results on the cells in suspension and in monolayer cultures show that the killing can occur in the presence and absence of the cartilage matrix. These results also show that anti-rat nasal septum IgG binds to the cell surface, fixes complement, and effectively activates the complement cascade to disrupt the cell membrane. Interestingly, the control antibody (normal rabbit immunoglobulin G) and complement also had a small (6%) effect on the viability of the chondrocytes grown in monolayer. This is consistent with results reported by other authors (Terasaki et al., 1962) that mature rabbit serum exerts a nonspecific cytotoxic effect on cells grown in tissue culture.

Two different antibody concentrations (7 mg/ml and 4 mg/ml) were used in the cytotoxicity experiments. The killing efficiency of the higher RNS antibody concentration was much greater (75%) than that of the lower RNS antibody concentration (35%). Although the experimental time period was one hour longer with the 7 mg/ml concentration, these results suggest that the cytotoxic effects of anti-rat nasal septum antibody might be concentration dependent. An additional finding which may support this implication was that the non-specific cytotoxic effect of normal rabbit IgG (control) was not seen with the lower concentration of antibody. Concentration-dependent cytotoxicity of the RNS/complement mixture would be expected due to the specific nature of antigen-antibody interaction. Physiologic processes involving specific membrane receptor sites generally exhibit concentration-dependent enzyme kenetics. Based on this assumption, the cytotoxic effect of anti-RNS antibody would be expected to increase with increasing concentrations of antibody up to a rate-limiting concentration. At the rate-limiting concentration all membrane antibody receptors would be bound with antibody and there would not be an increase in killing above this receptor saturation point.

The effect of anti-RNS antibody on protein synthesis demonstrates two important characteristics of the RNS antibody-antigen interaction. First, anti-RNS antibody did not grossly

alter protein synthesis over a three-hour time period in the absence of complement. This finding was demonstrated by the linear increase in protein accummulation seen in the cultures incubated with anti-RNS antibody without complement. This experiment also showed that replicate cultures incubated with the same antibody with complement had a similar increase in protein accumulation for the first two hours. However, after this initial linear increase the cells did not continue to synthesize additional proteins. This decrease in protein accumulation after two hours probably reflects the onset of the cytotoxic effect of the antibody and not a separate effect of the antibody on protein synthesis itself. Importantly, this experiment demonstrates that the cytotoxic effect of anti-RNS antibody is time-dependent. One possible explanation for the two hour lag period would be that it is during this time that the antibody is binding to the membrane receptors. This binding is essentially a passive diffusion process and thus would depend on both antibody concentration and time.

Plating efficiency was the only experiment conducted that did not show a difference associated with the interaction of the antibody and complement. One possible explanation of this observation is found in the experimental design. Half of the cultures used in this experiment were preincubated for three hours with RNS antibody and control antibody with complement. The remaining

chondrocytes were incubated for the same amount of time in the presence of the treatment and control antibody alone. At the end of this three hour pre-incubation, the medium was decanted. Any cells floating and presumably killed during this period were removed with the medium and not used to determine plating efficiency. Thus, the chondrocytes used to determine plating efficiency represent a preselected population of cells remaining attached to the surface of the culture flask and presumably not immediately killed by the complement enzyme system. Nonetheless, the results of this experiment do show that anti-RNS antibody binding to the cell membrane either killed or altered the cell's ability to adhere to the culture dish. Only 25% of the chondrocytes treated with anti-RNS antibody retained this ability compared to 66% of the cells treated with control antibody. This indicates that antibody binding alone did alter at least one membrane mediated cell function.

The combined results of the in vitro portion of this study indicate that anti-RNS antibody did exert cytotoxic effects on chondrocytes grown in cell culture. Further, this cytotoxicity was dependent on antibody concentration, time, and activation of the complement enzymatic cascade.

The in vivo portion of this study was designed to examine the effect of this cytotoxicity on growth and development in experimental rats injected with anti-rat nasal septum antibody.

Two control populations were utilized. One comprised a group of animals that received injections of isotonic saline; and a second group received injections of an IgG preparation isolated from normal rabbit serum (NRS). The purpose of this second control group was to rule out the possibility of any non-specific effects of the antibodies normally present in adult rabbit serum. Essentially the only difference between the second control group and the RNS treatment group was that the RNS antibody preparation contained antibodies specific for the rat's cartilaginous nasal septum, in addition to the antibodies normally found in mature rabbit serum. Statistical analysis of the effects of the anti-rat nasal septum antibody (RNS) on craniofacial growth were compared to both control groups. Statistically significant differences ($p \leq .05$) between saline and RNS treated animals were seen for 12 variables (see Results, Table XX pairwise comparison S-R). However, only three variables (is-i, AB-MX R, and O-MX R) evidenced a statistically significant difference between RNS and both control groups (see Table XX, pairwise comparisons S-R, N-R). The difficulty in demonstrating a significant difference between animals treated with NRS control and those treated with RNS antibody is probably a result of two factors. The first factor is primarily a statistical problem. There was a disparity in the size of the three treatment groups used for the final statistical analysis. The NRS group used for analysis had only 11 animals

with an acceptable lateral and dorsoventral cephalogram. This is compared to 19 animals in the RNS group and 16 animals in the saline control group. The statistical result of a smaller sample is a greater variation associated with the estimate of the sample population mean for any measured variable. This greater variation is evident when the standard deviations for each variable in the NRS treatment group are compared to the other treatment groups. In every case, the standard deviation for the NRS group is higher than for either of the other groups. Because of the increased variation associated with the NRS sample population mean, it is statistically more difficult to show a significant difference between that sample population mean and the other sample population means.

The other factor that further complicates analysis and interpretation of the cephalometric results pertaining to the NRS control group are the results of the *in vitro* cell viability studies. Normal rabbit serum in the presence of the complement enzyme system had a small (6%) cytotoxic effect on the chondrocytes grown in culture. As previously stated, other investigators (Terasaki et al., 1967) have reported that IgG fractions isolated from mature animal serum contain antibodies that will cross-react with epithelial and connective tissue components. Therefore, it is possible that some of the resulting reduction in craniofacial dimensions reported for the NRS control group are a result of

normal rabbit antibodies binding to the rat nasal septum. Together these two factors significantly complicate the interpretation of the experimental results pertaining to the normal rabbit serum (NRS) control group. Accordingly, the cephalometric results of the in vivo portion of this study will be discussed on the basis of significant treatment differences between either RNS and Saline, or RNS and NRS. When the treatment difference is significant for the variable when compared to both control populations this fact will be noted in the text.

Cephalometric radiographs were taken of each animal to record the effects of anti-rat nasal septum antibody on 60 craniofacial dimensions. Fifteen of these dimensions showed a small but statistically significant alteration in animals treated with anti-RNS antibody. The cartilaginous nasal septum has been implicated as a primary growth site and is considered by some authors (Scott, 1953; Sicher and Debrul, 1963) to provide a tissue separating force that causes translative growth movement of the midface. Importantly, 12 of the 15 significant measurements were located in the midfacial region of the animal. Nine of these measures reflect a relative reduction in the anteroposterior dimension of the snout. Premaxillary length (um-i) was significantly shorter ($p \leq .007$) when RNS treated animals were compared to saline controls. This reduction in length of the premaxilla is important because the nasal septum spans the entire length of this

bone and the septo-premaxillary ligament provides a fibrous attachment between the most anterior portion of the nasal septum and the premaxilla. Latham (1968) has suggested that this fibrous attachment between the nasal septum and premaxilla allows the nasal septum to "pull" the premaxilla forward and thus promote premaxillary growth. The reduction in the length of the premaxilla in animals treated with an agent cytotoxic for the nasal septum would support Latham's hypothesis. Another measurement that is of particular interest is variable is-i. This measurement spans the entire length of the nasal septum in the anteroposterior direction and can loosely be considered a measure of total snout length. The most posterior portion of the nasal septum extends to the region of the intersphenoidal synchondrosis (is) and attaches anteriorly to the premaxilla (i). The reduction in this measurement was significant at the highest level ($p \leq .001$) when RNS treated animals are compared to saline controls. In addition, this measurement was also significantly shorter ($p \leq .06$) when RNS treated animals were compared to the NRS control group. This reduction in snout length would support the contention that the nasal septum has an active role in the growth of the midface.

The seven remaining significant variables are measures of relative protrusion or retrusion of the midfacial bones. These variables (so-i, AB-UM R & L, O-MX R & L and IS-MX R & L) demonstrate a reduction in relative midfacial advancement in those animals treated with anti-RNS antibody. This reduction in ad-

vancement is also supportive of Scott's theory regarding the nasal septum as a primary force for the displacement of the midfacial complex downward and forward.

The finding that the majority of the significant measurements reflect anteroposterior changes in the rat midface has several possible explanations. First, the anatomic orientation of the rat's nasal septum is predominately anteroposterior and most of the postnatal growth of the rat snout is in length. Additionally, the generalized pattern of morphologic growth change is from a round to a more elongated facial form (Baer, 1954). Therefore, the effect of any treatment affecting midfacial growth would be most easily identified in this dimension. A second, more remote, possibility is that the antibody exerts a cytotoxic effect on the nasal septum in such a way as to affect predominately anteroposterior growth. There is, however, no biologic precedent suggesting that there are different causative factors which would allow for selective alteration of growth in the "forward" direction as opposed to "downward." Thus, it would seem prudent to consider that the reported reduction in anteroposterior growth of the midface reflects a generalized decrease in midfacial growth.

In addition to these anteroposterior changes one measurement of vertical change in the midface (jl-i) was found to be significantly shorter ($p \leq .05$) in RNS treated animals. Admittedly, this measure reflects a degree of anteroposterior change in

addition to its vertical component. In fact the orientation of this measurement is similar to the downward and forward growth vector described for the midface. Interestingly, there were no significant differences between the RNS group and the controls for any of the more direct measures of vertical nasal height ((pns-i)-nf, (pns-i)-n. It might be expected that if the nasal septum is the primary supporting structure of the nasal chamber, a cytotoxic agent directed against the nasal septum would cause some degree of collapse in the midface. In this experiment there was no indication of such an effect. However, decreases were observed in the RNS treatment group for two transverse measurements, bi-molar width (UM-UM) and bimaxillary width (MX-MX). The decrease in this dimension is subject to two possible interpretations. The first is based on Enlow's work (1975) on the characterization of growth fields in the face. Enlow has shown that many facial structures, the maxilla for example, increase (or decrease) in relative dimension by deposition and resorption in what is termed a "V" pattern. In the case of the rats treated with RNS antibody, the observed decrease in snout length would be accompanied by a decrease in width because the "V" (i.e., the snout) has been shortened at its posterior aspect. Theoretically, any change in the anteroposterior length of the snout would ultimately have to be effected by a disturbance in the depository fields, and these are generally located on the posterior aspect of the midfacial

bones. One other explanation of the observed decrease in width of the midface involves the maxillary midline cartilage of the rat. It is possible that the anti-nasal septal cartilage antibody affected other cartilaginous structures in addition to the nasal septum. In the rat there is hyaline cartilage between the maxillary bones. This cartilage is anatomically positioned in such a manner as to allow it to contribute to the expansile growth of the midface. Therefore, it is possible that an additional antibody mediated cytotoxic effect on this cartilage could account for the observed decrease in midfacial width. However, a cross-reactive cytotoxic effect for this cartilage, and not the other cephalic cartilages, is difficult to rationalize. It is probably more reasonable to assume that the decrease in midfacial width is a necessary anatomic compensation accompanying the decrease in length.

The three remaining cephalometric findings are difficult to interpret. The transcranial measurement (jl-ba) was found to be significantly larger in rats treated with normal rabbit immunoglobulin G (NRS). Whereas this change could reflect a more mature head form (more elongate) in the control population, this was not seen consistently when saline controls were compared to RNS treated animals. Similarly, changes in the two mandibular measurements (O-CO R and C-C) are not easily rationalized. As in the case of the transverse changes, it may be that the anti-nasal

septum antibody cross-reacted with the cartilage of the mandibular condyle and caused a cytotoxic effect. However, the pattern of reduction in mandibular dimension was not consistent enough to allow any conclusions regarding the effect of anti-RNS antibody on condylar cartilage or mandibular growth.

It was not expected a priori that the majority of the treatment effects would be confined to the midface. Brigham (1976) reported that nasal septal cartilage and epiphyseal cartilage were antigenically identical. In addition, he found that anti-nasal septum antibody would cross react with condylar cartilage antigens. Brigham's results suggest that anti-nasal septum antibody, when injected into growing animals, should affect growth at all the craniofacial cartilaginous sites. Cephalometric analysis of the results of this study did not confirm this expectation. There was also no evidence of any alteration in cranial base measurements in either the lateral or dorsoventral radiographs. This would indicate that neither the sphenoccipital synchondrosis or the intersphenoidal synchondrosal cartilage were affected by the antibody injections. There was also no statistically significant reduction in mandibular length, and such a reduction might be expected if anti-nasal septum antibody cross reacted with condylar cartilage. However, in the case of the mandible, there are practical difficulties in orienting the mandible in sacrificed animals with the consistency needed to

produce cephalometric radiographs of diagnostic quality. Therefore, as previously mentioned, it is difficult to draw any conclusions about the effect of the RNS antibody on mandibular growth or form.

There are several other factors which could explain the predominance of the treatment effects being found in the midface. The results of the in vitro cell viability studies suggest that the cytotoxic effect of the RNS antibody is concentration dependent. Therefore, any factor influencing the concentration of the antibody at the reactive site will necessarily modulate its effect on growth at that site. The nasal cavity is a highly vascular area. The rat uses the capillary network of the nasal passage for physiologic thermoregulation which necessitates that the nasal airway, and the nasal septum which lies within it, have a rich vascular network. Thus, antibodies which are carried in the blood stream would be available for diffusion from the capillaries to the nasal structures. Secondly, the nasal septum is a thin biconcave structure which would require diffusion of the antibody through only a few chondrocyte cell layers to exert its cytotoxic effect. In addition to these physical parameters which would allow localization of the antibody in the nasal septum, the midface as a region is highly susceptible to alteration by metabolic disturbances (McClure et al., 1979). Paulus (1976) showed that maternal ethanol inhalation caused a reduction in midfacial growth

and clefting of the palate. Hypothalmic disorders are also evidenced by midfacial retrusion (Ellyin et al., 1980; Friedman et al., 1979). These studies would imply that some non-specific changes in humoral factors affect growth in the midface, even when major growth disturbances are not evident elsewhere in the body. One final possibility which deserves further study is that the antibody directed against nasal septum while immunologically cross reactive with other cephalic cartilages is not cytotoxic to those structures.

The magnitude of the reduction in midfacial dimension, while statistically significant, was nonetheless small. A 6% reduction in snout length (um-i) was shown for animals treated with RNS antibody compared to saline controls. Several factors probably contributed to the magnitude of the observed effect. First, the amount of antibody directed specifically against the antigens of the nasal septum could not be determined because the IgG preparation used contained all the IgG normally found in rabbit serum in addition to the antibodies specific for nasal septum. Thus, it is possible that a monospecific antibody directed against nasal septum would cause a more dramatic reduction in snout length. Additionally, injection of a higher concentration of the polyspecific RNS antibody used in this study might also increase the observed effects on growth. Another factor which was not controlled in this in vivo study was the

effect of the mandible on maxillary growth. Latham (1970) offered a concept of "multiple assurance" in which he suggests that more than one factor may be responsible for providing the force necessary for facial growth. This concept draws support from the work of Petrovic et al. (1968, 1975). They have shown that mandibular growth can influence the growth of the maxilla via the incisor teeth. It is possible that a greater reduction in midfacial growth was not seen because of the extreme overbite of the incisors which allowed the mandible to carry the maxilla downward and forward. Either of these two factors would imply that by altering only one force involved in midfacial growth there would be only a proportionately small reduction in overall growth.

Another point that merits consideration is the developmental time period used for the in vivo study. Searls (1976) has reported that chondrocyte proliferation in the rat nasal septum in situ is greatest 5 days before birth. In this in vivo study the antibody was not administered to the pregnant rats, but, rather, the injections began on the first day postpartum. Searls' results suggest that administration of antibody to pregnant rats might produce a greater alteration in the offspring's midfacial development.

Finally, owing to the randomization of litters in the experimental design it was not possible to analyze the interaction between genetic and epigenetic factors. It is possible that

offspring from one litter can be more susceptible to the antibody's effect than those of other litters. This genetic susceptibility is evidenced in other growth disturbances. Achondroplastic and hypopituitary dwarves are known to exhibit a good deal of variation in their expression of the metabolic defect. An experimental design which included all treatments randomly assigned within pure litters would allow examination of this potential interaction and should be done in the future.

The combined results of the in vitro and in vivo portions of this study suggest that anti-cartilage antibodies offer a unique and exciting methodology for studying facial growth. The fact that anti-nasal septum antibody kills nasal septum chondrocytes in cell culture implies that there is a possibility for developing highly specific cytotoxic agents for each cephalic cartilage. The in vivo portion of this investigation represents a pilot study on the use of antibodies to modulate growth. The in vivo results presented indicate that growth of the midface can be altered by an antibody known to be cytotoxic for the nasal septum cartilage. Further studies now need to be undertaken in order to isolate the antigenic components of the nasal septum and the other caphalic cartilages. Once the antigens have been isolated, monospecific antibodies can be obtained by standard immunologic methods. These monospecific antibodies could prove to be a useful scientific tool for increasing the present understanding of growth control mechanisms operative at the cellular level.

SUMMARY AND CONCLUSIONS

Brigham (1976) demonstrated antigenic differences between bovine nasal septal cartilage and bovine condylar cartilage. Because of the importance of these cartilages in understanding craniofacial growth, additional studies were needed to determine the extent to which growth may be altered by antigen-antibody interaction. Palmer (1978) injected growing rats with anti-nasal septum immunoglobulin G (IgG) and demonstrated that the antibody was localized in the cartilage of the nasal septum *in vivo*. In addition, histologic examination of the tissue sections of the cartilaginous nasal septum of treated aninals showed evidence of cytopathic effects.

The present study examines the role of anti-cartilage antibodies in midfacial growth using both *in vivo* and *in vitro* experimental methodology. Rat chondrocytes were obtained from the cartilaginous nasal septum and grown in cell culture. The effect of anti-nasal septum antibody (RNS) on these cell cultures was assessed by monitoring protein accumulation, viability, and plating efficiency. In addition, the *in vivo* effects of anti-rat nasal septum IgG on craniofacial growth in rats from birth to 30 days was assessed by cephalometric radiography.

Results of this present study may be summarized as follows:

In Vitro (Effect of anti-nasal septum antibody on nasal septum chondrocytes grown in cell culture)

1. Anti-RNS IgG was bound to chondrocyte cell surface antigens as demonstrated by indirect immunofluorescent staining of chondrocytes in suspension. In addition, anti-RNS antibody was localized in the extracellular matrix as demonstrated by indirect immunofluorescent staining of monolayer cultures and cryostat tissue sections.

2. Anti-RNS IgG (7 mg/ml) and fresh complement caused 75% of the chondrocytes in monolayer culture to detach from the culture flask after a 3 hour time period. These unattached cells were presumably killed by the antibody/complement interaction.

3. Anti-RNS antibody (4 mg/ml) and fresh complement killed 35% of the chondrocytes in suspension as assessed by trypan blue exclusion.

4. Plating efficiency was reduced by 66% in chondrocytes treated with anti-RNS IgG both with and without complement compared to chondrocytes treated with control antibody with and without complement.

5. Total protein accumulations in chondrocyte monolayer cultures treated with anti-RNS antibody and complement levels off after two hours compared to cultures treated with RNS antibody alone which demonstrate a linear increase in protein accumulation over a three hour time period.

In Vivo (Injection of anti-RNS IgG into growing rats)

1. Premaxillary length (um-i) was significantly shorter ($p \leq .05$) in animals injected with anti-RNS IgG compared to control animals injected with isotonic saline.

2. Overall snout length (is-i) was significantly reduced ($p \leq .001$) in animals injected with anti-RNS IgG compared to saline injected controls and also significantly shorter ($p \leq .06$) than control animals injected with IgG isolated from normal rabbit serum.

3. Animals injected with anti-RNS IgG showed a significant ($p \leq .05$) midfacial retrusion when compared to saline injected animals as assessed by seven cephalometric measures (so-i, AB-UM R, AB-UM L, O-MX R, O-MX L, IS-MX R, IS-MX L).

4. Animals injected with anti-RNS antibody demonstrated a significant ($p \leq .05$) reduction in bimolar width (um-um) and bimaxillary width (MX-MX) of the midface compared to the saline injected control group.

5. Anti-RNS IgG injections resulted in a significant decrease ($p \leq .05$) in vertical height of the snout (jl-i) compared to the saline injected control group.

In conclusion, the results of this study demonstrate that anti-nasal septum IgG selectively binds to the cell surface of chondrocytes. More importantly, it is clear that the binding of this antibody causes a complement-dependent cytotoxic effect on nasal septum chondrocytes in cell culture. Preliminary results of the kenetics of this interaction suggest that this killing is probably time and dose-dependent.

Results of the <u>in vivo</u> portion of this study demonstrate that <u>in vivo</u> binding of anti-nasal septum IgG causes a statistically significant reduction in midfacial growth. Further, these results suggest that anti-RNS IgG could be a modulator of the growth process. The combined results of this study indicate that it may be possible to utilize anti-cartilage antibodies to effect a dose-dependent, tissue-specific, modulation of midfacial growth.

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