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ISOLATION OF ANTISERA SPECIFIC FOR FIBROBLAST-LIKE CELLS FROM EMBRYONIC CHICK CORNEA, HEART, AND SKIN

by

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For the Lord is a great God,
and a great King above all gods.
In His hand are the deep places of the earth:
the strength of the hills is His also.
The sea is His, and He made it,
and His hands formed the dry land.
O come, let us worship and bow down:
let us kneel before the Lord our maker.

The Ninety-fifth Psalm

LITERATURE REVIEW

Most of the fibroblasts of the body are derived from the neural crest mesenchyme and mesodermal mesenchyme (Figure 1). These two embryonic populations, refered to collectively by Hay and Revel (1969) as "secondary mesenchyme," give rise to almost all the connective tissue cells of the body. The cell types derived from this population therefore include chondrocytes, osteoblasts, as well as fibroblasts. The secondary mesenchyme contributes fibroblasts to primordia of the eye, lung, teeth, and heart, as well as to differentiating muscle, skin, walls of blood vessels, and the comb. Because of its importance in fibroblast differentiation, and the generation of connective tissue cells in general, the development of the primary and secondary mesenchyme is of primary concern.

Origin and Development of Primary and Secondary Mesenchyme

During vertebrate embryogenesis, cells from both mesoderm and ectoderm give rise to mesenchyme, a population of fibroblast-like cells and the embryonic forerunner of connective tissue (Holtfreter, 1968; Ham, 1974). The concept that two distinct lines of mesenchyme exist, the primary and secondary, in the trunk of the chick embryo arises principally from from morphological and ultrastructural observations (Trelstad et al., 1967; Hay, 1968; Hay and Revel, 1969).

The primitive streak is first visible six to seven hours after the egg is laid as an elongating growth center along the midline of the blastoderm (Spratt and Haas, 1965). At 25 hours after laying (Hamburger-Hamilton stage 8), the primitive streak occupies the posterior half of the body, four somites having

formed anteriorly (Hamburger and Hamilton, 1951). Along its length, the epiblast, or upper layer, gives rise, by invagination and migration of cells, to the mesoblast (middle layer) and perhaps also contributes to the hypoblast in the same manner (Spratt and Haas, 1965; Rosenquist, 1966; Trelstad et al., 1967). The epiblast is a simple pseudostratified columnar epithelium facing the vitelline membrane. The hypoblast is a simple squamous epithelium that lines the yolk cavity. The mesoblast is a mesenchyme; the cells are non-epithelial in the sense that although they derive from the basal surface of the epiblast, they have no free surface themselves and they migrate between the epithelial layers of the embryo (Hay, 1968). This original or first-formed mesenchyme has been named "primary mesenchyme" by Hay (1968) and Hay and Revel (1969). The primary mesenchyme eventually organizes itself into epithelial tissues, including the somites, lateral mesoderm, and nephrogenic mesoderm. The aggregation of the mesoblast into an epithelium in the chick embryo begins some distance anterior to the growth center or primitive streak (Spratt and Haas, 1965). Most, if not all, of the primary mesenchyme of the body axis of the chick embryo becomes aggregated into an epithelium (Hay, 1968; Hay and Revel, 1969).

In the embryo of 13 to 14 somites (stage 11, 45 hours of incubation), the newly formed posterior somites have a smooth, compact epithelial configuration. However, in the mid-region of the body, the older somites undergo dramatic changes. The cells in the ventro medial wall (sclerotome) seem to be pulled toward

the notochord (Hay, 1968). Despite the extensive juxtaluminal contact specializations (Trelstad et al., 1966), the ventromedial wall of the somite breaks apart resulting in the newly formed secondary mesenchyme. The dorsomedial wall of the somite retains its epithelial configuration, but the remainder of the somite epithelium facing the neural tube and notochord dissociates into sclerotome. Soon thereafter, a new ventromedial wall is created, the myotome. The dermatome, or dorsolateral wall of the somite, subsequently breaks up completely into mesenchyme. Mesenchymal cells also derive from the base of the lateral (coelomic) mesoderm and from the nephrogenic mesoderm during the same period (Hay, 1968; Hay and Revel, 1969).

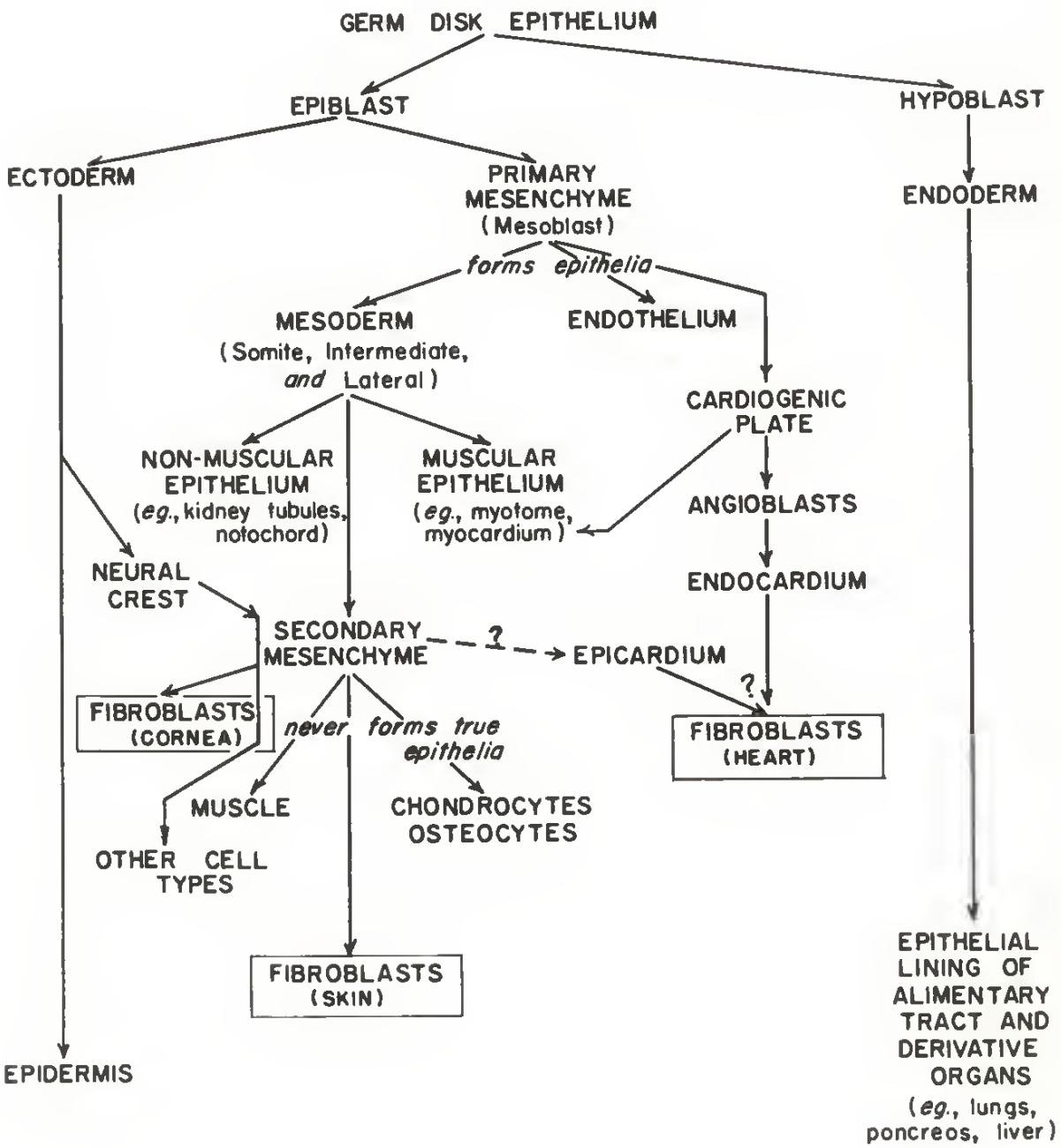
The neural crest, a second and major source of mesenchyme, starts to form along the body axis at the time the sclerotome dissociates (Hörstadius, 1969). The determination and differentiation of neural crest cells has been extensively reviewed by Hörstadium (1969), Weston (1970), and LeDouarin et al., (1977). In the region of the embryo anterior to the first somite, neural crest makes an extensive contribution to the secondary mesenchyme destined to form connective tissue, dermis, bone, cartilage, and other tissues of the head (LeLievre and LeDouarin, 1975).

In summary, the term secondary mesenchyme has been developed to refer to the mesenchymal cells which derive from the mesodermal epithelia and from the nerual crest because the fate of the late-formed, inward migrating cells is different from that of the primary mesenchymal cells (Trelstad et al., 1967). Most, if not all, of the inward wandering cells derived from

Figure 1. Presumed Embryonic Origin of Fibroblasts in Chickens (Hay, 1968)

EMBRYONIC ORIGINS OF FIBROBLASTS IN CHICKENS

(*modified from* Hoy, *1968*)



the epiblast along the primitive streak are destined to develop into epithelia, but secondary mesenchymal cells derived from the mesodermal epithelia and neural crest never give rise to true epithelia (Hay and Revel, 1969). They are destined to form connective tissue cells, ganglion cells, pigment cells, and muscle cells. Although some muscle is derived directly from epithelium (myocardium, myotome; Manasek, 1968, 1970), chondroblasts, osteoblasts, and fibroblasts are descendents of secondary mesenchyme.

Origin of Cornea, Heart, and Skin Fibroblasts

The presumed embryonic origins of cornea, heart, and skin fibroblasts are summarized diagrammatically in Figure 1. Numerous excellent publications and reviews of the literature describe cornea, heart, and skin development (Coulombre, 1961, 1965a,b; Bell, 1965; DeHaan, 1965; Woessner et al., 1967; Manasek, 1968, 1969, 1976; Parakkal and Matoltsky, 1968; Hay and Revel, 1969). Corneal fibroblasts probably originate from neural crest (Johnston, 1966; Hay and Revel, 1969; Johnston et al., 1974; Noden, 1975). Fibroblasts from dorsal skin dermis are believed to derive from the dermatome component of the somites (Murray, 1928). At least some of the heart fibroblasts originate in the epicardium. The epicardium consists of an outer simple epithelial layer and an underlying layer containing fibroblast-like mesenchymal cells surrounded by a collagenous extracellular matrix. The exact origin of the epicardium is not known (Manasek, 1968, 1969, 1970).

The Fibroblast

The fibroblast has long been identified and described histologically on the basis of its morphology. They are usually described as single, migratory cells of various shapes which occur interspersed through the connective tissues of most organs in all vertebrates. Maximow (1927) described fibroblasts as large, irregularly shaped cells with substantial cytoplasm surrounding an oval nucleus, and possessing long filopodial extensions of the plasma membrane. Maximow observed fibroblasts with smooth cell membranes in elongated, polygonal, or stellate forms. Despite these general morphological descriptions, the name "fibroblast" derives from the role that early microscopists correctly presumed such cells to play in fibrogenesis (Schwann, 1847). The close association of these cells with generally fibrous extracellular matrices (collagen and proteoglycans) was an early indication that fibroblasts are involved in fibrogenesis (Porter and Pappas, 1959; Gabbiani *et al.*, 1972; Ham, 1974). The use of modern techniques such as electron microscopy (Ross, 1968a,b) and autoradiography (Ross and Benditt, 1965; Ross, 1968a,b) confirmed the role of collagen biosynthesis by fibroblasts.

The numbers of fibroblasts in the vertebrate body, and the diversity of extracellular macromolecules which they synthesize make them extremely important for normal embryonic development as well as homeostasis of the adult. Although much is known regarding normal morphogenetic sequences in the differentiation of such connective tissue cell types as muscle cartilage, and bone *in vitro* and *in vivo*, little is known about the normal

steps in the differentiation of fibroblasts. Studies of differentiation of mesenchyme, fibroblasts, and other connective tissue cells has been focused primarily on the synthesis of extracellular molecules (see for review, Kulonen and Pikkarinen, 1973; Slavkin and Greulich, 1975; Lash and Burger, 1977). Such studies have demonstrated that fibroblasts are not the only connective tissue cells responsible for collagen formation as originally believed. Schlitz et al. (1973), observed that primary cultures of chondrocytes display patterns of collagen and glycosaminoglycan biosynthesis resembling those of presumed fibroblasts. Mesenchyme, fibroblasts, smooth muscle cells, chondrocytes, and osteoblasts have been found to synthesize a variety of types of collagen and glycosaminoglycans, and organize them into extracellular matrices which vary enormously in chemical composition and ultrastructure (Abbott et al., 1974; Levitt et al., 1974, 1975; Ross, 1975; Okayama et al., 1976; von der Mark et al., 1976; Hasty and Hay, 1977). Thus, the simple ability to synthesize collagen is no longer an indication of fibroblast or fibrocyte differentiation. Cell products once thought to be unique to certain connective tissue cell types are no longer so limited but are found among diverse cell populations.

Fibroblast-like cells from many organisms proliferate in vitro and have proven to be of great value in many diverse studies such as genetic diseases (Conrad et al., 1972; Dorfman et al., 1973; Dorfman and Matalon, 1976), viral transformation (Unkeless et al., 1973), and plasma membrane properties

(Kletzien and Perdue, 1975; Vaheri *et al.*, 1977). Presently, it is very difficult to distinguish *in vivo* between mesenchyme cells, fibroblasts, chondroblasts, osteoblasts, and other connective tissue cells. *In vitro* the problem is compounded by the tendency of some cell types that were non-fibroblastic *in vivo* to assume fibroblast-like characteristics *in vitro*. Fibroblast-like cells isolated from whole embryos or from several specific tissues are being grown *in vitro* as supposedly homogeneous populations. If such fibroblast-like cell populations are not alike when grown *in vitro*, their differences could have significant effects on the results of analyses which made use of such cells (Conrad, 1977b). Studies similar to those alluded to above have resulted in an informal dogma: 1) Fibroblast-like cells in culture are authentic fibroblasts, 2) Fibroblasts derived from different tissues of one animal are alike, and 3) Fibroblast populations are therefore homogeneous.

Recent data raises serious questions concerning the validity of these presumptions. Wilmer (1965), Coon (1966), Mayne *et al.* (1974), Ross (1975), and numerous other investigators have shown that many cell types which are not authentic fibroblasts or fibrocytes *in vivo* can be induced *in vitro* to change into cells morphologically, and in some instances biochemically, indistinguishable from authentic fibroblasts (e.g., chondrocytes, cardiac and smooth muscle, and some epithelial cell types). Thus, not all fibroblast-like cells observed in cell cultures exist *in vivo* as authentic fibroblasts. Efforts to alleviate the confusion over the authenticity of fibroblasts and fibrocytes

in vivo and these cells *in vitro* have not been totally successful. Wilmer (1965) proposed using the term "mechanocyte" to designate fibroblast-like cells seen *in vitro* regardless of their former cell type *in vivo*. However, the current usage of the term "fibroblast" in designating such cells is so firmly entrenched in the literature that proposing new terminology only adds to existing confusion. Conrad (1977a,b) has proposed that the terms "fibroblast" and "fibrocyte" be reserved to denote the authentic connective tissue cell type *in vivo*; the phrase "fibroblast-like cell" would be retained for cells *in vitro* whose *in vivo* cell type progenitors have not been identified rigorously. Conrad further suggested that the terms "fibroblast" and "fibrocyte" be used only to refer to states of differentiation of this cell type rather than to its ability or inability to divide. Similar distinctions are reserved for "chondroblasts" and "chondrocytes" as well as for "myoblast" and "myocyte." Indeed, perhaps no correlation should be drawn between states of differentiation and cell division, since previous work has shown clearly that chondrocytes, myocytes, and melanocytes can divide without losing their state of differentiation (Coon, 1966; Cahn and Cahn, 1966; Weinstein and Hay, 1970).

Early Evidence of Fibroblast Differentiation

Numerous early studies indicated that populations of fibroblast-like cells from different tissues are different. The experiments of Parker (1929, 1932a,b, 1933) represent one of the earliest attempts to determine if more than one type of fibroblast could be distinguished from the tissues of one animal.

Parker showed that from nine different tissues of an embryonic chick, one could isolate *in vitro* nine different populations of fibroblasts which were distinguishable from one another by their rates of outgrowth from an explant, the amount of acid they produced, their ability to digest a fibrin clot, and their ability to grow in media of low pH. McLoughlin (1961) demonstrated that embryonic chick mesenchyme from skin, heart, gizzard, and proventriculus, when combined *in vitro* with dermal epithelium, resulted in epithelial differentiation dependent upon the type of mesenchyme used. Such observed effects of heterotypic mesenchymes on surrounding tissues were believed to arise through activities of fibroblasts and their abilities to synthesize tissue-specific arrays of glycosaminoglycans (Meyer and Rapport, 1951; Balazs, 1970) and fibrous proteins (Dische *et al.*, 1958; Balazs, 1970). The mesodermal mesenchyme populations which surround different regions of the embryonic gut tube have been tested for their ability to support the morphogenesis and cytodifferentiation of epithelial cells. These experiments have demonstrated that different types of mesodermal mesenchyme exist along the gut tube (Bernfield *et al.*, 1973; Spooner, 1974) and that specific interactions between endodermal rudiments and mesenchyme are dependent upon extracellular elements synthesized jointly by mesenchymal and epithelial components (Bernfield *et al.*, 1972, 1973; Bernfield and Banerjee, 1972; Spooner, 1975; Slavkin *et al.*, 1977; see for review, Wessells, 1977).

The work of McLoughin, however, fails to prove the heterogeneity of fibroblast types; mesenchyme, as previously stated,

gives rise to a variety of connective tissue cells (e.g., chondroblasts, osteoblasts, etc.) any of which could synthesize the extracellular products responsible, at least in part, for the tissue interactions observed. Furthermore, the experiments of Parker take no precautions to exclude cells assuming fibroblastic morphology as a result of losing their specialized phenotypes *in vitro*. Thus, earlier efforts to demonstrate differences between pre-existing *in vivo* fibroblast populations in various tissues have been inconclusive.

The work of Parker and others has not gained wide recognition despite recent reviews (Movat and Fernando, 1962; Ross, 1968a,b) and symposia (e.g. Kulonen and Pikkarainen, 1973) dealing with the origin, structure, and differentiation of fibroblasts. As mentioned, there has been a tendency to regard fibroblasts as a single, homogeneous population of cells (Movat and Fernando, 1962; Green and Todaro, 1967; Owen, 1970). Recent studies continue to perpetuate this tendency. Different fibroblast-like subpopulations from human skin have been demonstrated *in vitro* with respect to lysosomal enzyme activity (Milunsky *et al.*, 1972) and with respect to testosterone metabolism (Kaufman *et al.*, 1975). Cultures of fibroblast-like cells made from whole chick embryos show consistent heterogeneity in the ability to produce Newcastle disease virus (Huppert *et al.*, 1974). Experiments by Schneider *et al.* (1977), demonstrated that lung fibroblast-like cells from human fetus and grown *in vitro* have shorter replication times, higher saturation densities, smaller cell volumes, decreased cellular RNA and protein content, and

a lengthened *in vitro* life span than compared to identically cultured fetal skin fibroblast-like cells. Ko *et al*. (1977), found that fibroblast-like cells display phenotypic heterogeneity when exposed to prostaglandins. Although each of these studies imply that tissue-specific fibroblast populations exist *in vitro* (and perhaps *in vivo*), none of these studies make efforts to remove nonfibroblastic cell types before initiating primary cultures. Thus, the differences observed could be explained by the initial existence of contaminating cell types rather than a difference in genuine fibroblasts.

Conrad (1977a) has demonstrated that fibroblast-like cell populations isolated from cornea, heart, and skin of embryonic chicks, and cultured in the virtual absence of nonfibroblastic cell types, differ *in vitro* in single cell morphology, social behavior of saturated cultures, saturation densities, and sensitivity to treatment with trypsin plus EDTA, or to EDTA alone. Biochemical studies of these same embryonic tissues have demonstrated definite differences in patterns of glycosaminoglycans synthesized by the three different fibroblast-like cell populations (Conrad, 1977b). Absher and Absher (1976) cloned populations of fibroblast-like cells and observed heterogeneity in average times between cell divisions, clone sizes, and generations per clone. However, experiments utilizing clones of "fibroblast-like cells" impose three serious disadvantages: 1) Present methods make it impossible to absolutely identify the cloned cell as an authentic (*in vivo*) fibroblast (with the possible exception of corneal stroma fibroblasts; Conrad, 1970b),

2) An average of 85% of the inoculated cell population dies (Conrad, 1970b) therefore, most of the isolated cell population is never examined, and 3) Clones of primary embryonic cells grow so slowly and must divide so many times that it is difficult to accumulate a sufficiently large population to analyze before effects of *in vitro* senescence appear (Beug and Graf, 1977).

Considerations for the Future Study of Fibroblast Differentiation

The previous discussion has considered briefly various aspects of fibroblast development, differentiation, and a review of the research studying these phenomena. Upon examination of the present state of knowledge of fibroblast differentiation, the answer to one question promises not only to lend new meaning to prior research, but also to stimulate new research on a variety of developmental problems. In view of technical and interpretive limitations placed on *in vitro* studies of fibroblast-like cells, how can such studies be accurately correlated to authentic fibroblast populations *in vivo*?

The present work proposes that immunological techniques offer the most direct approach to answering this question. The existence of stable differences between cultured fibroblast-like cells have been described. Such data suggest that these fibroblast-like cell populations also possess distinct antigenic differences which are stable *in vitro*. The possibility that cells from different embryonic tissues may possess qualitatively different surface constituents has been examined in numerous systems by immunological techniques (Dawkins and Halborow, 1972; Goldschneider and Moscona, 1972; Ikeda *et al.*, 1975; Bell *et al.*,

1976; Thompson *et al.*, 1976; Ishii *et al.*, 1977; Friedlander and Fischman, 1977; Vaheri *et al.*, 1977). The isolation of antibodies specific for different fibroblast-like cells grown *in vitro* is the first step in clarifying some otherwise anomalous data and allowing analyses of steps in fibroblast differentiation. Specific anti-fibroblast antibody would serve to supplement previous criteria demonstrating that fibroblast-like cells *in vitro* represent distinct and stably differentiated cell types. If tissue-specific antigenic differences between different fibroblast-like cells *in vitro* exist in corresponding fibroblast populations *in vivo*, a number of problems may be examined: 1) When do tissue-specific characteristics of each fibroblast population first appear during embryongenesis. 2) Do tissue-specific characteristics arise before or after fibroblasts arrive in the differentiating tissue. 3) Are the antigens expressed at a given stage of development (e.g. day 14) retained, lost, or altered during later development. 4) What, if any, role does the tissue (extracellular) matrix surrounding an immature or undifferentiated fibroblast play in determining what phenotype is expressed. 5) Considering the variety of characteristics for each fibroblast population, do these populations arise together during development. 6) Are there antigenic simularities shared between fibroblasts from different embryonic origins but not shared by fibroblasts of other origins. 7) Using fibroblast-specific antibody together with complement, what is the effect of removing (lysing) corneal fibroblasts at various stages of development. 8) What affect do anti-fibroblast antibodies have on cellular adhesion.

Such problems can be approached once a technique is available for monitoring tissue-specific differences between fibroblast populations *in vivo* during embryogenesis. The following work describes the isolation of antisera specific against 14 day chick embryo cornea, heart, and skin fibroblast-like cells grown *in vitro*.

ISOLATION OF ANTISERA SPECIFIC FOR FIBROBLAST-LIKE CELLS FROM EMBRYONIC CHICK CORNEA, HEART, AND SKIN

INTRODUCTION

Current evidence suggests that distinct populations of fibroblasts may differentiate in different tissues and continue to display their differences *in vitro* (Milunsky *et al.*, 1972; Huppert *et al.*, 1974; Kaufman *et al.*, 1975; Absher and Absher, 1976; Reddy *et al.*, 1976; Beug and Graf, 1977; Conrad *et al.*, 1977a,b; Ko *et al.*, 1977; Schneider *et al.*, 1977). These data are consistent with the conclusions of Parker (1932a,b) who observed that nine different tissues of an embryonic chick would give rise *in vitro* to nine different populations of fibroblast-like cells, distinguishable from one another by several criteria.

Fibroblast differentiation has proven difficult to study, however. First, except for early experiments of Parker, fibroblasts rarely have been isolated from different tissues of the same animal and systematically compared with one another under controlled conditions. Second, nonfibroblastic cell types frequently are included inadvertently with fibroblasts inoculated *in vitro* and then assume morphological and biochemical characteristics which make them appear similar to genuine pre-existing fibroblasts (Gaines, 1960; Holtzer *et al.*, 1960; Bryan, 1968; Chacko *et al.*, 1969; Anderson *et al.*, 1970). Third, the morphology of a single fibroblast population *in vitro* is difficult to describe except by matched photographic comparisons under controlled conditions with the morphology of fibroblasts from another tissue (Parker, 1929; Grossfeld *et al.*, 1955, 1957; Conrad *et al.*, 1977a). Fourth, the embryonic

secondary mesenchyme cells from which so many tissue populations of fibroblasts arise during normal embryogensis closely resemble fibroblasts themselves (Hay, 1968; Hay and Revel, 1969). Yet such mesenchyme gives rise to chondroblasts and myoblasts as well as to fibroblasts (Abbott et al., 1974). Moreover, in most tissues it is not clear when a fibroblast differentiates into a fibrocyte. Fifth, until recently, synthesis of collagen and glycosaminoglycans was thought to be a distinguishing characteristic of differentiated fibroblasts (i.e. fibrocytes) and such related cells as chondrocytes and osteocytes. It is now recognized, however, that such macromolecules also are made by several types of epithelial cells, embryonic mesenchyme, and smooth muscle (Dodson and Hay, 1971; Ross, 1971; Grant et al., 1973; Abbott et al., 1974; Levitt and Dorfman, 1974; Ross, 1975; Lash and Vasan, 1977). Thus, simple synthesis per se of collagens and glycosaminoglycans is no longer an acceptable criterion of fibroblast differentiation.

Several questions regarding fibroblast specialization remain unanswered: Are the tissue-specific characteristics of each fibroblast-type identified in vitro also displayed in vivo? When during development and where in the developing embryo do the tissue-specific characteristics of each fibroblast population first appear in vivo? To what extent do the fibroblasts derived from mesoderm differ from those that arise from the neural crest? These questions seem amenable to analysis with current immunological techniques.

Previous studies have shown that the fibroblast-like cells isolated from cornea, heart, and skin and grown *in vitro* behaved like three distinct and stably differentiated cell types (Conrad *et al.*, 1977a,b) and that the same populations might also display antigenic differences. The following data indicate that, even though these three populations are grown under identical culture conditions and in the relative absence of the nonfibroblastic cell types with which each population was normally associated *in vivo*, it is possible to isolate specific antisera which react with only one population and not with the other two, indicating that the populations are antigenically distinct from one another.

MATERIALS AND METHODS

Tissue isolation and cell culture. Tissues were dissected under sterile conditions from 14 day White Leghorn embryonic chicks. Embryonic age also was determined from Hamburger-Hamilton stages (Hamburger and Hamilton, 1951). Corneas, together with the surrounding limbus and scleral ossicles, were removed and rinsed three times in calcium-, magnesium-free saline G, pH 7.2 (CMF), containing 10% chick serum (v/v;CS). Heart ventricles were separated from auricles and major blood vessles, minced to small pieces and rinsed three times in CMF+CS. Back skin was freed of most feathers, minced, and rinsed three times in CMF+CS.

Fibroblast-like cells were isolated from tissues according to Conrad et al. (1977a). Whole corneas were trimmed free of all non-corneal tissue with iridectomy scissors under a dissecting microscope and transferred to another dish containing CMF+CS at 4°C. Trimmed corneas were digested at 37°C for 10 min in 0.25% collagenase (CLS II: Worthington, Freehold, NJ), 0.15% trypsin (1:250 Difco, Detroit, MI) in CMF followed by three 10 ml rinses in cold CMF+CS. Corneal epithelium and endothelium were removed by gentle scraping with an iridectomy scalpel under a dissecting microscope, and corneal fibroblast-like cells were released from the scraped corneas by a second period of digestion in fresh collagenase/trypsin solution (30 min, 37°C, on a gyratory shaker at 120 rpm (Conrad, 1970b)). The cell suspension then was mixed directly with an equal volume of cold nutrient medium (NM), centrifuged (1,500 rpm, 10 min, 20°C), and

resuspended in cold NM. Rinsed pieces of heart or skin were digested with collagenase/trypsin solution for 30 min at 37°C on the shaker. Digested tissues were dispersed by gentle pipetting. Resultant cell suspensions of heart and skin cells were filtered through seven layers of cheesecloth, mixed with an equal volume of cold NM, centrifuged as above, and resuspended in cold NM. Cell numbers were determined with a haemocytometer. Corneal cells, consisting of only stromal fibroblast-like cells (Conrad, 1970b), were plated into 60-mm culture dishes and incubated directly at 37°C. Heart cells, however, were mixtures of fibroblasts, endothelial cells, cardiac muscle cells, and perhaps smooth muscle cells from ventricular blood vessels. Skin cells were mixtures of fibroblasts and epithelial cells. Fibroblast-like cells of heart and skin were obtained by inoculating the cell suspension in culture dishes, incubating for 15 min at 20°C under an atmosphere of 5% CO_2-95% air, and then removing the floating and poorly attached cells by swirling and thorough aspiration. Fibroblast-like cells, but neither cardiac muscle or epithelial cell types, remained attached to the dishes under these conditions and were covered immediately with NM and incubated at 37°C. Nutrient medium was changed 18 h later (heart cultures were rinsed thoroughly in saline G, pH 7.2, and then fed) and every 48 h thereafter. Dishes were incubated at 37°C in humid chambers in an atmosphere of 5% CO_2-95% air. Using the protocol above, fibroblast-like cells from corneal stroma, heart ventricle, and back skin could be inoculated *in vitro* within a few hours following their isolation from the

embryo and appeared to be virtually free of nonfibroblastic cell types at all times of primary culture (Conrad et al., 1977a).

The NM used was Ham's F-12 containing (final concentrations) fetal calf serum (FCS; 10% v/v), sodium bicarbonate (14 mM), sodium ascorbate (0.54 mM), potassium salt of penicillin G (48 units/ml), and streptomycin sulfate (48 µg/ml, 0.033 mM). All sera and powdered F-12 were obtained from Grand Island Biological Co. (GIBCO, Grand Island, NY); all antibiotics were from Sigma Chemical Co. (St. Louis, MO).

Preparation of cell antigens. Primary cultures of cornea, heart, and skin fibroblast-like cells were grown in vitro for 14 days before being collected for use as antigens. But that time the cultures had been at saturation for approximately 4-5 days. Nutrient medium was removed, and the cells were rinsed three times with 5 ml of cold saline G without phenol red or antibiotics (CMF-A). After rinsing, 2 ml of fresh, cold CMF-A was added to each dish; the cells were scraped into suspension using a Nalgene policeman, dispersed with a Pasteur pipette, vortexed, and centrifuged (10,000 rpm, 30 min, 4°C). Pellets were resuspended in CMF-A to a final concentration of 2×10^6 cells/ml. Cell suspensions of each type (cornea, heart, and skin) were stored at -20°C until used as antigens.

Immunization. Stored cells were thawed and disrupted by multiple passes through a 25-guage needle. Six New Zealand white rabbits were immunized with the cell suspensions (2 rabbits for each fibroblast type). Rabbits were immunized subcutaneously with 2×10^6 cells mixed 1:1 with complete

Freund's adjuvant. A total of 2 ml of cell-adjuvant solution was given per injection. Five booster inoculations were given at two week intervals: The first three were given using 2×10^7 cells emulsified 1:1 with incomplete Freund's adjuvant. The final two injections consisted of 1×10^8 cells mixed 1:1 with incomplete Freund's.

Rabbits were bled prior to immunization and every seven days for fourteen weeks following the third inoculation. The pre-immunization serum (normal rabbit serum - NRS) was used as a control. For each fibroblast cell type, serum from one rabbit showing maximum titer was pooled. Sera from pre- and post-immunization bleedings were heat-inactivated (56°C, 30 min) and stored in small aliquots at -20°C.

Immunoabsorptions. Antibodies reacting with heterologous antigens were removed by the three-step immunoabsorption procedure described below.

a) Immunoabsorption of antisera with chick embryo homogenate. Antisera raised against cornea, heart, and skin fibroblast-like cells (anti-cornea/anti-heart/anti-skin antisera) were absorbed with homogenates of embryos from which the homologous tissue was first removed (for example, anti-cornea sera were absorbed wtih a homogenate of embryos from which the corneas had been removed). The purpose of this step was to enrich the antisera for tissue-specific antibodies.

Homogenates were made as follows from 14 day White Leghorn chick embryos under sterile conditions: Cornea, including scleral ossicles, were carefully removed from one group of

embryos. Whole hearts and major blood vessels leading to the heart were removed from a second group of embryos. From a third group of embryos, the wings, legs, and all remaining skin were carefully removed. Dissected embryos were rinsed three times in sterile CMF (1:1 w/v), then frozen and thawed once. Embryos were minced with scissors and then homogenized in a Virtis steel blade homogenizer in a vessel jacketed with ice water (5,000 rpm, 10-15 sec; Virtis Co. Inc., Farner, NY). Homogenizations were repeated using a Potter Elvehjem glass homogenizer with a Teflon pestle (10 strokes, 1,000 rpm, 4°C); sediment (bone, feathers, etc.) was discarded and the supernatant was frozen at -20°C for 24-48 h. Homogenates were thawed and then centrifuged (16,000 rpm, 30 min, 4°C). Supernatants were collected and centrifuged again as above. The final supernatant solutions were collected and stored at -20°C.

Quantitative precipitin tests were performed on each lot of antisera in order to determine the optimal ratio of antiserum to mix with homogenate (antigen) in order to achieve maximal precipitation of antibodies to heterologous antigens. After thawing, antisera and homogenates first were centrifuged (1,500 rpm, 15 min, 4°C) and then were combined at the optimum ratios. Mixtures were vortexed, incubated for 2 h at 37°C, vortexed again, and incubated at 4°C for 48 h. Precipitates formed during incubations were removed by centrifugation (16,000 rpm, 30 min, 4°C). The supernatants were collected, mixed with half the original optimal volume of homogenate, and then incubated and centrifuged again as above. The second

supernatants were collected and stored at -20°C. (Antiserum thus absorbed against embryo homogenates are termed 1° absorbed (e.g., 1° anti-cornea).)

b) Immunoabsorption of 1° absorbed antisera with heterologous fibroblast-like cells. To enrich the antisera for antibodies against tissue-specific fibroblast antigens, the 1° absorbed antisera next were absorbed with homogenates made from the two respective heterologous populations of fibroblast-like cells (e.g., 1° anti-cornea was absorbed with homogenates of heart and skin fibroblast-like cell cultures). Homogenates of cell cultures were prepared as follows: Fibroblast-like cells were prepared as described above and grown for 8-10 days in vitro (cultures at saturation). Nutrient medium was removed and dishes were rinsed twice with cold phosphate-buffered saline (0.15 M phosphate), pH 7.2 (PBS). Cells then were suspended in PBS using a Nalgene policeman and homogenized with a Potter Elvehjem homogenizer. Such homogenates were sonicated (Sonifer Cell Disrupter W185; Heat Systems Ultrasonics, Inc., Plainview, NY) under ice, vortexed for 30 sec, freeze-thawed three times, and centrifuged (16,000 rpm, 30 min, 4°C). Supernatants were used to perform secondary absorptions.

Quantitative precipitin tests were performed with antisera and heterologous cell homogenates. Absorption of each 1° absorbed antiserum with heterologous cell homogenates was performed in two steps. First, 1° absorbed antisera were mixed (at optimal ratios determined by precipitin tests) with one of the heterologous homogenates. Incubation, centrifugation, removal of precipitates, and subsequent absorption of the supernatants

with half the optimal volume of the same homogenate then occurred according to the same steps used to make the 1° absorbed antiserum. The resulting antiserum is termed 2° absorbed. In the second step, the 2° absorbed antisera were absorbed with the other heterologous fibroblast homogenate, using procedures identical to those of the first step. The resulting antiserum is termed 3° absorbed. In summary, by the steps above, antiserum prepared against corneal fibroblasts, for example, was absorbed first with homogenates of embryos lacking corneas, and then with homogenates of heart and skin fibroblast-like cells, thereby generating 3° anti-cornea antiserum.

Immunological techniques. The specificity of the antisera for cornea, heart, and skin fibroblast-like cells was tested by means of immunodiffusion, agglutination, cytotoxicity, and immunofluorescence.

a) Immunodiffusion. Absorbed and unabsorbed antisera were used in double immunodiffusion studies to detect specific antibodies against cornea, heart, and skin fibroblast homogenates. Agar gel immunodiffusion tests were performed in 1.0% agarose (Seakem Agarose-ME; Miles Laboratories, Elkhard, IN) in 0.1 M borate saline buffer, pH 8.2 and 0.01% sodium merthiolate. Diffusion plates were incubated at 20°C for 18 h in a moist chamber, rinsed in buffer for 36 h, dried, stained with 0.5% (w/v) Light Green SF Yellowish (Manufacturing Chemists, Norwood, OH) in acidic methanol (methanol 7: glacial acetic acid 1: distilled water 2; v/v), and photographed with a red lens filter. Control wells contained test antiserum absorbed with homologous

antigen (e.g., 3° anti-cornea antiserum plus cornea fibroblast-like cells: Pre-absorbed sera), NRS, CS.

b) Immune agglutination. Antisera were tested for their ability to agglutinate fibroblast-like cells prepared as described above from cornea, heart, and skin. Cell cultures were incubated for 10 min at 37°C with 0.5% trypsin (1:250 Difco), 0.1% ethylenediaminetetraacetic acid (2.5 mM, EDTA; Sigma Chemical Co.) in CMF. Cells were released from dish surfaces by gently pipetting the dissociating solution across the plate surface. Dissociated cells from each dish were pooled according to cell type, placed in chilled centrifuge tubes containing an equal volume of NM and centrifuged (1,500 rpm, 15 min, 20°C). Cells were resuspended in either CMF plus 10 mM EDTA or in NM (with or without 0.25% trypsin inhibitor, Trypsin Soybean Inhibitor; GIBCO), and then allowed to "recover" from trypsinization by incubation for 1 h in a gyratory water bath shaker (120 rpm) at 37°C (McClay and Baker, 1975; McClay et al., 1977). After such incubation, cells were kept at 4°C until used in agglutination assays. Total cell number was determined directly with a haemocytometer counting chamber; numbers of viable cells were determined from the proportion of cells excluding trypan blue (0.1% final concentration) after a 10 min incubation at 20°C. Cells were brought to a final concentration of 1×10^7 cells/ml with either CMF plus 10 mM EDTA or with NM.

Agglutination assays were performed on glass slides by mixing one volume of antiserum (serially diluted 1:2 with CMF plus 10 mM EDTA or with PBS) with one volume of cell suspension.

Slides then were then incubated 20 min at 4°C in a humid chamber and examined under phase optics. Manipulations were done at reduced temperatures, as much as possible, to minimize the risk of spontaneous aggregation of cells that might have obscured the effects of immune agglutination (Steinberg, 1962; Moscona and Moscona, 1967; Edwards and Campbell, 1971; Steinberg et al., 1973; McClay and Baker, 1975). Controls consisted of cell suspensions alone and cell suspensions mixed with pre-absorbed sera or with NRS. Antibody titer was defined as the reciprocal of the highest dilution of antiserum giving detectable agglutination.

c) Cytotoxicity assays. Anti-fibroblast antisera were tested for their ability to mediate complement-dependent cytolysis of homologous and heterologous cell populations. Cytotoxicity experiments were performed using cell suspensions or cells in monolayer. Cytolysis was quantitated by cellular ability to exclude trypan blue or to release radioactive sodium chromate ($Na_2{}^{51}CrO_4$).

1) Cytolysis of fibroblast-like cells in suspension. Single-cell suspensions were prepared by incubation of cell monolayers in trypsin-EDTA and their subsequent "recovery" effected as described above. Single cells (2×10^6 cells/ml) in 25 µl of NM and 25 µl antiserum (serially diluted 1:2) were mixed at 4°C in 5 x 50-mm glass tubes and incubated for 30 min at 37°C. At the end of the first incubation period 25 µl of rabbit serum (complement source, diluted 1:4 in PBS; Miles Laboratories, Inc.) was added to each tube and the preparations

were incubated a second time for 30 min at 37°C. After incubation, cells were placed on ice. To each tube, 25 µl of 0.4% trypan blue in physiological saline was added (0.1% final concentration). The number of surviving cells (cells excluding dye) was determined immediately using a haemocytometer. Each experiment included controls in which cells were incubated alone or with (a) NRS and complement, (b) complement alone, (c) antiserum only, (d) pre-absorbed antiserum and complement, and (e) antiserum and complement heated to 56±C for 30 min before use. Assays were performed three times, each in triplicate. The cytotoxic potency of the antiserum was expressed as a percentage of dead cells, Herberman et al., 1976):

$$\%Cx = \frac{(\text{Experimental} - \text{Control})}{(\text{Total Cells} - \text{Control})} \times 100$$

The experimental represents the mean number of dead cells in the presence of antiserum and complement. The total represents the mean number of total cells counted. The mean number of dead cells in the presence of NRS and complement constitutes the control value.

Cells in suspension were also assayed by measuring the release of radioactive sodium chromate (^{51}Cr). Fibroblast-like cells were collected in NM and suspended by the method previously used to collect cells for agglutination assays, including a 1 h "recovery" period at 37°C in a gyratory shaker bath (120 rpm). Thirty minutes after the beginning of the recovery period, cell suspensions were labeled by addition of $Na_2{}^{51}CrO_4$ in isotonic saline (specific activity 180.9 mCi/ml of cell suspension (1×10^7 cells/ml). Incubation continued in

the presence of isotope for another 30 min at 37°C. The cell suspension then was chilled, and the cells washed five times by repeated centrifugation in NM containing 10% heat-inactivated FCS. Cell suspensions were adjusted to 2 x 10^6 viable cells/ml as determined by trypan blue exclusion and haemocytometer counts. Fifty microliters of the cell suspension were incubated, in triplicate, with 50 µl of serial 1:2 dilutions of antisera at 37°C for 30 min. Fifty microliters of a 1:4 dilution of rabbit serum was added and the mixture incubated for 30 min at 37°C. After sedimentation of the cells by centrifugation (1,500 rpm, 15 min, 20°C), 50 µl of the supernatant was removed and the radioactivity measured in an automatic gamma spectrometer (Model 1185; Searle Analytic, Inc., Des Plaines, IL). Controls were identical to those used to assay cell suspensions. The percent cytotoxicity was calculated by the method of Rosenberg et al., (1977):

$$\%Cx = \frac{(\text{Experimental } {}^{51}\text{Cr Release} - \text{Spontaneous Release})}{(\text{Maximum Release} - \text{Spontaneous Release})} \times 100$$

Spontaneous release was determined with cells in the presence of NRS and complement, and experimental release was that seen in the presence of anti-fibroblast antiserum and complement. Maximum release represents the total counts per minute obtained after freeze-thawing (three times) cells.

2) Cytolysis of fibroblast-like cells in culture. In order to determine whether anti-fibroblast antisera could mediate cytolysis of cells that had not been treated with trypsin and EDTA, cytotoxic assays were performed directly on cells in

culture plates (Microtest plates, No. 3034; Falcon Plastics, Oxnard, CA). Fibroblast-like cells were isolated from embryos as described above and plated in either subconfluent (e.g., 200 cells/well) or confluent (e.g., 2,000 cells/well) concentrations. Cultures were incubated for 18 h under culture conditions described earlier. Serial 1:2 dilutions of antiserum were added to the wells, three replicates per dilution, in volumes of 5 µl per well. Sera were incubated with target cells for 30 min at 37°C, after which 5 µl of rabbit serum (diluted 1:4) was added to the wells. After incubation at 37°C in 5% CO_2-95% air for another 30 min, the medium was aspirated and the plates were gently washed five times (2 min per wash) in NM to remove dead cells. Plates were fixed for 5 min in ethanol (70% v/v) followed by 5 min in absolute ethanol:ether (1:1 v/v), aid-dried, examined under phase optics, and counted. The percent cytotoxicity was calculated as above. Controls were identical to those used to assay cell suspensions.

The cytolytic effect of antisera on cell monolayers was also assayed by ^{51}Cr release. Cell cultures grown as monolayers for 48 h in Falcon 3040 Microtest plates were washed once with fresh NM. Cells then were incubated in 100 µCi of $Na_2{}^{51}CrO_4$ per millileter of NM for 1 h at 37°C. Labeled cells were washed five times in NM plus 10% heat-inactivated FCS. To each well, 50 µl of 1:2 serially diluted antiserum was added and the plates incubated 30 min at 37°C. A second 30 min incubation follwed the addition of 50 µl of rabbit serum (diluted 1:4). After incubation, 50 µl of the incubation solution was removed and the

radioactivity counted. Total releasable radioactivity was determined by freeze-thawing samples containing cells and medium. Controls were identical to those used for cell suspensions. The percent cytotoxicity was calculated as described above.

d) Immunofluorescence of cultured cells. Specificity of the anti-fibroblast antibodies was tested by indirect immunofluorescence on cultured cells. Preparations of gamma globulin from immune and control sera were obtained by precipitation with 18% Na_2SO_4 followed by column chromatography on diethylaminoethyl (DEAE)-Sephadex (A-50; Pharmacia, Uppsala, Sweden) in 0.0175 M phosphate buffer, pH 6.4.

Corneal, heart, and skin fibroblast-like cell cultures prepared as described above were gently washed three times in PBS. Cells were fixed by flooding culture dishes with 70% ethanol (5 min, 20°C), then with ethanol:ether (1:1; 5 min, 20°C), followed by air-drying 30 min to 2 h (von der Mark and von der Mark, 1977). Designated areas of the dishes were covered with 10 µl of antibody and the plates incubated for 30 min at room temperature in humid chambers. The lowest concentration of anti-fibroblast antibody giving rise to specific fluorescence with homologous antigens was applied routinely to cell cultures. After incubation with anti-fibroblast antibody, culture dishes were rinsed three times with PBS and air-dried for 5-10 min. The designated areas then were covered with 10 µl of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (FITC-GAR; Miles Laboratories, Inc.) diluted 1:16

in PBS. FITC-GAR stained dishes were incubated in dark, humid chambers for 30 min at 20°C. After FITC-GAR staining, dishes were washed three times in PBS. The stained areas of each dish were covered immediately with a drop of freshly prepared glycerol:PBS (9:1, pH 7.2) and a cover glass. Preparations were examined immediately or stored in the dark for 24-48 h at 4°C.

Specimens were observed and photographed with a Zeiss Universal Photomicroscope II (Carl Zeiss, Inc., New York, NY) equipped for fluorescent microscopy. Photomicrographs were taken with Kodak Tri-X film (ASA 400; Eastman Kodak, Rochester, NY).

RESULTS

Absorption of Anti-Fibroblast Antisera

Populations of fibroblast-like cells isolated from the cornea, heart, and skin of 14 day embryonic chicks were grown *in vitro* as primary cultures and used as antigens for immunization and for testing antibody specificity. The populations used were previously shown to yield fibroblast-like cells virtually free of detectable nonfibroblastic cell types and yet distinct from one another as judged by cell morphology, social behavior at saturation, and patterns of glycosaminoglycan biosynthesis (Conrad *et al.*, 1977a,b). Antisera were produced by injecting these populations of fibroblast-like cells into rabbits. Each such unabsorbed antiserum reacted not only with its homologous fibroblast-type, as judged by immunodiffusion, agglutination, and cytotoxicity, but also with the other two heterologous fibroblastic populations. To reduce such heterologous reactivity, each antiserum was subjected to a three-step series of absorptions.

Antisera were absorbed first with homogenates of 14 day chick embryos from which the homologous tissue had been removed. The resulting (1°) antisera showed increased specificity for homologous fibroblast type, but reactivity with the heterologous fibroblast types was still present. These cross-reacting antibodies were removed by first absorbing 1° antisera with an homogenate of one heterologous fibroblast type (thereby yielding 2° antisera). After this absorption, quantitative

precipitation and cytotoxicity tests showed no remaining activity against the heterologous fibroblast type used for this absorption but such tests still detected activity against the remaining heterologous fibroblast type (Figures 2 and 3). The cross-reacting antibodies remaining in the 2° absorbed antisera were removed by absorption with the remaining heterologous cell homogenate. Thus, for example, 2° anti-cornea fibroblast antiserum still reacted with skin fibroblasts after being absorbed with heart fibroblast homogenates but when this 2° anti-cornea antiserum was then absorbed with homogenates of skin fibroblasts the resultant 3° antiserum reacted with cornea fibroblasts, but not with heart or skin fibroblasts. Similar results were found for the other two antisera tested.

The specificity of these antisera against fibroblast-like cells from cornea, heart, and skin was tested by means of double immunodiffusion, immune agglutination, cytotoxicity, and indirect immunofluorescence. The results described below suggest that the fibroblast-like cells present in each tissue are antigenically distinct from one another and can express these specific antigens even when they are grown *in vitro*, in the virtual absence of the non-fibroblastic cells with which each fibroblast population normally is associated *in vivo*.

Immunodiffusion

Antisera prepared against cornea, heart, and skin fibroblast-like cells were tested for cross-reacting antibodies by Ouchterlony double immunodiffusion (Figure 4). Unabsorbed

Figure 2. Quantitative precipitin tests for the absorbance of 1° absorbed anti-fibroblast antisera were conducted as described in Materials and Methods. LEFT: 1° anti-cornea antiserum was first absorbed with homogenates of skin fibroblast-like cells (○—○), incubated, and the supernatant isolated as described in the text. The resulting 2° absorbed anti-cornea antiserum was then absorbed with homogenates of heart fibroblast-like cells (●—●). (●--●), 1°NRS absorbed with heart homogenate; (○--○), 1° NRS absorbed with skin homogenate. MIDDLE: 1° anti-heart antiserum was first absorbed with homogenates of skin fibroblast-like cells (△—△), incubated and the supernatant isolated. The resulting 2° anti-heart antiserum was then absorbed with homogenates of cornea fibroblast-like cells (▲—▲). (▲--▲), 1° NRS absorbed with cornea homogenate; (△--△), 1° NRS absorbed with skin homogenate. RIGHT: 1° anti-skin antiserum was first absorbed with homogenates of cornea fibroblast-like cells (□—□), incubated, and the resulting 2° anti-skin antiserum then combined with homogenates of heart fibroblast-like cells (■—■). (□--□), 1° NRS absorbed with cornea homogenate; (■--■), 1° NRS absorbed with heart homogenate.

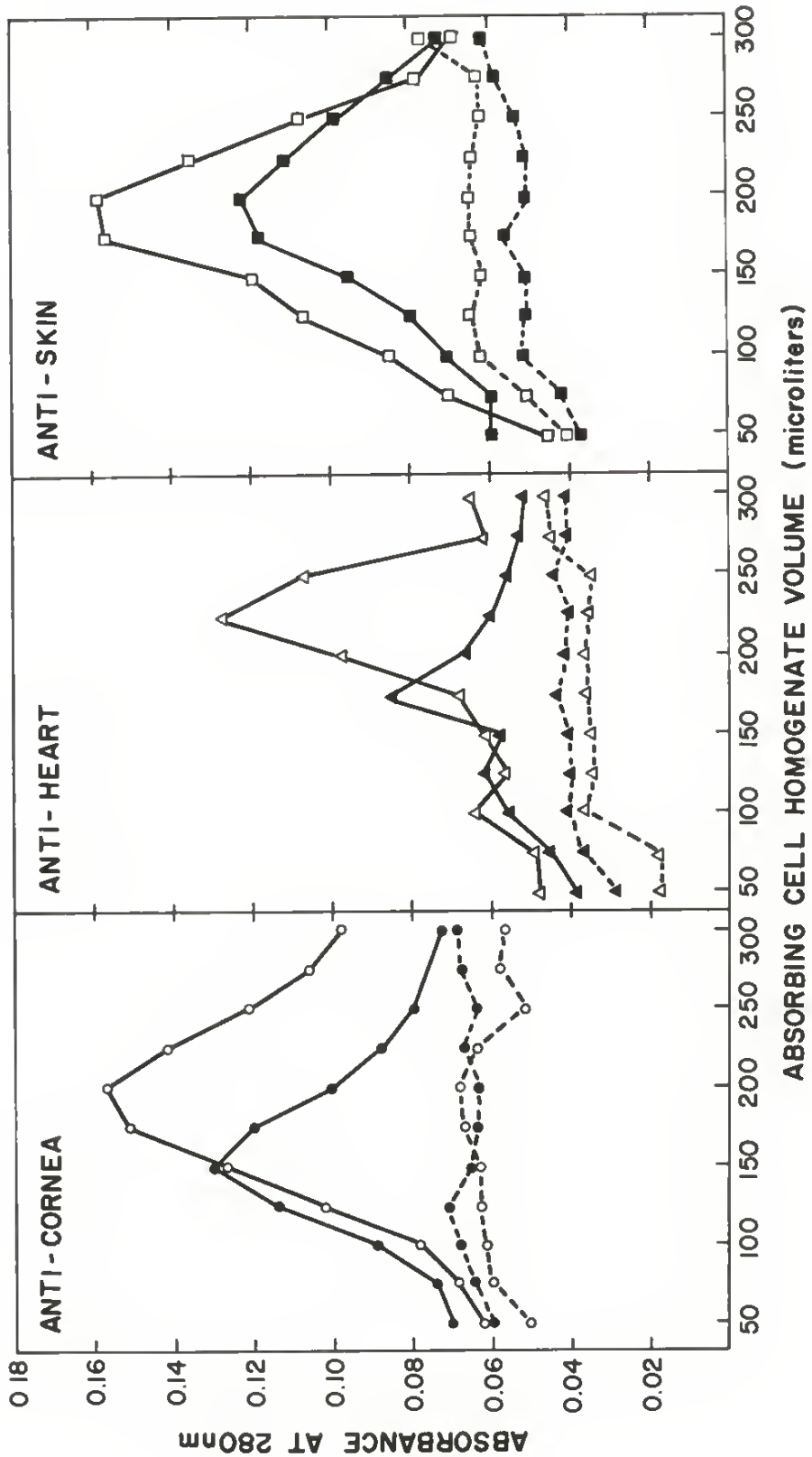

ANTI-CORNEA
ANTI-HEART
ANTI-SKIN
ABSORBANCE AT 280nm
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
50
100
150
200
250
300
ABSORBING CELL HOMOGENATE VOLUME (microliters)

Figure 3. Analysis of secondary (2°) absorptions of 1° absorbed anti-fibroblast antisera. Primary (1°) absorbed anti-fibroblast antisera were absorbed against one of the two heterologous fibroblast-like cell homogenates and then tested for cytotoxicity against the remaining heterologous fibroblast cell type in suspension. Absorptions, suspensions, and ^{51}Cr release procedures were as described in the text. TOP: 1° absorbed anti-cornea antiserum absorbed with: heart fibroblast-like cells (▲—▲), and tested against skin fibroblasts; skin fibroblast-like cells (■—■), and tested against heart fibroblasts; heart fibroblast-like cells (△—△), and tested against heart fibroblasts; skin fibroblast-like cells (□—□), and tested against skin fibroblasts. MIDDLE: 1° absorbed anti-heart antiserum absorbed with: cornea fibroblast-like cells (●—●), and tested against skin fibroblasts; skin fibroblast-like cells (■—■), and tested against cornea fibroblasts; cornea fibroblasts (○—○), and tested against cornea cells; skin fibroblasts (□—□), and tested against skin cells. BOTTOM: 1° absorbed anti-skin antiserum absorbed with: cornea fibroblast-like cells (●—●), and tested against heart cells; heart fibroblast-like cells (▲—▲), and tested against cornea cells; cornea fibroblast-like cells (○—○), and tested against cornea cells; heart fibroblast-like cells (△—△), and tested against heart fibroblasts.

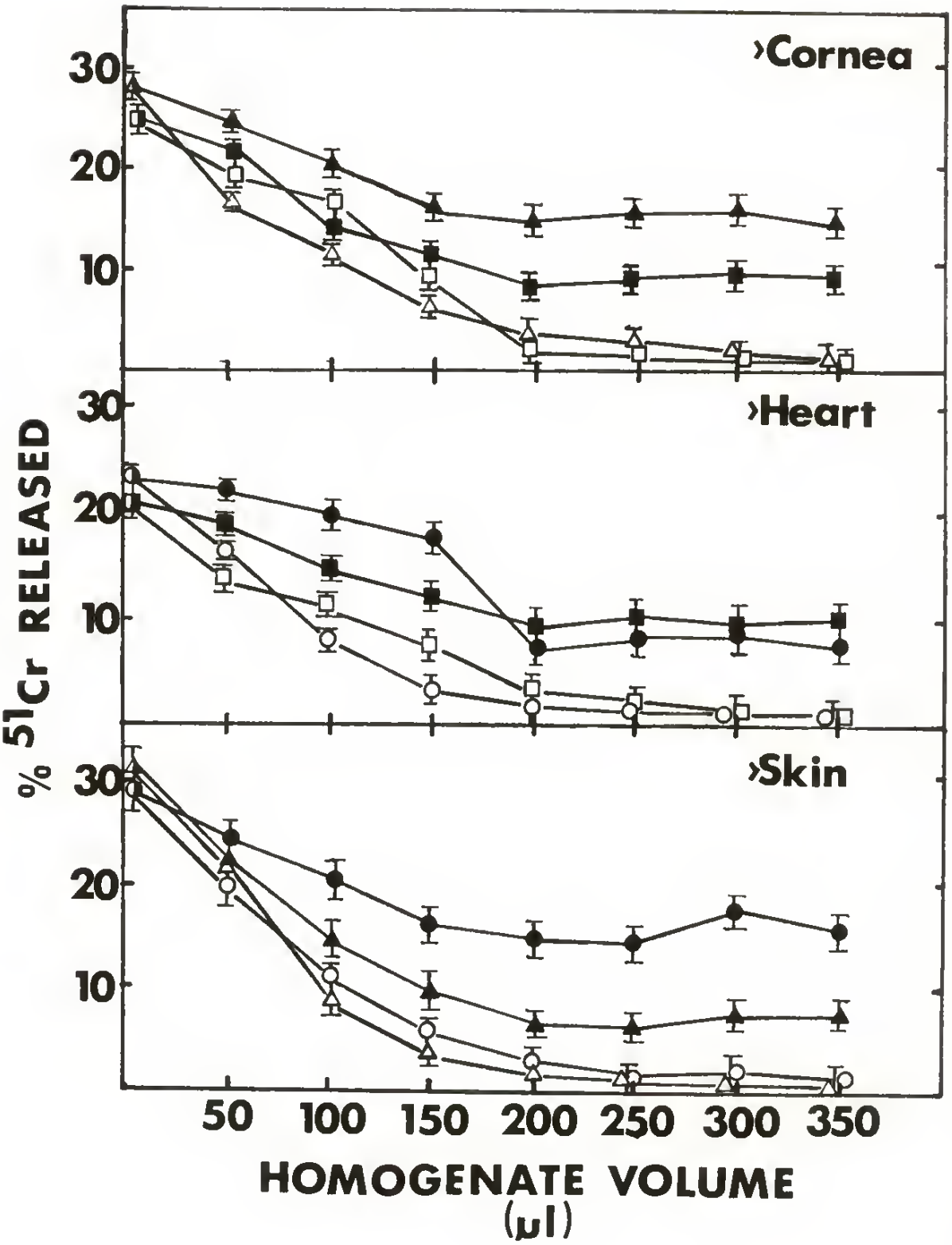
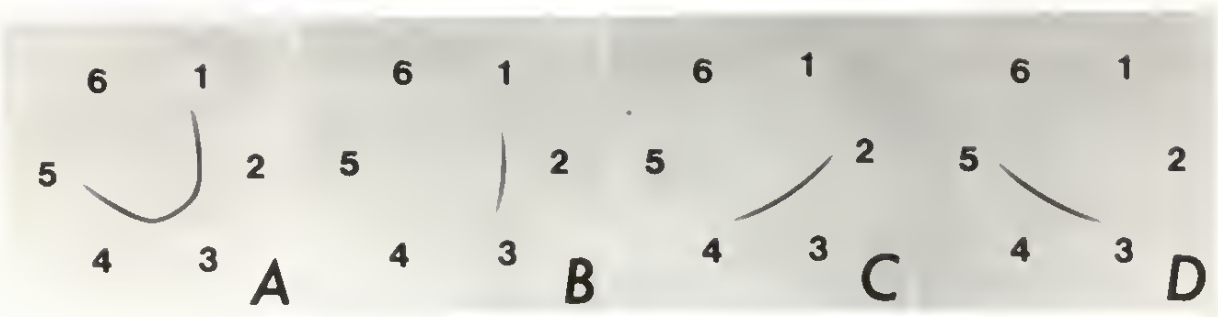

›Cornea
›Heart
›Skin
30
20
10
% 51Cr RELEASED
50
100
150
200
250
300
350
HOMOGENATE VOLUME
(µl)

Figure 4. Double immunodiffusion analysis of unabsorbed and 3° absorbed anti-fibroblast antisera versus homogenates of fibroblast-like cells grown *in vitro*. Antisera and homogenates were prepared as described in Materials and Methods. (A) Unabsorbed anti-cornea fibroblast antiserum in the center well. Peripheral wells contain normal rabbit serum (1), cornea fibroblast-like cell homogenate (2), heart fibroblast-like cell homogenate (3), skin fibroblast-like cell homogenate (4), fetal calf serum (same lot as used for culturing cells) (5), and chick serum (6). Results in 1A are identical to those obtained with unabsorbed anti-heart and anti-skin fibroblast antisera. (B) 3° anti-cornea fibroblast antiserum in the center well. Peripheral wells 1-5 are identical to those in A. Well 6 contains cornea fibroblast-like cell homogenate absorbed with 3° anti-cornea fibroblast antiserum. (c) 3° anti-heart fibroblast antiserum in the center well. Wells 1-5 are identical to A; well 6 contains heart fibroblast-like cell homogenate absorbed with 3° anti-heart fibroblast antiserum. (D) 3° anti-skin fibroblast antiserum in the center well. Wells 1-5 are as in A; well 6 contains skin fibroblast cell homogenate absorbed with 3° anti-skin fibroblast antiserum.


6 1
5 2
4 3
A
6 1
5 2
4 3
B
6 1
5 2
4 3
C
6 1
5 2
4 3
D

antisera formed precipitin lines with both homologous and heterologous cell homogenates (Figure 4A). Thorough absorption of such antisera with embryo homogenates minus the homologous tissue, followed by absorption with homogenates of both types of heterologous fibroblast-like cells removed cross-reacting antibodies. Thus, 3° anti-cornea fibroblast antiserum formed a single precipitin band versus homogenates of cornea fibroblast-like cells, but formed no detectable lines against homogenates of heart and skin fibroblast-like cells (Figure 4B). Similarly, 3° anti-heart antiserum gave a single band with its homologous cell homogenate (Figure 4C), as did 3° anti-skin antiserum (Figure 4D). Precipitin bands were not formed when 3° anti-fibroblast antisera were incubated with pre-immune rabbit serum, PBS, FCS, CS, or trypsin. Precipitin bands formed between 3° anti-fibroblast antisera and their homologous antigens were absent if the specific 3° antisera were first absorbed with homogenates of the homologous fibroblast type.

Immune Agglutination

The unabsorbed and absorbed antisera were tested for their ability to agglutinate suspensions of cornea, heart, and skin fibroblast-like cells. The standard assay used cells which were released from culture dishes by treatment with trypsin-EDTA, washed and suspended in NM, and allowed to recover for 1 h at 37°C in a gyratory shaker bath. Agglutinations were performed at 4°C. Unabsorbed antisera agglutinated homologous, as well as heterologous, fibroblast-like cells (Table I). However, after

TABLE I

AGGLUTINATION TITERS OF
UNABSORBED AND ABSORBED[a] RABBIT ANTISERA
AGAINST FIBROBLAST-LIKE CELLS FROM
EMBRYONIC CHICK CORNEA, HEART, AND SKIN

Source of Fibroblast-like Cells:	Agglutination Titers[b]		
	Cornea	Heart	Skin
Antisera Preparations			
Unabsorbed Anti-Cornea	1024	16	64
3° Anti-Cornea	1024	2	4
Pre-Absorbed Anti-Cornea	0	0	0
Unabsorbed Anti-Heart	64	256	32
3° Anti-Heart	0	256	1
Pre-Absorbed Anti-Heart	0	0	0
Unabsorbed Anti-Skin	64	32	1024
3° Anti-Skin	1	2	1024
Pre-Absorbed Anti-Skin	0	0	0
Normal Rabbit Serum	0	0	0
Fetal Calf Serum[c]	0	0	0
Chick Serum	0	0	0

[a]Unabsorbed antisera represents pooled antisera collected from rabbits immunized with one fibroblast cell type. Absorbed antisera represents 3° antisera, absorbed as described in the Materials and Methods. Pre-absorbed antisera represents 3° anti-fibroblast antiserum additionally absorbed with homologous antigen.

[b]Agglutination titer was defined as the reciprocal of the greatest antisera dilution tested which still resulted in antibody-dependent agglutination of the stated fibroblast type.

[c]FCS used was from the same lot used in the nutrient medium for cell culture.

absorptions described above, each 3° antiserum agglutinated only its homologous fibroblast type (Figure 5, A,B,C). These absorptions removed all detectable agglutinating antibodies to the heterologous cell suspensions (Figure 5, D,E,F). Cell suspensions alone, or cell suspensions in the presence of NRS, PBS, CS, or 3° anti-fibroblast antisera absorbed with homologous fibroblast type showed no ability to agglutinate cells (Table I).

In preliminary experiments, several techniques were investigated to obtain single-cell suspensions of fibroblast-like cells with minimal spontaneous aggregation (Table II). Fibroblast-like cells prepared as described in Materials and Methods consistently were in suspensions consisting almost entirely of single cells (occasional spontaneous aggregates usually consisted of only two to four cells per aggregate). Viability for the three cell-types was greater than 95% as judged by trypan blue exclusion. Cell suspensions tested with various antisera at various temperatures showed only nominal spontaneous aggregation at 4°C, consistent with previous work (Goldschneider and Moscona, 1972; Steinberg et al., 1973; McClay and Baker, 1975; Maslow, 1976).

Tests using double immunodiffusion failed to demonstrate precipitating antibodies to trypsin in the anti-fibroblast antisera. However, assays performed with cells which had not been allowed to "recover" from trypsinization for 1 h in the presence of NM alone or NM plus soybean trypsin inhibitor, showed reduced ability to agglutinate in the presence of antiserum. This may indicate that some cell surface antigenic

Figure 5. Immune agglutination of suspended cornea, heart, and skin fibroblast-like cells. Cells were released from culture dishes by treatment with trypsin-EDTA, washed in NM, suspended in NM, and allowed to recover 1 h at 37°C. Agglutinations were performed as described in the text. (A) Cornea fibroblast-like cells plus 3° anti-cornea antiserum diluted 1:32. (B) Heart fibroblast-like cells plus 3° anti-heart antiserum diluted 1:16. (C) Skin fibroblast-like cells plus 3° anti-skin antiserum diluted 1:32. (D) Cornea fibroblast-like cells plus 3° anti-skin antiserum diluted 1:32. (E) Heart fibroblast-like cells plus 3° anti-skin antiserum diluted 1:16. (F) Skin fibroblast-like cells plus 3° anti-cornea antiserum diluted 1:32. Scale line = 12 μm.

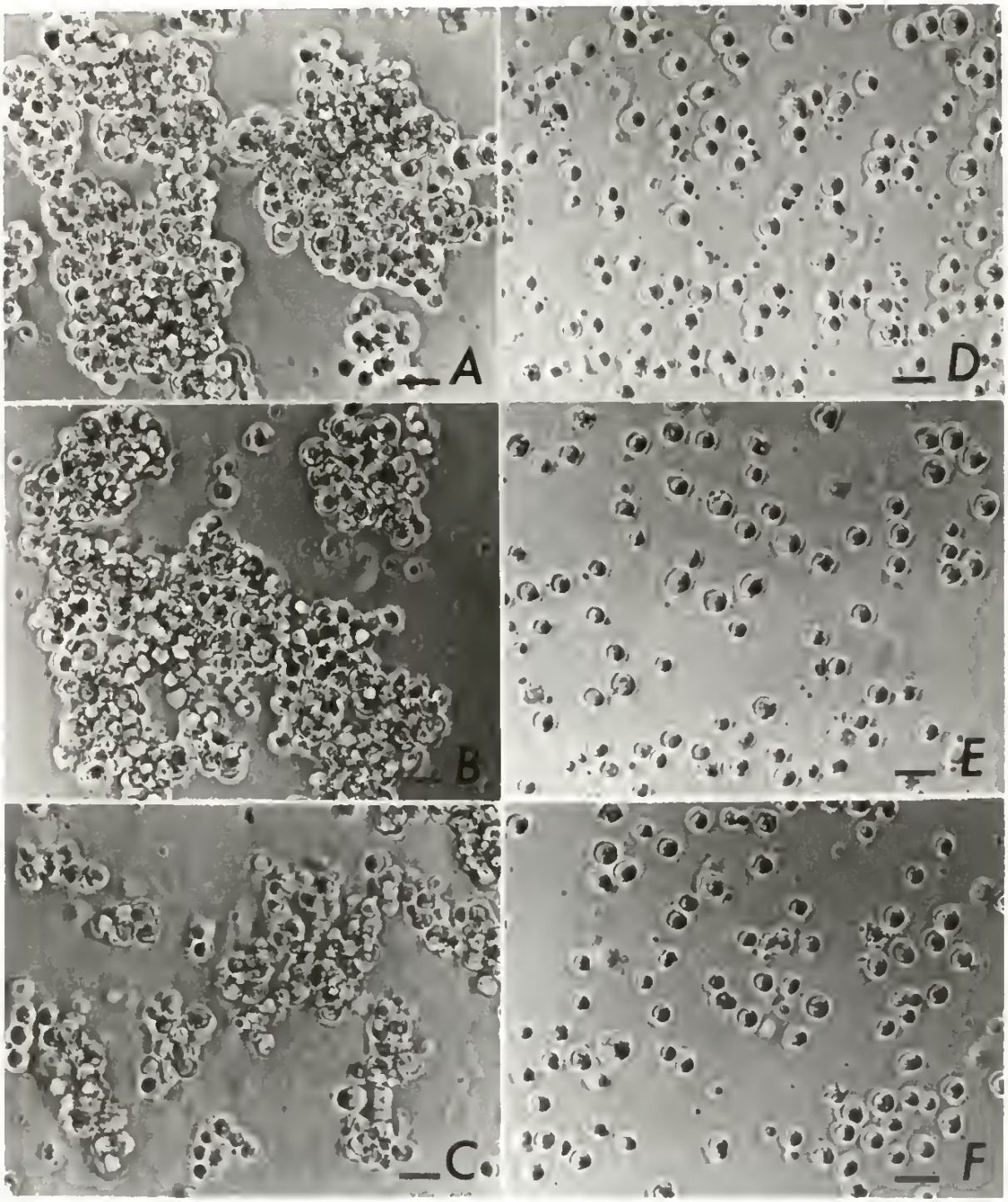

A
D
B
E
C
F

TABLE II

PREPARATION OF CELL SUSPENSIONS FOR IMMUNE AGGLUTINATION

Dissociation Technique[a]	Recovery or Fixation Conditions[b]	Medium	% Cell Viability[c] Cornea	Heart	Skin	Spontaneous Aggregation[d]	Immune Agglutination[e]
Trypsin-EDTA	37°C, 1h	NM	95.8	97.7	96.2	-	(e)
"	37°C, 1h	NM+ Soybean Trypsin Inhibitor	95.2	96.9	95.5	-	NDE
"	37°C, 1h	CMF+ EDTA	79.3	82.6	80.4	±	-
EDTA	37°C, 1h	NM	92.6	94.5	94.1	±	-
"	37°C, 1h	CMF	76.9	81.1	79.7	+	-
"	37°C, 1h	CMF+ EDTA	73.4	81.0	80.6	-	-
Trypsin-EDTA	20°C, 30min	3.5% Glutar-aldehyde	0	0	0	+ + +	NDE
"	20°C, 30min	3.5% Formal-dehyde	0	0	0	+ +	NDE
"	20°C, 30min	70% Ethanol	0	0	0	±	-

[a] Dissociation techniques described in Materials and Methods. Trypsin=0.5%; EDTA=0.1%.

[b] Temperature and times during recovery or fixation. Agglutinations done at 4°C. (See text)

[c] Cell viability determined after "recovery" by percentage of cells excluding trypan blue.

[d] Degree of spontaneous, nonimmune aggregation determined qualitatively. (-) single-cell suspensions, occasional clusters of 2-4 cells; (±) single cells with occasional clusters of 4-10 cells; (+++) substantial aggregation, only occasional clusters less than 4 cells.

[e] Effect of preparation on immune agglutination determined qualitatively; comparisons to standard preparation method (see text). (-) decreased responsiveness to specific antisera; (NDE) No detectable effect on immune agglutination.

sites involved in agglutination were susceptible to proteases, or that residual trypsin in the suspensions of freshly trypsinized cells tended to degrade the antisera (Cohen and Milstein, 1967), for either activity could reduce agglutination. The present results are in aggreement with those of Goldschneider and Moscona (1972) and McClay et al., (1977), who found that small amounts of trypsin added to anti-retina antiserum reduced the ability to agglutinate retina cells. These investigators found that the addition of equimolar amounts of trypsin inhibitor to the reaction mixture prevented the loss of agglutination activity. Therefore, a 1 h "recovery period" was adopted as a routine step in the preparation of cell suspensions to allow repair of trypsin-damaged membrane antigenic sites (McClay and Baker, 1975; Maslow, 1976) and dilution or inactivation of residual trypsin. Routinely, during "recovery," cells were incubated at 37°C in NM to allow biosynthesis, and kept in suspensions at 120 rpm, a gyratory speed sufficiently high to prevent cell aggregation. Recovery of cells in NM with or without soybean trypsin inhibitor resulted in lower spontaneous aggregation, high viability, and high specific immune agglutination in response to homologous antisera when cells were kept at 4°C following "recovery" and during the agglutination assay (Table II).

In some preliminary experiments, trypsin-EDTA was compared with EDTA alone for ability to produce single cell suspensions with minimum spontaneous aggregation and maximum responsiveness to homologous antiserum. Cells suspended with trypsin-EDTA were

more agglutinable than cells prepared with EDTA alone, when reacted with equal concentrations of antiserum. Proteolytic enzymes have been shown to unveil or activate cell surface antigens normally not accessible to antibodies (Burger, 1969; Moscona, 1971; Kleinschuster and Moscona, 1972; Friedlander and Fishman, 1977).

In other early experiments the effect of fixation of cells prior to agglutination also was examined. Cells were fixed in glutaraldehyde, formaldehyde, or ethanol for 30 min at 20°C and dialyzed against CMF before use in agglutination assays. None of these fixation methods offered any advantage over the methods using unfixed cells. The use of 3.5% glutaraldehyde increased spontaneous aggregation in the absence of homologous antiserum. Such aggregates obscured an accurate evaluation of the effect of glutaraldehyde on immune agglutination (Table II). Fixation of cells with 3.5% formaldehyde resulted in a similar increase in spontaneous aggregation, again resulting in no determined effect on immune agglutination. Fixation with 70% ethanol gave suspensions of both single cells and small aggregates (two to eight cells) but tended to reduce the effectiveness of antibody-dependent agglutination.

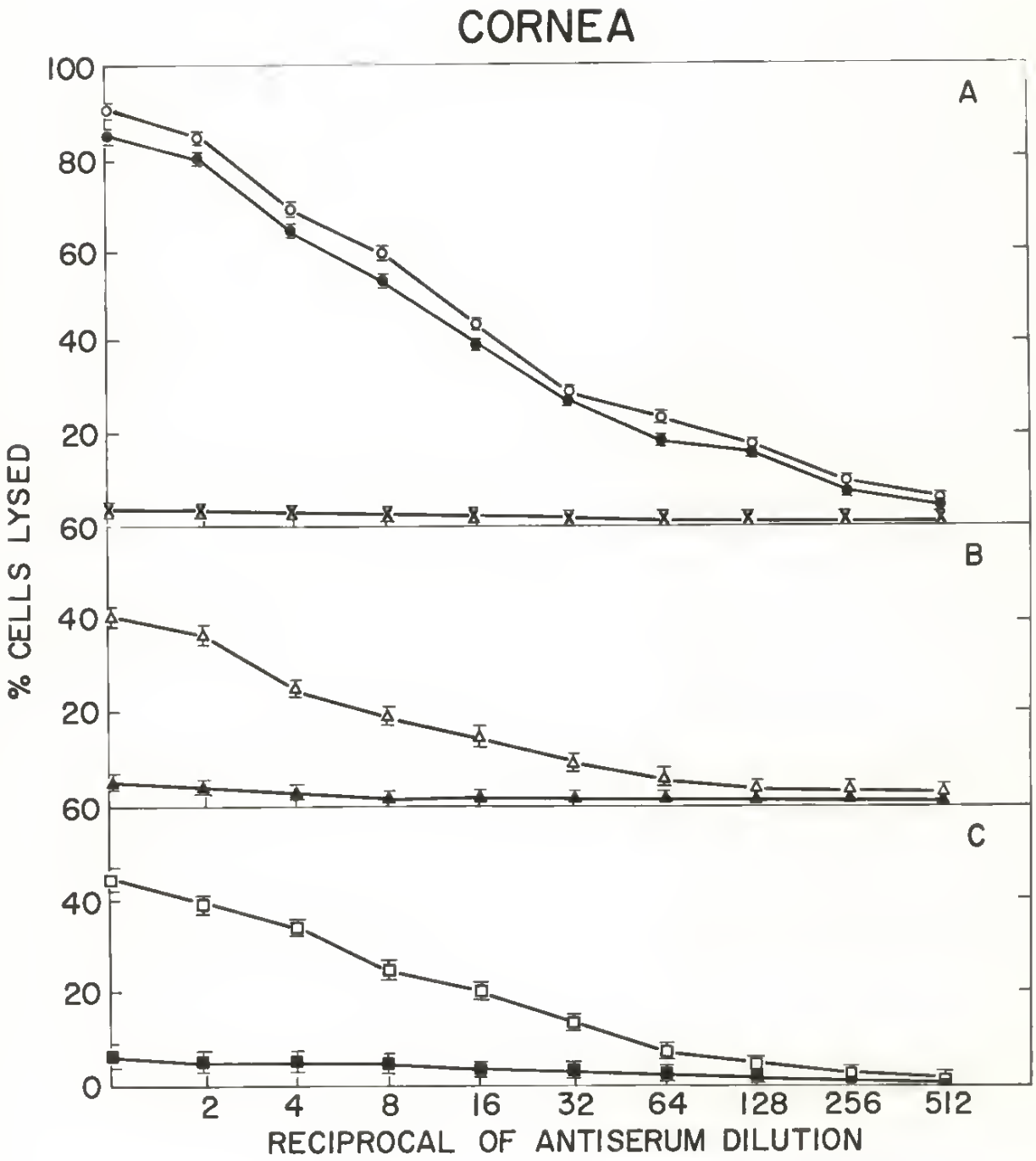
Cytotoxicity of Anti-Fibroblast Antisera

The ability of unabsorbed and absorbed anti-fibroblast antisera to kill fibroblast-like cells was used to further characterize antisera specificity. Microcytotoxicity and ^{51}Cr release assays were used to measure antiserum cytolytic effects on target cells in both suspensions and monolayer cultures.

Cells in suspension. Fibroblast-like cells, suspended and assayed as described in Materials and Methods, were greater than 95% viable prior to immune cytolysis. For cytotoxic quantitation, at each antiserum dilution a minimum of 1,000 cells were counted and the proportion absorbing the vital dye, trypan blue, were considered dead. The cytolytic effects of unabsorbed and 3° anti-fibroblast antisera on suspensions of fibroblast-like cells from cornea, heart, and skin are shown in Figures 6-8. The data indicate that each 3° antiserum kills only its homologous fibroblast type, and that these specific cytotoxic antibodies can be removed if each 3° antisera is absorbed against its homologous fibroblast type. Unabsorbed antisera not only kill the homologous fibroblast population but, to a lesser extent (about 40% with undiluted antisera), also kill the two heterologous populations. The three-step absorption procedure used to prepare the 3° antisera resulted in a minor decrease (usually less than 5% for undiluted antisera) in the cytolytic titer of each antiserum for its homologous fibroblast type.

In order to test these conclusions, cytotoxicity was measured by studying the ability of the antisera to cause release of ^{51}Cr from suspensions of fibroblast-like cells (Figures 9-11). The data confirm the results of the trypan blue experiments. The 3° antisera caused ^{51}Cr release only from their homologous fibroblast population and this ability was lost if the 3° antisera were absorbed against the homologous fibroblast type. Unabsorbed antisera caused release of ^{51}Cr from heterologous as

Figure 6. Cytolytic specificity of anti-fibroblast antisera on suspensions of cornea fibroblast-like cells prepared as described in Materials and Methods. Cells were incubated in the presence of antibody and complement and the number of dead cells were quantitated by absorption of trypan blue as described in the text. The percentage of cells lysed was calculated as a ratio of [(lysed cells - control)/(total cells - control)] x 100. Controls consisted of cells suspended in NRS and complement. (A) ●—● , 3° anti-cornea antiserum; O—O , unabsorbed anti-cornea antiserum; X—X, 3° anti-cornea pre-absorbed with cornea fibroblast-like cells. (B) ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum. (C) ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum. Graphs represent the mean data of three experiments ± S.E.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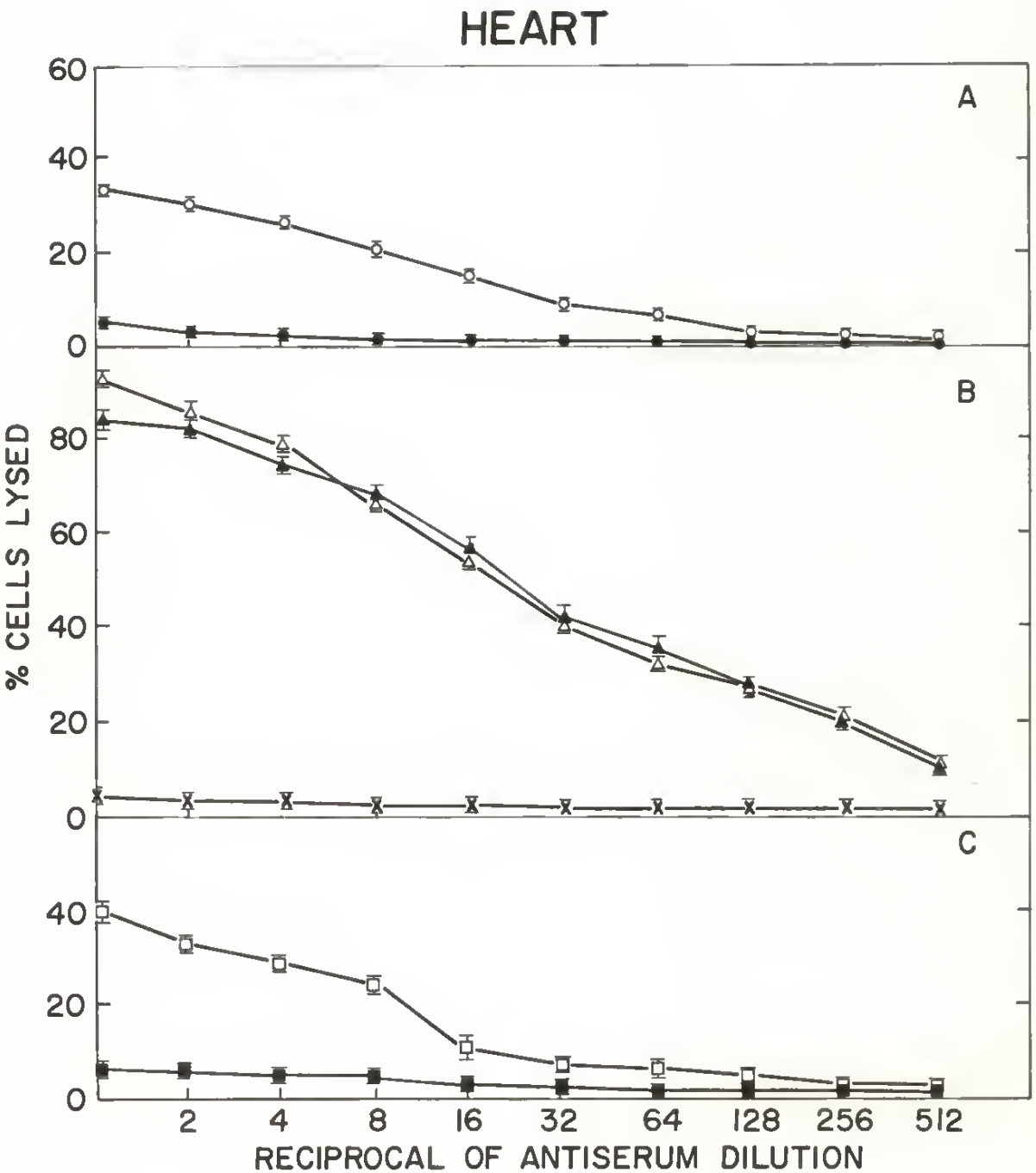
RECIPROCAL OF ANTISERUM DILUTION

Figure 7. Cytolytic specificity of anti-fibroblast antisera on suspensions of heart fibroblast-like cells prepared as described in Materials and Methods. (A) ●—● , 3° anti-cornea antiserum; O—O , unabsorbed anti-cornea antiserum. (B) ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum; X—X, 3° anti-heart pre-absorbed with heart. (C) ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum. Data represents the mean values from three experiments ± S.E.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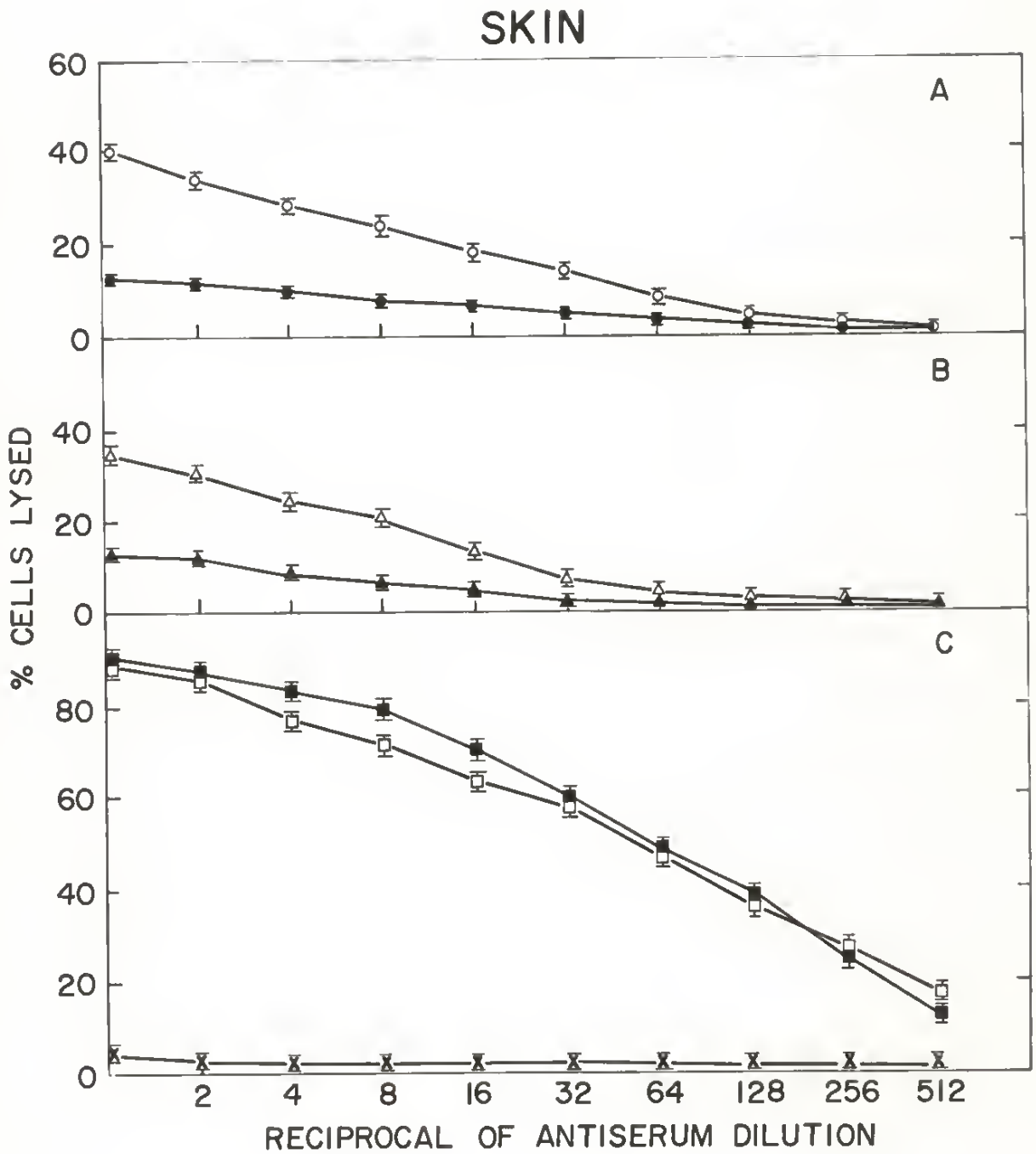
RECIPROCAL OF ANTISERUM DILUTION

Figure 8. Cytolytic specificity of anti-fibroblast antisera on suspensions of skin fibroblast-like cells prepared as described in Materials and Methods. (A) ●—● , 3° anti-cornea antiserum; O—O , unabsorbed anti-cornea antiserum. (B) ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum. (C) ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum; X—X, 3° anti-skin pre-absorbed with skin. Data represent the mean values from three experiments ± S.E.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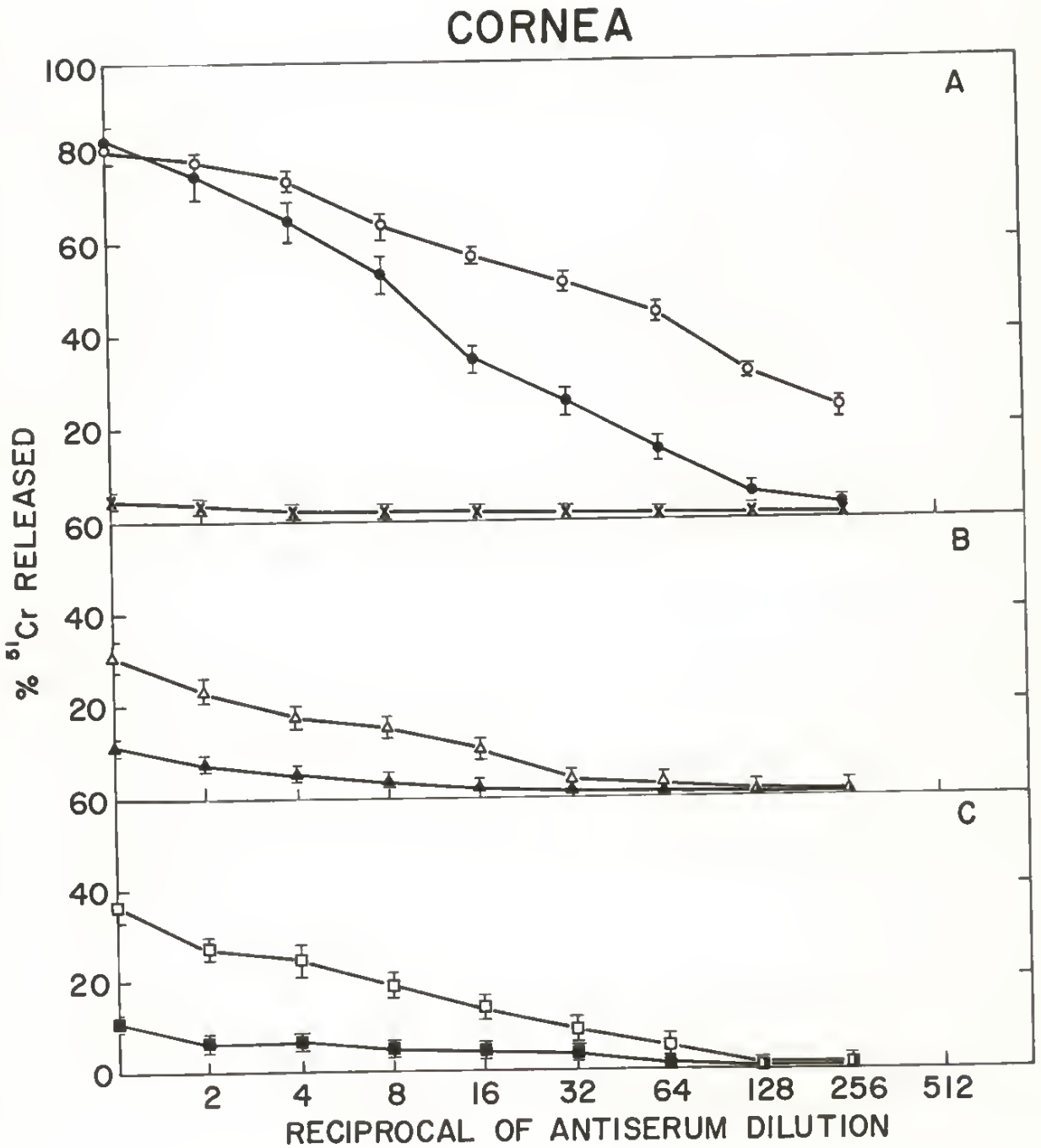
RECIPROCAL OF ANTISERUM DILUTION

Figure 9. Cytolytic specificity of anti-fibroblast antisera on suspensions of cornea fibroblast-like cells prepared as described in Materials and Methods. Cells were labeled by the addition of $Na_2{}^{51}CrO_4$ to a final concentration of 100 µCi/ml of cell suspension (1×10^7 cells/ml) for 30 min at 37°C. Cell suspensions were washed five times in NM, brought to a final concentration of 2×10^6 viable cells/ml, and incubated in the presence of antibody and complement. After the completion of the reaction, cells were centrifuged, the supernatant was collected, and the radioactivity measured in an automatic gamma spectrometer. The percentage of cells lysed was calculated as a ratio of [(experimental ^{51}Cr release) - (spontaneous release)/(maximum release) - (spontaneous release)]. Controls consisted of labeled cells suspended in NRS and complement, (see Materials and Methods). (A) ●—● , 3° anti-cornea antiserum; ○—○ , unabsorbed anti-cornea antiserum; X—X, 3° anti-cornea pre-absorbed with cornea. (B) ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum. (C) ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum. Graphs represent the mean data from three experiments ± S.E.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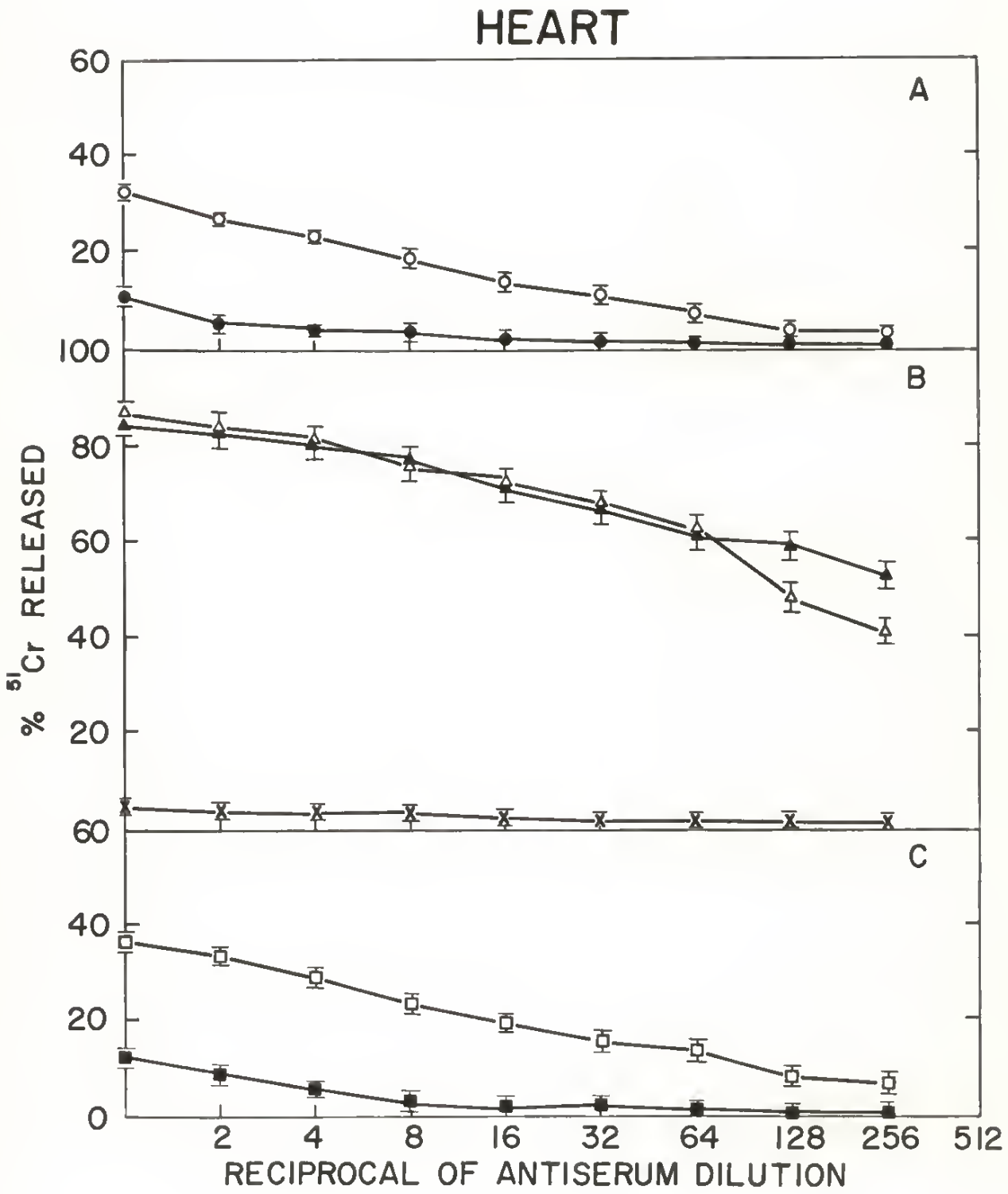
RECIPROCAL OF ANTISERUM DILUTION

Figure 10. Cytolytic specificity of anti-fibroblast antisera on suspensions of heart fibroblast-like cells prepared as described in Materials and Methods. Cytotoxicity measured by ^{51}Cr release as described in Figure 9 and text. (A) ●—● , 3° anti-cornea antiserum; ○—○ , unabsorbed anti-cornea antiserum. (B) ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum; X—X, anti-heart antiserum pre-absorbed with heart. (C) ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum. Data represents the mean values from three experiments ± S.E.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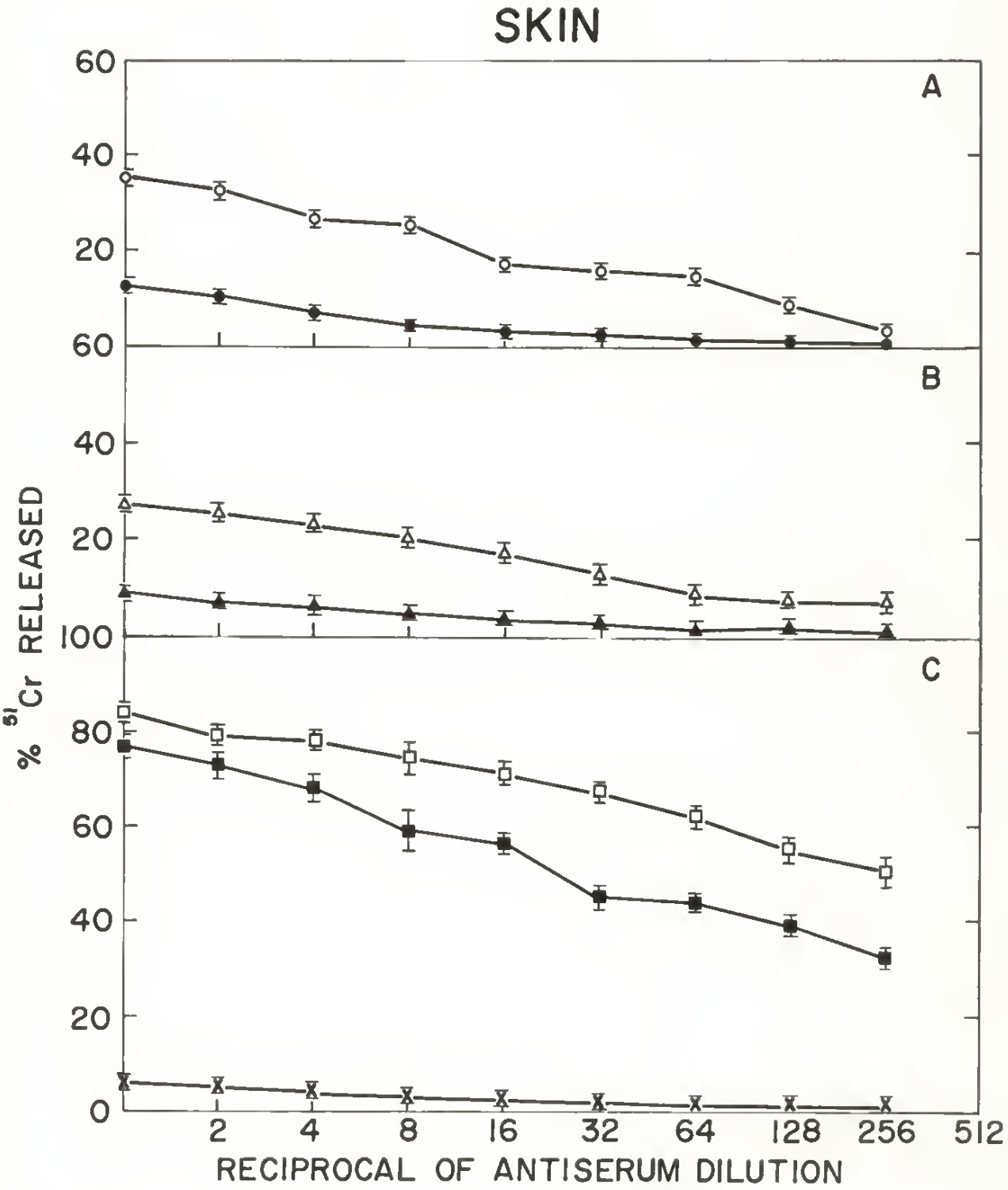
RECIPROCAL OF ANTISERUM DILUTION

Figure 11. Cytolytic specificity of anti-fibroblast antisera on suspensions of skin fibroblast-like cells prepared as described in Materials and Methods. Cytotoxicity measured by ^{51}Cr release. (A) ●—● , 3° anti-cornea antiserum; O—O , unabsorbed anti-cornea antiserum. (B) ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum. (C) ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum; X—X, 3° anti-skin antiserum pre-absorbed with skin. Data represents the mean values from three experiments ± S.E.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RECIPROCAL OF ANTISERUM DILUTION

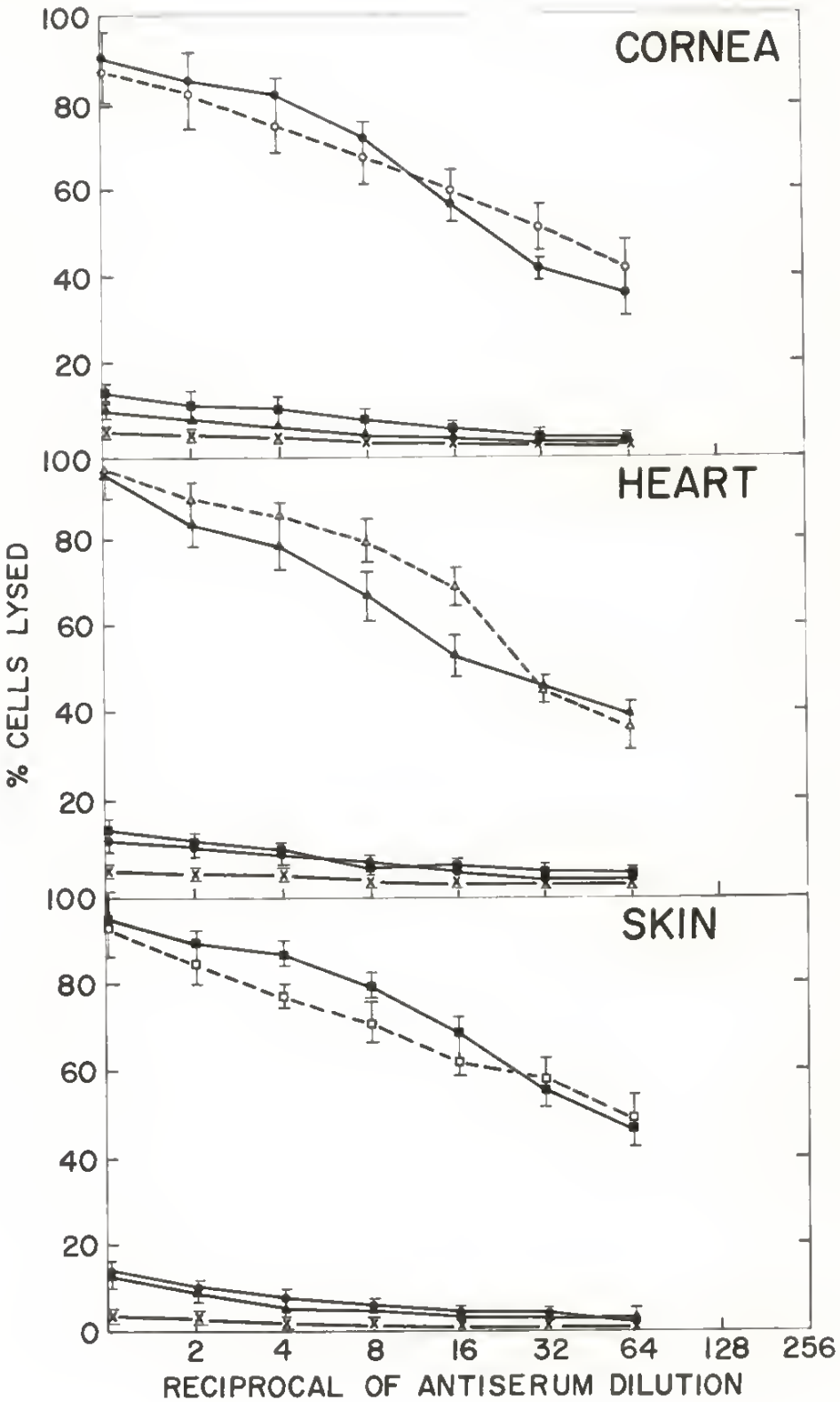
well as from homologous fibroblast populations. The absorption procedure resulted in slight decreases in the antiserum titers for each homologous fibroblast population.

Cells left attached to dishes. In order to determine whether antisera would cause cytolysis of cells that had not been treated with trypsin and EDTA, we performed cytotoxicity tests on cells that were left attached to culture dishes. Assays were performed on subconfluent and confluent cultures and measured by microscopy (Figure 12) or by ^{51}Cr release (Figure 13), as described in Materials and Methods.

Data in Figure 12 show similar results to those of antisera versus cell suspensions assayed with trypan blue. The 3° antisera versus cornea, heart, and skin fibroblast-like cells lyse only their homologous fibroblast type. Specific cytolytic antibodies are removed when the 3° antisera are absorbed against the homologous fibroblast type. The data from this type of assay suggest that the three-step absorption procedure removed antibodies reacting against heterologous fibroblast populations and resulted in a slight decrease in the cytolytic titer of each antiserum for its homologous fibroblast type.

Data in Figure 13 show similar results to those of antisera versus cell suspension assayed by ^{51}Cr release. Unabsorbed antisera were able to lyse homologous fibroblast populations and a lesser, but still marked ability to kill heterologous fibroblast-like cells. Cytotoxic antibody titer against the homologous fibroblast population was reduced slightly during absorptions.

Figure 12. Cytolytic specificity of anti-fibroblast antisera on subconfluent cultures of cornea, heart, and skin fibroblast-like cells. Cytotoxicity quantitated by microscopic counts as described in Materials and Methods. ●—● , 3° anti-cornea antiserum; ○—○ , unabsorbed anti-cornea antiserum; ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart antiserum; ■—■ , 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum. X—X, homologous 3° anti-fibroblast antisera pre-absorbed with homologous fibroblast-like cell homogenate. Mean data from three experiments ± S.E.


CORNEA
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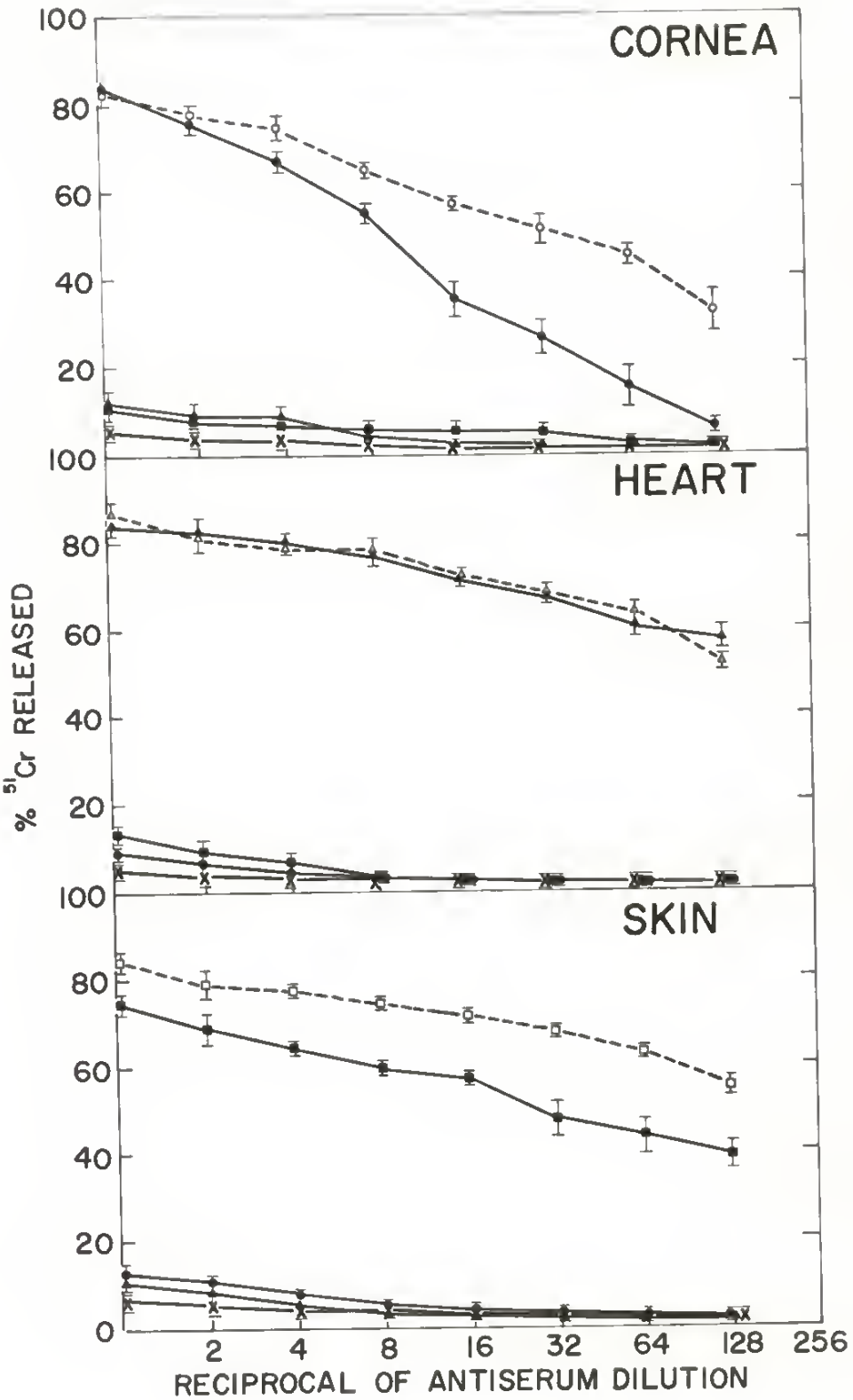
RECIPROCAL OF ANTISERUM DILUTION

Figure 13. Cytolytic specificity of anti-fibroblast antisera on confluent cultures of cornea, heart, and skin fibroblast-like cells and quantitated by release of ^{51}Cr. Assays conducted as described in the text. ●—● , 3° anti-cornea antiserum; O—O , unabsorbed anti-cornea; ▲—▲ , 3° anti-heart antiserum; △—△ , unabsorbed anti-heart; ■—■ 3° anti-skin antiserum; □—□ , unabsorbed anti-skin antiserum. X—X, homologous 3° anti-fibroblast antiserum pre-absorbed with homologous fibroblast-like cell homogenates. Mean data from three experiments ± S.E.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RECIPROCAL OF ANTISERUM DILUTION

An analysis of the data from visual counts and ^{51}Cr release experiments reveals no significant differenfes in the responses of subconfluent and confluent cultures, suggesting that cells in both log and stationary phases of growth are equally susceptible to cytolytic antisera. Moreover, comparison of Figures 12 and 13 indicates that there is a close correlation between the release of ^{51}Cr and the percentage of dead cells estimated by microscopic counts. Comparison of cytolytic assays performed on fibroblast-like cells in suspensions or in situ indicate that prior treatment of cells with trypsin-EDTA does not substantially affect antibody binding so long as "recovery" is allowed. It does not appear therefore, that cell specific antigenic sites are permanently lost or altered by treatment with trypsin-EDTA.

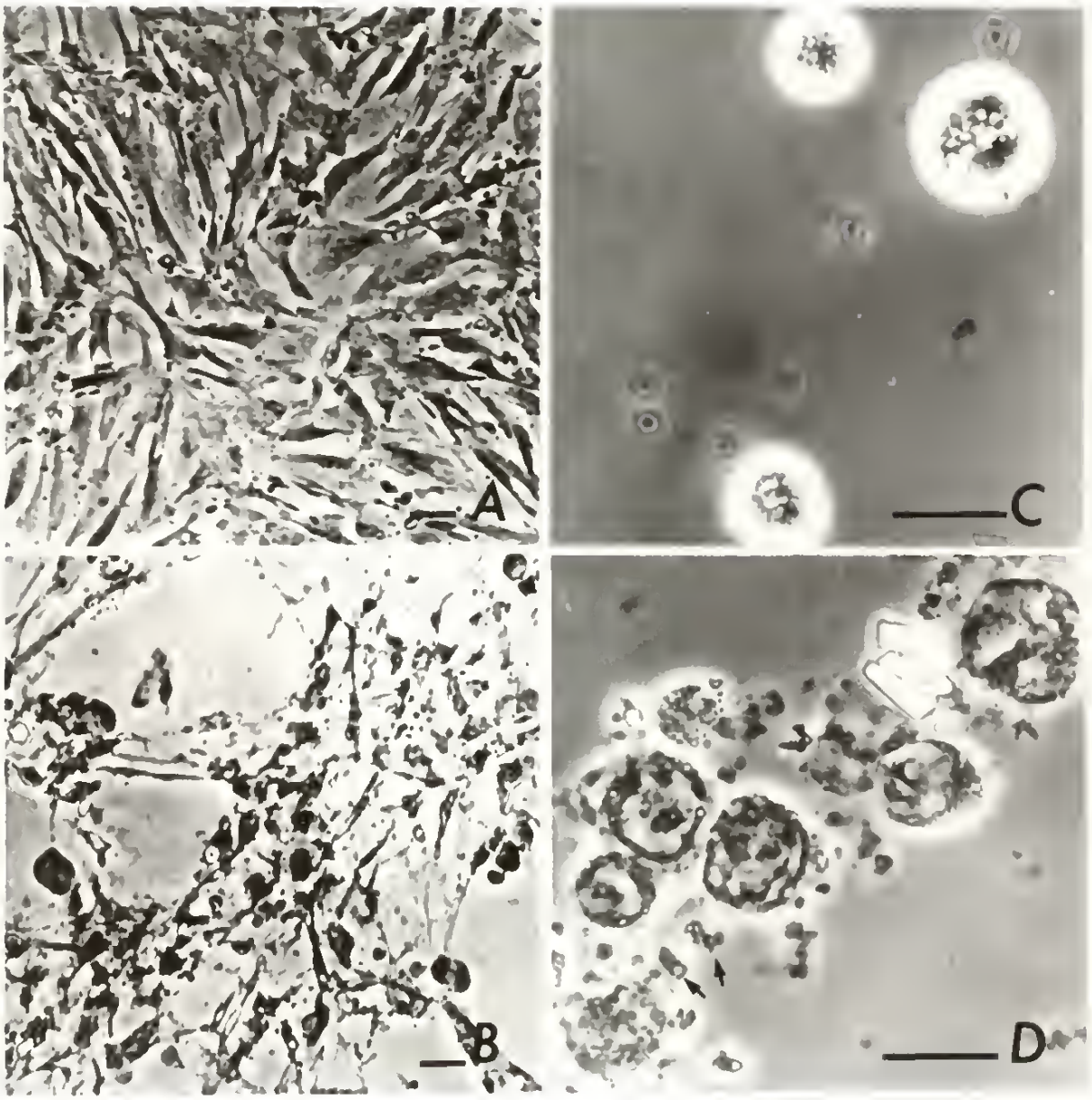
In cytotoxicity assays utilizing cell cultures, cells were counted before and after the cytolytic reaction. A minimum of 200 viable cells were required for a given microtest well to be used in the assay. Antisera and complement were added directly to the microtest chambers containing the cells; cultures were then incubated, washed, and counted as described. During the incubation with antisera, many cells detach from the substrata in an intact state, but later disintegrate. It has been shown previously that detached, but morphologically intact, fibroblasts cannot be recultured after treatment with specific antibody and complement, indicating that they are actually killed without lysis (Lundgren and Möller, 1969). In early experiments, the antiserum/complement solution was

collected after incubation with cells, mixed with trypan blue, and examined for viable detached cells. In all cases, all such floating cells absorbed the dye and were therefore considered dead (Figure 14). In other experiments, the antiserum/complement solution was recovered and subculturing of floating cells was attempted. In no instance did cells adhere to the substrata of culture dishes.

In microcytotoxicity assays, dead cells remaining on the culture dish substrata, after reaction with cytotoxic antibodies and complement, exhibited greatly altered morphology (Figure 14). In the presence of cytotoxic antisera, cells retracted their cell processes, rounded up, lost distinct nuclear morphology, and displayed increased granularity in the cytoplasm. The morphological differences between live and dead cells was always distinct.

Increased complement concentrations or increased incubation periods did not yield a marked increase in the percentage of dead cells. No differences were observed in the level of spontaneous lysis in cultures that did not receive complement, regardless of the incubation period in the presence and absence of antiserum. Treatment of cells (in suspension or in culture) with antiserum alone, complement alone, NRS, or NRS plus complement did not increase the number of dead cells over that in NM alone. Cells incubated in the presence of NRS and complement were routinely used as a control in calculations of cytotoxicity because maximum spontaneous lysis (although small) was shown under these conditions. The controls

Figure 14. Affect of cytotoxic anti-fibroblast antiserum on fibroblast-like cells in monolayer or suspension. (A) Four day cultures of embryonic chick skin fibroblast-like cells in NM, scale line = 20 μm. (B) Four day cultures of skin fibroblast-like cells incubated in the presence of 3° anti-skin fibroblast antiserum and complement as described in the text. Note the loss of cellular processes (filopodia, lamellipodia). Some cells have rounded up and absorbed the vital dye, trypan blue. Cells remaining on the substratum show marked abnormal morphology and cytoplasmic granularity. Scale line = 20 μm. (C) Skin fibroblast-like cells suspended in NM (suspensions prepared as described in Materials and Methods). Optic hallo obscures intact membrane of live cells. Scale line = 10 μm. (D) Skin fibroblast-like cells suspended in NM containing 3° anti-skin fibroblast antiserum and complement. Note reptured cell membranes and cellular debri (arrows). Darkened cellular cytoplasm indicates the absorbance of the vital dye trypan blue. Scale line = 10 μm.


A
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confirmed that the cytotoxic effects observed only arose from the combined effects of complement and reactive antisera.

In preliminary experiments, it was observed that if cells in subconfluent cultures were exposed to NM containing homologous 3° antiserum in the absence of complement, those cells never reached confluency. If the same cells were rinsed and given fresh NM, confluency still was not attained. However, if these same cells were dissociated from the substratum with trypsin-EDTA, resuspended in fresh NM, and placed in secondary culture, cells attached to the substratum and eventually reached confluent monolayers.

In summary, data from assays by immunodiffusion, immune agglutination, and two types of cytotoxicity assays on suspended cells as well as on cells left attached to culture dishes indicate that each 3° antiserum reacts specifically with only its homologous type of fibroblast-like cell. The data further suggest that fibroblast-like cells possess tissue-specific antigens.

Immunofluorescence

Indirect immunofluorescence staining showed that antisera prepared against cultured fibroblast-like cells contained antibodies to homologous and heterologous fibroblast-like cells grown *in vitro* (Table III). Absorption of antisera with heterologous antigens removed all detectable antibodies reacting to heterologous fibroblast populations but left high titers of antibody to homologous fibroblast-like cells (Table III, Figure 15).

TABLE II

IMMUNOFLUORESCENCE TITERS OF UNABSORBED AND ABSORBED RABBIT ANTISERA[a] AGAINST FIBROBLAST-LIKE CELLS FROM EMBRYONIC CHICK CORNEA, HEART, AND SKIN

Antisera Preparation	Immunofluorescence Titers Against Fibroblast-Like Cells Cultured *In Vitro*[b]		
	Cornea	Heart	Skin
Unabsorbed Anti-Cornea	1024	64	256
3° Anti-Cornea	256	1*	4*
Pre-Absorbed Anti-Cornea	0	0	0
Unabsorbed Anti-Heart	64	1024	128
3° Anti-Heart	1*	512	0
Pre-Absorbed Anti-Heart	0	0	0
Unabsorbed Anti-Skin	256	128	1024
3° Anti-Skin	2*	2*	512
Pre-Absorbed Anti-Skin	0	0	0
Normal Rabbit Serum	0	0	0
Fetal Calf Serum[c]	0	0	0
Chick Serum	0	0	0
Goat Serum	0	0	0

[a]Antisera, absorptions, and gamma globulin isolation described in Materials and Methods.

[b]Immunofluorescent titer was defined as the reciprocal of the greatest antibody dilution tested (1:1024) which resulted in specific fluorescence of the stated fibroblast type after the addition of FITC-GAR (1:8 in PBS). In most experiments, initial staining with homologous 3° antibody was proceded by incubating cells with normal goat serum (see Results).

[c]FCS used was from the same lot used in the nutrient medium for cell culture.

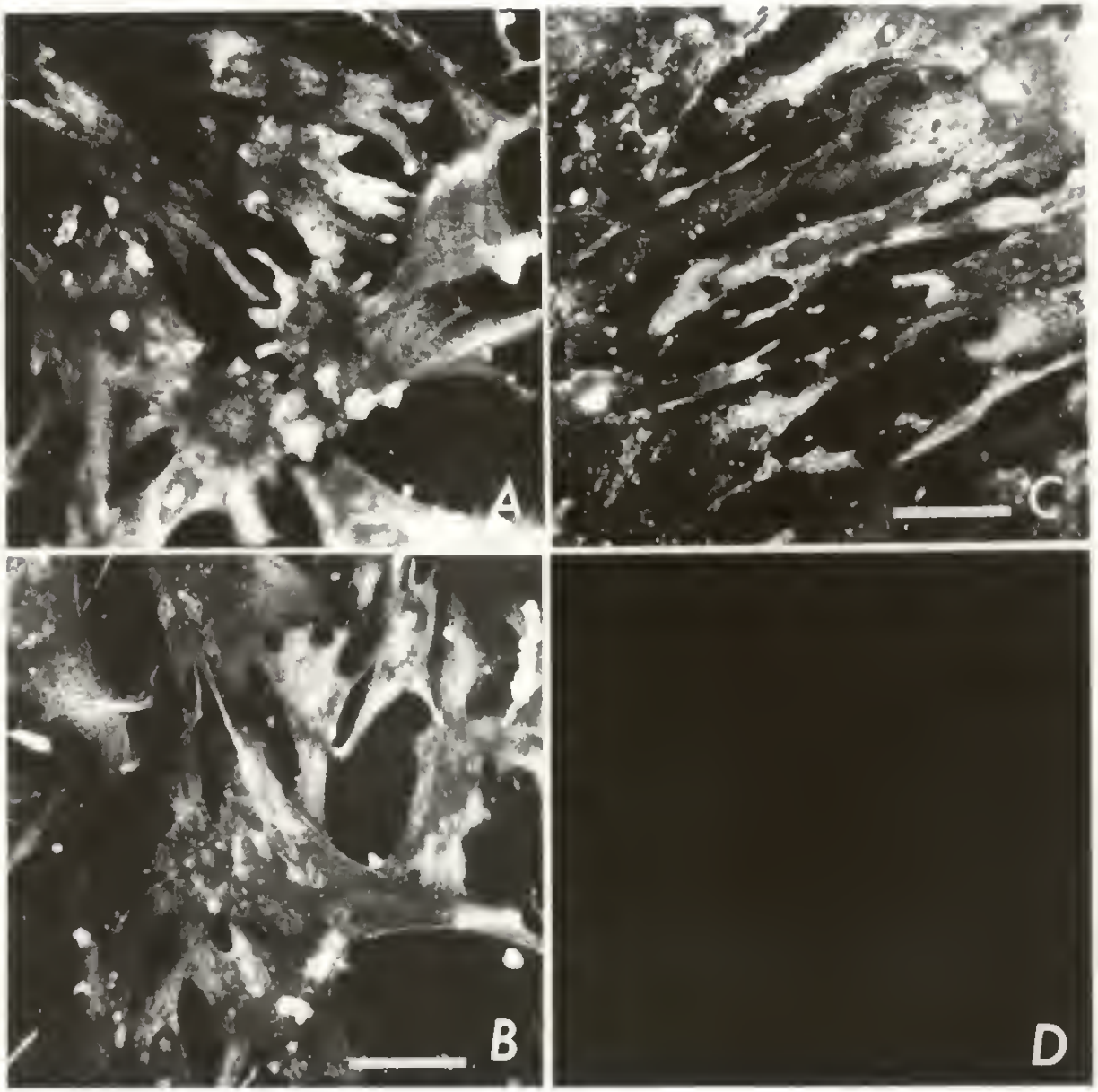
[d]The intensity of 3° antibody showing remaining activity against heterologous antigens was extremely low, in most cases ±, but are recorded as positive reactions. Homologous 3° antibody diluted 1:8 at no time showed reactivity with heterologous antigens.

Fibroblast-like cells used for immunofluorescence assays were cultured from three to seven days, depending upon whether subconfluent or confluent cultures were desired. Individual cells were seen most clearly in subconfluent cultures and displayed a slightly less intense overall staining than in confluent cultures. Confluent cultures displayed intense overall fluorescence and extensive extracellular matrices which further obscured individual cell detail.

Indirect immunofluorescence studies of cell cultures were performed using either unabsorbed or 3° antisera (without isolation or concentration of immunoglobulins), or with the gamma globulin fractions of the 3° antisera isolated by Na_2SO_4 precipitation followed by ion-exchange chromatography on DEAE-Sephadex. The use of gamma globulin (antibody) to stain cell cultures gave lower background fluorescence and reduced nonspecific staining. Improved specificity and lower background also were attained if cultures were treated first with normal goat antibody, followed by immune antibody and FITC-GAR.

Cells treated with homologous 3° antibody gave a bright, distinct pattern of fluorescence (Figure 15, A,B,C). No fluorescence was seen when cultures were treated instead with FCS, NRS, CS, or 3° antisera pre-absorbed with homologous antigen. Most of the cells examined displayed the same intensity of fluorescent staining. In general, the surface distribution of the fluorescence was finely granularand uniformly distributed over the cell surface. The 3° antibody solutions did

Figure 15. Indirect immunofluorescence of cornea, heart, and skin fibroblast-like cells. Cells were isolated from 14 day chick embryos and grown *in vitro* as described in Materials and Methods. The cultures shown were grown for 4 days *in vitro* to a concentration slightly below confluency. Cells were fixed and stained with antibodies as described in the text. (A) Cornea fibroblast-like cells stained with 3° anti-cornea antiserum diluted 1:16 in PBS. (B) Heart fibroblast-like cells stained with 3° anti-heart antiserum diluted 1:16 in PBS. (C) Skin fibroblast-like cells stained with 3° anti-skin antiserum diluted 1:16 in PBS. (D) Cornea fibroblast-like cells stained with 3° anti-skin antiserum diluted 1:8 in PBS. This control is representative of all controls using each 3° antiserum against its two heterologous cell types. (Printing time for the control photomicrograph was the same as used for the other figures.) Scale lines = 30 μm.


A
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did not react appreciably with the two heterologous populations of fibroblast-like cells (Figure 15, D).

Antibody was used in 1:2 serial dilutions in PBS to a final dilution of 1:1024. Titrations using 3° antibodies showed specific fluorescence for cornea, heart, and skin fibroblast-like cells up to and including the 1:256, 1:512, and 1:512 dilutions, respectively. The FITC-GAR conjugate consistently gave low background and reduced non-specific staining when used at dilutions of 1:16 in PBS. The test antibodies were applied routinely at concentrations (usually 1:8) which gave the brightest fluorescence with homologous cell cultures, while showing no reactivity with heterologous fibroblast-like cells.

DISCUSSION

Results of the present study show that fibroblast-like cells from embryonic chick cornea, heart, and skin are antigenically distinct from one another, even when grown *in vitro* in the relative absence of the nonfibroblastic cell types with which each population normally is associated *in vivo*. Thus, there appear to be stable antigenic differences between the fibroblast-like cells from different embryonic tissues.

Immunodiffusion, agglutination, cytotoxicity, and immunofluorescence analyses of anti-fibroblast antisera revealed that unabsorbed antisera reacted with both homologous and heterologous fibroblast cell types. Thorough absorption of the antisera with homogenates of heterologous tissues and heterologous fibroblast-like cells removed cross-reacting antibodies; the resulting 3° antisera displayed a high degree of specificity against the homologous fibroblast-like cells. Thus, anti-cornea fibroblast antiserum absorbed with non-corneal fibroblast-like cells and tissues, agglutinated and lysed only corneal fibroblast-like cells, suggesting reaction of the antibodies with cell-surface antigens. In addition, by immunofluorescence techniques, the antibodies reacted specifically with cornea fibroblast-like cells grown *in vitro*. Comparable results obtained with antisera against fibroblast-like cells from embryonic skin and heart confirm the existence of tissue-specific differences in the antigenicities of the cell surfaces of all three embryonic fibroblast-type cells.

When 2° antisera were tested versus heterologous fibroblast populations against which they had not yet been absorbed, antibodies against heterologous antigens were still detected (Figures 2 and 3). Thus, for example, absorbance of 1° anti-cornea sera with homogenates of heart fibroblast-like cells does not totally remove the ability of the serum to react with skin fibroblast-like cells. Similar results are obtained if the order of absorptions is reversed (i.e., 1° anti-cornea absorbed first with skin cell homogenate remains reactive against heart). Comparable data were obtained for 1° anti-heart and 1° anti-skin. Ikeda et al. (1975), in an immunofluorescent study of cornea development in the chick, made antisera against homogenates of whole cornea and determined that they still reacted with cornea even after absorption with liver, kidney, heart, or serum. However, when their anti-cornea antisera were absorbed with skin protein, all fluorescence was eliminated from the cornea. In contrase, the data presented here clearly show that antiserum against in vitro populations of cornea fibroblast-like cells absorbed with skin fibroblast homogenate retains its reactivity to cornea fibroblast-like cells. Tertiary (3°) absorbance of each antiserum, i.e., absorbance with homogenates of whole embryos minus the homologous tissue, as well as with homogenates of both heterologous fibroblast-types removes cross-reacting antibodies while failing to substantially reduce the reactivity of the antisera against homologous fibroblast-like cell populations. Finally, absorption of each 3° antiserum with its homologous fibroblast type eliminates

the specific activity against that cell population.

Taken together, these data suggest that some antigens detected on one fibroblast-cell population from one tissue also occur on heterologous cell types, whereas other antigens appear to be specific for one fibroblast-like cell population. Alternative interpretations of these results might include the possibility that fibroblast-like cells from different tissues possess not only qualitatively different surface constituents, but also unique topographical distribution patterns of antigenic sites on cell surfaces resulting in diverse specificities (Moscona and Moscona, 1967; Burger, 1969, 1977; Goldschneider and Moscona, 1972; Nicolson, 1972; Moscona, 1971, 1974; Moscona and Hausman, 1977). The qualitative and topographical diversity in antigenic sites may depend not only on cellular genetic regulatory mechanisms, but also on cell surface responses to factors in the extracellular milieu. Such factors may include cell-cell interactions (Levitt and Dorfman, 1974; Saxen et al., 1976; Wolpert, 1977; LeDouarin et al., 1977; Moscona and Hausman, 1977), cellular reactions with extracellular matrices (Meier and Hay, 1974, 1975; Hay and Neier, 1976; Hay, 1977; Lash and Vasan, 1977; Toole et al., 1977), response to hormones, cyclic nucleotides, or divalent cations (Rasmussen, 1977), or enzymatic reactions of cell surface enzymes with extracellular substrates (Roth et al., 1977) (see for review, Slavkin and Greulich, 1975; Wessells, 1977; Lash and Burger, 1977). However, the present experiments show the existence of antigenic differences between populations of fibroblast-like cells

cultured under identical conditions and in the relative absence of nonfibroblast cell types suggests that the antigenic specificities were established *in vivo* during differentiation and are retained stably *in vitro*.

Previous studies have demonstrated that fibroblastic cells in monolayer are more susceptible to immune cytolysis than are the same cells in suspensions (Ishii *et al.*, 1977). However, the present results indicate similar susceptibilities of cells left *in situ* as monolayers and cells released with trypsin-EDTA and allowed to "recover" in suspension culture for 1 h. (Differential susceptibilities among these cell types to dissociating agents have been detected from their ability to subsequently form secondary cultures; Conrad *et al.*, 1977a.) Trypsin-dissociated cells show reduced immune agglutination prior to a 1 h "recovery period." However, cells recovered from proteolytic treatment show increased immune agglutination over cells which are released with EDTA alone. Thus, trypsin, while disrupting some surface antigenic sites during dissociation of cells and requiring a repair period from that disruption, also may uncover new binding sites not exposed by EDTA treatment alone. Such new sites may occupy deeper locations in the cell surface or be complexed, thereby preventing antigen-antibody binding (Burger, 1969; Inbar and Sachs, 1969). This explanation assumes that trypsin-exposed sites are made available during immunization despite the fact that non-trypsinized cells were used as antigens. An alternate possibility is that sites are too sparsly distributed over the surface prior to

trypsinization to give optimum agglutination. Proteolysis may cause clustering of sites, thereby rendering cells more agglutinable (Nicolson, 1972; see for review, Maslow, 1976).

The present data are consistent with earlier studies demonstrating that fibroblast-like cell populations isolated from different tissues of a single organism are distinct from one another *in vitro* (Milunsky *et al.*, 1972; Kaufman *et al.*, 1975; Reddy *et al.*, 1976; Beug and Graf, 1977; Ko *et al.*, 1977; Schneider *et al.*, 1977). However, in none of the studies above, nor in the very early experiments of Parker (1932a,b) were nonfibroblastic cells removed before primary cultures were established, so the *in vivo* fibroblastic origin of the cells cultured is open to question. Recently, techniques have become available which eliminate (cornea) or almost eliminate (heart, skin) contamination with nonfibroblastic cell types. Using such techniques, fibroblast-like cells from cornea, heart, and skin of embryonic chicks have been shown to differ in individual cell morphology, social behavior at saturation, saturation densities, and sensitivity to trypsin-EDTA and EDTA alone (Conrad, 1977a), as well as in their patterns of glycosaminoglycan synthesized (Conrad, 1977b). The populations used in the present work for making and testing antisera were similarly freed of nonfibroblastic cell types before inoculation *in vitro*. Recent studies of the existence and distribution of fibronectin (Vaheri *et al.*, 1977) have dealt with antigens isolated from heterologous populations of embryonic fibroblast-like cells. Nevertheless, these studies offer no insight into the possible

existence of antigenic sites unique to tissue-specific fibroblast populations.

Besides offering further evidence for the heterogeneity of fibroblast-like cells, the isolation of fibroblast-specific antisera offers a valuable technique for studying fibroblast differentiation in vivo. Using histological sections of embryos, and specific antisera, future research will attempt to identify the location and developmental history of tissue-specific fibroblast antigens in vivo.

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ISOLATION OF ANTISERA SPECIFIC FOR FIBROBLAST-LIKE CELLS FROM EMBRYONIC CHICK CORNEA, HEART, AND SKIN

by

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ABSTRACT

Populations of fibroblast-like cells from 14 day embryonic chick cornea, heart, and skin were grown in vitro as primary cultures and found to have distinct antigenic differences. Corneal fibroblasts were obtained by dissection, whereas heart and skin fibroblast-like cells were separated from non-fibroblastic cell types by their rapid adhesion to substrata. Cultured cells were used as antigens. Antisera produced in rabbits were first absorbed against homogenates of embryonic chicks from which the homologous tissue was removed. Each such 1° absorbed antiserum then was absorbed against homogenates of the two respective heterologous fibroblast-like cell populations (2° and 3° absorptions). Resulting 3° absorbed antisera were tested for specificity by immunodiffusion, immune agglutination, immune cytotoxicity (trypan blue uptake and ^{51}Cr release), and indirect immunofluorescence.

Each 3° antiserum reacted only with its homologous type of fibroblast-like cell. Unabsorbed antisera reacted with both homologous and heterologous fibroblast-like cells, as did 1° absorbed antisera. Absorption of 1° antisera with homogenates of the two heterologous fibroblast-like populations removed antibodies against the heterologous populations without significantly reducing the 3° antiserum titer against the homologous fibroblast cell type. Moreover, absorption of 1° antisera with each of the two heterologous fibroblast-like populations removed antibodies not removed by the other. Thus, the fibroblast-like cells from cornea, heart, and skin

are antigenically different from one another *in vitro*. The stable antigenic differences detected may have arisen during the differentiation of these cells *in vivo*. Some of the tissue-specific antigens detected must occur on the cell surface.