Brief Note

Some Effects of Anti-Collagen Serum on Collagen Formation in Tissue Culture: A Preliminary Report.*

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It has been reported by Watson, Rothbard, and Vanamee (1) that antibody which will fix complement in the presence of homologous antigen can be induced in rabbits injected with a purified preparation of rat tail collagen; that this antibody can be removed by absorption with homologous but not heterologous collagen; and that the antibody is capable of interfering with normal collagen fiber reconstitution *in vitro*.

Studies by Porter and Vanamee (2) on collagen fibrogenesis demonstrated that the combined methods of tissue culture and electron microscopy could be used to advantage in the study of this problem. In subsequent work it was shown (3) that early fibrils are formed at the surface of the fibroblast; that they may bear a close relation to intracellular fibroglial fibers; and that they appear to reach their mature form extracellularly by accretion of further material from the ground-substance.

The possible role in collagen fibrogenesis of a particular cytoplasmic granule previously described in fibroblasts by many workers remains to be clarified. Gersh and Catchpole (4) concluded from their staining properties that these granules contain glycoprotein. This finding has been confirmed by Jackson (5), who has suggested further that they may have "fibrogenic properties." To date, the *in vivo* studies of Stearns (6) are the only microscopic observations which have been presented as evidence for the direct participation of these granules in the process of collagen formation.

The present study was designed to determine the effect of an antiserum prepared against chicken collagen on the fibrogenesis of chick collagen in tissue culture.

The antiserum was prepared by the method described by Watson *et al.* (1) for the preparation of antiserum to rat collagen, with slight modifications. Rabbits were immunized over a period of several months by the repeated intraperitoneal injection of a solution of purified chicken collagen prepared from fresh chicken tendons. Antibody was measured by complement fixation performed by the method used by Watson *et al.*, employing twofold serial dilutions of antigen. Only those antisera exhibiting a complement fixation reaction of two plus at 1:64 dilution of antigen, or greater, were included in the antiserum pools used in the tissue culture experiments. Anti-collagen

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serum was added in a final concentration of 20 per cent in Locke-bouillon medium to explants of dermis from 9 or 10 day old chick embryos mounted on formvar-coated coverslips in Maximow slides and incubated at 37.5°C. Several different experimental procedures were used. These included a single addition of antiserum initially, a single addition of antiserum after 48 hours' incubation, and multiple additions of antiserum at daily or 2 day intervals for 3 to 8 days. Observations were made with the light microscope on the living cultures, and on cultures fixed with alcohol-ether and stained by silver impregnation, hematoxylin and eosin, Masson's trichrome, and Mallory's connective tissue techniques. Observations were made with the electron microscope on cultures fixed with osmium tetroxide vapor, mounted and shadowed by standard methods. Control cultures to which 20 per cent pooled normal rabbit serum was added were included in all experiments. All sera were stored at −73°C.; and were heated to 60°C. for 30 minutes (to destroy possible non-specific cytotoxic substances) and passed through a Swinny filter before use.

Under the conditions of the experiments, marked changes were observed in the light microscopic and electron microscopic morphology of the cultures as the result of addition of anti-collagen serum to the medium. The changes observed were qualitatively the same in all experiments, but were most marked in those in which antiserum was introduced initially and repeatedly over several days. However, quite significant changes of lesser degree resulted when antiserum was added only once, either initially or after 48 hours' incubation.

The effects of the anti-collagen serum which have been noted may be briefly described as follows (see Figs. 1 to 4): Normal collagen fibrogenesis is markedly altered. The number of fibers seen is much reduced. The majority of those which are present are poorly formed and lack the clearly defined form and periodicity characteristic of collagen as observed in electron micrographs. Where the fibrils are discrete enough for measurement they show diameters of ~120 A as opposed to diameters of ~350 A for unit fibrils in control preparations (Figs. 1 and 2). A very small proportion of the fibrils in some preparations, however, do exhibit the typical morphology of the unit fibril of collagen. Numerous masses of amorphous material of widely varying size and shape are seen scattered throughout the cultures, many of them being adherent to fibers or cell processes. The larger of these masses are easily visible in the living preparations with the light microscope, and closely resemble other immune precipitates. In so far as has been determined, this material exhibits the tinctorial properties of collagen. It is impregnated by silver, stains pink with hematoxylin and eosin, green with Masson's trichrome stain, and blue with Mallory's connective tissue stain. In electron micrographs this amorphous material is seen to be commonly associated with bundles of poorly formed fibrils, and with bizarre aggregates characterized by a banded appearance with major striae spaced at 2000 to 2500 A.

In cultures containing normal rabbit serum many of the fibroblasts have been noted to contain numerous cytoplasmic granules distinct from mitochondria, fat vacuoles, or degeneration granules, and believed to be the same structures as those previously mentioned by others as perhaps being concerned in collagen fibrogenesis (5, 6). In our preparations (see Fig. 3) these bodies are seen to be generally round in shape, to vary from ap-

proximately 0.5 to 2.6 microns in diameter, and to occur in varying numbers per cell (from as few as three or four to as many as a hundred or more). Each granule appears to occupy a small vacuole, although this appearance may be secondary to fixation. They are observed in all parts of the cytoplasm except in the region of the central apparatus. They are easily visible in the living cell. In preparations fixed with alcohol-ether, they stain pink with hematoxylin and eosin, green with the Masson stain, and blue with Mallory's connective tissue stain. Their staining properties are the same as those of the amorphous masses described above, and, in addition, are similar to those of recognizable collagen organized in fibers. All fibroblasts in an outgrowth do not exhibit these granules at a given time, but in some preparations they may be discerned in the majority of cells. The number of granules is very greatly diminished by the absence of serum from the medium, which is also true for the number of collagen fibers formed. Furthermore, they are absent from, or are uncommon in, fibroblasts in cultures which have received antiserum, where the extracellular masses, exhibiting the same staining properties as the granules, are so numerous.

From the findings thus far it is believed that the detrimental effect on collagen fiber formation which results from the addition of antiserum is evidence in addition to the complement fixation reaction for the presence of antibody to collagen in the antisera used. The extracellular amorphous material seen in cultures to which antiserum has been added is thought in all likelihood to be an antigen-antibody complex. Because the cytoplasmic granules of the normal fibroblasts appear to have many of the staining properties of fibrous collagen, and the same staining properties as the amorphous masses, it is suggested that a substance of these granules is an immunologically related precursor or component of collagen, and that in the presence of antiserum this substance has reacted with antibody, resulting in the formation of the precipitate and in the disruption of the process of fiber formation. The site of this interaction may be at the cell surface as the substance of the granules leaves the cell; but it is also possible that the antibody penetrates the cell.

In summary, an antiserum to chicken collagen has been prepared by the immunization of rabbits with a purified preparation of collagen from chicken tendons. This antiserum has been observed to have a markedly detrimental effect on collagen fibrogenesis in tissue cultures of chick dermis, and to result in large amounts of an amorphous material which is thought in all likelihood to be an antigen-antibody precipitate. This material exhibits many of the staining properties of fibrous collagen, as do also the cytoplasmic granules observed in many fibroblasts grown in the presence of normal serum. From the findings presented it is believed that the described cytoplasmic granules play an important role in collagen formation, and that they may represent an intracellular precursor or component of the collagen fiber.

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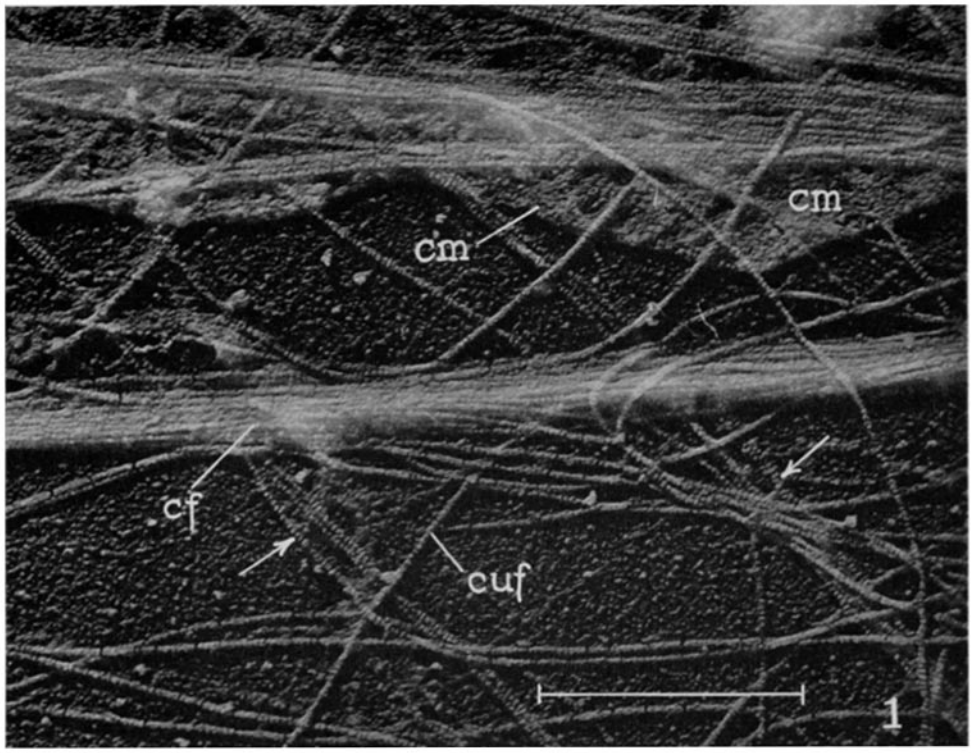
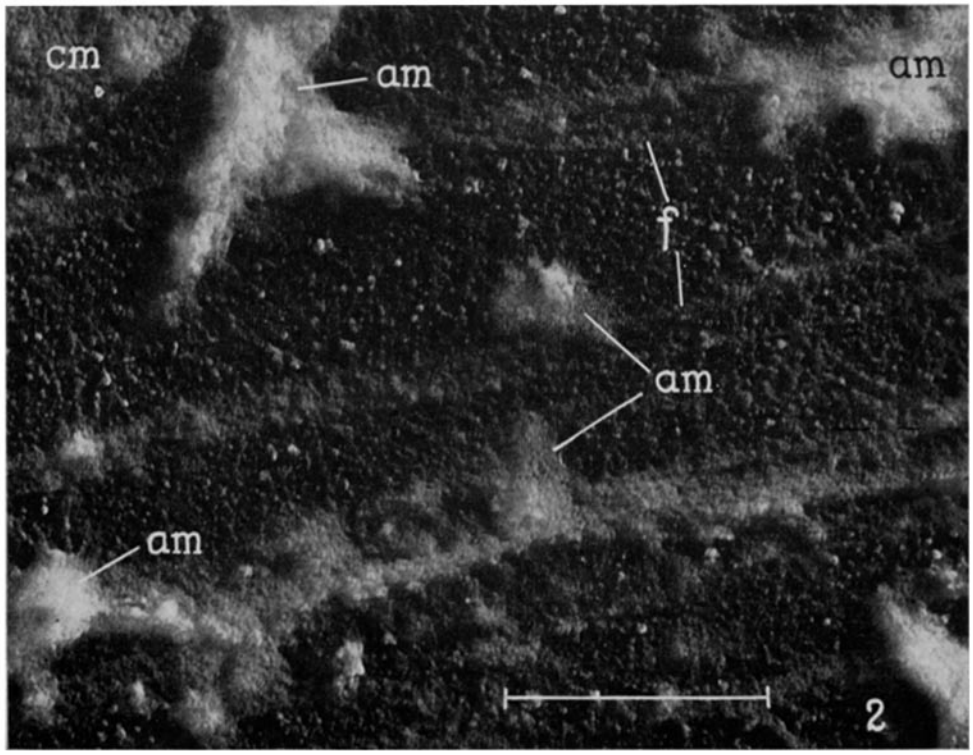
Explanation of Plates

Plate 99

Fig. 1. Electron micrograph of collagen fibrils in a culture of chick embryo dermis. The culture medium was Locke-bouillon for the first 48 hours of incubation, after which time 20 per cent normal rabbit serum was added. Age of culture at time of fixation: 96 hours. Typical unit fibrils of newly formed collagen (*cuf*), showing a periodicity of 220 A, are present (arrows). A small portion of the margin of a fibroblast is evident at *cm* across the top of the micrograph. A fiber bundle is indicated at *cf*. × 34,500.

Fig. 2. Electron micrograph of a portion of a tissue culture of chick embryo dermis grown in Locke-bouillon medium to which 20 per cent rabbit anti-chicken collagen serum was added after 48 hours' incubation. Age of culture at time of fixation: 96 hours. Note absence of clear-cut collagen fibers and presence of only irregular streaks of fibrous material (*f*) showing no evidence of periodicity. Several typical masses of amorphous material (*am*), many of which are contiguous with fibers, are present. A very small portion of a cell margin (*cm*) is visible at the upper left hand corner of the micrograph. × 34,500.





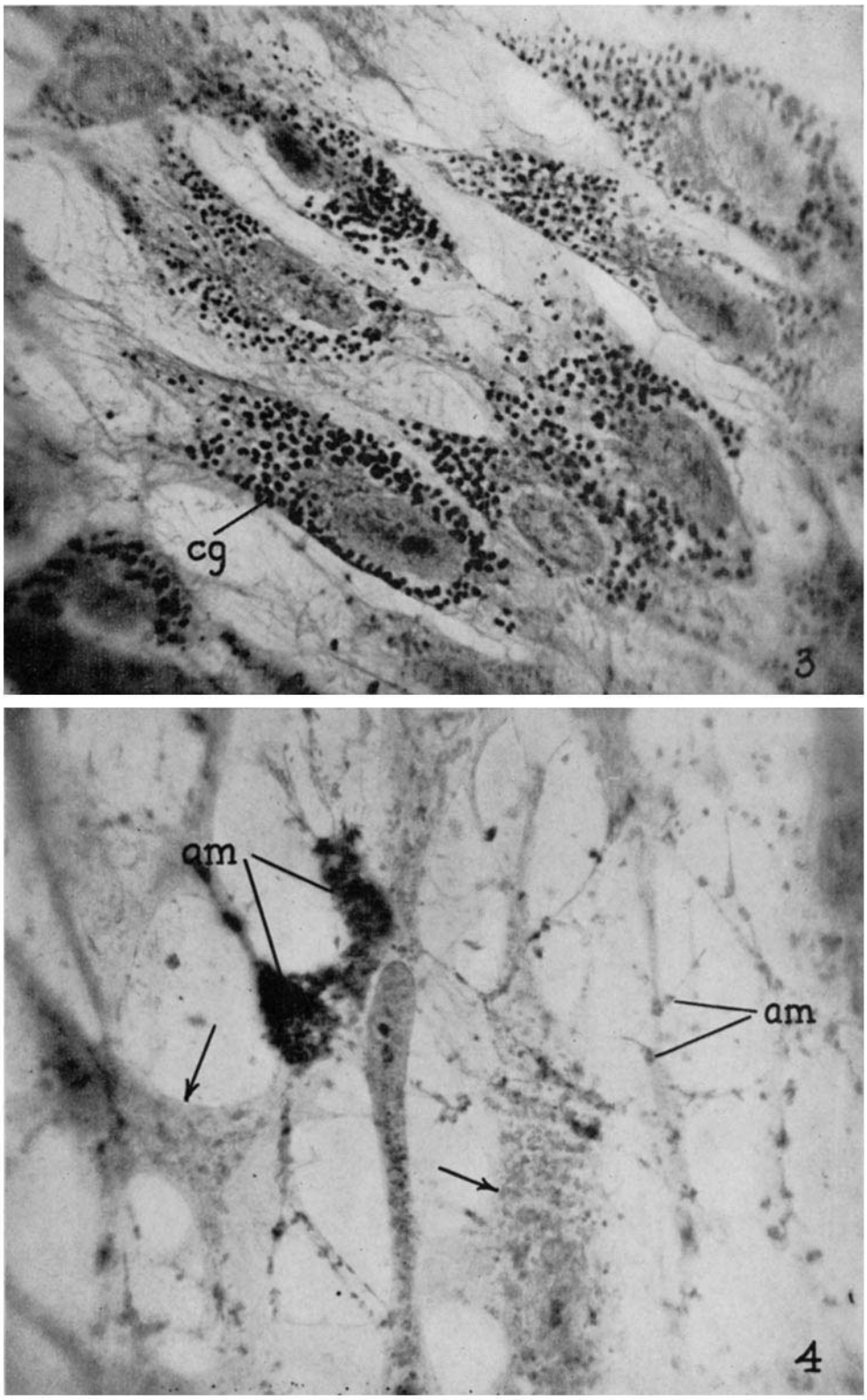
(Robbins *et al.*: Effects of anti-collagen serum on collagen formation)

PLATE 100

FIG. 3. Light photomicrograph of fibroblasts in tissue culture of chick embryo dermis in Locke-bouillon medium to which 20 per cent normal rabbit serum was added after 48 hours' incubation. Age of culture at time of fixation: 96 hours. Note numerous cytoplasmic granules (*cg*). A description of these is given in the text. Alcohol-ether fixation. Mallory connective tissue stain. × 1220.

FIG. 4. Light photomicrograph of tissue culture of chick embryo dermis in Locke-bouillon medium to which 20 per cent rabbit anti-chicken collagen serum was added after 48 hours' incubation. Age of culture at time of fixation: 96 hours. Note large amounts of extracellular material (*am*) apparently adherent to fibers and cell processes. This material has identical staining properties with hematoxylin and eosin, Masson's and Mallory's connective tissue stains, with those of the cytoplasmic granules in Fig. 3. A few faintly stained granules are visible in the cytoplasm of the fibroblasts in this field (arrows). Alcohol-ether fixation. Mallory connective tissue stain. × 1220.

The light photomicrographs were made by Mr. Julian A. Carlile.



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