A Tissue Culture Study of the Chick Fibroblast in Relation to Streptococcal Filtrates and Rheumatic Heart Disease

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Marked degeneration and disintegration of the chick embryo fibroblast was observed in tissue cultures after their exposure to sera of patients with active or inactive rheumatic heart disease or to the filtrates of beta hemolytic streptococci. Some tissue specificity was demonstrated; sera of the patients or the filtrates of the streptococci failed to affect contractility of the myocardial cells or secretion of the mesonephros. Sera of normal control humans or of patients with other so-called collagen diseases did not seriously affect the fibroblasts. Filtrates of nine other bacteria failed to alter the morphology of the fibroblasts unless severe toxicity was present and the tissue culture died.

This is a report on the response of chick embryo fibroblasts in tissue culture to filtrates of the beta hemolytic streptococci and to sera of patients with a rheumatic process. It was recognized that any bacterial filtrate contains many toxic antigenic substances and likewise that sera of patients with a rheumatic diathesis contain factors which might alter cells in tissue culture. Although previous investigation has directly or indirectly related rheumatic fever to the beta hemolytic streptococci, no causative role for the production of experimental or clinical rheumatic lesions by these organisms or any of their noncellular products has been clearly demonstrated.

Tissue culture affords an excellent technic for the study of living cells and their reactions to exogenous substances. Nephrotoxic substances in the sera of patients with glomerulonephritis have been demonstrated by an impairment of secretion of the embryo chick mesonephros. Immunologic testing for antitissue antigens and antibodies has been reported. The fibroblastic outgrowth of the chick embryo heart was employed in this study as test material for the observation of specific cellular derangement. Changes were observed in the fibroblasts when they were exposed to two apparently different substances, bacterial filtrates and sera of patients with active or inactive rheumatic heart disease.

Methods and Material

Bacteriology. (1) Six strains of streptococci obtained through the courtesy of Dr. Murray Streitfeld of the Bacteriology Department of the National Children’s Cardiac Hospital were used. Two of the strains were classified by the USPH as streptococcus group A, type 37, USPH strain SS 53 and group A (no reaction with many typing sera); the remaining three strains isolated from children with rheumatic fever were group A, type 6, group B and an ungrouped and untyped streptococcus. All were beta hemolytic. The sixth was a nonhemolytic streptococcus isolated from a stool culture.

(2) Filtrates from eight unrelated bacteria prepared in the same fashion were also studied. These included filtrates of Aerobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, an unknown species of bacillus isolated from a blood plate as contaminant, Staphylococcus aureus beta hemolytic and Proteus mirabilis.

(3) In preparing the filtrates, the inoculum was made into a standard medium consisting of: 1.5 ml. 0.2 phosphate buffer pH 7.4, 1.5 ml. 0.85 per cent saline, 1.0 ml. 1 per cent glutathione, 1.0 ml. 1 per cent sodium pyruvate, 5.0 ml. normal human serum. A trypticase, yeast extract type of filtrate, was used in preparation of the cholera filtrate.

(4) Filtrates were prepared after 10 ml. of the bacterial medium with its inoculum had been
TARI, E. 1.—Effect of Bacterial Filtrates on Chick Heart Explants in Tissue Culture (Living) (72 Hours)

Streptococcus B hemolytic
(1) Streptococcus Group A—Type 37...
(2) Streptococcus Group A...
(3) Streptococcus Group B...
(4) Streptococcus Group A—Type 6...
(5) Streptococcus...

Nonhemolytic Streptococcus...
Aerobacter aerogenes...
Escherichia coli...
Vibrio coli...
Pseudomonas aeruginosa...
Salmonella typhosa # 4327...
Bacillus—unclassified...
Staphylococcus aureus—B Hemolytic...
Proteus mirabilis...

In this and the other tables the conventions used are as follows: ++ = degree of positive response; + = questionable response.

Incubated at 37 C. for 24 hours, centrifuged for 15 minutes at 2,000 rpm and the supernatants filtered first through "F" grade fritted Corning glass filters and then through sterile "UP" grade glass filters. The cholera filtrate was filtered through two Ertell pads and concentrated so that it represented 10 times that of the original filtrate. All filtrates were maintained at 5 C. and kept at a neutral pH.

Preparation of Sera. Human sera were obtained under sterile conditions from normal male and female individuals, from random sampling of patients with rheumatic heart disease, active or inactive, and from patients with other forms of so-called collagen diseases. No anticoagulants were added to the blood and, upon retraction of the clot, the expressed sera were collected. All the filtrates and the patients' sera were kept and used repeatedly in the experiments.

Preparation of Tissue Cultures. The double coverslip method as described by Cameron was uniformly used. Hearts of 7 to 9 day chick embryos were cut into 12 to 14 fragments and two fragments placed on each coverslip in 2 drops of medium. The experiments were made in duplicate so that at least four explants were used per experiment. The control medium consisted of 2 drops of fowl plasma, 1 drop of Tyrode solution, 1 drop of 10 per cent chick embryo extract and 1 drop of normal human serum. In the experimental preparations the drop of normal serum was replaced by a drop of bacterial filtrate or of the patient's serum. To test the tissue specificity, cultures of chick embryo mesonephros containing phenol red were made.

The cultures were examined at intervals during 72 hours for the quality of contraction of the myocardial cells, the condition of the fibroblasts growing from the explant and the secretory activity of the mesonephros. Original filtrates and patients' sera were kept and the experiments repeated. Representative cultures were fixed in 5 per cent formalin solution and stained with hematoxylin and eosin for further study.

RESULTS

With the exception of two bacterial filtrates the various filtrates and sera did not seriously affect the viability of the cultures.

(A) Bacterial Filtrates in the Tissue Cultures. Filtrates of the hemolytic streptococci isolated from the throats of patients with rheumatic fever or typed as group A hemolytic all gave a uniform response. The fibroblasts became vacuolated, sometimes cytolysed, and the outgrowth was not luxuriant. The secretory activity of the mesonephros and the contraction of the myocardial cells did not appear to be affected (table 1). In the control cultures, the growth of the fibroblasts (fig. 1A) was orderly and normal, and mitosis was observed. No chromatin debris was seen. In experimental cultures with the filtrates of the beta hemolytic streptococci, sparse outgrowth and a marked disorganization of the fibroblasts were noted (fig. 1B). Most of the mitotic figures became abnormal; round, monocyteic types of cells with eccentric hyperchromatic nuclei appeared...
FIG. 1. (A) Fibroblasts grown in a normal medium; fixed, H. and E. stained tissue culture. Note mitotic figure in upper and lower portion of picture and orderliness of the cytoplasm. (x133) (B) Fibroblasts treated with filtrate of beta hemolytic streptococci; fixed and stained. Note vacuolation, chromatic dust in the lower portion of picture and the bizarre mitotic figure in the center of the figure. (x133)

FIG. 2. Appearance of fibroblasts after exposure to filtrates of beta hemolytic streptococci or sera of rheumatic patients. Note small hyperchromic nuclei with eccentric nucleoli, disorderly character of the cytoplasm and naked nuclei of fibroblasts. (x133)

FIG. 3. Effect of filtrate of beta hemolytic streptococci or sera of rheumatic patients on the cytoplasm of the fibroblasts: Note chromatic dust. (x133)
TABLE 2.—Effect of Filtrates and Sera on the Cytology of Fibroblasts in Tissue Culture, Fixed and Stained H. and E.

<table>
<thead>
<tr>
<th>Bacterial Filtrates</th>
<th>Vacuolation</th>
<th>Chromatic Dust</th>
<th>Contracted Degenerated Cells</th>
<th>Nuclear Changes</th>
<th>Abnormal Mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Filtrate without inoculum…</td>
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<td>(2) Group A—</td>
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<td>Type 6……………</td>
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<td>(3) Nonhemolytic strep……………</td>
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<th>Sera—Human</th>
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<tr>
<td>(1) Normal ………..</td>
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<td>(2) Active rheumatic………</td>
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<td>(3) Inactive rheumatic………</td>
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<td>(4) Hyperergy ………..</td>
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DISCUSSION

The demonstration of identical changes in fibroblasts in tissue culture exposed to sera of patients with rheumatic heart disease and filtrates of beta hemolytic streptococci confirms an inter-relationship which previously had been only suspected. However, the similarity of the fibroblastic response must be interpreted cautiously. It does not necessarily mean that the responsible agent in both test substances is the same.

The reaction of the fibroblasts to the filtrates of the beta hemolytic streptococci varied inversely with the dilution of the filtrates. A more profound degenerative reaction was produced with the greater than with the lower dilutions.

Other bacteria including the nonhemolytic streptococci failed to demonstrate any striking fibroblastic disorganization (table 1). Some of the filtrates, such as that of *Pseudomonas aeruginosa*, were universally toxic. The filtrate of nonhemolytic streptococci failed to produce any histocytologic changes comparable to the above (table 2).

(B) Human Sera in the Tissue Cultures.
Addition of normal sera from control subjects failed to produce any demonstrable effect on the explants or on the outgrowing fibroblasts. The sera of patients with diseases other than rheumatic fever failed to produce the fibroblastic change (table 3). The contracting myocardium was not influenced by the sera of any patients.

The cytologic changes in cultures containing the sera of rheumatic patients (table 2) were identical in every way with those seen in cultures with filtrates of the beta hemolytic streptococci. Rounded monocyte-like cells with eccentric hyperchromic nuclei were present. In addition, abnormal mitoses, spindle-shaped and naked nuclei, pyknotic nuclear remnants, vacuolated cytoplasm and nuclei and chromatin dust (figs. 2 and 3) were observed. The serum of one patient (T. R. table 3), with a mitral murmur but without a history of rheumatic fever produced no granularity or vacuolation of the fibroblasts.

* Initials of patients studied.

(fig. 2) and cystic nuclear areas could be seen. The cytoplasm became markedly vacuolated (fig. 1B). Chromatic dust and spindly, pyknotic and, at times, naked nuclei were observed (figs. 2 and 3). The bacterial filtrates were tested in dilutions of 1:2, 1:3 and 1:4. A more profound degenerative reaction was produced with the greater than with the lower dilutions.
dilution of the filtrate. Such a phenomenon may be similar to the "paradoxic zone phenomenon" of Eagle who was so designated because stronger concentrations of penicillin did not necessarily produce a reduction of bacterial activity. But he failed to demonstrate this reaction when he used the beta hemolytic streptococci. Another explanation for the paradoxic reaction of the 1:2 dilution of the beta hemolytic streptococci filtrates in tissue culture might be the so-called zones of minimal equivalents of an antigen-antibody combination. More work using tissue culture technics in conjunction with biochemical analyses will clarify this phase of the problem.

In the bacterial filtrates, the substance reacting on the fibroblast is presumably an exotoxin, perhaps streptolysin 0. Our fragmentary and, as yet, inconclusive data suggest that cholesterol added to the filtrates exerted a "protective" action, resulting in a less severe fibroblastic derangement. This reminds one of the protective action of cholesterol, noted by Hewitt and Todd against the lethal effect of streptolysin 0 in mice.

If the fibroblastic-reacting substance in the filtrate of the beta hemolytic streptococci is streptolysin 0 or any of the other exotoxins, it is unlikely that this would persist in the sera of patients long after cessation of the active disease. It must be remembered that the fibroblastic degeneration occurred whenever the sera of patients with inactive as well as active rheumatic heart disease were studied. Some of the patients with inactive rheumatic heart disease had had the last rheumatic recrudescence many years before. From the various sera-antibody studies, gamma globulin determinations, C-reactive protein or others, no prolonged reactions have been noted.

The in vitro fibroblastic specificity was further demonstrated by the failure of the sera of the patients with rheumatic heart disease to inhibit the secretory activity of the chick mesonephros in tissue culture or the contraction of the myocardium. Such a specificity of tissue antibodies for selectively affecting the cellular growth and function of a particular type of cell has been previously observed. The production of iso-antibodies and their role in the pathogenesis of disease has been previously advanced. Acute hemolytic anemias and thrombocytopenic purpuras are thought by many to be examples of the effect of auto-tissue antibody formation with resultant destruction of the cell. It is possible that a similar phenomenon occurs in patients with rheumatic heart disease.

Further critical investigation is necessary before a coherent theory on the pathogenesis of rheumatic fever can be safely advanced. The tissue culture technic offers a promising approach not only to the problem of pathogenesis but also to diagnosis and therapy of rheumatic fever.

SUMMARY AND CONCLUSIONS
1. A selective fibroblastic derangement was demonstrated in tissue cultures of chick embryo heart after exposure to filtrates of beta hemolytic streptococci.
2. Filtrates of nine bacteria other than the beta hemolytic streptococci failed to produce a selective fibroblastic derangement.
3. Identical fibroblastic derangement was noted following exposure of similar cultures to the sera of patients with active and inactive rheumatic heart disease.
4. Neither the filtrates of streptococci nor sera from patients with rheumatic heart disease affected the contraction of the myocardium or the secretory activity of the mesonephros.
5. Sera of patients with rheumatoid arthritis, glomerulonephritis, lupus erythematosus and a hyperergy response with joint manifestations failed to produce serious fibroblastic derangements.

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REFERENCES


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