INHIBITION OF A COLLAGENASE BY THE HUMAN GINGIVAL MICROBIOTA¹

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Interactions between the members of a complex indigenous microbiota could be critical determinants of the delicate biochemical balance between the cohabiting microorganisms and the tissues of their host, the status of which determines the relative states of health and disease (Dubos, 1954). The existence of such complex, but little understood, interrelationships in the oral cavity is evident (Bibby et al., 1938; Van Kesteren et al., 1942; Björnesjö, 1950; Thompson and Johnson, 1951). Although numerous antagonisms between members of the oral microbiota have been described, as manifested by inhibition of growth (reviewed by Rosebury et al., 1954), few instances have been reported of the inhibition of a specific biochemical activity of one microorganism by other microorganisms.

In experiments (Thonard and Scherp, 1957) investigating the mechanisms by which human collagen might be lysed during the development of periodontal disease, we incubated untreated gingival tissue with its indigenous microbiota in broth. Collagenolysis could not be demonstrated. In order to show that the gingival tissue contained enough collagen available for enzyme action to be detected by the methods used, control tests were made by adding Clostridium histolyticum or filtrates of its broth cultures. Unexpectedly, collagenolysis was undemonstrable in these tests also. However, when C. histolyticum filtrates were incubated with gingival tissue in the presence of antibiotics, i.e., when growth of the oral microbiota was suppressed, extensive collagenolysis was demonstrable regularly. Investigation of this anomaly forms the basis for the experiments reported here.

MATERIALS AND METHODS

Fresh human gingival tissue from patients suffering from periodontitis simplex² was obt-

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² Kindly supplied by Dr. Frederick J. Halik, Periodontist, Rochester, New York.
TABLE 1

Inhibition of Clostridium histolyticum collagenase by oral microbiota

<table>
<thead>
<tr>
<th>Series</th>
<th>Test</th>
<th>Collagenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A. Gingiva + indigenous flora + C. histolyticum filtrate</td>
<td>0/24*</td>
</tr>
<tr>
<td></td>
<td>B. Gingiva + indigenous flora + C. histolyticum</td>
<td>0/24</td>
</tr>
<tr>
<td></td>
<td>C. Gingiva (antibiotic sterilized) + C. histolyticum filtrate</td>
<td>21/23</td>
</tr>
<tr>
<td></td>
<td>D. Gingiva (antibiotic sterilized) + C. histolyticum</td>
<td>9/27</td>
</tr>
<tr>
<td>2</td>
<td>A. Tendon (ethox) + gingival flora</td>
<td>13/18</td>
</tr>
<tr>
<td></td>
<td>B. Tendon (ethoxy) + gingival flora + C. histolyticum filtrate</td>
<td>11/11</td>
</tr>
<tr>
<td></td>
<td>C. Tendon (antibiotic sterilized) + C. histolyticum filtrate</td>
<td>16/16</td>
</tr>
<tr>
<td></td>
<td>D. Tendon (antibiotic sterilized) + C. histolyticum</td>
<td>13/21</td>
</tr>
<tr>
<td>3</td>
<td>A. Gingiva (antibiotic sterilized) + x-toxin‡</td>
<td>2/2</td>
</tr>
<tr>
<td>4‡</td>
<td>A. Gingiva + filtrate of series 1A</td>
<td>10/11</td>
</tr>
<tr>
<td></td>
<td>B. Tendon + filtrate of series 1A</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>C. Gingiva + supernatant of series 1A</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>D. Gingiva + filtrate of series 1B</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>E. Gingiva + supernatant of series 1B</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>F. Gingiva + filtrate of gingival starter culture + C. histolyticum filtrate</td>
<td>4/6</td>
</tr>
</tbody>
</table>

* Number of tests showing collagenolysis/total number of tests.
† Sterilized by exposure to ethylene oxide vapor (Schultz-Haudt and Scherp, 1955).
‡ Purified collagenase of Clostridium perfringens kindly supplied by The Wellcome Research Laboratories, Beckenham, Kent, England.
§ All samples of tissue in this series were sterilized by antibiotics.

on ethylene-oxide-treated bovine tendon accorded with the results reported by Schultz-Haudt and Scherp (1955) and Thonard and Scherp (1957).

All cultures were incubated for 3 days at 37 C aerobically, except those inoculated with C. histolyticum, which were incubated anaerobically. The cultures were centrifuged and the supernatant fluids were analyzed for the presence of hydroxyproline-containing peptides as a measure of collagenolysis (Thonard and Scherp, 1957), in comparison with similar cultures not containing the collagenous substrate. In most of the tests recorded as positive, the amounts of hydroxyproline were many times that required as a criterion of minimal collagenolysis.

RESULTS AND DISCUSSION

The results of the tests in series 1A and 1B (table 1) indicate an inhibition of collagenase activity by cultures of the indigenous gingival flora. In the tests of series 1B, the growth of C. histolyticum in the presence of the oral microorganisms was confirmed microscopically. Conceivably, the anaerobic sporulating gram-positive bacilli observed might have originated from the gingival flora. This possibility was deemed unlikely, since Clostridium species occur very infrequently in cultures from the gingival sulcus (Roth and Myers, 1956). The presence of adequate amounts of collagen in the samples of gingiva and its susceptibility to C. histolyticum and Clostridium perfringens collagenases were evidenced by the positive results obtained in series 1C, 1D, and 3. Somewhat surprising was the low incidence of collagenolysis in those tests in which antibiologically sterilized tissues were incubated with cultures of C. histolyticum (1D and 2D). Microscopical and cultural examination of the negative tests in these two series either failed to disclose any viable bacteria or, in a few instances, revealed long filamentous bizarre forms of bacilli. The latter accord with Gardner’s (1940) observation of long filamentous forms of C. perfringens under the influence of penicillin in concentrations of the order of one μg per ml. Evidently in our experiments, even repeated saline washes over a period of several hours failed in many cases to elute the antibiotics from the tissues sufficiently to permit normal growth of C. histolyticum. Elution from tendon seems to have been more effective. Another factor in these experiments was the possibility that the production of collagenase by C. histolyticum was adversely affected by the TCY broth; however, comparison of collagenase production in TCY broth and the infusion broth used for the C. histolyticum filtrates failed to reveal any differences.

On first examination, the results of the tests with tendon sterilized by ethylene oxide (series 2A and 2B) seem to be inconsistent with those...
of the tests with gingiva. Previous studies (Schultz-Haudt and Scherp, 1955; Thomard and Scherp, 1957) have shown that exposure of collagen to ethylene oxide renders it susceptible to digestion by 70 per cent of cultures of the gingival flora from cases of periodontitis simplex. The results of series 2A confirm this observation. If the gingival flora inhibited the collagenase of C. histolyticum, collagenolysis should have occurred at the same rate in series 2B, whereas in fact it occurred in all tests. This discrepancy could be explained by the fact that C. histolyticum produces not only a specific collagenase but also two other proteases (MacLennan et al., 1958), of which only the collagenase seems to be inhibited by the oral microbiota. The increased incidence of collagenolysis in series 2B would accordingly be attributable to one or both of the nonspecific proteases acting in conjunction with oral microbial enzymes upon the collagenous substrate, which had been rendered more digestible by prior treatment with ethylene oxide.

To account for the complete lack of collagenolysis in series 1A and 1B, several explanations seemed possible: (a) the oral bacteria could be utilizing the collagenase metabolically or simply destroying it, (b) collagenase may not even have been produced by C. histolyticum in the presence of the oral bacteria, and (c) products of the oral bacteria could be inhibiting the collagenase. Additional experiments were undertaken to evaluate these possibilities (series 4, table 1).

After removal of samples to test for the presence of hydroxyproline, the remaining broth of the tests in series 1A was either filtered through Selas 02 filters or centrifuged at 3000 rpm for 20 min and then retested for collagenase activity against fresh gingival tissue or bovine tendon in TCY broth containing 200 i.u. of penicillin and 200 μg of streptomycin per ml. Collagenolysis occurred in 16 of the 17 retests (4A, B, C). Clearly, the oral bacterial cultures had not destroyed the collagenase.

Similar experiments with the remaining broth of the tests in series 1B showed collagenolysis in 9 out of 10 retests (4D, E). Evidently, C. histolyticum had produced collagenase while growing in the presence of the oral bacteria.

These results indicated that the oral microbiota effects a reversible inhibition of C. histolyticum collagenase. In order to test for the production of a soluble collagenase inhibitor, starter cultures in TCY broth were made as described previously, incubated aerobically for 48 hr at 37 C, and filtered through Selas 02 filters. A 7 ml portion of each filtrate was incubated for 3 days at 37 C with its homologous gingival tissue, which had been stored during the interim in saline at 4 C, plus 3 ml of filtrate from C. histolyticum. Sterility was maintained by a final concentration of 200 i.u. of penicillin and 200 μg of streptomycin per ml. Collagenolysis was obtained in 4 of 6 tests (4F). These results indicated that a soluble collagenase inhibitor is at best irregularly present in cultures of the gingival bacteria and that the mechanism of inhibition is intimately associated with the presence of the bacterial cells themselves.

The present observations recall the investigation of the antibacterial action of human saliva by Bibby et al. (1938), who reported: "The antibacterial agent effective against Micrococcus lysodeikticus is unable to pass a Berkefeld filter, is more closely associated with the salivary bacteria and cells and gives more definite evidence of its elaboration by bacterial cultures."

Although several instances of the inhibition of a specific activity of one organism by antagonistic effects of another organism have been recorded, we still have little knowledge as to how such effects are mediated. Gaté and Papacostas (1921) noticed that the symptoms of infection by Corynebacterium diphtheriae were much milder if Friedländer's bacillus was associated with the infection. However, with intramuscular injection in guinea pig thighs of mixed filtrates of these two organisms grown separately, no interference with the lethal effect of the diphtherial toxin was observed. On the other hand, when C. diphtheriae was grown in broth with Friedländer's bacillus or its culture filtrates, toxin production was decreased, presumably because growth of the C. diphtheriae was poor also. Alternatively, the inhibition of toxin production might have resulted from a disturbance of the iron metabolism of C. diphtheriae, which is now known to be a critical determinant of its toxin production (Pappenheimer, 1947). Hall and Peterson (1923) found that toxin production by Clostridium botulinum was markedly inhibited when it was grown in glucose broth with various aerobic acidogenic soil bacteria. The latter bacteria were shown also to destroy preformed botulinus toxin. Both the inhibition of toxin formation and the destruction of toxin were due not to the acidity as such but to undefined factors effective under acid conditions. Ramon et al. (1945) reported that filtrates of Bacillus
subtilis cultures inhibited the growth in vitro of a number of microorganisms, including staphylococci, Bacillus anthracis, and C. diphtheriae, and inhibited in vivo the action of their toxins. Similar filtrates inactivated the viruses of rabies and equine encephalomyelitis (Remlinger and Bailly, 1946a, b) probably due to unidentified proteases. Thompson and Johnson (1951) were able to show that the inhibition of growth of C. diphtheriae by Streptococcus mitis and other oral viridans streptococci correlated with the amount of hydrogen peroxide produced by the streptococci and that this inhibition could be reversed by catalase-producing staphylococci.

Elucidation of the mechanisms of antagonisms like those discovered in the present investigation, and determination of the significance of analogous reactions in the ecology of the oral flora will require extensive additional experimentation.

SUMMARY

When fresh human gingival tissue from patients suffering from periodontitis simplex was incubated for 3 days at 37 C in broth with its indigenous flora, plus either growing cells or culture filtrates of Clostridium histolyticum as sources of collagenase, no collagenolysis was detectable chemically. When the growth of the gingival microbiota was suppressed by antibiotics, the inhibition of collagenase was averted. The growth of the gingival microbiota did not destroy preformed collagenase or suppress its formation by C. histolyticum; rather, it effected a reversible inhibition of this enzyme. The formation of a soluble inhibitor of collagenase in cultures of the gingival bacteria could not be demonstrated; the inhibitory mechanisms required the continued presence of the bacterial cells. Altered collagen, in the form of bovine Achilles tendon sterilized by ethylene oxide, was lysed by 70 per cent of cultures of the gingival biota alone, and by all such cultures (presumably by proteases other than collagenase) when C. histolyticum filtrate was added.

REFERENCES


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