

Inhibition of Keratinocyte Proliferation by Extracts of *Actinobacillus actinomycetemcomitans*

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A heat-labile sonic extract of a nonleukotoxic strain of *Actinobacillus actinomycetemcomitans* caused a dose-dependent inhibition of proliferation of human keratinocytes. Exposure of cells to heat-labile sonic extract for periods as brief as 10 min produced significant suppression of [³H]thymidine uptake 72 h later. I propose that alteration of epithelial barriers may be one pathogenic mechanism of this organism.

Actinobacillus actinomycetemcomitans (Aa) exhibits numerous properties which may account for a major role in the pathogenesis of juvenile periodontitis (for a review, see reference 3). These properties include a strain-specific leukotoxin (1), an endotoxin (6), a nonendotoxic bone-resorbing factor (8), and a lymphocyte-inhibitory factor (15). This organism has also been shown to inhibit the proliferation of gingival fibroblasts (14; R. H. Stevens and B. F. Hammond, *J. Dent. Res.* **61**:193, 1982).

In periodontal pockets, Aa is normally in closer approximation to the cells of the junctional and sulcular epithelia than to fibroblasts. Since these cells comprise the initial barrier to the invasion of bacterial products, it is important to understand the response of epithelium to this organism. There is evidence that *Actinobacillus* sp. inhibit the proliferation of rat and human keratinocytes (H. Birkedal-Hansen, P. W. Caulfield, Y. M. Wanne-Muehler, and R. Pierce, *J. Dent. Res.* **61**:192, 1982; P. R. Kamen, *J. Dent. Res.* **61**:193, 1982; P. R. Kamen, *J. Dent. Res.* **62**:247, 1983), but important mechanisms of this response have not been described. The purpose of this report is to provide additional information concerning the mechanisms of epithelial cytotoxicity of a nonleukotoxic strain of Aa.

Keratinocyte cultures were obtained as described previously (5, 9). Briefly, 3T3 cells lethally irradiated with 3,000 rads were used as a feeder layer to support the growth and serial subcultivation of human foreskin keratinocytes while preventing the proliferation of dermal fibroblasts. For subcultures, adherent 3T3 cells were selectively removed by brief treatment with 0.02% Versene (GIBCO), leaving colonies of keratinocytes which were harvested by treat-

ment with 0.05% trypsin-0.02% EDTA. Cultures were grown in Dulbecco modified Eagle medium supplemented with 20% fetal bovine serum, 0.4% hydrocortisone, 10 ng of epidermal growth factor per ml, and 50 µg of gentamycin per ml and incubated at 37°C in an atmosphere of 5% CO₂. Cultures from passages two to four were used for experiments.

A dialyzed, lyophilized preparation of sonicate of Aa strain 652 was obtained from R. Stevens. For use in experiments, lyophilized sonic extract (SE) was reconstituted at the indicated concentrations directly with tissue culture medium and filter sterilized. To determine protein content (7), lyophilized SE was suspended in distilled water and filter sterilized. The protein content was 0.6 mg of protein per mg of SE.

For cell counting, keratinocytes were plated at a density of 10³ cells per well on 2.0-cm² tissue culture wells (Falcon) which had been inoculated 1 day previously with 4 × 10³ irradiated 3T3 cells. After 24 h, medium was replaced by fresh medium (control) or fresh medium containing the indicated concentrations of SE. At 48-h intervals, 3T3 cells were selectively removed and keratinocytes were harvested and counted in a hemacytometer.

To determine [³H]thymidine incorporation, 10⁴ keratinocytes were plated into 0.38-cm² flat-bottomed tissue culture wells (Falcon) inoculated 1 day previously with 4 × 10³ irradiated 3T3 cells. After 71 h, medium was replaced with fresh medium (control) or medium containing SE at the indicated concentrations. At indicated intervals, cells were pulsed for 4 h with 10.0 µCi of [³H]thymidine (20 mCi specific activity; New England Nuclear Corp.) per ml. The cells were washed thoroughly with Hanks balanced salt solution, detached with 0.2% trypsin-0.2%

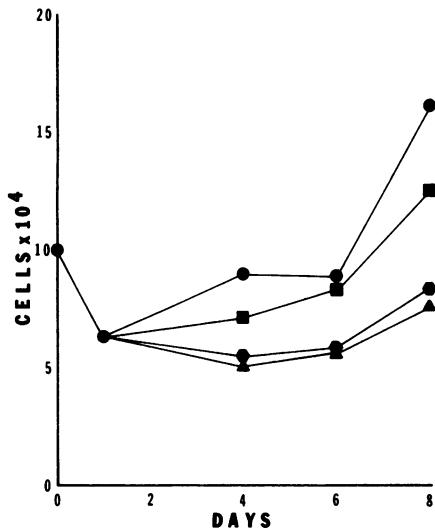


FIG. 1. Effects of Aa strain 652 on keratinocyte proliferation. Twenty-four hours after plating, human keratinocytes were incubated with medium containing 0 (●), 0.1 (■), 1.0 (◐), or 10.0 (▲) µg of 652 SE per ml for 7 days. All medium was changed on day 4. Cells were harvested at 48-h intervals and assayed by direct counts as described in the text. Each point represents the mean of duplicate cultures. The data shown represent three separate experiments.

EDTA, and harvested. The amount of incorporated label on the filter of the harvester was measured by liquid scintillation spectrometry.

Aa SE was a potent inhibitor of keratinocyte proliferation. This could be determined by direct observation of cultures, by cell counts, or by incorporation of [³H]thymidine. Differences in cell numbers could be observed 3 days after the addition of test agent (Fig. 1). There was only a slight increase in cell growth in cultures incubated with 1.0 or 10.0 µg of SE per ml by 7 days after the addition of test agent, whereas there was nearly a threefold increase in the number of control cells. SE at 0.1 µg/ml did not appear to significantly affect cell proliferation, whereas 1.0 and 10 µg/ml appeared to differ little in their inhibitory effects. Examination of culture medium in test cultures and viability determination with 0.4% trypan blue failed to demonstrate a direct cytotoxic effect of SE.

SE had a similar effect on [³H]thymidine incorporation (Fig. 2). We have previously demonstrated that lethally irradiated 3T3 cells do not incorporate [³H]thymidine significantly above background level by 4 days postirradiation (5). Incorporation of [³H]thymidine decreased during the 4-day test period in test cultures, whereas incorporation by controls increased significantly. Differences between test cultures and controls were highly significant, but there was

no significant difference between cultures challenged with 10.0 or 1.0 µg of SE per ml.

Heating at 90°C for 30 min destroyed the inhibitory effect of SE (Fig. 3). The addition of heated test agent at the time of plating to suspended cells or to adherent cells 72 h after plating did not inhibit cell proliferation as measured by the incorporation of [³H]thymidine.

Reversibility experiments showed that SE was quickly bound to keratinocytes and that the inhibitory effect could not be reversed even after thorough washing and reincubation in control medium (Fig. 4). Significant inhibition of [³H]thymidine incorporation was observed in cultures incubated with SE for as little as 10 min. Highly significant ($P < 0.001$), irreversible inhibition occurred after exposure for 24 h.

Our data indicate that an epithelial inhibitory factor can be added to the pathogenic properties of *Actinobacillus* sp. This confirms the previous findings of Birkedal-Hansen et al. (J. Dent. Res. 61:192, 1982) and Kamen (J. Dent. Res. 62:247, 1983).

The inhibitory factor appears to be different from the leukotoxin, since strain 652 is nonleukotoxic (3). Whether it is the same as the fibroblast-inhibitory factor described by Shenker et al. (14) and Stevens et al. (J. Dent. Res. 61:193, 1982) is not known. The fibroblast factor has a molecular weight of 150,000 and is chromatographically distinct from the lymphocyte-suppressive factor and the leukotoxin (14). The effects of the SE described here suggest that it is

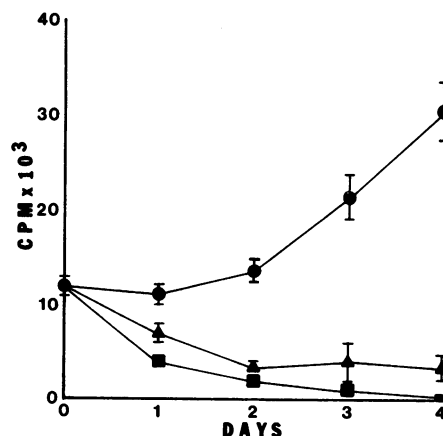


FIG. 2. Effects of Aa strain 652 on keratinocyte proliferation. Seventy-two hours after plating (day 0), human keratinocytes were incubated with medium containing 0 (●), 1.0 (▲), and 10.0 (■) µg of 652 SE per ml. DNA synthesis was assayed by incorporation of [³H]thymidine. Each point represents the mean value \pm standard error of triplicate assays. Data from all test cultures differed significantly ($P < 0.001$) from day 2 (Student's *t* test).

similar to the fibroblast-inhibitory factor. The SE was active in the range of 1.0 µg of protein per ml, was heat labile, bound quickly, and was not reversible after repeated washing.

We have previously reported keratinocyte inhibition from SE of *Bacteroides asaccharolyticus* (now *B. gingivalis*) (5) and *Capnocytophaga* sp. (Kamem, J. Dent. Res. 61:193, 1982), but high concentrations (50 to 100 µg of protein per ml) were required for significant effects. All strains of Aa examined so far are more potent, showing significant activity in the range of 1 to 10 µg of protein per ml. This seems more likely to be related to concentrations of bacterial products found in vivo.

Alterations of epithelial growth and/or function have profound effects on periodontal tissues. Plaque-derived products produce widening of intercellular spaces (12, 13, 16), resulting in increased permeability to bacterial antigens. Thinning and ulceration of pocket epithelium have been repeatedly shown to precede conversion of stable to aggressive lesions (2, 4, 10, 11). It is possible that infection with sufficient numbers of an epithelio-toxin such as Aa may be a "trigger" which disrupts the barrier of the junctional epithelium, resulting in rapid destruction of local tissues. Indeed, the capacity of this organism to violate tissue barriers has been demonstrated in a recent report (L. A. Christerson, B. Albin, J. Zamben, J. Slots, and R. J. Genco, J. Dent. Res. 62:198, 1983) showing the

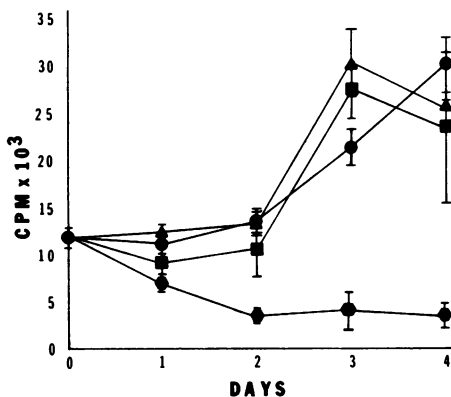


FIG. 3. Effects of heating on Aa 652 SE. Human keratinocyte cultures were incubated with 0 (●) or 10 µg of SE per ml. SE heated at 90°C for 30 min (▲) or unheated (●) was added to cultures 72 h after plating, when cells were attached to the culture well. Heated SE was also added to suspended cells at the time of plating (■). DNA synthesis was assayed by measuring incorporation of [³H]thymidine. Each point represents the mean ± standard error of triplicate experiments. There were no significant differences between heated samples and controls (Student's *t* test).

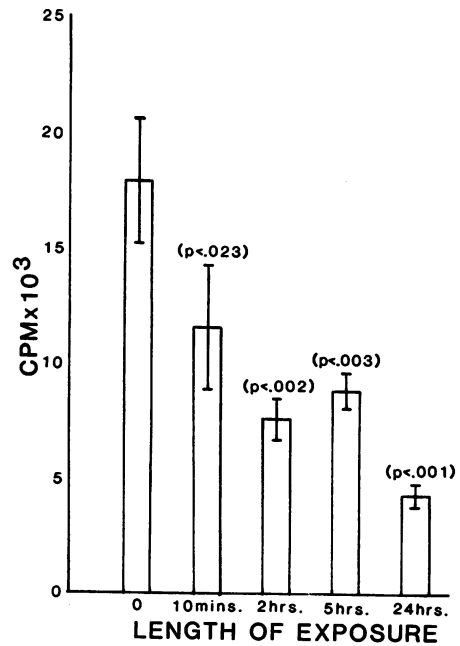


FIG. 4. Effect of washing on ability of Aa to inhibit keratinocyte proliferation. After 72 h of incubation, 10 µg of SE per ml was added to keratinocyte cultures. After the times indicated, cultures were washed four times with saline. On day 6 after cells were plated, DNA synthesis was assayed by measuring the incorporation of [³H]thymidine. Each point represents the mean ± standard error of triplicate experiments.

presence of *Actinobacillus* sp. in the connective tissue of juvenile periodontitis lesions.

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