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# Title
LYTIC EFFECTS OF SERUM AND MONONUCLEAR LEUKOCYTES ON ORAL EPITHELIAL CELLS IN RECURRENT APHTHOUS STOMATITIS

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# Abstract
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LYTIC EFFECTS OF SERUM AND MONONUCLEAR LEUKOCYTES
ON ORAL EPITHELIAL CELLS
IN RECURRENT APHTHOUS STOMATITIS

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RUNNING HEAD: Immune Lysis of Oral Epithelial Cells in RAS

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ABSTRACT

A radioisotope-release assay, utilizing $^{51}$Cr-labeled epithelial cells derived from nonkeratinizing oral mucosa, was developed to investigate in vitro cytolytic reactions correlating with recurrent aphthous stomatitis (RAS). The cytolytic effects of sera and mononuclear leukocytes from patients in the early stage of ulceration were compared with those from matched RAS-negative control subjects. RAS sera induced significantly more cytolysis than did matched control sera. Heating the RAS or control sera for 30 minutes at 56°C abrogated their cytotoxic activity. RAS mononuclear leukocytes, like their matched controls, showed no significant direct cytotoxicity. Heat-inactivated RAS or control sera acting in concert with RAS or control mononuclear leukocytes showed no consistent cytolytic effects. However, the heat-inactivated sera of some RAS patients, when combined with autologous mononuclear leukocytes, induced significantly more cytolysis than did either component acting alone. Thus heat-labile humoral factors and, in some cases, mononuclear leukocytes acting in concert with heat-stable serum factors are implicated in RAS-associated in vitro cytolytic reactions. These findings suggest that the effector mechanisms of such reactions include both complement-mediated and antibody-dependent cell-mediated cytotoxicity.
INTRODUCTION

Recurrent aphthous stomatitis (RAS), a common multifactorial inflammatory condition peculiar to man, is characterized by intermittent or continuous ulceration of nonkeratinizing oral mucosa (1-6). Broad spectra of oral involvement, associated extraoral findings, and response to treatment suggest that RAS is a heterogeneous condition which may represent the oral manifestations of a variety of diseases (7).

Nonetheless, during the last twenty years investigators have observed specific abnormalities related to both humoral (8-12) and cellular (3,10,13-19) immune responses in RAS patients; and, on this basis, some have proposed that the condition is primarily immunopathologic in origin. Dolby (15), using cell suspensions produced by trypsinizing specimens of keratinizing oral mucosa, observed the reduced survival of oral epithelial cells co-cultured for 24 hours with allogeneic RAS lymphocytes. This finding, based on the exclusion of supravital dye by viable epithelial cells, was interpreted to indicate direct RAS lymphocyte-mediated cytotoxicity of probable pathogenic significance. Rogers et al. (17), using similar methodology, have confirmed Dolby's observation and agreed with his interpretation.

A primary aim of this study was to determine whether the finding of RAS-associated in vitro oral epithelial cytolysis could be confirmed with an assay employing epithelial cells derived from
the more clinically-relevant nonkeratinizing oral mucosa, and a more objective, radioisotope-release method of assessing cell damage (20-22). If so, we proposed to use such an assay system to study the cytolytic effector mechanisms in more detail.

MATERIALS AND METHODS

Patients

Eleven patients, four males and seven females, experiencing at least six episodes of aphthous stomatitis per year, were selected for study. Ten had manifestations of minor RAS according to Cooke’s (5) classification. One of these (patient #4) gave a history of recurrent genital ulceration and has been previously diagnosed as having an incomplete form of Behcet’s Syndrome. Patient #2 had oral manifestations of major RAS and gave a history of associated extraoral findings. All patients were found to have CBC, serum iron, total iron binding capacity, whole blood folate, and serum vitamin B_{12} values within normal limits. Each patient was tested in conjunction with an age and sex-matched control subject determined to be RAS-negative by both history and examination. None of the patients or control subjects were being treated with any systemically administered anti-inflammatory medications or antibiotics at the time of testing.

Preparation of sera and mononuclear leukocytes

When an RAS patient reported with one or more ulcers of less than three days duration, blood specimens were collected asepti-
cally by antecubital venipuncture from both patient and matched control subject, and were processed immediately for assessment of cytolytic effects. RAS and control sera were separated from clotted venous blood, and an aliquot of each was heated for 30 min at 56°C. Heat-inactivated fetal calf serum (FCS) was obtained commercially (GIBCO Laboratories, Grand Island, NY). RAS and control mononuclear leukocytes were isolated from heparinized (15 U/ml) venous blood on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) gradients. They were washed twice in Hanks' balanced salt solution (HBSS) and resuspended in tissue culture medium 199 (GIBCO Laboratories, Grand Island, NY). Leukocyte viability, assessed by exclusion of 0.2% trypan blue dye, was routinely found to be in excess of 90%. Each suspension was adjusted to a concentration of 8x10⁶ viable cells per ml.

Preparation of suspensions of oral epithelial cells

Specimens of clinically normal nonkeratinizing oral mucosa were aseptically excised from patients undergoing surgical procedures involving the buccal mucosa, usually flap-releasing incisions made to facilitate the removal of impacted third molars. The excised tissue was rinsed in saline and placed immediately into cold, calcium and magnesium-free HBSS containing .05% trypsin and .02% EDTA (GIBCO Laboratories, Grand Island, NY). The specimen was trimmed to remove as much subepithelial tissue as possible, and then cut into small fragments. The minced tissue
was transferred to a solution of 0.25% trypsin in calcium and magnesium-free HBSS (GIBCO Laboratories, Grand Island, NY) and agitated in a fluted flask at 37°C for 2 hours. Batch harvesting of cells released from the mucosal tissue fragments was carried out by decantation at 30 min intervals. The harvested cell suspensions were diluted with HBSS supplemented with 20% heat-inactivated FCS and maintained at 4°C until termination of the trypsinization procedure. They were then washed twice in cold HBSS and resuspended in medium 199. Epithelial cell viability, assessed by exclusion of 0.04% trypan blue dye, was routinely found to be in excess of 75%.

**Radio-labeling of epithelial target cells**

At least 2×10⁵ oral epithelial cells, suspended in 1.0 ml medium 199 supplemented with 10% FCS, were incubated with 200 μCi of [⁵¹Cr] sodium chromate (New England Nuclear, Boston, MA) for 90 min at 37°C in a 5% CO₂ atmosphere. They were then washed twice in HBSS, resuspended in medium 199 with 10% FCS, and allowed to release tenuously incorporated label for 60 min at 37°C in 5% CO₂. Following a final washing, the cells were resuspended in medium 199 to a concentration of 5×10⁴ viable cells per ml.

**Cytolysis assay**

Each assay of ⁵¹Cr-release from labeled oral epithelial cells tested the cytolytic effector activities of the sera and/or leukocytes of a single matched pair of subjects. Ten experimental
groups, as indicated in Table 1, were tested. The maximum releasable $^{51}$Cr contained in the target cells was determined by incubation in a 0.5% solution of the membrane-lysing agent sodium dodecyl sulfate (SDS). The spontaneous release of label from the target cells was determined by incubation in medium 199 with 25% heat-inactivated FCS. Preliminary studies indicated that an effector:target ratio greater than 40:1 was necessary for detecting ADCC-like activity. The maximum and spontaneous radioisotope-release groups and the ten experimental groups were set up in triplicate in a 96-well round bottom microtiter plate (Linbro Scientific, Hamden, CT). Each well contained a total volume of 200 μl: 100 μl of $^{51}$Cr-labeled target cell suspension ($5 \times 10^3$ cells); 50 μl of the appropriate serum (FCS, RAS, or control); and 50 μl of medium 199 (in the cases of the spontaneous release and other serum-only groups), or 1% SDS (in the case of the maximum release group), or the appropriate mononuclear leukocyte suspension ($4 \times 10^5$ RAS or control cells). The plate was incubated for 12 hours at 37°C in 5% CO$_2$, and then harvested with a supernatant collection system (Skatron, Inc., Sterling, VA). The supernatants were analyzed for $^{51}$Cr content in a gamma radiation counter (Model 1185, Tracor Analytic, Des Plaines, IL). The measurement (in cpm) of radioisotope released in each group was expressed as the mean of the triplicates minus background. The percentage cytolysis within each experimental group was then calculated as follows:
Experimental - Spontaneous

Percentage Cytolysis = \[
\frac{\text{Experimental} - \text{Spontaneous}}{\text{Maximum} - \text{Spontaneous}} \times 100
\]

Statistical analysis

The differences between paired experimental groups were subjected to both parametric (paired-sample t test) and nonparametric (Wilcoxon signed-rank sum test) analysis. A result of P<.05 was considered significant.

RESULTS

Trypsinized oral epithelial cells were found to incorporate and retain sufficient $^{51}$Cr to permit the assessment of their specific lysis by the radioisotope-release method. The maximum release group supernatants were found to have activities on the order of $10^4$ cpm. And in nearly all cases, the spontaneous release of radioisotope during the 12 hour assay was less than 30% of maximum. Data were rejected and assays repeated on two occasions when the spontaneous release exceeded 30% of maximum.

Our data showed good precision and reproducibility. In each assay, the triplicate supernatant radioactivity measurements for each group were found to deviate less than 5% from their mean. Three matched pairs of subjects, incompletely tested during the assay-development phase of our work, were subsequently retested for inclusion in the study. The results derived on retesting were in close agreement with the limited, corresponding preliminary results.
In three assays, problems encountered in the preparation of the mononuclear leukocyte suspensions necessitated the deletion of groups containing RAS and/or control leukocytes. The calculated percentage cytolysis values for all other experimental groups are tabulated in Table 1.

**Direct cell-mediated cytolysis**

Groups V (n=8) and VI (n=9), containing heat-inactivated FCS with control and RAS mononuclear leukocytes, respectively, showed no appreciable cytolysis. Thus, we detected little or no direct cell-mediated cytotoxicity, and no significant difference between RAS and control mononuclear leukocytes as effectors in this assay system.

**Serum-mediated cytolysis**

Groups I (n=11) and II (n=11), containing control and RAS serum, respectively, showed appreciable cytolysis in most instances, with that in the RAS group being much greater than the matched control in 9 of 11 cases (Fig. 1). The mean of the difference values for the matched pairs was approximately 17. Both paired-sample t and signed-rank sum tests reveal the differences between RAS and control sera to be highly significant (P < .001). The absence of cytolysis in Groups III (n=11) and IV (n=11), containing heat-inactivated control and RAS serum, respectively, indicates that the observed serum-mediated cytolysis is heat-labile, a finding suggestive of complement-mediated cytolysis.
Serum-dependent cell-mediated cytolysis

Groups IX (n=6) and X (n=6), containing heat-inactivated control serum with control and RAS mononuclear leukocytes, respectively, showed no appreciable cytolysis. Likewise, Group VII (n=8), containing heat-inactivated RAS serum and control leukocytes, showed little or no cytolysis. However, Group VIII (n=9), containing heat-inactivated RAS serum and autologous leukocytes, showed considerable cytolysis, suggestive of antibody-dependent cell-mediated cytotoxicity (ADCC), in two of nine cases (patients #1 and #4). Comparison of Group VIII with Group VI, containing RAS leukocytes and heat-inactivated FCS, (Fig. 2) by both paired-sample t and signed-rank sum tests indicates that the differences between the two are not significant (.10 < P < .20).

DISCUSSION

Unlike previously published studies of RAS-associated in vitro oral epithelial cytolysis (15-17), our results suggest that humoral factors play an important role in this phenomenon. However, the natures of the effector mechanisms and targets of the cytolytic reactions await further clarification. A specificity study employing RAS-irrelevant target cells, and a study of the effect of complement reconstitution on the cytolytic activity of heat-inactivated sera would help to determine whether specific antibody and complement are involved.

The allogeneic relationship between humoral effectors and
epithelial target cells could be a factor in the observed serum-mediated cytolysis. However, in two pilot assays performed using an autologous system, we observed differences between RAS and control sera similar to those observed using allogeneic target cells. In view of this and the delayed healing of oral wounds observed in RAS patients (23), we opted to use an allogeneic system. Since both RAS and control subjects were allogeneic with respect to the target cells employed, any allogeneic effects would be randomly distributed among them. The finding of appreciable control serum-mediated cytolysis in nine of eleven cases may, in part, be a reflection of such randomly occurring reactions. However, the significant difference in magnitude between RAS and control serum-mediated cytolysis cannot be similarly explained.

The ADCC-like activity observed in the blood of some of our patients, while not a statistically significant correlate of early ulceration in this small sample, suggests another possible mechanism of action for humoral mediators of oral epithelial cytolysis. Given the heterogeneous nature of RAS patients, we believe that the two patients whose blood showed markedly elevated activity of this type may represent a noteworthy subpopulation. Furthermore, the relatively infrequent observations of such activity in our sample could be misleading since the demonstration of ADCC may be more dependent on the timing of specimen collection than is that of exclusively serum-mediated cytolysis. Indeed, Greenspan et al.
(24), using an RAS-irrelevant target cell coated with specific antibody, found significantly greater peripheral blood ADCC effector cell activity during early ulceration in 8 of 19 matched RAS and control pairs studied. They proposed that such activity might be an inverse function of the quantity of humoral blocking factors adsorbed to the potential effector cells in vivo. And the latter, they suggested, might be a function of the patient's stage of active disease at the time of blood collection.

Our findings do not support the reported correlation of direct cell-mediated cytolysis with active RAS (15-17). Significant differences in methodology might account for this discrepancy. In addition to using different target cells and a different method of assessing cell damage, we employed greater effector:target cell ratios and a shorter reaction time. The shorter reaction time might exclude the participation of direct monocyte-mediated cytolysis and other longer term cytolytic or cytostatic effects. It might also have resulted in a more limited recuperation of target cell surface antigens following experimental manipulation. Increasing the reaction time to 18-24 hours to enhance the sensitivity of our assay generally resulted in an unacceptably high spontaneous release of radioisotope from the labeled target cells. Our results do agree with those of Reimer et al. (25), who used autologous oral epithelial target cells derived from nonkeratinizing mucosa in a 4 hour $^{51}$Cr-release assay. These investigators
found no significant difference in direct cell-mediated cytolysis between samples of RAS patients and matched control subjects.

Consideration of the biological significance of our findings must take into account certain limitations imposed by the experimental design. First, our observations were limited to the in vitro behavior of experimentally manipulated cells and humoral effectors. Second, the cells and humoral factors assayed for cytolytic effector activity were obtained from samples of peripheral blood rather than from sites of developing lesions, which in the majority of cases remain localized to the oral mucosa. And third, the samples were collected subsequent to the induction of lesions, from patients with histories of longstanding intermittent or continuous oral epithelial breakdown.

Thus, the absence of detectable direct cell-mediated cytolysis in our in vitro system might be an indication that direct cell mediated killing plays little or no role in the pathogenesis of RAS. Or it could be a reflection of altered effector cell function in vitro, target cell alterations resulting from experimental manipulation, peripheral depletion of effector cells resulting from recruitment at developing lesion sites, and/or blockage of antigen-specific receptors on the effector cell surface by humoral factors adsorbed in vivo. Regardless, our findings and those of Reimer et al. (25), as well as the reported reduction in numbers of OKT3-positive cells in the peripheral blood and developing
lesions of RAS patients (26), make suspect the hypothesis that epithelial cytolysis mediated by cytotoxic T lymphocytes is the principal mechanism of ulcer formation in RAS.

Similarly, our finding of enhanced serum-mediated cytolysis might be an indication that humoral immunity plays an important role in the pathogenesis of a significant number of cases of RAS. Or it could be a reflection of the altered behavior in vitro of autoantibodies or other humoral factors present as an epiphenomenon of chronic oral ulceration. The latter is more consistent with evidence asserting no correlation between circulating antimucosal antibody titers and clinical disease activity (14,19). However, immunohistochemical observations of significant RAS associated IgG deposits in the spinous cell layer (10) and basement membrane zone (11) of the oral mucosa, and of increased numbers of OKM1-positive effector cells in the peripheral blood and developing lesions of RAS patients (26), lend support to the hypothesis that serum factors, sometimes acting in concert with mononuclear effector cells, play a role in the pathogenesis of RAS.
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\(^a\)Serum

\(^b\)Heat-inactivated serum (30 min at 56°C)

\(^c\)Mononuclear leukocytes
LEGENDS

Fig. 1. Percentage lysis of nonkeratinizing oral epithelial cells incubated for 12 hours in medium 199 with 25% serum from either RAS patients or age and sex-matched control subjects. Numbers above the bars indicate the amount by which the RAS exceeds the control serum value for each matched pair.

Fig. 2. Percentage lysis of nonkeratinizing oral epithelial cells incubated for 12 hours in medium 199 with RAS mononuclear leukocytes (effector:target = 80:1) and 25% heat-inactivated RAS or fetal calf serum. Numbers above the bars indicate the amount by which the RAS exceeds the FCS value for each pair.
ACKNOWLEDGMENTS

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REFERENCES
