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Antibody-based diagnostic for ‘refractory’ periodontitis

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Abstract

Objective—About 10–15% of US adults are ‘refractory’ to therapy for chronic periodontitis. Recently, studies suggest that these patients have elevated lysine decarboxylase activity in the sulcular microbiota. The aim of this study was to determine whether an elevated IgG antibody response to lysine decarboxylase, alone or with antibody to other bacterial antigens and baseline clinical measurements, would predict ‘refractory’ patients with high accuracy.

Methods—Chronic periodontitis patients were treated using scaling and root planing (SRP) followed by maintenance SRP and 3-monthly re-examinations. If there was a loss of mean full mouth attachment or more than three sites appeared with >2.5mm new loss within a year, the subjects were re-treated (modified Widman flap surgery and systemically administered tetracycline). If attachment loss as above recurred, the subjects were ‘refractory’. Baseline clinical measurements and specific antibody responses were used in a logistic regression model to predict ‘refractory’ subjects.

Results—Antibody to a peptide portion of lysine decarboxylase (HKL-Ab) and baseline bleeding on probing (BOP) prevalence measurements predicted attachment loss 3 months after initial therapy [pIAL = loss (0) or gain (1)]. IgG antibody contents to a purified antigen from *Actinomyces* spp. (A-Ab) and streptococcal d-alanyl glycerol lipoteichoic acid (S-Ab) were related in ‘refractory’ patients ($R^2 = 0.37$, $p < 0.01$). From the regression equation, the relationship between the antibodies was defined as linear (pLA/S-Ab = 0) or non-linear pLA/S-Ab = 1). Using pLA/S-Ab, pIAL and age, a logistic regression equation was derived from 48 of the patients. Of 59 subjects, 37 had 2–4mm attachment loss and were assigned as ‘refractory’ or successfully treated with 86% accuracy.

Conclusion—HKL-Ab facilitated an accurate prediction of therapeutic outcome in subjects with moderate periodontitis.

Keywords

actinomyces; antibodies; D-alanyl glycerol lipoteichoic acid; logistic regression; lysine decarboxylase; periodontitis; refractory

Chronic periodontitis (Armitage 1999) occurs when ‘classical’ periodontopathogens, such as members of the ‘orange’ and ‘red’ bacterial complexes described by Socransky et al. (1998),

increase in numbers and proportions in gingival sulci or periodontal pockets. Therapy lowers the numbers and/or proportions of these and other bacteria and prevents attachment loss except in about 10–20% of patients, referred to by some investigators as ‘refractory’ (Haffajee et al. 1997, Colombo et al. 1998a). The microbiota from ‘refractory’ patients includes more milleri streptococci (*Streptococcus anginosus*, *S. constellatus*, *S. intermedius*) *Capnocytophaga ochracea* and bacterial taxa uncommon in sulci, and less *Actinomyces naeslundii* and ‘classical’ periodontopathogens (Colombo et al. 1998a; 1999).

Recently, the gingival microbiota were found to inhibit the growth of cultured mammalian cells by depleting them of lysine—a nutritionally essential amino acid in mammals (Levine et al. 2001). *Eikenella corrodens* and *Capnocytophaga* spp. from healthy or diseased sulci (Socransky et al. 1998) are major sources of the responsible enzyme, lysine decarboxylase (Holmes et al. 1995). This enzyme is proposed to inhibit the turnover of the most coronally situated, dentally attached (DAT) cells (Levine et al. 2001), junctional epithelial basal cells that lie remote from capillaries (Salonen 1994). These cells are nourished by an interstitial fluid transudate that crosses the base of healthy sulci (Griffiths et al. 1997). When lysine decarboxylase converts lysine in this transudate to cadaverine and carbon dioxide, the DAT cells are proposed to starve from lack of lysine. The cells stop proliferating, causing the intermediate layer of junctional epithelium to become attenuated and weaken the epithelial barrier. The gingival crevicular fluid (GCF) exudate is induced and it enters the gingival sulcus, providing nutrients for ‘classical’ periodontopathogens (Socransky et al. 1998, Daly & Highfield 1996, Ramberg et al. 1996). The ‘classical’ periodontopathogens maintain the DAT cell growth inhibition by producing cytotoxic end-products of amino acid metabolism—short chain fatty acids, ammonia and hydrogen sulfide (Niederman et al. 1997). If the lysine decarboxylase activity in sulci is low, the influx of lysine in GCF will allow the DAT cell turnover to resume. Maintaining oral hygiene and low levels of bacteria in the sulcus or pocket will continuously retard the accumulation of ‘classical’ periodontopathogens and stop attachment loss. Conversely, if enzyme activity is high, DAT cell turnover will remain inhibited despite a reduced prevalence of ‘classical’ periodontopathogens (Colombo et al. 1999). These studies suggest that ‘refractory’ patients have elevated antibodies to lysine decarboxylase, an enzyme that is foreign to mammals (Papes et al. 1999).

Elevated serum antibody responses to many different oral bacteria (Colombo et al. 1998b), or selected antibodies and bacterial prevalences, distinguish ‘refractory’ from successfully treated patients (Colombo et al. 1999). Many human sera possess IgG antibodies that precipitate with an ornithine-rich antigen present in many *Actinomyces* spp. (Levine & Movafagh 1986, Levine & Miller 1991). The reduced prevalence of *A. naeslundii* serotype-2, a major source in plaque, suggests that refractory patients might have fewer actinomyces antibodies (A-Ab) that associate with effective oral hygiene (Levine et al. 1998) like increased *A. naeslundii* serotype-2 (Haffajee et al. 1997). Other antibodies precipitate d-alanyl esters attached to glycerol lipoteichoic acid (Levine 1982, Levine & Movafagh 1984). This latter specificity is always to d-alanine esters of LTA, irrespective of source. Increased milleri streptococci may therefore induce elevated amounts of these antibodies (S-Ab) in ‘refractory’ patients. The aim of this study was to determine whether an elevated IgG antibody response to lysine decarboxylase, alone or with antibody to actinomyces and d-alanyl LTA antigens and baseline clinical measurements, would predict the ‘refractory’ patients.

Material and methods

Patients and clinical data

Periodontitis patients were attending or referred to the Clinical Center for Periodontal Research at The Forsyth Institute, Boston. The selection, examination and treatment of these patients have been described in detail (Colombo et al. 1998a,b). Fifty-nine patients who consented to

receive treatment and follow-up examinations according to NIH guidelines were selected. They were healthy, at least 28 years of age and new or returning for maintenance therapy. They had at least 20 teeth present and exhibited at least eight sites with a pocket depth of >4mm and an attachment loss >3 mm. They were not pregnant or undergoing antibiotic therapy, or exhibiting any systemic condition that might affect treatment. Clinical measurements were obtained from six sites around each tooth: dichotomous measures of bleeding on probing and supragingival plaque accumulation, and measures of pocket depth (mm) and attachment loss from the cemento-enamel junction (mm). The examiners obtaining these measurements were calibrated against each other and did not provide therapy.

At the initial (baseline) visit, a 10-mL sample of venous blood was obtained for antibody measurements. Therapy consisted of full mouth scaling and root planing (SRP) under local anesthesia and was followed by instruction in home care procedures. Beginning 3 months after completing therapy, and continuing every 3 months for a full year, the subjects were recalled for reexamination, followed by maintenance SRP and reinforcement of home care procedures. Disease progression was defined as: (i) *mean* attachment loss within the 1-year period, as determined by the full mouth attachment level measurements taken pre- and post-therapy, or ii) more than three sites with >2.5mm new attachment loss even if *mean* attachment gain was present. Subjects found to have disease progression received further treatment consisting of modified Widman flap surgery and systemically administered tetracycline at the dosage of 250mg, 4 times a day for 28 days. These subjects continued to be monitored and to receive maintenance scaling and reinforcement of home care procedures every 3 months for an additional year. Successfully treated subjects had *mean* attachment level gain and not more than three sites with >2.5 mm new attachment loss 1 year after either SRP alone or the second phase therapy (surgery with tetracycline). 'Refractory' subjects demonstrated disease progression after both SRP and the second phase therapy.

Twenty-six control subjects were selected from patients seeking treatment at the University of Oklahoma College of Dentistry. The same guidelines were used, except that the subjects were all >20 years of age and had little evidence of attachment loss. They provided a 10-mL sample of blood at baseline and follow-up visits. The clinical measurements were obtained as described above and averaged from four sites around each of six marker teeth (Stallard et al. 1969). Therapy consisted of full mouth scaling and root planing (SRP) without local anesthesia and took no more than two visits a week apart. Patients were dismissed with oral and written instructions to follow proper home care procedures carefully. A single follow-up examination was performed after 5–7 weeks.

Antigen purification

S. intermedius, *S. constellatus* and *S. anginosus* are the milleri streptococci (Piscitelli et al. 1992) and contain d-alanine esterified to glycerol lipoteichoic acid (d-alanyl LTA) like *S. mutans* strain GS5 (Hogg et al. 1997). D-Alanyl LTA was purified from *S. mutans* GS5 as described previously (Levine 1982). The bacteria were grown in trypticase soy broth (BBL, Cockeysville, MD, USA) for 4 days. The spent culture fluid was concentrated 50-fold using a Spiral Regenerated Cellulose Column (Millipore Corp., Bedford, MA, USA) and passed over a Sephacryl S200 column in 0.05M Na acetate pH6.0 containing 0.4M NaCl (Levine 1982). D-Alanyl glycerol LTA-containing fractions were detected by immunoelectrophoresis, pooled, salt exchanged into 0.05 M acetate buffer pH 6.0, and eluted from an AGMP1 anion exchange resin (Biorad Laboratories, Hercules, CA, USA) with a NaCl gradient to 1.0M.

Various *Actinomyces* spp. possess an ornithine-rich antigen (Levine & Bush 1981, Levine & Miller 1991) which was purified from *A. naeslundii* ATCC 19246 (Levine & Movafagh 1986), a major component of the sulcular microbiota (Haffajee et al. 1997). After growth on trypticase soy broth, the spent culture medium was concentrated 10-fold using Spiral

Regenerated Cellulose. Antigen was precipitated by adding a 4-fold excess of ethanol (v/v), redissolved in 0.06m Tris HCl pH8 and eluted from a DEAE cellulose column with 0.20m NaCl. Antigen was detected by immunoelectrophoresis, the fractions concentrated to 20.0mL and applied to phosphocellulose (Whatman International Ltd, Maidstone, England) in 5mm Na formate pH3.0. After elution with Na formate to 5.0m, antigen-containing fractions were pooled, dialyzed into distilled water and separated over Sephacryl S200 in 0.15m NaCl buffered with 0.05m Tris HCl pH 8 (Levine & Movafagh 1986).

A peptide sequence at the catalytic center of *E. corrodens* lysine decarboxylase, ETHSTHKLLAAF-SQA, is identical in the inducible lysine decarboxylase from *Escherichia coli* and in lysine decarboxylases from *Hafnia alveoli*, *Moritella japonica* and *Vibrio vulnii* except for glutamine (Q) at position 3. In lysine/ornithine/arginine decarboxylase from *Pseudomonas aeruginosa*, the sequence is identical except for methionine (M) at position 8 (Altschul et al. 1997). The *E. coli* constitutive enzyme and *Salmonella typhimurium* inducible enzyme have three or more residues changed in the homologous sequence. Ornithine and arginine decarboxylases and all other proteins differ at more than three residues. The sequence was named HKL peptide because of its central sequence, and it was synthesized by standard methods described previously (Levine et al. 2001). Antibodies reacting with HKL peptide seem likely to react exclusively with lysine decarboxylase.

Antibody detection

Immunolon plates (Dynex Technologies, Chantilly VA, USA) were coated with 0.1-mL aliquots of 0.5m Na carbonate buffer pH9.6 containing 0.1mg/mL of purified actinomyces antigen, or 1.0mg/mL of HKL peptide. Alternatively, they were coated with 0.1-mL aliquots of 0.05m Na acetate buffer pH6.0 containing 0.1mg/mL of d-alanyl glycerol LTA. Blocking buffer, 0.5% w/v bovine albumin in physiological saline buffered with phosphate pH7 (PBS) or acetate pH6 for d-alanyl-LTA-coated wells, was added for 2h. The wells were then washed with PBS and incubated at 4°C overnight. Human serum from the respective clotted blood samples was diluted to 0.1mL with PBS containing 1.0% w/v albumin and 0.05% v/v Tween-20 (Sigma Chemical Co., St. Louis, MO, USA) and added to the wells for 1 h. After washing with PBS containing 0.05% v/v Tween-20, antihuman IgG alkaline phosphatase conjugate (0.1mL diluted 3000-fold with PBS containing 0.5% w/v albumin and 0.1 w/v percentage azide) was added for 30min. The wells were washed again, and developed with nitrophenyl phosphate as described by the manufacturer (Sigma). The color of the nitrophenol produced from each standard serum was graphed against the concentration of antigen-specific IgG in that serum, obtained by immunoprecipitation with the appropriate purified antigen (Levine 1982, Levine & Movafagh 1986). From these standard curves (Fig. 1), the concentration of the respective, and specific, IgG in each patient's serum was estimated.

The respective, standard human sera contained 0.43mg/mL of IgG to actinomyces antigen or 0.23mg/mL of IgG to D-alanyl glycerol LTA. The antibody to HKL peptide (HKL-Ab) was 0.36 mg/mL in goat antiserum. The absorbance from HKL peptide-coated wells after reaction with goat antiserum and antigoat IgG conjugate was compared with the reaction to a standard human serum and antihuman IgG conjugate. The results (not shown) indicated that the standard human serum contained 1.6 µg of HKL-reactive IgG per mL.

Relating antibody response to bacterial colonization

A. naeslundii and *E. corrodens* are, respectively, the major sources of actinomyces and lysine decarboxylase antigens in the sulcular microbiota, whereas D-alanyl LTA has no single major source. Boston subjects provided subgingival plaque from the mesiobuccal surface of each tooth from which the prevalence of *A. naeslundii* and *E. corrodens* were obtained using DNA probes and checkerboard DNA-DNA hybridization (Colombo et al. 1998a). The bacterial

colonization prevalence (proportion of tooth surfaces with detectable bacteria) at baseline and after 3 months was measured. The appropriate antibody content was regressed against the percent change in colonization prevalence.

Using antibody responses to predict 'refractory' patients

The therapeutic outcome of mild, chronic periodontitis is usually satisfactory (Haffajee & Socransky 1986, Cobb 1996) and none of four Boston patients with mild disease (<1.6 mm mean periodontal attachment lost at baseline) demonstrated disease progression after therapy. Thus, subjects were unlikely to be refractory to therapy unless they had >1.6 mm mean attachment loss at baseline.

Therapeutic outcome is a non-dichotomous variable and is predicted by using logistic regression analysis (Hall & Round 1994). Two outcomes were used: whether mean attachment was lost after initial therapy (initial attachment loss, IAL) and whether the patient was 'refractory' (disease progression despite therapy). Stepwise logistic regression was run using SAS software version 6.12 (SAS Institute Inc., Cary, NC, USA) and IAL or 'refractory' as the dependent variable. Equations were derived that described the probability of initial attachment loss (pIAL), or of disease progression within 12 months of all therapy being completed (pRefractory). Wald's chisquare was used to determine the upper and lower 95% confidence limits of the coefficient for each variable, which was retained in the equation if the odds ratio was greater or less than 1 with a probability (p) <0.15. The significance of the prediction model was derived from the likelihood ratio and Goodness-of-Fit test (Hosmer & Lemeshaw 1989).

$$pIAL = \frac{1}{(1 + e^{-(-5.4948 + 0.0237 * BOP + 2.3653 * HKLAB)})} \quad (\text{Eqn. A})$$

$$pRefractory = \frac{1}{(1 + e^{-(-5.0047 - 2.1285 * pIAL - 1.5211 * pLA/S - 0.0545 * Age)})} \quad (\text{Eqn. B})$$

$$pRefractory = \frac{1}{(1 + e^{-(-0.9508 + 0.0316 * BOP)})} \quad (\text{Eqn. C})$$

Eqn. A was obtained by stepwise regression of all the variables in Table 2. Predicted initial attachment losers and gainers were best separated when pIAL exceeded 0.35, the fraction of attachment losers 3 months after initial therapy (Table 2). Eqn. B was derived similarly from Table 3 and Eqn. C by using only baseline BOP in Table 3. Eqn B and Eqn C best predicted 'refractory' patients when pRefractory exceeded 0.62, slightly more than the actual fraction of 'refractory' patients in Table 3.

Results

Therapy, HKL-Ab response, *E. corrodens* colonization and initial attachment loss

The mean age of the Oklahoma and Boston patients was 32.0 and 49.4 years, respectively (standard deviations, 6.7 years and 11.7 years). The Boston patients did not gain significant attachment after therapy, whereas the Oklahoma patients did (Table 1). HKL-Ab concentrations were 1.0% of A-Ab and 0.1% of S-Ab concentrations. In the Oklahoma patients, none of the antibody concentrations changed following therapy: the mean measurement for each antibody pretherapy was essentially the same as obtained 5–7 weeks later (Fig. 2.). The

standard deviation of the respective means pre-therapy and after 5–7 weeks was 3.3% for A-Ab and S-Ab and 6.8% for HKL-Ab.

In Boston patients, the prevalence of *E. corrodens* was unrelated to antibody response at baseline. However, the patients with an increased HKL-Ab concentration exhibited an increased re-population of sites by *E. corrodens* 3 months after therapy (Fig. 3), although two outliers may have had bacteria other than *E. corrodens* producing this enzyme in their plaque (Holmes et al. 1995). Correspondingly, patients who lost mean attachment after initial therapy (initial attachment losers, IAL) had significantly more HKL-Ab and a significantly greater BOP prevalence than the patients who had gained mean attachment (Table 2). The highest mean HKL-Ab concentrations and greatest BOP prevalence were the most severely affected subjects, initial attachment losers (IAL) who were 'refractory' (Table 4). IAL who were successfully treated exhibited a mean HKL-Ab response resembling other successfully treated patients and 'refractory' patients who were not IAL and did not show attachment loss until 6–12 months after initial therapy.

Analysis of actinomyces and d-alanyl glycerol LTA antibody (A-Ab) responses

A-Ab concentrations were essentially the same in Oklahoma and Boston patients despite the differences in clinical measurements and therapeutic response (Table 1). Within the Boston patients, the A-Ab and S-Ab concentrations were, respectively, similar in initial attachment losers, 'refractory' patients, initial attachment gainers and successfully treated patients (Table 2 and Table 3). These antibody concentrations were unrelated to the age of the patient, the magnitude of attachment level change after initial therapy, the fraction of teeth showing bacterial plaque at baseline, or the change in plaque prevalence after initial therapy (results not shown). When the baseline *A. naeslundii* prevalence was >40% (12 patients), the A-Ab concentration (log ng/mL) was 4.08 ± 0.80 SD, but when baseline *A. naeslundii* prevalence was <40% (13 patients), the A-Ab concentration was 3.34 ± 0.48 SD ('t' statistic = 2.85, $p < 0.01$). On the other hand, with increasing A-Ab concentration, decreased *A. naeslundii* re-population was observed 3 months after therapy (Fig. 4; $r^2 = 0.23$, $p < 0.03$).

Correlation between A-Ab and S-Ab responses

A strong correlation between A-Ab and S-Ab responses was noted in 'refractory' but not successfully treated patients (Fig. 5). From the relationship in 'refractory' patients, an estimate of log ng/mL A-Ab was made from log ng/mL S-Ab. If the A-Ab estimate was less than +0.65 or more than -0.75 of the actual A-Ab concentration (log ng/mL), the relationship was predicted as linear (pLA/S-Ab = 0), otherwise it was predicted as non-linear (pLA/S-Ab = 1). These cutoffs optimized the difference between 'refractory' and successfully treated patients: Linear A/S responders accounted for 82.8% of 'refractory' patients but only 42.9% of successfully treated ones (derived from the data in Table 4), a significant difference ($\chi^2 = 6.5$, $p < 0.02$). The highest frequency of linear responders (pLA/S-Ab = 0) was greatest in the IAL/'refractory' patients who had the significantly increased HKL-Ab response (Table 4).

To the Boston and Oklahoma patients in Table 2 was added baseline data from seven Boston patients from whom IAL measurement were unavailable. Of these 58 patients, 38 were linear responders (pLA/S-Ab = 0) and had a mean baseline BOP prevalence of $49.4\% \pm 34.0\%$ SD. The remainder were nonlinear responders (pLA/S-Ab = 1) and had a mean baseline BOP prevalence of $29.3\% \pm 30.9\%$ SD, significantly different ('t' = 2.21, df = 56, $p < 0.04$).

Predicting 'refractory' patients (final outcome)

Table 3 shows that 'refractory' and successfully treated patients significantly differed by BOP prevalence, percent of predicted linear A/S-Ab response (pLA/S = 0) and percent predicted initial attachment losers (pIAL = 0), although age, plaque prevalence and percent initial

attachment losers were close to significant ($0.08 < P > 0.05$). Stepwise logistic regression indicated that only age, pIAL and pLA/S predicted 'refractory' response (Eqn. B, $p < 0.001$).

After solving Eqn. B, a correct prediction of 'refractory' response was found in patients with moderate periodontitis, 2–4mm mean attachment loss at baseline (sensitivity 86.4% and specificity of 86.7%, Table 5A). Mild periodontitis patients gave poor specificity (62.5%, Table 5B) and severe periodontitis patients gave poor sensitivity (60.0%, Table 5C). The seven patients who had only provided baseline data are included with the others in Table 5 (see legends). Two had <2mm attachment loss and one had >4mm. Of the remainder, three were diagnosed correctly and one incorrectly.

Discussion

It is proposed that lysine decarboxylase, primarily from *E. corrodens* and *Capnocytophaga* spp., initiates the inflammatory (GCF) exudate (Levine et al. 2001). If the activity of lysine decarboxylase in sulci is low, lysine in the GCF exudate will not all be converted to cadaverine and will therefore remain to minimize DAT cell growth inhibition. Despite the continuous presence of GCF, therapy and oral hygiene limit attachment loss by preventing the 'classical' periodontopathogens from increasing and their toxic end-products from inhibiting DAT cell growth. On the other hand, 'classical' periodontopathogens (and *A. naeslundii*) are depleted in the microbiota of 'refractory' patients, whose sulci are colonized by increased proportions of milleri streptococci, *C. ochracea* and uncommon bacterial taxa (Colombo et al. 1999). *E. corrodens* is a primary source of lysine decarboxylase, but *Cochracea* (Holmes et al. 1995) and some uncommon bacterial taxa are additional sources in 'refractory' patients. Deepened sulci have more cadaverine (Walters 1987), attributable to increased lysine decarboxylase activity.

The most 'refractory' subjects, those who had first lost attachment 3 months after initiating therapy (IAL) had the greatest level of antibody to lysine decarboxylase and the greatest re-colonization by *E. corrodens* 3 months following therapy. Indeed, Eqn. A, derived by stepwise logistic regression analysis, indicated that the initial attachment loss predicted by HKL-Ab concentration and BOP prevalence (pIAL) associated with 'refractory' response more strongly than initial attachment loss (IAL) obtained by direct measurement (Table 3). Thus, differences in the small amount of HKL-Ab seem to indicate differences in the response to initial therapy.

An elevated A-Ab response ($> 10\mu\text{g/mL}$; antilog of 4.0) associates with less plaque, gingivitis and caries in young adults (Levine et al. 1998), but indicated decreased recolonization by *A. naeslundii* following initial therapy and an uncertain final outcome. Although the effect of S-Ab concentration on re-colonization by a single species was not examined (see beginning of article), it strongly associated with A-Ab concentration across all 'refractory' patients. Because various *Actinomyces* spp. contribute to A-Ab response and various streptococci and other gram-positive bacteria to S-Ab response, the association in 'refractory' patients reflects the high likelihood of high antibody responses to many different bacteria in these patients (Colombo et al. 1998b, 1999). Although lysine decarboxylase is also produced by more than one oral bacterium, the small amounts of HKL-Ab are insignificant relative to the A-Ab and S-Ab responses.

The aim of therapy is often to reduce BOP and maintain it at low prevalence, for example, at less than 20% (Chapple 1997). In this study, the BOP prevalence 3 months after initial therapy was 24.8% in successfully treated patients, virtually unchanged from baseline (24.5% in Table 3) when the greatest decrease should have been evident. In 'refractory' patients, therapy significantly reduced mean BOP prevalence from 58.0% at baseline (Table 3) to 44.6% (t -test $p < 0.04$), but this is not even close to the recommended end-point prevalence. Indeed, even

professionally removing supragingival plaque weekly as a maintenance therapy did not reduce the mean BOP prevalence of periodontitis patients to below 20% (Ximenez-Fyvie et al. 2000). These results suggest that BOP may not be reduced to desirable levels in 'refractory' patients because lysine decarboxylase-associated inflammation cannot be significantly reduced by therapy.

Prediction Eqn. B indicates that the older a subject, the less likely is the response to therapy to be 'refractory.' In that equation, BOP contributed to pIAL and was independently related to pLA/S-Ab. Both these variables were used (with age) to predict therapeutic outcome with 86% sensitivity and specificity. In prediction, Eqn. C, BOP alone gave poor sensitivity (data not shown) as in previous studies (Armitage 1996). The predictions were the most accurate in the patients presenting with 2–4mm attachment loss. If attachment loss was <2mm, the patients were mostly successfully treated, even when predicted to be 'refractory' (poor specificity), whereas if attachment loss was >4mm, the patients were mostly 'refractory' even when predicted to be successfully treated (poor sensitivity). Anatomical and other changes decrease the efficacy of therapy in periodontitis patients with more than 4mm mean attachment loss (Haffajee & Socransky 1986, Cobb 1996), whereas the outcome of treating patients with 2–4mm attachment loss is unpredictable *a priori*. This test therefore seems useful because it detects patients who are 'refractory' to conventional therapy but have periodontitis that is successfully treated in 80–90% of affected subjects. Alternative therapies for refractory patients could be weekly professional plaque removal for 3 months (Ximenez-Fyvie et al. 2000), or possibly to use lysine decarboxylase inhibitors although they are not currently available (Levine et al. 2001).

Predicting 'refractory' patients

The three antigens described in this study are easily obtained and stable, although the d-alanyl glycerol LTA antigen requires storage at pH6.0 (Childs & Neuhaus 1980). Replacing the standard sera with IgG monoclonal antibodies that are specific for each antigen and covalently conjugated to peroxidase or alkaline phosphatase would provide commercial standards of known specific activity for measuring the human IgG antibody measurements more reproducibly than in this study. Because the number of variables at each step was small (two for Eqn. A and two additional ones for Eqn. B) and because compiled variables (pIAL and pLA/S) were used, confounding interactions and random variation were kept to a minimum. However, it is essential that a new set of subjects be examined to confirm the usefulness of Eqn A and Eqn B derived in this study, and to permit modifications that would improve their ability to predict 'refractory' patients in a less controlled setting.

Even with improvements, performing these immunoassays is not a chairside procedure. Unlike the mixed laboratory procedures reported previously (Colombo et al. 1999), there are many commercial laboratories providing serological testing. These laboratories normally receive serum samples that could be assayed with a kit containing antigens and standardized antibodies. The results could then be communicated to the periodontist within a few days.

As noted earlier, the need for a test for potentially 'refractory' disease would be the most useful for the patients with moderate periodontitis. Patients with minimal periodontitis typically respond well to periodontal therapies, so that the use of a diagnostic test for these subjects seems unwarranted. At the other extreme, subjects with advanced disease are recognized to be at risk for poor treatment response and the need for a diagnostic test for this group would also be questionable. Thus, the immunoassay is proposed for use in subjects with moderate periodontitis, i.e. subjects presenting with a mean attachment loss level of 2–4mm. In such subjects, the results of the antibody estimations would be used as follows to predict a poor-treatment response (disease progression). Eqn. A would be used to predict mean attachment loss 3 months after initial therapy (pIAL). A value of 1 would be assigned if pIAL < 35% and

0 if pIAL > 35%. The pLA/S-Ab response would be evaluated (see legend to Fig. 5). A value of 0 would be assigned if linear or 1 if non-linear. The probability of being 'refractory' would be derived from Eqn. B and if > 62%, the patient would be considered to be at high risk for poor response to periodontal therapy. As indicated earlier, it would be highly desirable to evaluate the antibody diagnostic in a new group of patients to validate its potential utility in a clinical practice setting.

Zusammenfassung

Diagnostik auf der Grundlage von Antikörpern bei ärefraktionärer Parodontitis

Zielsetzung—Etwa 10–15% aller US-amerikanischen Erwachsenen sind ärefraktionär in bezug auf Therapien bei chronischer Parodontitis. Studien neueren Datums legen den Schluss nahe, dass bei diesen Patienten in den Mikrobiota des Sulcus eine erhöhte Lysin-Decarboxylase-Aktivität vorliegt. Das Ziel der vorliegenden Studie war die Beantwortung der Frage, ob sich durch eine erhöhte IgG-Antikörper-Reaktion auf Lysin-Decarboxylase allein oder zusammen mit Antikörpern auf andere bakterielle Antigene und klinischen Messungen zu Baseline ärefraktionäre Patienten mit hoher Genauigkeit vorhersagen lassen konnten.

Methodik—Patienten mit chronischer Parodontitis wurden durch Scaling und Wurzelglätten (SRP; scaling and root planing) gefolgt von einer erhaltenden SRP-Behandlung und dreimonatigen Neuuntersuchungen behandelt. Bei Auftreten von Verlusten des durchschnittlichen Attachment-Niveaus im gesamten Mund oder bei mehr als drei Stellen mit >2,5 mm Neuverlust innerhalb eines Jahres wurden die Patienten erneut behandelt (modifizierter Widman-Lappen-Eingriff und systematische Gabe von Tetracyclin). Trat ein Attachment-Verlust wie oben beschrieben erneut auf, galten die Patienten als ärefraktionär. Zur Vorhersage ärefraktionärer Patienten wurden klinische Messungen zu Baseline und spezifische Antikörperreaktionen in einem logistischen Regressionsmodell eingesetzt.

Ergebnisse—Durch Antikörper auf einen Peptidteil der Lysin-Decarboxylase (HKL-AB) und Prävalenzmessungen bei Blutungen bei Sondierung zu Baseline (BOD; baseline bleeding on probing) konnte ein Attachment-Verlust drei Monate nach der ersten Therapie [pIAL=Verlust (0) oder Zunahme (1)] vorhergesagt werden. IgG-Antikörperanteile auf ein purifiziertes Antigen der *Actinomyces* spp. (A-Ab) und D-Alanyl-Glycerin-Liponsäure eines streptokokkalen Ursprungs (S-AB) waren bei ärefraktionären Patienten assoziiert ($R^2=0,37$, $p<0,01$). Auf der Grundlage der Regressionsanalyse wurde die Beziehung zwischen den Antikörpern als linear (pLA/S-AB=0) oder nicht-linear (pLA/S-AB = 1) definiert. Unter Verwendung von pLA/S-AB, pIAL und Alter wurde aus dem Profil von 48 Patienten eine logistische Regressionsgleichung erarbeitet. Von 59 Patienten hatten 37 einen Attachmentverlust von 2–4 mm; diese Patienten wurden mit einer 86%-igen Genauigkeit als entweder ärefraktionär oder erfolgreich behandelt eingestuft.

Schlussfolgerung—HKL-Ab ermöglichte eine präzise Vorhersage des Behandlungsergebnisses bei Patienten mit mässiger Parodontitis.

Résumé

Diagnostic de la parodontite réfractaire à partir des anticorps

But—Aux Etats-Unis, environ 10 à 15% des adultes sont réfractaires au traitement de la parodontite chronique. Des études récentes suggèrent que ces patients présentent une activité élevée de la lysine décarboxylase dans la flore sulculaire. L'objectif de cette étude était de déterminer si une réponse élevée des anticorps IgG de la lysine décarboxylase, seule ou avec

un anticorps à d'autres antigènes bactériens et des mesures cliniques initiales, permettrait de prédire avec une grande précision quels sont les patients réfractaires.

Méthodes—Les patients atteints de parodontite chronique ont fait l'objet d'un traitement par détartrage et surfaçage radiculaire (*scaling and root planing*, SRP), suivi d'un SRP de maintenance et de ré-examens trimesuels. Lorsqu'il y avait perte d'attache moyenne en bouche complète ou apparition de plus de trois sites avec une nouvelle perte >2,5 mm en l'espace d'un an, les sujets faisaient l'objet d'un nouveau traitement (opération par lambeau de Widman modifié et administration systémique de tétracycline). Lorsqu'une telle perte d'attache se répétait, les sujets étaient considérés réfractaires. Les mesures cliniques initiales et les réponses immunitaires spécifiques étaient utilisées au sein d'un modèle de régression logistique pour prédire quels sujets seraient réfractaires.

Résultats—Les mesures de prévalence de l'anticorps d'une portion peptidique de la lysine décarboxylase (HKL-Ab) et du saignement au sondage initial (*bleeding on probing*, BOP) permettaient de prédire une perte d'attache trois mois après la thérapie initiale [pIAL= perte (0) ou gain (1)]. Chez les patients réfractaires, les contenus des anticorps IgG d'un antigène purifié de *Actinomyces* spp. (A-Ab) et de l'acide streptococcal D-alanyl glycérol lipoteichoïque (S-Ab) étaient liés ($R^2 = 0,37$; $p < 0,01$). À partir de l'équation de régression, la relation entre les anticorps a été définie comme étant linéaire (pLA/S-Ab=0) ou non-linéaire (pLA/S-Ab=1). Une équation de régression logistique a été dérivée des valeurs de pLA/S-Ab, pIAL et de l'âge de 48 des patients. Parmi 59 sujets, 37 présentaient une perte d'attache de 2–4 mm et étaient considérés réfractaires ou traités avec succès avec une précision de 86%.

Conclusion—HKL-Ab facilitait la prédiction précise de l'issue du traitement chez les sujets atteints de parodontite modérée.

References

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 1997;25:3389–3402. [PubMed: 9254694]
- Armitage G. Periodontal disease: diagnosis. *Annals of Periodontology* 1996;1:37–215. [PubMed: 9118264]
- Armitage G. Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* 1999;4:1–6. [PubMed: 10863370]
- Chapple IL. Periodontal disease diagnosis: current status and future developments. *Journal of Dentistry* 1997;25:3–15. [PubMed: 9080734]
- Childs WC III, Neuhaus FC. Biosynthesis of d-alanyl-lipoteichoic acid: characterization of ester-linked d-alanine in the *in vitro*-synthesized product. *Journal of Bacteriology* 1980;143:293–301. [PubMed: 6772629]
- Cobb CM. Non-surgical pocket therapy: mechanical. *Annals of Periodontology* 1996;1:443–490. [PubMed: 9118268]
- Colombo AP, Haffajee AD, Dewhirst FE, Paster BJ, Smith CM, Cugini MA, Socransky SS. Clinical and microbiological features of refractory periodontitis subjects. *Journal of Clinical Periodontology* 1998a; 25:169–180. [PubMed: 9495617]
- Colombo AP, Haffajee AD, Smith CM, Cugini MA, Socransky SS. Discrimination of refractory periodontitis subjects using clinical and laboratory parameters alone and in combination. *Journal of Clinical Periodontology* 1999;26:569–576. [PubMed: 10487306]
- Colombo AP, Sakellari D, Haffajee AD, Tanner A, Cugini MA, Socransky SS. Serum antibodies reacting with subgingival species in refractory periodontitis subjects. *Journal of Clinical Periodontology* 1999;25:596–604. [PubMed: 9696261]

- Daly CG, Highfield JE. Effect of localized experimental gingivitis on early supragingival plaque accumulation. *Journal of Clinical Periodontology* 1996;23:160–164.
- Griffiths GS, Wilton JMA, Curtis MA. Permeability of the gingival tissues to IgM during an experimental gingivitis study in man. *Archives of Oral Biology* 1997;42:129–136. [PubMed: 9134125]
- Haffajee AD, Cugini MA, Dibart S, Smith C, Kent RLJ, Socransky SS. The effect of SRP on the clinical and microbiological parameters of periodontal diseases. *Journal of Clinical Periodontology* 1997;24:324–334. [PubMed: 9178112]
- Haffajee AD, Socransky SS. Attachment level changes in destructive periodontal diseases. *Journal of Clinical Periodontology* 1986;13:461–472. [PubMed: 3522651]
- Hall GH, Round AP. Logistic regression — explanation and use. *Journal of the Royal College of Physicians* 1994;28:242–246.
- Hogg SD, Whiley RA, De Soet JJ. Occurrence of lipoteichoic acid in oral streptococci. *International Journal of Systematic Bacteriology* 1997;47:62–66. [PubMed: 8995803]
- Holmes, B.; Pickett, MJ.; Hollis, DG. Unusual Gram-negative bacteria, including *Capnocytophaga*, *Eikenella*, *Pasteurella*, and *Streptobacillus*. In: Murray, PR., editor. *Manual of clinical microbiology*. Washington D.C: ASM Publications; 1995. p. 499-508.
- Hosmer, DW.; Lemeshaw, S. *Applied logistic regression*. New York: John Wiley & Sons; 1989.
- Levine M. Naturally occurring human serum precipitins specific for d-alanyl esters of glycerol teichoic acid. *Molecular Immunology* 1982;19:133–142. [PubMed: 7078554]
- Levine M, Brumley RL. Fast EL-ISA for measuring serum antibody responses. *Journal of Immunological Methods* 1989;119:211–215. [PubMed: 2723439]
- Levine M, Brumley RL, Avery KT, Owen WL, Komori N. Antibody to actinomyces and oral hygiene. *Journal of Dental Research* 1998;77:776. (IADR Abstract no. 1159).
- Levine M, Bush CM. Human serum precipitins to human dental plaque and oral bacteria. *Archives of Oral Biology* 1981;26:117–122. [PubMed: 6791620]
- Levine M, Miller FC. Use of monoclonal antibodies with neutralizing effects on toxic antigens from human bacterial plaque to detect specific bacteria by colony blotting. *Journal of Clinical Microbiology* 1991;29:2809–2816. [PubMed: 1757553]
- Levine M, Movafagh BF. D-Alanyl substituted glycerol lipoteichoic acid in culture fluids of *Streptococcus mutans* GS-5 and BHT. *Infection and Immunity* 1984;46:870–872. [PubMed: 6500717]
- Levine M, Movafagh BF. Analysis of the specificity of natural human antibody reactive to Actinomyces. *Molecular Immunology* 1986;23:255–261. [PubMed: 3086711]
- Levine M, Progulske-Fox A, Denslow ND, Farmerie WG, Smith DM, Swearingen WT, Miller FC, Liang Z, Roe BA, Pan H-Q. Identification of lysine decarboxylase as a mammalian cell growth inhibitor in *Eikenella corrodens*: possible role in periodontal disease. *Microbial Pathogenesis* 2001;30:179–192. [PubMed: 11312612]
- Niederman R, Zhang J, Kashket S. Short-chain carboxylic-acid-stimulated, PMN-mediated gingival inflammation. *Critical Reviews in Oral Biology and Medicine* 1997;8:269–290. [PubMed: 9260044]
- Papes F, Kemper EL, Cord-Neto G, Langone F, Arruda P. Lysine degradation through the saccharopine pathway in mammals: involvement of both bifunctional and monofunctional lysine-degrading enzymes in mouse. *Biochemical Journal* 1999;344:555–563. [PubMed: 10567240]
- Piscitelli SC, Shwed J, Schreckenberger P, Danziger LH. *Streptococcus milleri* group: renewed interest in an elusive pathogen. *European Journal of Clinical Microbiology and Infectious Diseases* 1992;11:491–498.
- Ramberg P, Furuichi Y, Volpe AR, Gaffar A, Lindhe J. The effects of antimicrobial mouthrinses on *de novo* plaque formation at sites with healthy and inflamed gingivae. *Journal of Clinical Periodontology* 1996;23:7–11.
- Salonen JI. Proliferative potential of the attached cells of human junctional epithelium. *Journal of Periodontal Research* 1994;29:41–45. [PubMed: 8113952]
- Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RLJ. Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology* 1998;25:134–144. [PubMed: 9495612]

- Stallard RD, Volpe AR, Orban JE, King WJ. The effect of an antimicrobial mouth rinse on dental plaque, calculus and gingivitis. *Journal of Periodontology* 1969;40:683–694. [PubMed: 4905177]
- Walters JD. Polyamine analysis of human gingival crevicular fluid. *Journal of Periodontal Research* 1987;22:522–523. [PubMed: 2963114]
- Ximenez-Fyvie LA, Haffajee AD, Som S, Thompson M, Torresyap G, Socransky SS. The effect of repeated professional supragingival plaque removal on the composition of the supra- and subgingival microbiota. *Journal of Clinical Periodontology* 2000;27:637–647. [PubMed: 10983597]

Fig. 1.

Standard curves for serum antibody measurement. Each antigen was immuno-precipitated against a standard serum, dilutions of which were also assayed for absorbance on each immunoassay plate as described previously (Levine & Brumley 1989). From the IgG antibody concentration of each standard serum and the dilutions on each plate, a graph of IgG concentration against absorbance at 410nm was obtained (see Methods). The IgG antibody concentration for each antigen was then calculated in each patient's serum using the linear portion of the appropriate curve. Bars indicate standard deviation. Standard curves are for A-Ab (Top Graph), S-Ab (Middle Graph), and HKL-Ab (Bottom Graph).

Fig. 2.

IgG antibody concentrations from the Oklahoma patients pre- and post-therapy. A-Ab ($\circ$), S-Ab ($\square$) and HKL-Ab ($\triangle$) concentrations (log ng/mL) were measured at baseline and 5–7 weeks after completing therapy. The observed relationship equation ($Ab_{\text{post-therapy}} = 1.03Ab_{\text{pre-therapy}} - 0.06$) and fraction of variance explained by the equation ($R^2 = 0.99$, $p < 0.001$) indicate that each antibody measurement from each patient was essentially the same before and after therapy.

Fig. 3.

Effect of HKL-Ab concentration on *E. corrodens* re-population 3 months after initial therapy. On the x axis, 0.00 means no change in prevalence after therapy, -0.50 and -1.00 mean a 50% and 100% decrease in prevalence and $+0.50$ and $+1.00$ a 50% and 100% increase in prevalence, respectively. Two outliers (unfilled circles) were excluded from regression analysis (see Results) and the remaining patients showed a strong association of HKL-Ab concentration with *E. corrodens* re-population ($R^2 = 0.46$, $p < 0.005$).

Fig. 4.

Effect of A-Ab concentration on *A. naeslundii* re-population 3 months after initial SRP. On the *x* axis, 0.00 means no change in prevalence (re-population frequency) after therapy, -0.50 and -1.00 mean a 50% and 100% decrease and +0.50 and +1.00 a 50% and 100% increase, respectively.

Fig. 5. Relationship between A-Ab and S-Ab in 'refractory' and successfully treated patients. Of the 55 patients with > 1.6mm attachment loss, 29 were 'refractory' and gave the regression equation: $A-Ab = 0.74 \cdot S-Ab + 0.41$ ($R^2 = 0.37$, $p < 0.001$). A-Ab was estimated from S-Ab and the estimated minus actual A-Ab was calculated. If more than -0.75 and less than $+0.65$, the patient was assigned a linear A-Ab/S-Ab relationship ($\bullet$). Otherwise the patient was assigned a non-linear relationship ($\circ$).

Table 1
Baseline and initial post-therapy measurements on 52 Boston and 26 Oklahoma patients

	Baseline		Initial post-therapy ^a		t-test
	Means	SD	Means	SD	
Minimal (Oklahoma)					
Plaque prevalence (%)	66.67	17.52	51.92	22.40	0.0109
BOP prevalence (%)	17.63	14.87	5.45	7.89	0.0006
PD mean (mm)	2.34	0.38	2.03	0.30	0.0020
AL mean (mm)	1.15	0.50	0.76	0.55	0.0097
A+Ab (log ng/mL)	3.70	0.55	3.69	0.57	^b n.s.
Moderate (Boston)					
Plaque Prevalence (%)	67.91	27.61	62.38	30.69	^b n.s.
BOP Prevalence (%)	43.37	36.80	36.11	33.88	^b n.s.
PD Mean (mm)	3.29	0.54	3.03	0.47	0.0088
AL Mean (mm)	3.16	1.16	3.03	1.11	^b n.s.
A+Ab (log ng/mL)	3.77	0.75	n.d. ^c	—	—

^a After 3months for Boston patients and after 5–7weeks for Oklahoma patients.

^b n.s., Not significant ($p>0.2$).

^c n.d., Not Done.

Table 2

Baseline and postinitial therapy measurements on 48 Boston and three Oklahoma patients who had lost or gained mean attachment (omitting four Boston patients with <1.6 mm attachment loss and seven with only baseline data)

Measure	18 Losers ^a		33 Gainers ^b		Difference
	Means	S.D.	Means	S.D.	
Age (years)	46.36	10.74	50.30	12.40	n.s. ^c
Plaque Prevalence (%)	71.66	32.16	68.15	24.58	n.s. ^c
BOP Prevalence (%)	61.20	39.70	32.77	29.93	0.0094
PD Mean (mm)	3.38	0.36	3.28	0.61	n.s. ^c
AL Mean (mm)	3.16	0.92	3.29	1.18	n.s. ^c
HKL-Ab, log ng/mL	1.72	0.37	1.52	0.30	0.0335
A-Ab, log ng/mL	3.73	0.74	3.69	0.74	n.s. ^c
S-Ab, log ng/mL	4.61	0.63	4.51	0.64	n.s. ^c

^a Losers score 0 and gainers score 1 when used as dependent variable in deriving Eqn. A.

^b Includes two patients who neither gained nor lost attachment.

^c n.s., Not significant ($p>0.2$).

Table 3

Baseline findings in patients who had > 1.6mm mean attachment loss, subset according to whether they were successfully treated or 'refractory' 1 year after the completion of all therapy

Measurement	27 'Refractory' ^a		21 Successful ^a		Difference
	Means	SD	Means	SD	
Age	46.64	11.59	53.19	11.21	0.0623
Plaque Prevalence (%)	76.31	29.65	59.87	22.72	0.0534
BOP Prevalence (%)	58.04	40.17	24.51	18.80	0.0015
Pocket Depth, Mean (mm)	3.40	0.38	3.28	0.68	^b n.s.
Attachment Loss, Mean (mm)	3.32	1.06	3.31	1.08	^b n.s.
S-Ab, log ng/mL	4.59	0.66	4.54	0.57	² n.s., ^b
A-Ab, log ng/mL	3.77	0.79	3.60	0.67	n.s.
HKL-Ab, log ng/mL	1.63	0.33	1.53	0.35	^b n.s.
% current smokers ^c	29.60	—	9.50	—	n.s. ^a
% initial attachment losers	48.15	—	19.05	—	0.0739 ^d
% predicted IAL	66.67	—	23.81	—	0.0028 ^d
% pLA/S-Ab	81.48	—	42.86	—	0.0135 ^d

^a 'Refractory' (score 0) and successful (score 1) when used as dependent variable in Eqns B and Eqns C.

^b n.s., $p > 0.2$.

^c Percentages are of 27 'refractory' or of 21 successfully treated patients.

^d Chi squares: for IAL was 3.2; for pIAL, 7.0; for pLA/S, 6.1.

Table 4

HKL-Ab response, initial attachment loss (IAL) and 'refractory' response

	IAL 'Refractory'	IAL Success	Not IAL IAL 'Refractory'	Not IAL Success
Numbers*	14	4	14	17
HKL-Ab Means	1.77 [§]	1.51	1.49	1.54
BOP	71.2	27.9	44.9	23.7
%A/S lineO0	92.9	25.0	71.4	47.1

* Additional patient's initial response to therapy is included (total of 49 subjects).

[§]'t' test indicates a significantly greater difference than all others ($p<0.02$).

Table 5
Sensitivity and specificity of predicted outcome

Predicted	Determined 'refractory'	Determined successful	Total
A. Moderate periodontitis: initial mean attachment level 2.0–4.0mm			
'refractory'	19 ^b	2	21
successful	3	13 ^b	16
Total ^a	22	15	37
B Mild periodontitis: mean initial attachment level 1.0–2.0mm			
'refractory'	2	3	5
successful	0	5	5
Total ^c	2	8	10
C. Severe periodontitis: mean initial attachment level >4.0mm			
'refractory'	3	2	5
successful	2	5	7
Total ^d	5	7	12

^a Includes four patients who had provided only baseline measurements.

^b Accuracy is the correctly predicted number as a fraction of total.

^c Includes the four successfully treated patients with attachment loss 1.2–1.6mm and six patients with attachment loss 1.6–1.9mm of whom two had provided only baseline measurements.

^d Includes one patient who had provided only baseline measurements.