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Feasibility of an HA2 Domain-Based Periodontitis Vaccine

A. A. DeCarlo,[1] Y. Huang,[2] C. A. Collyer,[3] D. B. Langley,[3] and J. Katz[2]*

Vaccine Research Division, Agenta Biotechnologies, Birmingham, Alabama 35253[1]; Department of Oral Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294[2]; and Department of Biochemistry, University of Sydney, New South Wales 2006, Australia[3]

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In a rat periodontitis model, preinoculation with the *Porphyromonas gingivalis* HA2 binding domain for hemoglobin provided protection from disease. Protection was associated with induced anti-HA2 immunoglobulin G (IgG) humoral antibodies. The IgG subclass ratios suggested that relatively lower Th2/Th1-driven responses were directly associated with protection when rHA2 was administered in saline.

The majority of adults worldwide will experience some level of periodontitis and could suffer from acute abscess formation, loss of teeth, and increased risk of other systemic complications associated with this chronic disease (1, 12, 19, 24, 32, 35).

During the disease process, serum antibodies directed at periodontal microorganisms naturally develop, but do not correlate strongly with the incidence and severity of the disease (5, 17). However, significant levels of serum antibodies leak from the microvasculature of the inflamed periodontium and might be harnessed to control periodontitis. Furthermore, while the local immune response has been implicated in periodontal destruction (10, 23, 31, 33, 36), antigen-specific responses within the tissue might also be manipulated for control of the disease.

Porphyromonas gingivalis is the bacterial species most widely and strongly implicated in the pathogenesis of periodontitis due to its association with disease incidence and severity (8, 18, 29, 30), and its broad range of abundant virulence factors (4, 6, 9, 26, 28, 34, 37). *P. gingivalis* is a gram-negative, anaerobic rod that requires exogenous porphyrin and iron for survival (3, 25), and in the inflamed periodontal pocket, hemoglobin would be a ready source of heme-associated porphyrin and iron. Hemoglobin binding in *P. gingivalis* has been clearly demonstrated by a TonB-dependent protein, HmuR (21, 27), and by the HA2 domain of gingipains, which are multidomain proteins thought capable of several functions related to heme acquisition and delivery to the organism (22). The HA2 domain has also been designated the hemoglobin receptor (HbR) domain protein (20). Previously, we specifically identified the site on the porphyrin ring that binds the HA2 domain and successfully expressed functional recombinant HA2 (rHA2) (7).

Analysis of anti-HA2 antibody in human serum with a monoclonal antibody specific for the HA2 domain has indicated a significant positive correlation between serum immunoglobulin G (IgG) anti-HA2 antibody titers and periodontal health in a sample of the general population (A. A. DeCarlo, M. Paramaesvaran, P. L. W. Yun, C. A. Collyer, and N. Hunter, unpublished data). The purpose of the present study was to investigate the use of rHA2 as an immunogen for a periodontitis vaccine.

The use of the rHA2 domain for diagnostic or therapeutic use is protected by International Patent Application no. PCT/AU00/00599, entitled "A method of prophylaxis and treatment and agents useful for same," and International Patent Application no. PCT/AU02/00102, entitled "Expression facilitating nucleotides," as filed in the name of The University of Sydney, NSW, Australia. The license for this patent technology is exclusively optioned to Agenta Biotechnologies, Inc., headquartered in Birmingham, Ala.

Methods. Conventional Fischer CD F(344) rats, 8 to 10 weeks old, were used in this study. The rats were not germfree, and no attempt was made to control their bacterial flora. All experiments were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

P. gingivalis ATCC 33277 was used to orally infect the animals as described previously (14).

The HA2 sequence of *P. gingivalis* 33277 (GenBank sequence [gb|U68468.1|PGU68468]) was cloned as previously described (7). HA2 was then subcloned with a C-terminal six-His tag via a thrombin cleavage site linker (LVPRG SHHHHHH), expressed in a modified *Escherichia coli* genomic background for high levels of expression by proprietary means (patent pending no. PR2825/01, Australia) and then recovered essentially as described previously (7). Gel filtration chromatography of the sample demonstrated that the rHA2 was predominately in an aggregated, multimeric state with roughly one-third as free dimer.

Experimental design. Based on preliminary data that subcutaneous (s.c.) immunizations of rHA2 in phosphate-buffered saline (PBS) with 50 μg of rHA2 per dose would provide optimal IgG and IgM responses, one group of eight rats was immunized s.c. on day 0 with 50 μg of rHA2 in 0.5 ml of PBS (group A). A second group of eight rats was sham immunized s.c. on day 0 with 0.5 ml of PBS only (group B). Based on preliminary data that three s.c. immunizations of rHA2 in saline stimulated serum anti-rHA2 IgG antibody levels approximately 40-fold beyond levels measured after only two immunizations, these groups were immunized similarly twice more, on days 14 and 28. A third group of rats was immunized s.c. on day 0 with 50 μg of rHA2 in 0.5 ml of complete Freund's adjuvant (FA) (group C). A fourth group of rats was sham

* Corresponding author. Present address: College of Dental Medicine, Nova Southeastern University, Rm. 7382, Dental, 3200 South University Dr., Ft. Lauderdale, FL 33328. Phone: (954) 262-1692. Fax: (954) 262-1692. E-mail: adecarlo@nova.edu.

TABLE 1. IgG subclass ratios in serum

rHA2 combination	IgG subclass ratio[a]		
	IgG1/IgG2a	IgG1/IgG2b	IgG2a/IgG2b
PBS			
Day 56	9.4 ± 3.1	436 ± 215	101 ± 52*
Day 84	3.8 ± 1.5	240 ± 160	64 ± 26*
FA			
Day 42	3.4 ± 1.1*	98 ± 38*	36 ± 14*
Day 70	3.0 ± 1.3	63 ± 20*	24 ± 6.1*

[a] Values are means ± standard errors. IgG1 and IgG2a suggest a Th2-type response, while IgG2b suggests a Th1-driven antibody subclass response in the rat. Asterisks represent significant differences between the two subclasses.

immunized s.c. on day 0 with 0.5 ml of complete FA only (group D). Two weeks later (day 14), group C was immunized again, this time with 50 μg of rHA2 in 0.5 ml of incomplete FA, while group D was sham immunized with incomplete FA. Serum samples were collected biweekly, and antibody levels were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (7, 14, 15).

Thirteen and 14 days after the last immunization of each group, rats were orally infected with *P. gingivalis* 33277 as previously described (14, 16).

Standardized radiographs of the rat mandibles were made after dissection, and the radiographs were analyzed in a blinded fashion by digital imaging methods as previously described (13, 14). The distance from the crest of the alveolar bone to the cemento-enamel junction (CEJ) of the teeth was measured at the mesial and distal parts of the first and second molars bilaterally.

Specific IgG against rHA2. Sham-immunized animals (PBS only, group B) demonstrated no measurable anti-rHA2 IgG antibodies (data not shown). Each of the animals immunized with rHA2 in PBS (group A), however, showed a sustained specific serum IgG antibody response throughout the 56-day postimmunization period (Fig. 1A). Three of the eight animals in group A demonstrated significantly higher anti-rHA2 serum IgG levels than the remaining five animals during the course of experimental periodontitis, from days 41 to 84 ($P = 0.005$, analysis of variance [ANOVA]).

As expected, the use of FA with the rHA2 immunogen (group C) stimulated a significantly stronger anti-HA2 serum IgG antibody response at all measured time points postinfection than did administration of rHA2 in PBS (group A) ($P = 0.04$, ANOVA) (Fig. 1B). There was no measurable anti-HA2 serum IgG in sham-immunized animals (group D) (data not shown).

Relationship of specific IgG levels with protection from bone loss. The effects of the adjuvant on bone height were negligible, because there was no significant difference between animals sham immunized with PBS (group B) and those sham immunized with FA (group D) ($P = 0.66$, Student's t test) (data not shown). Unexpectedly, there was no significant protection from bone loss in group C ($P = 0.89$, Student's t test), despite the relatively high specific antibody levels achieved (Fig. 2B). Within group C, there was no relationship between antibody responses and protection from bone loss (data not shown).

In contrast, analysis of crestal alveolar bone levels in group A demonstrated a protective effect of administration of rHA2 in PBS, which was attributable, at least in part, to the induction

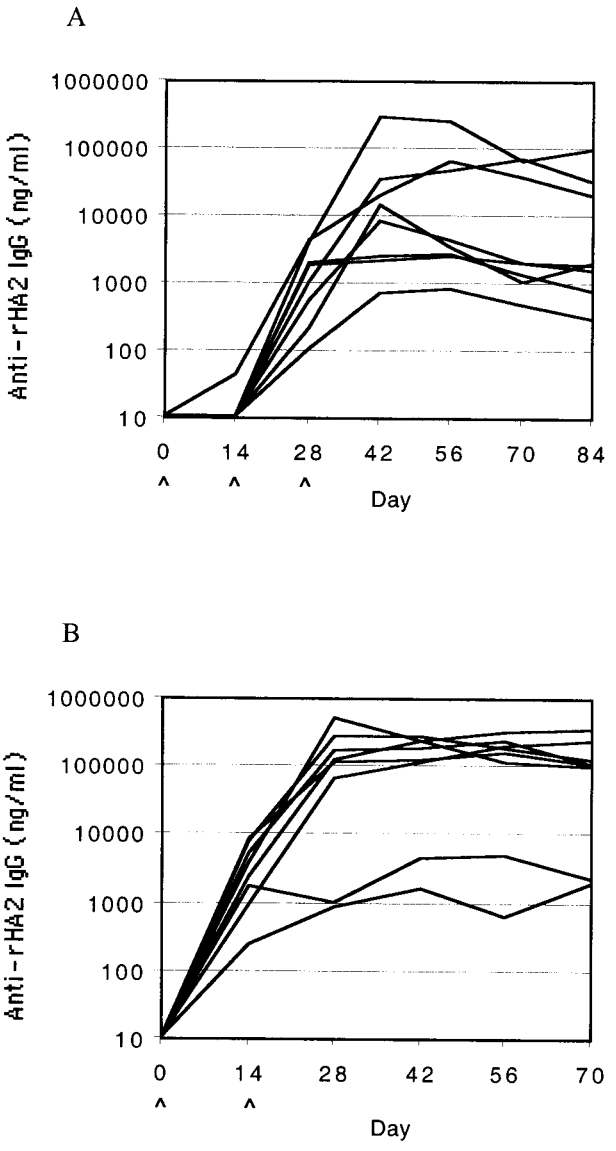

FIG. 1. (A) Anti-HA2 serum IgG antibody profiles of eight rats immunized three times s.c. with 50 μg of rHA2 protein in PBS (group A). (B) Anti-HA2 serum IgG antibody profiles of eight rats immunized twice s.c. with 50 μg of rHA2 protein in complete FA followed by incomplete FA (group C) (Arrowheads indicate the day of immunization.) Anti-rHA2 IgG serum levels were determined by ELISA as described in the text. Animals were infected orally with *P. gingivalis* on days 41 and 42 (group A) or days 27 and 28 (group C) of the experimental period.

of relatively higher anti-HA2 serum IgG antibody levels. By using an arbitrary cutoff level of 10 μg/ml, the animals in group A that sustained the highest serum anti-HA2 IgG responses (>10 μg/ml for 56 days postimmunization) (Fig. 2A, column a) lost significantly less alveolar bone than the remaining animals in group A (Fig. 2A, column b) or the remaining animals in group A and B combined (Fig. 2A, column c) (Student's t tests, $P < 0.05$). This significant degree of protection from bone loss was directly related to the attainment and maintenance of anti-rHA2 serum IgG levels >10 μg/ml. The differences in bone levels associated with high and low anti-HA2 serum IgG

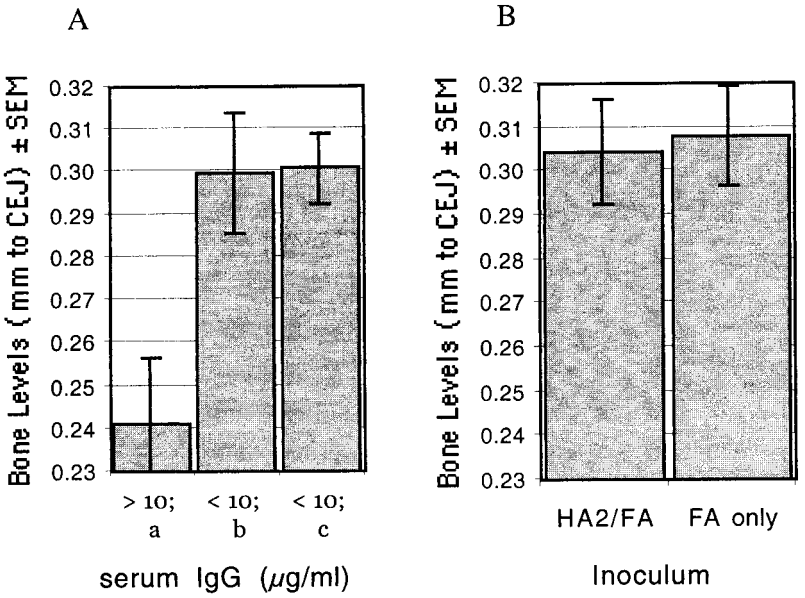

FIG. 2. (A) Higher anti-HA2 IgG levels resulted in less bone loss. Shown are mean crestal alveolar bone levels (millimeters to CEJ) in group A animals immunized with rHA2, which maintained serum IgG anti-HA2 antibody levels >10 μg/ml during the period of *P. gingivalis* infection (a); group A animals immunized with rHA2, which did not maintain serum IgG anti-HA2 antibody levels >10 μg/ml during the period of *P. gingivalis* infection (b); and all animals in group A that did not maintain serum IgG anti-HA2 antibody levels >10 μg/ml during the period of *P. gingivalis* infection, including sham-immunized animals (group B) (c). (B) rHA2 administration in FA provided no protection from alveolar bone loss. Shown are mean crestal alveolar bone levels (millimeters to CEJ) in group C animals immunized with 50 μg of rHA2 in complete and then incomplete FA (rHA2/FA) on days 0 and 14, respectively, and group D animals sham immunized similarly. All animals in each group were infected orally with *P. gingivalis*, and bone levels were measured as described in the text.

responses were significant in nested (or hierarchical) ANOVA ($P < 0.01$) whether analyzed by subject or by site.

Anti-rHA2 IgM was not associated with protection. A significant and sustained anti-HA2 serum IgM antibody response was induced in each of the rats immunized with rHA2 in PBS (group A) (Fig. 3A) and each of the rats immunized with rHA2 in FA (group C) (Fig. 3B). No anti-HA2 IgM antibodies were detected in the sham-immunized groups (groups B or D) (data not shown). Although strong and sustained, the anti-HA2 IgM serum antibody responses in groups A and C were not significantly related in any way to protection in this experimental periodontitis model (data not shown).

IgG subclass analysis. Antibody IgG1, IgG2a, and IgG2b subclass responses were measured in serum collected at two time points representing the middle and end of the experimental period. The use of FA with rHA2 (group C) produced significantly higher levels of serum anti-rHA2 IgG1, IgG2a, and IgG2b at both time points than those measured in group A ($P < 0.05$) (data not shown).

Anti-rHA2 IgG2a levels were significantly higher than the corresponding IgG2b levels in both groups A and C at each of the two time points measured (Table 1). These data suggested there were stronger Th2-type-driven (IgG1 and/or IgG2a) than Th1-type-driven (IgG2b) serum antibody responses to the rHA2 antigen in this rat model of periodontitis. However, the ratios were not significantly different within or between groups A and C.

High IgG antibody levels in the animals immunized with rHA2 in PBS (group A) were associated with high IgG2b antibody levels ($P = 0.002$, Student's t test). In contrast, higher IgG antibody levels in the animals immunized with rHA2 in FA (group C) were associated with significantly lower IgG2b levels ($F = 0.05$, linear regression) (data not shown).

In animals inoculated with rHA2 in PBS (group A), lower anti-rHA2 IgG1/IgG2b antibody ratios from both of the time points measured and lower IgG2a/IgG2b antibody ratios measured at the end of the experimental period were significantly associated with less periodontal bone loss ($F < 0.05$, linear regression) (Fig. 4). Together, IgG subclass analysis in group A suggested that IgG subclass antibody levels indicative of relatively lower Th2/Th1-driven anti-rHA2 responses were associated with protection from alveolar bone loss in this disease model. However, in rats inoculated with rHA2 in FA (group C), no relationship between subclass ratios and resulting alveolar bone loss was seen.

Conclusions. Administration of rHA2 s.c. in saline, without addition of adjuvant, stimulated an immune response, and one significant enough to provide some clinical protection from periodontitis. The present report is the first describing immunization and protection from periodontitis by delivery of a purified virulence protein target without the use of complete or incomplete FA.

Inclusion of FA (group C) in rHA2 administration prevented or eliminated the protective effect that was achieved by simply administering the rHA2 in saline (group A). This result is striking, since administration of rHA2 in FA produced significantly higher specific IgG and IgM antibody levels than administration of rHA2 in saline, yet resulted in no protection from periodontal bone loss, while administration in saline produced lower levels of these specific antibodies, yet resulted in clinical protection. Paradoxically, only the highest specific IgG

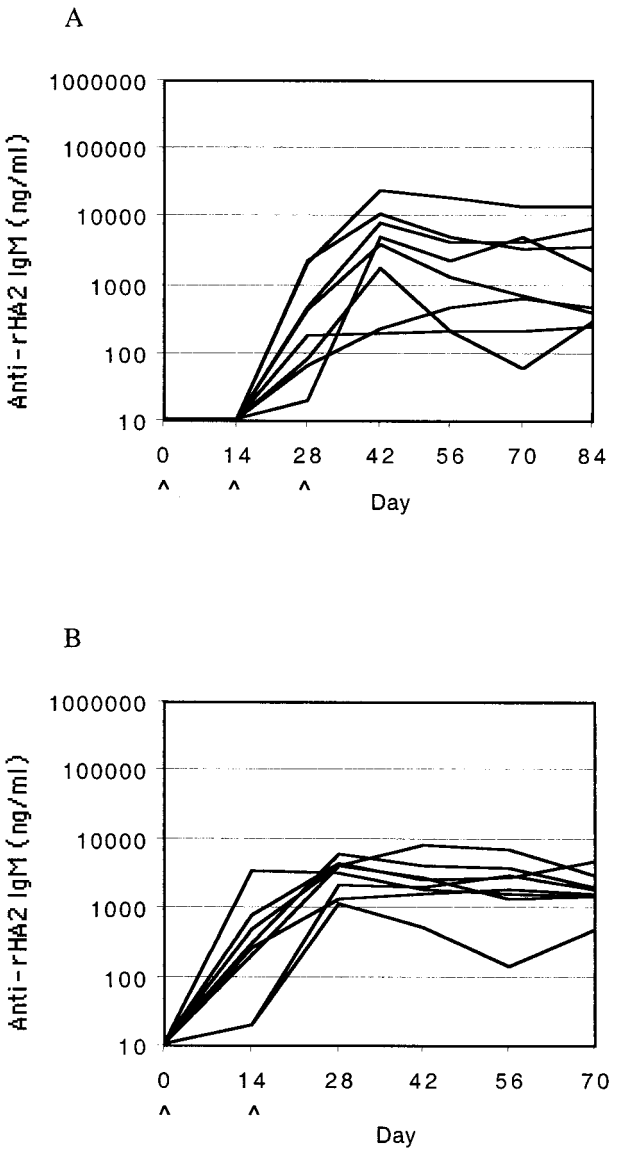

FIG. 3. (A) Anti-HA2 serum IgM antibody profiles of eight rats immunized three times s.c. with 50 μg of rHA2 protein in PBS (group A). (B) Anti-HA2 serum IgM antibody profiles of the eight rats immunized twice s.c. with 50 μg of the rHA2 protein in complete FA followed by incomplete FA (group C). Arrowheads indicate days of immunization. Anti-rHA2 IgM levels in serum were determined by ELISA as described in the text. Animals were infected orally with *P. gingivalis* on days 41 and 42 (group A) or days 27 and 28 (group C) of the experimental period.

levels within the group administered rHA2 in saline were associated with clinical protection. IgG subclasses were analyzed to address these conflicting results.

Profiles of IgG subclasses in the rat have been suggested to represent Th-type anti-HA2 responses with IgG1 and/or IgG2a representing a Th2-type-driven response and IgG2b representing a Th1-type-driven response (2, 11). In the group administered rHA2 in saline (group A), the ratio of either IgG2a or IgG1 to IgG2b subclass antibodies was decreased during the disease process in direct proportion to protection from periodontal bone loss measured at the termination of the experi-

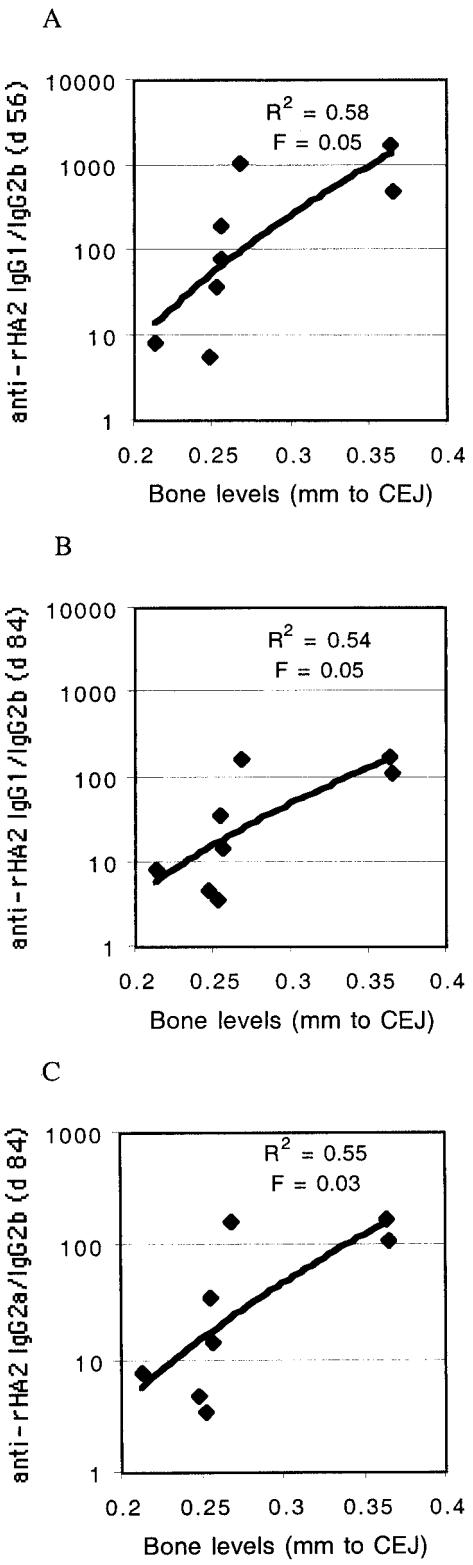

FIG. 4. Log-linear plots of anti-rHA2 IgG1/IgG2b (A and B) or anti-rHA2 IgG2a/IgG2b (C) ratios versus alveolar bone levels in each of the eight animals inoculated with rHA2 in PBS (group A). Charts depict subclass ratios from serum collected at either day 56 (A) or day 84 (B and C) of the experimental period. A solid line in each panel represents the trend line.

mental period. However, this was not the case for animals administered rHA2 in FA (group C), although they generally had subclass antibodies representing lower Th2/Th1-type ratios and significantly higher IgG2b levels at the two time points measured. Therefore, although it appears protection was related to higher humoral IgG response levels and relatively lower Th2/Th1-driven response ratios specific to rHA2, the mechanism of protection was more complicated, and the localized immune characteristics should be considered. While rHA2 administered in saline afforded some protection, well-known effects of FA such as prolonged antigen presentation, enhanced antigen uptake, and enhanced costimulation may have supported development of an overreactive or otherwise inappropriate specific and nonspecific localized immune response. The desired direction for manipulation of the host response is not clear (10, 23, 31, 33, 36) and may be antigen dependent.

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