RAC protocol 0311-614

Ad hoc Reviewer: Suzanne M. Michalek, Ph.D.

PI: Constance E. Stone, D.M.D., CCRI

Sponsor: Oragenics, Inc.

Technical Contact: Jeffrey D. Hillman, D.M.D., Ph.D.

RAC protocol 0311-614 (cont.)

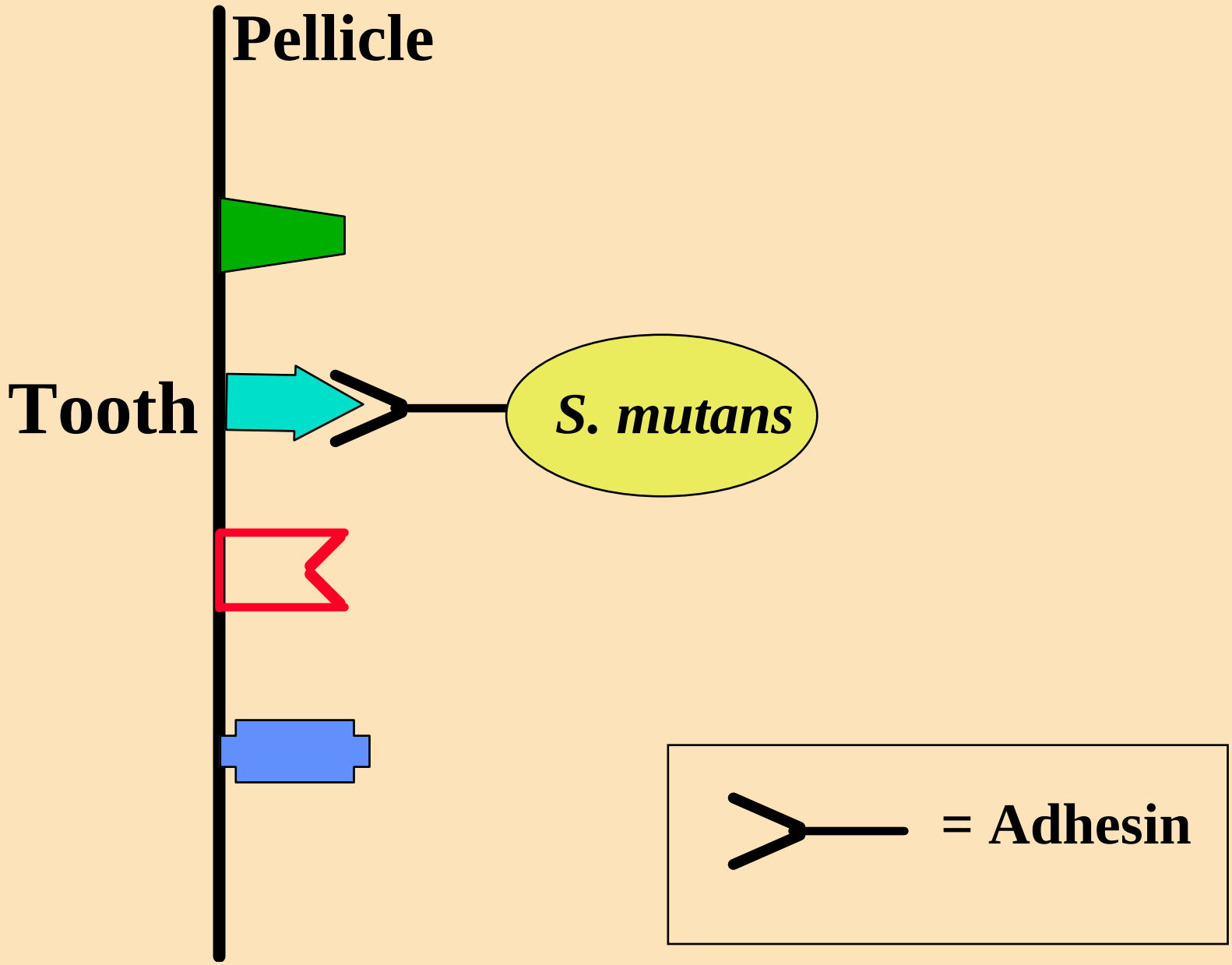
First Time in Human Safety Study of *Streptococcus mutans* Lactic Acid-Deficient Strain (A2JM) Administered in Conjunction with Twice-Daily Dose of D-Alanine Mouthwash in Healthy Adult Male Subjects for Replacement Therapy as an Aid in the Protection Against Dental Caries

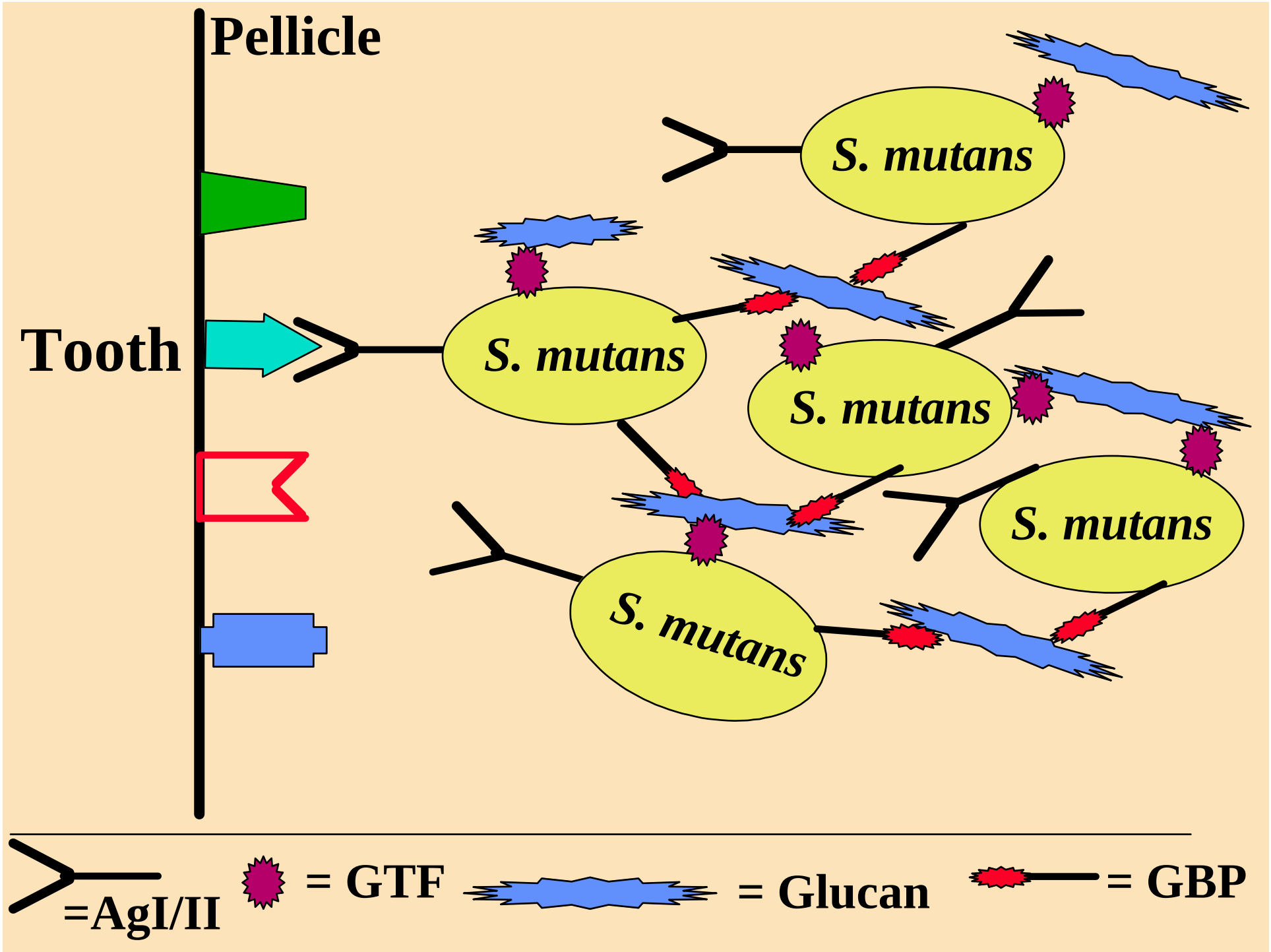
Background

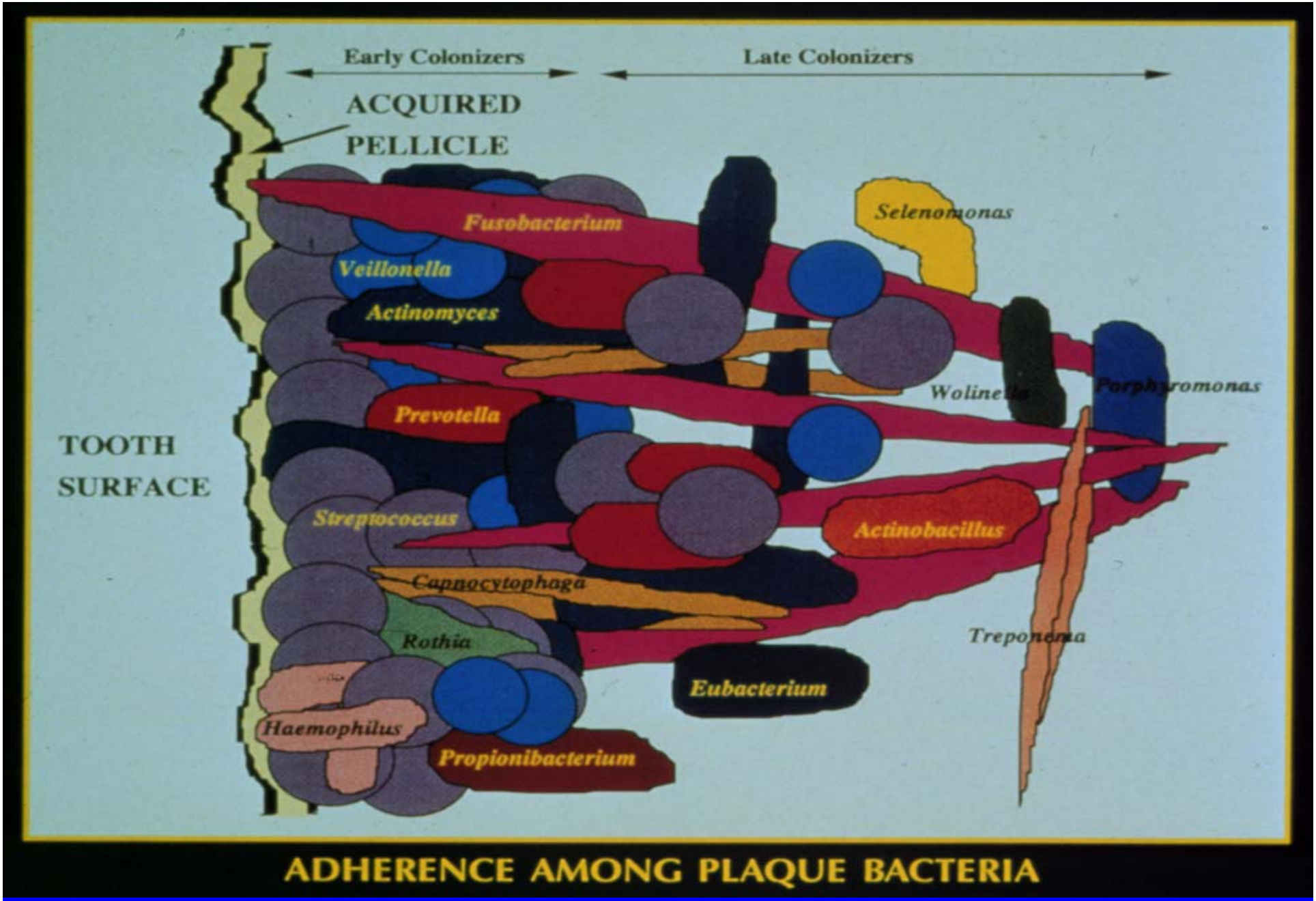
- This protocol addresses a major health problem - the ability to prevent dental caries.
- *Streptococcus mutans* A2JM was created using recombinant DNA technology for use as the effector strain for replacement therapy for the prevention of dental caries.
- A2JM does not produce lactic acid, a key product responsible for caries formation. Otherwise, A2JM is essentially identical to the *S. mutans* found in dental plaque of humans.

Mechanisms involved in *S. mutans* colonization and pathogenesis

- Sucrose-independent attachment (AgI/II)
- Sucrose-dependent reaction (glucosyltransferase)
- Bacterial metabolic activities with lactic acid production


Pellicle
Tooth
S. mutans
= Adhesin


Pellicle
Tooth
S. mutans
S. mutans
S. mutans
S. mutans
S. mutans
=AgI/II
= GTF
= Glucan
= GBP



From: Kolenbrander et al., FABEB J. 7:406, 1993.

Objectives

Primary Objective

- To assess the safety and tolerabilitity of *S. mutans* A2JM when given with a twice daily dose of D-alanine mouthwash.

Secondary Objectives

- To estimate transmission of A2JM.
- To estimate stability of A2JM.
- To assure A2JM can be eradicated after treatment.

Potential Concerns:

- Risks associated with administration of *S. mutans* A2JM
  - Effect of D-alanine on colonization/elimination
  - Effect of D-alanine on transmission/elimination
  - Stability of A2JM
- Risks of prolonged chlorhexidine rinse
  - Effect on *S. mutans*
  - Effect on oral microflora

Critique and Responses

(taken in order of written comments)

Critique and Responses

1. Why are only healthy males between the ages of 21 and 35 years being used to receive the effector strain?

Critique and Responses

1. Why are only healthy males, ages of 21 and 35 years being used?

The gender limitation is the request of the FDA, and age limitation is to facilitate enrollment. A2JM should behave the same in females or other age groups. The basis for the gender limitation stems from the evidence indicating that most children acquire *S. mutans* from their mothers during a window of infectivity between the ages of 2 and 4 years. Since this is a first-in-man study, we wanted to be sure to start modestly and then expand the treated population as we generate experimental data providing reassurance about safety.

Critique and Responses

2. The protocol indicates that the administration of A2JM to the subjects will involve oral swabbing of the occlusal surfaces. But how will the bacteria be applied to the interproximal spaces?

2. How will the bacteria be applied to the interproximal spaces?

A2JM will be supplied frozen in 5 ml amounts. After thawing to room temperature, ~ 3 ml will be uniformly applied (using a cotton swab) to the occlusal surfaces, with the remaining 2 ml distributed to the interproximal surfaces. The application will be performed gently, without pressure, in the areas adjacent to the gingival margins in order to minimize the possibility of bacteremia.

Critique and Responses

3. Is a 7-day experimental period sufficient time to establish the effectiveness of A2JM to ‘colonize’ the tooth surfaces of the subjects or to assess horizontal transmission?

3. Is a 7-day experimental period enough time to assess colonization of A2JM?

No, the effectiveness of A2JM to persistently colonize cannot be established in this short trial. Our principal intention in this study is to establish the safety of the A2JM effector strain. Later studies will be directed at establishing the efficacy using longer periods of exposure. (Cont.)

3. Is a 7-day experimental period enough time to assess transmission of A2JM?

The possibility for reversion and transmission to spouses are both a function of the numbers of A2JM present. From previous studies, we know that the numbers of A2JM will be highest during the first week following implantation. Thus, some useful preliminary data will be obtained regarding the genetic stability and transmissibility of the effector strain.

Critique and Responses

4. A secondary objective of this protocol is to estimate the transfer of A2JM from the subjects to spouse/partner. Based on the study design, a transfer of the effector strain would have to occur within the one-week test interval. This is a very short period of time. Animal studies have shown that the effector strain can persist in rats infected and not provided D-alanine. Is D-alanine a component of the diet provided to the rats? (Cont.)

Critique and Responses

4. (Cont.) The protocol and the response to Appendix M both indicate that D-alanine is not normally found in our diets. Therefore, what is the probability that a spouse/partner would become colonized with A2JM? What is the probability that subjects receiving a challenge with A2JM and not receiving D-alanine mouthwashes would become colonized?

4. Is D-alanine a component of the diet provided to the rats?

D-alanine is not found in measurable amounts in the diet. In the rat studies, D-alanine was provided in drinking water. Removal of D-alanine from the water did not lead to rapid elimination of A2JM. This may be due to *cross-feeding*, i.e. other bacteria in dental plaque provide the essential D-alanine for A2JM. (Cont.)

4. Is D-alanine a component of the diet provided to the rats?

(Cont.) Alternatively, since the rat is a coprophagic animal, the D-alanine may be provided by the feces. Nevertheless, the level of A2JM in the rats decreased significantly within weeks after removal of D-alanine from the water, indicating that the level of D-alanine provided by cross-feeding was suboptimal. (Cont.)

4. What is the probability that a spouse/ partner would become colonized with A2JM?

In discussions with the FDA, we agreed that it would be prudent to devise and implement a plan for use in the phase 1 trial that would reduce the possibility of effector strain transmission from treated to untreated subjects. A large, in-frame deletion mutation was introduced into the *dal* gene which encodes the enzyme, alanine racemase, that converts L-alanine to D-alanine. (Cont.)

4. (Cont.) What is the probability that a spouse/ partner would become colonized with A2JM?

D-Alanine is an essential component of the *S. mutans* cell wall. We found that A2JM cannot grow unless this amino acid is provided in the environment in sufficient amounts. Based on this data, transfer of A2JM to spouses may occur through kissing or sharing food or utensils. The first step to persistent colonization requires that an A2JM cell attach to the tooth surface. (Cont.)

4. (Cont.) What is the probability that a spouse/ partner would become colonized with A2JM?

In the absence of attachment, the bacteria will be swallowed and lost. Once attached, essential requirements for nutrients, pH, oxidation-reduction potential, and other factors must be met in order for the bacterium to persistently colonize. In the case of A2JM, there is the additional requirement for D-alanine cross-feeding. (Cont.)

4. (Cont.) What is the probability that a spouse/ partner would become colonized with A2JM?

The likelihood of all of these criteria being met is a direct function of the number of cells transferred, the so-called minimal infectious dose (MID). If we find that A2JM cannot be recovered from saliva of spouses, this would provide preliminary evidence that treated subjects do not supply the MID of A2JM during routine, daily contact. (Cont.)

4. (Cont.) What is the probability that a spouse/ partner would become colonized with A2JM?

If A2JM is identified in the saliva of spouses, longer observation periods than provided for in this study may be required to determine if A2JM is transiently or persistently present.

Critique and Responses

5. What effect will chlorhexidine treatment for 30 to 90 days have on the indigenous oral microflora?

5. What effect will chlorhexidine treatment have on the indigenous oral microflora?

Several studies were conducted in accordance with the American Dental Association Council on Dental Therapeutics (CDT) acceptance program guidelines for clinical studies to demonstrate safety and efficacy for chlorhexidine, which include requirements for a six-month double-blind, controlled clinical study with a placebo and/or active control. (Cont.)

5. (Cont.) What effect will chlorhexidine have on the indigenous oral microflora?

All studies showed a reduction in total plaque after six months of chlorhexidine treatment. From the reported microbiological data, the development of resistance or emergence of opportunistic or potentially pathogenic organisms did not occur, and some beneficial bacterial changes were reported, including reductions in *S. mutans*. (Cont.)

5. (Cont.) What effect will chlorhexidine for on the indigenous oral microflora?

Other studies found that a normal oral microflora re-established after intensive chlorhexidine treatment. The re-established plaque was characterized by low proportions of mutans streptococci. Acute and chronic side effects from established and recommended mouth-rinsing routines are extremely rare (FDI Commission, Interntl. Dent. J. 52:337-45, 2002).

Critique and Responses

6. Please clarify who will be doing the microbiology analysis of saliva samples from subjects and their spouses/partners for levels of total bacteria, total mutans streptococci and total A2JM? Who will determine the genetic stability of A2JM isolates from the subjects and what will this involve?

6. Who will do the microbiology analysis of saliva samples?

Saliva samples will be analyzed for levels of the indicated bacteria by the Periodontal Disease Research Center (PDRC) at the University of Florida. Dr. Clay Walker, Director of the PDRC, will oversee this portion of the program. (Cont.)

6. (Cont.) Who will do the microbiology analysis of saliva samples?

The concentration of total bacteria/ml of saliva will be calculated from colony counts of serially diluted saliva samples spread on trypticase soy agar medium containing 5% sheep's blood and incubated for 3 days at 37°C in an atmosphere consisting of 85% nitrogen, 10% carbon dioxide and 5% hydrogen. (Cont.)

6. (Cont.) Who will do the microbiology analysis of saliva samples?

Total mutans streptococci and A2JM will be determined from colony counts of serially diluted saliva samples spread on Oragenics' proprietary Screening/Selection (S/S) medium supplemented with D-alanine and incubated for 3 days at 37°C in candle jars. This medium is highly selective for mutans streptococci, and lactic acid-producing cells yield white colonies while A2JM yields bright red colonies. Counts of white and red colonies will be performed on plates containing appropriate numbers of colonies, and the concentrations of wild-type *S. mutans* and A2JM per ml of saliva calculated. (Cont.)

6. Who will determine the genetic stability of A2JM isolates?

With regard to testing the genetic stability of A2JM, the pertinent genetic markers are the mutations in *ldh*, *comE* and *dal*. Of these, only revertants of *dal* can be directly selected. A2JM is naturally resistant to a low level (1 μg/ml) of tetracycline. After taking an appropriate aliquot for performing the tests described above, the PDRC will spread 0.1 ml samples of saliva on 10 plates containing S/S medium supplemented with tetracycline, but containing no added D-alanine. The presence of tetracycline will prevent the growth of wild-type *S. mutans*, and, in the absence of added D-alanine, only dal^+ revertants of A2JM will be able to grow. (Cont.)

6. (Cont.) Who will determine the genetic stability of A2JM isolates?

Any colony that appears will be purified and tested to verify that it is a revertant of A2JM to D-alanine independence. The frequency of reversion will be calculated based on the total number of A2JM present per ml of saliva, as determined by plating serial dilutions of the saliva on S/S medium supplemented with both tetracycline and D-alanine. We have previously used this method successfully in determining the reversion frequency of *dal*$^{-}$ in the laboratory and during colonization of A2JM and rat models. (Cont.)

6. (Cont.) Who will determine the genetic stability of A2JM isolates?

There is no way to directly select for *ldh* and *comE* revertants. However, since the construction of the *ldh* and *comE* mutations in A2JM are very much the same as the *dal* mutation, the observed frequency of *dal* reversion can be considered as a good indicator for the reversion frequencies for these other mutations. To be thorough, white colonies that arise on S/S medium supplemented with D-alanine and tetracycline as described above will be tested for D-alanine dependency to determine if they are revertants of A2JM to *ldh*$^{+}$. As many as 10,000 colonies per patient per time point will be screened in this fashion, so that reversion rates $\geq 10^{-4}$ can be measured. (Cont.)

6. (Cont.) Who will determine the genetic stability of A2JM isolates?

Any indication of *dal* and *ldh* reversion in A2JM isolates, will be verified by Oragenics. Purified colonies of the isolates to be tested will be prepared by the PDRC, and they will serve as a source of template for colony polymerase chain reaction (PCR). Primers and PCR conditions have been perfected for this purpose, and the method has proven preferable to colony blot methods with regard to both precision of results and ease of performance. The sizes of the *dal* and *ldh* genes from each putative revertant will be compared to the sizes of the genes present in controls consisting of the wild-type parent, JH1140, and authentic A2JM from glycerol stabs.

Potential Concerns:

- Risks associated with administration of *S. mutans* A2JM
  - Effect of D-alanine on colonization/elimination
  - Effect of D-alanine on transmission/elimination
  - Stability of A2JM
- Risks of prolonged chlorhexidine rinse
  - Effect on *S. mutans*
  - Effect on oral microflora

Summary:

- **Risks associated with administration of *S. mutans* A2JM.**
  - **Effect of D-alanine on colonization/ elimination.**
    - Initial preclinical data suggest low risk regarding the use of D-alanine for colonization and its absence for elimination of A2JM from the oral flora. Longer term risk is unknown.

Summary:

- **Risks associated with administration of *S. mutans* A2JM.**
  - **Effect of D-alanine on transmission/ elimination.**
    - Initial preclinical data suggest low risk regarding the transmission of A2JM. Longer term risk is unknown.

Summary:

- **Risks associated with administration of *S. mutans* A2JM.**
  - **Stability of A2JM.**
    - Initial preclinical data suggest low risk regarding the stability of A2JM. Longer term risk is unknown.

Summary:

- **Risks of prolonged chlorhexidine rinse.**
  - **Effect on *S. mutans*.**
    - Initial preclinical data suggest low risk regarding the elimination of A2JM with chlorhexidine treatment and the subsequent establishment of the indigenous microflora. Longer term risk is unknown.

Summary:

- **Risks of prolonged chlorhexidine rinse.**
  - **Effect on oral microflora.**
    - Initial preclinical data suggest low risk regarding the effect of chlorhexidine treatment on the indigenous microflora. Longer term risk is unknown.