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Review

Production of secretory IgA antibodies in plants

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Abstract

Functional antibodies produced in tobacco plants were first reported over a decade ago (1989). The basic protocol used to generate these 'plantibodies' involved the independent cloning of H and L chain antibody genes in *Agrobacterium tumefaciens* vectors, the transformation of plant tissue in vitro with the recombinant bacterium, the reconstitution of whole plants expressing individual chains, and their sexual cross. In a 'Mendelian' fashion, a fully assembled and functional antibody was recovered from plant tissue in some double-transgenic plants. In mammalian cells, the antibody H and L chains are produced as precursor proteins that are translocated into the endoplasmic reticulum (ER), under the guidance of signal sequences. Within the ER, the signal peptides are proteolytically cleaved, and several stress proteins act as chaperonins to bind the unassembled antibody chains, and direct subsequent folding and tetramer formation. A similar process occurs in plant cells, and expression can be directed via signal sequences (even of foreign origin) into the aqueous environment of the apoplasm, or to be accumulated in other specific plant tissues, including tubers, fruit, or seed. Plants can facilely assemble secretory IgA, which is comprised of four chains, H and L chains, J chain and secretory component. Plant 'bioreactors' are expected to yield over 10 kg of therapeutic antibody/acre in tobacco, maize, soybean, and alfalfa [(Ann. NY Acad. Sci.)721(1994)235; (Biotechnol. Bioeng.)20(1999)135]. Compared with conventional steel tank bioreactors using mammalian cells, or microorganisms, the costs of GMP plantibodies are expected to perhaps one tenth. The differences in glycosylation patterns of plant and mammalian cell produced antibodies apparently have no effect on antigen-binding or specificity, but there is some concern about potential immunogenicity in humans. N-linked glycans of plants differ from human by having fucose-linked alpha 1,3 and the sugar xylose. No adverse effects or human anti-mouse antibodies (HAMA) have been observed in >40 patients receiving topical oral application of a plant produced secretory IgA specific to *Streptococcus mutans*, for the control of caries [(Nat. Med.)4(1998)601]. The progressive improvement of expression vectors for plantibodies, and purification strategies, as well as the increase in transformable crop species, is expected to lead to almost limitless availability of inexpensive (even edible forms of) recombinant immunoglobulins free of human pathogens for human and animal therapy, and for novel industrial applications (e.g. catalytic antibodies). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Apoplasm; Secretory IgA; Bioreactors; Plant biotechnology

1. Antibodies: an important drug-development platform

It has been almost 100 years since von Behring and Kitasato received the first Nobel prize for the discovery of passive immunotherapy and about 25 years since Köhler and Milstein first reported monoclonal antibodies generated by hybridoma technology for which they also shared a Nobel prize. In the 15 years since Mullis and coworkers described PCR, a number of discoveries and technologies have converged to produce a renaissance in antibody therapy of human disease. Antibodies as tools for research, useful for the prevention, detection, and treatment of disease have been revolutionized by these recent advances in antibody engineering ([4]). Antibodies now account for the single largest group of biotechnology-derived molecules in clinical trials, with a prospective market reaching expected to reach five billion dollars by 2005. According to the Pharmaceuti-

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cal Research and Manufacturers of America (see http://www.phrma.org), more than 20% of all biopharmaceuticals in clinical development are antibody products. One recent survey indicated that over 250 companies are working on over 700 therapeutic antibodies, 220 of which are in clinical trials ([4]) With this plethora of antibodies under development, a variety of production methods have been studied to increase efficiency and reduce costs. Among these, production in plant 'bioreactors' is perhaps the most promising. As discussed below, the development of plant production of the unique class of secretory IgA opens up a number of therapeutic targets previously not addressable using conventional antibodies.

2. Plant bioreactors for immunotherapeutic proteins

Progress over the past 10 years demonstrates that plants will be a facile and economic bioreactor for large-scale production of industrial and pharmaceutical recombinant proteins ([5–7,1,8–11]). Genetically engineered, transgenic plants have many advantages as sources of proteins compared with human or animal fluids/tissues, recombinant microbes, transfected animal cell lines, or transgenic animals. Among these are (a) production of raw material on an agricultural scale at low cost with the possibility, in some cases, of using the edible plant material directly; (b) reduced capitalization costs relative to fermentation methods; (c) rapid scale-up of production; (d) correct eukaryotic assembly of multimeric proteins such as antibodies (unlike bacteria); and (e) increased safety, since plants do not serve as hosts for human pathogens, such as HIV, prions, hepatitis viruses, etc. This final point is noteworthy, given the spread of foot-and-mouth disease and mad cow disease from the UK into Europe in the past year.

Potential disadvantages of plantibody production fall into three areas, environmental, adverse effects of antibodies produced in plants and process development. Concerns have been raised regarding the release of genetically modified plants into the environment with outcrossing to weeds or related wild species. Depending upon the plant species (e.g. corn) or targeted tissue (e.g. chloroplasts) this is not a substantial issue. Post-translational glycan-modifications of plantibodies do differ in minor ways from the glycans found on human proteins (see below). However, recent studies suggest that these glycans have limited immunogenicity ([12]) and topically applied antibodies have been shown to be remarkably safe ([10]). Even though antibodies accumulate to high levels in plant tissue, the processing of large quantities of biomass will be required to obtain metric tons of purified plantibodies. While this may require substantial front-end equipment, the costs are expected to be a fraction of fermentation.

Early investigators utilized strong generic promoters, such as the cauliflower mosaic virus 35S promoter giving protein expression throughout the biomass of the transgenic plant. For example, [13,14] showed that in *Arabidopsis*, antibody or Fab fragments bearing conventional leader sequences accumulate at the sites where water passes on its radial pathway towards and within the vascular bundle. A large proportion of these proteins are transported into the apoplast of *Arabidopsis thaliana*, possibly by the water flow in the transpiration stream. Recent work has focused on the use of novel promoters to control expression of transgenic proteins in specific parts of the plants (e.g. seeds) or specific organelles (e.g. chloroplasts) within a given plant cell. Numerous labs have shown transgenic protein accumulation in seeds of tobacco ([15]), corn ([16,17]), soybean ([18]) or barley ([19]). Antibodies produced in seed are remarkable for their stability. For example, full antigen-binding activity is retained after as long as 3 years at refrigerator temperature.

Homologous recombination is used to introduce a foreign gene into a spacer region between the functional genes of the chloroplast. This eliminates position effects of nuclear transformation, and gene silencing has not been a problem. [20] demonstrated that chloroplasts can process and assemble a fully assembled, disulfide bonded form of human somatotropin (expressed at 7% of total soluble protein (TSP)) and an oligomeric form of cholera toxin B (4–5% TSP). [11] have expressed foreign genes (up to 10 000 copies per cell) in tobacco chloroplasts, resulting in the accumulation of foreign proteins at up to 47% of the total soluble protein ([21]).

[22,23] (see Table 1) compared production levels of different antibody fragments in various plant cell compartments. Some fragments were unstable, probably due to folding. In contrast, when secretory IgA, known for its mucosal stability, is expressed in the secretory pathway, consistently high levels of expression are found ([10] Wycoff et al., unpublished).

Table 1
Representative expression levels of recombinant antibody forms in tobacco (in μg/g wet weight) [22,23,59,81]

Form of antibody	Cytosol	Endoplasmic reticulum	Apoplast
Membrane anchored scFv	Not done (n.d.)	n.d.	20
Bi-scFv	n.d.	80	10
ScFv	50	100	70
Fab	–	n.d.	10
$F(ab')_2$	–	n.d.	10
IgG	–	n.d.	50–150
sIgA	–	n.d.	50–500

Table 2
Representative immunotherapeutic proteins synthesized in transgenic plants

Recombinant protein	Plants	Protein production levels	Reference
sIgA Anti-*S. mutans*	Tobacco	200–500 (μg/g)	[3,64]
Hepatitis B surface antigen	Tobacco	0.01% TSP	[67,78]
Hirudin	Canola *Brassica napus*	0.30% seed protein	[53]
Growth hormone	Tobacco	Chloroplast—7% TSP; Nuclear—<0/01%	[20]
Norwalk capsid protein	Tobacco	0.23% TSP	[66]
Norwalk capsid protein	Potato	10–20 (μg/g)	[66]
Albumin	Tobacco	0.02% TSP	[69]
E. coli toxin [LT-B]	Potato	3–4 (μg/g)	[42,60,68]
Cholera toxin [CT-B]	Potato	30 (μg/g)	[48,49]
Alpha and beta hemoglobin	Tobacco	0.05% of seed	[53]
Mouse GAD67	Potato	150 (μg/g)	[63]
Protein C	Tobacco	<0.01% TSP	[53]

TSP, total soluble protein

Plants are attractive expression vehicles for numerous immunotherapeutic proteins (see Table 2). The most widely studied proteins have been antigens for use in oral vaccines and antibodies for passive immunotherapy. Among these, secretory IgA antibodies are known to have the highest levels of expression.

3. Issues regarding plantibody production

3.1. Plant antibody glycosylation

Questions have been raised regarding glycosylation of plant produced proteins. The major issues include immunogenicity, functional activity and pharmacokinetics. Glycosylation is important for Fc receptor binding. An antibody requiring this effector mechanism to be effective (e.g. certain anti-cancer antibodies such as herceptin or rituxin) will need to be evaluated for retention of functional activity after production in plants.

Cabanes-Macheteau et al., [24] reported the first detailed analysis of the glycosylation of a functional mammalian glycoprotein expressed in a transgenic plant. The structures of the N-linked glycans attached to the heavy chains of the monoclonal antibody Guy's 13 produced in transgenic tobacco plants (plantibody Guy's 13) were identified and compared with those found in the corresponding IgG1 of murine origin. As in mouse antibodies, both N-glycosylation sites located on the heavy chain of the plantibody Guy's 13 are N-glycosylated. However, the number of Guy's 13 glycoforms is higher in the plant than in the mammalian-expressed antibodies. In addition to high-mannose-type N-glycans, 60% of the oligosaccharides N-linked to the plantibody have beta(1, 2)-xylose and alpha(1,3)-fucose residues linked to the core Man3GlcNAc2.

These oligosaccharide linkages, not found on mammalian N-linked glycans, are potentially immunogenic and raise the possibility that plantibodies containing N-linked glycans may have a limited scope as parenterals or even when applied topically or orally, particularly in patients with severe food allergies. Food allergens bearing beta(1,2)-xylose and alpha(1,3)-fucose have been linked to specific IgE in serum and to biological activity, i.e. histamine release, in allergic patients ([25] and [26]). In contrast, the mere presence of serum IgE against cross-reactive carbohydrate determinants, which include the beta(1,2)-xylose and alpha(1,3)-fucose linkages to the core Man3GlcNAc2 of plant glycans, has been shown to be a poor predictor of clinical allergy ([27–29]). These results preclude generalities about the potential toxicity of plantibody glycans in humans.

When the mouse plantibody (mouse amino acid sequence and plant glycans) was used to immunize mice there was no, or only a minimally detectable, serum immune response ([12]). Thus, in the mouse at least, a 'self' primary protein structure, decorated with plant N-linked glycans, can be non-immunogenic. This plantibody has been applied topically in the mouth of humans with no detection of human anti-mouse antibodies ([3]).

Humanization of plantibody primary structures may go a long way to obviate the immunogenic potential of plant glycans and further genetic engineering may, if necessary, alter the glycan structures themselves. Most dramatically, aglycosyl antibodies can be created by altering the peptide recognition sequence for N-linked glycosylation (asn-X-Ser/Thr). High-mannose type glycosylation, which does not contain the core-linked xylose and fucose residues, may be favored by the addition of a C-terminal KDEL sequence ([30]) and the subsequent targeting of plantibodies to the proximal endoplasmic reticulum. Alternatively, the specific fucosyl ([31]) or xylosyl transferases, which operate in the *trans*-golgi, may be targeted for silencing. Of course, the KDEL sequence is not cleaved during protein pro-

cessing and the potential of a KDEL tag to alter immunogenicity, pharmacokinetics, etc. is not known.

3.2. Purification and process development

The potential for cost reduction when using genetically engineered green plants as bioreactors versus conventional pharmaceutical factories engineered with concrete and steel, favors the use of transgenic plants to produce recombinant proteins. Commercial-scale production of proteins from transgenic green plants will require the growth and processing of tons of biomass.

Minimalist approaches to large-scale process development will often be rewarded, even though they may appear simplistic. We found that grinding transgenic tobacco in water alone gave up to 70% of the expected immunoglobulin compared with tobacco ground in a buffer, poised at a specified pH, containing six components in addition to water; each component had been chosen for a particular, biochemically sound, reason. Yet this was evidence suggesting that perhaps not all of the components were vitally important and we now use a two-component buffer at a specified pH.

Commercial-scale production of recombinant proteins from plants will also benefit from the technology and equipment commonly used in the food and beverage industry. Grain mills and cole-slaw slicers will be useful off-the-shelf machinery for the initial processing of seeds and leafy tissue. The beverage industry has always been concerned with the clarification of juices and their phenolic content ([32]) and is a source of knowledge and equipment, both new and used. The chef's trick of delaying the browning of cut fruit by the addition of lemon acidulated water ([33]) has led us to formulate buffers to inhibit the 'tanning' of proteins in tobacco extracts.

Phenolics are a major concern when extracting most stems and leaves and they best removed early in the process. They may interact with proteins, and other extract components, via hydrophobic interactions, salt bridges, hydrogen bonding and by additional reactions to nucleophilic centers ([34,32,35]). These interactions can dramatically and irreversibly alter the properties of proteins. Fortunately, the majority of released phenolics are generally small in size, as well as water-soluble, and may be removed by tangential-flow ultrafiltration/diafiltration, which also serves to concentrate the considerably larger proteins of interest. In addition, other incompatible, water-soluble secondary metabolites, such as neonicotine (anabasine) and nicotine from tobacco, may also be removed by ultrafiltration/diafiltration.

One additional note of caution: since the processed plant or seed was probably grown in dirt, or some similarly unclean stratum, one may anticipate the presence of a diverse bioburden. Contamination of product with endotoxins and mycotoxins can be minimized by rapid processing and early filtration but process development must also always recognize the necessity to eliminate compounds, as well as the necessity to purify, concentrate and stabilize a product of interest. For production of parenteral antibody, GMP runs will need to be validated to demonstrate removal of endotoxin, mycotoxins, known toxins (e.g. nicotine from tobacco plants), plant DNA (and viruses), etc.

In summary, much has been learned from several plantibodies that have been purified to homogeneity for human clinical use. Purification and GMP compliance are no longer hurdles to clinical development of plantibodies.

3.3. Production costs

Cost benefits of plant production are enormous. For example, [5] calculated the cost of producing a recombinant protein in various agricultural crops. The cost estimate was based on the commodity price of the crop, the fraction of total protein in the crop, and the not unreasonable assumption that the recombinant protein accumulated to 10% of the total plant protein. Although crops with more protein content (e.g. soybeans, 40% vs. potatoes, 2%) are more cost effective these costs are 10–50-fold less than protein produced at high level in *E. coli* (i.e. at the rate of 20% total protein) (Larrick et al., unpublished data). Depending upon the use of the protein and the requirements for purification for in vivo pharmaceutical use, purification costs will obviously augment final product costs, however, at the hundred kilogram to metric ton level plant-produced proteins will provide obvious savings. In contrast to parenteral IgG antibodies, secretory IgA plantibodies for topical, mucosal or oral uses are expected to have greatly reduced purification costs.

4. Secretory IgA: production of a novel plantibody isotype

Although antibodies were first expressed in plants in the mid-1980s, (Stieger, Düring) by two German graduate students ([36]), the first publication was in Nature in late 1989 ([37]). Since then, a diverse group of 'plantibody' types and forms have been prepared (see Table 3). Originally, foreign antibody genes were introduced into plant cells by nonpathogenic strains of the natural plant pathogen *Agrobacterium tumefaciens* ([38]) and regeneration in tissue culture resulted in the recovery of stable transgenic plants. Although this initial work to generate multichain proteins required crossing of plants expressing each chain, more recent studies have shown that multiple chains can be introduced via a single biolistic transformation event ([39]Wycoff et al., unpub-

lished data), greatly reducing the time to final assembled plantibody.

This laboratory has focused on the production of secretory IgA (SIgA) plantibodies ([40]) (see Fig. 1). At the present time, plants offer the only large-scale, commercially viable system for production of this unique form of antibody as minimal levels are secreted by transfected mammalian cells ([41]) The levels of expression of SIgA in animal cell culture are SIgA is the most abundant antibody class produced by the body ($>60\%$ of total immunoglobulin). SIgA is secreted onto mucosal surfaces to provide local protection from toxins and pathogens (see Fig. 2). SIgA is comprised of four different protein chains: heavy and light immunoglobulin chains that form the antigen-binding hypervariable region, J chain that dimerizes two IgA molecules (SIgA has four antigen-binding sites) and secretory component that is derived from the mucosal epithelial cells. Dimeric IgA containing J chain derived from submucosal B cells binds to the epithelial cell polyimmunoglobulin receptor (PIG^R) that transports the IgA to the mucosal surface. Binding triggers transcytosis to the mucosal surface where a protease releases a portion of the PIG^R called secretory component, conveniently used to bind the SIgA. The secretory component protects the dimeric IgA from proteases and denaturation on the mucosal surface. Previously, it was not possible to obtain therapeutic quantities of this class of immunoglobulin. The recent availability of large amounts of secretory IgA plantibodies opens up a number of novel therapeutic opportunities for disorders of the mucosal immune system. These include therapies for intestinal pathogens such as hepatitis viruses, *Helicobacter pylori*, and enterotoxigenic *E. coli*, cholera, etc., respiratory pathogens such as rhinovirus and influenza, and genito-urinary sexually transmitted diseases (e.g. HSV, [18]) and contraception.

To date, a small number of products produced in plants have entered the clinic. These products include two antibodies, an oral vaccine ([42]) and pancreatic lipase. Clinical studies of the anti-EPCAM plantibody (co-developed by NeoRx and Monsanto) were discontinued due to significant gastrointestinal side-effects. The anti-*S. mutans* antibody is currently in Phase II trials as described below.

5. CaroRx™—an anti-*Streptoccocus mutans* SIgA to prevent dental caries

The most clinically advanced SIgA plantibody, called CaroRx™, recognizes and inhibits the binding of the major oral pathogen, *Streptoccocus mutans*, to teeth. In preliminary work, a series of in vivo passive immunization experiments was carried out in 84 human subjects using murine anti-*S. mutans* antibodies [43,44,45–47]. Topical application of anti-*S. mutans* antigen SA I/II MAbs prevented colonization of both artificially implanted exogenous strains of *S. mutans*, as well as natural recolonization by indigenous *S. mutans*. In these studies, the pathogenic *S. mutans* was replaced by endogenous flora (see Fig. 3).

The presence of the complement-activating and phagocyte-binding sites on the Fc fragment of the MAb was not essential for activity, because the $F(ab')_2$ portion of the MAb was as protective as the intact IgG, however, the Fab fragment failed to prevent recolonization of *S. mutans*. Prevention of recolonization was specifically restricted to *S. mutans*, as the proportion of other organisms, such as *S. sanguis*, did not change significantly. The surprising feature of these experiments was that protection from re-colonization by *S. mutans* lasted up to 2 years (J. Ma, personal communi-

Table 3
Representative antibody-derived molecules produced in transgenic plants

Antigen	Mab Form (# chains)	Plant species-comments	Reference
Substance P (neuropeptide)	Single domain (dAb) (1)	*Nicotiana*	[51]
Phytochrome	Single chain Fv (scFv) (1)	*Nicotiana*	[58,71]
Artichoke, mottled crinkle virus coat	scFv (1)	*Nicotiana*—viral protection	[77]
Abscisic acid	scFv (1)	*Nicotiana*—wilty phenotype	[50,72]
Root-knot nematode	scFv (1)	*Nicotiana*	[73]
Beet necrotic yellow vein virus	scFv (1)	*N. benthamiana*	[55,56]
B cell idiotype	ScFv (1)	*N. benthamiana*	[70]
Several	scFv (1)	*Nicotiana*, KDEL augments expression	[52,57,74,75]
Human creatine kinase	Fab; IgG (k) (2)	*Nicotiana Arabidopsis*	[54]
Transition-state analogue	IgG (k) (2)	*Nicotiana*	[37,61]
Fungal cutinase	IgG (k) (2)	*Nicotiana*	[80]
Carcinoembryonic antigen	IgG	Wheat, rice	[76,79]
Colon Cancer antigen	IgG	*N. benthiana*	[82]
Streptococcus mutans adhesin	IgG (k) and IgG/A hybrids (2)	*Nicotiana*	[65]
S. mutans adhesin	Secretory IgA/G (4)	*Nicotiana*	[40]
NP (4-hydroxy 3-nitro-phenylacetyl)	IgM (2)	*Nicotiana*	[36]

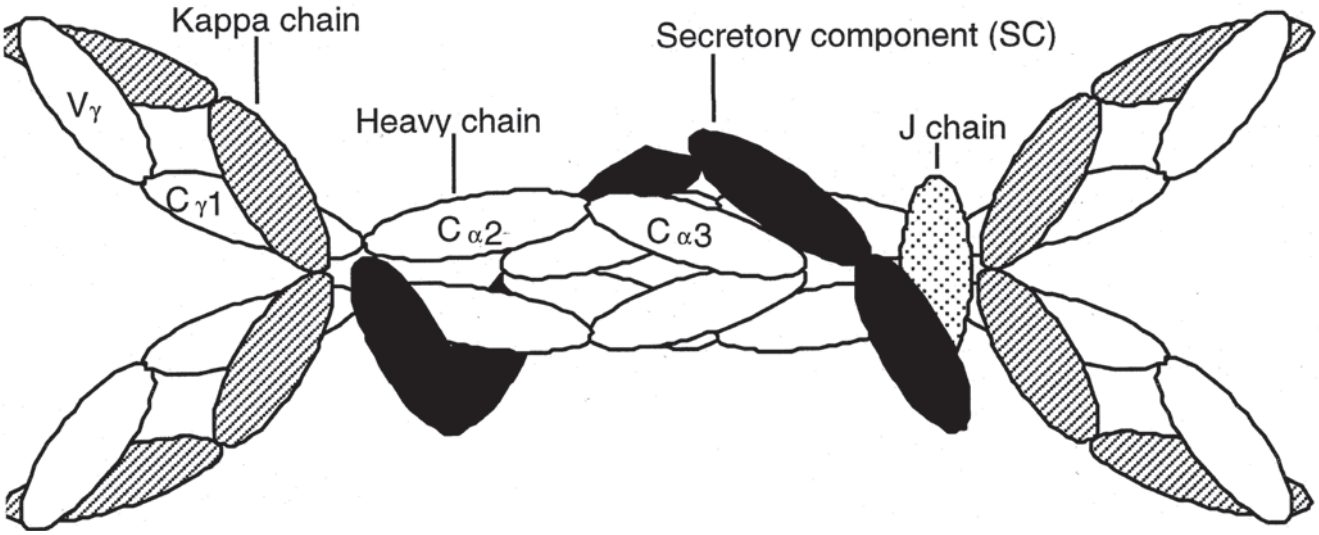

Fig. 1. The structure of secretory IgA.

cation), although MAb was applied for only 3 weeks and functional MAb was detected on the teeth for only 3 days following the final application of MAb. All studies indicated that this form of immunotherapy appears to be safe and well tolerated. The long-term protection could, therefore, not be accounted for by a persistence of MAb on the teeth, but may be due to a shift in the microbial balance in which other bacteria occupy the ecological niche vacated by *S. mutans*, resulting in resistance to recolonization by *S. mutans* (Fig. 3).

The antigen-binding V regions of the best murine Mab identified by [47], Guy's 13, has been used to create an SIgA plantibody produced in tobacco designated CaroRx™ (details in [40,3]). Levels of production of CaroRx™ in tobacco are up to 0.5 mg/g fresh weight. Future plans call for production of CaroRx™ in corn and other cereal grains.

CaroRx™ has been produced and purified from tobacco under GMP conditions for clinical testing in the UK and USA. CaroRx™ was engineered with an additional IgG CH2 domain to facilitate purification of the antibody by protein G affinity chromatography. A Poros™ Protein G affinity purification was used to obtain $>95\%$ pure CaroRx™ from green plant tissue.

Clinical evaluation of CaroRx™ in a pilot phase II trial has been completed at Guy's Hospital, London, UK ([3]). In this trial a functional comparison was made between CaroRx™ and the parent IgG monoclonal antibody Guy's 13. BIACORE analysis revealed that the affinity of the antibodies for purified *S. mutans* SA I/II was similar ($KD = 0.5–1.3 \times 10^{-9}$ M), however, CaroRx™ had 4-fold higher avidity (functional affinity), a not unexpected result given the tetravalent binding of the SIgA.

Using an experimental design similar to that used to demonstrate activity of the parent Mab, CaroRx™ gave specific protection against colonization by oral streptococci for over four months (details in [3]). In addition to this therapeutic endpoint, pharmacokinetics studies showed that in the human oral cavity, CaroRx™ survived for >3 days versus 1 day for the IgG antibody and multiple serum antibody samples were negative for human anti-mouse (HAMA) or anti-rabbit antibodies. There was no evidence of local or systemic toxicity of the topically applied plantibody.

These initial clinical studies demonstrate that topically applied anti-*S. mutans* SIgA plantibody (CaroRx™) is safe [no human anti-mouse antibody response (HAMA), no local or systemic toxicity] and prevents colonization by *S. mutans*, the major cause of human dental caries ([3]). Planet Biotechnology, Inc. has submitted an investigational new drug application (IND) to the US FDA and Phase I/II confirmatory clinical trials began at the School of Dentistry at the University of California in San Francisco in Autumn 1998. These trials were completed in Spring 2000. Details will be published elsewhere; however, initial analysis of the data indicates capacity to reduce the levels of *S. mutans* to clinically significant levels. Further confirmatory trials have been designed to optimize the treatment regimen.

6. Summary

Plants offer a cost-effective bioreactor to produce antibodies of diverse types. Recent studies demonstrate that secretory IgA, the predominant antibody isotype of the mucosal immune system, can be made in large quantities in plants. CaroRx™, the lead SIgA antibody being developed by Planet Biotechnology, Inc. has shown activity in pilot Phase II trials versus *S. mutans*, the major pathogen contributing to development of

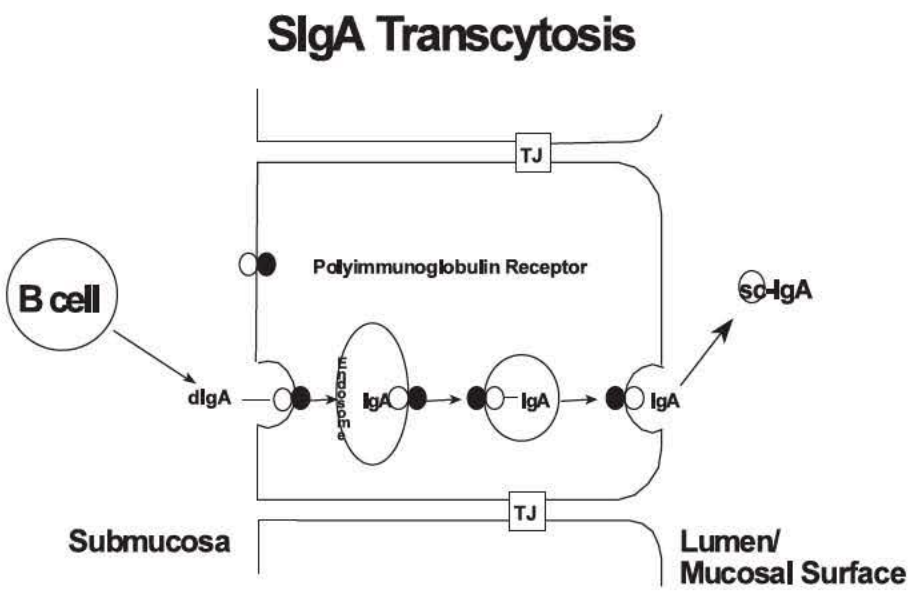

Fig. 2. SIgA is produced by two cell types. Submucosal lymphoid tissue produces dimeric IgA, which diffuses to the serosal side of the mucosal epithelial cells where it binds to the polyimmunoglobulin receptor. Secretory component (SC) is proteolytically-derived from the polyimmunoglobulin receptor.

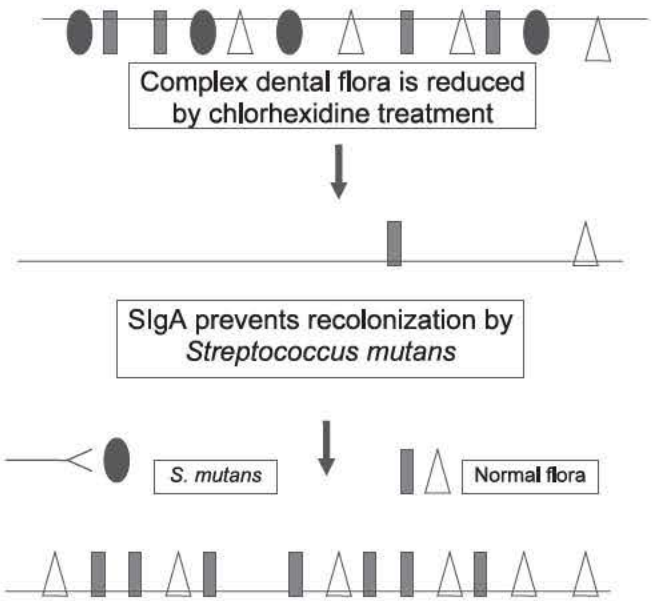

Fig. 3. Proposed mechanism of CaroRx™: the concept of ecological niche. After all flora have been reduced using oral anti-septic, chlorhexidine, a short treatment course with CaroRx™ provides an opportunity for normal flora to replace *S. mutans*.

dental caries. Numerous other SIgA plantibodies are in pre-clinical development.

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