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Note

The potential use of tomato lectin for oral drug delivery: 4. Immunological consequences

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Abstract

Previously, it has been shown that the non-toxic tomato lectin (TL) may have potential for oral drug delivery as both an intestinal bioadhesive and a drug carrier – it binds specifically to glycoproteins on the enterocyte surface, resulting in increased uptake of TL in vitro, and exhibits resistance to digestion in the gastrointestinal tract in vivo. In this study, the potential immunological consequences of the uptake of TL by the intestine were investigated by examining the ability of TL to be recognised by TL-specific antibodies after uptake in vitro and to elicit immune responses after uptake in vivo. After incubation in an improved everted gut sac system, TL or TL fragments were found to be able to react with anti-TL antibodies in an ELISA after intestinal processing and transfer into the serosal fluid. Feeding microgram quantities of TL to mice in vivo elicited high specific serum IgG responses and, to a lesser extent, specific intestinal IgA responses. The responses were dose-dependent. Preincubating TL with competing sugar gave measurable reductions in specific serum IgG and intestinal IgA levels. Whilst these results may limit the use of TL as an oral drug carrier for chronic delivery, the possibility is raised of using TL to promote immunization by the oral route.

Keywords: Tomato lectin; Oral drug delivery; Drug carrier; Antigenicity; Oral vaccine

We have been investigating the potential use of the non-toxic tomato lectin (TL) for oral drug delivery, as both an intestinal bioadhesive and a drug carrier. We have shown that TL binds avidly and specifically to glycoproteins on the enterocyte surface (Naisbett and Woodley, 1994a) and that this adhesion resulted in increased endocytic uptake of TL in an improved in vitro everted gut sac system (Naisbett and Woodley, 1994b). Because of this high rate of endocytic capture and in vitro and in vivo evidence of TL resistance to digestion in the gastrointestinal (GI) tract (Naisbett and Woodley, 1994b, 1995), it is possible that TL may have potential as an intestinal drug carrier. Drugs may be covalently coupled to TL, thus directing the drug towards an endocytic route of capture, the rate of which may be increased by specific bioadhesion.

However, if TL crosses the gut mucosa intact

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to some extent, as suggested by our early in vitro work, it may be able to enter the systemic circulation and elicit an immune response. This would have profound consequences for the use of TL as a drug carrier and limit its suitability, particularly for long-term therapy. Conversely, an immune response towards the lectin may give it potential as an adjuvant. In this study, we examine the ability of TL to be recognised by antibodies after uptake in vitro and to elicit immune responses after uptake in vivo. Non-radiolabelled TL was therefore incubated in an improved everted gut sac system and samples of serosal fluid were analysed for fragments of TL which were still reactive with anti-TL antibodies. Non-radiolabelled TL and BSA (bovine serum albumin, a control biodegradable protein of similar molecular weight) were also fed orally to mice and serum IgG and intestinal IgA responses towards the macromolecules were determined. These data will give an indication of the antigenicity of TL in vivo.

Tomato lectin was purified to homogeneity from the locular fluid of ripe tomatoes according to the method of Kilpatrick et al. (1983) as described by Naisbett and Woodley (1994a). Lectin activity was measured by the agglutination of untreated human erythrocytes (Kilpatrick and Yeoman, 1978). Polyclonal antibodies against purified TL were raised in rabbits according to the following protocol adapted from Reid (1988). Briefly, the animals were injected subcutaneously on day 1 with 1 mg of TL (in an emulsion of distilled water and Freund's complete adjuvant), days 14 and 28 (in incomplete Freund's adjuvant), and intramuscularly on day 42 (in phosphate-buffered saline). Blood was collected from the marginal ear vein on day 49, allowed to clot (1 h at 4°C) and serum obtained and stored at −80°C in the presence of 0.02% (w/v) NaN_3. The presence of specific anti-tomato lectin antibodies in the serum was determined by dot-blotting (data not shown).

Everted intestinal sacs were prepared using the improved method developed by Bridges (1980), described in detail in Naisbett and Woodley (1994b). Briefly, everted sacs of adult rat small intestine were incubated in 10.0 ml oxygenated tissue culture medium 199 (TC 199; ICN Flowlabs, Herts, UK) containing 10 μg/ml TL, at 37°C in an oscillating water bath for times up to 2 h. Sacs were removed at time points, blotted dry and the serosal contents collected and immediately frozen at −80°C. Gut sacs were digested individually in 25 ml of 1 M NaOH and samples were assayed for protein content (Lowry et al., 1951, as modified by Peterson, 1983).

After incubation of gut sacs with TL, samples of serosal fluid were analysed to measure the ability of the lectin fragments in the fluid to react with anti-TL antibodies. This gave an indicationof the ability of the lectin to retain its antigenicity during mucosal transfer. Antigenicity of the lectin fragments was measured by a 'sandwich' enzyme-linked immunosorbent assay (ELISA) in which ovomucin was used to 'capture' TL (LeVine et al., 1972; Merkle and Cummings, 1987). The amount of TL captured was then assayed using rabbit polyclonal antibodies raised against the lectin. The following protocol was a modification of that of Engvall (1980).

Each well of the microtitre plate was loaded with 250 μg of ovomucin (Sigma) in 50 μl of coating buffer (0.05 M carbonate buffer, pH 9.6, containing 0.02% (w/v) NaN_3) and incubated overnight at 4°C to allow the ovomucin to bind to the plate. After aspiration of the solution and thorough washing with PBS containing 0.05% (v/v) Tween 20 (ELISA buffer), known amounts of TL diluted in 50 μl ELISA buffer were added to triplicate rows of wells and allowed to bind to the ovomucin overnight at 4°C. After aspiration of the well contents and washing with ELISA buffer, the presence of TL in the wells was detected by incubation, firstly with anti-TL polyclonal antibodies raised in rabbits and, after further washings in buffer, biotinylated anti-rabbit IgG (1:1000 dilution) followed by streptavidin-biotin-peroxidase complex (1:1000 dilution). Anti-rabbit IgG and streptavidin complex were obtained from Amersham International Plc, UK. The presence of peroxidase was determined by incubation with phosphate-citrate buffer, pH 5, (50 ml) containing 20 mg *o*-phenylenediamine (Sigma) and 20 μl 30% (v/v) hydrogen peroxide. The reaction was stopped by the addition of 2 M

H_2SO_4 and the coloured end product was read at 492 nm on a Titertek [1] Multiscan Plus plate reader. Blank values were obtained using wells minus TL or wells minus ovomucin. A calibration curve was constructed, plotting absorbance against known amounts of tomato lectin (μg) and was used to calculate the amount of TL in the serosal fluid samples.

For in vivo studies, adult male Balb/c mice were lightly anaesthetized and administered on day 1 with 2.5, 5, 10, or 25 μg TL orally (by intubation into the stomach) in 0.5 ml of 0.1 M carbonate/bicarbonate buffer, pH 9.5, or intraperitoneally (i.p.) in 0.25 ml of sterile PBS. BSA, a control biodegradable macromolecule, was administered in a similar manner, both orally and i.p. TL was also preincubated with 75 μM *N*-acetylglucosamine tetramer as a competing sugar and doses of this 'blocked' lectin were fed to mice. A large excess of competing sugar (in molar terms) was used to ensure inhibition of lectin activity. On day 14, animals were killed by cervical dislocation and blood was obtained by cardiac puncture and allowed to clot for 1 h at 4°C. Serum was separated by centrifugation, NaN_3 (0.02% w/v) was added and samples were stored at −80°C until assay. After cardiac puncture, the gut was removed from the pylorus to the caecum and was immediately clamped at one end. The length of excised gut was filled with sodium chloride washing buffer containing Tris, EDTA and Tween 20 (De Aizpurua and Russell-Jones, 1988), clamped at the other end and kneaded gently. The contents were evacuated into a Luckham's tube and centrifuged (500 × *g*, 15 min, 4°C), to remove any debris. The supernatant was stored with 0.02% (w/v) NaN_3 at −80°C until assay.

To measure serum IgG and intestinal IgA production stimulated by oral or i.p. antigen, samples of serum and intestinal washings were incubated in ELISA plates. The microtitre plates were prepared coated with 1 μg/ml TL or 2.5 μg/ml BSA in coating buffer as described for ovomucin earlier. 50 μl serum or intestinal supernatant was added in column 1 of the appropriate microtitre plate. This was then serially diluted across the plate with ELISA buffer and incubated overnight at 4°C. Wells were washed in ELISA buffer as before and appropriate second antibodies were added; biotinylated anti-mouse IgG (1:1000 dilution) and biotinylated anti-mouse IgA (1:1000 dilution), (Amersham International Plc, UK). Incubations were then performed with streptavidin-biotin-peroxidase complex as described earlier and the absorbance in each well was measured on a plate reader at 492 nm.

Although previous work has shown that radiolabelled TL may be transcytosed in an everted gut sac system in a form which was trichloroacetic acid-precipitable and therefore likely to be of high molecular weight (Naisbett and Woodley, 1994b), it was unclear whether this trichloroacetic acid-precipitable fraction contained TL which was of sufficient size and/or shape to be recognised by antibodies or elicit an immune response. Non-radiolabelled TL was therefore incubated in an improved everted gut sac system and samples of serosal fluid were collected and analysed to determine whether any TL or TL fragments had retained the ability to react with anti-TL antibody

Table 1
Lectin content of serosal fluid after incubation with everted gut sacs

Time (min)	Mean amount of lectin in the serosal fluid (ng)		
	Observed ± S.E. [a]	Calculated [b]	Observed as % of calculated
15	0.014 (0.010) [c]	0.054	26%
60	0.053 (0.012) [c]	0.190	28%
120	0.098 (0.011) [c]	0.428	23%

[a] Determined by ELISA.
[b] Calculated using rate previously determined with radiolabelled tomato lectin.
[c] $n = 16$.

after incubation. Following the construction of a standard curve, correlating the amount of lectin with absorbance, the amount of lectin (or fragments) present in the serosal fluid which were still antibody reactive after incubation could be determined. The results are shown in Table 1. It can be seen that the amount of antibody-reactive TL in the serosal fluid increased with time to an average total of 0.098 ng per sac after 2 h. It was also possible, from knowing the rate of uptake of tomato lectin in the gut sac system at 37°C, to calculate the total amount of lectin expected to be found in the serosal fluid during in vitro uptake experiments. From previous work (Naisbett and Woodley, 1994b), it is known that the rate of lectin transfer (at a concentration of 2 μg/ml) into the serosal fluid was 1.7 ng/h per mg protein. It is also known from the same work that the rate of TL transfer into the serosal fluid was linear with increasing lectin concentration up to concentrations of 15 μg/ml. It was assumed therefore that, for the purpose of these experiments, the rate of transfer of lectin into the serosal fluid at a concentration of 10 μg/ml was 8.5 ng substrate/h per mg protein. As the total protein content of each sac and the incubation times were determined, it was possible to calculate the expected amount of lectin in the serosal fluid of each sac and compare it with the observed figure derived from the ELISA experiments. These data are also shown in Table 1.

The results from this experiment showed that TL or fragments of TL could still react with anti-TL antibodies after intestinal processing and transfer into the serosal fluid. The actual amounts of lectin determined from the ELISA are between 23 and 28% of the value of the calculated amount derived from uptake values of radiolabelled lectin. The calculated values take into account uptake of intact molecules and large and small radiolabelled fragments, as the figure was determined solely from radioactive counts. The ELISA however could only measure fragments which were still able to react with specific antibodies and which were still capable of binding to ovomucoid. The ELISA data may therefore be an

Table 2
(A) Serum IgG response to a single dose of oral antigen, (B) serum IgG response to a single dose of i.p. antigen and (C) intestinal IgA response to a single dose of oral antigen

μg antigen	Antibody titre								
	Tomato lectin			Tomato lectin + sugar			BSA		
	a	b	c	a	b	c	a	b	c
A									
2.5	16	32	32	0	2	0	N.D.	N.D.	N.D.
5	256	128	128	4	2	2	N.D.	N.D.	N.D.
10	512	512	512	32	64	32	0	0	0
25	1024	1024	2048	256	256	128	0	2	1
50	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2	2	4
B									
2.5	4000	4000	2000				N.D.	N.D.	N.D.
5	8000	8000	8000				N.D.	N.D.	N.D.
10	8000	16000	4000				0	8	4
25	16000	8000	16000				512	32	8
50	N.D.	N.D.	N.D.				128	128	64
C									
2.5	16	128	32	2	2	0	N.D.	N.D.	N.D.
5	64	256	128	4	8	1	N.D.	N.D.	N.D.
10	256	512	256	8	8	4	0	1	0
25	1024	1024	1024	8	16	16	1	0	0
50	N.D.	N.D.		N.D.	N.D.	N.D.	0	0	0

N.D., not determined. a, b and c represent three different mice for each antigen.

underestimate of the amount of antibody-reactive molecules. It was also assumed that native, unlabelled TL interacted and was endo- and transcytosed by the gut sacs in the same way and at the same rate as radiolabelled TL.

These results clearly demonstrate uptake of large fragments of TL, or intact TL, in vitro, which are able to bind ovomucin and are still recognised by anti-TL antibodies. Oral feeding experiments were performed to determine whether TL crossed the intestinal mucosa in vivo and stimulated a specific serum IgG response. Production of specific intestinal IgA was also measured to determine the extent of any local intestinal mucosal immune response against the lectin. BSA was fed to mice as a control protein. Groups of animals were injected intraperitoneally with the same antigens (TL or BSA) as positive controls. The results are summarized in Table 2A–C. The final antibody titre was derived from the lowest dilution of serum or intestinal washings which gave a positive absorbance value. The reciprocal of this dilution (titre) was then recorded as the antibody titre. An antibody titre of zero indicated no measurable response. It should be stressed that this was a pilot study and very small numbers of animals were used ($n = 3$). No statistical analyses were performed.

Table 2A and B shows the IgG response after oral and parenteral dosing of antigens. Oral dosing of as little as 2.5 μg of lectin gave a measurable serum IgG response in all three mice. Increasing concentrations of lectin resulted in an increase in antibody titre, but the response was variable. Intraperitoneal dosing of TL gave much higher circulating serum antibody levels, with an antibody titre of up to 4000 produced by 2.5 μg lectin compared with a maximum titre of 32 after oral administration of the same amount of lectin. After preincubation with competing sugar there was a measurable reduction in the levels of serum IgG after oral dosing. Mice fed 25 μg lectin gave a maximum titre of 2048, but this was only 256 with blocked lectin.

Oral administration of BSA gave a very poor serum IgG response. No antibody was detected after feeding 10 μg BSA, but two mice gave a weak positive response with 25 μg BSA and all three mice responded to some extent after feeding with 50 μg BSA. The i.p. route of administration gave higher serum IgG titres although one mouse failed to show any response with 10 μg of antigen. The results for BSA administration by either route were very variable.

Table 2C lists the levels of IgA detected in small intestinal washings in the same mice after oral administration of 'unblocked' and blocked lectin and BSA. Mice fed lectin orally showed relatively high intestinal IgA levels with one animal having a titre of 128 after an oral dose of only 2.5 μg lectin. Increasing the antigen concentration gave an increase in IgA titre. The strength of the response varied between animals. Intraperitoneal dosing of lectin resulted in virtually no detectable specific intestinal IgA, even after a 25 μg dose of antigen (data not shown). After blocking with competing sugar, there was a reduction in specific intestinal IgA.

BSA fed orally gave a negligible intestinal IgA response in two mice. In contrast with higher IgG serum antibody levels after i.p. injection, intraperitoneal BSA induced no specific intestinal IgA responses (data not shown).

Antigenicity is thought to be a major disadvantage when using proteins as drug carriers (Drobnik, 1989). If tomato lectin is to be used as a drug carrier for oral delivery, to adhere to the intestinal mucosal surface and/or to promote macromolecular drug uptake by endocytosis, then serum unresponsiveness towards the carrier is desirable. From the data presented here, it is clear that specific serum IgG antibodies to TL were raised following a single oral administration of very small amounts of antigen. This phenomenon is in itself unusual in that the induction of serum or mucosal antibody responses to orally administered antigens is often difficult and generally requires the administration of relatively large quantities of antigen, as the amount of antigen that is actually absorbed and capable of eliciting an immune response is usually low (De Aizpurua and Russell-Jones, 1988). Here, however, a single oral dose of 2.5 μg of TL was enough to elicit measurable serum IgG and intestinal IgA responses. This is in accordance with similar experiments performed by De Aizpurua and Russell-Jones

(1988), who were able to stimulate the humoral immune response using a minimum dose each of of *E. coli* pili and various plant lectins such as *Phaseolus vulgaris* lectin, concanavalin A and *Ulex europaeus* lectin.

The measured serum IgG response was dose-dependent; the greater the amount of ingested lectin, the larger the specific serum titre. Again, this is in accordance with De Aizpurua and Russell-Jones (1988), who obtained increasing antibody titres with up to 1000 μg antigen. It was impossible to calculate from these experiments the amount of antigen taken up by the intestine to produce this immune response and also to determine whether the lectin was intact or whether it existed as large fragments of partially degraded antigen. However, work by Kilpatrick et al. (1985) has shown that rats fed on a tomato lectin-rich diet passed faeces containing serologically detectable TL. This indicated that the TL could survive gastrointestinal transit in a form serologically indistinguishable from the native lectin. They also estimated the amount of TL reaching the circulation from the intestine as being 0.9% of the dose administered after 3 h.

Although it has been generally assumed that the mechanism by which antigen is taken up by the small intestine is primarily via non-specific sampling and subsequent transcytosis of the contents of the gut lumen by the 'M' cells that overly the Peyer's patches (McGhee et al., 1992), it is now thought that enterocytes themselves may also be responsible for the uptake and transcytosis of intact macromolecules. This has been demonstrated in our own work with gut sacs, which contained no Peyer's patches but which still transcytosed tomato lectin and other macromolecules such as polymers (Naisbett and Woodley, 1994b; Pató et al., 1994). According to Walker (1982), macromolecules such as HRP are preferentially taken up by M cells when low concentrations of HRP were present. At high antigen concentrations, however, Walker (1982) suggests that absorption is not restricted to M cells, but all epithelial cells absorb increasing amounts of luminal antigens. Pusztai (1989), however, suggests that the preferred route of uptake for luminal (food) proteins found in nanogram amounts in the sera of adults is through the M cells. Uptake of antigen by the enterocytes is of importance as enterocytes not only transcytose macromolecules in the apical to basolateral direction, but also express MHC class II antigens and may therefore play a role in sampling, processing and presentation of luminal antigens (Lazorová et al., 1993). It should also be stressed that the surface area of non-M cell epithelium is relatively large compared with the surface area represented by M cells (Ernst et al., 1988) and therefore even inefficient antigen processing and presentation by enterocytes may be functionally important.

It is interesting to note that the apical membrane of M cells binds a lectin, wheat germ agglutinin, avidly (O'Hagan et al., 1987). This lectin binds terminal sialic acid and oligomeric GlcNAc. Thus it may show some of the binding characteristics of tomato lectin which also binds oligomeric GlcNAc. If tomato lectin binds to M cells and/or enterocytes in vivo, it is possible that very low doses may be enough to elicit an immune response. De Aizpurua and Russell-Jones (1988), found that feeding bacterial adhesins and lectins which were all known to be intestinal adhesives, was remarkably efficient in inducing immune responses. Preincubation of TL with competing sugar did not completely abolish the induced immune response, but both the IgG and IgA responses against oral antigen were substantially reduced. This would indicate that blocked lectin cannot bind as avidly to M cells or enterocytes, resulting in less uptake and therefore a lower antibody titre, as demonstrated in vitro (Naisbett and Woodley, 1994b).

These results have implications for the potential of tomato lectin as a carrier or bioadhesive for oral drug delivery. Feeding of minute doses of lectin produced a high serum IgG titre. Whilst this implies that lectin can be transferred intact or as large fragments across the intestinal epithelium, and thus may be of some use as a drug carrier to enable drugs to be transported across the intestine, the fact that a serum response was raised against the lectin shows that it is immunogenic and therefore unsuitable for chronic delivery. Tomato lectin also elicits a measurable local intestinal immune response (IgA), which is

adapted to prevent the uptake of large amounts of antigen at mucosal surfaces and often limits the use of oral macromolecular drug delivery systems. However, this does not necessarily imply that TL uptake will be prevented on further feeding. Pusztai (1989) has shown that animals fed oral phytohaemagglutinin lectin (PHA) developed a powerful and selective humoral (IgG) response to the lectin, and booster effects were seen on subsequent further feeding. Thus, the neutralizing action of the local IgA was relatively ineffective and could not prevent systemic absorption of ingested PHA after repeated feedings.

This systemic and/or mucosal priming, however, does raise the possibility of using tomato lectin to promote oral immunization. TL can be taken up by the gut and transferred into the circulation where circulating antibodies can be raised against it. If a small peptide antigen (vaccine) could be attached to the lectin, it may be possible for the peptide to be taken up into the circulation and for antibodies to be induced towards it. As the lectin is highly immunogenic, this would increase the possibility of antibodies being raised to the attached peptide. Thus, the lectin would be used both as an oral immunoprophylactic agent and also as a carrier for vaccine-relevant antigens.

This has analogy with the highly immunogenic cholera toxin (CT), which is currently being investigated as an adjuvant for oral vaccine delivery. CT and its purified B subunit (BS) bind G_{M1} ganglioside and are potent oral immunogens, capable of stimulating strong mucosal secretory IgA and serum IgG antitoxin responses and immunological memory in the intestine (Holmgren et al., 1993). CT has also been shown to be a strong mucosal adjuvant, which does not seem to induce oral tolerance and which can markedly potentiate the immune responses to unrelated, weakly immunogenic protein antigens, given orally (Elson and Ealding, 1984; Lycke and Holmgren, 1986). This oral adjuvant effect of CT has now been demonstrated with HRP (McKenzie and Halsey, 1984), Sendai virus (Nedrud et al., 1987), and influenza virus (Chen and Stroeber, 1990). CT and BS are thought to be potent oral immunogens because of their lectin-like ability to bind avidly to the intestinal mucosa, and their ability to resist degradation by intestinal proteolytic enzymes. These are all properties demonstrated by tomato lectin and it would seem feasible to suggest that TL, whilst having a limited role as a drug carrier, may have potential as an immunological adjuvant for oral vaccine delivery.

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