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A Mutant Pertussis Toxin Molecule That Lacks ADP-Ribosyltransferase Activity, PT-9K/129G, Is an Effective Mucosal Adjuvant for Intranasally Delivered Proteins

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We examined the capacity of a genetically detoxified derivative of pertussis toxin (PTX), PT-9K/129G, to act as a mucosal adjuvant for an intranasally (i.n.) administered tetanus vaccine. Groups of mice were immunized i.n. with the nontoxic C-terminal 50-kDa portion of tetanus toxin (fragment C [Frg C]) either alone or mixed with PT-9K/129G, PTX, or cholera toxin (CT) or were immunized subcutaneously (s.c.) with an equivalent amount of Frg C adsorbed to alhydrogel. In response to a single immunization, mice receiving Frg C plus PT-9K/129G or CT i.n. and parenterally immunized mice developed high-titer (>20,000) anti-Frg C antibodies, whereas mice immunized i.n. with Frg C plus PTX or with Frg C alone seroconverted only after being boosted. The serum anti-Frg C response was dominated by immunoglobulin G1 (IgG1) in mice immunized with Frg C plus PT-9K/129G, with Frg C plus PTX, or s.c. In contrast, IgG1, IgG2a, and IgG2b contributed almost equally to the Frg C response when CT was the adjuvant. Anti-Frg C IgE was detected only in the sera of mice immunized i.n. with Frg C plus PTX and immunized s.c. with Frg C plus alhydrogel. High levels of IgA antibodies were present in nasal lavage fluid from mice immunized i.n. with Frg C plus PT-9K/129G, PTX, or CT but not in that from mice given Frg C alone i.n. or parenterally. The mucosal adjuvanticity of PT-9K/129G was manifested in inbred as well as outbred mice. A single i.n. dose of Frg C plus either PT-9K/129G or PTX (with high specific activity) was sufficient to protect all immunized mice from tetanus toxin challenge, in contrast to the case for mice that received Frg C alone i.n. We conclude that the pertussis toxin analog PT-9K/129G, which is devoid of ADP-ribosyltransferase activity, is a potent mucosal adjuvant for vaccines delivered via the respiratory tract.

The majority of pathogenic microorganisms initiate infection by attaching to the mucosal epithelial cells lining the gastrointestinal, oropharyngeal, respiratory, or genitourinary tract. Some pathogens, such as influenza virus, *Bordetella pertussis*, or *Vibrio cholerae*, may remain at or within the mucosal tissue. Others (for example, *Salmonella typhi* and hepatitis A virus) may possess mechanisms that allow them to penetrate into deeper tissues and disseminate systemically. The specific and nonspecific defense mechanisms of the mucous membranes provide a first-line defense against both types of pathogen. Nonspecific effectors include resident macrophages, antimicrobial peptides, lactoferrin, lysozyme, extremes of pH, bile acids, digestive enzymes, mucus, shedding of epithelial cells, flushing mechanisms (peristalsis, ciliary beating, and micturition), and competition from local flora (30).

As with the systemic immune system, cellular and soluble components participate in mucosal immunity (13, 22). One of the major differences in the immune responses engendered by the local and systemic lymphoid compartments is in the predominant antibody isotype produced. Immunoglobulin A (IgA) is the major immunoglobulin in the secretions, whereas IgG predominates in serum (13, 22). IgA is endowed with characteristics that make it well suited to the defense of the mucosae (17). Successful pathogens have evolved means to survive the nonspecific defenses present at the site they infect. The secretory immune system has been shown to play a major role in protecting against diseases caused by a number of bacterial and viral pathogens, and it is probably the predominant effector against pathogens restricted to mucosal surfaces (17). For organisms that spread systemically, specific local and systemic immune responses are probably needed for optimum immunity.

Therefore, stimulation of the local and systemic lymphoid compartments is required for effective immunization against many diseases. Unfortunately, current parenteral immunization regimes often elicit weak or undetectable secretory responses (13, 22). In order to effectively prime the mucosa-associated lymphoid tissue, the immunogen needs to be applied topically to the mucosal surface during the course of vaccination (13, 22). Unfortunately, most nonreplicating immunogens are poorly immunogenic when ingested or inhaled. Soluble proteins are particularly inefficient mucosal immunogens (13, 22). One of the major goals of modern vaccinology is to devise means of eliciting strong mucosal and systemic immune responses to mucosally delivered soluble proteins.

Although many soluble proteins are poor mucosal immunogens, some proteins, typically microbial components that can recognize and bind to receptors on the surface of eucaryotic cells, can elicit local and systemic immune responses (5). In

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particular, cholera toxin (CT) is the most potent mucosal immunogen known and is also a mucosal adjuvant that greatly enhances the responses to coadministered antigens (14). Minute quantities of CT are necessary for the adjuvant effect in mice (14). Unfortunately, 5 μg of CT fed to human volunteers was found to cause diarrhea, and a dose of 25 μg induced purging indistinguishable from that seen in classical cholera. Active CT is therefore unacceptable as a mucosal adjuvant in humans (19).

CT is a bipartite toxin consisting of an A protomer (CTA) and a B pentamer (CTB) (34). CTA is the enzymatic moiety of CT responsible for the covalent modification of host G proteins. CTB, which mediates the binding of CT to its receptor (ganglioside GM_1) on the surface of eucaryotic cells, is nontoxic and is also a good mucosal immunogen (34). CTB has been investigated as a mucosal adjuvant by many groups, with conflicting results (14). CTB obtained from commercial suppliers is prepared from CT and often contains trace quantities of CT, which could be responsible for the reported adjuvanticity of commercial CTB (14, 20, 38, 44). In fact, recombinant CTB and the B pentamer from the highly related *Escherichia coli* heat-labile toxin are immunogenic but are devoid of oral or nasal adjuvanticity, as is a mutant heat-labile toxin molecule (tested only orally) that does not possess ADP-ribosylating activity and no longer causes fluid secretion in rabbit ligated loops (4, 21, 38).

Pertussis toxin (PTX), like CT, has an AB_5 structure (36). Both CT and PTX are ADP-ribosylating (40, 41) toxins, but they have different cellular receptors and substrates (34). PTX can produce myriad biological effects, including adjuvanticity (23, 40). Parenterally administered PTX can potentiate IgE and delayed-type hypersensitivity to coadministered proteins (23, 41). PTX is one of the major protective antigens of *B. pertussis*, and inactivated forms of PTX form the basis of currently used and experimental acellular pertussis vaccines (31). A genetically detoxified form of PTX (PT-9K/129G) which lacks the deleterious properties of PTX but which can still bind to eucaryotic cells and is highly immunogenic in rodents and humans has been constructed (24, 28, 29, 31).

Using a murine intranasal (i.n.) immunization model, we have investigated the abilities of PTX and the genetically inactivated derivative PT-9K/129G to potentiate the immune response to a coadministered protein immunogen, the C-terminal 50-kDa portion of tetanus toxin (fragment C [Frg C]).

MATERIALS AND METHODS

Reagents. PTX was obtained from Calbiochem (Novabiochem, Nottingham, United Kingdom) and the National Institute for Biological Standards and Control (NIBSC) (Potters Bar, Herts, United Kingdom); CT was obtained from Sigma (Poole, Dorset, United Kingdom). The nontoxic PTX analog PT-9K/129G was prepared as previously described but was not stabilized with formalin (24). Recombinant Frg C was produced from *Pichia pastoris* as previously described (3). All proteins were diluted in phosphate-buffered saline (PBS). Unless otherwise stated, all other materials were obtained from Sigma.

Mice. Adult (8- to 16-week-old) female BALB/c and NIH:S mice were supplied by Charles River (Margate, Kent, United Kingdom) and Harlan Olac (Bicester, Oxon, United Kingdom), respectively.

Immunizations and sampling procedures. Mice were immunized subcutaneously (s.c.) with Frg C adsorbed overnight (4°C) to alhydrogel (0.25%) or i.n. with Frg C alone or Frg C mixed with PT-9K/129G, CT, or PTX. For i.n. immunizations mice were lightly anesthetized with halothane, and 30 μl of sample (15 μl per nostril) was applied to the external nares with a micropipette. Mice were immunized s.c. by injection of 100 μl of sample into the skin folds in the back of the neck. Serum was obtained by superficial venipuncture of the tail veins or via cardiac puncture. Nasal washes were obtained from killed animals by cannulating the orifice to the nasal cavity at the rear of the soft palate with a fine-tipped pipette (Alpha Labs, Hants, United Kingdom) and lavaging the nasal cavity with 1 ml of PBS plus 1% bovine serum albumin (BSA).

Measurement of antibody responses. Anti-Frg C and antitoxin-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well enzyme immunoassay/radioimmunoassay plates (Costar, High Wycombe, Bucks, United Kingdom) were coated with antigen (50 μl; 2.5 μg/ml in PBS; overnight, 4°C), washed three times with PBS containing 0.05% (vol/vol) Tween 20 (Sigma), and then blocked with PBS plus 1% BSA. After being washed, the plates were incubated with serial dilutions of nasal wash fluid or serum for 2 h at 37°C. All samples and reagents were diluted in PBS–0.05% Tween 20 plus 0.1% BSA. The plates were washed and incubated with biotin-conjugated goat anti-mouse IgA or IgG (Sigma) or, to determine IgG subclass or IgE responses, with biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b, IgG3, or IgE (Pharmingen, AMS Biotech, Witney, Oxon, United Kingdom) for 1 h at 37°C. Following washing, horseradish peroxidase-conjugated streptavidin (Dako, High Wycombe, Bucks, United Kingdom) was added, and bound antibodies were visualized by adding *o*-phenylenediamine substrate (0.04% *o*-phenylenediamine in citrate-phosphate buffer [pH 5] containing 0.01% H_2O_2). After color development, the reaction was stopped with 3 M H_2SO_4, and the A_{490} was read. Absorbance values were plotted against dilutions, and titers were determined as the reciprocal of the highest sample dilution giving an absorbance of 0.2.

Serum IgE responses. Total serum IgE was measured by capture ELISA with pairs of anti-murine IgE monoclonal antibodies and a murine IgE standard according to the instructions of the manufacturer (Pharmingen). The Frg C-specific IgE ELISA was carried out as for the IgG ELISA, except biotinylated anti-IgE was used instead of anti-IgG. The passive cutaneous anaphylaxis assay for anti-Frg C IgE was carried out according to the method of Ovary et al. (27). Briefly, the back of a rat (>300 g) was shaved, and 10-μl volumes of serial threefold dilutions of test sera and positive and negative control mouse sera in saline were injected intradermally. On the following day, 1 ml of a solution of 200 μg of tetanus toxoid per ml in 1% Evans blue was injected intravenously into the tail vein. After 10 to 15 min, the rat was killed, the skin on the back was removed, and the diameters of the blue spots on the internal surface of the skin were measured. The titer was determined as the reciprocal of the highest dilution of serum giving a spot with a diameter of >5 mm.

Tetanus toxin challenge. Mice were challenged with 0.01 μg (10 50% lethal doses) of purified tetanus toxin as previously described (7), and fatalities were recorded for 4 days.

TABLE 1. Primary and secondary serum IgG responses to Frg C alone and with PTX, PT-9K/129G, or CT in response to i.n. immunization

Treatment	Administration route	Serum IgG GMT[a] after dose: 1 Anti-Frg C	1 Anti-toxin[b]	2 Anti-Frg C	2 Anti-toxin
Frg C	i.n.	19	ND[c]	1,231	ND
Frg C plus PTX	i.n.	19	130	68,642[d]	1,088,532
Frg C plus PT-9K/129G	i.n.	3,162[d]	5,127	625,012[d]	2,060,992
Frg C plus CT	i.n.	5,529[d]	169,805	3,050,226[d]	24,792,471
Frg C plus alhydrogel	s.c.	74,025[d]	ND	1,206,370[d]	ND

[a] Geometric mean titer (GMT) calculated from the individual titers in sera from five mice per group.

[b] Responses were measured against either PTX, PT-9K/129G, or CT as appropriate.

[c] ND, not determined.

[d] Significantly higher ($P < 0.05$; Student's t test) than the result for mice immunized with Frg C alone i.n. For the purpose of the t test, the post-dose 1 Frg C i.n. serum was assigned a titer of the detection limit minus 1 (20 − 1 = 19).

RESULTS

Serum and secretory immune responses to Frg C, PT, PT-9K/129G, and CT in i.n. immunized mice. Groups of five adult female outbred NIH:S mice were immunized i.n. with 10 μg of Frg C alone or mixed with 3 μg of CT, PTX, or PT-9K/129G 25 days apart. A comparable group of mice were immunized twice s.c. with 10 μg of Frg C adsorbed to alhydrogel. Serum samples were taken 14 days after the primary immunization, and serum and nasal wash samples were taken 14 days after the boost. Antibody responses against each of the components were determined by ELISA.

Following a single immunization, anti-Frg C antibodies were not detected in the sera of mice immunized i.n. with Frg C or Frg C plus PTX (Table 1). In contrast, all mice receiving Frg C

combined with PT-9K/129G or CT had significant amounts of anti-Frg C antibodies in their sera. The levels of anti-Frg C antibodies were similar in these two groups. Mice immunized i.n. with Frg C and CT mounted a very strong serum IgG response to CT. Anti-PTX antibodies were present in mice receiving PT-9K/129G but were at very low levels in mice given PTX. The magnitude of the response to PT-9K/129G was considerably less than that to CT.

In response to boosting, some of the mice (two of five) that received Frg C alone i.n. seroconverted (Table 1). Mice in the group given Frg C plus PTX also exhibited an Frg C response following the second dose, and the titers were significantly higher than those in mice given Frg C alone, indicating that PTX had acted as an adjuvant. However, the anti-Frg C response was considerably lower than that of the mice given Frg C plus PT-9K/129G. In contrast, high anti-PTX titers were generated in mice given two doses of Frg C plus PTX, although these titers were lower than those in the mice receiving two doses of Frg C plus PT-9K/129G. The serum IgG anti-Frg C geometric mean titer for mice in the group given Frg C plus CT was higher than that for mice immunized twice with Frg C s.c.

IgG subtype specificity of serum response. The distribution of IgG subtypes specific for Frg C and the protein adjuvants in the sera of immunized mice was analyzed (Fig. 1 and 2). The contribution of the IgG1 and IgG2 subtypes to the anti-Frg C response was very similar in mice immunized i.n. with Frg C plus PTX and Frg C plus PT-9K/129G and s.c. with alhydrogel-adsorbed Frg C (Fig. 1). The majority of the anti-Frg C activity was located in the IgG1 subclass. IgG2b was the next-most-prominent subclass, followed by IgG2a. Specific IgG3 anti-Frg C antibodies were detected only in mice immunized parenterally. The profile of the Frg C IgG response was different in mice immunized i.n. with Frg C plus CT. There was similar anti-Frg C activity in the IgG1 and both of the IgG2 subclasses. As with the other groups, there were no specific IgG3 or IgE antibodies to Frg C detected.

The profile of the IgG subtype responses to PTX, PT-9K/129G, and CT was very similar to that of the anti-Frg C response, but there were some differences. A greater proportion of the anti-PTX activity was present in the IgG2a and IgG2b subclasses than was seen in the anti-Frg C response, but IgG1 antibodies still predominated. Also, there was anti-CT IgG3 present in the sera of mice given Frg C plus CT.

Serum anti-Frg C IgE responses. PTX is well-known to promote the induction of IgE responses to antigens coadministered parenterally. CT has recently also been demonstrated to increase the IgE responses to proteins in mice immunized i.n. (37) and orally (33). This ability has led to doubts about the suitability of such toxin adjuvants for use in humans. We analyzed pooled postboost sera for anti-Frg C IgE by ELISA and passive cutaneous anaphylaxis assay and for increases in total IgE by capture ELISA. No Frg C-specific IgE was detected in the sera of any of the groups by ELISA (data not shown). This is not surprising because of the known poor sensitivity of antigen-specific IgE ELISAs. Also, no rise in total serum IgE was found in any of the groups (data not shown). However, IgE anti-Frg C antibodies were detected by passive cutaneous anaphylaxis assay in the sera from mice in the groups given Frg C plus PTX and given Frg C plus alhydrogel s.c. (the titer was 30 for both groups) but not in the sera from mice given Frg C i.n., Frg C plus PT-9K/129G, or Frg C plus CT.

Respiratory IgA responses. The secretory responses in the nasal lavages of mice following the second immunization were studied. IgA anti-Frg C was present in the nasal lavages of all of the mice in the groups given Frg C plus PTX, Frg C plus PT-9K/129G, and Frg C plus CT (Fig. 3). As for the serum, the response was greatest in the mice that received CT as an adjuvant. There was very little Frg C-specific IgA recovered from the nasal cavities of the mice given Frg C alone, either i.n. or parenterally. In each group a single mouse exhibited evidence of an IgA response, and this was detectable only in undiluted nasal lavage. The corresponding IgA responses to PTX, PT-9K/129G, and CT were stronger than those against Frg C, and again the anti-CT response was predominant (Fig. 3).

Comparison of the i.n. immunogenicities and adjuvanticities of PTX and PT-9K/129G in inbred and outbred mice. To confirm the differences in immunogenicity and adjuvanticity that we observed between PTX and PT-9K/129G, we repeated the single-dose study with NIH:S mice and with PTX from a different supplier (NIBSC). To examine whether the genetic background of the host influences the adjuvanticity and immunogenicity of active and genetically inactivated PTX, we also studied the responses in an inbred strain (BALB/c). BALB/c mice were selected because Alonso et al. (1) reported that this strain could mount a serum response to parenterally administered PTX, whereas a strain with a different haplotype (C57BL/6) could not. Mice were immunized i.n. with 10 μg of Frg C alone or combined with 3 μg of PTX or PT-9K/129G as previously. Blood samples were taken 15 days later. To determine if the mice had developed protective immunity, they were challenged with tetanus toxin 22 days after immunization, and fatalities were recorded after 4 days.

In both BALB/c and NIH:S mice, the presence of PT-9K/129G provoked high titers of serum antibodies to Frg C (Table 2). In contrast to the results of the previous study, a single dose of PTX did have an adjuvant effect on the Frg C serum response in NIH:S mice as it did in BALB/c mice. In both strains of mice the combination of Frg C and PT-9K/129G induced a superior serum Frg C response compared with Frg C plus PTX, although the difference was not large. In BALB/c mice, in which the comparison was made, Frg C plus PT-9K/129G given i.n. was more effective at eliciting a serum Frg C response than s.c. immunization with Frg C adsorbed to alhydrogel. As was the case previously, NIH:S mice did not respond to a single 10-μg dose of Frg C given i.n. One of the three BALB/c mice immunized i.n. with Frg C alone developed a significant serum response.

Both strains of mice mounted a good serum response to PT-9K/129G and a weak serum response to PTX. In all cases (except in mice given Frg i.n. alone), NIH:S mice had higher titers in serum than similarly immunized BALB/c mice.

Mice were challenged with tetanus toxin to determine if the anti-Frg C antibodies elicited by i.n. immunization were protective. The results are shown in Table 2. All mice receiving Frg C plus PTX or PT-9K/129G were protected. The single BALB/c mouse that seroconverted following i.n. immunization with Frg C alone survived. The remaining BALB/c mice in this group, the similarly immunized NIH:S mice, and the naive control BALB/c mice all died.

DISCUSSION

In general, most soluble, nonreplicating antigens are poorly immunogenic when applied topically to mucosal surfaces (13, 22). The mucosal route of administration is usually required in order to engender mucosal immune responses and has the advantage over parenteral immunization regimens because it obviates the need for injections and the concomitant risk of blood-borne infection from reuse of needles. Therefore, in order to exploit the mucosal route for vaccination, there is a requirement for practical means of enhancing local and sys-

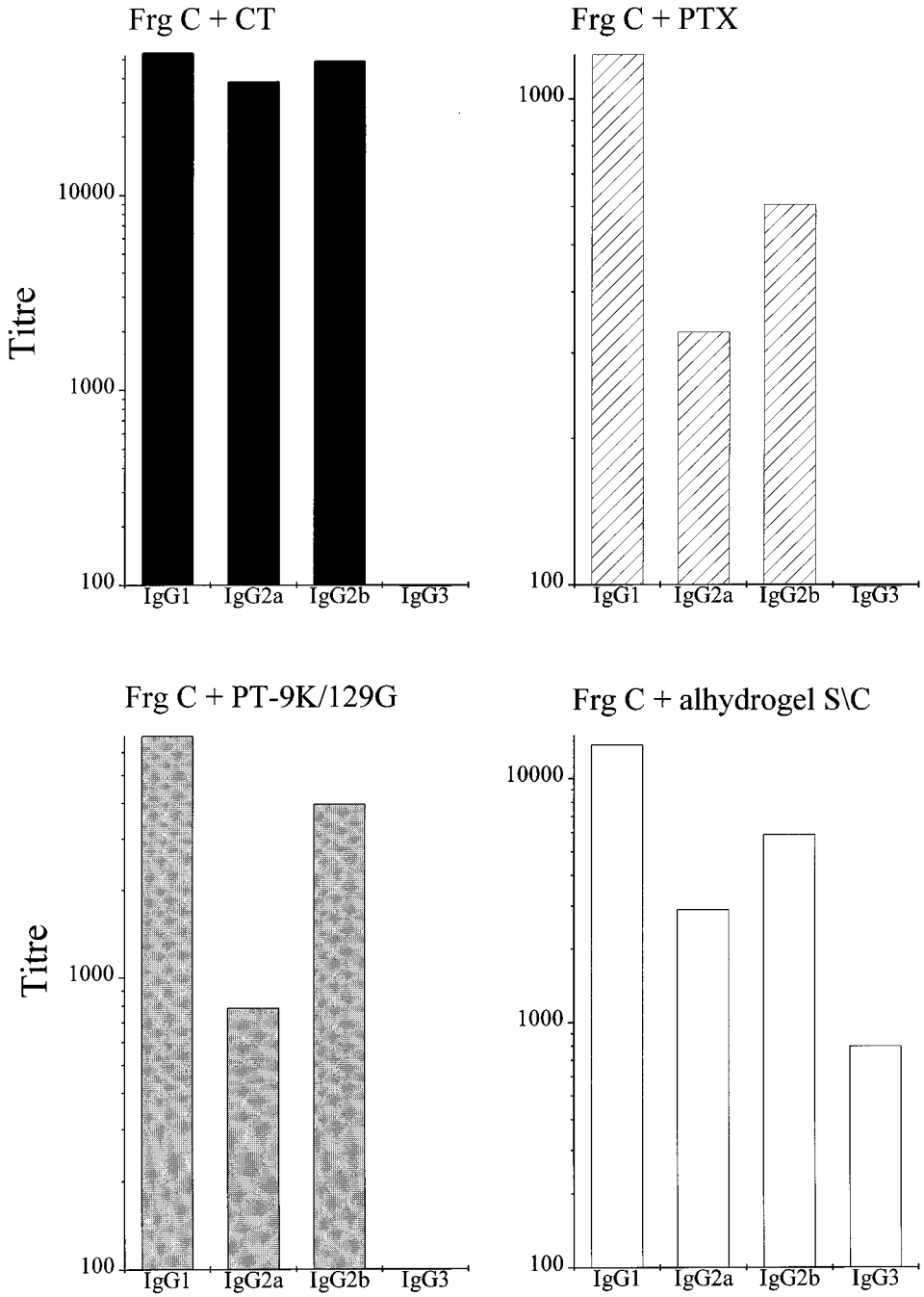

FIG. 1. Contributions of the different IgG subclasses to the serum anti-Frg C response in i.n. and s.c. immunized mice. The IgG subtype anti-Frg C responses were determined by ELISA with pooled postboost sera.

temic immune responses to mucosally delivered subunit vaccines. We investigated the potentials of enzymatically active PTX and inactive PTX (PT-9K/129G) molecules to enhance the local and systemic antibody responses to the protein Frg C coadministered to the nasal cavity of mice. Frg C is the carboxy-terminal 50 kDa of tetanus toxin. It is nontoxic, and parenteral immunization with Frg C provides protection against tetanus (7). CT is the most potent mucosal adjuvant known and was included in the study to provide a benchmark against which to judge other potential adjuvants. We used respiratory rather than oral immunization because we (4a) and others (12, 46) have found that many proteins are more immunogenic, in terms of generating both local and systemic antibody responses, when administered by the former route. There are probably several reasons for this, including the fact that the environment of the respiratory tract is less hostile to soluble antigens than that of the gastrointestinal tract. This may be particularly relevant to molecules like PTX, which is normally active in the respiratory tract. Furthermore, a number a biologically relevant peptides and proteins are absorbed more readily across the nasal mucosa than across the gastrointestinal mucosa (8, 16).

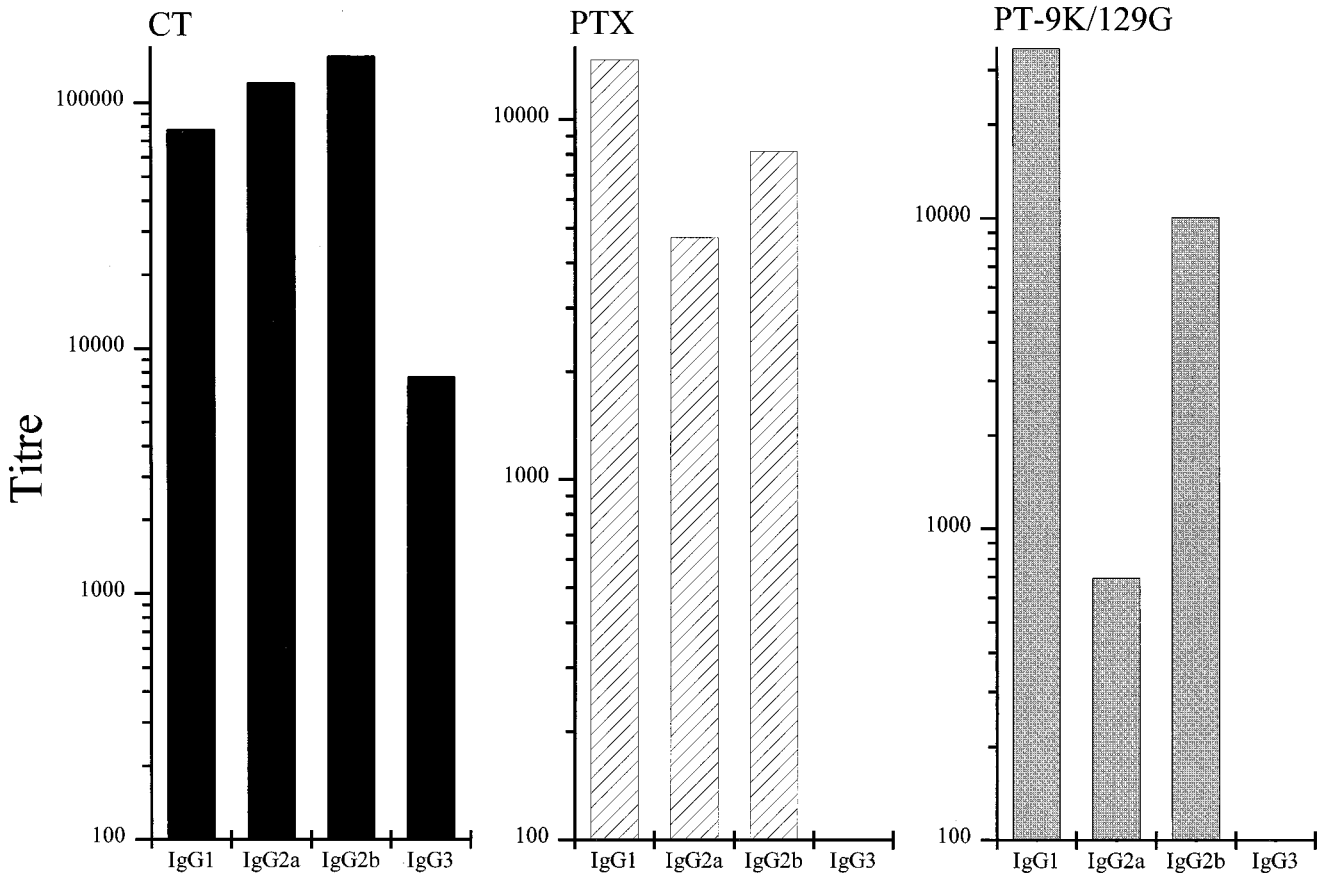

FIG. 2. Contributions of the different IgG subclasses to the serum CT, PT-9K/129G, and PTX responses in i.n. immunized mice. The IgG subtype anti-CT, anti-PT-9K/129G, and anti-PTX responses were determined by ELISA with pooled postboost sera.

We found that PT-9K/129G, which lacks ADP-ribosyltransferase activity, can greatly enhance the circulating and secretory antibodies to Frg C coadministered to the nasal cavities of both inbred and outbred mice. CT (as expected) and PTX also had an adjuvant effect on the immune response to Frg C in i.n. immunized mice. The adjuvant effect of PTX was more variable and depended on the source of the toxin. We found that the differences in the adjuvanticities of the two preparations of PTX correlated with their specific activities in assays for CHO cell clumping, agglutination of goose erythrocytes, and mitogenicity of murine splenocytes; PTX obtained from NIBSC was found to have four to five times greater specific activity than the material from Calbiochem in all assays (data not shown). A single i.n. immunization with Frg C combined with the more active preparation of PTX induced a serum anti-Frg C response, whereas two doses were needed to induce such a response if the less active PTX preparation was used. In all cases PT-9K/129G was superior to PTX as an adjuvant. Nevertheless, a single dose of 10 μg of Frg C mixed with 3 μg of either PT-9K/129G or PTX (NIBSC) was sufficient to induce protection against challenge with tetanus toxin in all immunized mice. In the case of BALB/c mice, the serum anti-PT-9K/129G titers in mice receiving Frg C plus PT-9K/129G were greater than those induced in mice immunized s.c. with Frg C adsorbed to alhydrogel. In contrast, the Frg C response in mice receiving Frg C alone i.n. was variable. In NIH:S mice a single i.n. immunization did not provoke a serum response in any of the animals, although some of these animals did seroconvert after a second dose. However, one of three BALB/c mice seroconverted and was protected from tetanus challenge after a single i.n. dose. We have previously found that it is possible to increase the proportion of mice responding to i.n. immunization with Frg C by increasing the dose, but we were unable to elicit serum anti-Frg C antibodies or protective immunity in all mice (unpublished observation).

Only mice immunized i.n. with Frg C combined with PT-9K/129G, PTX, or CT had high levels of IgA specific for Frg C in their nasal secretions. Despite eliciting high levels of circulating Frg C antibodies, s.c. immunization was ineffective at inducing a local antibody response to Frg C in the upper respiratory tract of mice. This is consistent with the findings of others that parenteral immunization is inefficient at eliciting secretory antibody responses.

Both PT-9K/129G and CT were good mucosal immunogens that evoked high-level serum antibody responses against themselves in response to a single i.n. immunization. The titers increased considerably following the second immunization. Good local antitoxin IgA responses were also elicited. The serum response to PTX exhibited a different pattern. In all cases, regardless of the PTX preparation or the strain of mice, the serum response to PTX was low or undetectable following a single i.n. immunization. This is despite the fact that PTX could boost the response to Frg C. However, following the second immunization, the serum anti-PTX response was very similar to that of PT-9K/129G-immunized mice. Others have reported that PTX may be a poor immunogen for antibody

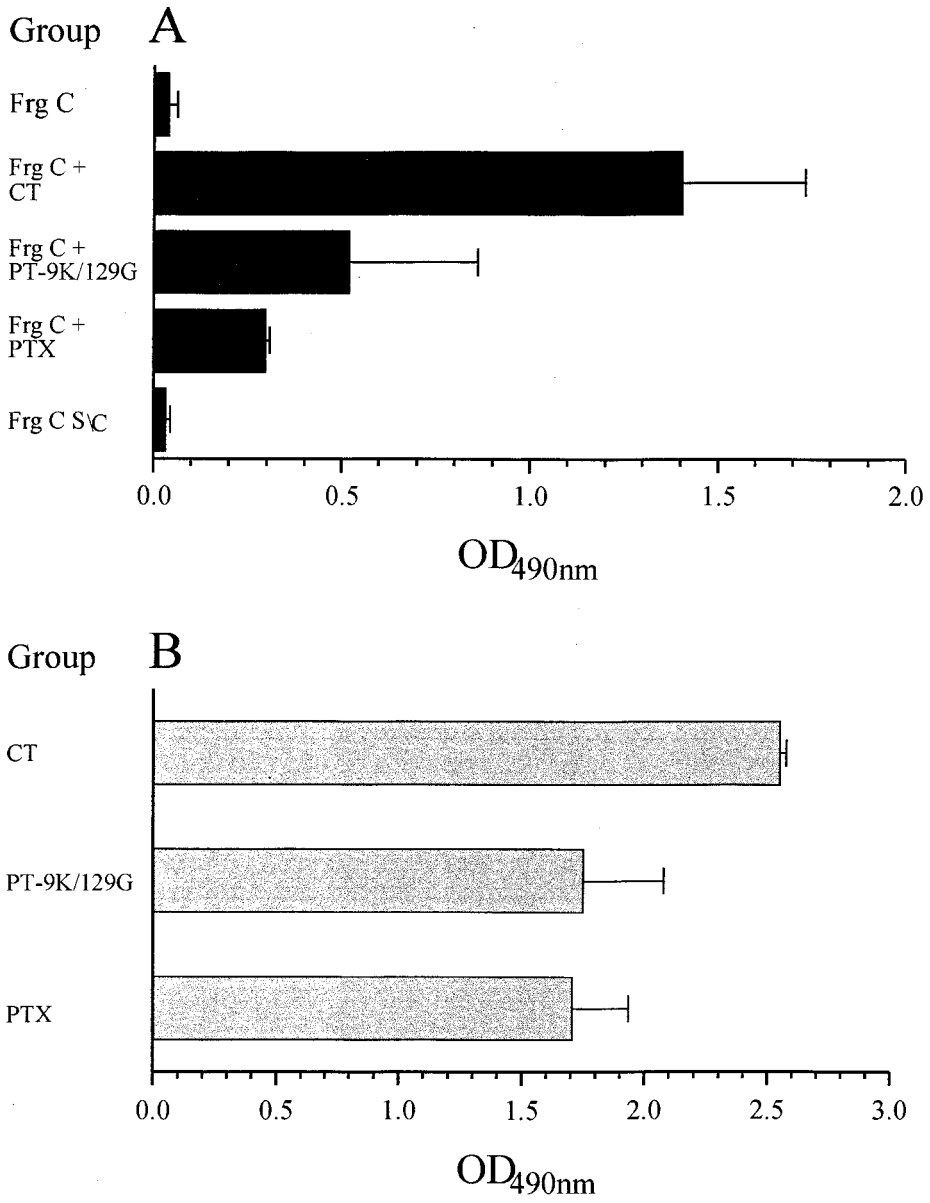

FIG. 3. Nasal IgA responses to Frg C and toxin adjuvants. Mice were immunized twice either i.n. with Frg C alone or mixed with PTX, PT-9K/129G, or CT or s.c. with Frg C adsorbed to alhydrogel. The nasal passages were lavaged 14 days after the second immunization. The IgA responses to Frg C (A) and the toxin adjuvants (B) were analyzed by specific ELISA. The optical densities at 490 nm (OD_{490nm}) generated by a 1/5 dilution of the nasal washes are shown. Each bar represents the mean for five mice +1 standard error of the mean.

responses compared with inactive toxin. Alonso et al. (1) found that C57BL/6 mice did not produce antibodies in response to multiple s.c. immunizations with PTX, whereas the mice did seroconvert when immunized with chemically detoxified PTX. On the other hand, BALB/c mice mounted antibody responses to both PTX and the detoxified PTX. Unfortunately, the response after the primary immunization was not measured. Conversely, both C57BL/6 and BALB/c mice exhibited a delayed-type hypersensitivity response to PTX, and this response was much stronger in C57BL/6 mice.

The profiles of the serum IgG subtypes specific for Frg C induced by i.n. immunization with PT-9K/129G and PTX as adjuvants were very similar to that of mice immunized s.c. with alhydrogel as the adjuvant. The IgG subtype hierarchy of the anti-Frg C response was also identical to that reported for parenterally administered antigens with *B. pertussis* as the adjuvant, namely, IgG1 > IgG2b > IgG2a (39). The response to Frg C and CT exhibited a different IgG profile. The IgG1, IgG2a, and IgG2b subtypes contributed almost equally to the response to both Frg C and CT in mice given Frg C plus CT. This is intriguing because recently it was reported that oral immunization with tetanus toxoid and CT or with tetanus toxoid alone selectively elicited $CD4^+$ T cells in both the Peyer's patches and spleen that had a Th2 phenotype, whereas parenteral immunization gave rise to a mixed Th1 and Th2 response (47). The contributions of the different IgG subtypes to the serum anti-tetanus toxoid and anti-CT response were not examined, but others have reported a preponderance of IgG1 reactive with ovalbumin in mice following oral immunization with ovalbumin plus CT (33). This supports the suggestion that

TABLE 2. Comparison of serum responses, seroconversion rates, and protection from tetanus challenge in i.n. immunized BALB/c and NIH:S mice

Mice and treatment	Serum anti-Frg C GMT[a]	Seroconversion to Frg C (no. positive[b])	Protection from tetanus (no. surviving[b])	Serum anti-PTX GMT
BALB/c				
Frg C	337	1	1	ND[c]
Frg C plus PTX	6,533	3	3	898
Frg C plus PT-9K/129G	15,527	3	3	671,371
Frg C s.c.	11,536	3	3	ND
None	<20	0	0	ND
NIH:S				
Frg C	<20	0	0	ND
Frg C plus PTX	36,096	3	3	1,868
Frg C plus PT-9K/129G	76,811	3	3	292,430

[a] GMT, geometric mean titer.
[b] Of three mice per group.
[c] ND, not determined.

the use of CT as an adjuvant favors the development of Th2-type responses. However, others have detected Th1 (as well as Th2-)-type cytokines, including gamma interferon, in unfractionated cultures of Peyer's patches and lamina propria cells from mice immunized perorally with CT plus keyhole limpet hemocyanin (15, 43). It has also been found that mice immunized i.n. with inactivated respiratory syncytial virus plus CT responded by producing serum anti-respiratory syncytial virus antibodies that were predominantly of the IgG2a subclass (32). CT did not affect the quality of the antibody response, because IgG2a was also the major subclass produced by mice immunized with inactivated or live respiratory syncytial virus alone (32).

It is possible that i.n. immunization allows more CT and antigen to reach the systemic immune system than does oral administration, which could account for the more balanced Th1 and Th2 responses seen in CT-immunized mice in this study. However, the spectrum of IgG subtypes elicited by i.n. immunization with Frg C plus PT-9K/129G or PTX is most typical of a Th2-type response.

PT-9K/129G did not stimulate an IgE response to Frg C in outbred mice when administered simultaneously i.n. This is in agreement with the finding that PT-9K/129G does not elicit IgE responses when given parenterally. This study demonstrates that purified PTX given i.n. promotes an IgE response to bystander antigens. The ability of parenterally administrated PTX to have an adjuvant effect on IgE responses is well documented (23, 41), indicating that the route of administration is not important for this effect.

We found that mice given Frg C plus CT did not have detectable anti-Frg C IgE in their sera. Others have recently reported that CT can promote IgE responses to chicken egg antigens (ovalbumin and hen egg lysozyme) coadministered orally (33) and i.n. (37). In both of these studies, the effect of CT on the IgE response was found to be both mouse strain and antigen dependent. This probably explains the difference between our and their results.

The adjuvant properties of PTX are well documented, but we know of only one study in which purified PTX has been investigated as an adjuvant for mucosally delivered proteins. Wilson et al. (44) found that PTX could have an adjuvant effect on the immune response to mixed CTB and keyhole limpet hemocyanin given perorally to mice. However, they never detected a local gut antibody response to PTX (in terms of the number of antibody-secreting cells), despite multiple oral immunizations with doses of up to 50 μg of PTX. Elson et al. (6) also found that PTX was not immunogenic when several doses were given orally to mice. The results of Wilson et al. (44) mirror ours in that PTX can exert an adjuvant effect while evoking only a poor antibody response to itself. However, i.n. boosting, unlike oral boosting, induced good serum and secretory responses to PTX. Wilson et al. (44) attributed the poor oral immunogenicity of PTX to low absorption of PTX from the gut. It is possible that PTX is taken up more readily from the nasal cavity than from the gastrointestinal tract. This is unlikely to explain the difference in the responses to PTX and PT-9K/129G that we observed.

There are numerous studies demonstrating that PTX can have an adjuvant effect on humoral and delayed-type hypersensitivity responses to coinjected antigens (23, 41). The mechanism(s) by which PTX exerts adjuvanticity is unknown but is thought to require an enzymatically active S1 subunit. Heat-killed whole cells of *B. pertussis* strains which had their S1 subunit gene deleted or which had an insertion in S1 resulting in a 90% drop in ADP-ribosylating activity did not enhance the serum antibody response to ovalbumin, whereas killed cells prepared from strains with wild-type PTX genes did (2). These results are in conflict with ours, but there are a number of differences in the two studies that could account for this. The PTX mutant studied by Black et al. (2) had four additional amino acid residues inserted into the S1 polypeptide, whereas the S1 protein of PT-9K/129G contains two amino acid substitutions (2, 28). The structure and the stability of their mutant PTX molecule were not studied by Black et al. (2). In contrast, PT-9K/129G has been demonstrated to form a stable holotoxin, and the S1 subunit is still recognized by a conformation-dependent protective monoclonal antibody (24). Black et al. (2) used whole cells of *B. pertussis*, whereas we studied purified molecules. This difference may be relevant to studies on immunopotentiation, because *B. pertussis* elaborates a number of factors, in addition to PTX, that have actions on leukocytes which may positively or negatively affect an immune response (41, 42). Finally, the route of administration may determine whether genetically inactivated PTX molecules are adjuvants or not. This may be because their effect is to increase antigen uptake across epithelial membranes (see below).

A characteristic shared by many mucosal immunogens is the capacity to bind to eucaryotic cells via recognition of molecules on the cell surface (5). Presumably, cell binding increases the retention time of the immunogen at the mucosal surface, facilitating uptake. However, eucaryotic cell binding per se does not guarantee mucosal immunogenicity (6, 45). Frg C itself

contains the receptor binding domain of tetanus toxin (11). It is not known whether Frg C or tetanus toxin can bind to the apical surface of the mucosal epithelial cells, but if it can, then this is obviously not sufficient to make it an efficient mucosal immunogen. The ability of an immunogen to bind to cells is not essential for PT-9K/129G to have an adjuvant effect on it, because we have found that PT-9K/129G can enhance the antibody response to a nonbinding protein (ovalbumin) (data not shown).

The only biological activities (other than immunogenicity) known to be shared by PT-9K/129G and PTX are T-cell mitogenicity and hemagglutination, both of which result from multivalent binding to eucaryotic cells mediated by the B subunit (25, 26). Also, PTX and its B subunit (and presumably PT-9K/129G) are mitogenic for human B cells (18). All of these activities are in vitro phenomena; there is no evidence with humans or animals that they occur in vivo, although they have been investigated only following parenteral immunization (29). This suggests that mitogenicity per se is not responsible for the adjuvant effect that we observed, but it is possible that binding to T or B cells or to other cells involved in the generation of an immune response may cause cell signalling events that can potentiate the immune response to coadministered immunogens. Another possibility is that PT-9K/129G exerts its effects on the epithelial cells lining the nasal cavity, rather than leukocytes, leading to increased uptake of other proteins. Such an effect has been reported for CTB, which has been shown to increase the electrical conductance and transepithelial flux of influenza hemagglutinin across isolated rabbit nasal mucosa (9, 10). We are currently investigating which if any of these mechanisms may be responsible for the mucosal adjuvanticity of PT-9K/129G.

Both CT and PTX are too toxic to be considered for use as mucosal adjuvants in humans. On the other hand, PT-9K/129G is nontoxic and highly immunogenic in humans and has been licensed as a component of an acellular pertussis vaccine in Italy (29). Also, our studies show that it does not promote IgE responses in outbred mice, so its use does not raise the concerns that the use of PTX and CT does. There should therefore be no barrier to investigating whether PT-9K/129G is a mucosal adjuvant in humans.

We used Frg C as a reporter antigen in this study because the mouse is a good animal model for studying tetanus immunity. However, our findings also have practical implications. Despite the existence of an effective vaccine against tetanus, over half a million people die from tetanus annually (35), with most of these deaths occuring in the third world (35). A tetanus vaccine that could be administered without needles and that could be stored without a cold chain (both PT-9K/129G and Frg C can be freeze-dried) would represent a considerable advance.

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